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Improved functions of human hepatocytes on NH_3 plasma-grafted PEEK-WC–PU membranes

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ABSTRACT

PEEK-WC–PU membranes were modified with an NH_3 glow discharge process to graft N-containing functional groups at their surface in order to improve the maintenance of human hepatocytes. Native and modified membrane surfaces were characterized with XPS, ToF-SIMS and WCA measurements. We have investigated morphological behaviour and specific functions of primary human hepatocytes on native and modified PEEK-WC–PU membranes in a small-scale gas-permeable bioreactor. N-containing groups grafted at the surface of the membranes improved the initial steps of adhesion and the maintenance of phenotype and differentiated functions of cells. Confocal microscopy of cell morphology evidenced human hepatocytes exhibiting a polygonal shape and organizing a 3D structure. The presence of CK19 positive cells, a marker of biliary duct epithelium, was also found on native and modified membranes. Liver specific functions, investigated in terms of urea production, albumin synthesis and diazepam biotransformation, were maintained at high levels up to 19 days, particularly on surface-modified membranes.

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1. Introduction

Biomaterials in tissue engineering and regenerative medicine should provide the necessary support for cell proliferation and maintenance of their differentiated functions. It is well known that cells interact *in vivo* among each other and with their microenvironment, e.g., with the proteins of the extracellular matrix (ECM), through receptors present over their membrane. This communication integrates and coordinates the various gene expression patterns that are crucial for tissue function and homeostasis. Various natural and synthetic materials have been used for culture of anchorage-dependent cells such as hepatocytes. Natural materials including components purified by ECM such as proteoglycan, fibronectin, laminin and collagens have been proposed as substrates for hepatocyte adhesion [1–3]. These materials have the advantages to favour interactions with cells through the integrins, but on the other hand their availability is limited, their costs are high and their composition is variable from batch to batch.

Synthetic polymeric materials are attractive because of their reproducible composition and their well defined and characterized nano and micro-structure. Polymeric semipermeable membranes with different physico-chemical and transport properties are appealing in tissue engineering and bioartificial organs since they share similarities with biomembranes, such as selective molecules transport, resistances and protection [4]. Furthermore, synthetic membranes can easily be mass produced with modulated morphological and physico-chemical properties for specific applications. We have developed a membrane from a polymeric blend of modified polyetheretherketone (PEEK-WC) and polyurethane (PU) as support for hepatocyte culture [5]. This membrane combines advantageous properties of both polymers (biocompatibility, thermal and mechanical resistance, elasticity) with those of membranes (permeability, selectivity and well defined geometry). Human hepatocytes cultured on such PEEK-WC–PU membranes showed adhesion efficiency comparable with that of cells cultured on natural substrates such as collagen [5].

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For this study we have modified PEEK-WC–PU membranes with an NH_3 glow discharge plasma aiming to graft nitrogenated functionalities at their surface, in order to investigate whether the maintenance of differentiated functions of human hepatocytes could be enhanced. Among several surface modification strategies, grafting of N-containing functional groups allows to increase the polarity of the surface and to have chemical groups typical also of proteins that could support cell adhesion and functions.

Low-temperature plasma modification (etching, deposition and grafting) processes are widely used to modify the surface of biomaterials, including membranes, with tunable density of surface functional groups, without altering their bulk [6,7]. Plasma treatments (grafting) with NH_3 or N_2 feeds and plasma deposition processes with N-containing monomer feeds (e.g., allylamine), can provide polymer surfaces with N-containing functional groups, whose distribution and density can be tuned with the plasma parameters, and depend also on ageing processes (hydrophobic recovery, surface oxidation, etc.). The surface density of $-NH_2$ groups plasma-grafted on polyethylene, for example, can be tuned among all other N-groups by changing NH_3/H_2 feed ratio, power input, sample position (glow-afterglow) and process duration [8]. Polar O- and N-groups generated on polymer surfaces are pursued to confer and improve "biological activity" and cell adhesion to materials [9–15].

We have plasma-grafted N-groups at the surface of PEEK-WC–PU membranes to improve adhesion and functions of human hepatocytes. Cell morphology and adhesion of hepatocytes were investigated on native and modified PEEK-WC–PU membranes, as well as cell phenotype and liver functions relevant in the case of organ replacement or regeneration.

