Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *86* (6) 555–559 (2021)
JSCS–5442
Note

NOTE

Cytotoxicity of amorphous calcium phosphate multifunctional composite coatings on titanium obtained by *in situ* anodization/anaphoretic deposition

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(Received 11 February, accepted 23 March 2021)

Abstract: The cytotoxicity of amorphous calcium phosphate (ACP) and chitosan lactate (ChOL) multifunctional and hybrid composite coatings on MRC-5 human lung fibroblast cell line was elucidated. ACP/TiO_2 and ACP/TiO_2/ /ChOL were deposited onto Ti by a novel *in situ* anodization/anaphoretic process at constant voltage. Cytotoxicity tests showed that there was no significant decrease in the survival of healthy MRC-5 cells exposed to composite samples without chitosan lactate, while there was an increase in the number of viable cells in the sample containing ChOL. These findings show that there was improved cell proliferation, differentiation and cell viability in the ChOL-containing sample, which makes ACP/TiO_2/ChOL coating a good candidate for applications in medicine and stomatology.

Keywords: cytotoxicity; dye exclusion test; colorimetric test with tetrazolium salts; amorphous calcium phosphate; chitosan oligolactate.

INTRODUCTION

Calcium phosphates (CP), amongst which amorphous calcium phosphate (ACP) and hydroxyapatite (HAp), along with TiO_2 layers on Ti, have found vast applications in preventive and regenerative medicine due to their excellent biocompatibility, nontoxic properties and ability to participate in the normal meta-

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https://doi.org/10.2298/JSC210211024P

bolism of organisms.[1–7] New research on chitosan oligolactate (ChOL) coated HAp particles for drug delivery indicated the advanced properties of this derivative.[4] The authors have already published results about the synthesis, physicochemical and bioactive properties of new ACP and ChOL-based multifunctional hybrid composite materials by novel *in situ* anodization/anaphoretic process.[1–3] However, in order to complete the research, there was a need for *in vitro* cytotoxicity tests of the obtained materials, in order to elucidate their biomedical compatibility. This note aims at reporting the cytotoxicity analysis to round up the research on these novel materials.

EXPERIMENTAL

ACP powder, as well as ACP/TiO_2 and ACP/TiO_2/ChOL composite coatings on Ti substrates, made using *in situ* anodization/anaphoretic deposition, were prepared as explained in a previous work.[2] Briefly, deposition of these coatings was performed at a constant voltage of 60 V for 180 s at 25 °C, starting from prepared suspensions.[2] Upon synthesis and prior to cytotoxicity tests, the appearance of the coatings were checked by FE–SEM, Tescan Mira 3 XMU FEG–SEM.

Studies on the cytotoxicity of the materials were performed on a human lung fibroblast cell line (MRC-5). The cells were grown attached to a plate (Costar, 25 cm^3) in Dulbecco's-modified Eagle's medium with 4.5 g L^{-1} glucose, 10 mass % fetal calf serum and antibiotic/antimycotic solution, all from Sigma. The temperature was maintained at 37 °C in a 5 % CO_2 (Heraeus) humidity saturated atmosphere. The cells were passaged twice a week and only viable cells in the logarithmic growth phase between the 3rd and 10th passage were used in the experiments.

For the dye exclusion test (DET), viable cells were trypsinized, resuspended and counted in 0.1 % Trypan Blue, then seeded on tested samples (2×10^5 mL^{-1}) in Petri dishes (Center well, Falcon) at 37 °C, with 5 % CO_2 for 48 h. Control samples had no tested material. After incubation, the cells were counted by an invert microscope (Reichert) in the counting chambers.

