Gelatin crosslinked with dehydroascorbic acid as a novel scaffold for tissue regeneration with simultaneous antitumor activity

Gelatin crosslinked with dehydroascorbic acid as a novel scaffold for tissue regeneration with simultaneous antitumor activity

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Abstract

A porous scaffold was developed to support normal tissue regeneration in the presence of residual tumor disease. It was prepared by gelatin crosslinked with dehydroascorbic acid (DHA). A physicochemical characterization of the scaffold was carried out. SEM and mercury porosimetry revealed a high porosity and interconnection of pores in the scaffold. Enzymatic degradation provided 56% weight loss in ten days. The scaffold was also evaluated *in vitro* for its ability to support the growth of normal cells while hindering tumor cell development. For this purpose, primary human fibroblasts and osteosarcoma tumor cells (MG-63) were seeded on the scaffold. Fibroblasts attached the scaffold and proliferated, while the tumor cells, after an initial attachment and growth, failed to proliferate and progressively underwent cell death. This was attributed to the progressive release of DHA during the scaffold degradation and its cytotoxic activity towards tumor cells.

(Some figures may appear in colour only in the online journal)

1. Introduction

Tissue regeneration represents an important issue, mainly with regard to tumor disease, where tissue damage may be due to the disease's progression or may be caused by chemotherapy, radiotherapy or chirurgical resections. To this purpose, porous scaffolds have been used either of natural [1, 2] or synthetic origin to favour cell attachment, cell growth and differentiation [3, 4]. It has been highlighted that in order to promote tissue regeneration the scaffold needs to hold suitable porosity and pore interconnectivity [5] in order to maintain mechanical integrity while undergoing biodegradation. At the same time, it also needs to sustain cell proliferation and new extracellular matrix deposition [6–8]. Several authors have investigated the use of different porous scaffolds, especially for bone regeneration purposes [9–12]. They mainly focused on the

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scaffold's ability to promote the regeneration of healthy tissues but have rarely addressed the possibility that prevention of the attachment and proliferation of tumor cells is possible at the same time [13]. Indeed, it is well known that tumor cells may persist in the body after antitumor therapy and may cause, over time, a relapse of the disease.

In this work we prepared and evaluated a novel porous scaffold based on gelatin crosslinked with dehydroascorbic acid (DHA), where the antitumor activity relied on the DHA released following scaffold degradation. Gelatin was chosen as it is one of the most regularly used biomaterials for the preparation of porous scaffolds, due to its innocuous nature and its bioadhesive properties ascribable to both its biological characteristics and the presence of the RGD sequence involved in cell attachment [14]. Usually, in order to obtain stable gelatin scaffolds at physiological temperatures, chemical crosslinking agents such as butadiene or glutaraldehyde are typically used, whose toxicities are well known [14]. In this study DHA was used as the gelatin crosslinking agent due to the presence of two ketone groups on its molecule linking the amine groups of gelatin. Besides its ability to crosslink gelatin, the DHA released due to scaffold degradation also provides antitumor activity. Indeed the antitumor activity of DHA has been reported either due to its reduction to ascorbic acid (AA), once it has entered the cells by the glucose transport mechanism, or due to its intrinsic activity as an inhibitor of the kinases, IKKβ and IKKα [15]. Moreover the DHA conversion to AA is expected to promote collagen deposition and thus connective tissue regeneration [16]. In particular this work aims to demonstrate that this novel gelatin–DHA scaffold may be suitable to induce healthy cell adhesion and growth with simultaneous antitumor activity. Regeneration should be favoured by the bioadhesive properties of gelatin, which support cell attachment and the formation of extracellular matrixes [16]. The antitumor activity should correlate with the DHA release following scaffold degradation.

2. Materials and methods

2.1. Scaffold preparation

Gelatin type III from bovine skin, 225 Bloom (Sigma Aldrich) was solubilised in water (20% w:v) at 40 °C. 200 ml of this solution was added to 100 ml of a DHA solution (10% w:v) in water. After stirring for 6 h at 80 °C a gel was obtained which was collected on a filter and then washed thoroughly with water. To remove unreacted DHA, the gel was placed in a beaker with 200 ml of water at 37 °C. Water was replaced every hour after being analysed by HPLC for its DHA content until no presence of DHA could be detected. The HPLC analysis was performedusing a Phenomenex Luna C18 analytical column (100 mm × 4.6 mm i.d., 3 μm) fitted with a Brownlee RP-18 precolumn (15 mm × 3.2 mm i.d., 7 μm). The mobile phase consisted of 0.05 M sodium phosphate, 0.05 M sodium acetate, 189 μM dodecyltrimethylammonium chloride and 36.6 μM tetraoctylammonium bromide in 25:75 methanol:water, (vol:vol) pH 4 [17]. The elution rate was 0.5 ml min^{-1}. UV-detection was carried out at 264 nm [18, 19].

