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Cytocompatibility testings using cell culture modules fabricated from specific candidate biomaterials via injection molding

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1. Introduction

According to the Council Directive 93/42/EEC of the European communities concerning medical devices, biomaterials have to be compatible with contacting tissues, cells and body fluids. In recent years, in vitro methods for assaying biomaterials have gained in importance owing to the growing concern over the use of animals for biomaterials testing (Helmus et al 2008; Murray Peter et al 2007; Yliperttula et al 2008). At present a number of standardized in vitro methods can be used to simulate and predict biological reactions to biomaterials when placed into or on tissues in the body. Significant effort is therefore being focused toward developing predictive and quantitative, but also simple and reliable methods of testing using cultured cells. Usually the cells are cultured and exposed to defined sections of the sample material in standard cell culture vessels like dishes, flasks of multiwell plates. In these test setups the cell behavior might be influenced not only by the sample material but also by the material of the cell culture vessel (e.g. polystyrol, polycarbonate). To overcome this problem it might be helpful to perform the in vitro-biocompatibility tests in cell culture vessels which are made from the sample material itself. The study aimed for testing the applicability of 24-multiwell cell culture inserts, fabricated from a sample polymer itself by thermoplastic injection molding, for cell compatibility test according to the international standard ISO 10993-5. Poly(ether imide) (PEI) was used as sample material because it can be functionalized covalently and because it can be processed both by injection molding and by formation from solution. Because polyimides shows excellent engineering properties, thermal stability, dielectric strength, mechanical strength and chemical resistance, they are used as catheder tubing (Lynch 2008). PEI is a membrane-forming polymer (Albrecht et al 2005; Richardson et al 1993), which has attracted interest as a biomaterial for e.g. blood purification devices (Albrecht et al 2006), organ-assist, wound closure (Trimpert et al 2006) in the last years. Investigations concerning the biocompatibility revealed that PEI supports the attachment and growth of a variety of cells (Krasteva et al 2002; Richardson et al 1993; Seifert et al 2002).

For a test set-up 24-multiwell cell culture inserts made of polystyrene (PS) and polycarbonate (PC), which were used as reference materials because of their widely-use for cell culture and biomaterial testing, and of PEI were fabricated by injection molding. Aim of this test set-up was to compare the surface properties of the inserts with commercial available ones and to investigate cell growth and morphology. The inserts were constructed to be inserted in any commercially available 24-well plate and thus can be used in any standard testing instrument or handling device. Besides the main goal of preparing inserts from the biomaterial to be tested, it is also possible to use inserts of different materials within one well-plate which offers a high flexibility compared to the well-plate made from one material. The surface was characterized via profilometry and contact angle measurements. The contact angle could not be compared to a commercially available well-plate because they are plasma-treated for optimized cell adhesion and proliferation. Besides physicochemical surface investigations, cell morphology, functional integrity of the cell membrane, and mitochondrial activity were used as parameters for cytocompatibility testing.

After the above described test set-up, it was aimed to provide a cup surface, which was similar to the membranes prepared in previous papers (Albrecht et al 2007). Thus a proof-of-principle of preparing cups with a rougher surface and its covalent surface modification was investigated because both surface roughness and surface hydrophilicity can influence cell attachment and growth. For this proof of principle, PEI inserts with a membrane-like surface have been produced through a non-solvent induced phase separation procedure in analogy to flat sheet membrane fabrication. PEI can be modified at the surface easily to vary hydrophilicity and surface charge resulting in increased selectivity at the biointerface (Trimpert et al 2006). The imide moiety of PEI readily reacts with nucleophiles leading to the formation of a new covalent bond while maintaining chain integrity. This reaction works especially well with amine nucleophiles such as poly(ethylene imine) (Albrecht et al 2007) under alkaline conditions allowing to tailor the polymer surface for the aimed application.

Material and Methods

Polymers

All polymers were provided from commercial suppliers (PC: Makrolon® 2805, Bayer, Germany; PS: PS Type 158K, BASF, Germany; PEI: ULTEM® 1000, General Electric, USA). Figure 1 shows the chemical structures of the polymers:

Insert Figure 1

Injection moulding

Injection moulding was performed with a Thermo Electron device (Karlsruhe, Germany) with a divisible heated mould, maximum shot volume of 5.5 ml (capacity of removable the injection unit) and maximum operating temperature of 350 °C. The device has two controlled heating zones and a maximum injection force up to 12.000 N. The heating time for the injection nozzle (from 20 to 240 °C) is less than 6 min. The parameters used to form the 24-multiwell cell culture inserts from PC, PS, and PEI by injection moulding are summarized in table 1.

