Culture of porcine Vascular Wall - Mesenchymal Stem Cells on 3D biodegradable highly porous mineral doped Poly (α-hydroxy) acids scaffolds

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Abstract

Background Vascularization is a crucial factor when approaching any engineered tissue. Hybrid biomaterials composed of biodegradable synthetic polymers and inorganic materials showed suitable properties for biomedical application. Being Vascular Wall – Mesenchymal Stem Cells derived from pig thoracic aorta (pVW-MSCs) an excellent in vitro model to study vascular remodelling due to their strong angiogenic attitude, the aim of the present study was to demonstrate the angiogenic potential of experimental highly porous scaffolds based on polylactic acid (PLA) or poly-e-caprolactone (PCL) doped with calcium silicates (CaSi) and dicalcium phosphate dehydrated (DCPD), namely PLA-10CaSi-10DCPD and PCL-10CaSi-10DCPD.

Results The results obtained clearly demonstrated that the cells colonized the scaffolds and were metabolically active. Moreover cells, grown in these 3D systems, showed the typical gene expression profile they have in control 2D culture, although with some main quantitative differences. DAPI staining and immunofluorescence assay confirmed cellular presence on both scaffolds, however pVW-MSCs cultured in PLA-10CaSi-10DCPD showed an individual cellular growth, in contrast, in PCL-10CaSi-10DCPD scaffolds pVW-MSCs grew in spherical clusters.

Conclusion In conclusion, the property of the experimental scaffolds demonstrated the suitability of the scaffolds to be colonized by vascular stem cells for new vessels formation and a potential application in tissue regeneration for biomedical purpose.

Background

Vascularization is a crucial factor when approaching any engineered tissue (1). A proper vascular network should allow the transport of oxygen, nutrients and growth factors, as the diffusion limit to maintain viable cells is only 400 μm (2). Moreover, vascular network should also act as a transport system for hormones, waste products, toxic and non-functional substances (3), and should be a source of growth factors locally produced by endothelial cells, such as pro-angiogenic morphogenetic factors (4)

In post-natal life, vascular remodeling is main due to angiogenesis process. This process takes place through cell recruitment and cell differentiation with the involvement of growth factors such as vascular endothelial growth factor (VEGF) and Fibroblast growth factor–2 (FGF–2) (5).

It has been proposed that many tissue-specific stem cells/progenitors could be involved in neoangiogenesis (6). Among Vascular Stem Cells (VSCs), Vascular Wall-Mesenchymal Stem Cells (VW-MSCs), residing in the wall of the large vessels, present excellent characteristics in inducing new vascularization (7)(8) (9).

A new method to isolate and culture vascular wall mesenchymal stem cells (pVW-MSCs) from the tunica media of pig thoracic aorta was described previously (10). These cells have excellent pro-angiogenic features either for their ability of differentiating in endothelial cells and the capacity to sustain a capillary

network (11). Moreover, recently it has been demonstrated a pro angiogenic features of pVW-MSCs secretome (12). Overall pVW-MSCs are candidates as an excellent model for translational medicine thanks to the high similarity between human and pig also in the field of stem cells.

Biodegradable synthetic polymers such as polylactic acid (PLA) and poly-e-caprolactone (PCL) are currently used for scaffold design (13–15). Moreover, hybrid biomaterials composed of biodegradable synthetic polymers and inorganic materials showed suitable property for biomedical application, also in the field of engineered tissue.

Interestingly it has been demonstrated that some ions are reported to have a significant role in neo-angiogenesis, including Silicon (Si). Silicon is an important element for angiogenesis induction during new bone formation, and for the acceleration of bone regeneration (5,16).

Bioactive calcium silicate-based materials (CaSi) may provide interesting advantages in this context in relationship to their chemistry as expose silanol groups and release silicon (16–18). CaSi demonstrated bio interactive properties (18,19) and the ability to induce the differentiation of different population of cells, such as orofacial bone mesenchymal stem cells (20), cementoblasts (21), pulp cells (19) and oral derived periapical cyst mesenchymal stem cells (22). The combination of calcium phosphates, such as dicalcium phosphate dehydrate (DCPD) to CaSi materials demonstrated to enhance their biological properties and apatite-forming ability (23) (18).

Moreover, when used as filler in a polymeric matrix, the high alkalinizing ability may counterbalance the acidic degradation products synthetic poly-α-hydroxyl polymers (24,25). These properties explain their role as filler in tissue engineering. Different scaffolds were designed for angiogenic and osteogenic purposes using bioactive calcium silicates (26).

Recently, highly porous PLA and PCL scaffolds doped with bioactive calcium silicates and calcium phosphates have been produced (24,25). PLA mineral doped scaffolds demonstrated the ability to be colonized by oral derived mesenchymal stem cells and to stimulate their shift through osteogenic lineage (22). Highly porous PCL mineral doped scaffolds showed interesting biointeractive properties and the ability to nucleate apatite, enhancing pure PCL biological characteristics (25).

The aim of the present study was to evaluate the ability of pVW-MSCs to colonize experimental highly porous Poly-α-hydroxyl based mineral doped scaffolds, and to express their angiogenic potential.

PLA- and PCL-based scaffolds doped with CaSi and DCPD were tested in a 3D *in vitro* model and traditional 2D culture was used as control.

Results

Surface porosity evaluated by the morphometric analysis on ESEM images

The mean surface porosity of PLA scaffolds, evaluated on three random areas at 500× and 1000× was 45.49% and 51.08%, respectively (Figure 1a). PLA–10CaSi–10DCPD scaffolds showed a mean porosity, evaluated on three random areas at 500× and 1000× magnifications was 31.51% and 26.94%, respectively (Figure 1b). PCL scaffolds mean surface porosity, evaluated on three random areas at 500× and 1000× magnification, was 51.58% and 52.22%, respectively (Figure 2a). PCL–10CaSi–10DCPD mean surface porosity, evaluated on three random areas at 500× and 1000× magnifications, was 41.42% and 41.65%, respectively (Figure 2b).

Cytocompatibility

To investigate the pVW-MSCs ability to colonize biomaterials, we quantified the cell seeding efficiency (CSE) (Figure 3). The results indicated a significant efficiency rate already at 24 h, both for PLA or PCL matrix (≥ 90%), the presence of additives did not influence the cell seeding efficiency.