2.2. Characterisation of the scaffold

Total porosity of the freeze dried scaffold was measured by mercury pycnometry (MP) [28]. The morphology of the scaffold without cells was processed by high resolution scanning electron microscopy (HRSEM), and coupled to an electron dispersive spectroscopy, (EDS, Oxford System). For HRSEM analysis, scaffold samples of 5 × 1 × 3 mm were fixed with a solution of 2.5% glutaraldehyde in a 0.1 M phosphate buffer (Sigma Aldrich, St. Louis, USA) for 2 h at 4 °C and subsequently post-fixed with 1% OsO4 in a 0.1 M phosphate buffer (Società Italiana Chimici, Roma, Italy) for 1 h at room temperature (RT). After several washes in the 0.15 M phosphate buffer, the samples were dehydrated in an ascending alcohol series and critical point dried (CPD 030, Balzers, Leica Microsystems GmbH, Wetzlar, Germany). The samples were then metal coated with a thin layer of carbon/platinum (CPD 030, Balzers, Leica Microsystems GmbH, Wetzlar, Germany) and observed under field emission in-lens scanning electron microscopy (FEISEM,JSM 890, Jeol Company, Tokio, Japan) with 7 kV accelerate voltage and 1 × 10–11 mA. HRSEM images were analyzed by Image pro plus software (Media Cybernetics, MD, USA).

2.3. Water uptake

The water uptake of the scaffold was determined by placing a known amount of scaffold in phosphate buffer saline (PBS) pH 7.4 at 37 °C for 10 days. Every day the scaffold weight was determined and the medium changed. The water uptake at each time point was expressed as: WUPt = (Wt-W_0)/W_0 where W_0 was the initial weight of the dry scaffold and Wt the weight of the soaked scaffold at each time point. The experiment was repeated several times and the values of water uptake were expressed as the mean $\pm$ standard deviation ($n = 10$).

2.4. Degradation of the scaffold

To gain information on the scaffold's ability to undergo degradation in body fluids, its weight loss in MEM medium containing lysozyme was measured over time. Lysozyme is known as the major enzyme in human serum responsible for enzymatic degradation of biodegradable materials [20]. The experiments were carried out by using 1 ml MEM medium (Invitrogen, San Giuliano Milanese, Italia) supplemented with 10% FCS, 100 IU ml^{-1} penicillin–50 μg ml^{-1} streptomycin and 1.6 μg ml^{-1} lysozyme which corresponded to the lysozyme concentration in human serum [21]. The scaffold samples (5 × 1 × 5 mm) were placed individually in capped vials and, after recording their initial weight (W_0), 1 mL of MEM containing lysozyme was added to each vial and maintained at 37 °C in static incubation. The MEM–lysozyme solution was refreshed daily to ensure continuous enzyme activity. Each day the samples were taken from the medium, rinsed with deionized water, freeze dried and weighed. The weight loss was expressed as the percentage of the scaffold weight remaining after lysozyme treatment: Wr% = (Wt/W_0) × 100. W_0 denoted the initial weight of the scaffold and Wt the remaining weight of the scaffold at time (t).

The values were expressed as the mean $\pm$ standard deviation ($n = 3$).

2.5. Release of DHA from the scaffold

The release of DHA from the scaffold was evaluated both in the presence of cells and without cells. Scaffold samples ($5 \times 1 \times 5$ mm, 59 mg $\pm$ 0,04) were placed in 96 well plates either without cells or in the presence of cells, which were seeded upon their surfaces (30.000 cells/well). They were incubated for 1, 2, 5, 7 or 10 days in the presence of 100 μl of MEM, supplemented with 10% FCS, 100 IU ml^{-1} penicillin–50 μg ml^{-1} streptomycin. At each time point the medium was collected, 200 μl of acetonitrile was added and the medium was centrifuged at 5000 rpm for 10 min to separate proteins. The supernatant was collected and analysed by high performance liquid chromatography-mass spectrometry (HPLC) for its DHA content using the same conditions reported in section 2.1. Scaffolds without cells and cells seeded on the plates without scaffolds were used as blanks at the experimental time points.

