

Final Draft
of the original manuscript:

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In: Journal of Biomaterials Science - Polymer Edition (2009) Springer / Brill

DOI: 10.1163/156856209X427005

Novel polymer blends for the preparation of membranes for biohybrid liver systems*

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* In memoriam of our colleague Guenter Malsch, who passed away on December 24, 2006

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ABSTRACT

It was found previously that membranes based on copolymers of acrylonitrile (AN) and 2-acryloamido-2-methyl-propansulfonic acid (AMPS) stimulated greatly the functionality and survival of primary hepatocytes. In those studies however, the pure AN-AMPS copolymer had poor membrane forming properties resulting in quite dense rubber-like membranes. Hence, membranes with required permeability and optimal biocompatibility were obtained by blending the AN-AMPS copolymer with poly (acrylonitrile) homopolymer (PAN). The amount of PAN (P) and AN-AMPS (A) in the blend was varied from pure PAN (P/A-100/0) over P/A-75/25 and P/A-50/50 to pure AN-AMPS copolymer (P/A-0/100). A gradual decrease of molecular cut-off of membranes with increase of AMPS concentration was found, which allows tailoring membrane permeability as necessary. C3A hepatoblastoma cells were applied as a widely accepted cellular model for assessment of hepatocyte behaviour by attachment, viability, growth and metabolic activity. It was found that the blend P/A-50/50, which possessed an optimal permeability for biohybrid liver systems, supported also the attachment, growth and function of C3A cells in terms of fibronectin synthesis and P450 isoenzyme activity. Hence, blend membranes based on a one to one mixture of PAN and AN-AMPS combine sufficient permeability with the desired cellular compatibility for application in bioreactors for liver replacement.

Keywords:

Bioartificial liver, C3A cells, Fibronectin, P450, Synthetic Membrane

Running Headline: Novel polymer membranes for biohybrid liver

INTRODUCTION

Biohybrid or bioartificial organs (BHO) are designed for the support or replacement of human organs with malfunctions. A widely accepted bioengineering concept for the design of such a device consists of a membrane with "passive" separation or filter functions covered with living organ cells providing active transport and metabolic functions [1]. Membranes separate normally the blood side from organ cells as a protective barrier against the immune system and provide mechanical support for the cells [2]. Hence, the membranes for BHO have to fulfil a number of requirements such as good compatibility for blood (i), sufficient permeability for oxygen, nutrients, metabolites and smaller proteins (ii), retention of immunogenic components (iii), and support of adhesion, survival and function of organ cells (iv) [3, 4]. So far most of the synthetic membranes used in BHO have been developed for other biomedical applications, such as haemodialysis or blood oxygenation [5]. Since these membranes are optimized to prevent adsorption of proteins and adhesion of cells it is not surprising that organ cells tend to dedifferentiate and lose rapidly their function if cultured on such a membrane [5, 6, 7]. To overcome this limitation extracellular matrix proteins have been used to coat the membranes and to improve their cell-contacting properties [8, 9]. However, this complicates further the production of BHO and bears also some risk to transmit infections or to become immunogenic [10]. Hence, a membrane support which fulfils the requirements listed above without the need of additional procedures before immobilization of cells is highly desirable.

It has been shown that the adhesion, functionality and survival of hepatocytes are dependent on a number of physicochemical surface parameters. For example, several authors could demonstrate that an increased wettability of materials may improve functionality and survival of cells [11, 12]. Wettability however is a complex property which depends on many other surface factors including chemical composition of the material, the surface charge, the amount of adsorbed ions or molecules, and – to a certain extent – the surface topography. Moreover, these surface properties may have

their own influence on the biological response [13-16]. Accordingly, it was shown that a range of copolymers with increasing content of the hydrophilic, non-ionic monomer N-vinylpyrrolidone supports interaction and functional activity of hepatoblastoma cells [13]. On the other hand copolymers containing primary amine groups or **sulfonate** groups though being moderately or highly wettable did not have such a promoting effect on hepatocytes [14]. It should be noted that copolymers bearing sulfonate groups have been also used in the past as membrane materials for haemodialysis because of their relatively good blood compatibility [15, 16]. The low thrombogenicity and complement activation of membranes produced from acrylonitrile copolymerized with sodium methallylsulfonate has been demonstrated both in vivo and in vitro [16, 17]. Hence, copolymers with sulfonate groups may have some advantages for blood contacting applications but are obviously less suitable as support for anchorage-dependent cells. One surprising finding in this context however was that a copolymer based on acrylonitrile (AN) and 2-acryloamido-2-methyl-propansulfonic acid (AMPS) as co-monomer stimulated the functionality and survival of primary hepatocytes to an extent exceeding by far that of other copolymers mentioned above [18]. This copolymer contains a sulfonate group but possesses also an amide function. In these initial studies however, a pure AN-AMPS copolymer was used with rather poor membrane forming properties, moreover requiring a non-woven as support and led to quite dense membranes (water permeability $< 0.5 \text{ l m}^{-2} \text{ h}^{-1} \text{ kPa}^{-1}$) with a cut-off below 40 kDa. Since the requirements for permeability [1] and the fact that the porosity of membranes has also separate effect on hepatocyte behaviour [19, 20] an increase of the pore size was highly desirable. Unfortunately, the variation of AMPS concentration in the copolymer was ineffective on this parameter, except at low but inefficient co-monomer contents. Therefore, we sought to blend the AN-AMPS copolymer with poly(acrylonitrile) (PAN) homopolymer to prepare membranes by phase inversion having the required permeability and optimized compatibility for liver cells. Apart from the previous studies performed with primary cells [18], a well described C3A hepatoblastoma cell line was applied here, which is a widely accepted cellular model for the assessment of hepatocyte

interaction. The efficiency of cell-substratum and cell-cell interactions was studied via immunofluorescence for vinculin and E-cadherins. The overall morphology and proliferation of viable C3A cells was investigated by vital staining and colorimetric assays, while their functional activity was compared using the markers such as the cytochrome P450 and the FN synthesis. A supporting effect of AMPS on hepatoblastoma cells interaction was found with increasing the AMPS amount in the blends. At the same time we could tailor the membrane permeability via controlling the AMPS content. Thus a blend with a one to one mixture of PAN and AN-AMPS still provided a high functional activity of cells but had also a sufficient permeability ($> 0.5 \text{ lm}^{-2}\text{h}^{-1}\text{kPa}^{-1}$) with a cut-off of 220 kDa. Details of this study are presented herein.

