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Pore size distribution and blend composition affect in vitro pre-vascularized bone matrix formation on poly(vinyl alcohol)/gelatin sponges

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Abstract:	This study was aimed at identifying compositional and architectural (pore size and distribution) parameters of biocompatible scaffolds, which could be best suitable for both osteoblasts and endothelial cells to produce optimized 3D co-cultured constructs. Spongy scaffolds were prepared using poly(vinyl alcohol) (PVA) and gelatin (G) at different weight compositions (PVA/G range: 100/0 - 50/50 w/w) via emulsion and freeze-drying. The higher gelatin content, the larger volume occupied by higher size pores. Human umbilical vein endothelial cells and human mesenchymal stromal cells were independently differentiated on the scaffolds to select the best candidate for the co-culture. The results of metabolic activity and histology on single platforms showed both cell- and material-type dependent outcomes. PVA/G 80/20 scaffolds were finally selected and allowed the formation of mineralized matrix containing organized endothelial-like structures. This study highlighted the need for systematic investigations on multifactorial parameters of scaffolds to improve vascularized bone substitutes.
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cover letter here. Please note, if you are submitting a revision of your manuscript, there is an opportunity for you to provide your responses to the reviewers later; please do not add them to the cover letter.	It is my great pleasure to submit to Macromolecular Materials and Engineering on behalf of my co-authors, the revised version of our original manuscript entitled “Pore size distribution and blend composition affect in vitro pre-vascularized bone matrix formation on poly(vinyl alcohol)/gelatin sponges” by J.G. De la Ossa, L. Trombi, D. D’Alessandro, M.B. Coltelli, L.P. Serino, R. Pini, A. Lazzeri, M. Petrini, and S. Danti. Our research work relies on identifying compositional and architectural (pore size and distribution) parameters of biocompatible scaffolds, which could be best suitable for both osteoblasts and endothelial cells to produce optimal 3D co-cultured constructs via tissue engineering. Understanding the role played by physico-chemical and architectural parameters of porous scaffolds on both bone cells and endothelial cells can allow the development of in vitro pre-vascularized autologous bone substitutes, which may be functional and viable soon after implantation. Indeed, it has been shown that different cell types have different affinity towards specific scaffold features, including physico-chemical and architectural cues, and this undisclosed aspect can challenge the optimal scaffold choice. Little systematic work has been performed to reveal which scaffold parameters specifically affect cellular behaviors in co-cultured systems, in which bone and endothelial cells are ultimately required to synergize. Our study proposes a first systematic screening of some the parameters involved which may pave the way to understand and possibly predict such a complex cellular interplay. In particular, in this Communication we demonstrated that PVA/Gelatin 50/50 w/w, which accounted for the largest volume fraction of higher size pores, sustained endothelial, but osteoinduced-hMSC viability, thus suggesting that other parameters than pore size, as invoked by Karageorgiou and Kaplan (Biomaterials, 2005), possibly in combination, play key roles for achieving functional bone tissue engineering. I confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal, but only as a conference abstract. Hoping that you may find our contribution innovative, interesting, and suitable for publication in Macromolecular Materials Engineering. We took into account the comments provided by the Reviewers. Yours sincerely, Serena Danti
Do you or any of your co-authors have a conflict of interest to declare?	No. The authors declare no conflict of interest.

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Communication

Pore size distribution and blend composition affect *in vitro* pre-vascularized bone matrix formation on poly(vinyl alcohol)/gelatin sponges[a]

Dedicated to the memory of Prof. Michele Lisanti (1950-2017).

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This study was aimed at identifying compositional and architectural (pore size and distribution) parameters of biocompatible scaffolds, which could be best suitable for both osteoblasts and endothelial cells to produce optimized 3D co-cultured constructs. Spongy scaffolds were prepared using poly(vinyl alcohol) (PVA) and gelatin (G) at different weight compositions (PVA/G range: 100/0 - 50/50 w/w) via emulsion and freeze-drying. The higher

[a] **Supporting Information** is available online from the Wiley Online Library.

gelatin content, the larger volume occupied by higher size pores. Human umbilical vein endothelial cells and human mesenchymal stromal cells were independently differentiated on the scaffolds to select the best candidate for the co-culture. The results of metabolic activity and histology on single platforms showed both cell- and material-type dependent outcomes. PVA/G 80/20 scaffolds were finally selected and allowed the formation of mineralized matrix containing organized endothelial-like structures. This study highlighted the need for systematic investigations on multifactorial parameters of scaffolds to improve vascularized bone substitutes.

