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Chitosan/PEGDA based scaffolds as bioinspired materials to control *in vitro* angiogenesis

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ABSTRACT

In the current work, our purpose was based on the assessment of bioactive chitosan (CS)/Poly(ethylene glycol) diacrylate (PEGDA) based scaffolds ability to stimulate *in vitro* angiogenesis process. The bioactivation of the scaffolds was accomplished by using organic (BMP-2 peptide) and inorganic (hydroxyapatite nanoparticles) cues. In particular, the properties of the materials in terms of biological response promotion on human umbilical vein endothelial cells (HUVECs) were studied by using *in vitro* angiogenesis tests based on cell growth and proliferation. Furthermore, our interest was to examine the scaffolds capability to modulate two important steps involved in angiogenesis process: migration and tube formation of cells. Our data underlined that bioactive signals on CS/PEGDA scaffolds surface induce a desirable effect on angiogenic response concerning angiogenic marker expression (CD-31) and endothelial tissue formation (tube formation). Taken together, the results emphasized the concept that bioactive CS/PEGDA scaffolds may be novel implants for stimulating neovascularization of tissue-engineered constructs in regenerative medicine field.

1. Introduction

In the tissue-engineering field, angiogenesis represents a key phase in bone regenerative processes [1]. Indeed, bone is a vascularized tissue thanks to the spatial and temporal proximity between capillary vessels and bone cells, with the aim of maintaining skeletal integrity [2]. Nearly all tissues require a regular vascularization and blood supply to support their functions. Specifically, in skeletal development and repair, angiogenesis has an essential role in events like endothelial cell migration, proliferation and differentiation [3]. Consequently, changing in the vascularization processes characterized by hypoxia, lack of nutrients, accumulated waste products, altered biochemical signalling and cell recruitment could lead to failure of tissue balance and consequent inhibition of tissue regeneration. Regarding bone healing, an adequate vascularization has been appeared to be critical to ensure the bone implant success [4]. Different studies reported that the correlation between angiogenesis and other processes, such as osteogenesis and inflammation, ensure a successful bone repair [5]. In the context of tissue engineering, novel approaches take care of offering future therapeutic opportunities and possibilities to repair and regenerate bone tissue [6]. In this way, to study possible materials and devices with bone regenerative properties, one of the approaches may be to introduce bioactive signals, genes or growth factors able to enhance vascular and endothelial cells viability to prolong the lifespan of construct engineered vessels [7]. In particular, the knowledge behind the cellular processes involved into angiogenesis can help to develop novel and innovative treatments to promote bone fracture healing and support tissue vascularization, thus accelerating the healing response and tissue regeneration. In this scenario, Chitosan (CS), a natural polymer, has gained attention thanks to its inherent ability to combine with other bioactive molecules such as Bone Morphogenic Protein-2 (BMP-2) peptide, to enhance the biological response [8]. Previous studies reported that porous scaffolds composed by CS/PEGDA and bioactivated with biomimetic signals (organic and inorganic), possess osteoinductive activity in the local bone fractures treatment [9]. Starting from several studies that report CS materials used for vascular tissue applications

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aiming to reproduce vascular construction similar to the native vessels by combining biodegradable scaffolds with cells [10], it is very interesting to consider these composites as alternative way for treating problems associated to blood vessels necrosis. Obviously, the understanding of critical angiogenic mechanisms requires peculiar *in vitro* models capable to simulate main angiogenic steps so as to assess therapeutic agents' efficacy (that up-regulate or down-regulate specific angiogenic mechanisms). Notably, many models often attempt to mimic main steps of the angiogenesis to evaluate whether and how materials can influence cellular function, in terms of angiogenic biological response [11]. As it was widely reported, endothelial cells (EC) take part in several biological events including physiological haemostasis, capillary vessel permeability and response to physiological and pathological stimuli [12]. Therefore, the cells could be useful in pre-vascularized or endothelialized constructs to obtain successful tissue substitutes and improve vascularization in osteoporotic site [13]. On the basis of these works that have highlighted the correlation between angiogenesis and osteogenesis [14], our goal was to develop and study the effect of bioactive CS/PEGDA scaffolds on angiogenesis process. Here, the potential of bioactive CS/PEGDA scaffolds on angiogenesis induction by evaluating cell migration, proliferation and tube formation on HUVECs based *in vitro* model, was investigated.

2. Experimental section

2.1. Scaffold preparation and bioactivation

The CS/PEGDA scaffolds used in this work were designed and developed in collaboration with the University of Salento [9]. Briefly, scaffolds were synthesized combining physical foaming and microwave curing processes. Chitosan was purchased from Sigma Aldrich and used as received (Medium molecular weight, molecular weight of 190–310 kDA, deacetylation degree of 75–85%, and a viscosity of 200–800 cPs 1 wt% in 1% acetic acid at 25 °C, Brookfield). CS solution was prepared by dissolving chitosan in acetic acid (AA) at concentration of 0.1 M (AA, 99%) solution (1.5%wt). The mixture was stirred for 90 min to guarantee a homogeneous viscous polymer solution; successively, fixed amounts of PEGDA (40% w/v, average molecular weight of 700 g/mol) were introduced in CS solution and stirred for about 2 h. Pluronic F127 (0,4% w/w) was used as specific physical blowing agent, in order to obtain a homogeneous distribution of air bubbles in a stable form. Several washings of resulting samples were done in acetone to remove the possible unreacted PEGDA and dried in a vacuum oven. The bioactive scaffolds composed by 60/40 w/v ratio of CS and PEGDA (60CS40P), were obtained through inorganic and organic treatments, respectively as described by Soriente *et al* [9]. In this way, the process allows to obtain biomineralized scaffolds with hydroxyapatite (HA) deposition on the scaffolds surface (CS-BIOM) and scaffolds bioactivated with BMP-2 peptide (2.7 μM, CS-BMP2). According to preliminary biological studies performed on BMP-2 peptide at different concentrations, the peptide amount was determined [9]. The sterilization was carried out in Ethanol at 70% under UV ray for 2 h.

2.2. Scaffold characterization: morphological investigations

60CS40P -based scaffolds were characterized by using Scanning Electron Microscopy (SEM, JEOL 6310) and Microcomputed tomography (MicroCT) analyses (Bruker Skyscan 1172, Kontich, Belgium) as reported in previous paper [9].

