INNOVATIVE TEST OF NON TRANSPARENT MATERIAL CYTOTOXICITY

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Abstract

The paper describes an innovative method of determining cytotoxicity rate for non-transparent materials. Cytotoxicity is a very important property of materials in terms of their use in health care. It means that all materials with a targeted use in a field of interaction with tissue have to be tested for cytotoxicity. Therefore it is necessary to have a quick and reliable method of determination available. Innovative method for testing cytotoxicity of non-transparent materials is based on monitoring cell-material interaction directly on its surface. Animal cells are inoculated on the surface of a material and cultivated there for approximately three successive generations. The cultivated cells are fluorescently stained so that they are visible in the incident light. Afterwards an analysis of surface coverage of material by cells is accomplished by the means of semi-automatic segmentation software. Image recording ready to be applicable for further analysis is an advantage of this method. The innovative aspect of the method is in utilization of fluorescence for capturing images of cell colonization of the surface and subsequent semi-automatic determination of the colonization surface which guarantees objective and reproducible results.

Keywords: Titan alloys, cytotoxicity, cell-alloy interaction, cell segmentation

1. INTRODUCTION

Newly emerging and developing materials with totally different physical properties designed mainly for medicine and biotechnologies, require formation and development of new more detailed methods of testing their cytotoxicity, which is ability of cells or chemicals to destroy other cells. If materials do not show any cytotoxicity, we can consider so called cytocompatibility as chemical and material tolerance in a biological environment. Material does not have to be cytotoxic, nor it has to show some of mutagenic elements, and still it may not be suitable because of a disturbed relation of cells towards tested material. It depends mainly on the cell ability to accept a material or its surface and on the way how cytoskeleton elements react as for their regeneration, and in the case of a positive reaction, the question is if and how cell division [1] is maintained or limited. Recently, the methods of cytotoxicity testing are established by CSN EN ISO 10993 norm in several versions in Czech republic [2]. These usually are methods utilizing material leach and its influence on the cell layer or contact of the material with the cell layer, alternatively contact of cells with an edge of a non-transparent material [2]. The main disadvantage of these methods is that the direct interaction of cells on the surface of a non-transparent material, as well as eventual morphological changes of cells after a longer contact with the tested surface cannot be observed. Nevertheless, the base on which the cell lines are attached, is very important for their growth and very often this is the determining factor whether the cultivation will be successful or not [3]. Colonies of the cell population adhered on the surface of a transparent material can be observed by a microscope with a phase contrast on transparent materials only. The newly developed method of cytotoxicity in non-transparent materials enables to obtain data showing how cell population colonizes the surface of a non-transparent material with the help of a microscope with incident light. The semi-automatic analysis of the image of surface with the grown-up cell colonization enables to determine the cell-sample surface interaction quickly and exactly.
2. METHOD DESCRIPTION

It is necessary to carry out sterilization process of the tested samples of material that are mostly delivered as non-sterile by methods according to manufacturer’s or supplier’s recommendations. The sample has to be properly and unmistakably marked. The shape of material sample should be preferably cylindrical with tested horizontal upper surface parallel to the bottom surface which touches the bottom of a culture vessel (plastic boards with several holes, Petri dish). Tested samples are put into the cultivation vessel in sterile environment, and a chosen cell line with the culture medium is inoculated on the surface of the material sample. The cultivation vessel with samples is then placed into an incubator for a determined period of exposition. The incubator should be set to an optimal temperature for mammal cell cultivation, which in this case are 37°C and air mixture (95%) and CO₂ (5%) [3,4]. After the exposition period is over, the cultivation vessel is removed from the incubator and the cells on the surface or the material are subsequently fixed and stained so that it is possible to make an image record of the surface of material. By the means of the microscope with incident light the image records of each sample of material surface are made by the scanning method. The image records are preceded and area of cell colonization is defined with the help of the software. Data are statistically evaluated and the rate of cytotoxicity of material is determined on the basis of the colonized area.

2.1. Individual steps of the method

Time schedule of the experiment including the used cell line to the given number of samples is drafted before beginning of the experiment. It is necessary to choose a suitable cultivation vessel according to the size of the samples, e.g. plastic boards with several holes for cultivation of tissue cultures (Fig. 3Chyba! Nenalezen zdroj odkazů.)