Key words: tissue engineering; scaffold; bioartificial; endothelial cells; mesenchymal stem cells

1. Introduction

Restoration of bone defects represent a widespread clinical problem occurring as a consequence of several pathologies, such as trauma, tumor excision, chronic osteomyelitis, non-union, avascular necrosis and spinal fusions.[1] If the defect size is critical, bone regeneration cannot occur spontaneously, leading to the necessity of surgical strategies that avail themselves of bone substitutes; usually tissue grafts.[2] Under innovative tissue engineering approaches, autologous cells can be grown *in vitro* on biocompatible scaffolds based on synthetic and/or biologic biomaterials and transplanted back to the patient, thus reconstructing autologous bone with no need for tissue explants.[3] Jeopardized failures of bone tissue-engineered replacements have recently been reported to deal with unsuccessful post-implant neo-vascularization.[4] Indeed, the lack of an efficient vascular supply after implantation may put at serious risk the survival of the transplanted cells. Therefore, the development of non-surgical strategies able to promote microvasculature have become a primary goal in bone tissue engineering.[5,6,7] It has been pointed out that *in vivo* formation of new blood vessels depended on the ordered interaction of endothelial cells with different cell types, including mesenchymal stromal cells (MSCs).[8,9,10] Among the various strategies studied to foster the establishment of a functional vascular network in bone substitutes, co-cultured systems, in which endothelial cells already coexist with bone cells at the time of implantation, have been the subject of investigations in the last decade.[5] Recent work has focused upon setting up and studying three dimensional (3D) co-cultured systems to comprehend the mechanisms underlying cell cross-talk with the ultimate purpose to empower post-implant bone survival and integration.[5] Independently of these studies, it has been highlighted that bone cells, as well as other cell types, showed different affinity towards diverse architectural cues of biomaterial scaffolds, pore size.[11] However, little systematic work has been performed to reveal which scaffold parameters specifically affect cellular

behaviors in co-cultured systems, in which bone and endothelial cells are ultimately required to synergize.

This study was aimed at defining compositional and architectural features of biocompatible spongy scaffolds which could be best suitable for both osteoblasts and endothelial cells, in order to produce optimized 3D co-cultured constructs. Spongy scaffolds were prepared from emulsions of a synthetic biocompatible polymer, poly(vinyl alcohol) (PVA), and a biopolymer, gelatin (G), at different weight compositions (PVA/G range 100/0 - 50/50 w/w). Human umbilical vein endothelial cells (HUVECs) and human MSCs (hMSCs) were independently cultured and differentiated on all the sponge types to select the individual cell-type affinity and to ultimately identify the best scaffold candidate for the co-culture.

Understanding the role played by physic-chemical and architectural parameters of porous scaffolds on bone cells and endothelial cells can pave the way to developing *in vitro* pre-vascularized autologous bone substitutes which may be functional and viable soon after implantation.

3. Results and Discussion

Biomaterial-based substitutes for large bone defects offer the advantages of reproducibility and biosafety, thus being promising alternatives to bone grafts under a tissue engineering approach. Owing to the high costs necessary for a personalized therapy, it is important to define the scaffold parameters that could best promote both formation and survival of bone substitutes after surgery, the latter depending on the neo-vascularization of the implanted cellular constructs.[4] As a consequence, the selection of the optimal scaffold for bone regeneration must take into account its capability of synergizing the interactions between bone cells and endothelial cells. However, different cell types may show different affinity

towards specific scaffold features, including physico-chemical and architectural cues, and this undisclosed aspect can challenge the optimal scaffold choice.[11]

This study was aimed at investigating HUVEC and osteo-differentiated hMSC response towards PVA/G sponges produced in a composition range in which G weight content was increased from 0% up to 50%. Varying this parameter affected the scaffolds through physico-chemical and architectural changes and ultimately resulted in different specifications by the two cell types. An *ab initio* selection of those scaffold parameters can elucidate endothelial-bone cell cross-talk mechanisms that would ultimately release best reliable *in vivo* implants with increased success rate, which are key enabling factors in personalized therapy.

