European Journal of Pharmaceutics and Biopharmaceutics 73 (2009) 50–58

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

VEGF-controlled release within a bone defect from alginate/chitosan/PLA-H scaffolds

Beatriz De la Riva [a], Christian Nowak [b], Esther Sánchez [a], Antonio Hernández [c], Michaela Schulz-Siegmund [b], Martina K. Pec [a], Araceli Delgado [a,*], Carmen Évora [a,*]

[a] Department of Chemical Engineering and Pharmaceutical Technology, University of La Laguna, La Laguna, Spain
[b] University of Leipzig, Pharmaceutical Technology, Leipzig, Germany
[c] Traumatology Service, Hospitén Rambla, Santa Cruz de Tenerife, Spain

ARTICLE INFO

Article history:
Received 18 November 2008
Accepted in revised form 28 April 2009
Available online 12 May 2009

Keywords:
Vascular endothelial growth factor (VEGF)
Alginate
Chitosan
Poly(lactide)
Bone regeneration

ABSTRACT

VEGF and its receptors constitute the key signaling system for angiogenic activity in tissue formation, but a direct implication of the growth factor in the recruitment, survival and activity of bone forming cells has also emerged. For this reason, we developed a composite (alginate/chitosan/PLA-H) system that controls the release kinetics of incorporated VEGF to enhance neovascularization in bone healing. VEGF release kinetics and tissue distribution were determined using iodinated (^{125}I) growth factor. VEGF was firstly encapsulated in alginate microspheres. To reduce the high *in vitro* burst release, the microspheres were included in scaffolds. Matrices were prepared with alginate (A-1, A-2), chitosan (CH-1, CH-2) or by coating the CH-1 matrix with a PLA-H (30 kDa) film (CH-1-PLA), the latter one optimally reducing the *in vitro* and *in vivo* burst effect. The VEGF *in vitro* release profile from CH-1-PLA was characterized by a 13% release within the first 24 h followed by a constant release rate throughout 5 weeks. For VEGF released from composite scaffolds *in vitro*, bioactivity was maintained above 90% of the expected value. Despite the fact that the *in vivo* release rate was slightly faster, a good *in vitro–in vivo* correlation was found. The VEGF released from CH-1 and CH-1-PLA matrices implanted into the femurs of rats remained located around the implantation site with a negligible systemic exposure. These scaffolds provided a bone local GF concentration above 10 ng/g during 2 and 5 weeks, respectively, in accordance to the *in vivo* release kinetics. Our data show that the incorporation of VEGF into the present scaffolds allows for a controlled release rate and localization of the GF within the bone defect.

1. Introduction

Neovascularization is an essential factor for the successful integration of bioengineered tissues. In this context, several approaches have been used to improve or induce the formation of vasculature at the target site. Angiogenesis, the growth of new capillary blood vessels from pre-existing host vasculature, is also involved in the initiation of fracture healing [1]. Vascular endothelial growth factor (VEGF) stimulates proliferation and migration of endothelial cells that mediate vessel sprouting and, together with their endothelial progenitors, neovascularization, essentially necessary for the transport of metabolic products, oxygen and nutrients. Preclinical and clinical investigations have shown that therapeutic administration of angiogenic factors to the sites of ischemia can improve regional blood flow [9]. Thus, an appropriate transient delivery of VEGF *in situ* is indicated to activate a cellular response leading to successful vascularization [10,11]. Although VEGF is predominantly known for its role in vessel formation, it is also implicated in the promotion of endochondral and intramembranous ossification in bone growth [2,3], playing an important role in the recruitment, survival and activity of bone forming cells [4–6]. Evidences that VEGF directly stimulates migration and differentiation of primary human osteoblasts have been recently reported [7,8].

However, when angiogenic factors are released too rapidly or in an uncontrolled manner, an increase of adverse effects can be expected, such as severe vasculature leakage leading to edema and hypotension [12,13]. In addition, owing to the potent mitogenic nature of VEGF, systemic exposure may promote undesired vascularization in non-target sites as well as enhance pathological conditions like tumor growth and retinopathies in distant areas.

These potential risks could be avoided by using controlled release formulations of recombinant growth factors (GFs), which would not only deliver precisely restricted quantities but also localize the GF in the ischemic tissue region. Delivery systems of different materials as carriers for VEGF have been investigated. VEGF alone has been incorporated into poly(lactide-*co*-glycolide) (PLGA) systems that allow for sustained delivery of biologically active VEGF *in vitro* and enhanced neovascularization *in vivo* [14–17].

* Corresponding authors. Av. Astrofísico Francisco Sánchez s/n, Facultad de Farmacia, 38200 La Laguna, Tenerife, Spain. Tel.: +34 922 318507; fax: +34 922 318506.
E-mail addresses: adelgado@ull.es (A. Delgado), cevora@ull.es (C. Évora).

0939-6411/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2009.04.014

Combined angiogenic (VEGF) and osteogenic factors (BMP-4) with human bone marrow stromal cells in a scaffold system were shown to promote bone formation at an ectopic site [18]. VEGF and platelet-derived growth factor (PDGF) in a PLGA scaffold [19] or in alginate hydrogels [20] stimulated vessel formation.

Many of these approaches are based on incorporating GFs in appropriate biomaterials. Such combinations can provide controlled release into the local microenvironment and yield desirable concentrations over periods ranging from days to months.

The delivery of multiple factors involved in tissue formation could mimic the conditions of the tissue regeneration. However, it may be necessary to develop systems capable of controlling the kinetics of every single GF, taking into account that GF activities take place in an orchestrated, time-controlled manner.

In this regard, we developed a composite (alginate/chitosan/PLA-H) system capable of controlling VEGF release kinetics within the bone to enhance neovascularization in bone healing. Our combination of polymers and VEGF provides controlled release into the local microenvironment of the implantation site, avoiding the systemic exposure to the growth factor.

2. Materials and methods

VEGF and ^{125}I-VEGF were purchased from Chemicon and PerkinElmer, respectively. Chitosan (Protasan® UP CL 213) and alginate (Pronova® UP MVG) were purchased from Novamatrix Biopolymer (Norway), end-group uncapped poly(lactide) (Resomer® R203 H; PLA-H 30 kDa) was from Boehringer Ingelheim KG (Germany).

2.1. Formulation of VEGF-alginate microspheres

Alginate microspheres were prepared by an emulsification/internal gelation method. Briefly, microspheres were prepared by adding heat sterilized $CaCO_3$ (0.375%, w/w) to 5 ml of an aqueous alginate solution (3%, w/v) and stirring the mixture at 60 rpm for 30 min (AGV-8 Brunsen with R1002 screw-type paddle, IKA). Then, 300 μl of a solution of 1% BSA in PBS containing 7.5 μg VEGF/^{125}I-VEGF (0.5–2 μCi) was added, and the mixture was stirred at 60 rpm for another 30 min. Each fraction of 1 ml of the resulting alginate dispersion was injected via syringe into 5 ml of heat sterilized medium-chain triglycerides (Miglyol-812®), the latter being stirred at 1150 rpm. After 5 min of emulsification, 0.5 ml of glacial acetic acid in Miglyol-812® (1:100) was added, followed by stirring for another 5 min to permit $CaCO_3$ dissolution. Oil-dispersed microspheres were recovered by washing with 2.5 ml of sterile $CaCl_2$ solution (50 mM). The oil phase was removed and the microparticles were washed again two times with $CaCl_2$ solution and then with water and *n*-hexan. Finally, the particles were freeze-dried and kept at 4 °C until use.