After DET, cytotoxicity was investigated from the aspect of cell recovery using colorimetric test with tetrazolium salts (MTT test). Upon incubation, the cells were sieved from the tested substances into fresh medium and viable cells were seeded (5×10^3 per 100 μL) in quadriplicate in a 96-well microtiter plate. Plates with seeded cells were tempered at 37 °C in a 5 % CO_2 humidity saturated atmosphere for the next 48 h. Then 10 μl of freshly prepared MTT solution was added to each well and incubation was continued for the next 3 h (at 37 °C, 5 % CO_2). Post incubation, 100 μL of 0.04 mol L^{-1} HCl in 2-propanol was added to each well. The absorbance was determined immediately after incubation on a microtiter plate reader (Multiscan, MCC/340) at 540 and 690 nm. Medium with MTT served as a control.

RESULTS AND DISCUSSION

The FE-SEM images are shown in Fig. 1 indicating that the synthesized coatings cover the Ti surface evenly, as expected from previous research.[1,2]

The coatings consist of agglomerated nanoparticles smaller than 100 nm in size. ACP/TiO_2 agglomerates are larger in size, having a rougher surface in comparison to the ACP/TiO_2/ChOL coating. This morphology enabled high level adhesions of the coatings to the substrate.[2]

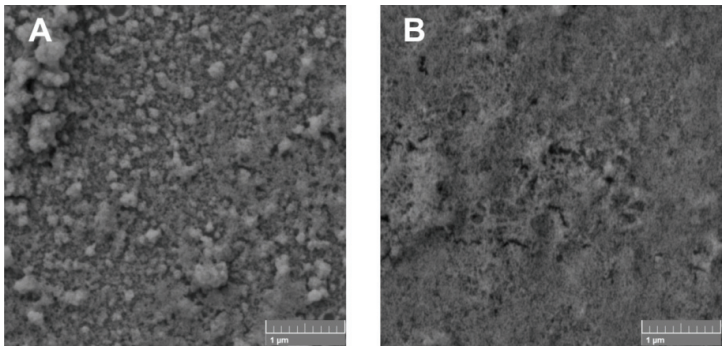

Fig. 1. FE–SEM images of: a) ACP/TiO_2 and b) ACP/TiO_2/ChOL hybrid multifunctional composite coatings on Ti.

The results of cytotoxicity test of DET and MTT assays for MRC-5 cells on Ti, ACP/TiO_2 and ACP/TiO_2/ChOL composite samples are shown in Fig. 2. Cytotoxicity, *CI*, in the DET assay was calculated as $CI = 100(1–N_s/N_k)$, and for MTT assay as $CI = 100(1–A_s/A_k)$, where N_k is the number of cells in the control, N_s is the number of cells in the test samples, A_k is the absorbance of the control and A_s is the absorbance of the test samples.

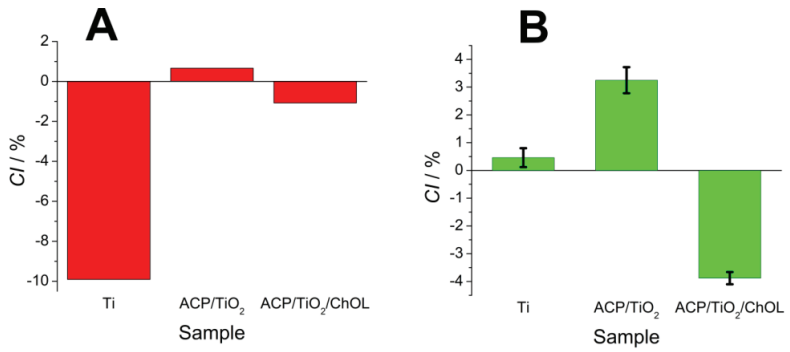

Fig. 2. a) DET cytotoxicity after 48 h and b) MTT cytotoxicity after 48 h + 48 h of recovery for MRC-5 cell line challenged by Ti, ACP/TiO_2 and ACP/TiO_2/ChOL samples.

