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Abstract: Polyelectrolyte complexes (PEC) of chitosan and ulvan were fabricated to study ALP mediated formation of apatitic minerals. Scaffolds of the PEC were subjected to alkaline phosphatase (ALP) and successful mineral formation was studied using SEM, Raman and XRD techniques. Investigation of the morphology via SEM shows globular structures of the deposited minerals, which promoted cell attachment, proliferation and extracellular matrix formation. The PEC and their successful calcium phosphate based mineralization offers a greener route of scaffold fabrication towards developing resorbable materials for tissue engineering.

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Dear Editor,

Carbohydrate Polymers

We are honored to submit our manuscript entitled "Ulvan-Chitosan Polyelectrolyte Complexes as Matrices for Biomineralization" for your consideration.

The article focuses on the development of bioresorbable scaffolds for tissue engineering purposes, where the aim was to evaluate a combination of the two different types of charged polymers namely ulvan and chitosan, whose presence could be beneficial to each other. This is a sequential study of the already published work on the mineralization of UV crosslinked ulvan methacrylate scaffolds. In this follow-up study the rationale is to evaluate a greener material as compared to our previously published material, ulvan methacrylate since, exposure to UV irradiation in some cases is known to cause significant degradation of materials. The polyelectrolyte complex of ulvan and chitosan is fabricated to act as a bioresorbable template for the enzyme, alkaline phosphatase to allow nucleation and mineral crystal formation.

Carbohydrate Polymers offer its audience an elegant combination of materials and their interaction with cells for various applications. We hope that even this manuscript with its interdisciplinary approach will be interesting laying more insight into our series of publications related to enzymatic mineralization on polymer matrices.

This article is not under consideration elsewhere and is not published on web as well.

We hope the article will meet the high standards of the journal of Carbohydrate Polymers.

With best regards,

Mamoni Dash

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Highlights

- In the present study, it is aimed to investigate the role of enzymatic mineralization on polymeric structures bearing both cationic and anionic groups.
- Polyelectrolyte complexes of ulvan and chitosan are evaluated as matrices for biomimetic mineralization using ALP.
- Successful mineralization by ALP is observed which improved cellular activity.

Ulvan-Chitosan Polyelectrolyte Complexes as Matrices for Biomineralization

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ABSTRACT

Polyelectrolyte complexes (PEC) of chitosan and ulvan were fabricated to study ALP mediated formation of apatitic minerals. Scaffolds of the PEC were subjected to alkaline phosphatase (ALP) and successful mineral formation was studied using SEM, Raman and XRD techniques. Investigation of the morphology via SEM shows globular structures of the deposited minerals, which promoted cell attachment, proliferation and extracellular matrix formation. The PEC and their successful calcium phosphate based mineralization offers a greener route of scaffold fabrication towards developing resorbable materials for tissue engineering.

Keywords: Ulvan, Chitosan, Scaffolds, Enzyme, Biomineralization, Alkaline Phosphatase

1. INTRODUCTION

Biomimetic mineralization is the process of preparing inorganic crystals in the presence of organic molecules. Biomimetic mineralization attracts interest since it usually leads to crystals with multiscale ordered structures, which are rare in natural minerals. Besides, it is also an easy way to synthesize novel organic/inorganic hybrids. $CaCO_3$, $BaSO_4$, $BaCO_3$, and hydroxyapatite (HAP) are the mostly investigated minerals synthesized via biomimetic mineralization. In the field of biomedical research, polymer scaffolds or hydrogels that mineralized apatite had improved osteoconductivity and osteoinductivity compared to unmineralized polymers (Habibovic & de Groot, 2007). Several polymers have been investigated for this application such as chitosan(Mamoni Dash et al., 2015; Timothy E. L. Douglas et al., 2013; Lišková et al., 2015), ulvan(Mamoni Dash et al., 2014), silk(Samal, Dash, Chiellini, et al., 2014; Samal, Dash, Declercq, et al., 2014), gellan-gum(T. E. L. Douglas et al., 2014), poly(ethylene glycol)(Phadke, Zhang, Hwang, Vecchio, & Varghese, 2010), bacterial cellulose (Zimmermann, LeBlanc, Sheets, Fox, & Gatenholm, 2011), poly(L –lactic acid)(Ruhé et al., 2005) and polycaprolactone(Koupaei &

Karkhaneh, 2016). In each case, some type of biological apatite was formed, such as calcium-deficient HAP(Zimmermann et al., 2011), dicalcium phosphate dihydrate (DCPD)(Costa, Dixon, & Rizkalla, 2012), carbonated apatite(Costa et al., 2012),or HAP(Phadke et al., 2010). In this study, polyelectrolyte complexes (PEC) of ulvan and chitosan are evaluated as matrices for biomimetic mineralization. PEC's are mixtures of positively and negatively charged polyelectrolytes blended at the molecular level. Ulvan is an anionic sulphated polysaccharide that is water soluble and semi-crystalline in nature(Mamoni Dash et al., 2014), which can be obtained by extraction from the cell-walls of the green seaweeds belonging to Ulvales (Ulva and Enteromorpha sp.).(Morelli & Chiellini, 2010) The natural availability of ulvan represents a source of abundant and economic renewable resources(Morelli & Chiellini, 2010) with minimal concerns regarding toxicity towards host organisms. Sulfated, rhamnose, xylose, glucuronic and iduronic acids are the main constituents of ulvan (Figure 1)(Mamoni Dash et al., 2014). Ulvan has been reported as anticoagulant, antioxidant, antitumor and immune modulator. Chitosan is a polysaccharide composed of ß-(1,4)-2-acetamido-2-deoxy-**d**-glucose and ß_-(1,4)-2-amino-2- deoxy-**d**-glucose units, is a deacetylated form of chitin(Mamoni Dash, Chiellini, Fernandez, Piras, & Chiellini, 2011; M. Dash, Chiellini, Ottenbrite, & Chiellini, 2011; Mamoni Dash, Piras, & Chiellini, 2009). This natural cationic polymer, offers unique properties; it is biologically renewable, biodegradable, biocompatible, non-antigenic, non-toxic, and biofunctional(Lupascu et al., 2015). Chitosan has been proven to accelerate wound healing, stimulate the macrophage activity, and inhibit the growth of tumor cells and posses antimicrobial properties (R. Muzzarelli et al., 1990; R. A. A. Muzzarelli, 2011). PEC's involve anionic and cationic side chain reactions on their macromolecular backbone (Schwarz, Richau, & Paul, 1991). Therefore, ulvan and chitosan as oppositely charged macromolecules can potentially form a polyelectrolyte assembly.