2.6. Primary culture of human fibroblasts

Dental pulps were obtained from human third molars extracted for orthodontic reasons. Informed consent was obtained from patients. Pulp explants were dissected in small pieces (3–4 mm^3) and cultured in MEM medium, supplemented with 10% FCS and 100 IU ml^{-1} penicillin–50 μg ml^{-1} streptomycin. The human pulp fibroblasts (HPFs) obtained were cultured at 37 $^\circ$C in a humidified atmosphere of 5% CO$_2$. Cells from passages 3 to 10 were utilized for the following experiments.

2.7. Cell viability in the presence of the scaffold

HPFs and MG-63 cells were seeded onto the surface of scaffold samples ($5 \times 1 \times 5$ mm, 59 mg $\pm$ 0.04) contained in 96-well plates. The cells were seeded at a density of 30 000 cells/well and cultured in MEM medium supplemented with 10% FCS and 100 IU ml^{-1} penicillin–50 μg ml^{-1} streptomycin at 37 $^\circ$C in a humidified atmosphere of 5% CO$_2$. After 1, 3, 5 and 7 days, the cell viability was determined by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. 10 μl of MTT solution in PBS (0.5 mg ml^{-1}) was added at each well and incubated for 4 h at 37 $^\circ$C. Scaffolds without cells were used as blanks. After solubilisation with MTT solvent (0.1 N HCl in isopropanol), the optical density was read at 570 nm by a Microplate Reader (Model 680, Biorad Lab Inc, California). MTT data were reported as the mean ($\pm$ S.D.) of triplicate experiments.

As a comparison, an MTT assay was also performed on MG-63 and HPFs seeded without the scaffold at a density of 20.000 cells/well and cultured with complete MEM medium, supplemented with 0.10 μM, 0.20 μM, 0.25 μM; 0.30 μM of DHA for 1, 3, 5, and 7 days.

2.8. HRSEM of the cells cultured on the scaffold

To evaluate the cell adhesion on the scaffold surface and the cell morphology, HRSEM analysis was carried out on the
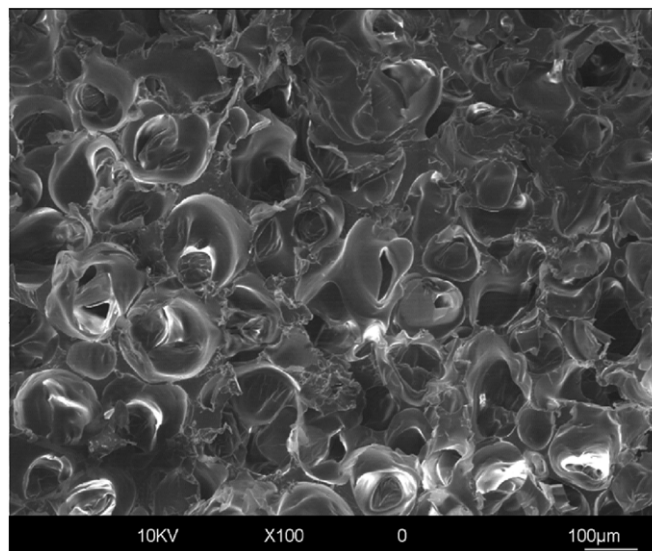
Figure 1. HRSEM analysis of gelatin–DHA scaffold (bar: 100 μm). The average diameter pore size is 109.41 μm. Ten measures of pore sizes were performed on ten HRSEM images of magnification $100 \times$.

scaffold samples containing cells seeded and cultured by the same method reported in section 2.5. At each experimental point the scaffold samples were fixed with a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer (Sigma Aldrich, St. Louis, USA) for 2 h at 4 $^\circ$C and subsequently post-fixed with 1% OsO4 in 0.1 M phosphate buffer (Società Italiana Chimici, Roma, Italy) for 1 h at RT. After several washes in a 0.15 M phosphate buffer, the samples were dehydrated in an ascending alcohol series and critical point dried (CPD 030, Balzers, Leica Microsystems GmbH, Wetzlar, Germany). The samples were then metal coated with a thin layer of carbon/platinum (CPD 030, Balzers, Leica Microsystems GmbH, Wetzlar, Germany) and observed under HRSEM (JSM 890, Jeol Company, Tokio, Japan) with 7 kV accelerate voltage and 1×10^{-11} mA.