2.3. Biological characterization of the scaffolds

2.3.1. Cell culture: isolation and maintenance of human umbilical vein endothelial cells (HUVECs)

ENDOGRO™ HUVECs were provided at passage 1 from Millipore (USA) and have been quality tested in low-serum media without phenol red. After harvesting, cells were grown in cell culture polystyrene flasks T75 cm^2 pre-coated with a solution of purified human fibronectin (Fnc, 2 mg/mL) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. ENDOGRO™ medium for human endothelial cells was used. The medium consisted of 0.2% ENDOGRO-LS supplement, 5 ng/mL rhEGF, 10 mM L-glutamine, 1.0 mg/mL hydrocortisone hemisuccinate, 0.75 U/mL heparin sulfate, 50 mg/mL ascorbic acid, 5% FBS. The detachment of cells was obtained by incubation with trypsin (Gibco, 0.05% trypsin with 0.02% EDTA in PBS) and then residual trypsin was inactivated by addition of culture medium.

2.4. Analysis of CS/PEGDA based scaffolds effect on HUVECs viability

2.4.1. Qualitative analysis: live-dead assay

To study scaffolds cell viability a qualitative *in vitro* live/dead assay (Invitrogen) was performed. 1×10^3 HUVECs were put in a 48-well plate with 5% CO_2 and 95% air at 37 °C. The assay allowed to distinguish living from dead cells by using calcein AM/propidium iodide. Calcein AM stained living cells in green, propidium iodide stained dead cells in red. Bioactive and non-bioactive 60CS40P -scaffolds were incubated with ENDOGRO medium for 24 h. Then, the cells were incubated with the collected conditioned media and incubated for 24 h. Later, the detached cells were removed by washing with PBS 1×; cells were treated with calcein AM (2.5 μL) and propidium iodide (10 μL) at 37 °C for 15 min. Subsequently, cell culture was washed with PBS 1× thrice. To assess cell morphology and spreading pattern interaction of HUVECs with CS-scaffolds, cells were observed under a confocal laser microscopy (LSM510, CarlZeiss) and/or fluorescent microscope. Images of two sites per well with microscope and digital camera (10× objective) were taken.

2.4.2. Calcium release from CS-based scaffolds

Intracellular calcium concentration regulates several physiological and pathological events in the cellular environment [15]. Here, *Ortho-Cresolphthalein complexone* (CPC) method was used to quantify the calcium released in the medium from scaffolds [16]. In this way, scaffolds were sterilized by using Ethanol 70% and washed thrice in PBS 1×. Then, bioactive and not bioactive 60CS40P-scaffolds (50 mg/mL) were moved in 96 multi-well plate with two different culture media: ENDOGRO™ and DMEM 1×, respectively. The media contain different calcium amount, ENDOGRO™ contains 2.5 mM, meanwhile DMEM 1× contains 0.25 mM. After 24 h, all CS-scaffolds conditioned media were cleared with centrifugation. Resulting supernatants were assessed at 1 and 4 days.

2.4.3. Cell viability test on 3D scaffolds

To assess 60CS40P -scaffolds effect on angiogenesis, HUVECs at passage 5 were used. Scaffolds were sterilized in 70% Ethanol (2 h) under UV-ray and then pre-wetting them in medium (2 h). Culture medium during experimental time was replaced every 2–3 days. The experiments using ENDOGRO™ medium products specific for human endothelial cells were carried out. The cells, at density of 1×10^3/50 μL of medium, were seeded onto scaffolds in 96-well plate. For cytotoxicity assay, after the initial incubation of a drop of cell suspension (2 h) for inducing cell adhesion, 150 μL of cell-culture medium were added to each well and the plate was incubated for 1, 3 and 7 days. Cell metabolic activity was analyzed using PicoGreen® dsDNA reagent and kits (Invitrogen), useful to measure nucleic acid concentration, according to the manufacturer's instructions. Cell viability was evaluated at 1, 3 and 7 days of cell culture. At these time points, samples were washed in PBS 1× and added to them lysis buffer. Then, freezing (−80 °C) and thawing (37 °C) cycles were performed. For standard curve preparation, serial dilutions of dsDNA stock, at final concentrations of 0–2000 ng/mL were prepared. Then, 100 μL of working solution to samples or standards was added. The fluorescence was measured at room temperature (RT) after 2–5 min and in dark condition, through

a UV–Vis spectrophotometer at wavelengths of 450 nm (Victor X3, Perkin-Elmer, Italy).

2.5. Cell migration

2.5.1. Scratch wound assay

The migration capability of HUVECs was assessed by using a scratch-wound assay [17]. To estimate the potential of 60CS40P -scaffolds on cell migration, a scratch-wounded HUVECs monolayer was utilized. Briefly, cells to confluence (80%) into 12-well tissue culture using ENDOGRO medium were seeded. Mitomycin (10 μg/mL) as proliferation inhibitor was added to confluent cells before doing the scratch. Confluent HUVECs were scrape-wounded with a sterile pipette tip (p200) for one perpendicular linear scratch. A blank line perpendicular to the scratch, painted under the bottom of the plate, ensured recording of the same wound area per well. The dead cells were removed by washing the wells with PBS 1× and then conditioned media were added to each well. After the scratch, wound closure was monitored collecting digitalized images at 4 and 18 h. Images of two sites per well through an inverted microscope (Nikon TE200) and digital camera (Olympus DP72) (5× objective) were captured. To estimate cell migration, three scratched areas representative images from each well were captured. The wound areas reduction was analyzed using *Image J1.48i* software [18]. Four replicates per condition were used and three experiments were done.

2.6. Tube formation

2.6.1. In vitro Matrigel-based formation assay

To reproduce a later angiogenic stage and study the possibility to use 60CS40P-scaffolds as angiogenesis stimulators or inhibitors, a Matrigel-based model was used. The tubes formation was determined in 48-well plates coated using Matrigel (Corning®) for 1 h at 37 °C. HUVECs at passages 5 were harvested by trypsinization and seeded onto or within Matrigel. At the same time point, 60CS40P-scaffolds conditioned media were added to the wells. The formation of tubes was evaluated at 4 and 8 h through inverted microscope (Leica). At specific time points, tubule development was observed and the degree of formed tubule was quantified using *Angiosys 2* analysis program.