PEC's of ulvan and chitosan are biofunctionalized by employing the natural enzyme alkaline phosphatase (ALP) as mineralization inducer and the osteogenic cell activity of these scaffolds were

evaluated. Mineralization and particularly enzymatic mineralization has been a topic of research interest over the last decade (Timothy E. L. Douglas, Gassling, et al., 2012; Rauner, Meuris, Zoric, & Tiller, 2017; Saito, Fujii, Soshi, & Tanaka, 2006). A number of investigations have been reported on matrices like chitosan(M. Dash et al., 2011), fibrin(Timothy E. L. Douglas, Gassling, et al., 2012) (Gassling et al., 2013) (Timothy E. L. Douglas, Messersmith, et al., 2012), silk(S. K. Samal, T. Gheysens, & R. Cornelissen, 2014) etc. In the present study, we have aimed to investigate the role of enzymatic mineralization on polymeric structures bearing both cationic and anionic groups. In some of our recent works with enzymatic biomineralization, we have studied the behaviour of both positively and negatively charged polymers individually. Herein, we performed a follow-up study, where the aim was to evaluate a combination of the two different types of charged polymers, whose presence could be beneficial to each other. This is a sequential study of the already published work on the mineralization of UV crosslinked ulvan methacrylate (UMA) scaffolds(Mamoni Dash et al., 2014) wherein the rationale is to evaluate a greener material as compared to UMA since, exposure to UV radiation in some cases is known to cause significant degradation of materials. UV radiation might cause photooxidative degradation, which results in breaking of the polymer chains, produces free radical and reduces the molecular weight, leading to loss of mechanical properties. Mineralization includes advantages such as enhancing bioactivity post implantation(Mamoni Dash et al., 2014; Mamoni Dash et al., 2015), osteoblastic differentiation through increased stiffness (Olivares-Navarrete et al., 2017) and enhanced binding of growth factors which stimulate bone healing.(Vo, Kasper, & Mikos, 2012) The evaluation of such matrices could lead to tissue engineered bioactive bioresorbable scaffolds for bone tissue engineering.

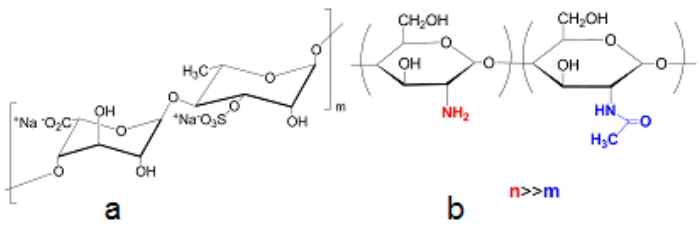

Figure 1. The chemical structure representing a) the main disaccharide repeating unit of ulvan, α-L-Iduronic acid (1→4) α-L-Rha 3S→1(Mamoni Dash et al., 2014) b) chitosan.

2. EXPERIMENTAL SECTION

2.1 Materials. Ulvan batch in powder as extracted from *Ulva armoricana* was kindly supplied by CEVA within the framework of the EU-founded project BIOPAL. The number average molecular weight of ulvan (Mn=60000 g/mol) was determined by using size exclusion chromatography. Pullulan standards (Polymer Laboratories, UK) were used to obtain the calibration curve (range 6000-400000 g/mol). Chitosan (M_w=108 kDa, M_w/M_n=2.4, deacetylation degree (DD)=92% (Piras et al., 2014)was purchased from Sigma–Aldrich, Milan, Italy. Bovine intestinal ALP (specific activity: ≥ 10 DEA units/mg, P7640) and calcium glycerol phosphate (50043) were obtained from Sigma (Sigma Aldrich, Belgium). Ulvan polysaccharide from *Ulva armoricana* was kindly supplied by CEVA (Mw = 60 KDa).

2.2 Preparation of Ulvan-Chitosan Polyelectrolyte complexes (UC PEC) Cylindrically shaped PEC hydrogels containing ulvan (60% w/w) and chitosan (40% w/w) were prepared into a 24-well tissue culture plate. 0.600 mL of ulvan solution in deionized water (30 mg/mL) was added to 0.012 mg of chitosan powder contained into each well. Acetic acid 1% (v/v) was poured into each solution to dissolve chitosan powder and the resulting mixture was vigorously stirred until gelation occurred. The hydrogels were left standing at room temperature for 24 hours and then freeze dried under vacuum (0.04 mbar) at -50°C to remove the excess of water.

2.3 Polymer Scaffold Characterization.

2.3.1 Hydrogel Swelling Degrees

Freeze dried scaffolds (approximately 50 mg) were prepared according to the procedure reported into the previous paragraph and used without any further purifications. The dry PECs were weighed

and then immersed in 0.1M PBS (pH 7.4) at room temperature. The samples were weighed at regular time intervals after removal of excess surface liquid by blotting with a soft tissue. The water uptake of the PECs was established by calculating their Swelling Degree % (SD%) as:

$$SD\% = [(Ws - Wd)] / Wd \times 100$$

where W_s represents the weight of the swollen sample taken at each time interval and W_d represents the weight of the dry sample taken at the beginning of the experiment. The experiments were performed in triplicate and the SD% reported as the mean value.

2.3.2 FT-IR analysis

FT-IR spectra of the dried scaffolds were recorded as KBr pellets (1/100 mg) in the range 4000–400 cm^{-1} by using a Jasco FT-IR 410 spectrophotometer with a resolution of $4cm^{-1}$. Each spectrum was recorded after 16 scans.

2.3.3 Mineralization of the Scaffolds. The scaffolds were incubated in a solution of ALP for 30 minutes. Three different concentrations of ALP were used in this study (5, 25 and 50 mg/mL). The ALP treated PEC scaffolds of ulvan chitosan will be denoted as UC 5, UC 25 and UC 50 respectively. The ALP-soaked scaffolds (5x5 mm^2 for cell culture and 10x10 mm^2 for mechanical analysis) were subsequently incubated in a mineralization medium (5 mL and 10 mL respectively) containing 0.1 M calcium glycerophosphate (aq) at 37°C. The mineralization medium was refreshed every day. After 7 days of mineralization, scaffolds were rinsed thrice in Milli-Q water to remove residual calcium glycerophosphate and subjected to lyophilization after freezing at -20°C.

2.3.4 Scaffold Morphology. Scanning Electron Microscopy (Hitachi S-3400N) was used to analyze the morphology of the scaffolds. Images were obtained in low vacuum mode (20Pa) to avoid image

distortions, using back scattered electrons. Elemental analysis was performed on a Peltier cooled dry EDS system (Thermo Scientific Noran System 7, energy resolution < 125eV).

2.3.5 Study of Scaffold Mass Increase. The mass increase of the scaffolds at different time points after the incubation in calcium glycerophosphate was calculated using previously established equation:

Mass increase (%) = $(m_t - m_0)/m_0 * 100$

Where, m_t = the mass of scaffolds after incubation at time t. m_0 = the original mass of scaffolds before incubation and mineralization.

The final mass increase was calculated by using the same formula as above but m_t is the dry mass of the scaffolds after mineralization.