Metabolic activity

In order to measure the metabolic activity of pVW-MSCs grew on scaffolds, a MTT based assay method was performed on cells 24, 48 and 72 hours post seeding. Our results demonstrated that pVW-MSCs maintain a metabolically active state when seeded on all the materials, nevertheless, after 48 hours cells seeded on PLA and PLA–10CaSi–10DCPD showed higher metabolic activity compared to cells seeded on PCL or PCL–10CaSi–10DCPD (Figure 4).

Effect of biomaterials on pVW-MSCs markers

pVW-MSCs cultured in PGM in presence of different scaffolds expressed all the studied genes (Figure 5). Moreover, qPCR analysis revealed a statistical significant downregulation of α-SMA in pVW-MSCs cultured on PCL scaffolds, in relation to the pVW-MSCs cultured under standard 2D condition (CTR) (Figure 5). The presence of CaSi and DCPD additives restored the basal level. On the contrary PDGFR-β was always decreased in both scaffolds in presence of CaSi and DCPD.

pVW-MSCs distribution on different scaffolds

DAPI staining and immunofluorescence assay for alpha-tubulin confirmed cellular presence on both kind of scaffold. Interestingly, a difference in cellular distribution was observed. In PLA–10CaSi–10DCPD scaffold pVW-MSCs grew individually with interconnection by cytoplasmic prolongations (Figures 6c, e), in contrast, in PCL–10CaSi-DCPD scaffolds, pVW-MSCs grew in spherical cluster (Figures 6d, e). The same distribution was observed also in pVW-MSCs cultured on the non-doped scaffolds (data not shown).

Discussion

Hybrid biomaterials composed of biodegradable synthetic polymers and inorganic materials showed suitable property to use for biomedical application, also in the field of engineered tissue (13,26).

Biodegradable synthetic polymers such as based on poly-α-hydroxyl acids (as PLA and PCL) are currently used for scaffold design due to their biocompatibility, degradation into non-toxic components, long shelf-life, easy process ability, low cost and possibility to be customized/adapted to the surgical site (13,15). Highly porous Poly-lactic acid scaffolds doped with bioactive calcium silicates and calcium phosphates have been produced (24) and demonstrated the ability to be colonized by oral derived mesenchymal stem cells and to stimulate their shift through osteogenic lineage (22). Calcium silicates/poly-e-caprolactone 3D printed scaffolds were produced for dental pulp tissues revascularization purposes and showed good dental pulp stem cells proliferation and osteogenic differentiation (27)

Being Vascularization a crucial factor when approaching any engineered tissue, new scaffolds, able of promoting local angiogenesis, could represent a multi-approach strategy to obtain best result in engineered tissue.

In the present study the scaffolds showed a uniform surface porosity. Both PCL scaffolds formulations (pure PCL and mineral doped polymer) revealed higher surface porosity than PLA formulation. These values were in line with a recently published paper (24,25)

Several studies demonstrated that mineral doped formulations revealed the ability to release biologically relevant ions (Ca and OH) and to nucleate apatite on its surface. Both ions are of particular interest for new bone tissue formation, as Ca ions act as a powerful signal for mineralizing cells (28,29) and alkaline environment demonstrated to increase apatite nucleation (30).

Si is an important element for angiogenesis induction during new bone formation and for the phases of bone regeneration (5,16). Indeed Si, was localized in active calcification sites (osteoid) of young bone (31).

Si from CaSi (such as bioglasses, ackermanite, wollanstonite) induce angiogenesis by increasing gene expression of pro-angiogenic cytokine (VEGF and FGF) (17,28) and up-regulate downstream signaling of nitric oxide synthesis, genes and activity (14,16,32). Several CaSi (akermanite and wollanstonite) stimulate a significant increase in the secretion of angiogenic growth factors from fibroblasts and can induce the infiltration of a great number of blood vessels into tissue engineering scaffolds (32).

In the present study pVW-MSCs have been cultured on 3D porous scaffolds based on PLA and PCL doped with high amounts (20% w/w) of reactive biointeractive mineral fillers (CaSi and DCPD). The used vascular stem cells derived from the thoracic aorta (10) represent an excellent model to test the angiogenic/anti angiogenic potential of different substances (33).

The results presented in this paper clearly demonstrate that both experimental PLA- and PCL-based scaffolds support pVW-MSCs growth and colonization; pVW-MSCs grown on all the scaffolds were metabolically active throughout the experimental time (72 hours). Interestingly, at 48 hours, pVW-MSCs cultured on PLA based scaffold showed an increase of metabolic activity respect to PCL based scaffold regardless of the presence of the additives. The difference in metabolic profile showed for PLA based scaffolds must be further investigated, considering that a different metabolic activity could represent a stem cells differentiation fate towards different specific lineages (34).

We previously demonstrated (11) that pVW-MSCs cultured in 2D are characterized by a typical gene expression profile including mesenchymal stem cell markers, such as CD90 and pericyte markers such as: alpha-SMA, PDGFR-βand NG2. Similarly, pVW-MSCs cultured on all the experimental 3D scaffolds, continued to express these markers suggesting the possibility of a successful long-lasting colonization. However, some main differences in the expression level of the above mentioned genes has been revealed: PLA and PCL scaffolds determined a reduction of alpha-SMA, while the same scaffold with calcium silicates (CaSi) and dicalcium phosphate dihydrate (DCPD) mineral fillers restored the alpha-SMA expression level. Otherwise, PDGFβ-receptor was significantly reduced in scaffolds enriched with reactive minerals CaSi and DCPD. These results are particularly attractive and further investigation increasing time of culturing could explain if the different level of alpha-SMA and PDGFβ-receptor expression could be associated with different spontaneous differentiation.

The distribution of pVW-MSCs cultured on the experimental 3D scaffolds showed an individual cell growth on PLA–10CaSi–10DCPD whilst on PCL–10CaSi–10DCPD cells grown as compact clusters, which resemble the spheroids that could be obtained by hanging drop culture of pVW-MSC. The difference in alpha-SMA gene expression could be related to this different distribution, in fact is well known that MSCs phenotype is dramatically changed when culture condition shifts from 2D to 3D and also depend on the approaches utilized to generate the spheroids (35), while the differences in PDGR-□□seems to be related to different to the presence of mineral addictives.

