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A hydrophobic perfluoropolyether elastomer as a patternable biomaterial for cell culture and tissue engineering

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ABSTRACT

We present a systematic study of a perfluoropolyether (PFPE)-based elastomer as a new biomaterial. Besides its excellent long-term stability and inertness, PFPE can be decorated with topographical surface structures by replica molding. Micrometer-sized pillar structures led to considerably different cell morphology of fibroblasts. Although PFPE is a very hydrophobic material we could show that PFPE substrates allow cell adhesion and spreading of primary human fibroblasts (HDF) very similar to that observed on standard cell culture substrates. Less advanced cell spreading was observed for L929 (murine fibroblast cell line) cells during the first 5 h in culture which was accompanied by retarded recruitment of $\alpha_v\beta_3$-integrin into focal adhesions (FAs). After 24 h distinct FAs were evident also in L929 cells on PFPE. Furthermore, organization of soluble FN into a fibrillar ECM network was shown for hdF and L929 cells.

Based on these results PFPE is believed to be a suitable substrate for several biological applications. On the one hand it is an ideal cell culture substrate for fundamental research of substrate-independent adhesion signaling due to its different characteristics (e.g. wettability, elasticity) compared to glass or TCPS. On the other hand it could be a promising implant material, especially due to its straightforward patternability, which is a tool to direct cell growth and differentiation.

1. Introduction

Perfluoropolyethers (PFPEs) are characterized amongst other things by chemical inertness, solvent and high temperature resistance, low friction coefficient, hydrophobicity, lipophobicity and in particular very low surface energy [1]. Due to these diverse characteristics, the scope of possible applications for PFPE is very broad. Besides the well known usage of PFPE fluids as lubricants, they can be employed as moldable materials in soft lithography [2–5] or in microfluidics [6]. In the biomedical field a PFPE-based material has so far only been used as a substrate for the fabrication of corneal implants [7–9]. Biocompatibility and long-term biostability have been shown during animal tests and Phase 1 clinical trials [10].

Nevertheless, in some cases in order to support cell growth the surface wettability of the applied PFPE material was purposely increased, e.g. by copolymerizing a zwitterionic monomer and PFPE macromonomer [11].

Other fluoropolymers such as polyvinylidene fluoride (PVDF) or polytetrafluoroethylene (PTFE, Teflon®) are already commonly used in clinical applications (e.g. suture material, vein grafts). PVDF is a commercially available, partially fluorinated homopolymer and shows excellent biocompatibility for many cell types [12–14]. While PVDF is a moderately hydrophobic material, with a water contact angle of approximately 81°, PFPE with a water contact angle of 110 ± 2° displays a much higher hydrophobicity.

This high water contact angle and the low surface energy of the PFPE-based material are due to the high fluorine content of the perfluorinated polymer: the chemical groups at the surface constitute of –CF2– and –O–, which are presumably not or much less available for H-bonding than surface chemical groups in hydrogenated analogues. Hence, surface chemistry and wettability are interconnected properties because it is the hydrogen bonding of water to surface functional groups that most profoundly influences wettability (see, as examples Refs. [15,16]).

Generally the *in vivo* biocompatibility and *in vitro* cytocompatibility of a material is dependent on the amount, type and accessibility of adsorbed proteins, the so-called “adsorption profile”. Based on this adsorption profile cell adhesion and the subsequent cellular processes are taking place [17,18].

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Protein adsorption is a highly complex process which is determined by the material surface properties and the relative protein concentrations in the solution. After an initial, fast, rather concentration-dependent adsorption, the adsorption rate decreases in relation to the number of available binding sites becoming progressively more dependent on the protein–surface affinity; the protein layer is subject to competitive exchange and reorganization [19,20]. Surface properties such as the surface wettability as well as the display of functional, polar and charged groups are key mediators of the protein–material affinity (see Ref. [21] for review).

On less wettable materials, the hydrophobic interactions are generally stronger, which often leads to a significant change in the protein conformation (secondary structure rearrangement) on the surface, which can be considered as denaturation of the protein. The appropriate availability of specific binding sites of adhesion-mediating proteins such as fibronectin (FN) and vitronectin (VN) for the cells' surface receptors (integrins) can thereby be negatively affected [22,23]. For example, it was shown that, whereas the amount of FN adsorbed to substrates displaying different chemical groups at their surfaces (self-assembled monolayers, SAM) surfaces followed the trend $NH_3^+ > CH_3 > COO^- > OH$, on the latter surface the protein retained the highest binding activity while the CH_3-terminated SAM surface induced the greatest degree of conformational change [22,24]. Consequently, the following trend of cell adhesion (FA assembly and $\alpha_5\beta_1$ expression): $OH > NH_2 = COOH > CH_3$ was found [25]. In addition, on more hydrophobic surfaces the most abundant serum protein albumin and other non-adhesive proteins are less frequently displaced by adhesion-mediating proteins (e.g.VN or FN) due to irreversible adsorption [26,27]. As a consequence cell adhesion and spreading is often observed to be delayed and less pronounced on hydrophobic materials [28]. Nevertheless, certain cell types, such as macrophages, are predominantly cultured on very hydrophobic substrates (e.g. Teflon®) as those materials prevent inflammatory activation of the cells [29].