The results of comparative DET testing of Ti, ACP/TiO_2 and ACP/TiO_2/ /ChOL composite samples for cytotoxicity towards MRC-5 cells are shown in Fig. 2a. Pure Ti and ACP/TiO_2/ChOL samples showed negative cytotoxicity, *i.e.*, there was an increase in the number of viable cells compared to the control. The survival rate of healthy MRC-5 cells on the composite containing ChOL in relation to the control is 101.1 % in the DET test. ACP/TiO_2 did not exhibit a significant cytotoxic effect (<5 %) toward the examined cell line after 48 h. In general, the ACP/TiO_2 samples showed negligible inhibitory effects on healthy MRC-5 cells.

Comparing the obtained results of the DET (Fig. 2a) and MTT assay (Fig. 2b), it could be noticed that the findings are consistent and do not collide with each other. Both the DET and MTT tests showed that there was no significant reduction in the survival of healthy MRC-5 cells in the ACP/TiO_2 composite sample, whereas Ti showed increased cytotoxicity in the MTT assay. Hence, both ACP/TiO_2 and Ti can still be classified as non-cytotoxic to the MRC-5 cell line.[4,8,9]

Besides DET, samples having an ACP/TiO_2/ChOL coating also showed negative cytotoxicity in the MTT test. A more pronounced recovery of MRC-5 human lung fibroblasts cells was observed in comparison to control sample after 48 h of recovery. From these results, it could be concluded that not only the ACP/TiO_2/ChOL multifunctional composite coating is non-cytotoxic, but the presence of ChOL in the coating improves cell proliferation, differentiation and cell viability.

Based on the obtained results, it could be concluded that both composite materials used in the studies are non-cytotoxic to the cell lines used, and that 5 mass % of ChOL has a positive effect on the non-toxicity of the material.

CONCLUSIONS

The cytotoxicity of Ti substrate, ACP/TiO_2 and ACP/TiO_2/ChOL biomaterials-coated Ti for possible application in medicine and stomatology was tested in the reported research. Cytotoxicity tests showed that there was no significant decrease in the survival of healthy MRC-5 cells exposed to the Ti substrate and ACP/TiO_2 composite coatings, whereas an increase in the number of viable cells for the ACP/TiO_2/ChOL coating was noticed. Improved cell proliferation, differentiation and cell viability in the presence of ACP/TiO_2/ChOL was found. Based on the presented results and previously published findings on the physicochemical and bioactive properties of ACP/TiO_2/ChOL composites, it could be concluded that further development of this material and potential preclinical studies would be largely justified.

Acknowledgement. This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grants No. 451-03-9/2021-14/200026 and No. 451-03-9/2021-14/200175).

ИЗВОД

ЦИТОТОКСИЧНОСТ МУЛТИФУНКЦИОНАЛНИХ КОМПОЗИТНИХ ПРЕВЛАКА ОД АМОРФНОГ КАЛЦИЈУМ-ФОСФАТА НА ТИТАНУ ДОБИЈЕНИХ *IN SITU* АНОДИЗАЦИЈОМ/АНАФОРЕТСКИМ ТАЛОЖЕЊЕМ

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Приказани су резултати испитивања цитотоксичности мултифункционалних и хибридних композитних превлака на бази аморфног калцијум-фосфата (ACP) и хитозан-олиголактата (ChOL) на титану према MRC-5 ћелијској линији хуманих фибробласта плућа. ACP/TiO_2 и ACP/TiO_2/ChOL су исталожени новим *in situ* поступком анодизације/анафоретског таложења под константним напоном од 60 V током 180 s на 25 °C. Тестови цитотоксичности су показали да није дошло до значајног смањења преживљавања здравих MRC-5 ћелија код композита који не садржи ChOL, док је код оних са додатим ChOL дошло до повећања броја одрживих ћелија. Резултати показују да је побољшана пролиферација, диференцијација и одрживост ћелија у узорку са ChOL, што овај узорак чини добрим кандидатом за примену у медицини и стоматологији.

(Примљено 11. фебруара, прихваћено 23. марта 2021)

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