Overall with the present paper we demonstrated the possibility of culturing pVW-MSCs on 3D biodegradable highly porous mineral doped poly-α-hydroxy acids scaffolds. pVW-MSCs spontaneously colonize these biomaterials and maintain a general undifferentiated phenotype in the first 72h of culture with slight differences with control 2D culture. However, further studies, with longer growth kinetics, are needed to investigate the differentiated fate of these cells grown on these 3D culture system.

Being VW-MSCs an excellent *in vitro* model to study vascular remodelling, due to their strong angiogenic attitude, the possibility of culturing pVW-MSCs on these biodegradable scaffolds represents a strategy for biomedical tissue engineering.

Conclusions

In conclusion, the property of the experimental scaffolds to be colonized by vascular stem cells demonstrated the potential of the scaffolds to induce the formation of new vessels and their potential

application in tissue regeneration.

Methods

Materials

Polycaprolactone (PCL) (average Mn 45000 g/mol, Sigma-Aldrich, Milan, Italy) was received in pellet form and purified via dissolution in $CHCl_3$ (15% wt/vol) and reprecipitation in a large excess of cold MeOH, in order to eliminate residual polymerization catalysts.

Poly(L-lactic acid) (PLA) (MW = 65000 g/mol, Ingeo^{TM} biopolymer PLA 4060D, Natureworks LLC, Blair, NE, USA) was received in pellet form and purified via dissolution in $CHCl_3$ (10% wt/vol) and re-precipitated in a large excess of cold MeOH, in order to eliminate residual polymerization catalysts.

Methanol (MeOH), ethanol (EtOH, 99.8%), 1,4-dioxan (DIOX) and chloroform ($CHCl_3$, HPLC grade) all from Sigma Aldrich (Milan, Italy) were used as received without further purification.

Dicalcium phosphate dihydrate (DCPD; $CaHPO_4{\cdot}2H_2O$) powder (Sigma-Aldrich, Steinheim, Germany) and/or calcium silicate (CaSi) powders (Aalborg, Denmark), prepared by melt-quenching technique and milling procedures, as previously reported (19), and composed of dicalcium silicate, tricalcium silicate, tricalcium aluminate, and calcium sulfate, were added to PLA or PCL.

All plastic used for standard culture condition were from BD-Falcon (Corning, NY USA). Antibiotic–antimycotic, Dulbecco's phosphate buffered saline (DPBS), DAPI staining solution were purchased from Gibco-Life Technologies (Carlsbad CA, USA). Trypsin–EDTA solution1X, MTT based assay were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pericyte Growth Medium was purchased from Promocell (Heidelberg, Germany). NucleoSpin RNA kit was purchased from Macherey-Nagel GmbH & Co. KG (Düren, Germany).

TIPS Scaffolds preparation

The porous polymer scaffolds were prepared by TIPS starting from PLA or PCL solutions in DIOX (3.5% wt/vol). CaSi and DCPD were added to the polymer solution in powder form in 10% by weight with respect to PLA or PCL.

Homogeneous dispersions were obtained by sonicating the mixtures for 3 hours using the ultrasonic processor UP50H (Hielsher, 50 watts, 30 kHz), equipped with the sonotrode MS2 (made of titanium, tip diameter 2 mm). After mixing, solutions were placed inside disposable aluminum dishes of 60 mm in diameter, and cooled at –18°C. After 18 h, the frozen samples were extracted from the holders and fully immersed in EtOH bath precooled at –18°C, where they were kept for 48 h, with solvent refresh every 3 h. At the end of the extraction procedure, the porous scaffolds were taken out of the freezer and completely

dried under vacuum (24). Six disks (diameter 60 ± 1 mm, thickness 10±0.1 mm) per composition were prepared.The prepared scaffolds were: PLA, PLA–10CaSi–10DCPD, PCL, PCL–10CaSi–10DCPD.

Surface porosity evaluated by the morphometric analysis on ESEM images

The scaffolds were examined using an environmental scanning electron microscope (ESEM, Zeiss EVO 50; Carl Zeiss, Oberkochen, Germany). Specimens were placed directly onto the ESEM stub and examined in wet conditions without any previous preparation (the samples were not coated for this analysis) at low vacuum (100 Pascal) in Quadrant Back-Scattering Detector (QBSD) mode, using an accelerating voltage of 20 kV, working distance 8.5 mm, 0.5 wt% detection level, 133 eV resolution, amplification time 100 µs, measuring time 60 s.

ESEM images were analyzed through Image J program (National Institutes of Health, Bethesda, USA) to evaluate the scaffolds porosity, in terms of percentage. Surface porosity was calculated as the ratio between the most black areas (micropores) and the total examined area (24,36). For each scaffold three measurements were performed in three different areas at 500× and 1000× magnification. For each magnification, the mean value was recorded.

Isolation and culture of primary porcine Vascular Wall Mesenchymal Stem Cells

Primary porcine Vascular Wall Mesenchymal Stem Cells (pVW-MSCs) were isolated from the thoracic aorta of female 3-months-old pigs (Large White) euthanized for other experimental purposes, following the published methods previously described (11,37): briefly, young commercial hybrids of Sus scrofa (4 males–aged 4–5 wk, 7 ± 0.5 Kg live weight), born at the ASA Unit (DIMEVET, University of Bologna), were enrolled in the study. Piglets were bred under the lactating sow till 28 d, then weaned and kept in a multiple box for young piglets, temperature was kept at 28 ± 1 °C with adequate ventilation and humidity in relation to the young age. Surgical procedures were carried out during the morning in the surgical theatre of the DIMEVET facilities. Animal received an i.m. bolus of tiletamine-zolazepam (5 mg/kg) 10 min before induction; general anesthesia was achieved using sevoflurane with an induction mask. Animals were then sacrificed with a single bolus (0.3 mL/kg) of Tanax (embutramide/mebezonium iodide/tetracaine hydrochloride; Msd Animal Health Srl). All procedures on pigs were reviewed and approved in advance by the Ethical Committee of the University of Bologna (Bologna, Italy) and were then approved by the Italian Ministry of Health (Protocol number n.43-IX/9 all.37; 20/11/2012).. Cells were grown and expanded not beyond till passage (P) 6 in Pericyte Growth Medium (PGM—Promocell). All the experiments described in this paper were performed with cells at the third passage (P3), cultured in Pericyte Growth Medium (PGM).

