Biokompatibilnost z metodo superkritičnega ogljikovega dioksida procesiranih poli(laktid-ko-glikolida) in poli(ε-kaprolaktona) na primarnih človeških osteoblastih

Biocompatibility of supercritical carbon dioxide processed poly(lactide-co-glycolide) and poly(ε-caprolactone) assessed with primary human osteoblasts

Avtor / Author

Ustanova / Institute

Barbara Dariš[1], Polonca Ferk[2], Elena Markočič[3], Željko Knez[3]

[1]Univerza v Mariboru, Medicinska fakulteta, Inštitut za biomedicinske vede, Maribor, Slovenija; [2]Univerza v Mariboru, Medicinska fakulteta, Katedra za farmakologijo in eksperimentalno toksikologijo, Maribor, Slovenija; [3]Univerza v Mariboru, Fakulteta za kemijo in kemijsko tehnologijo, Laboratorij za separacijske procese, Maribor, Slovenija

[1]University of Maribor, Faculty of Medicine, Institute of Biomedical Sciences, Maribor, Slovenia; [2]University of Maribor, Faculty of Medicine, Department of Pharmacology and Experimental Toxicology, Maribor, Slovenia; [3]University of Maribor, Faculty of Chemistry and Chemical Engineering, Laboratory for Separation Processes, Maribor, Slovenia

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Naslov za dopisovanje / Correspondence
Prof. Dr. Željko Knez
Univerza v Mariboru, Fakulteta za kemijo in kemijsko tehnologijo
Smetanova ul. 17, Maribor, Slovenija
Telefon +386 22294461
E–pošta: zeljko.knez@um.si

Izvleček

Namen: *V študiji smo preverjali biokompatibilnost poli(laktid-ko-glikolid) (PLGA) in poli(ε-kaprolakton) (PCL) na primarnih človeških osteoblastih (HOB).*

Metode: *Za preizkušanje proliferacijske aktivnosti celic smo uporabili test WST-8 (Water-soluble Tetrazolium salts). Stopnjo diferenciacije HOB smo ocenjevali s testom aktivnosti alkalne fosfataze.*

Rezultati: *Proliferacijska aktivnost HOB je bila na penah PLGA in PCL zmanjšana v primerjavi s PLGA in PCL, na konstruktih pen PLGA in PCL s hidroksiapatitom (HA) pa zvečana glede na proliferacijsko aktivnost celic na penah PLGA in PCL.*

Zaključek: *Aktivnost alkalne fosfataze na PLGA, PCL in peni PCL se ni razlikovala od kontrole. Na konstruktih pen PCL s HA*

Abstract

Purpose: *In the present study, poly (lactide-co-glycolide) (PLGA) and poly (ε-caprolactone) (PCL) combined with hydroxyapatite (HA) were processed with supercritical CO_2 ($scCO_2$) to obtain macroporous foams.*

Methods: *The in vitro biocompatibility and mineralization of the foams was assessed with primary human osteoblasts (HOB).*

Results: *The WST-8 (Water-soluble Tetrazolium salts) test for cell proliferation activity and alkaline phosphatase (ALP) activity assay were used to estimate HOB proliferation and differentiation respectively. The proliferation activity of HOB on foamed PLGA and PCL decreased compared to non-foamed polymer, while on PLGA and PCL foamed constructs with hydroxyapatite (HA), the proliferation activity increased compared to the foamed PLGA and PCL.*

Conclusions: *The ALP activity on*

je bila aktivnost alkalne fosfataze manjša v primerjavi s kontrolo, medtem ko se na peni PLGA in konstruktih pen PLGA s HA aktivnost alkalne fosfataze ni izrazila.

PLGA, PCL and foamed PCL was similar to the control, being less intense on PCL foamed constructs with HA, while foamed PLGA and PLGA foamed constructs with HA showed no evidence of ALP activity.

