Synthesis and Biocompatibility Evaluation for a Phosphorylated Polyvinyl Alcohol Derivative

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Polyvinyl alcohol and phosphorylated functionalized derivatives are widely used in tissue engineering and regenerative medicine. The present work aims to characterize chemically and biologically two types of samples based on phosphorylated polyvinyl alcohol. Synthesis and biocompatibility assays for a porous polymer scaffold based on PPVA reticulated by glutaraldehyde and foamed by $CaCO_3$ in different ratios are shown above. Swelling index for the PPVA hydrogel goes up with the decrease in reticulation agent that has been used (glutaraldehyde) for foam production. A minimal recorded pH shift had no influence for the further development of in vitro cell assays. Moreover, samples immersed into SBF for 48h maintained their mechanical stability. Contact inhibition assay and imaging performed for 72 hours demonstrated a good cell proliferation in contact with the material sponge-like samples. Osteoblast-like cells recorded important viability in contact with both type of foam samples. The proposed PPVA-$CaCO_3$ composite mainly conditioned as a foam represent a cheap and feasible solution for a biocompatible osteoconductive porous scaffold destined to tissue engineering.

Keywords: *polyvinyl alcohol, hydrogel, phosphorylation, porous polymer scaffold, osteoblast-like cell*

Polyvinyl alcohol (PVA) represents a synthetic polymer with important biomedical applications, including drug-delivery systems, molecular adsorbents, flame-retardants, ion-exchangers. PVA based hydrogels are being used as scaffolds for cell proliferation and differentiation, thus getting useful as support for regenerative medicine and tissue engineering [1-5]. PVA was also extensively used to produce nanostructured polyethylene-glycol, hyaluronic acid or dextran based hydrogels [6]. PVA functionalization by phosphorylation have been demonstrated as a support for in vitro calcium phosphate mineralization [7, 8]. PVA foams are hydrophilic and unstable in aqueous environment thus requiring reticulation by glutaraldehyde – which is however toxic for the cells. PVA applications in tissue engineering are quite limited by its reduced ability to induce protein/cell adhesion [9, 10]. However, numerous OH groups in PVA may be useful in polymer functionalization.

The aim of the present work was to develop synthesis and biocompatibility assays for a porous polymer scaffold based on PPVA reticulated by glutaraldehyde and foamed by $CaCO_3$ in different ratios. Foam samples were chemically characterized and biologically tested in the presence of osteoblast-like cells.

Experimental part

PVA was partially phosphorylated using H_3PO_2 instead of H_3PO_4. Chemical characterization of the phosphorylated polyvinyl alcohol (PPVA) samples was performed by FTIR (Fourier Transformate Infrared Spectroscopy) using a Vertex 7 spectrometer (Brucker Ltd Co).

PPVA foam samples preparation

Powdered $CaCO_3$ and PPVA in different amounts have been added in 100mL distilled water and mechanically stirred on a boiling water bath for 1h. The mixture was cooled down at room temperature and added by a stoechiometrically equal volume of 5mM HCl. The mixture was intensely stirred to dissolve $CaCO_3$ and to generate CO_2 bubbles. The resulted bubbles were sequestered in the foam by snap-freezing the mixture at -80°C for 4 hours. Foam samples were thawed at room temperature and cut in 1x1x1 cm cubes in hydrated state. Sample cubes were placed in aqueous glutaraldehyde (GA) solution at 20°C for 72h. Then samples were washed 5 times in distilled water for 24h each time. Prior to biological assays, foam samples were dried and hydrated for 24h in sterile phosphate saline buffer (PBS, Sigma #D8662).

Foam samples were cut in sterile conditions under laminar flow in the IInd Class cell culture hood using a scalpel. Each sample was approximate to a cube with 1 cm edges. Prior to placement in cell culture, all samples were sterilized by UVA in a transilluminator for 30 minutes.

Sample hydration was performed by incubating cubic foam samples in 1 mL culture media used for cell proliferation and assay in each well of a 24-well plate. At 24h from incubation, medium was removed and samples were carefully placed in a 6-well plate.