2.3.6 Raman Spectroscopy. Fourier transform Raman (FT-Raman) spectra were performed on a NXR FT-Raman Module. The samples were pressed in a suitable gold coated sample holder and a laser power of 0.35 W was used to collect the scans.(Samal, Dash, Chiellini, Kaplan, & Chiellini, 2013) 1500 scans were collected at a resolution of 4 cm^{-1}.

2.3.7 Thermal Analysis by Thermogravimetry (TGA). Thermogravimetric analyses were performed using a TA Instruments Series TA 2950 and results were analyzed using Thermogravimetric Analyzer Software (Universal Analysis 2000). Sample weights of 9-13 mg were used and scanned at 10°C·min^{-1}. A temperature range between 30-900°C under a 60 mL·min^{-1} flow rate of nitrogen was used for the analysis(Samal et al., 2013).

2.3.8 Crystal Structure Elucidation by X-ray Diffraction (XRD). XRD technique was used to investigate the crystallographic structure of the samples. X-ray diffractometer with CuKα-radiation (PW 3710, 50 kV, 40 mA) was used. Samples were fixed to a position of 2.5º and scanning the detector between 5º 2θ and 40º 2θ with a counting time of 5s/step and step-size of 0.01 º2θ.

2.3.9 Biological Investigations.

2.3.9.1 Cell culture and seeding. Pre-osteoblastic MC3T3-E1 cells were purchased from the American Type Culture Collection (CRL-2593). Cells were maintained and expanded in Alpha Modification Minimum Essential Medium containing 10% fetal bovine serum, 100U/ml penicillin, 0.1 mg/ml streptomycin (Invitrogen) in humidified 5% CO_2 at 37° C. Confluent MC3T3 cells at passage 25 were trypsinized (0.25% trypsin-EDTA), centrifuged, re-suspended in culture medium and counted. Subsequently $1 \cdot 10^5$ cells, initially dispersed in 20 µl of culture medium, were seeded onto the scaffolds in a 24 well plate. After 3 hours of incubation, 980 µl culture medium was added to the scaffolds. In order to assess the osteogenic differentiation, 24 hours after cell seeding the scaffolds were incubated in osteogenic medium (culture medium supplemented with 10 mM β-glycerolphosphate and 0.3 mM ascorbic acid). Biological investigations were carried out at days 7 and 14 after seeding. The medium was replaced every 48 hours. Cells grown on tissue culture plates were used as control.

2.3.9.2 Alkaline phosphatase (ALP) activity. Alkaline phosphatase activity ALP was determined in culture using MC3T3-E1 cells grown onto the prepared scaffolds at day 7 according to previously established protocols.(Gazzarri et al., 2013) Briefly, treated scaffolds were washed three times with DPBS, treated with lysis buffer, containing Triton X-100 (0.2%), magnesium chloride (5 mM) and trizma base (10 mM) at pH 10, for 15 min at 4° C. Cell lysate was centrifuged for 15 min at 10,000 rpm at 4° C. 20 ul of supernatant were collected and incubated with p-nintrophenyl phosphate at 37° C for 30 minutes. The reaction was stopped by the addition of 2M NaOH. The absorbance at 405 nm was measured with a UV-vis spectrophotometer. The amount of ALP was

calculated against a standard curve and normalized to total protein content. ALP activity was expressed as nanomoles (nmol) of p-nitrophenol produced per minute.

2.3.9.3 Total protein. Cellular protein content was measured with a BCA protein assay kit (Pierce, USA) was used to determine the cellular protein content following a protocol of St-Pierre *et al*(St-Pierre, Gauthier, Lefebvre, & Tabrizian, 2005). Cell lysate was incubated with bicinchoninic acid solution for 2 hours at 37° C and the absorbance were measured at 565 nm in a microplate reader. A standard curve using bovine serum albumin was used to generate a standard curve.

2.3.9.4 Quantification of total collagen production. Quantification of total collagen produced by cells seeded on scaffolds was carried out at day 7. The detailed protocol has been described by Gazzari *et al*.(Gazzarri et al., 2013) Briefly, medium was removed and samples were were rinsed in DPBS. Scaffolds were then incubated for 1 hour with a solution of Direct Red 80 dye (Sigma) prepared in picric acid (0.1%). Dye excess was removed by washing the samples with 10 mM HCl. Bound stain solution was obtained with the incubation 0.1 N NaOH. The absorbance of the dye was read at 540 nm. Known concentrations of collagen type I, filmed on glass slides, were prepared and used for the standard curve. The films were then fixed and treated as the samples.(Junqueira, Bignolas, & Brentani, 1979)

2.3.9.5 Confocal Laser Scanning Microscopy (CLSM): CLSM investigation was performed to evaluate the morphology of the osteoblasts grown on scaffolds on day 14 of the culture according to a protocol by Gazzari et al.(Gazzarri et al., 2013). Briefly, scaffolds with cells were fixed for 1 hour in 3.8% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 for 10 min. The Nikon Eclipse TE2000 inverted microscope (Nikon) and 60X oil immersion objective was employed for samples investigations. Argon Ion Laser (488 nm emission) and a laser diode (405 nm) were used to excite FITC and DAPI fluorophores respectively. Images were captured with Nikon EZ-C1 software applying identical instrumental settings for each sample, and were further

processed with the GIMP (GNU Free Software Foundation) image manipulation software and merged with Nikon ACT-2U software.

2.3.9.6 Histological Analysis After cell culturing, cell/scaffold constructs were rinsed with PBS, fixed with 4% phosphate (10 mM) buffered formaldehyde (pH 6.9) (4°C, 24 h), dehydrated in a graded alcohol series and embedded in paraffin. Sections (5-7 µm) were made and stained with hematoxylin & eosin (H&E) and mounted with mounting medium (Cat.No. 4111E, Richard-Allan Scientific).

Statistical analysis. Data were obtained from triplicate samples and are presented as mean ± standard deviation. Statistical comparison was performed using one-way analysis of variance (ANOVA), and significance was defined at $p < 0.05$ (*) and $p < 0.001$ (**).

3 RESULTS AND DISCUSSION

3.1 Fabrication of Ulvan-Chitosan PEC

Ulvan represents an anionic polysaccharide containing carboxylates and sulfate esters as negatively charged groups in the repeating disaccharide units (Figure 1). The combination between ulvan and chitosan, as cationic polysaccharide, in mild acidic conditions led to the formation of a polyelectrolyte complex as evidenced by the rapid gelification of the mixture occurring upon vigorous mixing. The PECs were prepared by using ulvan as 60% w/w and chitosan as 40% w/w as optimal feeding composition to theoretically balance the number of positive and negative charges involved in the formation of the complexes and obtain hydrogels stabilized by strong electrostatic forces. FT-IR analysis of UC-PEC confirmed the occurring of electrostatic bonds between the ammonium groups of chitosan and the carboxylate groups of ulvan (Figure 2a).