- Purifying and sterilization of tested samples – The samples are soaked in absolute ethanol for approximately 2 hours and then rinsed with distilled water thoroughly or autoclaved for 20 min at 121°C according to their composition, or subjected to UV radiation for 30 minutes.

- Cultivation of cell lines - The cell lines come from European Collection of Cell Cultures and are cultivated according specific requirements of each cell line. The cell line is selected according to requirements of the contracting authority of material sample (MG63 – Human osteosarcoma, L929 – Mouse fibroblast, HeLa – Human cervix epithelial carcinoma) Fig. 3. Within one experiment (also in case of a larger number of samples) inoculation dose of the selected cell line from a single cultivation bottle under the same cultivation conditions is used. This minimizes experiment error caused by a different behaviour of cells cultivated under different conditions.

- Calculation of inoculation dose – Burker chamber

- Types of cell line: examples of cell lines are show at Fig. 1

![Cell cultures](image-url)

Fig. 1 Illustration of cell cultures used for cytotoxicity testing. On the left - MG63, in the middle – L929, on the right - HeLa
Fig. 2 Sample image obtained by a microscope with phase contrast with cell line inoculated into Burker chamber for determining calculation of inoculums

- Inoculation of cell line on the surface of the sample - Cell line is inoculated (see Fig. 2) into a measured amount of cultivation medium and transferred to the surface of material sample with the density of 3 500 cells per a centimeter of the square area of cultivation vessel (Fig. 3). This is followed by exposition period of 72 hours in a thermostat at 37°C, 5% CO₂ and 90% air humidity (Fig. 3)

Fig. 3 Left - Cultivation 12 well plate, middle - Cultivation 48 well plate, right - thermostat suitable for cultivation of cell lines

- Termination of experiment after exposition period – Cultivation medium is aseptically suctioned from the cultivation vessel and afterwards the sample is gently rinsed with PBS solution (phosphate buffer solution) with pH 7.3 twice.

- Fixation and staining of the cell growth on the samples – The samples are fixed by acetic acid solution and methanol in a ratio of 1:3 for 5 minutes. The solution is then removed and the samples are rinsed with PBS solution twice and eventually with distilled water once. Afterwards the samples are stained by a usual staining technique for tissue cultures. Typically, the classical staining technique (Giemsa Romanowski and May-Grunwald) is used, possibly fluorescent stain Fluorescein can be used, too. It is possible to observe fluorescently stained cell colonies on the surface of non-transparent material with the help of a standard fluorescent microscope with incident light.

- Field of view record of each sample – use of the microscope with incident light for making 32 images from the surface of each sample by the method of scanning. This number of images guarantees the possibility of statistic evaluation of the experiment. Magnification of the microscope is 20 – fold.
Fig. 4 Coverage of non-transparent material by cell population stained with fluorescent stain without (left column) and with labeling (right column) of cell structures using the software – red color indicate cell colony borders, yellow color indicated the holes in cell colony.

Statistical evaluation:

1) Basic set evaluation (values from 32 image records) according to Gauss division, with the help of t-test of obliquity and pointedness, using 5% of the significance level and critical values 2.042. This enables us to find out if the set is distributed in Gauss way, as well as rejection or non-rejection of zero hypotheses.

2) Evaluation of non-parametric Kolgomorov-Smith test for two independent selections. For minor selections (when n is less or equals 40) we use 5% significance level with critical value 11. By the means of Kolgomorov-Smith test we evaluate if the separate samples differ [5].

On the grounds of the above mentioned statistic tests we evaluate the percentage of the cell colonized material surface Fig. 4. The size of the field of view of the obtained image record is stated in pixels and taken as 100% value. Colonization of cell population in the field of view of the image record, adherent on the sample surface is x%. E.g. if the size of the field of view of the record is 1310720 pixels, i.e. 100%, then by occupation of cell population adherent on the sample surface in the given record with the value of 994880 pixels, the percentage of the cell colonized surface of material is 75.90%.

Not only cytotoxicity but also tolerance of cells towards the tested material sample is evaluated. This is expressed in the percentage of colonization which indicates the quantity of tolerance in percentage.