INTRODUCTION

Bone grafts are currently used to fill bone defects caused by disease or trauma, such as bone fractures, infections and tumors. Traditional approaches involve the use of autogenous or allogenic bone, although the limited availability (autogenous bone) and possible tissue rejection (allogenic bone) have resulted in the search for other methods to generate new bone grafts for bone substitution (1). These new synthetic materials need to match the physical and chemical properties of the host bone tissue in order to provide a microenvironment for cell-matrix interactions that mimic the biological environment. Furthermore, the materials must be biodegradable, biocompatible and eosteoconductive (2, 3). Poly(lactide-co-glycolide) (PLGA) and poly(ε-caprolactone) (PCL) are biodegradable polymers with widespread use in medicine (4–7). Different processing techniques have been used to optimize the characteristics of these polymers for biomedical applications. In order to further enhance these substrates for bone regeneration, addition of hydroxyapatite (HA) can promote the adhesion and growth of osteoblasts (4, 8), while the foaming of the polymers enables the development of porous structures suitable for three-dimensional colonization, proliferation and differentiation of cells (6, 9). Among the foaming techniques, supercritical CO_2 ($scCO_2$) foaming is a very promising technique for synthetic polymer processing, since porous scaffolds are made without the use of organic solvents that are potentially harmful for cells. Furthermore, this foaming technique allows controlling the size and distribution of the pores through proper selection of processing conditions, mainly solubilization pressure (gas concentration in polymer), foaming temperature and depressurization rate (9, 10). Despite several studies on the $scCO_2$ foaming of PLGA, PCL and their composites with HA have already been performed (6, 9–13), further optimization is required to tune the osteoinductive and osteoconductive properties of the scaffolds which would enhance the attachment, proliferation and differentiation of cells both in vitro and in vivo. Furthermore, there is a lack of research focused on the biocompatibility of such polymers on primary human osteoblast cells. In our study, we used a modified technique of gas foaming with $scCO_2$ in order to obtain polymeric and composite three-dimensional scaffolds suitable for human osteoblast growth and differentiation. For this purpose, we used PLGA, PCL and their composites with HA. The biocompatibility of these biomaterials before and after foaming was tested in vitro on primary human osteoblasts. Here, we report the preliminary results of the study.

MATERIALS AND METHODS

PLGA (50/50 DL-lactide/glycolide) copolymer (MW = 220,000 g/mol) was provided by Purac (The Netherlands). PCL (MW = 80,000 g/mol) and HA were obtained from Sigma-Aldrich (Germany). Carbon dioxide (CO_2) was obtained from Messer, Slovenia. All reagents were used as received without further purification.

Polymer discs preparation

PLGA and PCL were separately processed by compression molding in a stainless-steel die using 1 MPa pressure and shaped into 12 mm discs of 2–3 mm thickness. .

Hydroxyapatite composite materials

Both polymers were separately mixed with HA at a weight ratio of 1:1 in the presence of $scCO_2$, which was used as a plasticizer. After mixing, the composite materials were subsequently shaped into discs as previously described.

Foam preparation

Foams of PLGA and PCL, as well as their corresponding composites with HA, were obtained using the gas foaming technique in the absence of solvents. Briefly, the substrate was saturated with $scCO_2$, followed by rapid depressurization at constant temperature (pressure quench). The samples were then shaped into discs (surface area 1,86 cm2), fixed in cylindrical polyethylene molds and then placed in a high pressure autoclave. The autoclave was sealed and placed in an oil bath to reach a working temperature of 313 K. After the working temperature was reached, the vessel was filled with CO_2 to a 20 MPa pressure. The samples were kept at the described temperature and pressure for 6 hours, allowing the saturation of the polymer with CO_2. Once the pressure was decreased, the samples were spontaneously foamed. The foamed samples where then removed from the vessel and dried in air for 2–3 days at room temperature in order to allow the remaining CO_2 to diffuse from the substrate. The foams were then cut into discs using a surgical knife. The pore sizes of PLGA foams (PLGA_f), PCL foams (PCL_f), PLGA composite foams (PLGA-HA_f) and PCL composite foams (PCL-HA_f) were 50–100 µm, 100–180 µm, 10–50 µm and 100–120 µm, respectively (Fig. 1, Fig. 2).

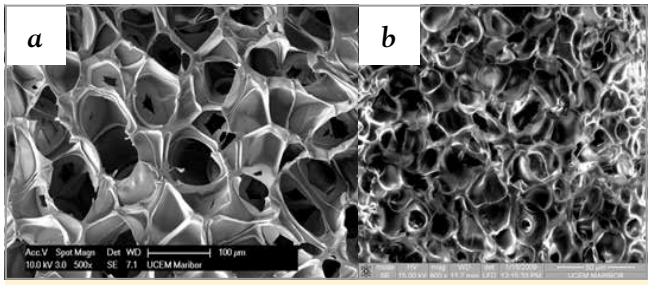

Figure 1. *Morphology of foamed poly(lactide-co-glycolide) (PLGA) samples: a) PLGA foam (PLGA_f); b) PLGA-hydroxyapatite composite foam (PLGA-HA_f).*

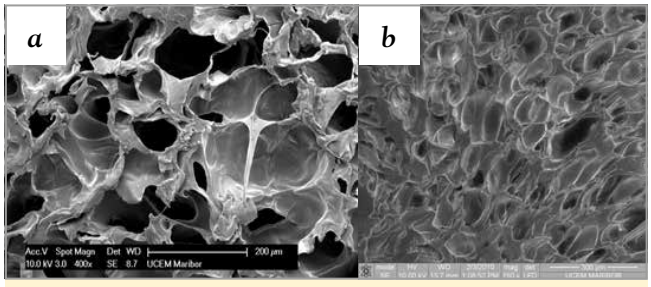

Figure 2. *Morphology of foamed poly(ε-caprolactone) (PCL) samples: a) PCL foam (PCL_f); b) PCL-hydroxyapatite composite foam (PCL-HA_f).*