In tissue engineering, the scaffolds behave as engineered matrices that provide primary structural support for 3D bone tissue formation by enabling bone extracellular matrix (ECM) fundamental processes. PVA is a synthetic polymer, water soluble and nonhazardous, which is used in several biomedical applications.[12] It can be processed into several structures, blended with proteins, and stabilized via physical and/or chemical crosslinking, thus acting as a biostable material in the human body. Previous tissue engineering studies have focused only on specific compositions of PVA/G hydrogels and sponges without any systematic characterization.[13,14,15,16,17]

The scaffolds produced in this study were analyzed using Fourier transmission infrared (FTIR) spectroscopy to assess cross-linking and chemical effects of increasing G content in the PVA/G blends.[18] The general pattern of the PVA spectrum was in agreement with those reported in literature (**Figure 1 A**).[19] The higher G content, the stronger intensity of the 1635 cm^{-1} and 1535 cm^{-1} (amide I and amide II bands) typical of polyamides. By increasing G content, only minor changes in the PVA band profile were observed, with the tendency of the spectrum to increase in complexity showing several large bands in the range 1000-1500 cm^{-1}.[20] Cross-linking with glutaraldehyde (GTA) did not alter significantly the PVA infrared spectrum. Differently, in presence of G, spectral changes were observed by comparing the

uncross-linked with the cross-linked counterparts (**Figure 1 B, C**). For example, in cross-linked PVA/G 70/30 sample, a very weak shoulder at 1710 cm^{-1} appeared due to stretching C=O of GTA (**Figure 1 B**). Moreover, an increase in the band at 1130 cm^{-1} was present that has been attributed to C-O-C groups formation due to cross-linking reaction.[21] In the PVA/G 50/50 sample, the shift from 1513 cm^{-1} to 1535 cm^{-1} of the G band was evident (**Figure 1 C**). This change reasonably occurred following linkage formation, as also observed in GTA cross-linked collagen samples.[22] Moreover, in all the blends the double peak, typical of the uncross-linked PVA/G system at 1442 cm^{-1} and 1407 cm^{-1} caused by the overlapping of the bending CH_2 of PVA and G, became a unique peak centered at an intermediate wavelength for the cross-linked system. This is in agreement with the reaction of G with GTA, characterized by the presence of $-CH_2$, and thus leading to an alteration of the $-CH_2$ bending profile. Finally, the reaction of GTA with the $-NH_2$ groups of lysine residues allowed CH=N aldimine groups to be formed, with a characteristic absorption at 1450 cm^{-1}.[18] Reasonably, the formation of this linkage is also responsible of the slight material yellowing after GTA cross-linking (**Figure 1**). FTIR analysis confirmed the compositional differences and the occurrence of cross-linking in all the samples (**Figure S1**).

Porosity and pore interconnectivity are among the most important characteristics of a scaffold, because they greatly influence cell migration and molecule diffusion, finally facilitating the formation of bone ECM and vascularization processes. In general, large pore sizes can accommodate cell aggregates and ECM molecules, while small pore sizes play a role in fostering small molecule trafficking, such as nutrients, oxygen and catabolic products. In this view, the production of scaffolds containing several pore size classes is highly desirable to generate an optimal microenvironment for cell growth and differentiation. In particular, owing to large osteoblast size, migration requirements and transport phenomena, pores in the 100-300 μm class and above are recommended to allow new bone ECM and capillary formation.[23]

Under SEM, the inner structure of all the samples was found to consist of large pores of spherical-like morphology provided with smaller intra-poral openings, thus providing proof of interconnectivity (**Figure 2 A**). The porosimetric analysis ~~highlighted~~ of macropores ranging in 0.08 – ~~300~~ 125 µm, ~~with~~ highlighted a different distribution depending on PVA/G composition (**Figure 2 B**). In particular, the volume filled by the 30 – 100 µm pore class increased with increasing G content up to 83% ± 1% in PVA/G 50/50, to the detriment of 10 – 30 µm and~~–~~ 0.08~~–~~–10 µm classes. Conversely, the smaller 10~~–~~–30 µm pore class was predominant (59% ± 10%) in PVA/G 100/0 (**Figure 2 B**). This finding can be attributed to the foaming effect of G. After hydration in saline and culture media, PVA/G sponges were stable over week-times.