2.7. Immunofluorescence (CD31) expression in HUVECs culture

Immunofluorescence analysis for studying the potential of 60CS40P-scaffolds on HUVECs differentiation and tube formation through CD31 expression after 7 days of cell culture was realized. The experiment with all 60CS40P-scaffolds conditioned media in contact with HUVECs, was performed. The staining was carried out using endothelial cell adhesion molecule-1 (CD31) antibody (BioLegend) on HUVECs after fixation with formalin solution 10%, permeabilization with Triton 0,1% and blocking with bovine serum albumin BSA 0,5% (Sigma-Aldrich). Later, specific marker protein FITC anti-human CD31 antibody (1:200 dilution) was incubated overnight at 4 °C. After, to remove the excess staining, the cells were washed in PBS 1× and the nuclei were stained with 4′, 6-diamidino 2-phenylindole (DAPI, Molecular Probes) 1 μg/mL at 37 °C for 15 min. Then, after several washings, the samples by using confocal laser microscopy (Leica TCS SP8 confocal microscope) were observed.

2.8. Statistical analysis

All quantitative experiments were performed in triplicate and the results as mean ± standard deviation (SD) were expressed. Statistical analysis was undertaken using GraphPad Prism®, version 5.00 (GraphPad Software, La Jolla California USA, www.graphpad). Data were analyzed using a Student's *t*-test, a one-way ANOVA, with a Bonferroni post-test (parametric methods). Group differences of

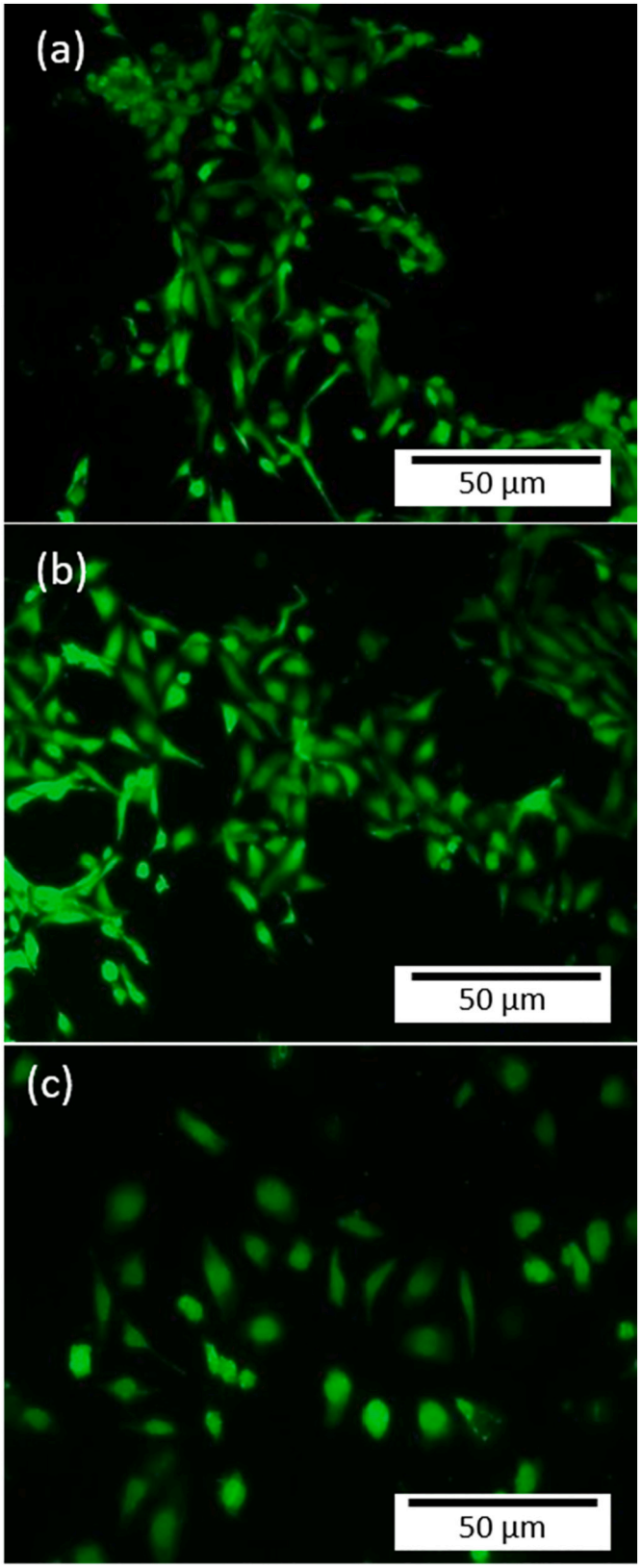


Fig. 1. Fluorescent microscopic images of live (green)/dead (red) staining for HUVECs in contact with neat CS (a), CS-BIOM (b) and CS-BMP2 (c) conditioned media after 24 h. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

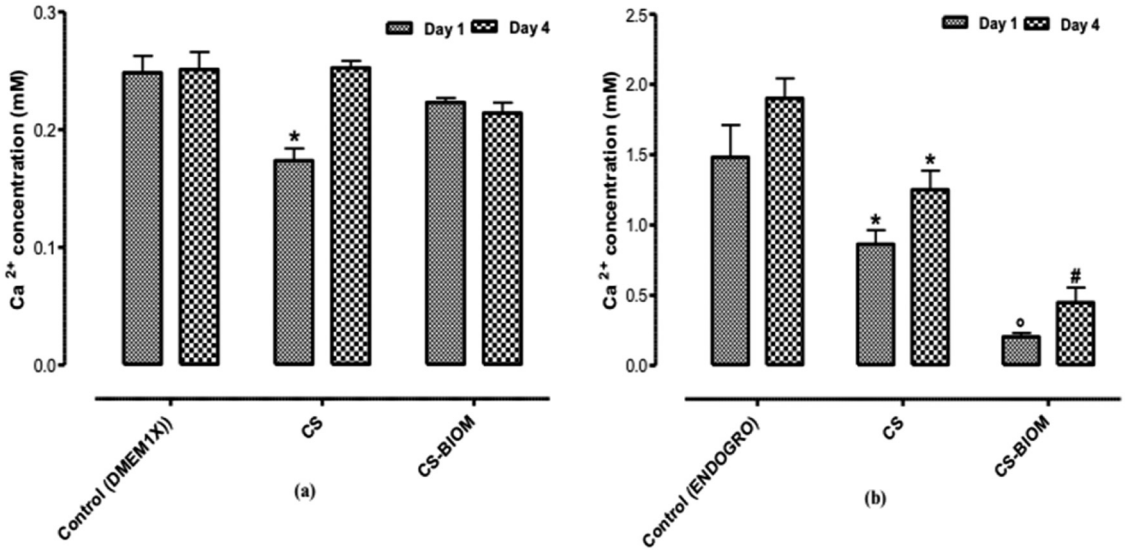


Fig. 2. Calcium concentration (mM) released from CS and CS-BIOM scaffolds conditioned with DMEM 1× (a) and ENDOGRO™ (b) media for 1 and 4 days. The results, expressed as Ca^{2+} concentration (mM) are the mean ± SEM of three experiments. *p < 0.05, °p < 0.01 and #p < 0.001 *vs* control (DMEM 1×, ENDOGRO).