Insert Table 1 here

The cross-section of the test sample is given in figure 2. The inserts were manufactured with an upper diameter of 16.3 mm, and had an inner lower diameter of 10.5 mm. The height of the inserts was 17.8 mm and the wall thickness 1.0 mm.

Please insert Fig 2 here

Prior testing all samples were sterilized by gas sterilization using ethylene oxide (gas phase: 10 % ethylene oxide, 54 °C, 65% relative humidity, 1.7 bar, gas exposure time: 3 h, aeration phase: 21 h).

Preparation of cups with a membrane-like structure (PEI-mem cups)

40 µl NMP was added to the PEI- cups. The cups were heated for 10 Min to 80 °C and finally immersed into a water bath for 24h. The cups were washed with water (2 days) and then placed into a water bath of 80 °C for 2 x 1h.

Surface modification of a membrane-like structure (Pei-PEI-mem cups)

The cups were modified with a 2% solution of Pei (poly(ethylene imine), Aldrich, M_n ~ 423) in water at 70 °C for 120 min. The cups were washed with water (2 days) and then placed into a water bath of 80 °C for 2 x 1h.

Surface characterization

The wettablity of the samples was determined by measuring water contact angles (CA) using the captive bubble method: an air bubble was injected with a stainless steel needle from a syringe onto the inverted surface of the cell culture insert bottom. The diameter of the contact area between the surface and the bubble was always larger than 2 mm in diameter. Advancing and receding contact angle measurements were performed with a goniometer (Carl Zeiss, Germany) by stepwise withdrawing/adding of air from/to the captured bubble. CA measurements were realized at room temperature with a Drop Shape Analysis System DSA 100 (KRÜSS, Hamburg, Germany). At least ten measurements of different bubbles on at least two different locations of the insert bottom were performed and averaged to yield the contact angles and their standard deviation.

Profilometry

Surface profiles of the inner bottom of the PC-, PS-, and PEI-inserts were obtained with an optical profilometer type MicoProf 200, equipped with a CWL 300 (Fries Research & Technology GmbH (FRT)) chromatic white-light sensor. Data acquisition was performed with the software AQUIRE (ver. 1.21), the evaluation with software MARK III (ver. 3.8b).

The maximal resolution of the roughness is lateral 1-2 µm, in z-direction specified as ≈ 5 nm. The surface profile and the roughness (Ra, Rq) respectively were analysed by scanning a region from the border to the centre of the sample. Each analysis was performed on an area of 4x1 mm^2 (4000 lines per image, 250 dots per line, 300 Hz). The raw data were corrected for the sample tilt (subtraction of a plane), smoothed by a median filter and modified to the region of interest, removing invalid data,

Acid-Orange-Test

The amine content of Pei modified membrane-like bottoms of the inserts was determined with the Acid-Orange II assay by assuming that one amine group will form a complex with one Acid-Orange II. As comparison non modified PEI inserts with membrane-like surface were used. Four samples of each insert forms were measured to determine an average value of the amine content. The surface pH of samples were adjusted by 3x30 min shaking in distilled water with a pH=3. Incubation of the inserts with Acid-Orange II (500 µmol, pH=3, adjusted with HCl) were performed by shaking over night at room temperature (RT). After washing with 3x30 min in distilled Water (pH=3) the dye was desorbed in distilled water with a pH= 12 (adjusted with NaOH) under shaking at RT for 30 min. The amount of adsorbed Acid-Orange II was determined by the optical density of the solution with a SpectraFluorPlus spectrometer (Tecan, Crailsheim, Germany) at 492 nm and quantified by means of a calibration curve.

SEM measurement

The morphology of the PEI-mem insert was investigated by scanning electron microscopy. The dry cup was cut into slices and was then saturated with liquid nitrogen and freeze fractured. A high resolution sputter coater Polaron SC7640 (Quorum Technologies Ltd, UK) was used for coating with gold/palladium (80/20) (EAP 101, Gatan, USA). The prepared samples were investigated using a Gemini field emission scanning electron microscope, type Supra 40 VP (Zeiss, Germany) at an acceleration voltage of 3 kV.

