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Effects of the surface modification of polyurethane substrates on genotoxicity and blood activation processes

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ABSTRACT

The aim of this study was to determine the mutagenic and thrombogenic potential of a material composed of a thin coating deposited on a polymeric substrate. In this work, a surface was modified in a manner that would mimic the function of cellular niches. Finally, the surfaces should actively capture and differentiate progenitor cells from the blood stream. Thin films with 10 to 500 nm thicknesses were deposited by unbalanced, pulsed DC magnetron sputtering on smooth polyurethane. Such high energy conditions led to a stiffening of the polymer surface layers by pseudodiffusion during the initial stages of film growth. Both the high intrinsic film stress due to high energy film growth and the huge difference in the elastic properties of the films and polymer substrates resulted in hierarchical and self-adapting nanowrinkling. Surface modifications of synthetic materials for future use in regeneration of the circulatory system must be tested in terms of their thrombogenicity and mutagenicity. Point mutations in many cases can lead to many serious haematologic complications. Genotoxicity was determined by testing for reverse histidine mutations in selected strains of *Salmonella typhimurium*. The analysis was performed in the presence and absence of metabolic activation system S9 containing liver microsomal fraction of rats. Based on these results, no mutagenicity of the tested material was observed. The interaction of blood and the material under dynamic conditions was described. Blood from above the analysed surface was collected after the test, and the quality of the blood was assessed along with the type of cellular response to the surface. In the obtained results of the coagulation processes, it was found that the tested material reduced the process of platelet activation under hydrodynamic conditions in comparison to the control material, polyurethane.

1. Introduction

The surface modification of thin layers of polymers allows for the formation of new features while maintaining or only slightly changing the physical properties of the polymer [1]. The surface modification of medically useful polymers has focused on improving the stability of materials in direct contact with tissue [2,3]. The adhesion of a polymer layer to a substrate is one of the most important factors in classifying a material for biomedical engineering.

Progress in the field of biomedical engineering is possible as a result of the effective combination of materials science and cell biology [4]. Stem cells are capable of self-renewal and have a high potential to differentiate into other cell types. Each stem cell is able to proliferate, self-renew and differentiate by asymmetrical division [5–7]. Each cell that grows and matures has its own niche. Each niche is the spatial structure of the cells and extracellular matrix.

This creates a distinctive microenvironment that maintains the cell and transmits cellular processes [8–10]. To reconstruct niche-like structures, surface wrinkling is considered. To reduce the overall strain energy in layers subjected to compressive stress, surface "wrinkling", which is a common deformation of the substrate surface and layer, is observed [9]. "Wrinkling" is the formation of sinusoidal elevations on a surface, but it does not cause a loss of adhesion to the substrate. The main mechanism of wrinkling is uplifting of the substrate or subsurface areas of the substrate. The reason for the folding of the deposited layer is mechanical instability.

Each material that is newly designed for a clinical purpose needs to be analysed in the context of a particular destination. As already mentioned, this work focuses on designing new, challenging materials that would capture progenitor cells from the blood stream in vivo, influencing their differentiation. In this case, the requirements are complex and starting from the beginning of the material development process, it is necessary to eliminate the risks of creating mutations or initiating sudden blood activation and aggregation processes.

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The AMES test was developed in the 1970's by Bruce Ames, Professor of Biochemistry at UC-Berkeley, as a fast and sensitive assay of the ability of a chemical compound or mixture to induce mutations in DNA. The AMES test is used to determine the potential of compounds to induce mutations in bacterial strains Point mutations that are present in the histidine operon of certain bacteria (*Salmonella typhimurium*) do not allow the bacteria to synthesize the corresponding amino acid (His − organisms). Therefore, they are unable to grow in media that lacks these amino acids. If the mutagenic agent has an effect, the change of the reading frame of gene mutations can cause reversion and restore prototrophy, which is observed as the ability to grow in media lacking histidine. Since the designation of genotoxicity is carried out using prokaryotic organisms, it is necessary to use an exogenous metabolic activation system in the assay, which partially simulates in vivo conditions in mammals. The bacterial reverse mutation test is therefore used as a preliminary screening test of genotoxicity and, in particular, the ability of compounds to induce point mutations.

The developed materials had to exhibit excellent haemocompatibility, eliciting low thrombogenic and immune responses. Materials were selected with respect to the possibility of creating a suitable environment for the differentiation of endothelial cells under dynamic blood flow conditions. The paper presents results of the process optimization regarding cellular niche-like structure formation based on genotoxicity and haemocompatibility analysis.