2.9. Statistical analysis

All values in the figures and text are expressed as mean $\pm$ SD of N experiments (with $N \geqslant 3$). Statistical data analysis was carried out using Student's t-test. Data sets were examined by analysis of variance, and P values less than 0.05 were considered statistically significant.

3. Results

3.1. Characterisation of the scaffold

The microstructure of the gelatin–DHA scaffold is shown in figure 1. The presence of a very porous structure in which the pores are uniformly distributed in the solid matrix and are separated with thin walls can be seen. The pore size varied between 90 and 200 μm. The pore size distribution appeared almost unimodal. The total porosity obtained by MP was 56 $\pm$ 3.9.
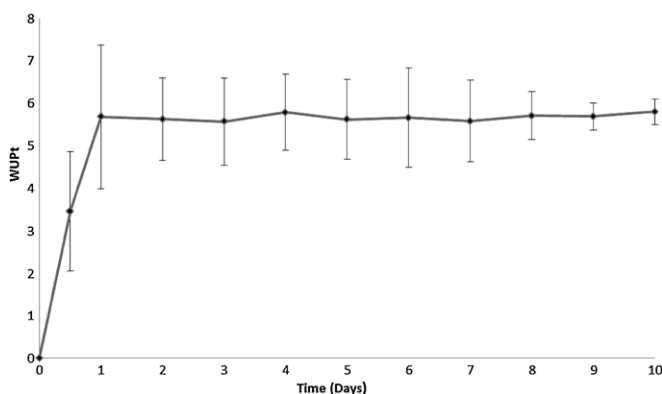
Figure 2. Water uptake of gelatin–DHA scaffold. The experiments were carried out for 10 days. Data were expressed as mean of ten experiments $\pm$ SD (*$P < 0.05$).

3.2. Water uptake

The water uptake was quite efficient, providing a final weight of the hydrated scaffold about 6-fold its initial dry weight. The maximum water uptake corresponding to the maximum hydration ability of the scaffold was obtained only after 1 day and lasted for the whole duration of the experiment (figure 2).

3.3. Degradation of the scaffold

The scaffold degradation provided a progressive weight loss with a degradation rate almost constant over time, up to day 9. Between day 9 and 10 a sharp increase in the degradation rate was observed (figure 3), which preceded the scaffold dissolution taking place at day 10.

3.4. Release of DHA from the scaffold

The DHA released from the scaffold was characterized by a rate which progressively increased over time (figure 4). The presence of cells did not significantly influence the DHA released as no significant difference was observed between the different systems (figure 4).

3.5. Cell viability

The viability of the tumor cells in the scaffold strongly decreased over time to reach complete cell death at day 7. In contrast to this, the viability of the normal cells was highly improved in the scaffold. A progressive increase in viability was observed over time reaching 2.5-fold the viability of the control at day 7 (figure 5).

HPFs incubated in MEM medium without any scaffold and with different concentrations of DHA showed a high and constant cell viability during the treatment (figure 6(*a*)), while MG63 showed a decrease of cell viability after 5 days from the start of the treatment (figure 6(*b*)).

3.6. Cell adhesion

HRSEM showed the presence of HPFs inside the macroporosity of the gelatin–DHA scaffold after 24 h of cell culture (figure 7(*a*)). Cells showed a round shape morphology,
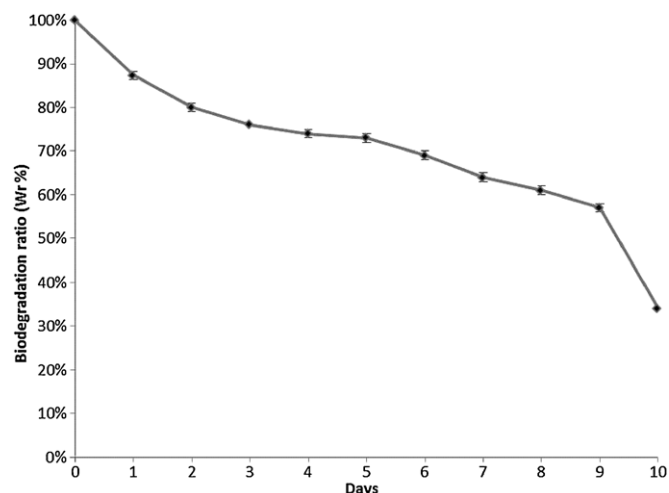
Figure 3. Gelatin–DHA scaffold degradation. The experiments were carried out for 10 days. Data were expressed as mean of three experiments $\pm$ SD (*$P < 0.05$).