MATERIALS AND METHODS

Polymer Synthesis

Beside a poly (acrylonitrile) homopolymer, a copolymer of acrylonitrile (AN) and the anionic co-monomer 2-acrylamido-2-methyl-propane sulfonic acid (AMPS) was prepared (see Fig. 1). The synthesis of these polymers has been described in detail elsewhere [21]. Specifically for this study, the basic AN monomer (Merck, Darmstadt, Germany, >99 % purity) was inert fractional distilled before use, whereas the comonomer AMPS (Sigma-Aldrich, Taufkirchen, Germany, 98 % pur.) was applied without further purification. N,N-dimethylformamide (DMF, Sigma-Aldrich, 99,8 % pur.) was inert vacuum distilled at 40 mbar/b.p.60°C before application and used as aprotic solvent in polymer synthesis. The free radical initiators 2,2'-azoisobutyronitrile (AIBN, recrystallized twice from ethanol at 30°C) or ammonium peroxidsulfate (APS, fractional crystallized from water at 40°C) were used in homo- (AIBN) and copolymerisation (APS), respectively. The precipitated AN-AMPS copolymer was dried up to a constant weight and

characterized for its composition by elemental analysis and number-average molecular weight by membrane osmometry.

Additional, for the preparation of blend membranes commercial PAN (Hoechst Celanese, Germany) with a high molecular weight was used as purchased.

Membrane formation

Membranes were formed from solutions of homopolymers, copolymers and polymer blends in DMF by application of two different techniques both using a non-solvent-induced phase inversion process. Water was used as coagulant. Membranes with specific permeability were first fabricated at a lab scale. A thin layer of the polymer solution (300 μm thick) was spread by a casting knife onto a polished glass plate and immediately coagulated in water at room temperature (22°C). The residual solvent was removed by discontinuous rinsing of the membrane with deionised water. The separation properties of these prepared membranes are summarized in Table 1.

In a second set of experiments when appropriate conditions were already established to control permeability, larger membrane quantities were fabricated by pilot equipment. Here the polymer solution was cast in a continuous process onto a textile support (pure polymer membranes) or directly onto the stainless steel band (blend polymer membranes) and transported by that steel belt into the precipitation bath. A casting slit of 300 μm was applied to distribute the polymer solution on the band.

After a discontinuous washing (over night) the membranes were tempered in water for 10 min. Details of the preparation conditions as well as the separation properties of the prepared and further investigated membranes are listed in Table 2.

Membrane permeability

The separation properties of the membranes were determined using an ultrafiltration stirred cell (type GN 10-400, Berghoff, Germany), first with distilled water (water permeability), and second with an aqueous solution of different dextran test substances (mixture of dextrans with molecular weights from 650 to 1.410.000) for cut-off determination. The molecular size distribution of dextrans in permeates, retentates and in the original solutions were determined using gel permeation chromatography (GPC; Shimadzu, Germany). From the results of GPC the cut-off was calculated assuming a logarithmic normal distribution of the pore size. The cut-off corresponds to the inflectional tangent of the logarithmic normal distribution extrapolated to a rejection of 100 %. These parameters are shown in Tables 1 and 2.

Chemical composition of membrane surfaces

The composition of membrane surface was characterized by X-ray photoelectron spectroscopy (XPS). The elemental analysis of the membrane surface and the binding states were determined by AR-XPS (take-off angle resolved XPS) measurements using a Kratos Axis 165 instrument (Kratos Analytical, UK). A monochromatic Al K_{α} beam source (1486.6 eV) was applied. A magnetic lens and electrostatic lenses were used for focusing emitted electrons into the analyzer. The signal was averaged over a spot size of 0.3 mm x 0.7 mm.

Contact angle measurements

Water contact angle measurements were conducted to estimate the wettability of the membranes. Contact angles (CA) were measured with a goniometer (Carl Zeiss Jena, Germany) using the captive bubble method with water as test liquid. The diameter of the contact areas between surface and bubble was always greater than 3 mm. At least 10

measurements of different bubbles, on at least 3 different locations, were averaged to calculate each CA value.

Atomic Force Microscopy

The surface topography was investigated by Atomic Force Microscopy (AFM). For that purpose a multimode AFM (Nano Scope IIIA, Digital Instruments, Santa Barbara, CA, USA) was used in contact mode with a commercial silicon cantilever to analyse the polymer membrane surface immersed in distilled water.

Cell culture

C3A cells (a human hepatoblastoma cell line, supplied by ATCC, USA) were grown in Eagle MEM supplemented with 10% foetal bovine serum (FBS), 1% sodium pyruvat and 10% antibiotic/antimycotic solution (all purchased from Sigma-Aldrich) in a humidified incubator at 37⁰ C in the presence of 5% CO₂. For the experiments membranes were cut into discs of 13 mm diameter, sterilized with 70% ethanol and washed with sterile water. To avoid floating in the medium the membranes were tightly attached to the bottom of the wells using glass rings. The cells were harvested with trypsin/EDTA and suspended in Eagle MEM with 10% FBS. For the experiments they were plated at a density of 5x10⁴ cells per well, using 24 well tissue culture plates (Costar, Corning, NY, USA), containing the membranes.

Immunofluorescence microscopy

C3A cells were cultured for 3 days as described above and than fixed with 3% paraformaldehyde (Sigma-Aldrich) in phosphate buffered saline (PBS, pH 7.4) for 10 min, permeabilized with 0.5% Triton X-100 (Merck) in PBS for 5 min and saturated with 1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 15 min.

To detect focal adhesions, the samples were incubated with monoclonal anti vinculin antibody (Sigma-Aldrich) for 30 min at room temperature, followed by 30 min incubation with Cy² conjugated goat anti-mouse IgG (Jackson Immunoassay Laboratories, West Grove, PA, USA) as secondary antibody.

To visualize cell-cell contacts (adherent junctions), the permeabilized samples were incubated with monoclonal anti E-cadherin antibody (Dianova, Hamburg, Germany) for 30 min and further processed with the same secondary antibody as above. Finally, all the samples were mounted with Mowiol, viewed and photographed with an inverted confocal laser scanning microscope LSM 510 (Zeiss, Germany).

Cell viability and proliferation

To visualize the overall morphology and viability of growing C3A cells they were stained with fluorescein diacetate (FDA; Sigma-Aldrich) after 1 and 3 days of culture. FDA is a non-fluorescent compound that is internalized by living cells and transformed by cellular esterases into a fluorescent product that was shown to correlate well with hepatocytes functionality [22]. For that purpose 5 µl FDA (from 5 mg/ml stock in acetone) were added to each well. After 2 min the samples were washed with PBS and directly viewed and photographed with LSM.

The proliferation of C3A cells was determined with a lactate dehydrogenase (LDH) assay obtained from Roche (Penzberg, Germany). The LDH assay is a colorimetric method originally developed for the quantification of cell death, based on the measurement of LDH activity released from the damaged cells. Here we applied this system to measure the LDH activity after total cell lysis (via addition of 1% Triton X-100) thus quantifying the total amount of cells [23] at different times of incubation, e.g. cell proliferation. The later was assayed after 1, 3 and 5 days of culture. Briefly, after the incubation times indicated, the medium was removed and cells were lysed with 0.5 ml 1% Triton X-100 by shaking the samples for 1 h. The resulting lysates were centrifuged at 2000 g for 5 min

using Biofuge[®] (Heraeus/Kendro, Langensfeld, Germany) and further processed as described in manufacturer's protocol. Finally the absorbance was measured with a Spectra Fluor Plus plate reader (Tecan, Crailsheim, Germany) at 492 nm. All results are presented as optical density (OD). Each experiment was quadruplicated.