2.2. Scaffold fabrication

Alginate or chitosan scaffolds were fabricated by freeze–drying a dispersion of VEGF/^{125}I-VEGF-loaded alginate microspheres (7 mg) in 150 μl of 1% alginate or 1.5% chitosan aqueous solution. For cross-linking, the obtained alginate or chitosan cylindrically shaped matrices were incubated for 30 s in 50 mM $CaCl_2$ and 1 M NaOH, respectively. After two wash steps with deionized water, the scaffolds were frozen in liquid nitrogen and again lyophilized. These scaffolds were named A-1 for alginate and CH-1 for chitosan preparations.

A modification of these two groups of scaffolds was elaborated by immersion of the previously obtained alginate or chitosan matrices in another 150 μl of 1% alginate or 1.5% chitosan aqueous solution. Subsequently, these matrices were freeze-dried, cross-linked, washed and again freeze-dried, as mentioned earlier. These scaffolds were named A-2 and CH-2, respectively.

A third type of scaffolds was prepared by immersion of the CH-1 scaffolds in a 50% PLA-H 30 kDa methylene chloride solution (CH-1-PLA) for 1 min and by subsequent overnight desiccation at room temperature.

All the scaffolds were stored at 4 °C until use.

2.3. Physical and morphological characterization

Non-radioactive particles or scaffolds were used for physical and morphological characterization.

2.3.1. Particle size and size distribution

Particle size of freshly produced, freeze-dried and swollen alginate microspheres were determined using a zoom stereo microscope (Hund Wetzlar SM33) combined with a CD camera (Nikon DS-2 M) and an imaging software (Nikon NIS-Element AR 2.30). The freshly produced and swollen alginate microspheres were treated with 100 μl of a methylene blue solution for 2 min. The methylene blue solution was removed, and 2 ml of sterile water was added to the microspheres. The size distribution of each batch was calculated from a sample of 300 microspheres.

2.3.2. Scanning electron microscopy (SEM)

SEM images of scaffolds and their cross-sections were analyzed by scanning electron microscopy (Jeol JSM-6300). SEM photomicrographs were used for morphology, microstructure and porosity evaluation of the constructs before and during the *in vitro* release assays.

2.3.3. Mercury porosimetry

Pore diameter distribution, total pore volume and the porosity of the freeze-dried scaffolds, were measured at the beginning and during the *in vitro* assays, using a mercury intrusion porosimeter (Autopore IV 9500, Micromeritics Instrument Co.). Determination of the porosity was based on the relationship between the applied pressure and the diameter of the pores into which mercury intrudes. According to the Washburn equation:

$$D = (-4\gamma \cos\theta)/P$$

where P is the applied pressure (runner from 0.5 to 58000 psia), D is the pore diameter, γ is the surface tension of the mercury (484 dynes/cm), and θ is the contact angle between mercury and the pore wall (taken as 141°).

2.3.4. Gel permeation chromatography (GPC)

GPC (Waters® chromatograph) relative to polystyrene standards (Tokyo Soda Ltd.) was used to determine the average molecular weights of the PLA-H before and during the *in vitro* release experiments. Four columns in a series with different pore sizes (Ultrastyragel) were used. Tetrahydrofuran was used as solvent (1 ml/min).

2.4. Encapsulation efficiency and ^{125}I-VEGF distribution

VEGF encapsulation efficiency was determined by the measurement of radioactivity levels in three aliquots of microspheres using a gamma counter (Cobra® II, Packard). The obtained radioactivity levels per weight of microspheres were related with the initial amount of ^{125}I-VEGF. The total radioactivity of each scaffold was counted to determine VEGF loading. The spatial distribution of ^{125}I-VEGF in the systems was assessed by cutting the scaffolds into three sections (upper, medium and low) and measuring the radioactivity of each section in the gamma counter.

2.5. ^{125}I-VEGF In vitro release experiments

^{125}I-VEGF *in vitro* release assays were carried out in polypropylene tubes (Sarstedt) containing α-MEM medium, supplemented with 2% FBS (Gibco) and 0.02% sodium azide at 37 °C, in a 5% CO_2 atmosphere and relative humidity of 95%, under slow orbital shaking at 75 rpm (Orbital shaking platform POS-300, Grant-bio). Briefly, 7 mg of VEGF/^{125}I-VEGF alginate microspheres or each of the fabricated scaffolds was incubated in 2 ml of release medium. At specific time intervals, 0.25 ml of medium were withdrawn and replaced by fresh solution. The radioactivity of the samples was measured using a gamma counter (Cobra® II, Packard). The cumulative ^{125}I-VEGF released from the formulations (microspheres and scaffolds) was calculated as percentage of the total encapsulated ^{125}I-VEGF.

In parallel, the radiolabeling stability of ^{125}I-VEGF in the release medium was checked by thin-layer chromatography (TLC). A solution of ^{125}I-VEGF (1μCi/ml) in release medium was incubated in the above-mentioned conditions. At specific time intervals, an aliquot (7 μl) was spotted on a plastic silica gel (60 F_{254}, Merck) (0.9 × 8 cm) stripe, and the chromatography was carried out with 85% methanol in water along 5 cm. Once finished, the band was cut into three parts (starting point, medium and front) that were measured for radioactivity in a gamma counter (Cobra® II, Packard). With this chromatographic system, the free ^{125}I-reaches the front (R_f = 1), and the ^{125}I-VEGF is retained at the starting point (R_f = 0).

2.6. VEGF bioactivity

To determine the biological activity of the VEGF released from the composite scaffolds, a cell-based *in vitro* activity assay was carried out.

Human umbilical vein endothelial cells (HUVECs), kindly donated by Dr. Federico Díaz-González, were expanded on gelatine-coated cell culture plastic in complete culture medium containing Medium 199 with Hanks' BSS, 100 mg/l L-glutamine, 25 mM HEPES and 1.4 g/l $NaHCO_3$ (BioWhittaker), supplemented with 20% of the defined fetal bovine serum Gold (PAA), 50 mg/ml endothelial cell growth supplement (ECGS from Becton Dickinson), 3,33 UI/ml porcine heparin (Sigma), 50 UI/ml penicillin and 50 μg/ml streptomycin.

During the activity assay, HUVECs were cultured in basal culture medium that is culture medium as described above except for omitting ECGS and heparin supplementation. 5×10^3 cells per well were seeded in 150 μl of basal medium in gelatine-coated 96-well plates. Then, 150 μl of adequately diluted media directly obtained from alginate microspheres or CH-1-PLA matrices after a release period of 1 and 7 days or of defined VEGF standard solutions, prepared in basal medium at 0–4 ng/ml, were added. The VEGF concentrations in the collected media were calculated using the release kinetics obtained from the *in vitro* release study described above. Additional amounts of these media were stored at 4 °C and used to change the assay medium on day 3 after seeding.

On day 5, cells were quantified using the colorimetric XTT tetrazolium assay (Roche Molecular Biochemicals) following the manufacturer's instructions. Briefly, the tetrazolium salt XTT is metabolized by mitochondrial dehydrogenases of viable cells to formazan dye. Numerical values of color densities are obtained in an enzyme-linked immunosorbent assay plate reader (BioRad) at a wavelength of 490 nm/reference 690 nm.