FTIR-ATR Measurement

To estimate the modification of the membrane-like PEI inserts with Pei, Fourier-transformed infrared spectroscopy was used. The bottoms of the inserts were measured in a single beam, attenuated total reflection modus with 100 scans per measurement at the MagnaIR™ Spectrometer 550 (Nicolet, USA). The determination of the Pei modification is possible due to the transition of the imide into amide groups and the structural elements of the modifier. For data interpretation, the spectra were normalised to the adsorption of the C-O valence of the ether bridge at 1230 cm^{-1} which should be unaffected by the modification.

Cell culture

According to the international standard EN ISO 10993 cytotoxicity testings were performed with cells derived from normal subcutaneous areolar and adipose tissue of a 100-day-old male C3H/An mouse (L929, ATCC, Germany). For cell cultivation DMEM (ATCC, Germany) supplemented with 10% horse serum (ATCC, Germany) and 1% penicillin/streptomycin (Invitrogen, Germany) was used. Cell cultures were maintained in a humidified atmosphere with 5% CO_2 in air at 37 °C (Heraeus Incubator, Germany). Every second day, the culture medium was changed. When the confluence of the cells was about 80%, secondary cultures were realised. A rinsing of primary cultures was carried out with a phosphate buffered saline (PBS). The cells were then subdivided by trypsination using trypsin-EDTA solution (trypsin 0.05% and ethylene-diaminetetraacetic acid 0.02%). The cellular seeding of the inserts was started from these secondary cultures.

Cytotoxicity testing

Each of the PC-, PS-, and PEI-based 24-multiwell cell culture inserts was seeded with L929 cells (5000 cells/cm^2). After an incubation time period of 48 h at 37 °C in a humidified

atmosphere with 5 % CO_2 and the mitochondrial activity of the cells was measured by use of a tetrazolium compound which when reduced produces a colored formazan product (MTS) that is soluble in tissue culture medium and absorbs maximally at 490 nm. The compound is reduced due to the mitochondrial activity in the cell (CellTiter® 96 Aqueous Assay, Promega, Germany). Additionally the activity of the cytoplasmatic enzyme lactate dehydrogenase (LDH) which is released by damaged cells was measured by use of a colorimetric assay in the cell culture supernatant (Cytotoxicity Detection Kit (LDH), Roche, Germany) to evaluate the integrity of the cell plasma membrane. The cytotoxicity tests were performed in accordance to the supplier's instructions and in conformity with the EN DIN ISO standard 10993-5. The number of cells on the samples was calculated as mean cell number from five different fields of view.

Immunhistochemistry

Fluorescent staining used to visualise vinculin, F-actin and DNA/nuclei of the cells on the inner surface of the bottom of the PS- PC-, and PEI 24 multiwell cell culture inserts. The cells were fixed with paraformaldehyde (3% v/v) and pre-treated with Triton X-100 (0.5% v/v). Vinculin and F-actin were immunhistochemically visualised (vinculin: polyclonal rabbit anti-vinculin IgG, 1:50, polyclonal goat anti-mouse IgG, conjugated with Cy2, 1:20; F-actin: BODIPY® 558/568 phallodin, 1:20), and dsDNA/nuclei was stained using DRAQ5™. From every sample five different fields of view were analysed with a confocal laser scanning microscope ((LSM 510 META, Zeiss, Germany).

Statistics

Data are reported as mean value ± standard deviation for continuous variables, or as selective frequency for categorical variables, and analyzed by Student's t-test. A p value of less than 0.05 was considered significant.

Results

Surface characterization

The samples show differences in surface wettability. The most hydrophobic material was PC, followed by PS. The latter, PS, is characterized by a high value of hysteresis – the difference between the advancing and receding water contact angle, indicating a high surface heterogeneity. PEI revealed to be moderately hydrophobic.