with the cell membrane covered by thin microvilli connected with their migratory behaviour (figure 7(*a*)). After 5 days of cell culture, HPFs showed a fibroblastic like morphology with several thin cytoplasmic extroflessions, which allowed a strong interaction with the scaffold surface (figure 7(*b*)).

MG63 cells colonized the scaffold surface and its macroporosities after 24 h of cell culture (figure 7(*c*)). They showed a round shape morphology as described for HPFs. After 5 days of cell culture, they still showed a round shape morphology, suggesting the lack of a strong adhesion to the scaffold surface (figure 7(*d*)). The cell and scaffold surfaces were covered by several bubbles and cell debris which suggest a strong cell sufferance (figure 7(*d*)) in agreement with the reduction of cell viability.

4. Discussion

Porous scaffolds for tissue regeneration have been the subject of several studies, especially in the presence of tumor disease, to promote connective tissue regeneration in defective areas caused by radiotherapy, chemotherapy, chirurgical resection or reabsorption by tumor activity. Until now attention has been focused mainly on the physicochemical characteristics of the scaffolds such as mechanical strength, degradation rate and porosity in order to find out suitable supports for cell proliferation and differentiation. The possibility for a scaffold to promote normal cell proliferation and differentiation, while preventing tumor cell development, has rarely been addressed until now in spite of its great importance in medicine as a part of the antitumor approach. Indeed, scaffolds for tissue regeneration are mainly employed in patients already treated for the presence of tumor disease. In this case, the possibility that residual tumor cells remain in the body after antitumor treatment strongly exposes the patient to the threat of a disease relapse if these cells infiltrate the scaffold and proliferate. In this work we prepared a novel scaffold based on gelatin crosslinked with DHA and evaluated its ability to promote normal cell proliferation, while preventing
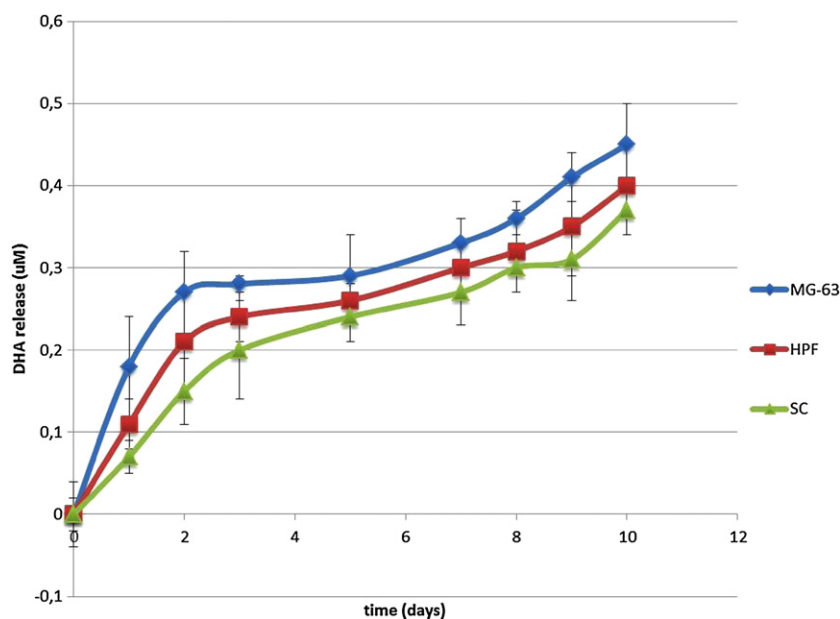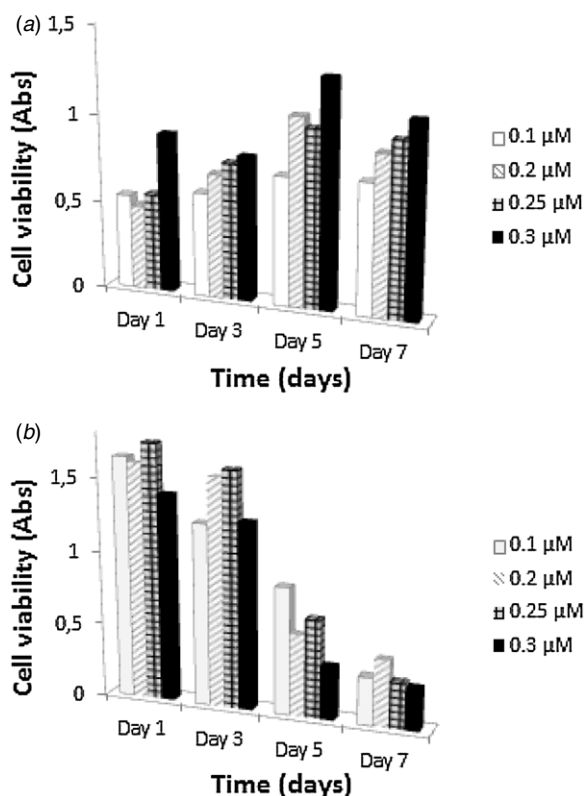
Figure 4. Release of DHA from gelatin–DHA scaffold containing cells compared to the gelatin–DHA scaffold without cells. Data were expressed as mean of three experiments $\pm$ SD (*$P < 0.05$).
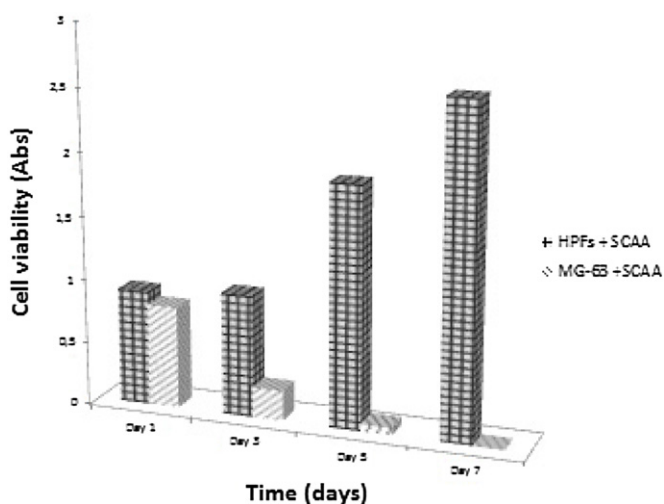