The used crosslinking and post-treatment method prevented residual GTA-induced toxicity.[13,14,15,16] PVA/G scaffolds were cultured *in vitro* to assess the scaffold interactions with HUVECs and osteo-differentiated hMSCs, separately, ~~in order~~ to select the best candidate for the co-culture. The results of metabolic activity indicated an enhanced viability of HUVECs inside PVA/G 70/30 with respect to all the other compositions ($p < 0.05$), but 50/50 (p = n.s.) (**Figure 3 A, B**). PVA/G 50/50 showed the second highest value that was not statistically different from that detected in PVA/G 80/20 (p = n.s.). Differently, hMSCs cultured inside PVA/G sponges displayed a statistically significant drop in metabolic activity along the osteoinduction time in PVA/G 50/50 ($p < 0.05$). The best suitable PVA/G composition for hMSCs was 80/20, followed by 70/30, the former being significantly increased with respect to the initial time point ($p = 0.03$) and the highest at the endpoint among all ($p < 0.05$), but 70/30 (p = n.s.). Unlike in PVA/G 80/20, in 70/30 the hMSC viability at the endpoint did not increase from the initial value (p = n.s.). An important aspect to take into account when *in vitro* generating bone is the presence and morphology of the mineral matrix.[24] A morphologic analysis highlighted that well evident mineral nodules were found in PVA/G 80/20 and 70/30 (**Figures 3 C and S2**). Basing on the fact that good mineralization and

osteogenic cell viability are both fundamental aspects to create a functional bone substitute, and that in our experimental plan HUVECs were to be seeded onto pre-generated bone constructs, PVA/G 80/20 was finally chosen for the co-culture. However, PVA/G 70/30 was to be considered a similarly suitable candidate. Interestingly, PVA/G 50/50 that accounted for the largest volume fraction of high size pores did not sustain hMSC viability, thus suggesting that other parameters than pore size affected bone formation. In fact, the results of metabolic activity and histology on single cellular platforms clearly showed both cell- and material-type dependent outcomes. It can be hypothesized that the combination of the polymeric blend composition, its specific pore size distribution, and other topological and physical aspects, altogether influenced each cell type with microenvironmental hints.

Finally, using PVA/G 80/20, after 23 osteoinduction days, viable MSC/scaffold constructs were seeded with HUVECs and differentiated using Matrigel® to obtain endothelial tubes. The results of the co-cultured constructs are shown in **Figure 4**. Cell~~s laden~~ular rings onto the pore surface and resembling tube-like structures could be frequently observed (**Figure 4 A, C**). These cells were negative to von Kossa staining and positive to vascular endothelial growth factor – 2 (VEGFR-2) immunostaining, which revealed endothelial cells. Differently, osteoblasts were found located in large aggregates and embedded in mineral nodules (**Figure 4 A, C, D**). It has been reported that co-culture of endothelial cells with osteoblasts derived from MSCs enhanced tube formation *in vitro* on 3D scaffolds due to the production of cytokines and angiogenic growth factors, with scaffold composition and structure affecting this cellular interplay.[25,26] As an example, the addition of silk fibroin nanofibers to poly (D,L-lactic acid) salt-leached scaffolds with similar porosity and pore size distribution, which were otherwise highly suitable for co-culture, affected endothelial cell and osteoblast response in a inhibitory way.[26] The work of Stoppato and co-workers has nicely shown that either a synergistic or depletive behavior of endothelial and bone cells can be observed depending on certain scaffold architectural and compositional features. However, a systematic screening of

all the parameters involved is needed to understand and possibly predict such a complex interplay.