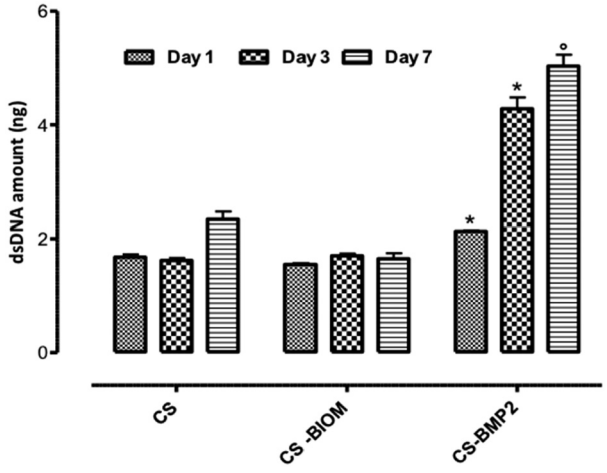


Fig. 3. HUVECs proliferation through PicoGreen assay was evaluated. Cells were incubated with CS, CS-BIOM and CS-BMP2 scaffolds for 1, 3 and 7 days of cell culture. Quantitative results, (DNA amount, ng/mL) represent the mean ± SEM of three experiments. *p < 0.05; °p < 0.01 *vs* CS in ENDOGRO medium.

p < 0.05, p < 0.01, p < 0.001 were considered statistically significant.

3. Results

3.1. Scaffold characterization: morphological investigations

The physico-chemical and morphological investigations of CS/PEGDA scaffolds were reported in a previous study [9]. However, here a briefly description of morphological results was reported. In particular, morphological characterization performed through SEM and micro-CT analyses demonstrated that 60CS40P scaffolds show an interconnected and homogeneous structure with pores size ranging from 20 to 300 μm and a porosity of 97.9%, respectively as previously reported [9]. This porosity is due to the specific CS/PEGDA ratio where the presence of PEGDA allows to obtain a stable structure with good cell infiltration [9].

3.2. HUVECs viability: live-dead assay

To investigate the response of CS/PEGDA scaffolds on HUVECs viability a live-dead assay was performed. Microscopy images showed that all CS/PEGDA scaffolds conditioned media do not compromise HUVECs survival and cellular spreading (Fig. 1). Moreover, a considerable difference in HUVECs morphology between all CS/PEGDA-scaffolds (CS) was observed. In particular, CS/PEGDA scaffolds bioactivated with BMP-2 peptide (CS-BMP2) confer a different HUVECs morphology than other samples (neat CS and CS-BIOM). This effect is probably ascribable to expression of proteins (VE-cadherin) involved in cell differentiation, which induces an increasing in HUVECs permeability [19].

3.3. Calcium release from CS-based scaffolds

Ortho-Cresolphthalein complexone (CPC) method suggested that CS/PEGDA scaffolds do not release cytotoxic calcium concentration for HUVECs viability. In particular, bioactive and non-bioactive CS/PEGDA scaffolds contain a lower calcium amount than DMEM 1× (Fig. 2a) and ENDOGRO media (Fig. 2b). This behaviour could be explained considering the higher adsorption capability of CS/PEGDA scaffolds [20].

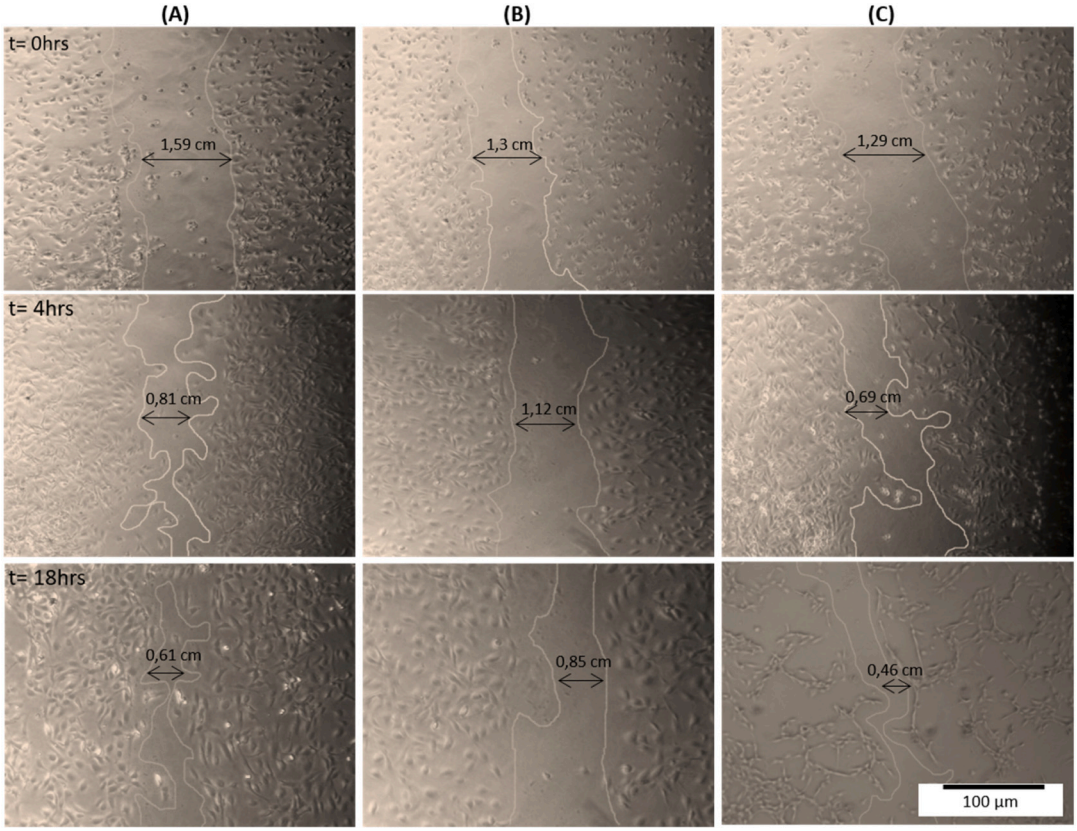
3.4. Effect of CS-based scaffolds on cell proliferation, migration and tube formation

3.4.1. HUVECs proliferation

To estimate the angiogenic behaviours of the biocomposites, HUVECs represent a valid tool useful to study angiogenesis [21]. On this basis, our goal was to test *in vitro* proliferation of HUVECs on neat and bioactive CS/PEGDA scaffolds at specific time points (*i.e.* 1, 3 and 7 days). The results have suggested that bioactive and non-bioactive scaffolds supported cell proliferation over culture time (Fig. 3). Moreover, CS-BMP2 scaffolds showed a better proliferation than CS-BIOM scaffolds. This behaviour, probably, was due to potential hydroxyapatite effect to influence differentiation mechanisms of HUVECs [21].