Cell seeding efficiency assay

All the scaffolds were sterilized by 30 min of incubation in absolute EtOH followed by three washes in DPBS (30 min each one), then cubic-shaped sections of about 1 mm on each side were produced with the help of scalpel blades. 30 sections were suspended in 500 ul of culture medium (Pericyte Growth Medium) then pVW-MSCs were seeded on different biomaterials (PLA–10CaSi–10DCPD and PCL–10CaSi–10DCPD) following a published method (38) with some main adaptations. The biomaterials were drop-seeded with 100 µl of a concentrated cell suspension containing 4 x 10^5 or 8 x 10^5 cells. The microcentrifuge tubes were placed on a rocker oscillating at 30 rpm for 2 hours to allow for initial cell attachment then fresh cell culture medium was slowly added to each tube and cells were cultured for additional 24, 48, 72 hours in static condition at 38.5°C at 5% CO2. Cell-free biomaterials were incubated under same conditions and used as control.

Cell seeding efficiency (CSE) was estimated using an indirect method (38) after 24, 48 and 72 hours. Briefly the CSE was calculated based on the number of unattached cells that still present in the medium using the following equation CSE (%) = (1-$cells_u$/$cells_i$) x 100, where $cells_i$ is the number of cells initially seeded and $cells_u$ is the number of unattached cells in the residual medium and in DPBS used for rinsing cell-seeded biomaterials. Unattached cells were counted by hemocytometer in three different aliquots of medium collected from each sample.

Metabolic cell activity assay

The metabolic pVW-MSCs activity was monitored at 24, 48 and 72 hours using MTT based assay (Sigma) following the manufacturer's instruction, with some main adaptation. Ten sections were removed at each experimental point, then scaffold sections were washed twice with DPBS and then 20 µl of MTT substrate was added and recovered for 4 hours at 38,5 °C at 5% CO_2.After that, 200 µl of solubilisation solution was added and, after 30 min, the solution was vigorously mixed and the absorbance at a wavelength of 570 nm was measured spectrophotometrically, with the background substraction at 690 nm.

RNA extraction and qPCR

RNA extraction was performed using TRI Reagent (Molecular Research Center) and NucleoSpin

Briefly, culture medium was removed at 72 h and different scaffolds where washed with DPBS (twice); 500 ml of TRI Reagent (Molecular Research Center, Inc.) was added and the materials where lysed by using TH Tissue Homogenizer (Omni International GA, 30144 United States). Following, 100 µl of chloroform was added to the suspension and mixed well. After incubation at room temperature (10 min), samples were centrifuged (12000 g for 10 min) and the aqueous phase was recovered. An equal volume of absolute ethanol was added and the resulting solution was applied to the Nucleo spin RNA Column,

RNA was finally purified according to the manufacturer's instructions. After spectrophotometric quantification (Denovix, Denovix Inc., Wilmington, NC) total RNA (500 ng) was reverse-transcripted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) in a final volume of 20 µl. Swine primers were designed using Beacon Designer 2.07 (Premier Biosoft International, Palo Alto, CA, USA). Primer sequences, expected PCR product lengths and accession numbers in the NCBI database are shown in Table 1.

To evaluate gene expression profiles, quantitative real-time PCR (qPCR) was performed in CFX96 (Bio-Rad) thermal cycler using a multiplex real time reaction for reference genes (GAPDH, glyceraldehyde–3-phosphate dehydrogenase; HPRT, hypoxanthine guanine phosphoribosyl transferase; β-Actin), and using Taq-Man probes and SYBR green detection for target genes (CD90, α-SMA, NG2 and PDGFR-b). All amplification reactions were performed in 20 µL and analyzed in duplicates (10 µl/well). Multiplex PCR contained: 10 µl of iTaqMan Probes Supermix (Bio-RAD), 1 µL of forward and reverse primers (5 µM each) of each reference gene, 0.8 µl of iTaq-Man Probes (5 µM) of each reference gene, 2 µl cDNA and 2.6 µL of water. The following temperature profile was used: initial denaturation at 95°C for 30 seconds followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds.

The SYBR Green reaction contained: 10 µl of IQSYBR Green Supermix (Bio-RAD), 0.8 µl of forward and reverse primers (5 µM each) of each target gene, 2 µl cDNA and 7.2 µl of water. The real-time program included an initial denaturation period of 1.5 min at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 30 s, followed by a melting step with ramping from 55°C to 95°C at a rate of 0.5°C/10 s.

The specificity of the amplified PCR products was confirmed by agarose gel electrophoresis and melting curve analysis.

The relative expressions of the studied genes were normalized based on the geometric mean of the three reference genes. The relative mRNA expression of tested genes was evaluated as fold of increase using the $2^{-\Delta\Delta CT}$ method (39) referred to control (pVW-MSCs cultured in flask under standard 2D culture condition).

DAPI staining and immunofluorescence

To test cellular distribution in the different scaffolds, pVW-MSCs cultured for 72 hours were processed for labeling DNA in fluorescence with DAPI staining and alpha-tubulin immunofluorescence. In particular biomaterials were washed twice in DPBS then were fixed ON in cold 4% Formaldehyde Solution in PBS, pH 7,4. Each sample was transferred into a 25% sucrose (Sigma-Aldrich, MO, USA) solution in PBS at 4°C for 24 hours to get cryoprotection. Finally, samples were embedded and freezed in OCT (Sakura Finetek, CA, USA). Ten micrometers sections were cut at a Leica CM1950 cryostat (Leica, Wetzlar, Germany) mounted on microscope's slides and stainined with DAPI Staining Solution (Thermo Fischer). For immunofluorescence staining slides were completely dried at room temperature (RT), washed three times in PBS 1X for 5 minutes, permeabilized with Triton X–100 0,1% in PBS 1X for 1 hour then washed three

times in PBS 1X for 5 minutes. For aspecific sites blocking slides were treated with 10% Normal Goat Serum in PBS 1X for 1 hour at RT then incubated ON in a humidified chamber with an anti alpha-tubulin antibody (Clone TU–01, Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:250 in PBS 1X. In negative controls the primary antibody was omitted. At this point samples were washed three times in PBS 1X, incubated with anti-mouse FITC conjugate antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:100 in PBS 1X for 1 hour at RT in a humidified chamber. After two washes for 5 min in PBS 1X and one wash in distilled water for 5 minutes, coverslips were mounted on slides with Fluoreshield with PI (Sigma-Aldrich, St. Louis, MO, USA). Photomicrographs were obtained using a Nikon digital camera installed on a Nikon epifluorescence microscope (Nikon Inc., Melville, NY, USA).