Scale of tolerance:

1) Intolerant – up to 10% of the colonized area
2) Slightly tolerant – up to 20% of the colonized area
3) Medium tolerant – up to 50% of the colonized area
2.2 Cell area determination - software

Manual determination of the area covered by the cells on the surface of the material is very time consuming because the expert has to manually label the areas occupied by cells in the image. Typical experiment for biocompatibility test consists of 32 images of cell colonization. Usual duration of the cell border determination is several minutes per image (5-20 minutes, depending on the number of cells and structure of the material). Therefore the specialized software for cell area determination was developed. The software is based on semi-automatic cell/background segmentation method and the assumption of the intensity difference between labeled cells (fluorescence microscopy) and surface of the material. The microscopic images obtained from the method typically contain a lot of noise in the form of fluorescing particles that were stained during the process of cell staining. Because of the fluorescence method used for cell colonization observation we can expect that the intensity of the cells will be much brighter that the intensity of those on the stained surface of the material. These prepositions allowed us to use simple methods for objects segmentation based on intensity tresholding and objects filtration based on the object size. The process of image processing is depicted on the Fig. 5.

![Fig. 5 Scheme of the semi-automatic cell area determination. Input of the method is single image and output is the number of pixels covered by the cells and detected borders of the cells. The borders can be manually modified by the expert to change the areas covered by the cells.](image)

One of the problems of cell colonization of the material surface is the non-homogenous fluorescence response of the stained cells on the non-flat surfaces. The absolute intensity of the cell fluorescence can vary based on the position and orientation of the cell grown on the structured surface. This is one of the reasons of the non-homogenous response often in a single cell. The determination of the cytotoxicity level is critically depended on the precision of the cell area determination and therefore the result of the software cell segmentation has to be verified by the expert for every image (see Fig. 6).
For this task, also fully automated methods exist. Software CellTracker [6] uses the watershed transformation to identify individual cells. We also tested our software SegmentationTool [7] which was originally developed for phase contrast microscopy images analysis. Due to its properties our software would be also used for fluorescence image analysis. It uses images taken in different moments and by subtracting them it is capable to find the movements of the cells. Only simple operation as tresholding, blurring and morphological operation are used in a specific way therefore it is fast.

3. EVALUATION OF THE METHOD AND TESTING

The described method of determining cytotoxicity was verified on actual material samples used in stomatology research (a commercial research contract). Materials for dental implantology – TiGr2 (pure Titanium), TiGr5 (Ti6Al4V) and TNT (Ti3 6Nb4Ta) were evaluated at the Stomatologic Research Centre in Brno. Percentage of tolerance was 68.88% in the sample TiGr2, 74.01% in the sample TiGr5, and 77.46% in the sample TNT. The above mentioned tested samples and their alloys are used as cytocompatible in dental implantology and medicine, which was also confirmed by this method with the result of tolerance in interaction with the cell line as tolerant.
The method was verified on other types of samples as well (Titanium alloys with different applied coatings, ceramic composites), primarily in the field of biological tolerance towards materials for medical use as well as for studying primary steps in colonization of benthic organisms in hydrobiology. Recently, inter-laboratory testing of the method is being carried out with the aim of verifying its reproducibility following the procedure defined in this article.

4. CONCLUSION

The paper introduces the innovative method for cytotoxicity of non-transparent materials. The method of analyzing cell culture interaction on the surface of material allows quantitative and qualitative analysis of the mentioned interaction with screening of cell colonization of the material surface. The method consists from standardized approach of cell culture cultivation, fluorescence staining and analysis of microscopy images directly on the surface of the material. The method uses semi-automatic method of cell colony growth determination. The method is based on simple thresholding and object size filtration which is suitable for daily usage in the laboratory practice. The approach provides the objective and fast analysis of material cytotoxicity. Besides the basic analysis, the advantages of this method are numerous further screenings (e.g. frequency of mitosis, morphological atypia), which are possible thanks to the acquired image record and its re-evaluation. The method is being further developed in a way that allows capturing the time lapse image of dynamics of cell proliferation on the surface of material and full automatic image processing.

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LITERATURE