3.4.2. Cell migration: scratch-wound assay

The wound healing effect of all CS/PEGDA scaffolds on HUVECs migration, using a scratch assay model was evaluated. Specifically, for



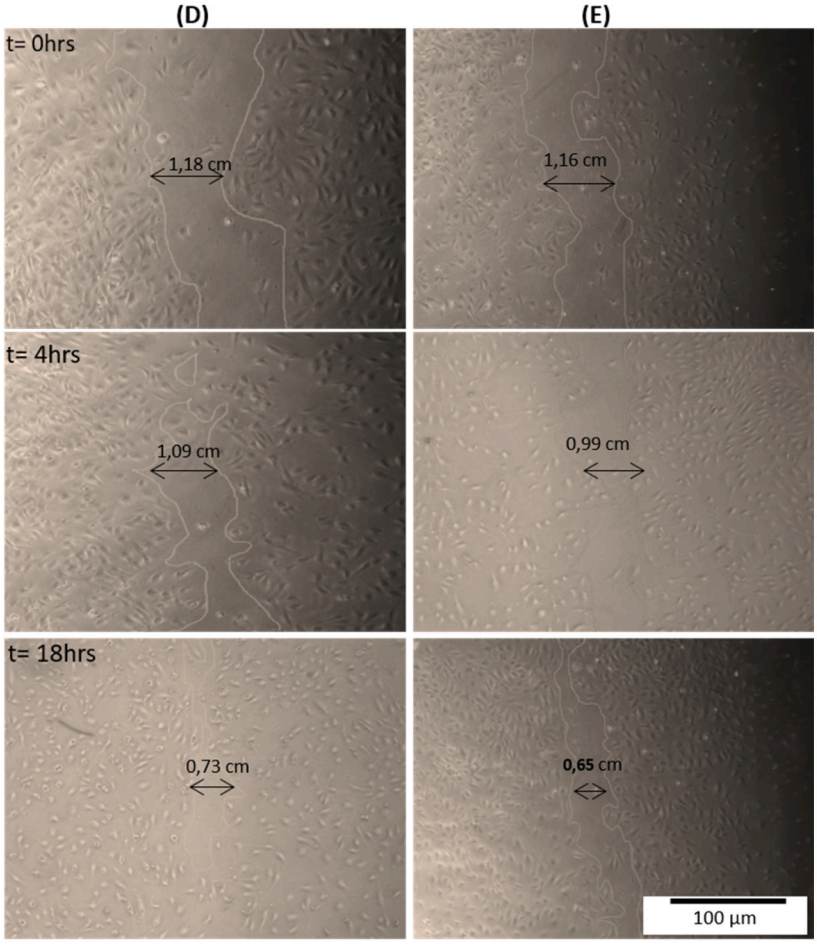


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Fig. 4. Optical images show scratch of HUVECs obtained after treatment with neat CS (A), CS-BIOM (B) and CS-BMP2 (C) conditioned media compared to positive control VEGF (20 ng/mL) (D) and HUVECs (E). Reduction of scratch area was observed at 4 and 18 h of cell culture. Optical images are representative of three experiments. Scale bar = 100 μm.

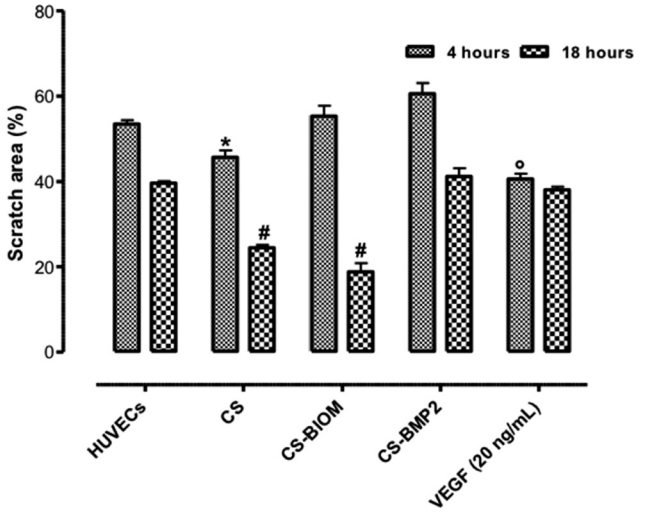

Fig. 5. Effect of CS-scaffolds on the migratory activity of HUVECs after 4, 18 h of culture. Quantitative analysis of cell migration, in contact with CS, CS-BIOM, CS-BMP2 scaffolds conditioned media, as compared to control (HUVECs), positive control (VEGF 20 ng/mL) was reported. Data are expressed as scratch area percentage (%) at 4, 18 h in the wounded area normalized to 0 h. Bars represent the mean ± S.E.M. of three experiments. $^{\#}p < 0.001$; $^{\circ}p < 0.01$; $^{*}p < 0,05$ *vs* control (HUVECs) in ENDOGRO medium.

the experiment Mitomycin used as proliferation inhibitor allows to study the CS/PEGDA scaffolds conditioned media effect on cell migration. Qualitative analysis suggested that the scaffolds possess the ability to induce HUVECs migration (Fig. 4) by promoting a reduction of wounded area at 0, 4, 18 h of cell culture.

In addition, quantitative results have confirmed the scaffolds capability to stimulate and support HUVECs migration. Precisely, quantification of HUVECs migration process in contact with CS/PEGDA scaffolds conditioned media, have suggested that the materials stimulated cell migration thus reducing the scratch area (20%) compared to control (HUVECs) and positive control (VEGF 20 ng/mL). In particular, the best scratch area reduction for neat CS and CS-BIOM were obtained at 18 h of cell culture (Fig. 5).