The viability of cells indicated by the dehydrogenase enzyme activity found in metabolically active cells during culture was measured with the MTS assay (CellTiter 96[®] AQueous Non-radioactive Cell Proliferation Assay, Promega Mannheim, Germany). This assay utilise a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfohenyl)-2H-tetrazolium, MTS) and an electron coupling reagent (phenazine methosulfate). MTS is chemically reduced by the cells into formazan, which is soluble in tissue culture medium. Thus, the measurements were carried out in supernatants at 492 nm with a Spectra Fluor Plus plate reader. Results are given as OD. Each experiment was quadruplicated.

Fibronectin Synthesis

A standard competitive ELISA assay kit (QuantiMatrix[™] Human Fibronectin, ECM 101, Chemicon, Temecula, CA, USA) was applied to study the amount of fibronectin (FN) secreted from C3A cells into the supernatant. The results received as OD at 450 nm were compared to the standard curve to calculate the corresponding quantities of FN in the samples in ng/cm². All measurements were done in quadruplicates.

Cytochrome P-450 activity

The metabolic function of C3A cells was measured by activity of the cytochrome P-450 isoenzyme CYP1 A2 that is involved in the conversion of 7-ethoxycoumarin. The measurement of 7-ethoxycoumarin-O-deethylation (ECOD) was carried out as described elsewhere [24]. Briefly, C3A cells were grown on the membranes for 1, 3 and 7 days. Then the medium was replaced by culture medium containing 250 nmol/ml 7-

ethoxycoumarin (Sigma-Aldrich), that will be deethylated by the liver cells to 7-hydroxycoumarin. After 24 h the supernatants were collected and deep-frozen immediately. For the measurement, 0.9 ml of thawed supernatants was added to 3.83 ml of ice-cold mixture of isoamyl alcohol and diethylether (1:6 v/v) and the samples were vortexed for 1 min. Then 2.5 ml from the upper phase was removed and added to 1.75 ml 0.2 m glycine/NaOH buffer (pH 10.4) and vortexed again for 1 min. Finally, the fluorescence of the ice-cold aqueous phase was measured at excitation 360 nm and emission 465 nm using Spectra Fluor Plus plate reader. For the quantification, a calibration series with different concentrations of 7-hydroxycoumarin (Sigma-Aldrich) up to 10.5 nmol/ml in PBS was processed in the same way.

Statistical Analysis

Statistical analyses were performed with GraphPad InStat software. The data were first analysed for Gaussian distribution. Significance tests were performed using Student's t-test for the different sets of data. A value of $p < 0.05$ was considered to be statistically significant. All the data are expressed as means with standard deviations.

RESULTS

Membrane characterization

The results of water permeability measurements and cut-off determinations of first trials to control the permeability of the membranes during the lab-scale experiments are given in Table 1. Overall, the membranes from AN-AMPS copolymers with different concentration of the polymer solutions possessed a relatively low water flux, which was gradually reduced with increase of the polymer concentration in the casting solution. The resulting membranes possessed cut-offs less than 40 kDa, which can be considered as insufficient permeability for membranes in biohybrid liver systems as the albumin-bound

toxins cannot permeate. A further reduction of the copolymer concentration during formation was not feasible because of the poor mechanical properties of the resulting membranes. Hence, as an alternative approach, the blending of AN-AMPS copolymer with PAN homopolymer was chosen because membranes made of the latter polymer possessed good mechanical and permeation properties. As shown in Table 1 as well, permeability and cut-off of the membranes could be varied by blending AN-AMPS copolymer with PAN homopolymer one to one and a further reduction of the polymer concentration in the casting solution. These experiments resulted in the obtaining of membranes with the required permeability and mechanical strength. Based on these investigations were selected the preparation conditions for larger scale production of membranes to be used for surface characterization and biological investigations. The preparation conditions and the resulting separation characteristics of these selected membranes are listed in Table 2. The data show that we succeed to obtain the desired variation of separation properties.

The chemical composition of membrane surfaces was studied by XPS. Table 3 shows that the elemental composition of the surface was not equal to the theoretical bulk composition of the polymer blends. Obviously P/A-50/50 had a surface composition, which was very close to that of P/A-0/100. It must be noted that pure poly(acrylonitrile) contained about one percent oxygen, which probably due to some impurities of the sample. It could also indicate an oxidation of a small quantity of nitrile groups during storage of membranes in water. However, the increase of oxygen with addition of increasing quantities of AN-AMPS copolymer goes along with the presence of oxygen in the AMPS monomer. On the other hand the expected increase in sulfur content was not observed. Only 0.3 percent sulfur were measured for all AMPS containing membranes, which might be due to the limited sensitivity of XPS measurements. On the other hand a lower content of the nitrogen content was observed in the blends P/A-75/25 and P/A-50/50 if compared with the theoretical values. This indicates an enrichment of the more hydrophilic AN-AMPS copolymer on the aqueous interface of the membrane surface. The

appearance of a chloride peak in P/A-100/0 and a silicone peak in P/A-50/50 should be also due to some impurities of the membranes.

Results of water contact angle measurements are shown in Table 4. Compared to the moderately wettable PAN (P/A-100/0), the AN-AMPS copolymer P/A-0/100 presented significant increase in the surface wettability, which was evident by the significantly reduced receding water contact angle. A rather unexpected finding was however, that the blend membranes, P/A-75/25 and P/A-50/50, were extremely hydrophilic because advancing and receding water contact angle were 0 °.

Figure 2 summarizes the results from AFM measurements representing the overall surface morphology of a typical scanning area of 5 µm x 5 µm. Obviously membranes with higher AN-AMPS copolymer content (P/A-50/50 and P/A-0/100) were smoother at a first glance. However, if the quantitative roughness values were considered as depicted in Table 5 it became evident that the blends (P/A-75/25 and P/A-0/100) had much higher roughness values if compared to the pure PAN and AN-AMPS polymers.