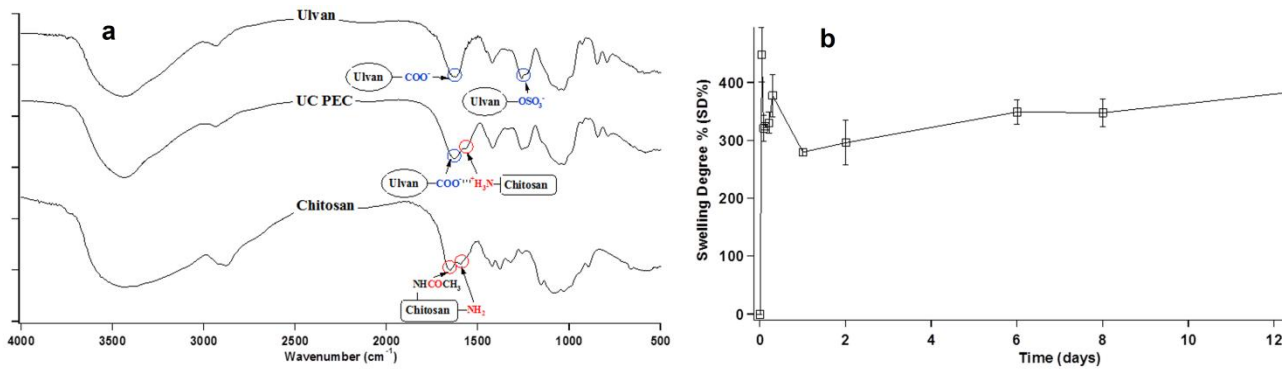

Figure 2 a) FT-IR spectra of ulvan, chitosan and UC PEC b) Swelling degree experiment of UC-PEC scaffold carried out in PBS solution (0.1M, pH 7.4).

The absorption bands relevant to the groups involved in the polyelectrolyte complex formation were highlighted in blue (ulvan) and red (chitosan). Indeed the absorption bands relevant to the asymmetrical stretching vibration of carboxylate group of ulvan (1622 cm^{-1}) and the N-H bending vibration of amine group of chitosan (1596 cm^{-1}) were found shifted in the spectrum of UC-PEC respectively to 1564 cm^{-1} and 1635 cm^{-1} representing a valid evidence of formation of the polyelectrolyte complex(Lawrie et al., 2007).

The amount of water absorbed by polymeric scaffolds intended to be used in biomedical applications such as tissue engineering and regenerative medicine represents a crucial parameter that needs to be evaluated to state their feasibility as successful biomaterials(Slaughter, Khurshid, Fisher, Khademhosseini, & Peppas, 2009) . The water uptake capability of UC-PEC was evaluated by swelling experiments in simulated body fluid conditions through the immersion of the dried scaffolds in PBS solution (0.1M, pH 7.4) for 14 days. The SD% curve showed a maximum value reached after 1h of immersion followed by a smooth decrease to a constant equilibrium value reached after 1 day of immersion (Figure 2b). The swelling capability of UC-PEC resulted reduced to one tenth compared to that reported for ulvan-based hydrogels obtained by UV crosslinking (Morelli & Chiellini, 2010) due to the neutralization of the negatively charged carboxyl and sulphate groups of ulvan by the ammonium ions of chitosan. However the SD% values recorded at

equilibrium conditions were suitable for supporting the activity of a polymeric scaffold in biomedical applications.

As we expected the composition of ulvan (60% w/w) and chitosan (40% w/w) used in the formulation of the PEC provided the most stable scaffolds as evidenced macroscopically by visualizing the shape and the texture of the PECs at different compositions after being immersed in PBS solution (0.1M, pH 7.4) for 14 days (Figure 2c).

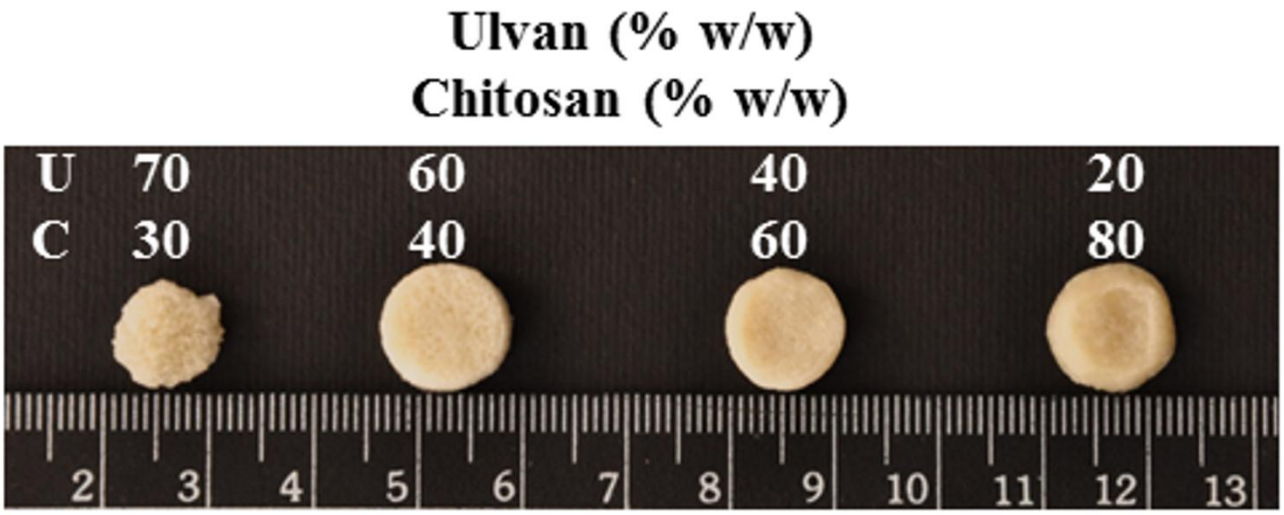

Figure 2c. UC-PEC scaffolds at different composition after 14 days of immersion in PBS solution (0.1M, pH 7.4). The amount of ulvan and chitosan used for the preparation of the UC-PECs are reported inside the picture as w/w %.

3.2 Physical-chemical characterizations of biomineralized PEC scaffolds.

The physicochemical characterizations include an indirect measure of the weight increase of the mineralized scaffolds, Raman spectroscopy, TGA. The dry mass percentage is a representation of the ratio of the weight percentage of the scaffolds before and after mineralization as shown in Figure 3a. The dry mass percentage increased upon increasing the ALP concentration from 0 to 50 mg mL^{-1} after 7 days of incubation in calcium phosphate medium. An approximate increase of 250% was observed in the UC scaffold weight when treated with 50 mg mL^{-1} of ALP. The presence

of phosphate groups was done through Raman spectroscopy, which revealed a strong peak at 958 cm^{-1} characteristics for the P−O stretching mode (ν1) of the phosphate group (Figure 3b). The peak is observed only in samples treated with ALP and is proportional to the to the concentration of the ALP. The X-ray diffractograms of the mineralized scaffolds also showed a similar trend. (Figure 3 c). A broad reflection peak was seen at 32° 2θ for UC 25 and UC 50 but not for UC 5. The formations of nano-sized crystals are responsible for the broad peak instead of a sharp peak in XRD. The thermal property of the mineralized scaffolds was analysed by TGA Figure. 3d). The scaffolds show a continuous weight loss. In TGA derivative, 1st degradation peak in the temperature range of 30–130 °C corresponded to the equilibrium moisture of the samples. The scaffolds containing ALP possessed mineral, showed higher residue content at 700 °C (Figure 3d) and different degradation pattern with an extra shoulder at around 230 °C. This is due to the presence of minerals compared to unmineralized scaffolds. The amount of residue gave a clear indication of the presence of the inorganic mineral content in the mineralized scaffolds. There was no difference in the residue amount with the change in the concentration of ALP.