3.4.3. Tube formation quantification

As mentioned before, angiogenesis consists of different steps as cell migration, proliferation and remodelling in the process of tube formation [22]. To evaluate these stages of angiogenesis, HUVECs and Matrigel were used on *in vitro* tube formation model. In fact, HUVECs undergo capillary-like tube formation on Matrigel component. The results have suggested that CS/PEGDA based scaffolds promoted HUVECs tube formation at 4 and 8 h. Indeed, qualitative analyses have suggested the possibility to use CS-based materials for promoting tube formation of HUVECs (Fig. 6).

Fig. 6 shows the typical appearance of tubes on Matrigel for each CS/PEGDA based scaffolds at 4 and 8 h (neat CS, CS-BMP2 and CS-BIOM) as compared to negative and positive controls (HUVECs and VEGF 20 ng/mL), respectively. Furthermore, the angiogenic activities of scaffolds by quantitative evaluations of tube formation were confirmed. In particular, bioactive and non-bioactive scaffolds are capable to modify angiogenic parameters expressed as total tube length, branches and junction's quantification (Fig. 7).

3.5. Effect of CS-based materials on CD31 expression in HUVECs culture

In order to study the effect of CS/PEGDA scaffolds on CD31 expression, an important marker of angiogenesis, a confocal microscopy investigation was done at 7 days. HUVECs confocal images fluorescently labelled with CD31 antibody (green) and DAPI (blue), have shown a higher fluorescence intensity of green signal for CS-scaffolds bioactivated with BMP-2. Thus, CS-BMP2 induced a higher CD31 expression compared to CS-BIOM and neat CS-scaffolds (Fig. 8).

4. Discussion

Angiogenesis includes several physiological and pathological processes and allows to generate new capillary blood vessels. Specifically, during bone implant the vascular system development is essential and angiogenesis takes part in bone regenerative processes [23]. Indeed, it has been reported that during bone implant, inflammation response, microbial infections and blood vessels necrosis occur [24]. In this context, the knowledge of specific biomaterials able to promote angiogenesis could provide a promising diseases treatment. For this purpose, our goal was to assess the potential of two bioactive signals on CS/PEGDA scaffolds to obtain a potential platform which combine different biological activities. Starting from our study that suggested the ability of bioactive CS/PEGDA scaffolds to modulate osteogenesis by promoting proliferation of mesenchymal stem cells and differentiation in osteoblast [9], this study focused on the assessment of bioactive functional scaffolds on HUVECs migration, proliferation and tube formation to reproduce *in vivo* conditions. This study was possible thanks to the structural properties of scaffolds, in terms of stability and porosity obtained by the combination of CS and PEGDA at specific ratio [9] that allow cell migration and tube formation. The results have underlined that bioactive scaffolds promoted a good cell proliferation over culture time even though it is lower comparable to non-bioactive control group. In particular, CS/PEGDA scaffolds bioactivated with hydroxyapatite (BIOM) showed a comparable cell proliferation to neat CS/PEGDA-scaffolds. Meanwhile, CS/PEGDA scaffolds bioactivated with BMP-2 peptide induced a better cell proliferation at 1, 3 and 7 days than biomineralized (CS-BIOM) and neat CS (Fig. 3). This behaviour could be due to the presence of organic bioactive signal which promotes cell proliferation by activating several receptors involved in many events [25]. Moreover, despite calcium concentration is involved in cell disruption and integrity, calcium amount released from CS/PEGDA scaffolds was assessed. The experiment has pointed out that scaffolds don't exert any cytotoxic effect on HUVECs viability. Then, HUVECs migration was studied by scratch assay, even if it cannot consider as substitute of *in vivo* studies as a final proof of the angiogenic efficacy. In particular, all CS/PEGDA scaffolds induced HUVECs migration (Figs. 4 and 5) by reducing scratch area at specific time points (4, 18 h). In details, the best results were obtained with neat CS and CS-BIOM at 18 h (quantitative data). This result was supported by qualitative analysis, where the scratch area reduction over culture time was obtained for all CS/PEGDA scaffolds, compared to positive control (VEGF 20 ng/mL). Furthermore, tube formation study of HUVECs has established that all scaffolds induce tube formation at 4 and 8 h. It has been reported by current studies, BMP-2 plays a pivotal role in vascular development [26]. Indeed, BMP-2 promotes vascularization and angiogenesis thanks to its ability to stimulate Id1 and p38 MAPK pathway. In the context of modular proteins, the incorporation of BMP-2 peptide is a great strategy to engineer biomaterials in tissue engineering field [27]. On this basis, we have hypothesised that scaffolds bioactivated with BMP-2 peptide activate proangiogenic stimuli and mechanisms.