Cell lines and culture methods

The experimental design included human osteosarcoma (SaoS2) cell line cultures. Cells were purchased

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from PromoCell Austria, proliferated in 75cm^2 sterile, cell culture ready flasks, and splitted using McCoy5A culture media supplemented by 15%FCS (fetal calf serum), 1% Glutamax and 1% antibiotic/antimycotic. SaoS2 cells were detached by TrypLE trypsin (2mL for 3 min in a 75cm^2 cell culture flask), while trypsin inactivation was induced by 10 mL complete culture media. Cells were gently mixed to detach them from clumps and counted by an automated cell counter (Countess Invitrogen) prior to plate dispensing. SaoS2 osteosarcoma cells have been added at a density of 1x10^6 cells/well. Plates with samples and cells were incubated for 24, 48 and 72h at 37°C 5%CO_2 and 99.6% humidity.

Cell viability/proliferation assays

CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used to assess ATP levels in the cells applied and proliferated on the samples. Manufacturer re-commendations and concentrations have been used.

Swelling test

Swelling test was performed for both sample types. Dried samples from both foams (PPVA:$CaCO_3$ 1:1 and 2:1) weighting 15 to 20mg were immersed in water or saline for different durations. Water or saline excess was removed by filter paper. The swelling degree was evaluated according to formula W% (Weight swelling degree) = $100(W_1-W_0)/W_0$, where W_1 signals the hydrated foam and W_0 the dried status.

PPVA foam morphology

Pore size in foam samples represents an important element for a polymer support to be used as scaffold for normal cell lines. Average pore size should range between 150-500μM to allow a convenient cell migration into the scaffold. To estimate pore size, both in hydrated and dried forms, samples were explored by scanning electron microscopy following freeze-fracture procedure. Samples were snap-frozen in liquid nitrogen following treatment with cryoprotective agent, and then fractured to expose the foam core. Exposed fractured foam surface was vacuum-sprayed and covered by a thin gold-layer to improve shadowing and contrast. Samples were examined by a FEI Quanta 3D scanning electron microscope. All measurements have been performed by using the FIJI_ImageJ open source software [11,12].

pH evaluation

*p*H variations in simulated body fluid (SBF) were assayed in a mixture performed according to Tas, Kokubo and Takadama [13, 14]. The mixture at *p*H 7.4 included NaCl (0.327g, 112 mM), $NaHCO_3$ (0.113g, 27mM), KCl (0.018g, 5mM), $Na_2HPO_4.2H_2O$ (0.009g, 1mM), $MgCl_2.6H_2O$ (0.015g 1.5mM), 1M HCl (<0.15mL), $CaCl_2$ (0.013g, 2.5mM), Na_2SO_4 (0.003g, 0.5mM), Tris buffer (0.303g, 50mM). Samples from both foam compositions (PPVA:$CaCO_3$ 1:1 and 2:1) were incubated in SBF for 48h at 37°C in the 5% CO_2 incubator.

Results and discussions

FTIR analysis for PPVA reticulated by GA showed a specific absorption band on ν=1642cm^{-1}. This event is supposed to be the result of water removal on the polymer chain, with polyenes formation [15,16,17]. Absorption band for phosphate group is associated with ν = 1170-1350 cm^{-1} [15,18] Cyclization and reticulation can affect the vibrations in the phosphate group which is found at ν=1178cm^{-1} for the current samples (fig. 1). Maintaining low levels for the absorption band on C=O bonds at ν=1723cm^{-1} advocates for a complete reaction of the aldehyde groups in glutaraldehyde (GA) with the hydroxilic groups (O-H) in PVA. Moreover, extended absorpion band for C-O bond at about 1100cm^{-1} for plain PPVA is replaced by a wider absorption band (ν=1040-960cm^{-1}) for reticulated PPVA; this event can be related to the C-O bonds or acetal rings (C-O-C) as being formed during GA reticulation step [19].