4. Conclusions

FTIR analysis confirmed the compositional differences and the occurrence of cross-linking in PVA/G sponges. The porosimetric analysis highlighted macropores ranging in 0.08 ~~– 300~~ 125 µm, with different distributions depending on PVA/G composition. Specifically, the higher gelatin content, the larger volume occupied by higher size pores. The results of metabolic activity and histology showed both cell- and material-type dependent outcomes. PVA/G 80/20 scaffolds were finally selected for the co-culture and allowed the formation of mineralized matrix containing organized endothelial-like structures. Interestingly, PVA/G 50/50, which accounted for the largest volume fraction of higher size pores, sustained HUVEC, but osteoinduced-hMSC viability, thus suggesting that other parameters than pore size play undisclosed roles for achieving functional bone tissue engineering.

Supporting Information

Supporting Information is available from the Wiley Online Library

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Figures

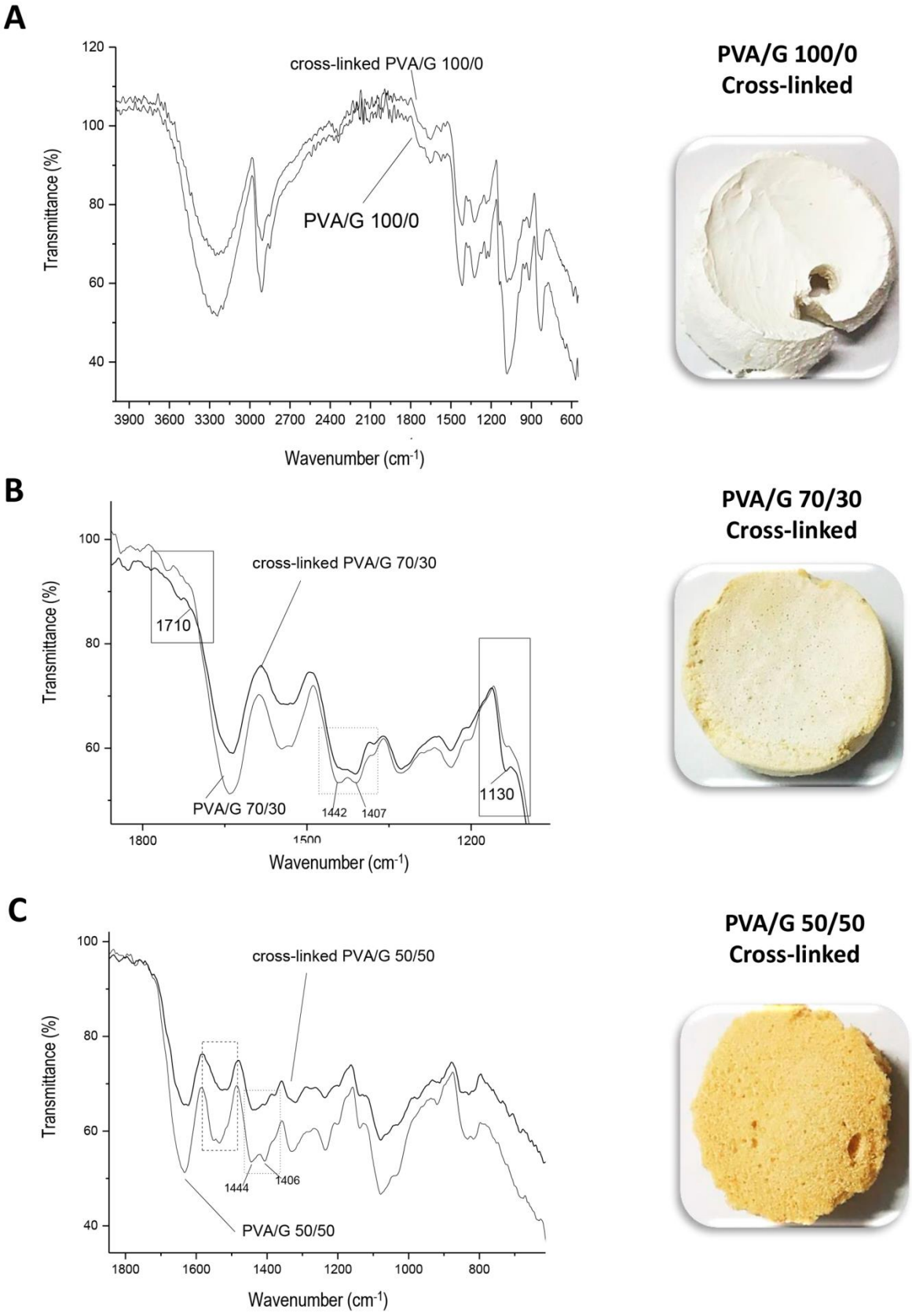

Figure 1. Representative FTIR spectra (cross-linked and uncross-linked) and respective pictures of: (A) PVA/G 100/0, (B) PVA/G 70/30, and (C) PVA/G 50/50 w/w.