Figure 5. Cell viability of MG-63 and HPFs seeded on gelatin–DHA scaffold up to 7 days. Data were expressed as mean of three experiments $\pm$ SD (*$P < 0.05$). Abs: absorbance

Figure 6. Cell viability of HPFs (*a*) and MG-63 (*b*) seeded without scaffolds up to 7 days. Data were expressed as mean of three experiments $\pm$ SD (*$P < 0.05$). Abs: absorbance

tumor cell development. Gelatin has been selected for its proven ability to play the role of scaffold for supporting cell attachment and proliferation and due to its bioadhesive properties ascribable to the presence of the RGD sequence involved in cell attachment and formation of intercellular matrix [22]. DHA has been used as the crosslinking agent for gelatin due to the presence of two ketone groups on its molecule which may form covalent bonds with the amine groups of gelatin. Crosslinking is indeed necessary to obtain stable gelatin scaffolds at physiological temperatures. To this purpose bifunctional ketones or aldehydes such as butadione or glutaraldehyde are generally used as crosslinking agents but their release following scaffold degradation usually raises concerns about scaffold toxicity [23]. In contrast, the DHA

released by scaffold degradation is not expected to raise toxicity concerns as the biocompatibility of ascorbic acid and its derivatives is well documented [24]. In addition DHA release may provide antitumor activity and favor collagen
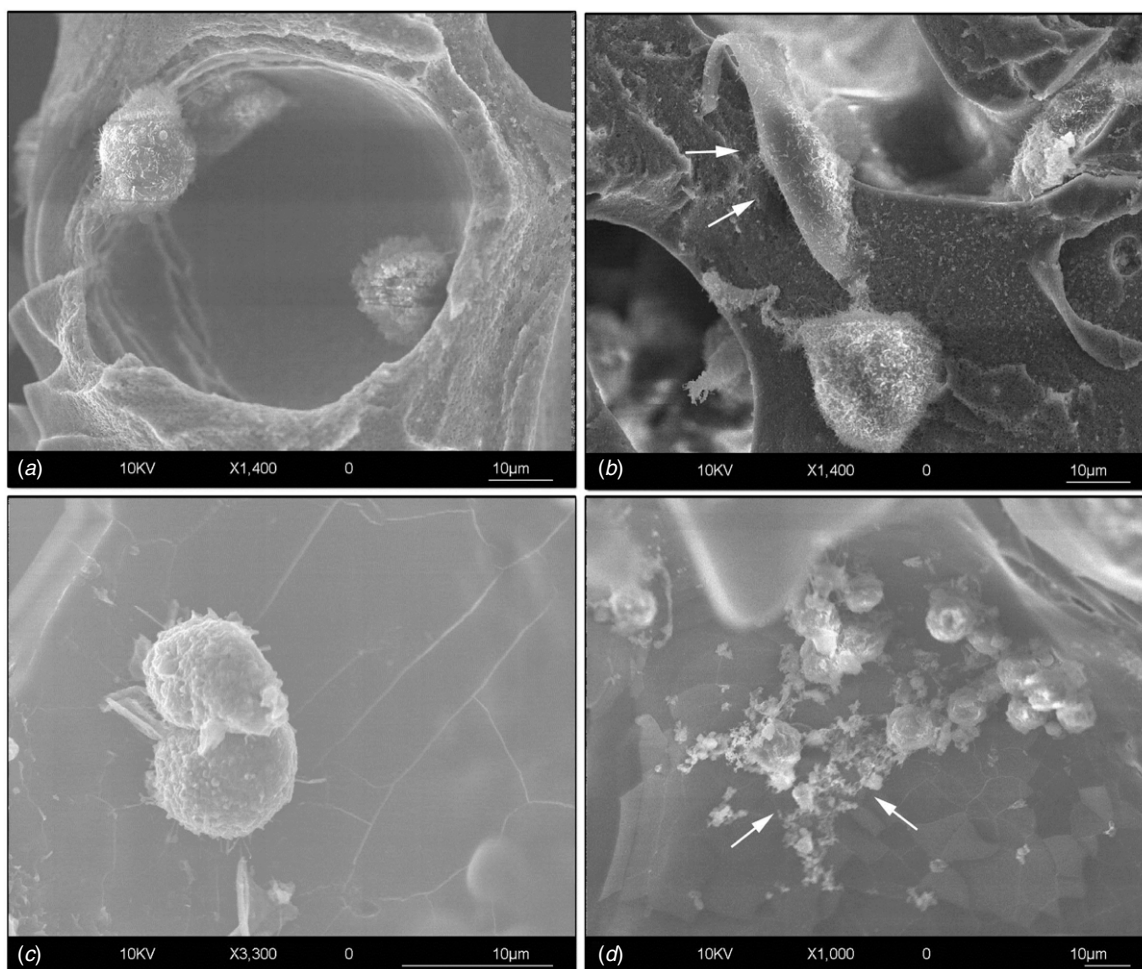
Figure 7. HRSEM of MG-63 cells and HPFs seeded on gelatin–DHA scaffold for 24 h and for 5 days. (*a*) HPFs after 24 h of cell cultures. Porosity of the scaffold is colonized by cells (bar: 10 μm). (*b*) After 5 days of cell culture HPFs showed a fibroblast like morphology. Thin filaments raising from the cell membrane allowed the interaction with the scaffold surface (white arrows) (bar: 10 μm). (*c*) MG-63 cells cultured for 24 h on gelatin–DHA scaffold. Cells showed a round shape morphology (bar: 10 μm). (*d*) MG-63 cells after 5 days of cell culture. Cell membrane and scaffold surface were covered by several cell debris (white arrows) suggesting cell death (bar: 10 μm).