We present here the systematic study of a pure PFPE-based elastomeric material for cell culture applications. Substrates made out of the PFPE-based biomaterial are prepared by UV-initiated radical crosslinking via methacrylate end-groups, which results in densely crosslinked bulk material. Very smooth PFPE substrates are prepared by casting the prepolymer against a smooth (silicon) surface. Unlike the non-deformable, stiff cell culture substrate TCPS (tissue culture polystyrene), PFPE is an elastomeric material with a Young's modulus of ~2 MPa [30]. As correlations between substrate stiffness and cell reactions have only been found in the Pa to kPa range we assume that the elasticity difference of PFPE compared to TCPS has no significant effect on cell adhesion and growth [31,32].

Another aspect with extremely high relevance for cell–biomaterial interactions in general and therefore essential for the successful design of tissue engineering constructs, is the material's surface topography. It has been known for almost 100 years that cells respond to topographic cues [33]. Not only cell morphology, size and migration can be determined by substrate nano or micro-topography, also proliferation, the protein expression profile and even differentiation of stem cells were shown to directly correlate with the displayed surface structures [34,35]. In addition, we have recently shown that μm-sized patterns can induce cell adhesion to intrinsically non-adhesive substrates [36]. Eventually, we have also shown that the inflammatory response of primary human macrophages could be altered by defined micrometer surface structures [37].

By virtue of fabricating PFPE substrate by crosslinking of liquid prepolymers the material is easily patternable. Topographic surface structures can be introduced by soft lithography techniques such as (nano)imprinting or replica molding. Transfer of patterns from structured silicon masters to PFPE samples can be reproduced down to nanometer sizes [3,38,39]. The possibility of straightforward patterning is a unique advantage of PFPE, especially compared to other fluoropolymers such as PVDF and PTFE.

The aim of this study is to show the great potential of PFPE as a new material for several biological applications. On the one hand we test its applicability as an *in vitro* cell culture material in comparison with standard cell culture substrates such as TCPS. On the other hand we analyze it with regards to possible biomedical applications by comparing PFPE to an already established fluor-containing biomaterial, PVDF.

A systematic study of the *in vitro* cytocompatibility of the PFPE-based substrates is presented. Cytocompatibility generally comprises non-cytotoxicity and the overall cell response to the material. Cytotoxicity is concerned mainly with leaching of chemicals from the material, which are toxic to cells cultured on the material (direct cytotoxicity) or to cell cultured on standard surfaces under the influence of conditioned medium (indirect cytotoxicity). Further aspects of material-dependent cell behavior which are relevant for the evaluation of the *in vitro* cytocompatibility are amongst others, cell adhesion, spreading (morphology), proliferation, apoptosis and intracellular changes in gene expression or protein synthesis.

We evaluate the adhesion behavior of primary human dermal fibroblasts (HDFs) and two murine fibroblast cell lines (L929 and 3T3). L929 cells are well accepted for standard cytocompatibility experiments, while primary cells' responses *in vitro* resemble more closely those in the natural environment (*in vivo*). Besides analyzing quantitatively cell adhesion and morphology as important aspects of cytocompatibility, we study FN fibrillogenesis and the formation of adhesion sites in more detail. The involvement of different integrin receptors in initial cell adhesion is analyzed using a fibroblast cell line (M-3T3) expressing β_3-GFP-integrin [40]. In addition we highlight the unique advantage of the material – the straightforward topographic patterning – by presenting the influence of surface topography on cell adhesion and morphology.

2. Materials and methods

2.1. Material fabrication and characterization

2.1.1. Fabrication of smooth and micro-patterned PFPE substrates

The PFPEdiol (hydroxyl-terminated, linear perfluoropolyether) was kindly provided by Solvay Solexis (Milan, Italy). The functionalisation of the PFPEdiol with methacrylate end-groups to yield the crosslinkable perfluoropolyether dimethacrylate (PFPE DMA) was performed according to a procedure adapted from Lensen et al. [39].

Micro-patterning of PFPE was achieved by replica molding from silicon masters. The masters, exhibiting μm-sized structures (round holes and lines) on their surfaces were purchased from Amo GmbH, Aachen, Germany.

The UV-curable PFPE mix was prepared by dissolving 1 g of PFPE DMA dissolved in 0.5 ml of Freon (Merck, Darmstadt, Germany) in the presence of 10 mg (1 wt%) of benzoin methyl ether as a photoinitiator (PI). After homogenization, the solvent was evaporated under a mild stream of nitrogen. For the fabrication of substrates, a droplet of the PFPE (circa 15 μl) was dispensed on a silicon master, a cover glass (diameter: 12 mm) was placed on top and samples were cured with UV-light ($\lambda = 365$ nm; ~1.20 mW/cm^2) under a nitrogen atmosphere for 30 min. After curing, the (~50 μm thick) PFPE films were peeled from the master and the glass. To clean the substrates from possible, residual photoinitiator they were extracted by methanol for 48 h (change of solvent after 24 h) and afterwards placed in 24-well suspension culture plates (Greiner BioOne, Frickenhausen, Germany). Prior to the cell culture experiments the samples were rinsed three times with sterile water and Dulbecco's Phosphate Buffered Saline (PBS) (PAA, Cölbe, Germany).

2.1.2. Preparation of PVDF substrates

PVDF foils (thickness: 0.05 mm) were purchased from GoodFellow (Bad Nauheim, Germany). Samples with a diameter of 12 μm were punched out and transferred to 24-well suspension culture plates. Prior to the cell culture experiments the samples were rinsed three times with sterile water and subsequently three times with sterile PBS.