While PPVA is a matrigel able to include and deliver water-soluble particles, it is important to determine the foam sample behavior related to water/saline solutions. Present results show comparative data according to PPVA/$CaCO_3$ ratio. PPVA is highly hydrophilic and its swelling is occurring instantly. Even if most of the water volume is retained by the sample in the first 10-30 minutes from hydration onset, the process is ongoing and lasts for at least 24 h (fig. 2). The swelling index was more important in saline than in water.

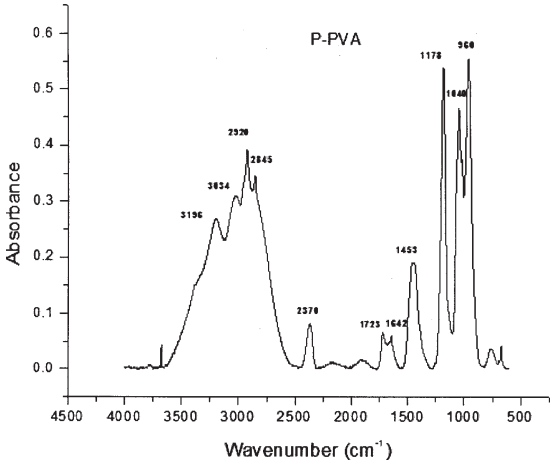

Fig. 1. FT-IR spectra for PPVA reticulated by GA

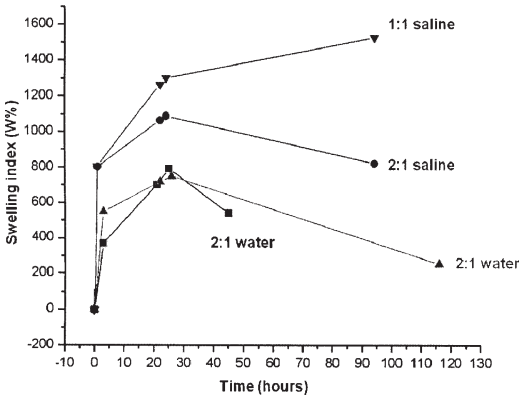

Fig. 2. Swelling test for PPVA in water and saline for PPVA:$CaCO_3$ 1:1 and 2:1 samples

Swelling index for the PPVA hydrogel goes up with the decrease in reticulation agent that has been used (glutaraldehyde) for foam production. The swelling threshold is reached at 3 hours for the highest reticulation degree. Beyond this 3h-threshold, mainly for lower reticulation degree foams, the swelling index is reduced (fig. 3).

Pore size imaging, performed by scanning electron microscopy, demonstrated a pore size reduction for the partially hydrated foam samples. Image analysis regarding pore size distribution demonstrated a higher homogeneity for the sample 1:1 than for 2:1. However, pore

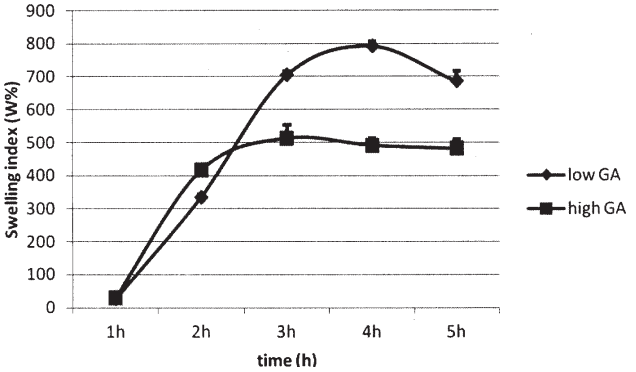

Fig. 3. Swelling index for PPVA foam samples (W%) according to reticulation degree