Bioactivity of the released VEGF from the scaffolds was determined by comparing the induced cell duplication with standard VEGF (cell proliferation due to equivalent amounts of VEGF added directly to the culture medium). Untreated control cells served to verify the growth stimulatory effect of VEGF on HUVECs.

2.7. In vitro behavior

2.7.1. Microsphere swelling behavior

Swelling behavior of the freeze-dried microspheres over periods of up to 9 days was assessed after incubation in the release medium. Swelling capacity was determined by sizing freeze-dried and swollen particles as described earlier (Section 2.3.1).

2.7.2. Scaffold water uptake and mass loss

Scaffolds were incubated in 2 ml of release medium under the same conditions as in the release assays. Water uptake and weight loss were determined gravimetrically. At specific time points, three samples of each formulation were withdrawn, blotting away excess water and weighted. The scaffolds were freeze-dried and the final weight recorded. The percentages of water uptake and mass loss were calculated from Eqs. (1) and (2), respectively:

$$\text{Water uptake } (\%) = [(W_w - W_d)/W_d] \times 100 \quad (1)$$

$$\text{Mass loss } (\%) = [(W_0 - W_d)/W_0] \times 100 \quad (2)$$

where W_w is the wet weight of the swollen sample at time t, W_d is the weight of the dried sample at time t, and W_0 is the initial weight of the sample.

2.8. Animal experiments

All the experiments were carried out in conformity with the EC. Guideline (86/609/CEE) on care and use of animals in experimental procedures. Furthermore, the animal experiments were previously approved by the local committee for animal studies of the University of La Laguna.

2.8.1. Surgical procedure: bone defect

The surgery to produce the bone defect was performed as previously described [21]. Briefly, male Wistar rats (250–280 g) were anaesthetized intramuscularly with ketamine (75 mg/kg) and xylazine (10 mg/kg), and their right hind legs were shaved and disinfected. A vertical external parapatellar incision was made in the knee. Then, a dislocation of the patellar tendon and quadriceps was performed to allow access to the femoral condyles. A hole in the intercondylar space was made with a 1.8 mm dental burr to reach the medullar cavity. The scaffold was inserted in the damaged femur and the patella and patellar tendon were reduced. The surgical wound was closed with stitches and disinfected, and an IM injection of magnesium metamizol was administered to reduce post-surgical pain. After recovery from the operation, the animals were allowed for free movement, food and water uptake.

2.8.2. In vivo release assays

The VEGF release kinetics from the scaffolds inserted in the femur of the rats was followed up by two measurement methods:

a) Non-invasive method: The ^{125}I-VEGF remaining at the defect site was monitored periodically by measuring the radioactivity at the femur defect using an external probe-type gamma counter (Captus®, Nuclear Iberica), as previously described and validated [22]. Briefly, a collimator of 2 cm in diameter was positioned at the end of the detector to focus on the femur area of the anaesthetized rat, where the scaffold was implanted. At each sampling time point, five readings were taken, and the ^{125}I emission peak (maximum 27 keV) was integrated for the cumulative events registered over 1 min. The highest and the lowest values were excluded, and the mean of the others were taken as the remaining radioactivity at each specific time point. After recovering from anaesthesia, the animal was allowed for free movement in its cage. These experiments were carried out

on five rats. Measurement of the total administered dose was considered at time point 0 (100%).

b) Invasive method: radioactivity measurements were carried out using a well-type gamma counter (Cobra® II, Packard). For each sampling time point, three rats were sacrificed, the femur extracted and the implants removed. The femurs freed of soft tissues were divided into three pieces: distal metaphysis (DM), proximal metaphysis (PM) and diaphysis (D). In order to determine VEGF bio-distribution, muscles around the femur, thyroid and blood samples were also collected. The radioactivities of all samples were measured in the ^{125}I energy range (15–75 KeV) for 30 s.

3. Results

3.1. Scaffold characteristics

VEGF-alginate microspheres were prepared with a fixed VEGF/alginate loading of 50 ng/mg. VEGF encapsulation efficiency of alginate microspheres was 50.7 ± 9.5%.

The prepared scaffolds were cylindrical in shape, the weight range 8.3 ± 0.55 mg for A-1 to 13.7 ± 1.21 mg for CH-1-PLA, and the resulting size was between 2.5 × 4 mm for A-1 and 3.8 × 5.2 mm for CH-1-PLA.

The radioactivity measured in the three sections (upper, medium and low) of the different scaffolds was around 30–35% each, indicating a homogeneous distribution of the ^{125}I-VEGF-loaded microspheres throughout the scaffolds.

3.2. Particle size and swelling behavior

Particle size is an important parameter as it can directly affect drug release. The volume diameters corresponding to cumulative 50% and 90% of particles for freshly produced, freeze-dried and swollen alginate microspheres are reflected in Fig. 1. During the freeze drying process, the microspheres lost almost 50% of their size. After 1 day of incubation in release medium, the diameter of the freeze-dried microspheres regained the size of their original, freshly produced status. Until day 9, no further significant changes in size were observed.

3.3. In vitro release

The release kinetics of the growth factor encapsulated within the microspheres and incorporated in the differently prepared scaffolds was subsequently analyzed using ^{125}I-VEGF. The resulting data of radiolabeling stability exhibited an increase of free ^{125}I-VEGF of around 15% in the 5 weeks that the assay lasted.

The release kinetics of VEGF pre-encapsulated in alginate microspheres before scaffold preparation was studied. The VEGF was released with an initial rapid rate of approximately 75% during the first 3 days, followed by a slower release rate for the remaining duration of the experiment until more than 90% was released (after 2 weeks) (Fig. 2).

The growth factor release kinetics from alginate microspheres incorporated into alginate or chitosan matrices was assessed to determine whether this approach would allow for a delayed release. The alginate scaffold (A-1) VEGF release profile was similar to that obtained from alginate microspheres (Fig. 2), the percent released during the first three days was reduced to around 65%, and approximately 90% was released within the first two weeks. The analysis of the VEGF delivered from chitosan sponges, CH-1 and CH-2 as well as from the alginate scaffolds A-2 showed that the burst portion of the release profile was similar to all three formulations. The initial release rate was notably reduced to around 25% in the first 24 h, and only a slight difference was noted in the sustained portion of CH-1 and A-2. Around 70% of the GF was released within 2 weeks. On the contrary, during the sustained phase, VEGF release from CH-2 was slower compared to the other two scaffolds, A-2 and CH-1 (Fig. 2), due to the higher amount of chitosan in the CH-2 composition.