2. Materials and methods

2.1. Membrane preparation

Native membranes were prepared from a blend of modified PEEK-WC (patented by Zhang et al. [16] and provided by the Institute of Applied Chemistry, Changchun, China) i.e., poly(oxa-1,4-phenylene-oxo-1,4-phenylene-oxa-1,4-phenylene-3,3-(isobenzofurane-1,3-dihydro-1-oxo)-diyl-1,4-phenylene) and PU by means of the inverse phase technique by using the direct immersion-precipitation method as previously described [5]. The PEEK-WC is obtained by polycondensation reaction between 4,4-dichlorobenzophenone and phenolphthalein [16].

2.2. Membrane modification

2.2.1. Plasma processes

Plasma pre-treatments with H_2 and treatments with NH_3 processes were performed in a pyrex plasma reactor, described in detail elsewhere [17], equipped with two internal steel electrodes in parallel plate configuration. The H_2 pre-treatment was found efficient to slow down the ageing of the modified membranes after the N-grafting process, as it will be explained in the next. Discharges were ignited between the radiofrequency (RF, 13.56 MHz; ENI-ACG-10 generator, and impedance matching network) driven upper electrode and the flat lower internal electrode, 7 cm far, ground, used as substrate holder. H_2 generated with a HG200 Claind Hydrogen Generator and 99.999 % purity NH_3 (Air Liquide) was fed trough electronic MKS mass flowmeters. A rotative pump was used to keep the pressure constant during the processes (base pressure 10^{-3} Torr); the pressure was controlled with an MKS baratron. The two plasma processes were performed in sequence, without opening the reactor in between, in the following experimental conditions: pre-treatment: 10 sccm H_2 flow rate; 200 m Torr pressure; 30 W power; 1 min; treatment 10 sccm NH_3 flow rate; 200 m Torr pressure; 20 W power; 1 min.

Plasma parameters of both processes were optimized in order to maximize the grafting extent of N-groups, measured by the N/C ratio of the modified PEEK-WC–PU surface, and to slow down the hydrophobic recovery of the modified polymer and retain with time the surface properties gained [17]. Soon after the plasma modification, processed membranes were stored in polystyrene boxes and used for cell culture experiments within 8 days.

2.2.2. Surface characterization

The surface composition of the membranes was examined within 1 h after the plasma process and 7–15 days after by means of X-ray photoelectron spectroscopy (XPS), in order to evaluate their ageing. A Thermo VG Scientific XPS instrument (monochromatic AlKα X-rays source; 1486.6 eV, 100 W, 400 μm spot size) was used, at a take-off angle of 53° with respect to the normal to the sample surface (sampling depth 6 ± 2 nm). Survey (0–1100 eV Binding Energy, BE) and high-resolution spectra (C1s, N1s, O1s) were recorded. Error bars resulted from measurements performed on 3–5 different spots on the same substrate, on 3–5 substrates of the same kind. C1s spectra were best-fitted with 6 components: C0 (BE 285.0 ± 0.2 eV, reference; C–C, C–H); C1 (BE 286.4 ± 0.2 eV; C–O–C, C–OH, C–N); C2 (BE 287.6 ± 0.2 eV; C═O, O–C–O, N–C═O); C3 (BE 289.2 ± 0.2 eV; COOH, COOR, O–CONH); C4 (BE 289.9 ± 0.2 eV; shake up, aromatic structures) and C5 (BE 291.8 ± 0.2 eV; shake up, C═O).

Sessile drop water contact angle (WCA) measurements were performed in static mode with a CAM 200 contact angle instrument (KSV Instruments LTD, Helsinki, Finland) equipped with a photocamera. 2 ml drops of double distilled water were used. WCA values were measured within 1 h after the surface modification process, and many times during 2–3 weeks. The CAM software of the instrument was utilized to fit the shape of the drop and determine the WCA tangent with the Young–Laplace equation. Error bars resulted from measurements performed on 3–5 different spots per substrate, on 2–4 substrates of the same kind.