Statistical analysis

Three primary cell cultures derived from three different animals were used. Data represent the mean ± SD (or ± range of expression for qRT-PCR) of the three biological replicates and all test were performed in a technical duplicate. The data were analyzed by One-way analysis of variance (ANOVA) followed by the Tukey post hoc comparison Test. Differences of at least $p<0.05$ were considered significant. Statistical analysis was carried out by using GraphPad Prism 5.0 software.

Abbreviations

pVW-MSCs: porcine Vascular Wall-Mesenchymal Stem Cells; VSCs: Vascular Stem Cells; FGF–2: Fibroblast Growth Factor–2; VEGF: Vascular Endothelial Growth Factor; PDGF: Platelet Derived Growth Factor; DPBS: Dulbecco's phosphate buffered saline; PGM: Pericyte Growth Medium; PLA: polylactic acid; PCL: poly-e-caprolactone; CaSi: calcium silicates; DCPD: dicalcium phosphate dehydrated; DIOX: 1,4-dioxan; ESEM: Environmental Scanning Electron Microscope; QBSD: Quadrant Back-Scattering Detector (QBSD); CSE: Cell Seeding Efficiency; MTT: 3-(4,5-dimethylthiazol–2-yl)–2,5-diphenyltetrazolium bromide; qPCR: quantitative; DAPI: 4',6-diamidino–2-phenylindole, FITC: fluorescein isothiocyanate; PI: propidium iodide; Polymerase Chain Reaction; GAPDH, glyceraldehyde–3-phosphate dehydrogenase; alpha-SMA: alpha- smooth muscle actin

Declarations

Ethics approval

All procedures on pigs were reviewed and approved in advance by the Ethical Committee of the University of Bologna (Bologna, Italy) and were then approved by the Italian Ministry of Health (Protocol number n.43-IX/9 all.37; 20/11/2012).

Consent for publication

Not applicable

Availability of data and materials

The material analysed during the current study is available from the corresponding author on reasonable request.

Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

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Authors’ contributions

MGG and MF concepted and designed the research; CB., FZ., AZ., RS., DV, ME., PF., FF performed experiments; MF and CB. analysed data; MGG., MF, CB., PF., FF and CC. interpreted results of experiments; CB and FZ prepared figures; MGG., MF and CB drafted the manuscript; MGG approved final version of the manuscript and all authors have read and approved the manuscript.

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Figures

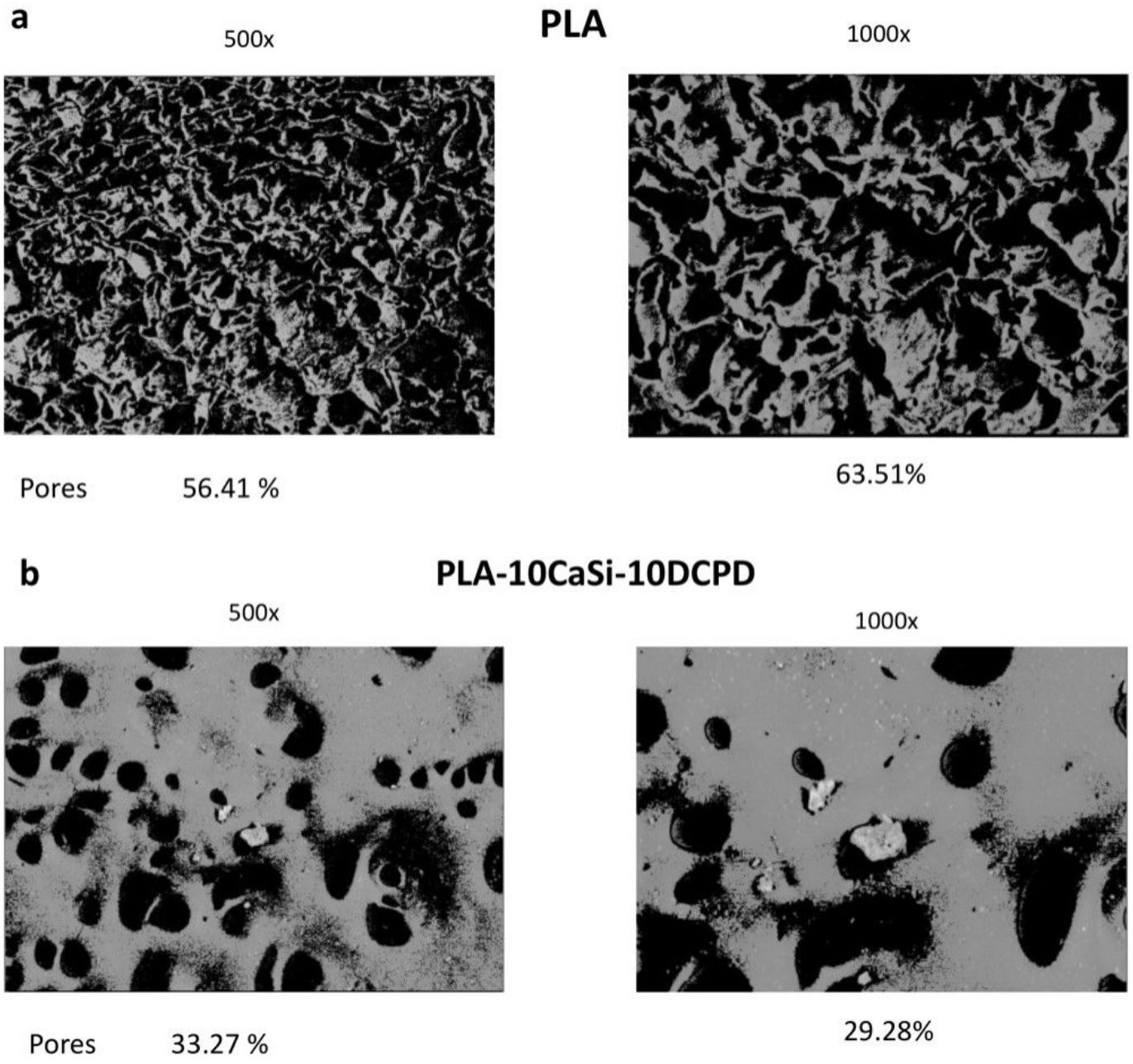

Figure 1

Surface porosity evaluation of PLA (a) and PLA-10CaSi-10DCPD (b) scaffolds on one random area at 500x and 1000x magnifications. Mineral doped formulation revealed a more compact surface, with lower surface porosity values when compared to pure PLA formulation.