The VEGF release rate from alginate microspheres included into PLA-H 30 kDa-coated CH-1 scaffolds (CH-1-PLA) was lower compared to the other preparations (Fig. 2). The burst release was reduced to approximately 13% in the first 24 h, and around 65% was delivered in the following 5 weeks of the experiment. The cumulative drug release was fitted to a zero-order model (Eq. (3)), maintaining a release rate of 1.58%/day.

$$\%\ \text{released} = 14.78 + 1.58\ \text{days},\ R^2 = 0.9788 \quad (3)$$

3.4. VEGF bioactivity

A cell-based assay was used to evaluate the biological activity of VEGF released from the scaffolds. Controls included untreated cells and HUVECs treated with media containing known amounts of VEGF (standards). Experimental wells were exposed to the media collected after incubation with VEGF-loaded alginate micro-

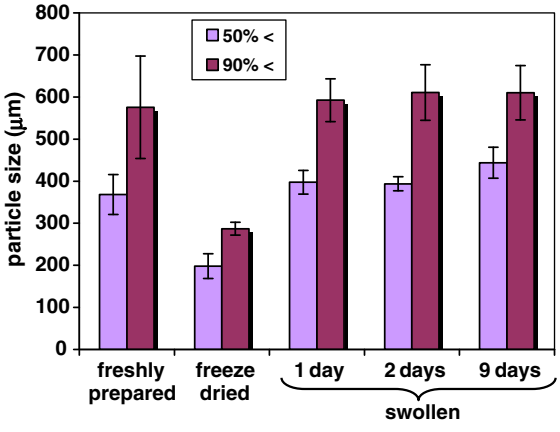

Fig. 1. Diameters corresponding to the cumulative 50% and 90% of alginate microsphere size distribution. Determinations were done with freshly prepared and freeze-dried microspheres and after incubation of the latter ones for 1, 2 or 9 days in aqueous medium (n = 3).

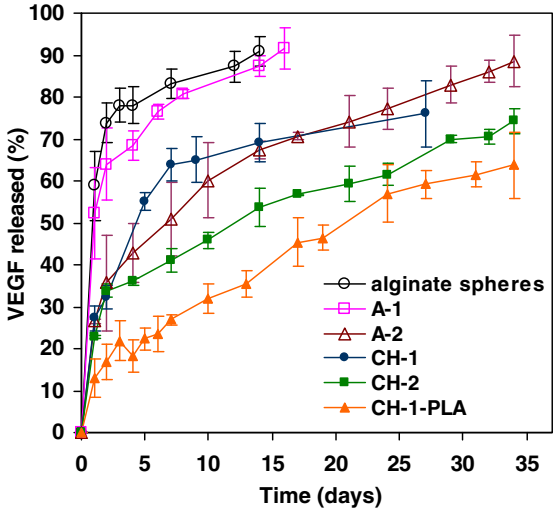

Fig. 2. *In vitro* release profiles of ^{125}I-VEGF-loaded alginate microspheres alone or incorporated in different scaffold preparations.

spheres, which constitute the first step in manufacturing any of the formulations, or to release media from CH-1-PLA scaffolds, as an example for the most complex elaboration process. A linear, dose-dependent response of endothelial cell proliferation was obtained from the VEGF standards (Fig. 3a). As in the study by Patel et al. [23], growth factor bioactivity was expressed as the percentage of the observed cell growth upon exposure to the release media with respect to the expected cell proliferation (calculated from the dose-dependent response to the standard VEGF dilutions). The biological activity of VEGF was maintained above 90% of the expected bioactivity at all times (Fig. 3b). Hence, no significant loss of VEGF bioactivity through the preparation processes of the scaffolds could be observed.

3.5. Scaffold water uptake, mass loss and porosity

The high water adsorption capacity of chitosan was very evident in the formulations of CH-1 and CH-1-PLA (Fig. 4). The PLA-H coating modulated both water uptake and mass loss throughout the release assays. The results demonstrate the influence of these parameters on the release profile of VEGF from chitosan formulations.

Despite the higher mass loss of CH-1 matrix during the release experiment, the homogenous VEGF distribution remained unchanged. The radioactivity measured in the three sections (upper, medium and low) after incubation in release medium during 3 and 5 weeks was in the range of 30–35% of the ^{125}I-VEGF remaining in the scaffolds.

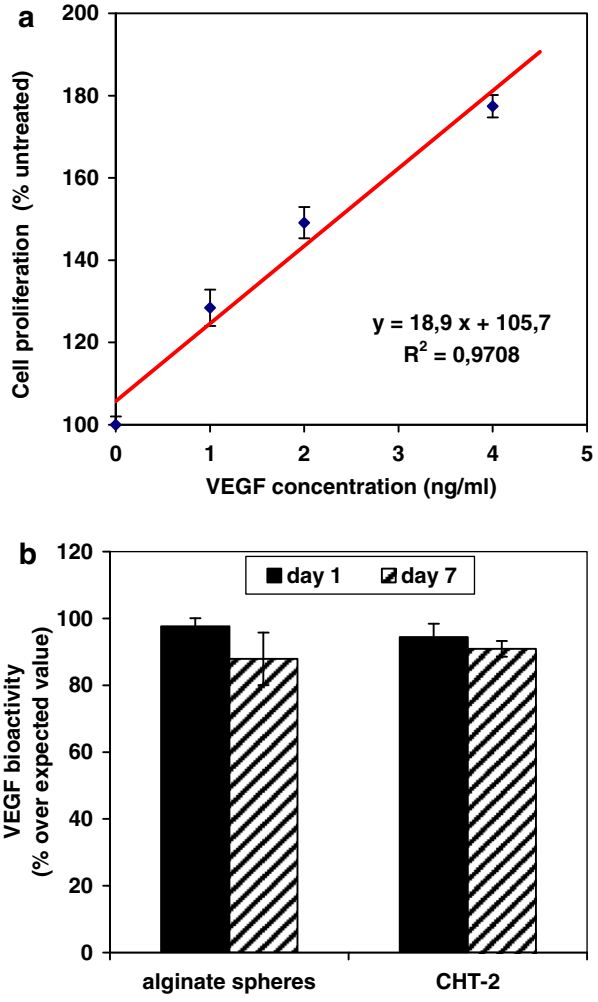

Fig. 3. VEGF bioactivity after release from composite scaffolds. (a) Dose-dependent response on cell growth for samples receiving standard VEGF concentrations. (b) VEGF bioactivity from release media expressed as percentage of the observed cell growth with respect to the expected standard activities ($n = 3$).

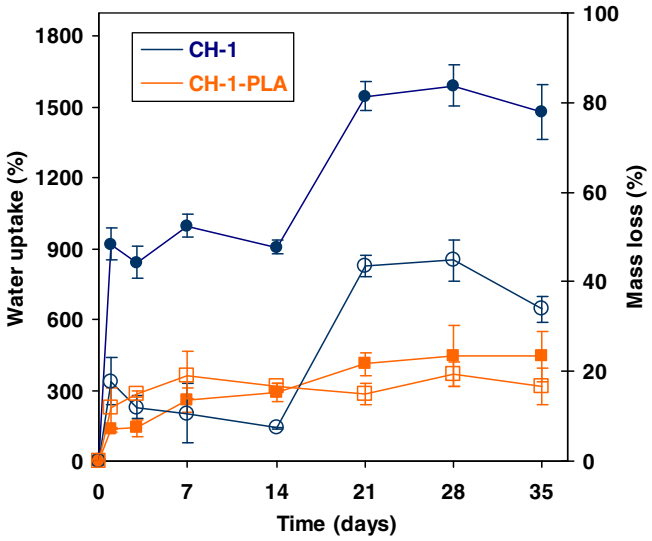

Fig. 4. Water uptake (solid symbol) and mass loss (open symbol) profiles from CH-1 (circles) and CH-1-PLA (squares) scaffolds.

SEM pictures show the increase of porosity of CH-1 and CH-1-PLA during the release experiment (Fig. 5). The porosity of the PLA-H coating increased slowly and constantly during the release experiment, whereas the inner structure remained practically unchanged. These results fit well with the decrease in the average molecular weight (Mw) detected by GPC. The Mw of PLA-H was reduced from 25 kDa to 20 kDa within 3 weeks, and to 17 kDa at the end of the release assay. By contrast, CH-1 exhibited a fast and profound increase in porosity as shown in Fig. 5.