Please insert Table 2

The roughness of each insert was determined with an optical profilometer. All pure inserts show more or less a plan surface with comparable Ra values in the lower nm-scale (Ra between 30-100 nm). At the same time the Rq values differ more strongly. The reach values for the PC inserts of Rq = 0,25 µm, PS with Rq = 0,40 µm and Rq = 1,00 µm for PEI. The Ra and Rq values of the prepared inserts are comparable to a commercial 24-well plate. Compared to the inlay of the injection moulding, which contacts the bottom, the values are in the same range for the Ra values and 1-2 magnitudes higher for the Rq, indicating that the roughness is not totally determined thereby.

Please insert Figure 3 here

The induced membrane-like PEI bottom structure is like suggested less plain. The Ra and Rq values increase in the order of up to two magnitudes to Ra = 0.834 µm, Rq = 1.853 µm for the unmodified and Ra = 2.527 µm, Rq = 4.568 µm for the Pei modified surfaces respectively.

Evaluation of the cell morphology:

The transparent nature of the PC-, PS-, and PEI-inserts enabled microscopical analysis with a standard inverse cell culture microscope (Axiovert 40C, Carl Zeiss, Germany). The maximum magnification was 20-fold and limited by the thickness of the insert-bottom (1.0 mm). Figure 4 shows the morphology of the L929 cells on the inner surface of the bottom of the PS- PC-, and PEI inserts 48 hours after cell seeding. The cells were able to proliferate and to spread on all polymers. According to the United States Pharmacopeia metric, which is based on changes of the cell shape, the frequency and size of intracellular vacuoles and the homogeneity of the cell layer, the PS-, PC-, and PEI-based 24-well inserts were evaluated as non-toxic. However, the number of adherent cells 48 hours after seeding on PC and PEI was significantly lower ($p<0.05$, PC: 9.7 ±3.7 / mm^2; PEI: 10.4 ±2.9 / mm^2) than seen on PS (16.4 ±6.2 / mm^2).

Please insert Figure 4 here

The PS-, PC-, and PEI-based 24-well inserts proved also to be suited for immunhistochemical studies with a confocal laser scanning microscope. Figure 5 shows L929 cells on the inner surface of the bottom of the PS- PC-, and PEI inserts after fluorescent staining of F-actin and vinculin. Vinculin is known as a membrane-cytoskeletal protein in focal adhesion plaques that is involved in linkage of integrin adhesion molecules to the actin cytoskeleton. Two days (48 hours) after seeding L929 cells in the inserts differences in the vinculin assembly could be noted. In the L929 cells seeded on PC vinculin was almost homogenously distributed in the cell body. In contrast the vinculin of the L929 cells which grew on PS and PEI was more peripheral concentrated. The staining of F-actin showed no difference between the cells on the different inserts.

Please insert Figure 5 here

Like shown in figure 6 the mitochondrial activity of the cells seeded in the PC-, PS-, and PEI-inserts showed no significant difference ($p<0.01$). Also the extracellular activity of the cytoplasmatic enzyme LDH was not significantly different in the cell culture supernatant of the cells 48 h after cell seeding in them in the PS-, PEI- and PC-inserts.

Please insert Figure 6

The formation of a membrane-like morphology is visualized in Fig. 7. The finger pore of the membrane is clearly shown in the SEM which is a morphology which was also reported for the flat-sheet membranes (Albrecht et al 2007). In the bottom of the picture, the bulk structure of the original PEI insert is visualized. The rougher surface of PEI-mem compared to the original PEI insert has only a minor effect on the contact angle is lower than the contact angle reported for the flat-sheet membrane. Again in similarity to the membranes, the modification of PEI-mem with Pei strongly reduces the contact angle without influencing the membrane morphology. Due to the surface tension in the membrane forming process, the membrane-like bottoms are more convex and several bubbles are needed to measure the values. The determination of the hysteresis is thus not possible. Furthermore the covalent modification is revealed in the ATR-FTIR showing a decrease of the imide bands' intensity at 1720 cm^{-1} while a strong increase of the amide peaks at 1640 cm^{-1} and 1550 cm^{-1} can be found. An estimation of the amine group content of the Pei-PEI-mem by the acid orange assay showed an amine content of 220 $nmol*cm^{-2}$. These results are accordance with flat-sheet membranes.