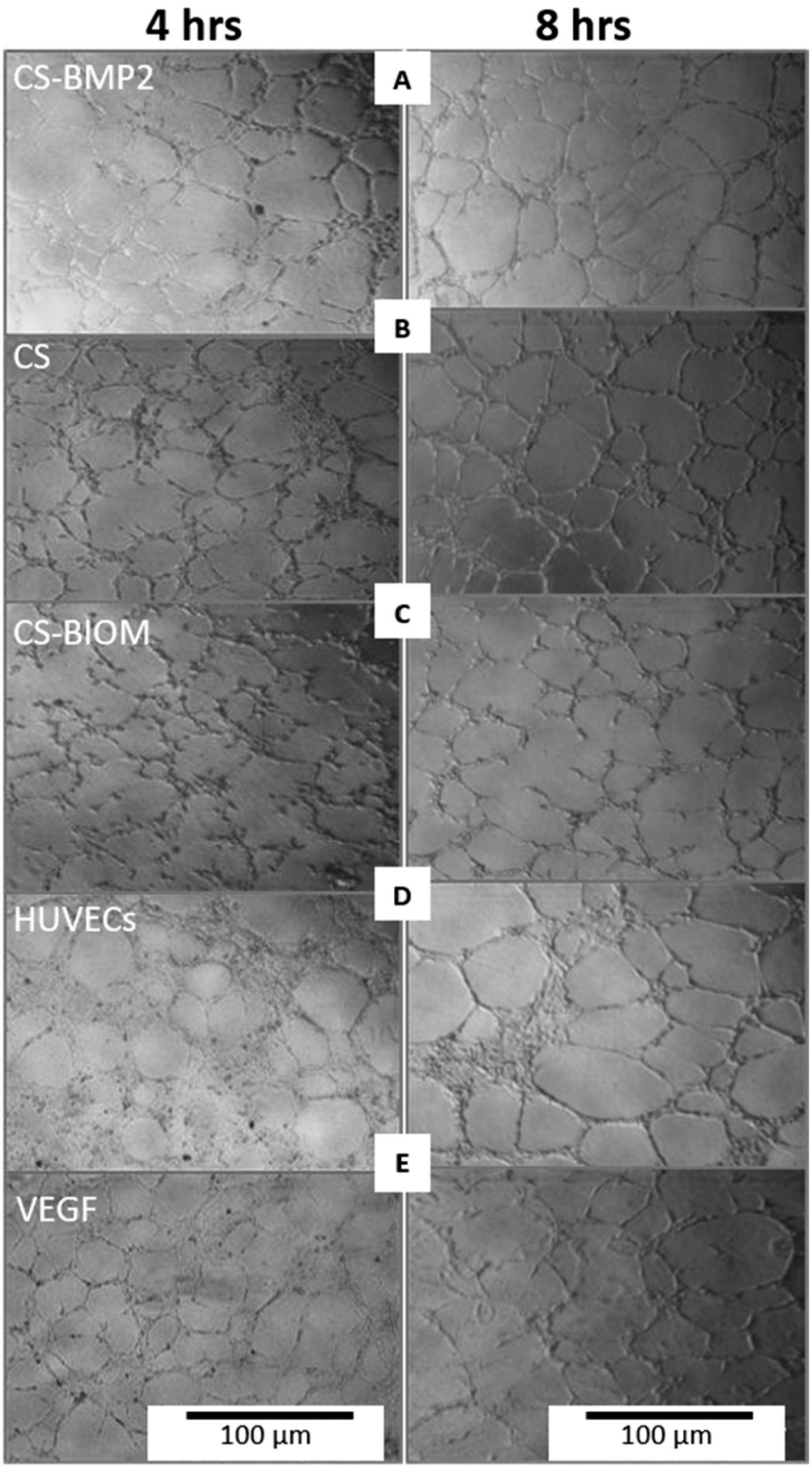

Fig. 6. Optical images of HUVECs tubes at 4 and 8 h of incubation time of CS-BMP2 (A), neat CS (B), CS-BIOM (C), HUVECs seeded on Matrigel (D), VEGF (20 ng/mL) (E). Scale bar = 100 µm.

Indeed, the results have highlighted that CS-BMP2 stimulated HUVECs migration, proliferation and the tube-like structures formation by cells cultured on Matrigel than CS/PEGDA scaffolds without bioactivation. Furthermore, HUVECs morphology changed in presence of CS/PEGDA scaffolds bioactivated with BMP-2. This behaviour could be due to expression of specific proteins involved in cell differentiation such as CD31 (PECAM-1). In literature, it is reported that BMP-2 stimulates phosphorylation of Erk-1/2, Smad 1/5, and increases Id1 expression. Indeed, Erk-1/2 regulates several cellular functions of endothelial cells including proliferation and tube formation related to vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and angiogenin expression [28].

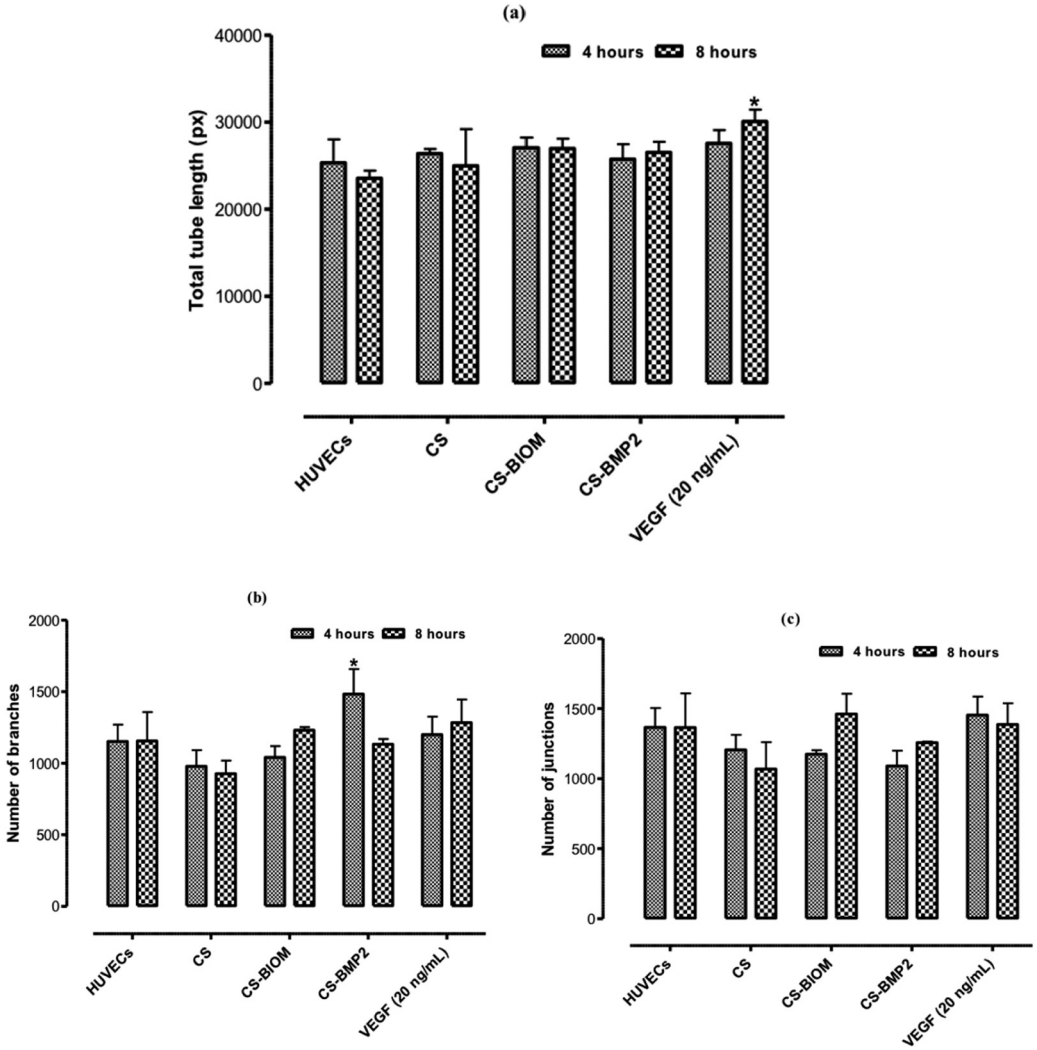

Fig. 7. Quantification of tube formation in CS, CS-BIOM, CS-BMP2 conditioned media compared to HUVECs seeded on Matrigel and VEGF (20 ng/mL). Results, relative to cells growing on Matrigel are expressed as a mean of total tube length (a), number of branches (b) and junctions (c) after 4, 8 h of cell culture. Results are the mean ± SEM of three experiments (in triplicates). *p < 0.05 *vs* control (HUVECs) in ENDOGRO medium.