The above mass loss, SEM pictures and PLA-H degradation results correspond with the initial and subsequent evolution in porosity and pore size distribution in both CH-1 and CH-1-PLA matrices, as determined by mercury porosimetry (Fig. 6). The originally high porosity of the CH-1 matrix (85.9%) was reduced to 53.1% by the PLA-H film. Additionally, a smaller pore size and narrower pore size distribution were determined in the CH-1-PLA scaffolds. In the release medium, porosity and pore size distribution increased for both CH-1 and CH-1-PLA but in different modes. The total porosity of the CH-1 matrix increased to 93.3% within 3 weeks and then stayed stable until week five. However, a slight shift of pore size distribution to bigger sizes was detected. Similarly, the porosity of CH-1-PLA matrix increased to 81.5% during the first 3 weeks, and pore distribution also shifted towards a bigger size. After 5 weeks, a dramatic increase of small pores in the PLA-H coating was found, which was reflected in the increase in porosity which reached 86.6% (Fig. 6). This can also be observed in the SEM pictures (Fig. 5).

3.6. In vivo release

VEGF release kinetics from three formulations was monitored by the non-invasive method after scaffold implantations in femurs of rats. Three formulations CH-1, CH-2 and CH-1-PLA were selected; the criteria used to select a formulation for *in vivo* assessment were based on the *in vitro* release profile, mainly the small burst release.

The ^{125}I-VEGF remaining in the matrix was measured externally during 2–5 weeks, depending on the release rate. VEGF pre-encapsulated in alginate microspheres and incorporated in the chitosan

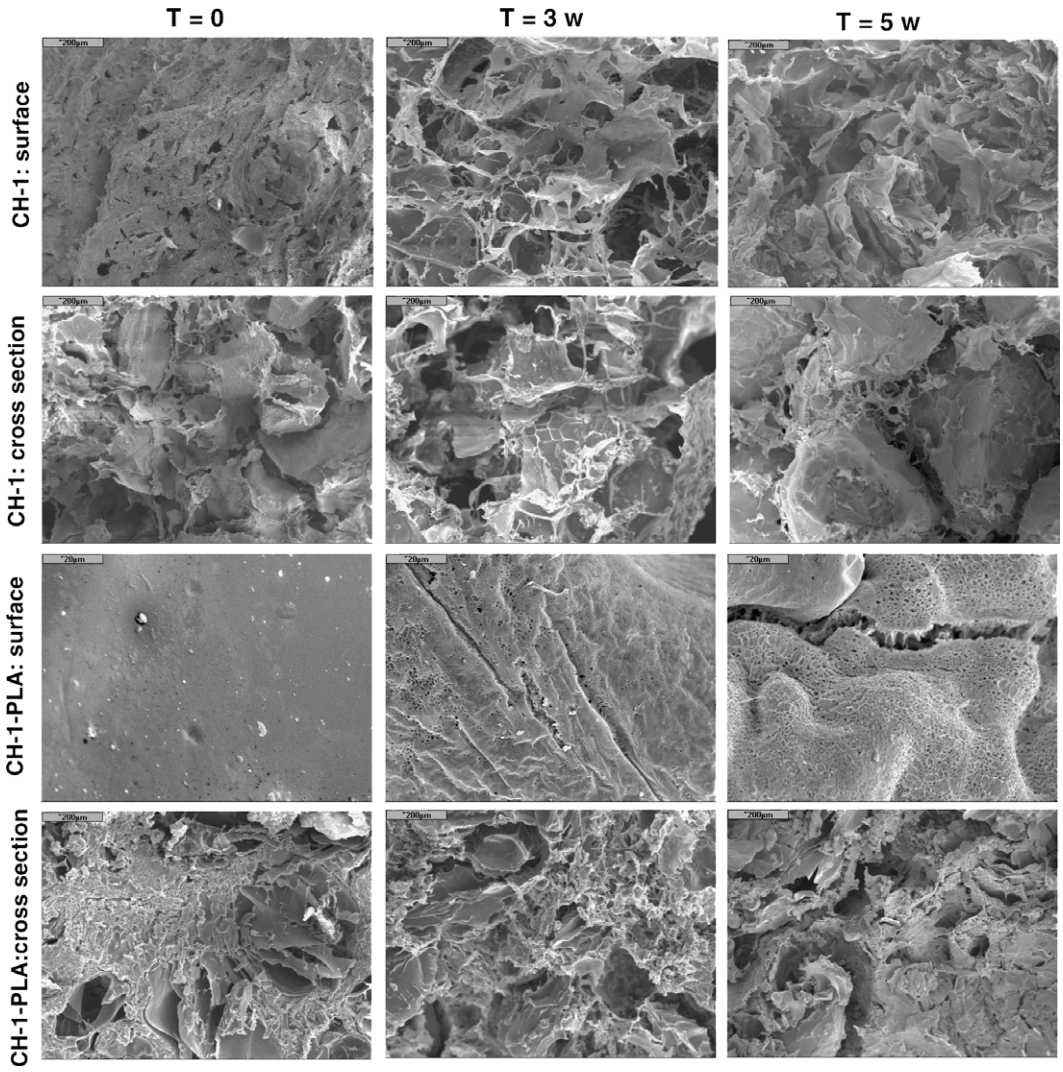

Fig. 5. Scanning electron micrographs of surfaces and cross-sections of CH-1 and CH-1-PLA implants before ($T = 0$) and after 3 ($T = 3$w) and 5 ($T = 5$w) weeks of *in vitro* release. All magnifications are 100×, except for CH-1-PLA surface pictures which are 1000×.

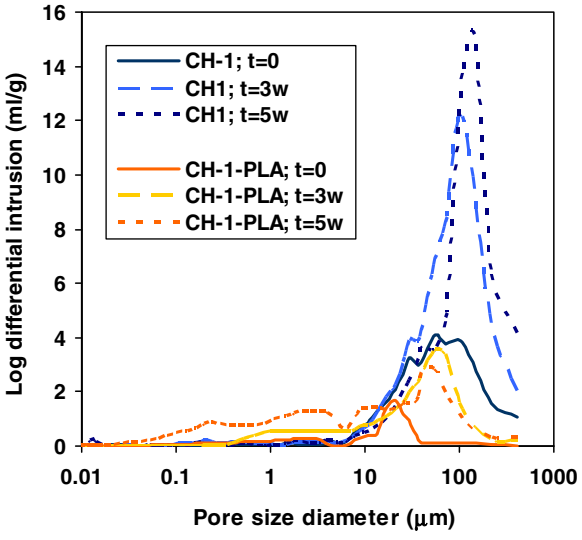

Fig. 6. Evolution of the porosity and pore size distribution in CH-1 and CH-1-PLA scaffolds during *in vitro* release. Data were obtained before ($t = 0$), after 3 ($t = 3$w) and 5 weeks ($t = 5$w) of the assay.

matrices, CH-1 and CH-2, but not coated with PLA-H coating led to a rapid burst release of approximately 50% of the GF within the first 24 h (Fig. 7), which was notably greater than that measured *in vitro* (Fig. 2). After the first day, CH-1 and CH-2 revealed different *in vivo* release profiles (Fig. 7). The VEGF in the CH-1 scaffolds was rapidly delivered; around 80% was released in less than 1 week. Afterwards, no more GF loss from the implantation site was detected. After the burst release, VEGF in CH-2 showed a very slow release phase during the first week, followed by a faster release of 1.7% per day during the last two weeks. A release of approximately 80% of the matrix-encapsulated VEGF was achieved.