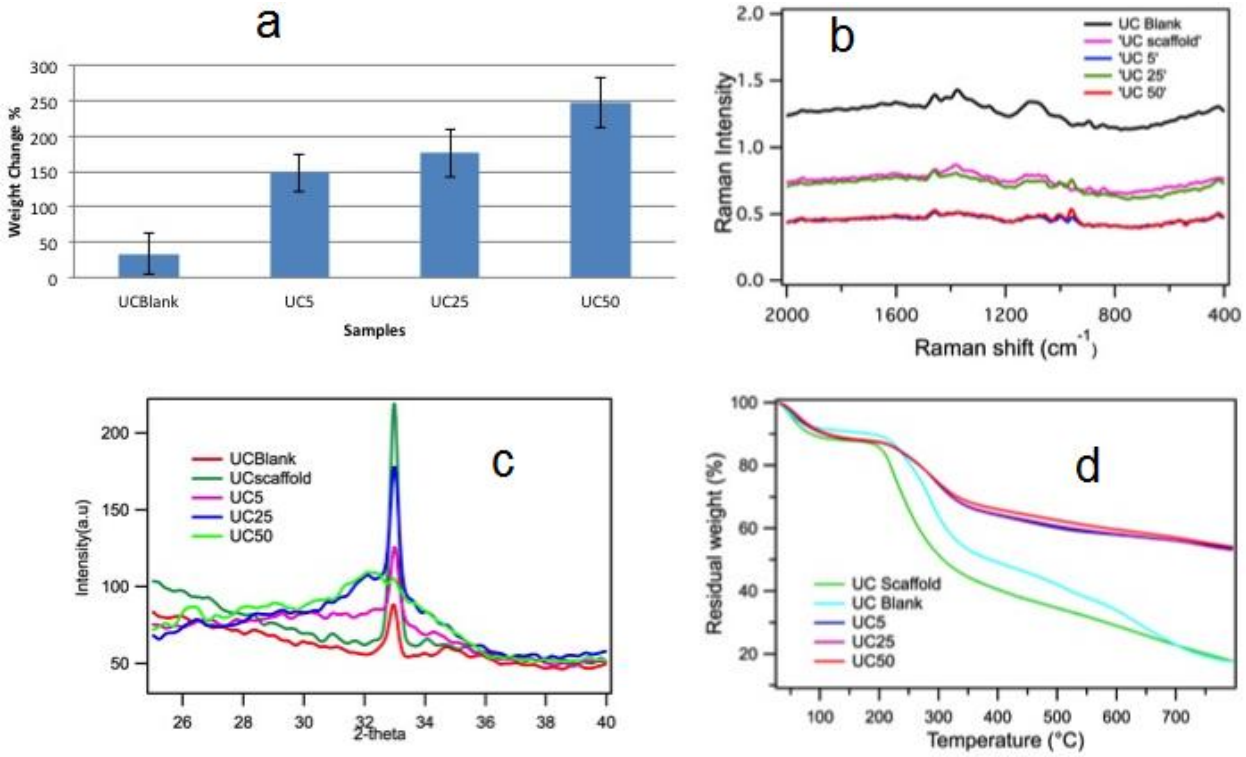

Figure 3. a) weight change in dry mass after 7 days of mineralization of UC PEC scaffolds b) Raman spectra of ulvan scaffolds mineralized in the presence of different ALP concentrations, c) X-ray diffraction patterns of mineralized ulvan scaffolds with different ALP concentrations (the blank scaffold was unmineralized), d) TGA curves of native and mineralized PEC scaffolds.

3.3 Morphological Analysis of the mineralized scaffolds

The morphology of the mineral deposits in the mineralized PECs was done using SEM (Figure 4) and their chemical composition was determined using EDS. The blank scaffolds showed only lamellar structures and no presence of any kind of deposits while the mineralized scaffolds showed the presence of mineral deposits. Small rounded clustered structures are seen as mineral deposits in the mineralized scaffolds. The Ca: P ratio in the mineralized scaffolds varied from 1.1 to 1.7 with increasing enzyme concentration. The blank scaffold also showed a high Ca:P ratio of 1.5 possibly from the remaining glycerol phosphate solution after washing of the scaffolds.

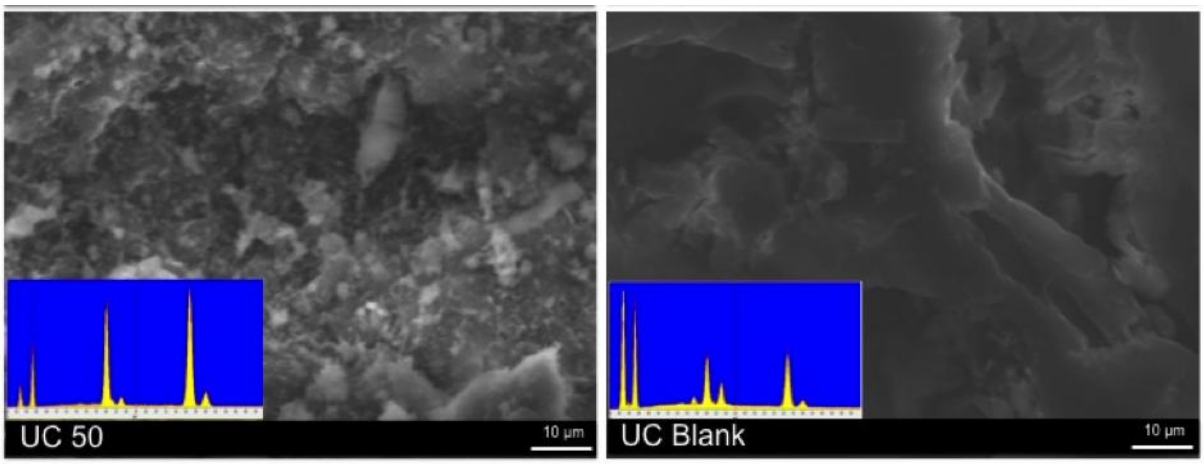

Figure 4. SEM images of UC50 (left) and UC Bank (right).