Additionally, many studies reported that the receptors of BMP-2 promote HUVECs hyperpermeabilization by internalizing and c-SrC-phosphorylating VE-cadherin which causes an increasing in cell permeability [29]. Therefore, our study suggested that all CS/PEGDA scaffolds were not cytotoxic and CS bioactivated with BMP-2 change HUVECs morphology by stimulating angiogenesis in term of cell migration, proliferation and tube formation.

5. Conclusions

The findings have revealed that CS/PEGDA scaffolds provide a positive response to HUVECs. Indeed, bioactive and non-bioactive scaffolds are capable to control *in vitro* angiogenesis. In particular, organic bioactive signal (BMP-2) on CS/PEGDA scaffolds surface allows to promote better angiogenesis process compared to biomineralized (BIOM) and neat CS/PEGDA scaffolds. Indeed, the work encourages the research concerning the use of bioactive CS/PEGDA scaffolds as potential implant for bone regeneration and repair by enhancing osteogenesis and angiogenesis simultaneously in tissue engineering field.

CRediT authorship contribution statement

Alessandra Soriente: Conceptualization, Methodology, Biological Investigations, Data curation, Writing - original draft, Writing - review & editing.

Soledad Pérez Amodio: Methodology, Biological Investigations, Supervision, Writing - review & editing.

Ines Fasolino: Conceptualization, Methodology, Writing - original draft, Writing - review & editing.

Maria Grazia Raucci: Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing.

Christian Demitri: Material preparation, Writing - review & editing.

Elisabeth Engel: Resources, Writing - review & editing.

Luigi Ambrosio: Resources, Writing - review & editing.

Declaration of competing interest

The authors have declared that there is no conflict of interest.

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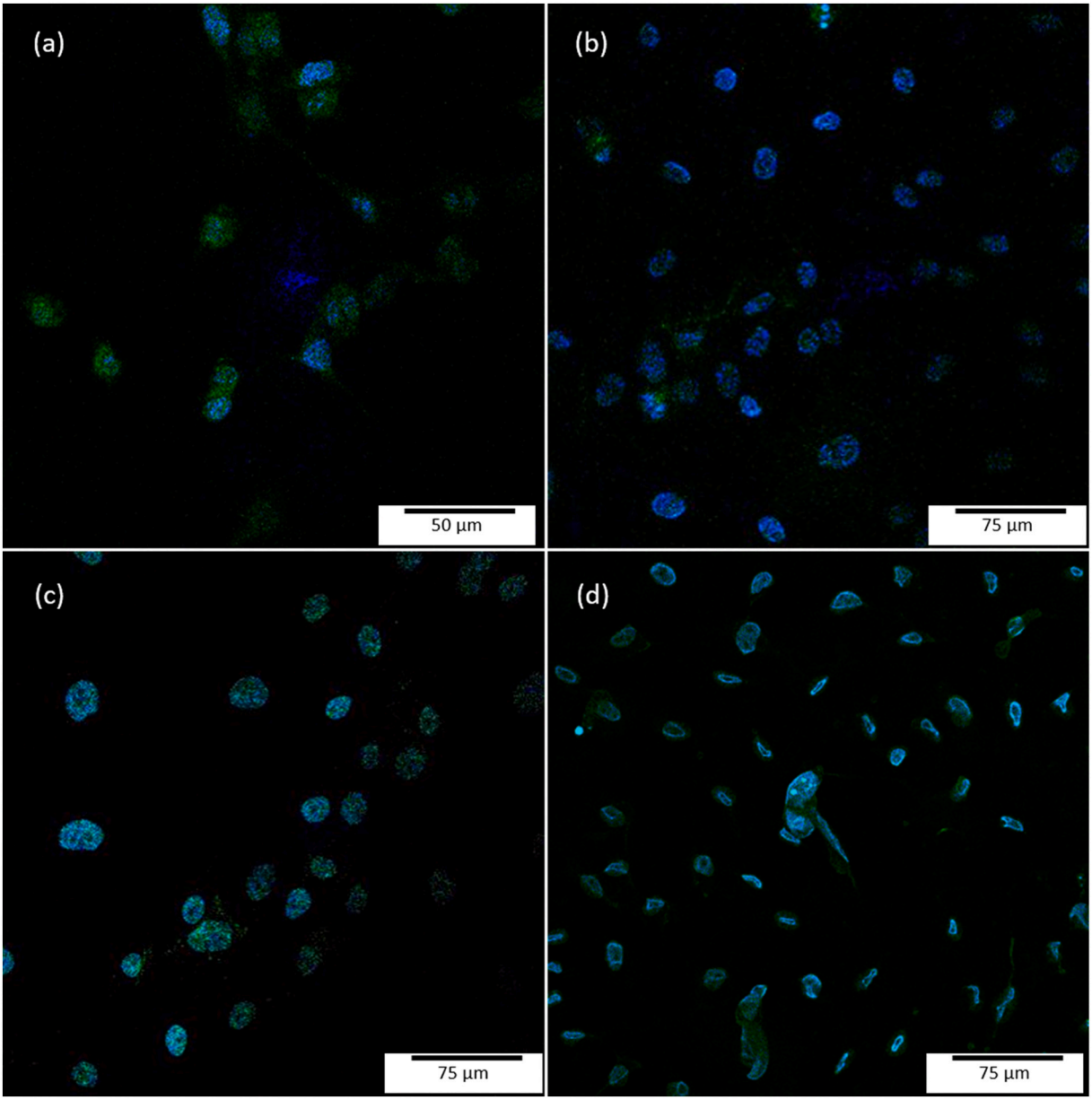

Fig. 8. Confocal images of CD31 labelling of HUVECs for neat CS (b), CS-BIOM (c) and CS-BMP2 (d) based scaffolds compared to control HUVECs (a) at 7 days of cell culture. CD31 (green) and nuclei (DAPI) signals were observed. Scale bar = 50 and 75 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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