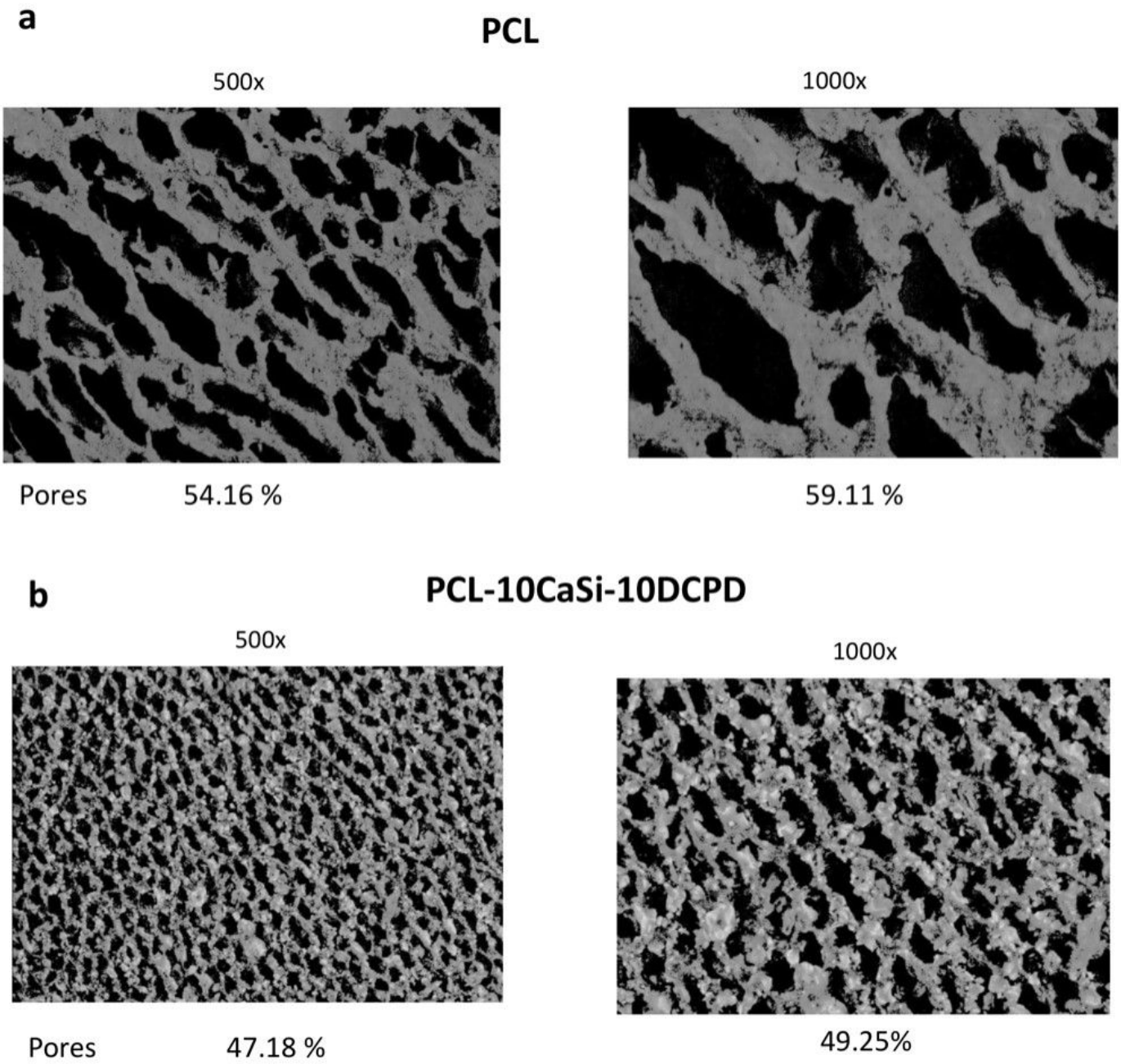

Figure 2

Surface porosity evaluation of PCL (a) and PCL-10CaSi-10DCPD (b) scaffolds on one random area at 500x and 1000x magnifications. PCL scaffolds reveal a more regular surface when compared to PLA scaffolds. CaSi and DCPD mineral fillers are widely distributed on the scaffold surface, partially occluding the pores.