Table 1: Atomic percentage of carbon, oxygen, phosphorus and calcium on the UC mineralized scaffolds

Sample	Calcium (at %)	Oxygen	Phosphorus	Sulfur	Ca:P

UC blank	10.7	78.5	7.2	3.6	1.5
UC 5	14.9	71.5	13.0	0.6	1.1
UC 25	15.4	72.0	12.4	0.2	1.2
UC 50	22.7	62.8	13.4	1.1	1.7

3.4 Mechanical properties of biomineralized PEC scaffolds

The complex moduli of scaffolds mineralized in ALP solutions of different concentrations were determined using rheology. The results are depicted in Figure 5. The mineralized samples UC 5, UC 25 and UC 50 displayed a significantly higher complex modulus than the ALP-free control (UC blank) as well the unmineralized PEC scaffold.

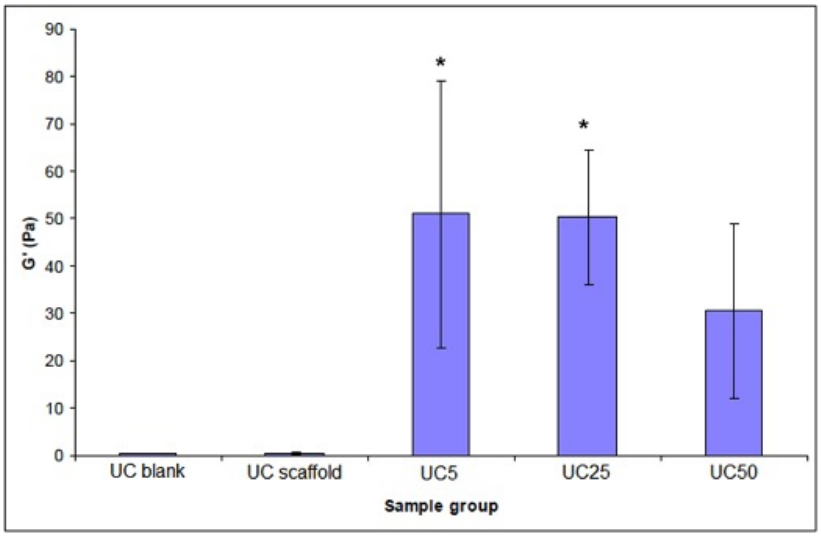

Figure 5. Rheological measurements of complex modulus of mineralized UC PEC scaffolds, *****$p < 0.05$ relative to UC Blank.

3.5 Biological evaluation of the biomineralized PEC scaffolds

The results of cell proliferation of MC3T3-E1 cultured on ulvan-chitosan polyelectrolyte complexes (UC) are shown in Figure 6a. At day 7 and 14 of culture, enzymatically treated samples showed a

cell proliferation significantly higher with respect to the non-enzymatically treated sample (UC blank) ($p < 0.001$). However, the cell proliferation of MC3T3-E1 on all the typologies of UC samples resulted significantly lower with respect to the control.

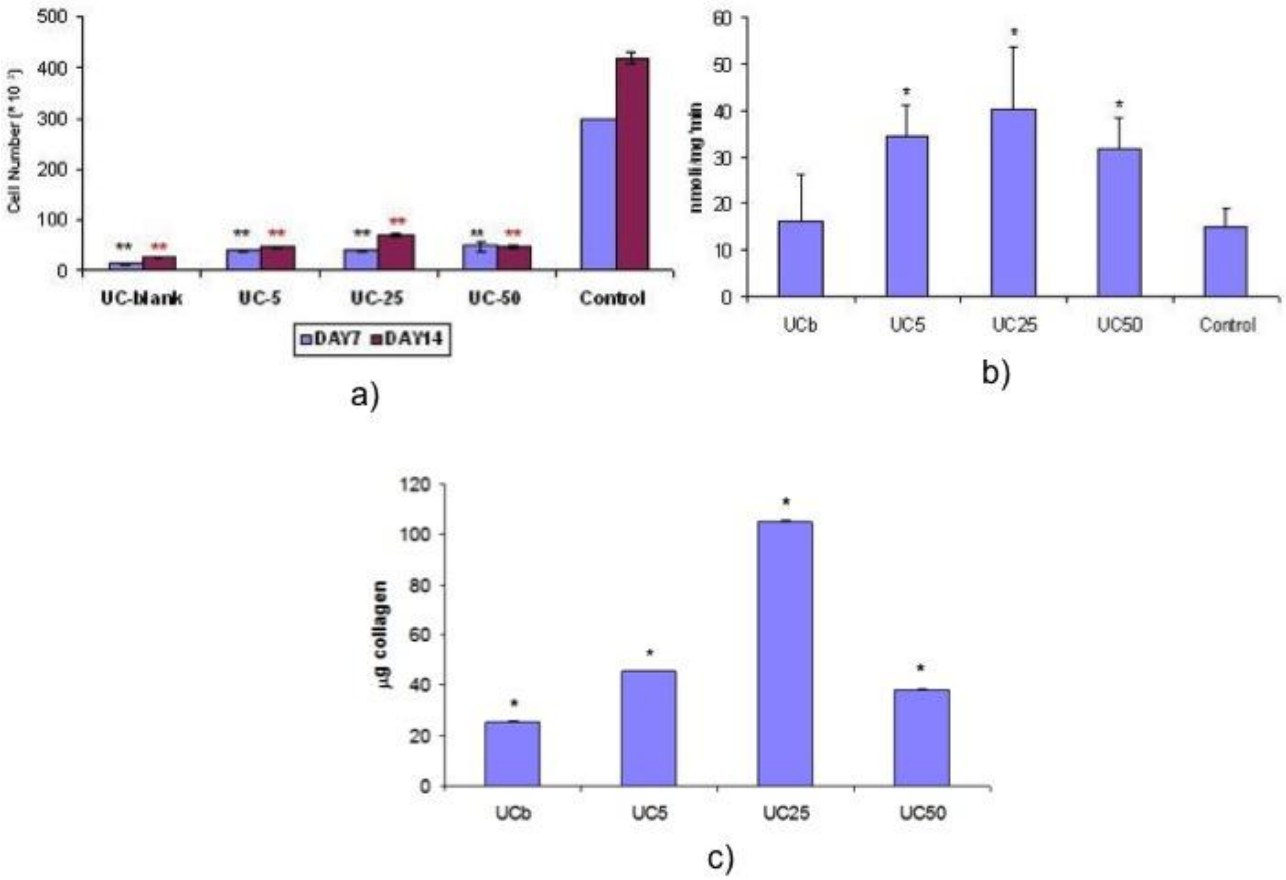

Figure 6. a) Cell Proliferation of MC3T3 E-1 cultured UC PEC scaffolds ** (black) Significant at $p<0.001$ with respect to control at day 7. ** (red) Significant at $p<0.001$ with respect to control at day14=, b) Alkaline phosphatase activity of MC3T3 E-1 cells grown on UC PEC's. * Significant at $p<0.05$ with respect to control at day 7, c) Collagen production obtained from MC3T3 E-1 cells cultured onto UC PEC scaffolds. *Significant at $p<0.05$ between different samples at day 7.