Please insert Figure 7

Please insert Figure 8

Discussion

The increasing life expectancy and continued medical advances are main promoters for rapidly growing scientific field of polymer-based biomaterial research followed by new biotechnological strategies to test the material biocompatibility. In the present study inserts for 24-well plates fabricated of the biomaterial polymer itself by thermoplastic injection molding proved to be suited for reproducible and fast cytotoxicity testings. The PC-, PS-, and PEI-based 24-well inserts allowed studying the cell morphology via transmitted light and laser scanning microscopy, and colorimetric testing of the mitochondrial activity and of the functional integrity of the outer cell membrane. The tests could be performed in strong accordance to the international standard 10993 and guaranteed no influence of secondary materials on the test results.

PC and PS were chosen as reference polymers for the study because they represent the most commonly used polymers for cell culture vessel fabrication (Zaehrlinger 2008). Both materials revealed no signs of cytotoxicity after formation into the 24 multiwell inserts. Two days after seeding L929 cells in the inserts fabricated from both polymers no significant difference in the extracellular activity of the cytoplasmatic enzyme LDH and the mitochondrial activity as well was detectable. Via transmitted light microscopy revealed no changes in the cell morphology could be found that hint for cytotoxic effects of the materials. However, immunhistology showed a homogenous vinculin distribution of the cells seeded on PC and a more peripheral vinculin assessment in the cells seeded on PS. Additionally the number of adherent cells 48h after seeding on PS was significantly higher than seen on PC. These results show, that PS and PC, which are established as standard polymers for cell tissue plate fabrication, can reveal different effects at least on the vinculin skeleton arrangement of cells and the cell adherence. It is well known from the literature, that the wettability and hydrophilicity of materials respectively can influence cell adherence. Hydrophilicity provides low interfacial free energy resulting in reduced protein adsorption and cell adhesion (J Wang

2005; Wang et al 2004). However, contact angle measurements revealed comparable wettability of the PS and PC surface in our tests. The differences in the number of adherent cells, and also the differences in the arrangement of the vinculin skeleton might be linked to the different surface roughness of the PS- and PC-inserts. The higher surface roughness of the PS insert might have supported cell adhesion more than the lower rough PC surface. The lower roughness of PC is also supposed to be causal for the re-arrangement of the vinculin skeleton of the L929 cells which grew on in the PC inserts. Vinculin, a protein localized at the cytoplasmatic face of cell–matrix and cell–cell adhesions (Burn & Burger 1987), is required for strong cell adhesion. The arrangement of the vinculin cytoskeleton in the peripheral parts of the cells nearby the cell membrane (Geiger 1979) like seen on the cells which were seeded on PS might have become changed to a more homogenously distributed vinculin arrangement because of the beneficial cell adhesion capabilities of the rougher PC surface.

The cells which were seeded in the PEI-inserts showed after 48 h of cultivation no significant different morphological features, extracellular LDH-activities and mitochondrial activities compared to the inserts made from the reference polymers PS and PC. Interestingly the vinculin cytoskeleton arrangement was comparable to the cells which were grown on PS and the number of adherent cells was also higher on PEI than seen on PC. These results hint for a PEI surface that is more comparable to PS than to PC.

The limitations of the PC-, PS-, and PEI-inserts are given by the thickness of the insert bottom, which predetermines the quality of microscopical analysis. For a more detailed analysis of the inner cell structures magnifications higher 40 fold should be applicable. Further studies will show the use of the “polymer-specific” inserts also for tests with primary cells, and with modified surface characteristics also including physical modifications, chemical modifications, and radiation.

Besides investigating the PC-, PS-, and PEI-inserts, also a proof of principle concerning the preparation of PEI-inserts with a membrane-like surface structure and its surface modification was established and showed to be very similar to the findings of flat-sheet membranes. The membrane-like structure of the insert has been verified by SEM and profilometry. The covalent modification of the surface was possible in analogy to the previously reported results on flat sheet membranes and could be verified by contact angle measurements, by ATR-FTIR spectroscopy, and determination of the amine group content. Thus it seems possible to tailor inserts in analogy to the original biomaterial to be tested concerning surface morphology and chemical composition and thus to overcome the disadvantage of the standard PS cell culture vessel influencing the biomaterial testing. Furthermore the inserts offer the flexibility of to systematically investigate inserts of different materials within a standard 24-well-plate, which is a possibility not found for well-plates produced from one material.

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