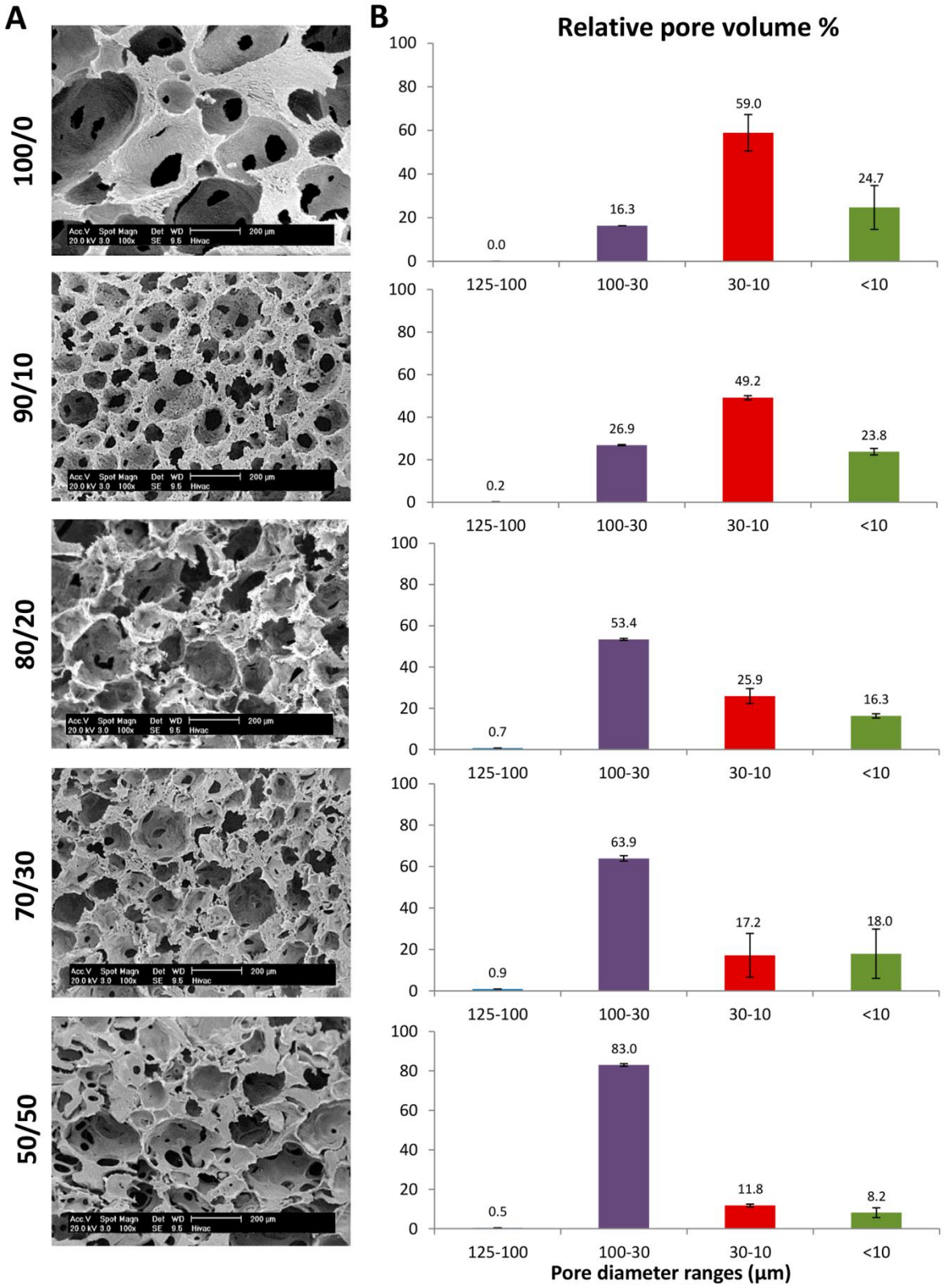

Figure 2. Results of SEM (A) and mercury intrusion porosimetry (B) for all the produced sponges showing pore morphology and pore size distribution, respectively.