The ALP activity of MC3T3-E1 grown on UC PEC scaffolds was monitored at day 7 of culture (Figure 6b). Cells grown on UC enzymatically treated samples expressed values of ALP activity significantly higher with respect to the control and to the untreated sample (UCb) ($p < 0.05$), although the cell number on TCPS was found significantly higher with respect to the UC PEC's.

The ALP activity indicates an early beginning of the differentiation process towards an osteoblastic phenotype. . The synthesis of a collagen correlates with the expression of osteoblast phenotype in MC3T3-E1 cells (Franceschi & Iyer, 1992). Collagen production by preosteoblast cells cultured on UC PEC was measured and quantified at day 7 of culture. This gives an indication regarding the expression of the osteoblast phenotype. The results highlighted ~~a highlighted~~ a significantly higher collagen production ($p < 0.05$) for cells grown in particular on sample UC 25 (Figure 6 c), although good collagen values were observed for all the enzymatically treated samples in comparison to blank (UC blank).

3.6 Cell morphology investigation by confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy was performed at day 7 to investigate the cell adhesion, morphology and cytoskeleton organization. Cells were stained for F-actin and nuclei with FITC-phalloidin and DAPI respectively. Figure 7 shows the morphology and distribution of cells on UC PEC's. In agreement with the cell proliferation results a low number of cells was detected onto non-enzymatically treated sample (UCb). The cells cultured on enzymatically treated UC5 and UC50 samples showed an improved adhesion and spreading as confirmed by the presence of fusiform structures well distributed and in contact with each other via cellular extensions (Figure 7b, 7d). Moreover, MC3T3 cells grown on UC25 sample displayed also a cuboidal shape, typical of differentiated osteoblasts (Figure 7c), suggesting its suitability in promoting osteogenic differentiation of pre-osteoblast cells as confirmed by results of ALP activity and collagen production.

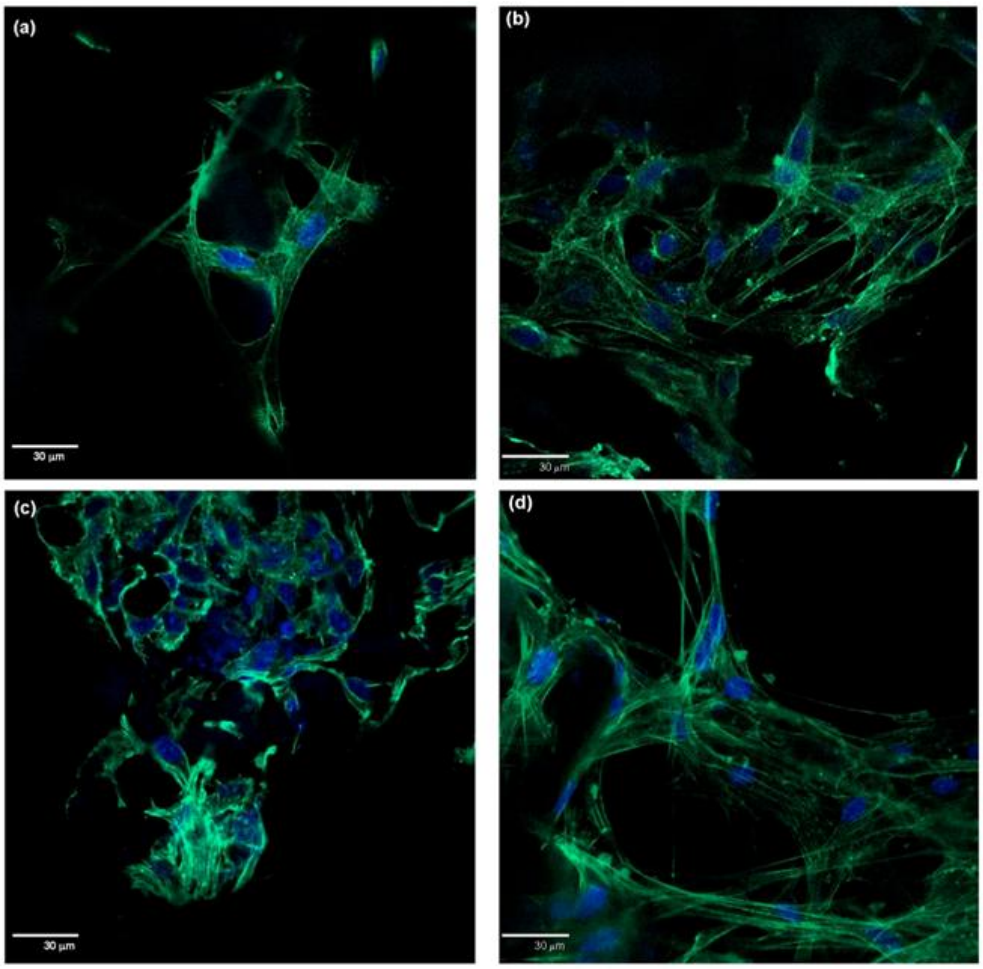

Figure 7. CLSM micrographs of MC3T3 E-1 cells cultured on UC blank (a), UC5 (b), UC25 (c), UC50 (d) scaffolds.

Cross-sections of cell/scaffold constructs at day 14 and day 21 post-seeding in osteogenic medium are presented in Figure 8. A dense tissue layer is formed at the edge of the mineralized UC PEC scaffolds (Fig 10 b, b', c, c', d, d'), in comparison to the blank samples untreated with ALP (fig. 8 a, a'). after 14 days of culturing. The blank scaffolds (Fig 8 a', e') show the presence of non-attached clustered cells. Cells cultured on the mineralized scaffolds followed the contours of the scaffolds in all cases. Cells were able to colonize the edge of the scaffold and formed an extracellular matrix layer. The size of this adherent tissue layer varies between 40 and 100µm. However, the centers of the scaffolds were not colonized in this time period. After 21 days of culturing the cell/scaffold

constructs, the same trend could be observed as is shown in fig 8 for blank scaffolds (fig 8 e, e) and mineralized scaffolds (fig 8 f, f’, g, g’, h, h’).