2. Materials and methods

2.1. Thin film deposition

The deposition of the coatings was performed by Physical Vapour Deposition (PVD) (for a detailed description, see [11]). Before deposition, we cleaned the substrates polyurethane TPU (Advan-Source Biomaterials Chronothane™ P) ultrasonically with ethanol and dried them afterwards under vacuum. After mounting the substrates parallel to the sputter target surface at a distance of ~120 mm, we pumped the vacuum chamber down to at least 4×10^{-3} Pa. Prior to the coating deposition, we used anode layer ion source Ar etching to clean the surfaces of oxide layers. Then, we used unbalanced, pulsed DC magnetron sputtering (80 kHz pulsing, 2000 W power, pyrolytic carbon target with 99.95% C content from Schunk, Bad Goisern, Austria) to deposit the a-C:H coatings in Ar and Ar + N_2 atmospheres, respectively, at 3×10^{-1} Pa. Growth of a coating of thicknesses 100 nm was performed at room temperature (25 °C) with less than 5 °C heating during deposition in plasma. This temperature rise was measured by thermocouples on substrate positions in test batches for the process setup. Surface analysis was performed by atomic force microscopy. The scratch tests were done with two indenters. The first, Rockwell C geometry, round radius 20 μm. The tests were performed within the maximum permeability range of 0.2; 0.5; 2 and 5 N. The tests due to the low thickness of the coatings were carried out with a Rockwell custom indenter with a round radius of 20 μm. Initially, for all samples, crack formation was observed on the coating surfaces - L_{c1}. The second, Berkovich's geometry. This geometry introduces much greater contact stress to the coating due to its small round radius, 100 nm. The applied load was 0.2; 0.5 and 1 N.

2.2. Genotoxicity

2.2.1. AMES test

The AMES test MPF™ Penta I (mutagenicity test) was developed by the Xenometrix company. The main features of the test are as follows:

- full compliance with OECD guidelines no 471,
- form of a complete set of bacterial strains and media ready to use,
- strict quality control (genotype and phenotype) of the tested bacteria strains of *Salmonella typhimurium* conducted by the manufacturer, and
- ability to analyse the results automatically.

The bacterial strains used in the study are *Salmonella typhimurium* TA98 and TA1537. These strains are designed to detect mutations in reading frames. *S. typhimurium* strains have GC base pairs at the primary reversion site. Strains meet the requirements of the OECD 471 for testing chemicals (Table 1).

The bacteria were subjected to exposure at 6 concentrations of the test compound for exposure times in media containing an appropriate amount of histidine (*S. typhimurium*) to allow for an average of two cell divisions. After the exposure step, the cultures were diluted in medium containing a pH indicator without histidine and distributed in equal amounts between 48 wells of a 384-well plate. Within two days, the cells where a functional gene was restored for the amino acid increased and formed colonies. The metabolism of bacterial colonies, lowering the pH of the medium, caused a change in the colour of the substrate in the well. The number of wells containing the reversion mutation colonies was counted for each dose and compared with the zero dose control.

Each dose was tested in triplicate to allow for statistical analysis of the obtained data. After exposure to the test compound, an increase in the number of colonies with reversion mutations in comparison to controls at a zero dose indicated that the compound expressed mutagenic effects in the AMES MPF™ Penta I test. The potential mutagenic substances were assessed either directly or in the presence of rat liver S9 fraction. Rat liver S9 homogenate is a supernatant fraction obtained from an organ (usually liver) homogenate by centrifuging at 9000*g* for 20 min. 9000*g* supernatant (S9), and microsomal fractions are presently used to characterize the in vitro metabolism.

The upper concentration of the compound that can be used in the assay prior to the screening test is based on the lowest concentration of compound that is insoluble in a given solvent. This test is used to evaluate the cytotoxicity properties of soluble concentrations.

For determination prior to screening, it is possible to apply any one of the bacterial strains, but not all strains correspond to a similar sensitivity to toxic substances. The strain TA98 is suggested in accordance with the instructions of the AMES MPF ™ Penta I test for strains incubated in semi-liquid substrates (Xenometrix).

The investigated material, a-C:N, in the form of a thin coating was placed in a test tube followed by the addition of the appropriate volume of the extraction medium (0.1 g of sample for 1 mL of medium). The completely submerged tested material was kept at 37 °C for 72 ± 2 h. After the extraction, the medium was transferred to a new tube and within 24 h was analysed using the AMES test.

The ISO 10993-12 considers 3 types of solvents, two of which must be chosen to carry out the extraction. These are (1) polar solvents, (2) non-polar solvents, and (3) additional solvents. Since it is not possible to use a non-polar solvent in the AMES MPF test, the first and third groups were chosen. When choosing an extraction medium, the following assumptions were taken under consideration:

- compatibility with the AMES MPF method for assessing genotoxicity (both solvents used),
- physiological conditions of the implantation place (0.9% v/w NaCl), and
- solvents with increasing ability to dissolve hardly water-soluble chemicals (DMSO).

In accordance with the manufacturer's procedure, test bacterial strains were inoculated by transferring a 25 μL slurry colony suspended in 200 mL of growth medium to 10 mL of growth medium with ampicillin, if required. The culture was shaken overnight at 250 rpm with access to open air at 37 °C. The incubation was completed in time when OD 600 gained a value of 2.0. The optical density was measured at 600 nm.