Adhesive Phenotype of Hepatoblastoma Cells

The adhesive interactions of C3A cells were characterized by the development of focal adhesion complexes, visualized by vinculin, and the formation of adherent cell junctions monitored by E-cadherin staining. It should be noted here, that both structures were studied after three days of culture (Fig. 3) as they were weakly expressed after one day (not shown). An improved adhesive interaction was observed for P/A-75/25 and 50/50 membranes. Typically small vinculin-positive clusters of focal adhesions were visible beneath and at the periphery of cell aggregates, pronouncedly on P/A-50/50 membrane, while these anchorage structures were weaker expressed on pure PAN (P/A-100/0) and particularly AN-AMPS copolymer (P/A-0/100) membranes. Figure 3, right panel, shows cell-cell cohesions visualized by the expression of E-cadherins, which were almost absent in C3A cells plated on pure PAN (P/A-100/0). With increasing the AMPS content in the

polymer they tended to concentrate along the cell-cell borders, again pronouncedly on P/A-50/50 membrane. On pure AN-AMPS copolymer (P/A-0/100) however, C3A cells seemed to lose their adhesive structures because of the rather diffuse distribution of both vinculin and E-cadherins.

Overall Cell Morphology and Viability

The overall morphology of viable C3A cells cultured on different PAN and AN-AMPS copolymer membranes was visualized by FDA staining. Representative pictures are shown in Figure 4. After one day of culture (left panel) the cells on pure PAN tended to attach well, but in small aggregates, apart from the AN-AMPS blend membranes P/A-75/25 and P/A-50/50, where they were randomly dispersed and looked better spread. On pure AN-AMPS (P/A-0/100) however, the C3A cells neither attached nor spread well (the lower picture). The overall morphology of C3A cells underwent considerable changes within three days of culture. The cells obviously grew well on all membranes as their number increased. However their morphology was quite different, particularly between pure PAN and P/A-75/25 from one side, and P/A-50/50 and P/A-0/100, from the other. They tended to grow in aggregates although their overall amount still followed the differences in the initial cell attachment.

The capability of different membranes to support growth of hepatoblastoma cells over the period of 7 days was quantified using LDH assay. As shown in Figure 5 A all the membranes promoted cell growth, as their amounts increased regularly with advancing the incubation time. Significant differences were found between membranes with low AMPS content (P/A-100/0 and P/A-75/25), where the LDH content was higher, compared to those on P/A-50/50 and P/A-0/100. A slightly different finding was made if the MTS assay was applied, which reflects the metabolic activity of cells. Here at day 7, as Figure 5 B demonstrates, the metabolic activity was significantly decreased on the blend P/A-75/25 and P/A-50-50 membranes if compared to pure PAN and AN-AMPS (P/A 0/100) copolymer membranes.

Fibronectin Synthesis

FN is the main adhesive protein in the biological fluids that is normally synthesized in the liver. It was therefore interesting to follow its secretion *in vitro* when C3A cells were in contact with different membranes, thus assuming it reflects also their functional state. As shown in Figure 6, the amount of secreted FN significantly varied between the membranes, and these differences were particularly pronounced in the later cultures (7 days), when significantly greater quantities of FN were quantified on P/A-50/50 and P/A-0/100 membranes, but without significant difference between each other.

ECOD Assay

The functional behaviour of cells on the membranes was further compared measuring their detoxifying activity by ECOD assay (Fig. 7). The measurements showed that this activity of C3A cells was significantly higher after one day of cultivation on the homo- and pure copolymer membranes, P/A-100/0 and P/A-0/100, respectively, if compared to the other two blend membranes. In the course of cultivation up to 7 days the activity was increasing on all membranes. At days 3 and 7 however, no significant differences in ECOD activity could be detected between the membranes.

DISCUSSION

Recently we developed a number of polymer membranes based on PAN copolymerized with different hydrophilising co-monomers representing improved surface properties for culture of cells [13, 14, 17, 18, 20, 21]. In one of the papers we discovered that polymerization of acrylonitrile with AMPS resulted in a copolymer, which specifically supported hepatocyte function and survival [18]. However, this copolymer had poor

membrane forming properties resulting in quite dense membranes with rubber-like mechanical properties. Hence, a major task of this work was the development of AMPS containing membranes with tailored separation profile and mechanical properties that is combined with a sufficient compatibility for hepatocytes. It was found that blending of AN-AMPS copolymer with PAN is a feasible approach to obtain membranes with optimized mechanical stability and desired transport properties that fit to the requirements of biohybrid organs [1, 2, 4].

In fact, the permeability of membranes could be adjusted by the ratio between PAN homo-polymer and the AN-AMPS copolymer in the blend, as well as, by varying their concentration in the casting solution. Specifically, blending of PAN with AN-AMPS copolymer was performed at ratios 75/25 and 50/50 percent. If the surface composition of these blends was analyzed by XPS survey scans and high resolution spectra, an enrichment of the more hydrophilic AMPS component on the membrane surface was indicated. Such enrichment can occur during the membrane formation due to the precipitation of the polymer solution in contact with water as consequence of the different water tolerance. Phase separation of the more hydrophilic copolymer requires a higher water fraction in the ternary system – non-solvent/water/polymer - than for the moderately hydrophilic PAN homopolymer. Insofar the rate of phase demixing is delayed resulting in an enrichment of the hydrophilic blend component on the surface of the polymer-rich phase forming the pore walls [26]. Therefore the copolymer with charged sulfonate groups may be enriched at the water-polymer interface during the phase separation. Similar observations were made with blend systems from polyacrylonitrile and polyvinylpyrrolidone [27], polyimides and polybenzimidazole [28], or in the addition of so-called surface-modifying macromolecules [29]. Supporting evidence for an enrichment of the hydrophilic AN-AMPS copolymer came also from AFM measurements showing that blend membranes had a higher surface roughness than the pure PAN (P/A-100/0) and AN-AMPS (P/A-0/100) membranes. A non-homogenous distribution of the hydrophilic AN-AMPS copolymer in the blend might be the reason for this. It is

noteworthy that similar observations were made for other polymer systems showing that a demixing of polymers in the blend leads to surface structures in the nanometer scale [30]. This effect can explain also the extreme wettability of the blend membranes that probably affected their biological properties in comparison to the pure PAN or AN-AMPS. In addition, the surface roughness and porosity of membranes was also increased in the blends, which may contribute to a stronger wetting behaviour [31].

The biological experiments were aimed to confirm the assumption that blends with AMPS are suitable for hepatocyte interaction that was in line with our previous study [18]. The investigations however, were further extended to learn if the biological response of hepatoblastoma cells can be controlled to some extent by the quantity of AN-AMPS in the blend. If all the biological results are summarized no simple correlations between cell morphology, growth, and functional activity may be concluded. In general, we found that the plating efficiency visualized by FDA after 24 hours did not differ significantly between the membranes except on pure AMPS where the cells tended to detach. However, a clear tendency for an improved cell spreading on the blended P/A-75/25 and P/A-50/50 membranes was observed. It was also found that spreading of cells on the blend membranes was accompanied by a stronger expression of focal adhesion complexes as shown by vinculin staining. Surprisingly E-cadherins were also well organized on these samples. Thus, the higher hydrophilicity and roughness seemed to affect positively the initial cellular and intercellular interaction, which is in line with our previous and also other studies [11, 12, 19, 20]. If good adhesion and spreading of cells is considered as prerequisite for subsequent cell growth [32] than different results were obtained. At the 7th day the best cell growth was observed on P/A-100/0 followed by P/A-75/25, as indicated by LDH assay. The FDA staining also supports this finding although more cells could be seen on all membranes, moreover representing flattened morphology. A smaller number of cells were detected on P/A-0/100 with a more round phenotype suggesting diminished cell-substratum interaction. However, the expression and organization of E-

cadherins was better on the blends, particularly on P/A-50/50, i.e. the same membrane where the development of focal adhesion complexes was superior.