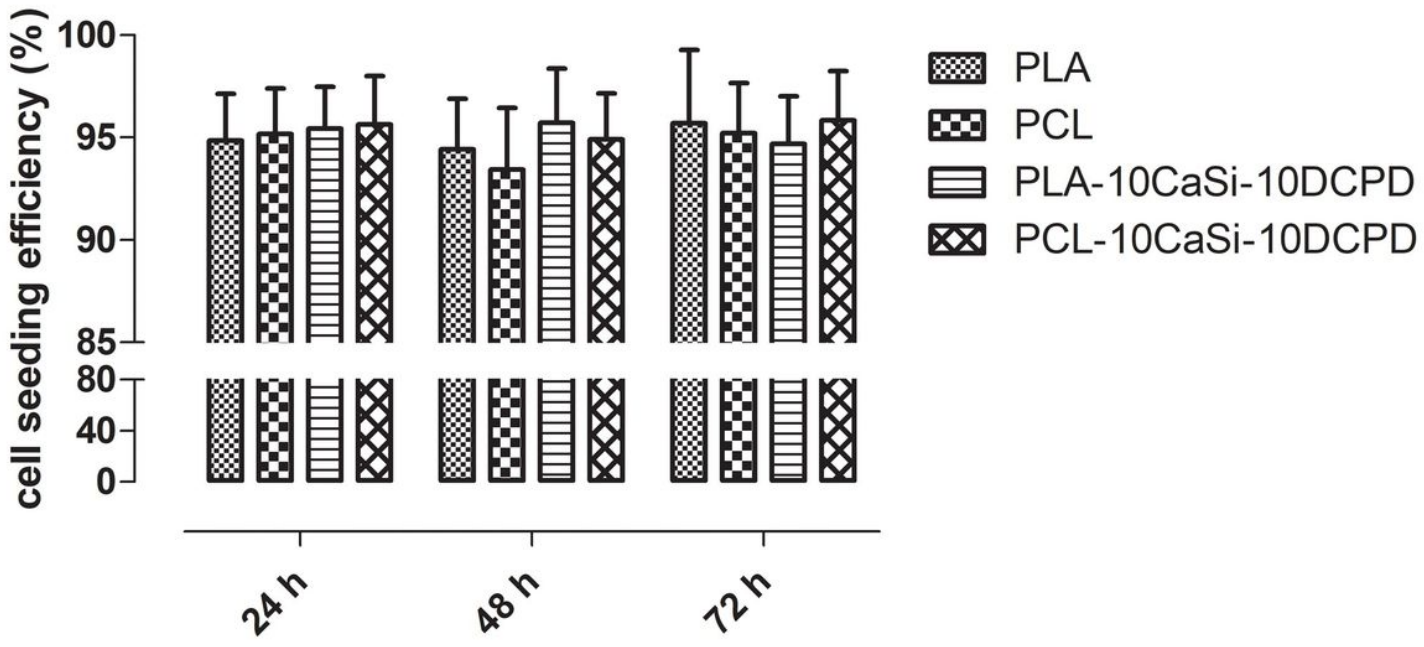

Figure 3

Cell seeding efficiency. Cell seeding efficiency was performed by an indirect method. After 24 hours from the seeding cells did not attach to the scaffolds were quantified and cell seeding efficiency was calculated by the equation: CSE (%) = (1-cellsu/cellsi) x 100. Data represent the mean±SD of three independent biological replicates (n=3) and were analysed using one-way ANOVA followed by the Tukey's post hoc comparison. No differences were detected among different scaffolds.

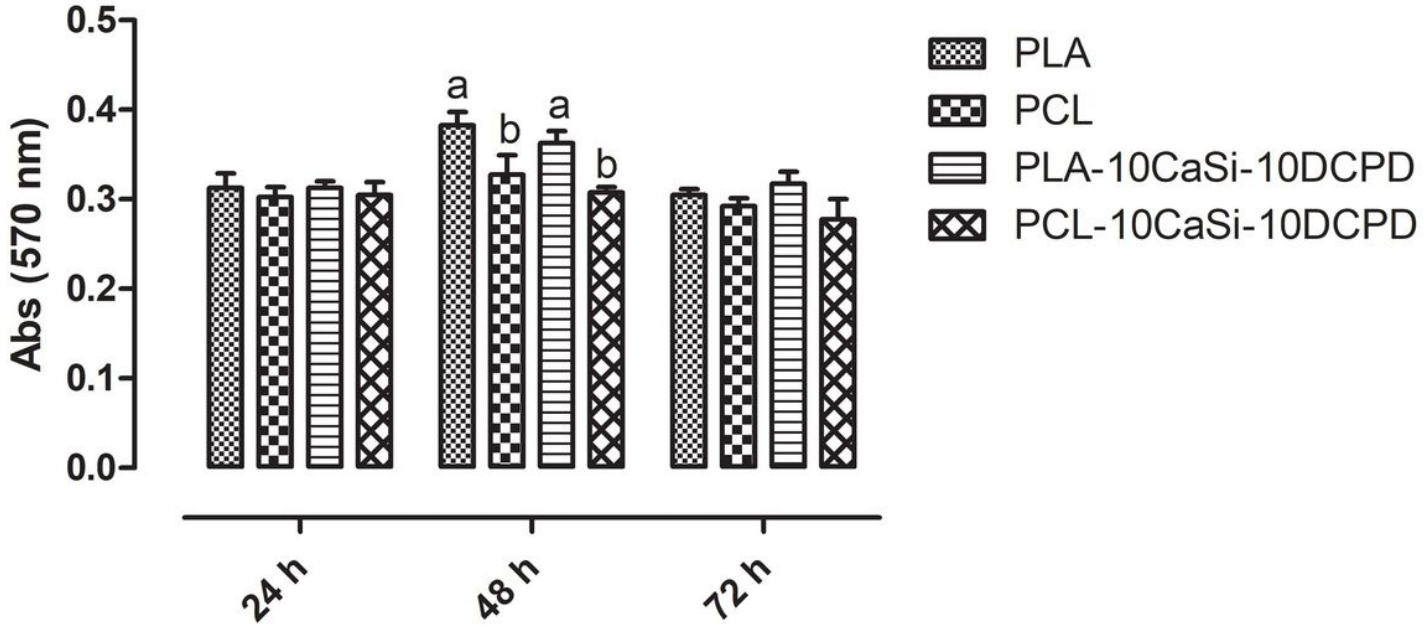

Figure 4

Metabolic activity of pVW-MSCs seeded on different scaffolds after 24, 48 and 72 hours of culture was evaluated by MTT based assay. Data represent the mean±SD of three independent biological replicates (n=3) and were analysed using one-way ANOVA followed by the Tukey's post hoc comparison. Different letters indicate significant difference $p<0.05$.

