**Hemijska Industrija**

**Ref. No. 659**

**Title: Validation of a novel perfusion bioreactor system in cancer research**

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**Answers to Reviewers**

We thank the Reviewers for valuable comments and suggestions. We have accepted all suggestions and we believe that we have answered to all of the questions so that the manuscript is now fairly improved. Please find detailed answers to the Reviewers below and the revised manuscript in which all changes are marked in yellow.

**Reviewer #1:**(reviewers' comments are rewritten in **boldface)**

**1. Please indicate the number of technical replicates involved in the procedures where possible**

We thank the Reviewer for this suggestion and we have added the numbers of technical replicates.

**2. Figure 7. As sodium citrate appears to be critical for cell recovery (and viability as well) from microfibers, Authors should discuss a potentially occurred selection within SiHA cell population, considering their “resistance” to chemical agents (and culture conditions). This would be particularly interest for ant-cancer drug approaches. Also, would adjustment of sodium citrate concentration contribute to better cell recovery? Please state whether the cells in 2D monolayer group were also detached by sodium citrate. Please use the same y-axis in presented graphs.**

We thank the Reviewer for these comments and contemplation. Indeed, sodium citrate was shown to induce cytotoxic effects on different cell lines such as a prostate cancer cell line (PC3), gastric cell lines BGC-823 and SGC-7901, stomach adenocarcinoma cell line (AGS) in dose- and time- dependent manner (Garland et al., 1989, Lu et al., 2011, Xia et al, 2018, Caiazza et al 2019). In addition, effects of sodium citrate on cell viability were shown to depend on the cell growth phase (Lee et al., 1991).

The sodium citrate solution was used only for dissolution of microfibers while the cells in 2D monolayer were detached by Trypsin/EDTA solution. In future studies, we will definitely investigate the effects of sodium citrate on cells only (without alginate hydrogels) and we believe that optimization of sodium citrate concentration and exposure time or using other agents for microfiber dissolution could probably improve the cell viability.

Discussion on possible effects of sodium citrate is now added in the revised manuscript.

We have corrected the y-axis in Figure 7.

**3. Did Authors try to dissociate cell aggregates formed upon/due to cultivation in microfibers?**

We thank the Reviewer for this suggestion and we agree that aggregate dissociation could influence the values obtained in this study. However, in this study we were not dissociating cell aggregates since they were very small and the procedure for aggregate dissociation should be optimized first. The cell aggregates could be dissolved in a solution with optimal Trypsin/EDTA concentration, while the time of trypsinization could affect the cell viability. In future, longer studies where formation of larger aggregates could be expected, aggregate dissociation will be indeed required for obtaining correct results.

**4. Throughout the text: Please uniform the abbreviations, indicated clearly which medium was used and which supplements were added.**

We thank the Reviewer for this suggestion and we have uniformed the abbreviations and clearly added medium composition throughout the text.

**Reviewer #2 (**reviewers' comments are rewritten in **boldface)**

**1. In Materials and Methods section state the magnification at which micrographs were taken;**

We apologize for this omission and we have now added the microscope magnification in the revised text:

“Microfiber diameters were measured by using an optical microscope DM IL LED Inverted Microscope, Leica, (Leica Microsystems, Germany) at a 10x magnification.“

**2. In Materials and Methods section, subsection 2.5. refers mainly to characterization of cell culture in alginate microfiber not characterization of microfibers itself and this should be changed in the title of the subsection;**

We thank the Reviewer for this comment and we have changed the title of the subsection 2.5. as:

“Characterization of cells immobilized in alginate microfibers”

**3. Explain why concentrations of 1.5 % w/w alginate and cell density 4 x 106 cells cm-3 were chosen for cell culturing.**

We have selected the alginate concentration according to our previous studies of alginate microfibers production by the extrusion technique (Stojkovska et al., 2018), as well as immobilization of murine stromal cells from bone marrow and bovine chondrocytes in alginate microbeads (Osmokrovic et al., 2006, Stojkovska et al., 2010). Investigation of optimal cell density within microfibers was out of scope of this study but according to the literature (Cacciotti et al, 2017) and our preliminary studies the density of 4 x 106 cells cm-3 was chosen. We have added this information in the revised manuscript.