ToF-SIMS analysis was performed with an ION-TOF (IV) ToF-SIMS system equipped with a 25 keV cluster metal ion source operating with Bi^{3+} primary ions. Spectral analyses have been obtained from $250 \times 250\ \mu m^2$ spots in high mass resolution burst mode (resolution $M/\Delta M > 6000$). The total ion beam dose was limited to less than 1×10^{12} ions/cm^2, within the static SIMS regime.

2.3. Human hepatocytes culture

Primary human hepatocytes (Lonza Sales Ltd, Basel, Switzerland) isolated from non-transplantable tissue of young single donors were used for cell culture experiments. The purity of isolated hepatocytes is 95% and nonparenchymal cells are present in a very low percentage (5%). Cryopreserved human hepatocytes were quickly thawed in a 37 °C water bath with gentle shaking. Then, the cell suspension was transferred slowly into a tube containing 30 ml of cold hepatocyte culture medium (HCM™, Lonza Sales Ltd, Basel, Switzerland), and centrifuged at 50g at 4 °C for 5 min. The HCM™ is constituted of hepatocyte basal medium (HBM™, Lonza Sales Ltd, Basel, Switzerland) together with all the components provided in HCM™ bulletkit® (Lonza Sales Ltd, Basel, Switzerland): epidermal growth factors, insulin, ascorbic acid, transferrin, hydrocortisone 21-hemosuccinate, bovine serum albumin–fat acid free 2% (BSA–FAF) and gentamicin sulphate 50 μg/ml amphotericin B 50 ng/ml. The cell pellet was suspended in HCM™ and tested for the cell viability by Trypan blue exclusion.

Human hepatocytes were seeded at a final concentration of 2.5×10^5 cells/cm^2 on native and plasma-grafted PEEK-WC–PU membranes previously conditioned with hepatocyte culture medium supplemented with bovine serum albumin–fat acid free 2% (BSA–FAF), in a small-scale gas-permeable bioreactor system [18]. Native and modified membranes were located in tight contact with the gas-permeable membrane of the bioreactor, to ensure optimal transfer of CO_2, O_2 and H_2O vapour in the culture chamber. Cells were incubated at 37 °C in a 5% CO_2; 20% O_2 atmosphere (v/v) with 95% relative humidity in hepatocyte culture medium containing 2% BSA–FAF for the first 24 h, thereafter under serum-free conditions for the total culture time. Experiments were performed in the presence of diazepam 10 μg/ml in the culture medium to evaluate the ability of cells to perform drug biotransformation functions, in particular the elimination of diazepam and the formation of its metabolites.

The morphology of the cells cultured on native and modified membranes was assessed by means of Scanning Electron Microscopy (SEM) and Laser Confocal Scanning Microscopy (LCSM).

Liver specific cellular functions were investigated in terms of albumin production and urea synthesis.

2.4. Hepatocyte fixation for SEM

Cells cultured for 19 days on native and modified membranes were prepared for SEM analysis by fixation in 3% glutaraldehyde and 1% formaldehyde in PBS, followed by post-fixation in 1% osmium tetroxide and progressive ethanol dehydration.

2.5. Hepatocyte staining for LCSM

The morphological behaviour of human hepatocytes cultured on native and modified membranes was investigated at 7, 13 and 19 days of culture with Laser Confocal Scanning Microscopy (LCSM) after cytoskeleton and ECM protein immunostaining. Samples were rinsed with PBS, fixed for 15 min in 3% paraformaldehyde in PBS at room temperature (RT), permeabilized for 5 min with 0.5 % Triton-X100 and saturated for 15 min with 2% Normal Donkey Serum (NDS).

To visualize vinculin, a mouse monoclonal antibody raised against human vinculin (Santa Cruz Biotechnology, Santa Cruz, CA) and a Cy™3-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, Cambridge, UK) was used. Actin was stained with phalloidin Alexa 488 conjugated (Molecular Probes, Inc, Eugene, OR). To visualize laminin a rabbit polyclonal antibody raised against laminin α-4 of human origin (Santa Cruz Biotechnology, Santa Cruz, CA) and a Cy™2-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Europe Ltd,

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