With respect to the CH-1-PLA formulations, the PLA-H coating reduced the release rate similarly as *in vitro*. The burst release declined; around 20% was delivered within the first 24 h, with a subsequent sustained release rate of 15% per week during the first 3 weeks. VEGF was released slowly during the last 2 weeks; approximately 7% per week was released to give rise to almost 80% of total release by the end of the experiment at week 5 (Fig. 7).

The invasive method was used to analyze the distribution of the VEGF released from CH-1 and CH-1-PLA in femur, surrounding muscle, thyroid glands and blood (Fig. 8). VEGF concentration was higher at the site of the implant and then declined with distance from the implantation site.

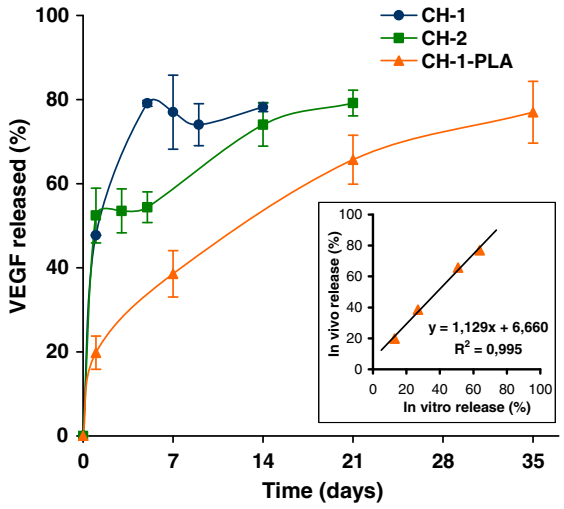

Fig. 7. ^{125}I-VEGF release profiles from femur-implanted CH-1, CH-2 and CH-1-PLA scaffolds. Inset: *in vitro–in vivo* VEGF correlation obtained from CH-1-PLA implants.

With CH-1 sponges maximum VEGF concentrations were achieved at day 5 in the distal metaphysis, the area next to implantation site. From there on, the levels decreased. The concentrations found in diaphysis and proximal methaphysis were lower. Twenty-four hours following implantation, VEGF levels in diaphyses were around 20 ng/g and then declined with time. VEGF concentrations in proximal metaphyses stayed around 3 ng/g during the two weeks of assay duration (Fig. 8). On the contrary, the results obtained with CH-1-PLA implantations revealed that the maximum VEGF concentrations in distal metaphysis and diaphysis (the zones closest to the scaffold) were achieved 3 weeks after implantation. The VEGF levels were higher than 7–10 ng/g of bone tissue throughout the 5 weeks of the experiment. On the other hand, VEGF concentrations in muscle, blood and thyroids obtained with both scaffolds were very low and did not differ significantly from the background of the gamma counter.

4. Discussion

Therapeutic approaches to replace diseased or damaged bone tissue depend on complex tissue regeneration processes directed by the spatially and temporally synchronized presentation of growth factors. To date, it is known that bone regeneration is a highly coordinated process. The development of new bone tissue significantly benefits from the strategies that mimic concomitant interactions among various factors involved in this process. The efficacy of these approaches will highly depend on the delivery of defined quantities of growth factor to the target tissue and on the duration of a sustained release of the GFs. However, the specific, exogenous GF presentation kinetics to the remaining cells to stimulate a bone regeneration process is not well fixed yet. The results presented in this study demonstrate that different VEGF release kinetics can be achieved by scaffold material manipulations.

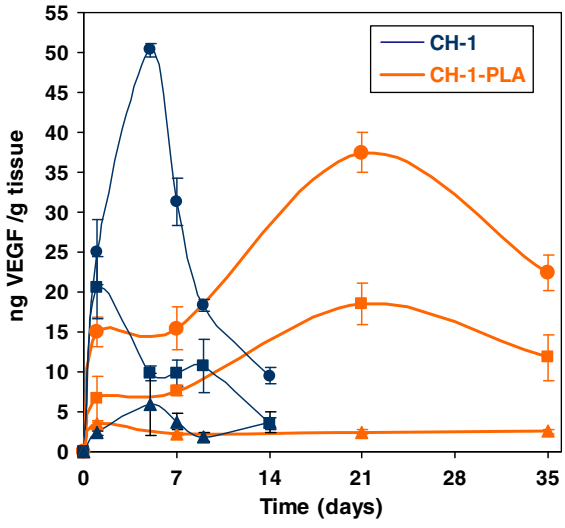

Fig. 8. ^{125}I-VEGF concentrations achieved in the different areas of rat femurs after CH-1 and CH-1-PLA implantation. Distal metaphysis (●), diaphysis (■) and proximal metaphysis (▲) zones were analyzed.

It has been reported that alginates as well as BSA and salts have a stabilizing effect on VEGF [24–28]. Our results are in accordance with the results of these authors. More than 90% of the VEGF released from calcium alginate microspheres proofed to be bioactive. The encapsulation yield of VEGF within the microspheres was around 50%. An electrostatic attraction is expected between VEGF and alginate carboxylate groups which could affect encapsulation efficacy and VEGF release kinetics. This alginate-VEGF interaction has been studied in detail and modified by Gu et al. [28], shielding the charges of alginate and consequently manipulating the encapsulation yield and release kinetics. In the present study, VEGF was released from alginate microspheres in a non-controlled manner (Fig. 2). Possible explanations for the observed burst release involve factors related to the preparation process such as a high BSA content, relatively short cross-linking time as well as a porosity induced by gas foaming during the cross-linking. On the other hand, the high water uptake capacity of alginate microspheres, the rapid desorption–diffusion of VEGF associated with the surface of the microspheres and the increase of porosity created by the release of BSA could also favor a burst release. According to the previous research reports [28,29], variations in the kinetics of protein release from alginate microspheres into different release media indicate the importance of media choice. For this reason, FBS-supplemented cell culture medium was used as release medium in this study.

With the objective of improving VEGF release, reducing the burst effect and extending the release period, VEGF alginate microspheres were included within alginate or chitosan matrices. VEGF loss during the matrix preparation process was neglected. The results exhibited a strong modification of the VEGF release profile with both materials, characterized by an important reduction of the burst effect with A-2, CH-1 and CH-2 (Fig. 2). As the VEGF *in vitro* release profiles from all three formulations could be potentially useful in bone regeneration, they were tested *in vivo*. Because of the high burst release detected in the first two determinations with A-2 (data not shown), experiments were abandoned. Conversely, the release profiles for CH-1 and CH-2 were completed as shown in Fig 7. The differences in release rates observed *in vitro* versus *in vivo*, a higher burst and a faster release rate *in vivo* may be related to blood flow rates. In the *in vitro* experiments, alginate microspheres as well as chitosan matrices showed high swelling capacity (Figs. 1 and 4) that could affect the VEGF release kinetics. *In vivo*, the same swelling behavior was expected in a dynamic environment. The dissolved and easily available VEGF could be rapidly removed, and consequently fresh protein continuously washed away by the blood flow. In addition, the described process might be more pronounced during the surgery and post-surgery period explaining the notable increase of the burst portion *in vivo* (Fig. 7).