Cell cultures

Primary human osteoblasts (HOB) were purchased from PromoCell® (Heidelberg, Germany) and cultured as recommended by the supplier in Osteoblast Growth Medium (PromoCell, Germany). Cells were plated in T25 culture flasks (TPP, Switzerland) and incubated at 37°C with 5% humidified CO_2. Media was replaced every 3–4 days. All trials were carried out using 4th cell passage. The cells were detached using Detach Kit (PromoCell, Germany) and seeded on the different test substrates. Cells grown on cell culture plastic were used as control.

Cell proliferation assay

To evaluate cell proliferation, osteoblasts were seeded at a density of 2 x 104 cells/well in 24-well plates (TPP, Switzerland) containing polymer discs and polymer and composite foams. The cell growth was assessed after 5 days of culture under standard conditions by a WST-8 assay (PromoKine, Germany) according to the manufacturer's recommendations. Briefly, 50 µl of the WST-8 solution and 500 µl of fresh complete media were added to each well. The mixture was incubated for 1 hour at 37°C. After the incubation period, the extent of reaction was measured at an absorbance of 450 nm using the Infinite® 200 PRO microplate reader (Tecan, Switzerland).

Alkaline phosphatase assay

Osteoblasts were seeded at a density of 2 x 104 cells/well in 24-well plates (TPP, Switzerland) containing polymer discs and polymeric based foams. The alkaline phosphatase activity was histochemically determined after 5 days of culture by treatment with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma Fast™BCIP/NBT; Sigma Aldrich, USA) according to manufacturer's instructions. At the specified time point, osteoblasts were washed with phosphate buffered saline solution (PBS), fixed with 10 % formalin (Sigma Aldrich, USA) and then stained using

BCIP/NBT substrate solution. The stained samples were visualized using a phase contrast microscope (Leica DMI 6000B; Leica, Germany). The percentages of cells stained positive were estimated in duplicates and the mean values were calculated.

Statistical Analysis

The average values of tetraplicates were calculated and standard deviations were calculated unless otherwise specified. Statistical differences between the groups were determined using SPSS version 20.0 software (SPSS, Inc., Chicago, IL, USA), with an independent samples t-test. $P < 0.05$ was considered as the threshold value indicative of statisically significant differences.

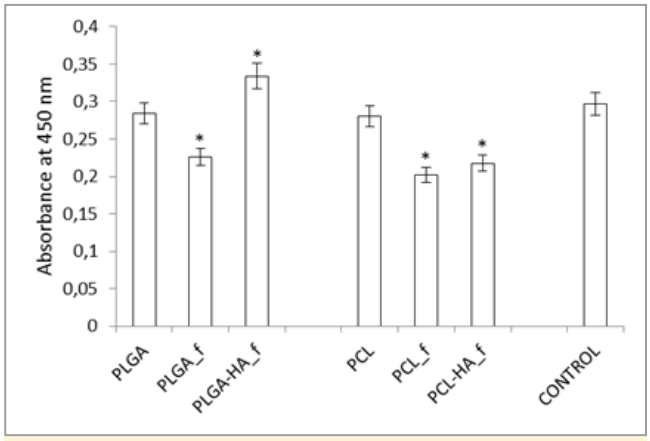

Figure 3. *The biocompatibility on primary human osteoblasts for poly (lactide-co-glycolide) (PLGA), poly (ε-caprolactone) (PCL) and their composites with hydroxyapatite (HA) before and after $scCO_2$ foaming. Proliferation activity of HOB.*
*Average absorbance values of tetraplicates and standard deviations are shown: * = statistically significant differences ($P < 0.05$), PLGA_f = foamed PLGA; PLGA-HA_f = PLGA-HA composite foam; PCL_f = foamed PCL; PCL-HA_f = PCL-HA composite foam.*

RESULTS

Cell proliferation assay

As reported in Figure 3, in all cases the results were comparable to the control.
Foaming of PLGA and PCL resulted in polymer structures that caused decreased proliferation activity of HOB compared to non-foamed PLGA and PCL. Higher HOB proliferation activity was observed on foamed composites with HA compared to foamed PLGA and PCL.

Alkaline phosphatase assay

The results of the alkaline phosphatase staining test on non-foamed PLGA and PCL, and on PCL_f were similar to the ones obtained for the control; the staining was less intense on PCL-HA_f, while on PLGA_f and PLGA-HA_f samples no alkaline phosphatase staining was observed (Table 1).