Since cell-cell contacts are important for the functioning of hepatocytes [33] one would expect a maximal metabolic activity of cells on P/A-50/50. However, when measured by MTS assay the later was significantly greater at day 7 on the homopolymer membranes P/A-100/0 and P/A-0/100, but not on the blended membranes, although the anticipated number of cells as shown by FDA at day 3 and from LDH assay were not corresponding. If one keeps in mind that cell numbers were relatively high on P/A-100/0, the higher metabolic activity there can be explained easily. On P/A-0/100 however, the cell number was lower, which indicates that AMPS rather promotes the metabolic activity of C3A cells. On the other hand, when the functional activity of hepatoblastoma cells in terms of their ability to secrete FN was measured it was superior on the membranes with higher AMPS content, highest on P/A-50/50 and followed by P/A-0/100. It is noteworthy, that FN is the main adhesive glycoprotein in the biological fluids and it is particularly important for the interaction of cells with foreign materials [32-34]. Thus secreted FN might be responsible for an improved interaction of cells, i.e. as more FN is secreted the better interaction of cells might be expected later on such a membrane. In this respect, it can be stated that the blend P/A-50/50 provides sufficient conditions for hepatocyte functionality assessed by both P-450 activity and FN secretion.

In summary, one can conclude that membranes made of P/A-50/50 represent the desired transport properties combined with a promoting effect on the cellular functional activity. In line with our previous findings [18] and literature evidences for a good blood compatibility of AMPS [25], our results suggest that this particular membrane is extremely promising as cellular support in biohybrid liver systems.

ACKNOWLEDGEMENT

The authors thank Dr. Andreas Holländer from Fraunhofer Institute for Applied Polymer Research in Potsdam-Golm for XPS characterization of membranes.

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Figure Captions

Figure 1: Chemical structure formulas of acrylonitrile (AN) monomer and the anionic co-monomer 2-acrylamido-2-methyl-propane sulfonic acid (AMPS)

Figure 2: AFM images of the overall surface morphology of a typical scanning area of 5 μm x 5 μm .

Figure 3: Formation of focal adhesion complexes in C3A cells visualized by vinculin and of adherent cell junctions visualized by E-cadherin staining after 3 days of culture. Size of the scale bar: 50 μm

Figure 4: The viability and growth of C3A cells cultured on different polymer membranes visualized by FDA staining after 3 days of culture. Size of the scale bar: 100 μm

Figure 5: The capability of different membranes to support growth of hepatoblastoma cells over the period of 7 days quantified using LDH (A) and MTS (B) assays

Figure 6: Secretion of fibronectin by C3A cells *in vitro* in contact with the different membranes quantified by QuantiMatrix assay

Figure 7: Activity of 7-ethoxycoumarin-O-deethylation by C3A cells *in vitro* in contact with the different membranes quantified by ECOD assay

Table 1: Characteristic data of the separation ability of AN-AMPS copolymer (CP) and PAN/AN-AMPS blend membranes

<i>Membrane composition</i>	<i>Polymer concentration in casting solution</i> [wt.%]	<i>Separation ability</i>	
		Water permeability [$\text{lm}^{-2}\text{h}^{-1}\text{kPa}^{-1}$]	Cut-off [kDa]
AN-AMPS ¹⁾ -CP	22.5	0.01	7
AN-AMPS ¹⁾ -CP	20	0.08	19
AN-AMPS ¹⁾ -CP	17.5	0.16	23
AN-AMPS ¹⁾ -CP	15	0.22	40

1:1 blend	15	0.7	40
1:1 blend	13	1.1	320
1:1 blend	12	1.6	270
1:1 blend	10	3.2	470

¹⁾ AN-AMPS - poly(2-acrylonitrile-co-acrylamido-2-methyl propane sulfonic acid)

Table 2: Preparation conditions of the investigated flat membranes and their characteristic separation data

Membrane identifier	Designation	Preparation conditions				Membrane properties	
		Non-woven	C _{polymer} [wt.%]	Preparation rate [mm ⁻¹]	Annealing temp. [°C]	Water permeability [lm ⁻² h ⁻¹ kPa ⁻¹]	Cut-off [kDa]
P/A-100/0	Pure PAN ¹⁾	+	15	4	90	5.2	1200
P/A-75/25	3+1 blend	-	10	2	50	2.2	590
P/A-50/50	1+1 blend	-	10	2	50	0.85	220
P/A-0/100	Pure AN-AMPS ²⁾ -CP	+	15	4	50	0.16	40

¹⁾ poly(acrylonitrile)

²⁾ poly(acrylonitrile-co-2-acrylamido-2-methyl propane sulfonic acid)

The membrane identifier includes the ratio of poly(acrylonitrile) (P) and poly(2-acrylonitrile-co-acrylamido-2-methyl propane sulfonic acid) (A) on a weight basis, e.g. P/A-75/25 is a polymer blend with 75 wt.% poly(acrylonitrile) and 25 wt.% poly(acrylonitrile-co-2-acrylamido-2-methyl propane sulfonic acid).

Table 3: Actual and theoretical composition of elements (atom-% in polymer) in the surface of the investigated membranes examined by XPS

<i>Membrane identifier</i>	<i>Elemental composition (atom-% in polymer)</i>									
	<i>actual</i>						<i>theoretical</i>			
	C	N	O	S	Si	Cl	C	N	O	S
P/A-100/0	75,2	23,2	1,4			0,2	75,00	25,00		
P/A-75/25	75,4	22,8	1,5	0,3			74,45	24,63	0,74	0,18
P/A-50/50	76,0	19,9	3,4	0,3	0,3		73,91	24,27	1,46	0,36
P/A-0/100	75,2	21,2	3,2	0,3			72,94	23,63	2,74	0,69

Table 4: Water contact angles of investigated flat membranes measured by captive bubble method

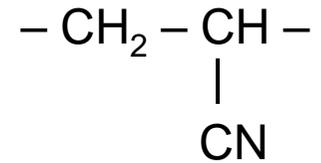
<i>Membrane identifier</i>	<i>Contact angle (°)</i>	
	<i>advancing</i>	<i>receding</i>
P/A-100/0	59,5 ± 3,1	43,0 ± 2,9
P/A-75/25	0	0
P/A-50/50	0	0
P/A-0/100	47,3 ± 1,7	13,8 ± 1,9