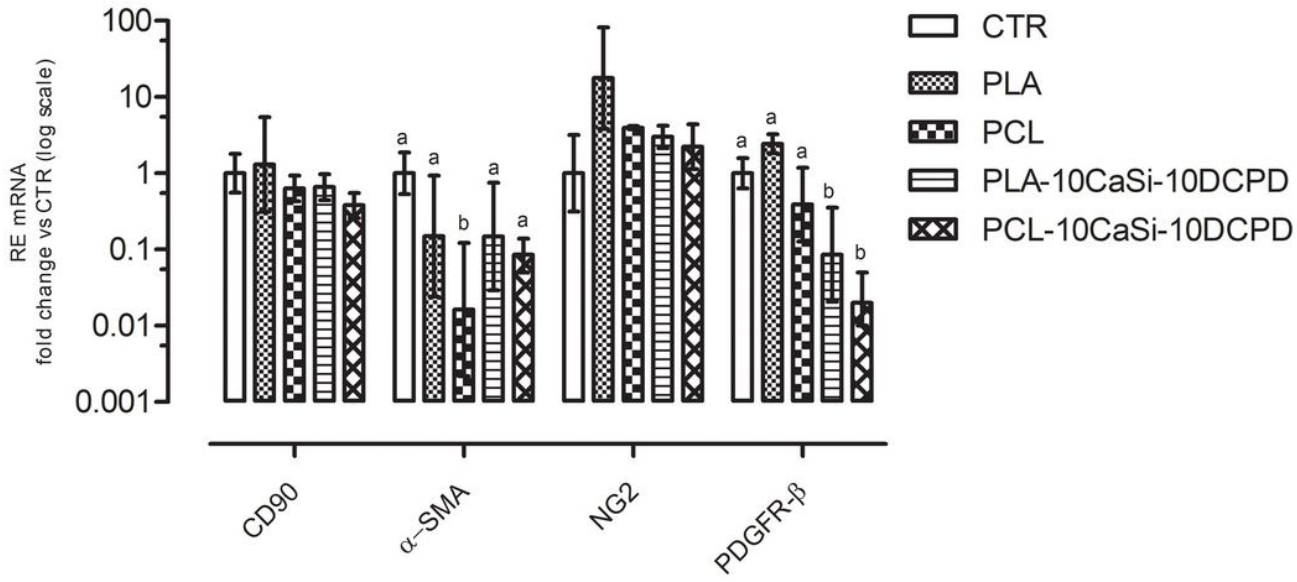

Figure 5

CD90, αSMA, NG2 and PDGFR-β gene expression in pVW-MSCs evaluated after 72h of culture in presence of different scaffolds or in 2D standard culture condition (CTR). Data represent mean ± range of relative expression (RE) of three biological replicates (n=3). Data were analysed using one-way ANOVA followed by the Tukey's post hoc comparison test. Different letters above the bars indicate significant differences ($p<0.05$).

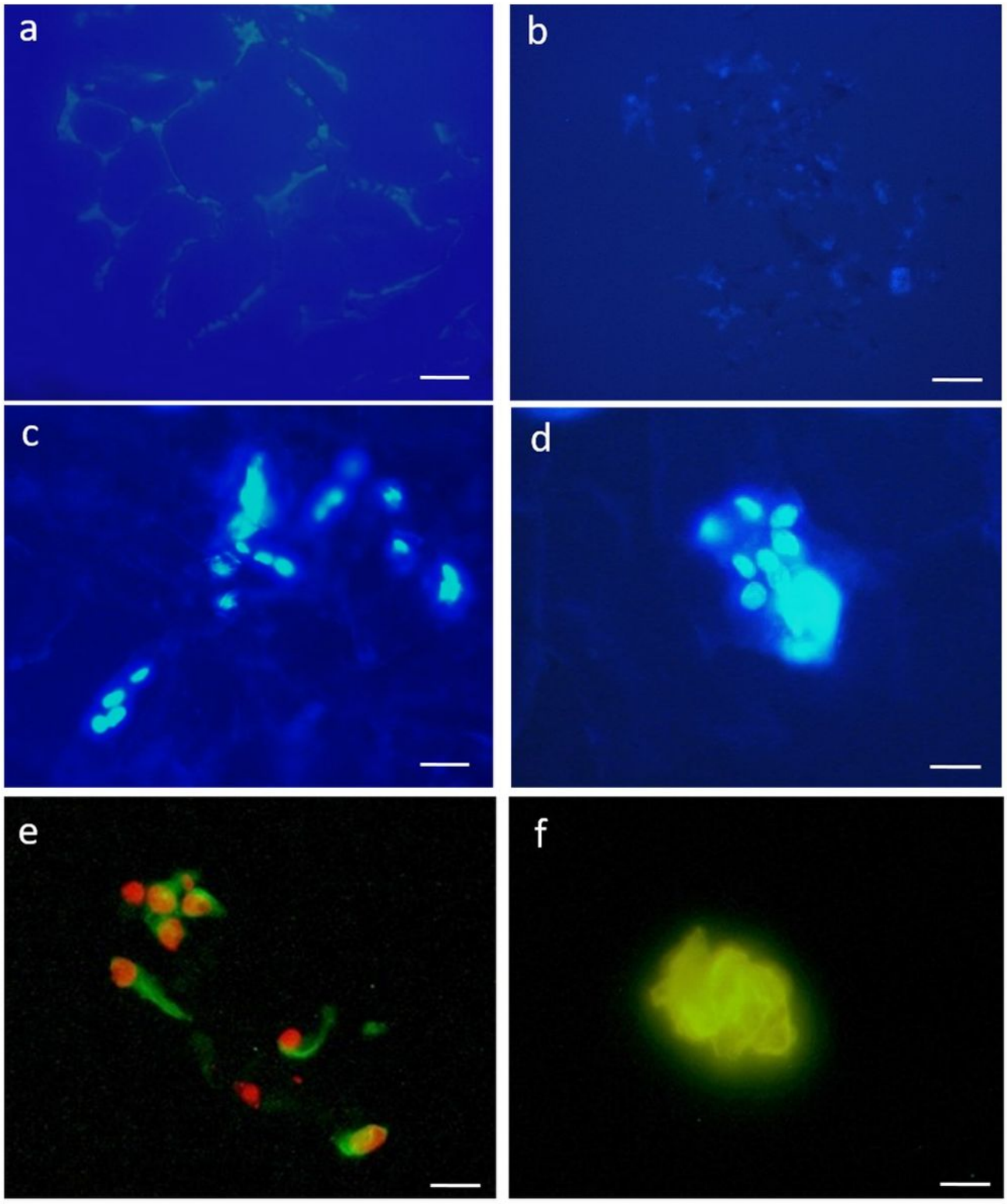

Figure 6

DAPI staining (a-d) PLA-10CaSi-10DCPD (a) and PCL-10CaSi-10DCPD (b) without cells and in the presence of cultured cells (72h): PLA-10CaSi-10DCPD (c), PCL-10CaSi-10DCPD (d). Immunofluorescence analysis for alpha-tubulin of cultured cells (72 h) on PLA-10CaSi-10DCPD (e) and PCL-10CaSi-10DCPD (f). Scale bar = 10 μm for a, b and 20 μm for c-f.

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