DISCUSSION

Current strategies to substitute injured or damaged bone seek the use of novel biomaterials that match the microenvironments found in native tissue. An important aspect in the design of these materials is to have excellent biocompatibility as well as biodegradability. Furthermore, since bone is composed mainly of calcium phosphate in the crystallographic form of HA, the presence of HA in the materials may render osteoconductive properties, which are essential for the regeneration of bone. For this purpose, in the present study, biocompatible and biodegradable PLGA and PCL foams were prepared and further enhanced by the incorporation of HA. In order to assess their biocompatibility as well as their ability to induce cell mineralization, HOB cells were seeded on the materials to observe cell-material interaction.

The present study used $scCO_2$, which poses several advantages over other foaming techniques. For instance, the current technique avoids the use of organic solvents, which reduces cell viability and limits the possible incorporation of biological molecules within the structure of the polymers to be used as a drug delivery vehicle. Furthermore, the plasticizer effect significantly reduces the melting temperature and the viscosity of the polymer, allowing excellent incorporation and homogenous dispersion of the ceramic filler (14). This was mainly related with the high pressure of CO_2, which allowed high amounts of gas to be absorbed into the substrate (15, 16).

Table 1: *The biocompatibility on primary human osteoblasts for poly(lactide-co-glycolide) (PLGA), poly(ε-caprolactone) (PCL) and their composite with hydroxyapatite (HA) before and after $scCO_2$ foaming. Alkaline phosphatase assay results presented as the percentage of cells stained positive (mean values).*

Sample	PLGA	PLGA_f	PLGA-HA_f	PCL	PCL_f	PCL-HA_f	Control
Alkaline phosphatase intensity (%)	77	0	0	69	75	53	88

PLGA_f = foamed PLGA; PLGA-HA_f = PLGA-HA composite foam; PCL_f = foamed PCL; PCL-HA_f = PCL-HA composite foam.

The foaming of PLGA and PCL with $scCO_2$ resulted in a macroporous structure that decreased cell proliferation activity compared to the control. Similar results were previously obtained by Zhu et al. (17) using human hepatoma cell line (Hep3B) on $scCO_2$ processed PLGA. Tayton et al. (18) seeded mesenchymal stem cells on $scCO_2$ foamed poly(DL-lactide-co-glycolide) (PDLLGA) materials, showing increased cell number. Nevertheless, despite the use of similar materials, the previous study presented different processing methods and obtained different polymeric features. The results changed after the incorporation of HA into $scCO_2$ PLGA and PCL foams, showing increased cell proliferation, hence proving the potential of HA to stimulate the proliferation of bone related cells (3, 8, 9, 19–21). The rationale mainly relates to the surface properties of HA, which is known to have a hydrophilic nature, which differs to the hydrophobic nature of synthetic polymers; this is believed to enhance adhesion and proliferation of cells (3, 21). Furthermore, since its chemical composition is close to that of bone mineral, HA is expected to play a significant role in bone regeneration (19, 22). The physical and mechanical properties of PCL/HA composite scaffolds synthesized in $scCO_2$ were already evaluated; however, no biocompatibility studies were made (23).

Regarding the ALP activity, the results showed that the ALP activity of HOB cells was positive on non-foamed PLGA, which confirms the observations from previous studies (4). On the other hand, HOB cultured on $scCO_2$ foamed PLGA and $scCO_2$ foamed PLGA-HA expressed no alkaline phosphatase activity. Opposite to our results, previous studies showed an increased ALP activity expressed by the cells exposed to foamed polymers (13, 24, 25). Nevertheless, the results are not completely comparable since one of the studies presented PLGA foams fabricated by a solvent-casting particulate-leaching technique (25), while two other studies used different polymers (poly-D-lactic acid and PDLLA) (13, 24). Additionally, we observed high alkaline phosphatase activity on HOB exposed to non-foamed PCL, which is in accordance to previous findings (3). The ALP activity of HOB cells seems to be maintained on foamed PCL, while the addition of HA into foamed PCL resulted in a slight decrease of ALP activity.

In conclusion, according to our knowledge, this is the first study that has tested the biocompatibility of PLGA and PCL processed with our modified $scCO_2$ foaming method with human primary osteoblast cells. Proliferation activity of HOB on foamed PLGA and PCL decreased compared to non-foamed polymers and increased on PLGA and PCL foamed constructs with HA compared to foamed PLGA and PCL. The ALP activity of cells cultured with non-foamed PLGA and PCL, and on foamed PCL was similar to the control, whereas it was less intense on PCL foamed constructs with HA and was not present on foamed PLGA and PLGA foamed constructs with HA. Nevertheless, these results are preliminary and further research is already in progress to better understand the behavior of the polymeric and composite biomaterials in the presence of human cells.

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