Table 5: Quantitative mean roughness (Ra) values of membrane surfaces estimated by AFM measurements

	<i>Ra in nm</i>	
<i>Membrane identifier</i>	<i>Scanning area</i>	
	<i>25 μm</i>	<i>50 μm</i>
P/A-100/0	16,6 \pm 2,6	33,7 \pm 5,5
P/A-75/25	69,6 \pm 46,5	139,5 \pm 108,4
P/A-50/50	28,4 \pm 18,1	72,4 \pm 53,1
P/A-0/100	4,9 \pm 4,6	8,6 \pm 8,0

Monomers

AN – acrylonitrile



AMPS – 2-acrylamido-2-methyl-1-propane sulfonic acid

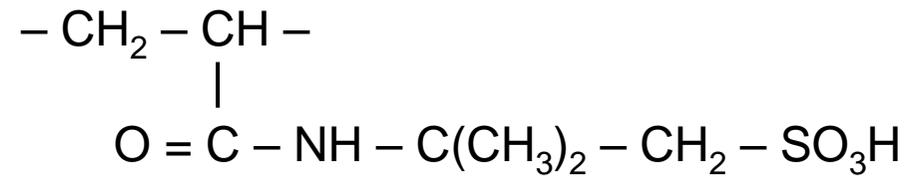


Figure 1

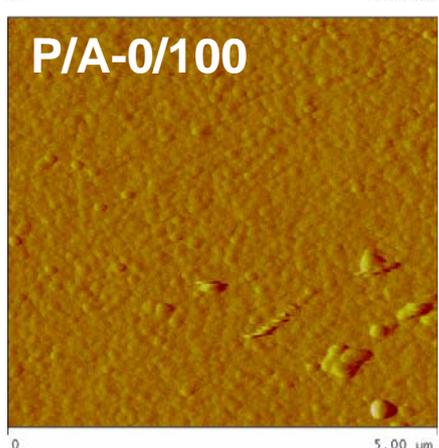
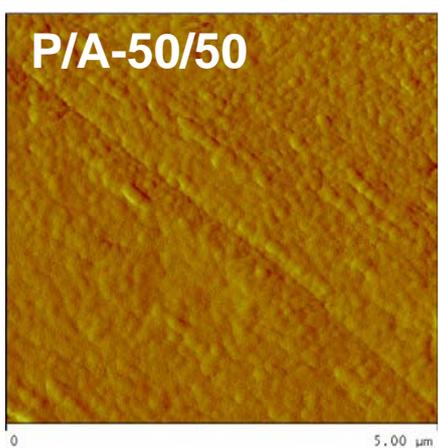
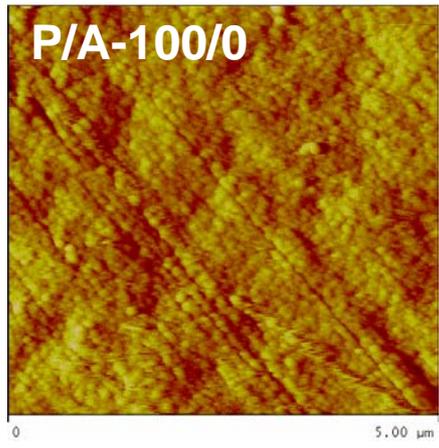
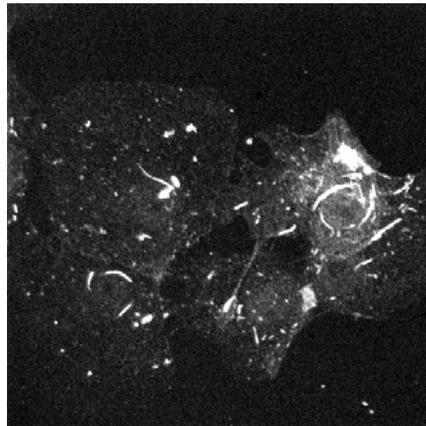
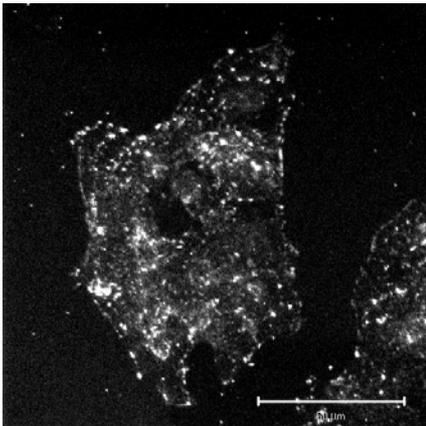


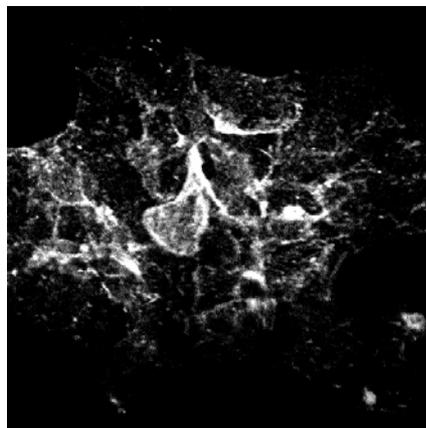
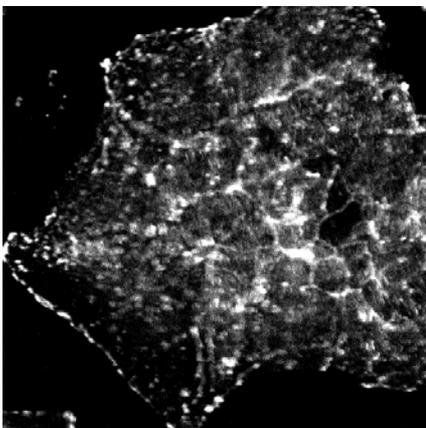
Figure 2

Vinculin

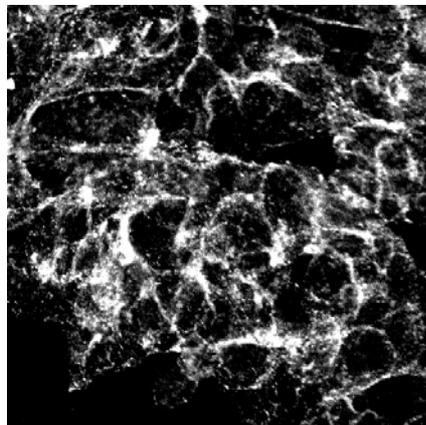
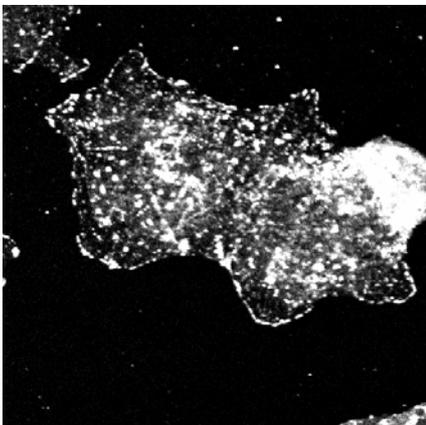
E-cadherin