deposition for connective tissue formation. The antitumor activity of DHA is both intrinsic due to inhibition of the kinases IKKβ and IKKα [15] and it is also provided by its reduction to ascorbic acid inside the cells after DHA absorption through the glucose transport mechanism [15, 25]. As it concerns the physicochemical characteristics of this novel gelatin–DHA scaffold, HRSEM indicated the presence of pores uniformly distributed in the solid matrix whose dimensions correlated with the range 90–200 μm, which has been recognized as being suitable for use in tissue regeneration (figure 1) [26]. Also its total porosity correlated with the best characteristics of the scaffolds used for tissue regeneration, being higher than 50% the total volume. Moreover the hydrophilic nature of both gelatin and DHA made the scaffold very hydrophilic. Indeed the water uptake was quite efficient, providing, after only 1 day, a 5.68-fold increase of the scaffold weight (figure 2). The scaffold hydration ability together with a controlled porosity are considered key features for cell attachment and proliferation as they allow the scaffold to become a gelled tridimensional environment upon hydration able to mimic the extracellular matrix. High levels of scaffold

hydration make cell attachment and growth easier due to an increase in cell diffusion in the gelled structure which favours cell spreading and colonization. The gelatin–DHA scaffold was also stable towards matrix disaggregation in an aqueous environment in the absence of hydrolytic enzymes. Indeed, once it reached its complete hydration it maintained a constant weight for the whole length of the experiment as indicated by the plateau in figure 2. In contrast to this, in the presence of hydrolytic enzymes such as lysozyme a decrease in the scaffold weight was obtained over time due to degradation. During degradation hydrolysis of the DHA crosslinked gelatin takes place with diffusion of the hydrolized residues from the gelled matrix towards the external aqueous environment. As long as degradation takes place the release of the hydrolysed chain residues to the aqueous environment provides a progressive weight decrease of the gelled matrix which is usually about constant over time as long as the matrix integrity is maintained. When the matrix collapses due to the advancement of degradation a sharp weight loss is obtained followed by matrix dissolution. The degradation of the DHA-gelatin scaffold in the presence of lysozyme

took place at a constant rate until day 9. Between day 9 and 10 a sharp weight decrease was observed, indicative of the matrix collapse (figure 3). At day 10.5 complete scaffold dissolution was observed. The DHA release is a consequence of the scaffold degradation. Indeed, during degradation both the peptide bonds of gelatin and the crosslinking bonds of DHA with the amine groups of gelatin are hydrolyzed providing, other than chain residues, free DHA molecules. The free DHA molecules are released towards the external aqueous environment at a rate depending on both their concentration and diffusibility inside the gelled matrix. At the start of degradation the free DHA's concentration and diffusibility are expected to be very low, causing the low numbers of hydrolyzed bonds, but as the degradation proceeds more bonds are hydrolyzed. This is providing that more free DHA molecules are inside the gel and also provide a looser gelled network favouring DHA diffusion. As a consequence the rate of DHA release is expected to increase over time according to the model proposed for drug release from matrices undergoing bulk biodegradation [27, 28]. The DHA release from the gelatin–DHA scaffold is indeed characterized by an initial slow release, whose rate increases over time (figure 4). The presence of cells on the scaffold did not significantly influence the DHA release pattern both in terms of released concentration at each time point and kinetic behaviour (figure 4). Nevertheless the DHA release provided different effects on normal and tumor cell proliferation. In the scaffold containing fibroblasts the DHA release strongly enhanced cell proliferation, with the cell viability being about 2-fold the control at day 5 and further improving at day 7 (figure 5). The improvement of fibroblast proliferation has been correlated to the DHA ability to promote connective tissue formation [16]. Scaffold biodegradation after 10 days is compatible with start of tissue regeneration [29–31]. In contrast to this, the DHA release from the scaffold containing the tumor cells significantly inhibited cell growth and proliferation, with the cell viability being about 80% the control at day 1, about 6% at day 5 and 0% at day 7 (figure 5). The inhibition of tumor cell growth has been correlated to both the intrinsic antitumor activity of DHA and its reduction to AA inside the cells [15]. HRSEM images showed a facilitated attachment of HPF cells, their progressive migration and adaptation inside the scaffold, and an initial MG-63 cells colonization at day 1 with a subsequent decrease in cell number, in accordance with the viability studies.

5. Conclusions

A novel scaffold based on gelatin crossliked with dehydroascorbic acid has been prepared to promote healthy tissue regeneration while preventing tumor cell proliferation. The antitumor activity of dehydroascorbic acid released from the scaffold during degradation prevented proliferation of tumor cells while promoting normal fibroblasts growth and proliferation due to its ability to favour connective tissue regeneration. This peculiar combination of gelatin and dehydroascorbic acid represents a novel approach in the use of scaffolds for tissue regeneration, especially in patients who underwent antitumor therapy as the possible persistence of

residual tumor cells in the body may allow their attachment and proliferation in the scaffold, thus exposing the patient to the risk of a relapse of the disease.

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