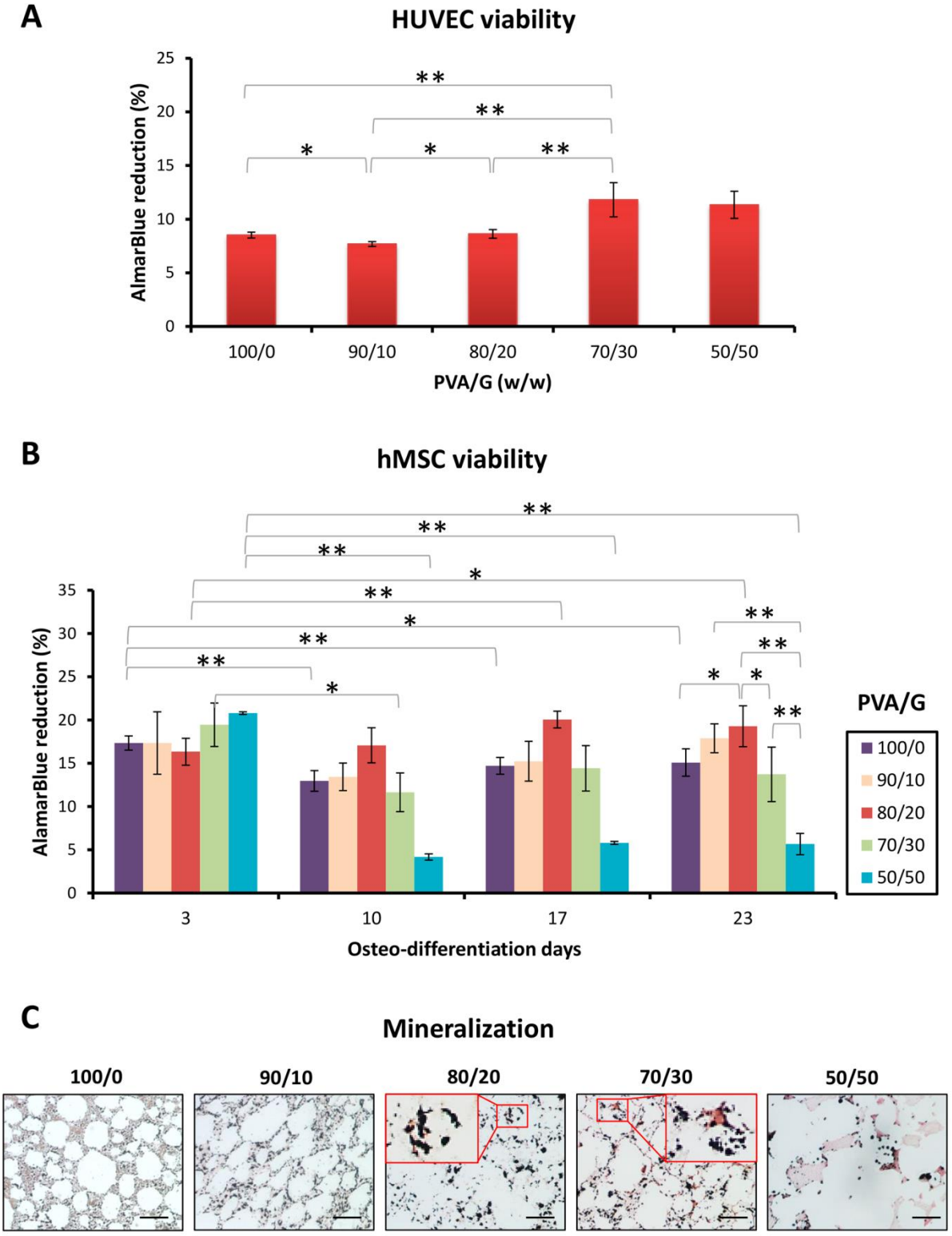

Figure 3. Biological results: cell viability (A,B) and von Kossa staining (C) for HUVECs (A) and osteoinduced hMSCs (B,C) in all the produced scaffolds. (C) Calcium deposits are stained in black; scale bar is 100 µm.

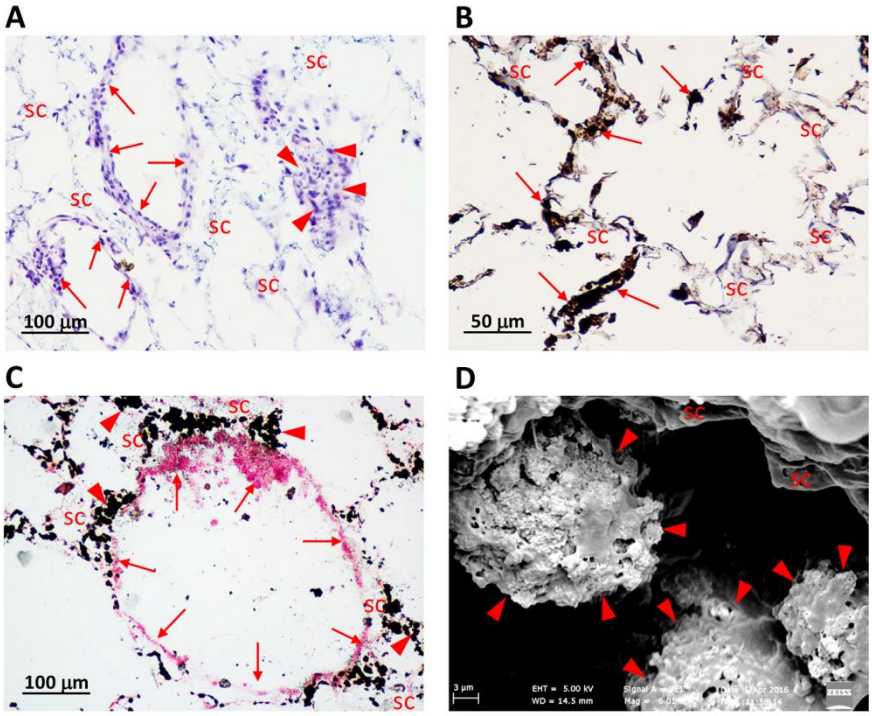

Figure 4. Results of the co-cultured construct: PVA/G 80/20 scaffold cultured with osteoinduced hMSCs and HUVECs: (A) H&E staining showing cell nuclei in blue and cytoplasms in pink; (B) VEGR-2 immunoreaction in dark brown showing endothelial cells; (C) von Kossa staining showing calcium deposits in black and cells in red; and (D) SEM analysis showing osteoblast-like cells and mineral-like matrix deposition. Arrows point to endothelial cells, ~~while~~ arrowheads to osteoblasts~~.~~, "sc" is scaffold material.

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PVA/gelatin scaffolds were produced via emulsion and freeze-drying from 100/0 to 50/50 w/w compositions. Changing composition affected pore size distribution and biological response of endothelial cells and mesenchymal stromal cells, including metabolic activity and differentiation. PVA/gelatin 80/20 scaffolds allowed the formation of mineralized matrix containing organized endothelial-like structures. Systematic investigations on physico-chemical and architectural features can improve vascularized bone substitutes.

De la Ossa J.G., Trombi L., D'Alessandro D., Coltelli M.B., Serino L.P., Pini R., Lazzeri A., Mario P., Danti S.*

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ToC figure