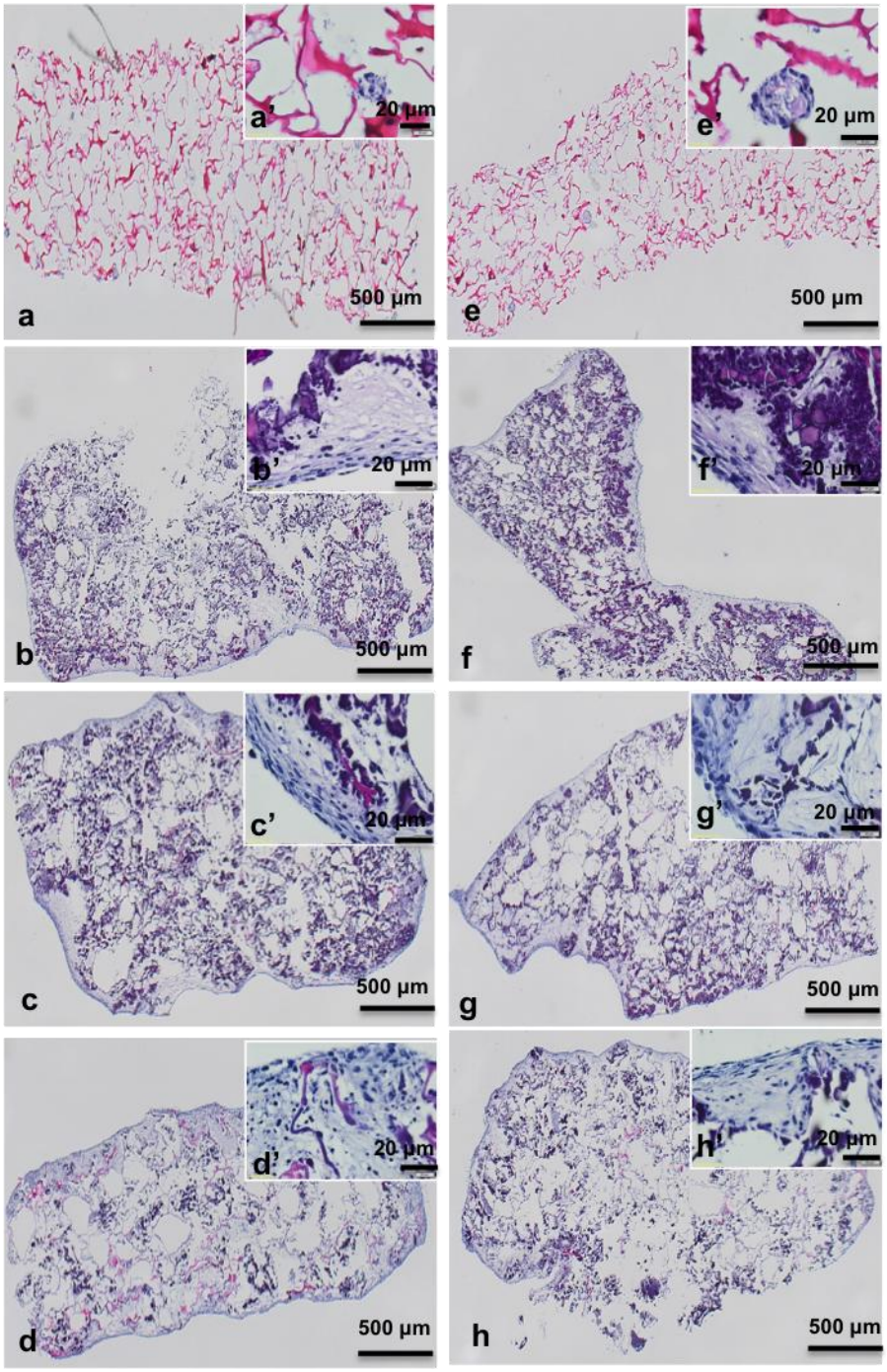

Figure 8. Influence of mineralization on the cell behavior in and the colonization of the scaffolds (----- 500µm, — 20 µm). Histological analysis (H & E staining) of 3D scaffolds in osteogenic medium at day 14 and day 21 post-seeding. At day 14, a, a') UC blank, b, b’) UC 5, c, c') UC 25, d, d’) UC 50. At day 21, e, e') UC blank, f, f’) UC 5, g, g') UC 25, h, h’) UC 50.

4. DISCUSSION The results described in this work are a comprehensive study on mineralization ability of ALP on PEC of ulvan and chitosan. Traditional mineralization approaches involve alternate soaking of scaffolds in simulated body fluid (SBF) while this study is based on enzyme induced mineralization and is a continuation of our ongoing investigation of mineralization on different polymeric matrices. ALP induced mineralization and the characteristic properties of the materials thereof have been previously investigated on a cationic polymer chitosan, anionic polymer gellan gum, and proteins such as fibrin etc. In this particular study, we investigated a polyelectrolyte complex consisting of chitosan as a cationic polymer and ulvan as an anionic polymer. The PEC was made with a 60/40 ratio of ulvan and chitosan to form a stable gel by balancing the opposite charges involved. The presence of the individual polysaccharides in the PEC scaffold was done by the major bands in the infrared region, namely the band at the 1596 cm^{-1} from the amine groups and a band at 1622 cm^{-1} from the carboxylates. The polysaccharide gels swell to a maximum of around 400% in SBF medium. This swelling potential is lower as compared to the UV crosslinked ulvan scaffolds mostly due to the neutralization of charges, which lowers the uptake of water. These scaffolds were further mineralized using three different concentrations of ALP as mineral inducing enzyme. A higher concentration of the enzyme leads to more mineral deposits as indicated by the weight change data. SEM revealed the morphology of the formed minerals as rounded aggregates with a Ca:P ratio varying from 1.1 to 1.7. The XRD results also indicated that a certain concentration of the enzyme is able to deposit minerals with a Ca:P ratio close to hydroxyapatite in a similar polymeric matrix. Confocal laser scanning microscopy indicated that enzymatically treated samples showed improved adhesion and proliferation with cubical shaped cells. Cells seeded on the enzymatically-mineralized matrix formed a dense extra cellular matrix at the edge of the scaffold as observed with histology. The cell proliferation, ALP activity and collagen production indicate that UC 25 samples show higher activity than the UC 50 samples. As compared to the UV crosslinked ulvan matrix, there is not much difference in the characteristic of the mineralized scaffolds, hence it can be inferred that irrespective of the matrix property, ALP is able to induce

minerals and the formed minerals support cell proliferation, differentiation and matrix formation in the scaffolds. However, such PEC's offer better alternative compared to UV crosslinkable ulvan as a matrix since it is greener route of developing resorbable scaffolds.

5. CONCLUSIONS

The study was performed as a follow-up investigation of the previously published result of ulvan methacrylate as a matrix for enzyme induced mineralization. A comparatively greener route was followed to develop gels of ulvan. In this study ulvan and chitosan were employed to form a polyelectrolyte complex and the resulting scaffolds were treated with ALP and calcium phosphate minerals were successfully deposited. The PEC's were stable and could be confirmed by FTIR. Mineralization on the PEC scaffolds was done using three different concentrations of ALP which deposited calcium phosphate minerals with Ca:P ratio ranging from 1.2 to 1.7 which varied in relation to the concentration of ALP used for mineralization. The mineralized scaffolds were non-toxic and promoted cell adhesion and differentiation towards an osteogenic phenotype. The cells formed a dense extracellular matrix as revealed by the histological analysis. These scaffolds could successfully find application as resorbable scaffolds and can be a better alternative in some aspects compared to UV crosslinked ulvan matrix.

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The authors declare no competing financial interest.

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