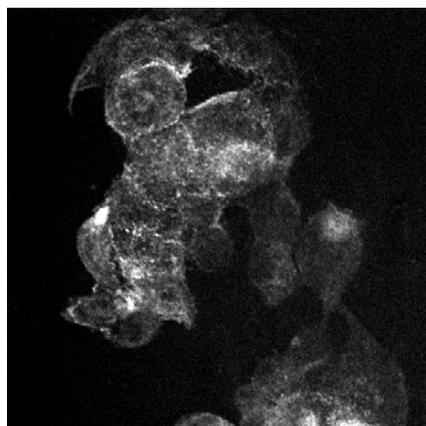
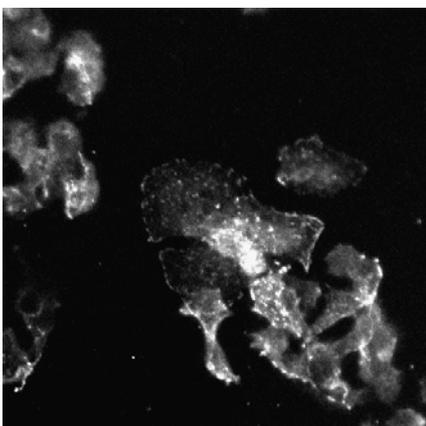
P/A-100/0



P/A-75/25



P/A-50/50



P/A-0/100

Figure 3

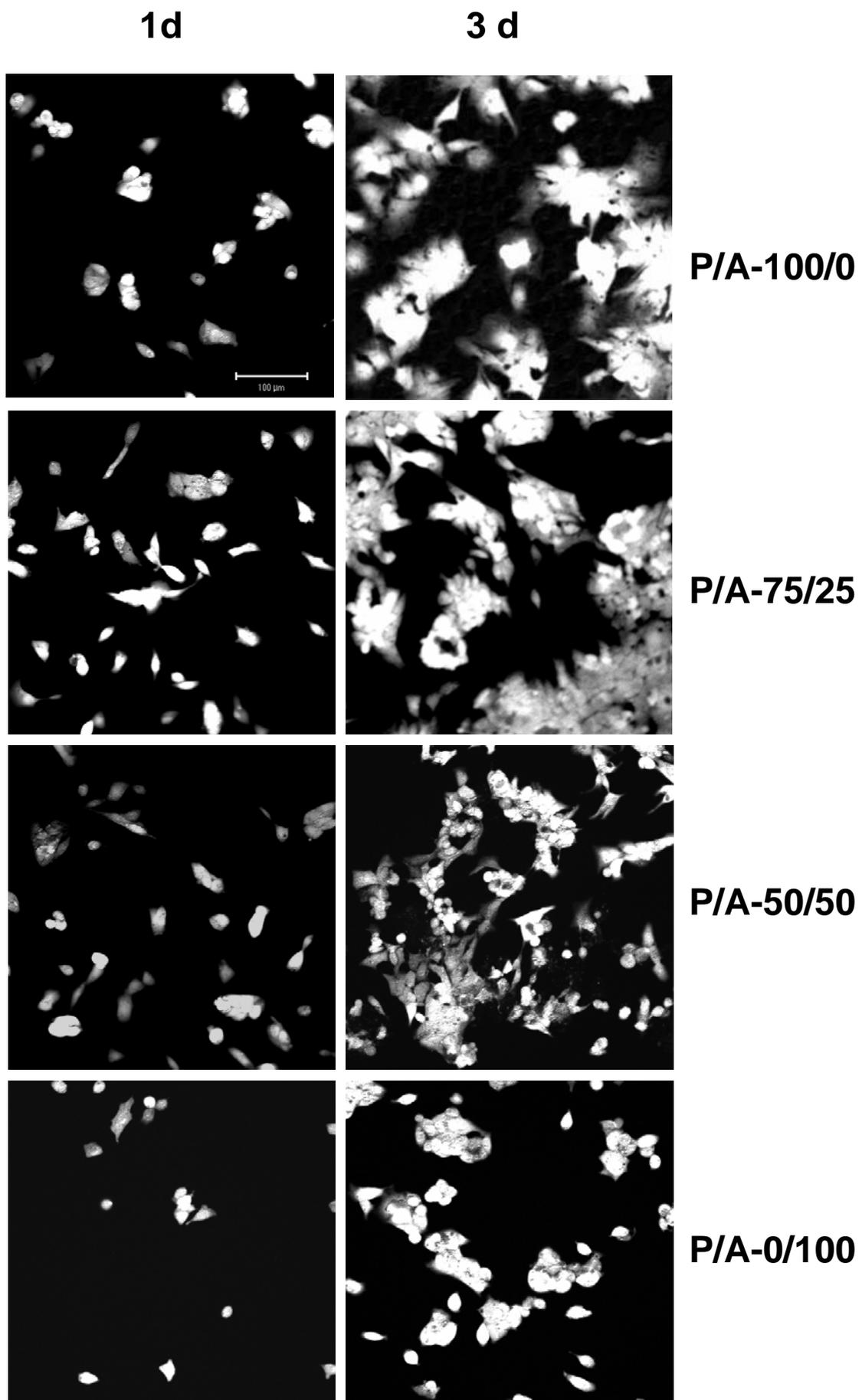
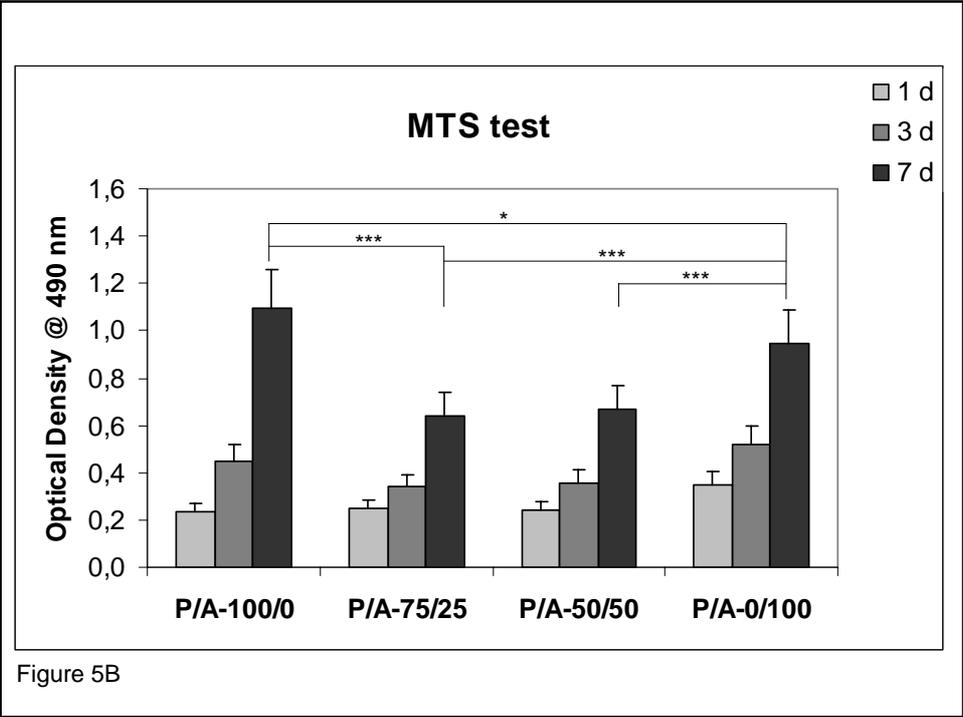
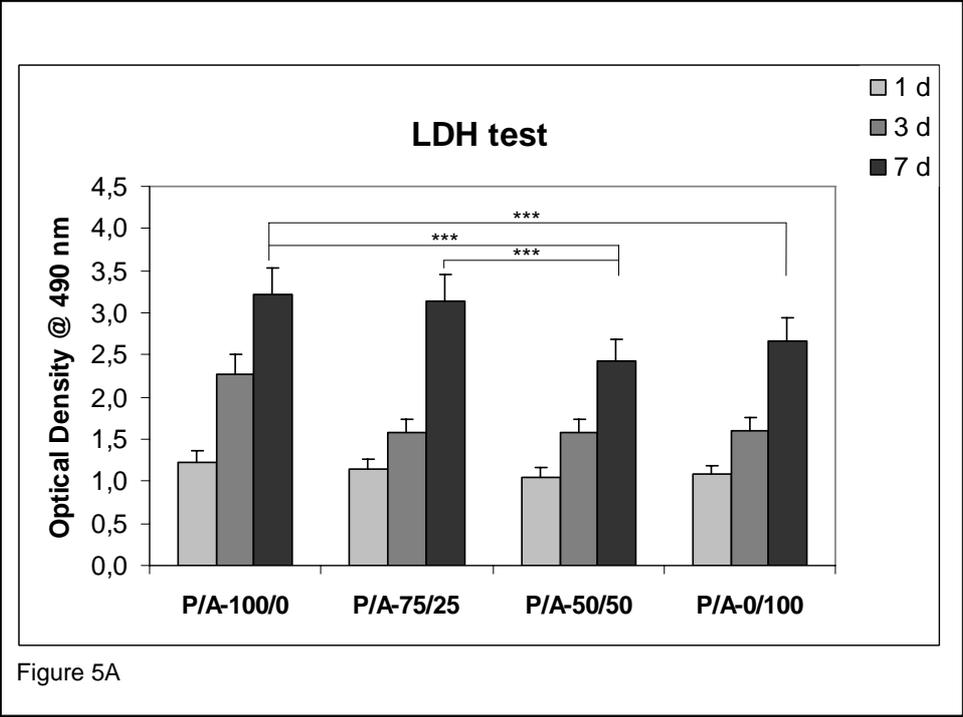