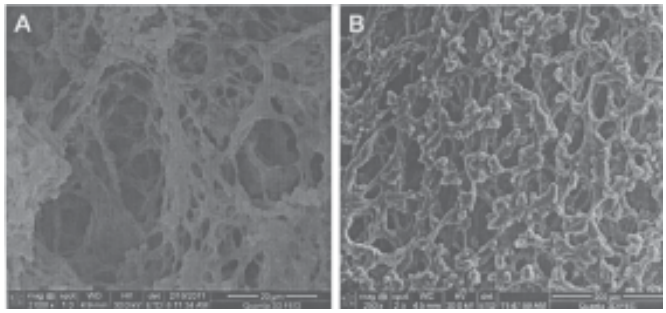

Fig. 4. Pore size in foam samples (A – PPVA:$CaCO_3$ 1:1; B – PPVA:$CaCO_3$ 2:1)

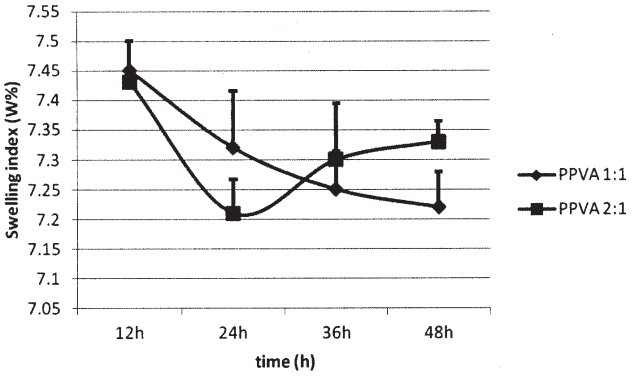

Fig. 5. *p*H variations in the two foam samples in SBF for 12-48h

interconnecting bridges were thicker for PPVA 2:1, representing an enhanced support for cell adhesion (fig. 4). The average pore size ranged from 59-141μm for PPVA 1:1 to 2-51μm for PPVA 2:1. For a better cell penetration into the foam, the pore size should exceed 30μm, indicating the PPVA 1:1 as a better candidate for a larger pore design (by varying the HCl:$CaCO_3$ ratio during foaming process).

As a general observation, pore distribution in the foams is quite uniform, but with pore size and shape variations. Pore size is also dependent on the hydration degree of each foam type, and this should be considered in respect to cell morphological profile that is intended to be applied on this biomaterial.

*p*H values showed slight acidic shift following foam samples immersion for 48h in SBF. Samples from PPVA 1:1 decreased the *p*H for the SBF to 7.22 while the PPVA 2:1 sample decreased the *p*H only to 7.32 (fig. 5). This minimal *p*H shift has no influence for the further development of in vitro cell assays. Moreover, samples immersed into SBF for 48h maintained their mechanical stability.

Contact inhibition assay and imaging performed for 72 hours demonstrated a good cell proliferation in contact with the material sponge-like samples. Cell density seems to improve in close contact with both samples (fig. 6).

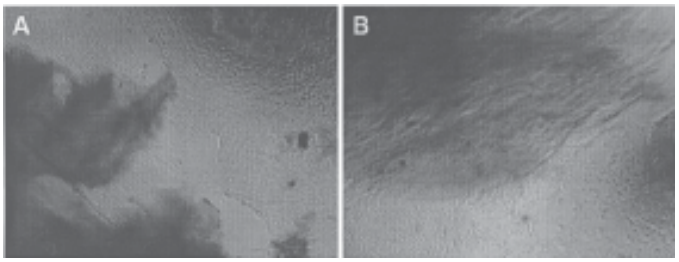

Fig. 6. SaoS2 cells in contact with foam samples (A – PPVA:$CaCO_3$ 1:1; B – PPVA:$CaCO_3$ 2:1); Phase contrast microscopy, 100x.