The CH-1-PLA scaffolds successfully released VEGF *in vitro* and *in vivo*. In comparison with the non-coated formulation (CH-1), burst effect was strongly reduced and VEGF constantly delivered during 5 weeks. The zero-order *in vitro* release kinetics (Fig. 2) ob-

tained in this study is not frequently found in the related literature for proteins and specifically for growth factors. The typical *in vitro* curves are characterized by an initial release [30–31] followed by a negligible, sustained and incomplete growth factor release. Recently, *in vitro* VEGF linear sustained release for a short time (8 days) [32] or long post-burst period has also been reported [17].

The PLA-H coating reduced the swelling rate, mass loss and the porosity of the scaffold, and consequently diminished the VEGF release rate. The PLA-H allowed for water entry, with a progressively and slowly increasing water uptake to reach around 300% by the end of the experiment (Fig. 4). The mass loss behavior was similar to the water adsorption. Within the first week, a loss of around 20% was detected. Then, a plateau was achieved and maintained throughout 5 weeks (Fig. 4). The *in vitro* behavior of the same formulation (CH-1) without the PLA-H coating was totally different: the chitosan gave way to a huge water uptake, the structure swelled, and the pore size increased without important mass loss from the third week on. According to the mass loss results and SEM pictures, the swelling process did not damage the scaffold structure during the first two weeks (Figs. 4 and 5). However, an important loss of the CH-1 structure occurred after the second week but did not affect VEGF release kinetics as most of the GF had already been released (Fig. 2). The CH-1-PLA scaffolds were protected by the PLA-H coating that prevented scaffold damage and allowed for slow scaffold degradation (Figs. 4 and 5). Thus, to achieve a modulation of the VEGF release from the chitosan scaffold, it was mandatory to reduce the high water uptake. Our data suggest that the VEGF release mechanism from this scaffold is likely to be governed by diffusion through the film that progressively increases its porosity (Fig. 6), resulting in a combined diffusion–erosion release mechanism.

We expected the VEGF *in vivo* release kinetics from CH-1-PLA to be controlled by the PLA-H coating the same way as *in vitro*. The PLA-H prevented the high initial release of VEGF observed with the non-coated formulation, because the entrance of the blood flow was also controlled by the polymer film. VEGF was continuously delivered throughout the 5 weeks at a release rate slightly faster than *in vitro*. Eighty percentage of the total VEGF contents in the scaffold was released during this period. The *in vitro–in vivo* correlation was very good with a slope closes to 1 (Fig. 7), a fact which also supports a shared *in vitro–in vivo* release mechanism.

Additionally, the bioactivity of the *in vitro* released VEGF was also assessed. For VEGF released from CH-1-PLA, as well as from alginate microspheres, the bioactivity was maintained over 90% of the expected value (Fig. 3b). Consequently, the bioactivity was not affected by the process of scaffold preparation. These results confirm the protective effect of alginate and BSA on growth factor bioactivity [24–28].

The VEGF levels obtained from different tissue samples showed that the GF remained located around the implantation site with a negligible systemic exposure (Fig. 8). The quantity of VEGF available in the bone defect should induce neovascularization and bone formation. Outside the bone tissue, VEGF concentrations did not differ from the background. The VEGF release kinetics was found to be perfectly reflected on the tissue level (Figs. 7 and 8). The bone peak VEGF concentration obtained from implantation of the fast formulation (CH-1) was achieved on the 5th day. Moreover, VEGF levels above 10 ng/g tissue were maintained throughout the 2 weeks of the assay. By contrast, due to the continuous VEGF supply from the CH-1-PLA scaffold, sustained VEGF concentrations were maintained for a longer time period, i.e. during at least 5 weeks, in the bone defect. In general, GF levels that can be achieved in a target defect diminish rapidly because of the short half-life of the GF. Therefore, the way to maintain effective concentrations of VEGF is the release of the GF in a controlled manner from a delivery system.

Vascularization of tissues has been reported by several authors with VEGF only or VEGF combined with other GFs using a variety of delivery systems and doses [16–20]. However, it is important to highlight the dual capacity of VEGF as its primary action is the formation of new blood vessels but it also exerts a direct influence on bone formation and remodeling [4,5]. Neither of the cited studies provided a quantitative analysis of the *in vivo* VEGF release kinetics in a bone defect. However, the work of Ennett et al. [16] which was carried out in subcutaneous tissue, needs to be mentioned in this context. The results obtained with VEGF incorporated into a PLGA system by these authors are comparable with those obtained in the present study; protein concentration was maintained above 10 ng/ml during 3 weeks at a distance of 2 cm from the implantation site. However, the VEGF dose used by these authors was substantially higher (3 μg) than the dose used in the present study (175 ng). This explains why the concentrations achieved in the subcutaneous tissue were higher mainly during the first hours.

Therefore, as our understanding of the bone forming process evolves, it is necessary to establish the optimal *in vivo* VEGF release kinetics not only with respect to local concentrations but also with respect to the duration of action of VEGF to be maintained in the damaged tissue. In the present work, we suggest formulations that may be useful to achieve physiologically relevant VEGF profiles in bone repair.

5. Conclusions

In summary, VEGF pre-encapsulated in alginate microspheres was embedded in alginate sponges, chitosan sponges and in chitosan sponges coated with a PLA-H coating to control the release of the GF. The active VEGF, released from chitosan uncoated sponges, diffused directly in the aqueous medium, whereas the one released from the matrix of the coated scaffold was concentrated in the matrix/film interface, abandoning the system in two ways: diffusion through the pores and diffusion through the polymer coating film itself. VEGF was released *in vitro* from the chitosan PLA-H-coated scaffolds at zero-order kinetics with a minimal initial burst. Importantly, a good *in vitro–in vivo* correlation with a slope very close to 1 was also achieved. The uncoated and coated scaffolds provided a bone local GF concentration above 10 ng/g during 2 and 5 weeks, respectively, in accordance with the *in vivo* release kinetics. This study demonstrates that the VEGF levels in bone created by a loaded implant at the site of the defect depend on the release kinetics provided by that delivery system.

Acknowledgements

This work was supported by the Ministry of Science and Technology (Project SAF 2005-02753 and MAT2008-02632/MAT) and Accion Integrada (HU 2005-0009). We gratefully acknowledge Ada María Herrera García and Dr. Federico Díaz-González for HUVEC preparation and donation.

References

[1] M.R. Hausman, M.B. Schaffler, R.J. Majeska, Prevention of fracture healing in rats by an inhibitor of angiogenesis, Bone 29 (2001) 560–564.
[2] H.P. Gerber, N. Ferrara, Angiogenesis and bone growth, Trends Cardiovasc. Med. 10 (2000) 223–228.
[3] N. Ferrara, Vascular endothelial growth factor: basic science and clinical progress, Endocr. Rev. 25 (2004) 581–611.
[4] J. Street, M. Bao, L. deGuzman, S. Bunting, F.V. Peale Jr., N. Ferrara, H. Steinmetz, J. Hoeffel, J.L. Cleland, A. Daugherty, N. van Bruggen, H.P. Redmond, R.A.D. Carano, E.H. Filvaroff, Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover, Proc. Natl. Acad. Sci. 99 (2002) 9656–9661.
[5] H. Eckardt, M. Ding, M. Lind, E.S. Hansen, K.S. Christensen, I. Hvid, Recombinant human vascular endothelial growth factor enhances bone healing in an experimental nonunion model, J. Bone Joint Surg. Br. 87 (2005) 1434–1438.