Figure 4



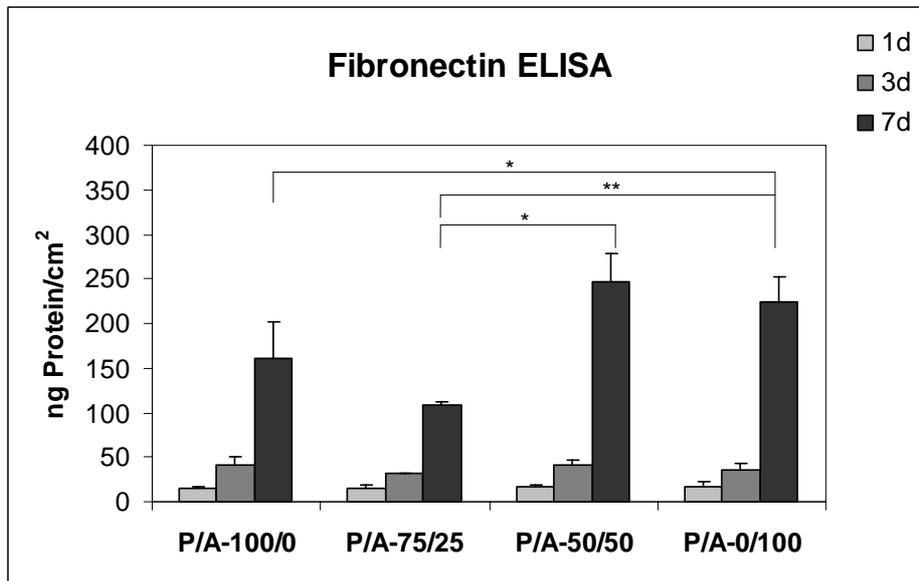


Figure 6

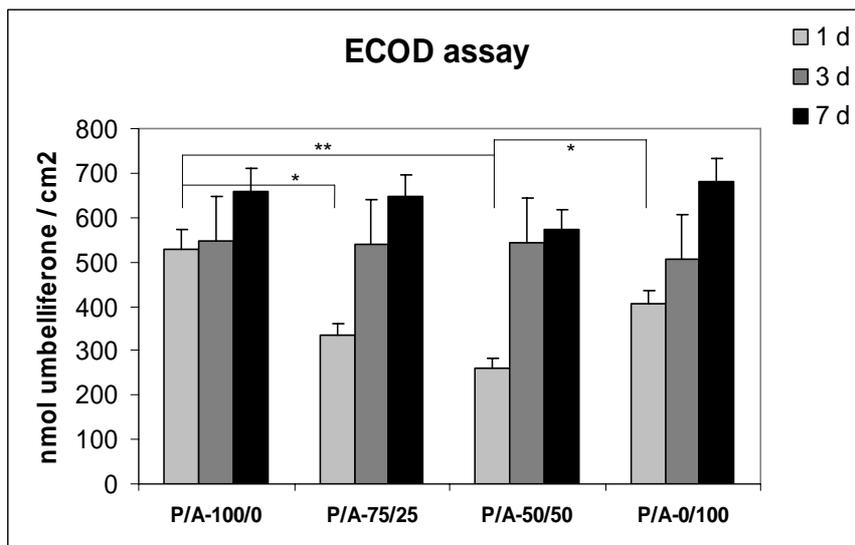


Figure 7