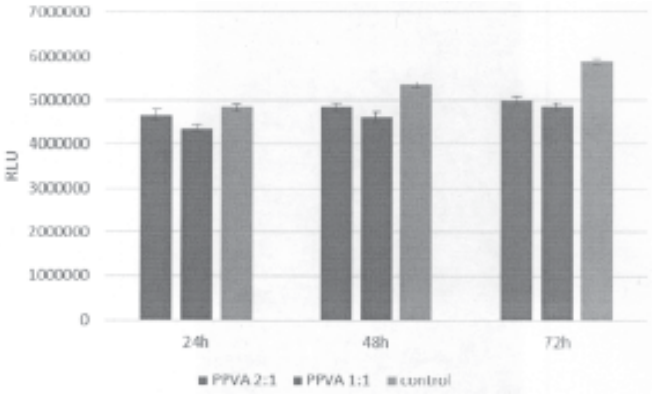

Fig. 7. Comparative SaoS2 viability on both PPVA foam samples, performed by CellTiter Glo (ATP metabolic assay)

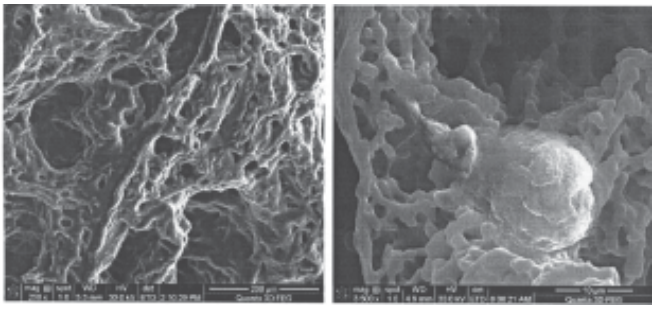

Fig. 8. Osteoblast like-cells on PPVA 2:1 samples in scanning electron microscopy

SaoS2 cells recorded important viability by ATP assay in contact with both type of foam samples (fig. 7) Viability for PPVA 2:1 ranged from 97.84% at 24h to 92.45% at 72h; the slight viability recorded dim was consistent with the slight time-related *p*H shift to values lower than 7.2. SaoS2 cell viability in contact with PPVA 1:1 samples ranged from 96.77% at 24h to 90.31% at 72h. Viability rate was better in samples from PPVA 2:1; however osteoblast-like cell viability in contact with both samples was elevated at 24 and 72h, compared to the control.

Cells grown and proliferated on sample surface were detected by scanning electron microscopy, for the PPVA-$CaCO_3$ 2:1 sample only, regarding its best biocompatibility (fig. 8).

In figure 8 SaoS2 osteoblast-like cells cand be observed growing on the PPVA 2:1 sample, demonstrating not only cell-adhesive properties of this surface but also a good biocompatibility.

The present work proposed the synthesis, chemical and biological characterization of a PPVA-based foam intended to represent a porous biodegradable scaffold for osteoblast-like cells as a further support for bone-cartilage tissue engineering. Some recent papers evaluated PPVA biocompatibility in various formulations and combinations as a scaffold for tissue engineering. PPVA-hydroxylapatite composite was obtained as a compact block by thermal

preparation and phase separation, being subsequently hydrated [20]. Despite the details regarding preparation for this scaffold, no biocompatibility assay was performed. Lactic acid-esterified PPVA was prepared as films that have been characterized by MRI spectroscopy, X-ray crystallography and FTIR while biocompatibility assays included only the MTT assay [21]. Electrospun nanofibers from PPVA have been assayed as a potentially osteoconductive and osteoinductive material (colloids surf). The proposed composite is based on PVA, a synthetic polymer widely used for biomedical applications. PVA phosphorylation induces a special functionalization and a 7-fold increase of hydrophilicity [17-20]. The idea behind the experiment was to generate a microporous PPVA scaffold, produced by an original protocol and to assay its biocompatibility against osteoblast-like cells.

Conclusions

In this study was presented the obtaining and biocompatibility assays on a porous PPVA-$CaCO_3$ composite. Samples included two PPVA-$CaCO_3$ ratios 1:1 and 2:1. Osteoblast-like cells showed excellent viability in contact with both type of foam samples, while ATP levels indicated very good metabolic cell activity in contact with samples. pH evaluation in SBF showed minimal acidic shift for both samples, with no influence for the further development of in vitro cell assays. The proposed PPVA-$CaCO_3$ composite mainly conditioned as a foam represent a cheap and feasible solution for a biocompatible osteoconductive porous scaffold destined to tissue engineering.

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Manuscript received: 15.03.2015

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