[6] S.X. Hsiong, D.J. Mooney, Regeneration of vascularized bone, Periodontology 41 (2006) 109–122.
[7] U. Mayr-Wohlfart, J. Waltenberger, H. Hausser, S. Kessler, K.P. Gunther, C. Dehio, W. Puhl, R.E. Brenner, Vascular endothelial growth factor stimulates chemotactic migration of primary human osteoblasts, Bone 30 (2002) 472–477.
[8] M. Orlandini, A. Spreafico, M. Bardelli, M. Rocchigiani, A. Salameh, S. Nucciotti, C. Capperucci, B. Frediani, S. Oliviero, Vascular endothelial growth factor-D activates VEGFR-3 expressed in osteoblasts inducing their differentiation, J. Biol. Chem. 281 (2006) 17961–17967.
[9] A.H. Zisch, M.P. Lutolf, J.A. Hubbell, Biopolymeric delivery matrices for angiogenic growth factors, Cardiovasc. Pathol. 12 (2003) 295–310.
[10] T.W. King, C.W. Patrick, Development and *in vitro* characterization of vascular endothelial growth factor (VEGF)-loaded poly(DL-lactic-coglycolic acid)/poly(ethylene glycol) microspheres using a solid encapsulation/single emulsion/solvent extraction technique, J. Biomed. Mater. Res. 51 (2000) 383–390.
[11] A.H. Zisch, U. Schenk, J.C. Schense, S.E. Sakiyama-Elbert, J.A. Hubbell, Covalently conjugated VEGF-fibrin matrices for endothelialization, J. Control. Rel. 72 (2001) 101–113.
[12] M.D. Hariawala, J.R. Horowitz, D. Esakof, D.D. Sheriff, D.H. Walter, B. Keyt, J.M. Isner, J.F. Symes, VEGF improves myocardial blood flow but procedures EDRF-mediated hypotension in porcine hearts, J. Surg. Res. 63 (1996) 77–82.
[13] I. Vajanto, T.T. Rissanen, J. Rutanen, M.O. Hiltunen, T.T. Tuomisto, K. Arve, O. Närvänen, H. Manninen, H. Räsänen, M. Hippeläinen, E. Alhava, S. Ylä-Herttuala, Evaluation of angiogenesis and side effects in ischemic rabbit hindlimbs after intramuscular injection of adenoviral vectors encoding VEGF and LacZ, J. Gene. Med. 4 (2002) 371–380.
[14] T.P. Richardson, M.C. Peters, A.B. Ennett, D.J. Mooney, Polymeric system for dual growth factor delivery, Nat. Biotechnol. 19 (2001) 029–1034.
[15] M.C. Peters, P.J. Polverini, D.J. Mooney, Engineering vascular networks in porous polymer matrices, J. Biomed. Mater. Res. 60 (2002) 668–678.
[16] A.B. Ennett, D. Kaigler, D.J. Mooney, Temporally regulated delivery of *VEGF in vitro* and *in vivo*, J. Biomed. Mater. Res. A 79 (2006) 176–184.
[17] S.D. Patil, F. Papadmitrakopoulos, D.J. Burgess, Concurrent delivery of dexamethasone and VEGF for localized inflammation control and angiogenesis, J. Control. Rel. 117 (2007) 68–79.
[18] Y.C. Huang, D. Kaigler, K.G. Rice, P.H. Krebsbach, D.J. Mooney, Combined angiogenic and osteogenic factor delivery enhances bone marrow stromal cell-driven bone regeneration, J. Bone Miner. Res. 20 (2005) 848–857.
[19] R.R. Chen, E.A. Silva, W.W. Yuen, D.J. Mooney, Spatio-temporal VEGF and PDGF delivery patterns blood vessel formation and maturation, Pharm. Res. 24 (2007) 258–264.
[20] X. Hao, E.A. Silva, A. Månsson-Broberg, K.H. Grinnemo, A.J. Siddiqui, G. Dellgren, E. Wärdell, L.Å. Brodin, D.J. Mooney, C. Sylvén, Angiogenic effects of sequential release of VEGF-A165 and PDGF-BB with alginate hydrogels after myocardial infarction, Cardiovasc. Res 75 (2007) 178–185.
[21] E. Sánchez, M. Baro, I. Soriano, A. Perera, C. Evora, *In vivo–in vitro* study of biodegradable and osteointegrable gentamicin bone implants, Eur. J. Pharm. Biopharm. 52 (2001) 151–158.
[22] J.J. Delgado, C. Evora, E. Sánchez, M. Baro, A. Delgado, Validation of a method for non-invasive in vivo measurement of growth factor release from a local delivery system in bone, J. Control. Rel. 114 (2006) 223–229.
[23] Z.S. Patel, H. Ueda, M. Yamamoto, Y. Tabata, A.G. Mikos, In vitro and In vivo release of vascular endothelial growth factor from gelatin microparticles and biodegradable composite scaffolds, Pharm. Res. 25 (2008) 2370–2378.
[24] K.P. Claffey, W.O. Wilkison, B.M. Spiegelman, Vascular endothelial growth factor. Regulation by cell differentiation and activated second messenger pathways, J. Biol. Chem. 267 (1992) 16317–16322.
[25] M.C. Peters, B.C. Isenberg, J.A. Rowley, D.J. Mooney, Release from alginate enhances the biological activity of vascular endothelial growth factor, J. Biomater. Sci. Polym. Ed. 9 (1998) 1267–1278.
[26] A. Kawada, N. Hiura, S. Tajima, H. Takahara, Alginate oligosaccharides stimulate VEGF-mediated growth and migration of human endothelial cells, Arch. Dermatol. Res. 291 (1999) 542–547.
[27] M.H. Sheridan, L.D. Shea, M.C. Peters, D.J. Mooney, Bioadsorbable polymer scaffolds for tissue engineering capable of sustained growth factor delivery, J. Control. Rel. 64 (2000) 91–102.
[28] F. Gu, B. Amsden, R. Neufeld, Sustained delivery of vascular endothelial growth factor with alginate beads, J. Control. Rel. 96 (2004) 463–472.
[29] L.A. Wells, H. Sheardown, Extended release of high pI proteins from alginate microspheres via a novel encapsulation technique, Eur. J. Pharm. Biopharm. 65 (2007) 329–335.
[30] K.W. Lee, J.J. Yoon, J.H. Lee, S.Y. Kim, H.J. Jung, S.J. Kim, J.W. Joh, D.S. Lee, S.K. Lee, Sustained release of vascular endothelial growth factor from calcium-induced alginate hydrogels reinforced by heparin and chitosan, Transplant. Proc. 36 (2004) 2464–2465.
[31] L.W. Norton, E. Tegnell, W.M. Toporek, *In vitro* characterization of vascular endothelial growth factor and dexamethasone releasing hydrogels for implantable probe coatings, Biomaterials 26 (2005) 3285–3297.
[32] F. Gu, R. Neufeld, B. Amsden, Sustained release of bioactive therapeutic proteins from a biodegradable elastomeric device, J. Control. Rel. 117 (2007) 80–89.