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Starch-poly-ε-caprolactone Microparticles Reduce the Needed Amount of BMP-2

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Abstract BMP-2 is currently administered clinically using collagen matrices often requiring large amounts of BMP-2 due to burst release over a short period of time. We developed and tested a novel injectable drug delivery system consisting of starch-poly-ε-caprolactone microparticles for inducing osteogenesis and requiring smaller amounts of BMP-2. We evaluated BMP-2 encapsulation efficiency and the in vitro release profile by enzyme-linked immunosorbent assay. BMP-2 was rapidly released during the first 12 hours, followed by sustained release for up to 10 days. We then evaluated the osteogenic potential of dexamethasone (standard osteogenic induction agent) and BMP-2 after incorporation and during release using an osteo/myoblast cell line (C2C12). Alkaline phosphatase activity was increased by released BMP-2. Mineralization occurred after stimulation with BMP-2-loaded microparticles. A luciferase assay for osteocalcin promoter activity showed high levels of activity upon treatment with BMP-2-loaded microparticles. In contrast, no osteogenesis occurred in C2C12 cells using dexamethasone-loaded microparticles. However, human adipose stem cells exposed to the microparticles produced high amounts of alkaline phosphatase. The data suggest starch-poly-ε-caprolactone microparticles are suitable carriers for the incorporation and controlled release of glucocorticoids and growth factors. Specifically, they reduce the amount of BMP-2 needed and allow more sustained osteogenic effects.

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Introduction

Optimal biologic and microenvironmental conditions are needed for optimal bone regeneration. Treatment of complex fractures and other bone defects caused by high-energy trauma, disease, developmental deformity, revision surgery, and tumor resection have been improved due to advances in surgical techniques, orthopaedic implants, and perioperative management [12, 16, 25, 27, 38, 52]. However, detrimental bone healing conditions (eg, comorbidities such as diabetes mellitus, infections, improper vascular supply, etc), suboptimal surgical techniques, or biomechanical instability can result in impaired healing capacity (eg, nonunions, large remaining bone gaps) [39]. Such defects create major surgical and socioeconomic challenges [10].

Therefore, considerable efforts are currently underway in the field of bone regeneration.

Tissue engineering (TE) has emerged as a possible novel alternative to traditional medical technology, improving treatments for human diseases and disorders. TE involves the culture of living cells, using a support material (eg, scaffold) in combination with biologically active molecules (eg, growth factors [GFs]) [15]. Releasing bioactive molecules for the support of cells, allowing their growth and differentiation into specialized cell types, is important [28].

During the differentiation process of stem cells, various hormones and cytokines regulate osteoblast differentiation. Among these, bone morphogenetic proteins (BMPs) are probably the most important GFs involved [6, 7, 50]. BMPs regulate osteogenesis at two different levels: (1) the commitment of skeletal progenitor cells and (2) the maturation of osteoblasts in postnatal development [54]. BMPs, and more specifically BMP-2, have potent effects in inducing osteoblast differentiation of different cell sources [14, 19, 20, 46, 53]. Several studies have demonstrated dexamethasone (DEX) as a non-GF can induce terminally differentiated osteoblasts [18, 36, 42].

Although direct application of these bioactive molecules can be beneficial, repeated administration and high doses are often required due to their short half-lives (ie, 7–16 minutes for BMP-2 [33] and 10–15 hours for BMP-7 [21]). Simultaneously, the difficulty of the proper delivery to the specific injury site causes a need for controlled delivery techniques [43] that will ensure constant levels of BMP-2 at the desired site for an extended time period. Osteoinducing factors, such as BMP-2, must be delivered to the site of interest in the correct concentration. Therefore, their sustained delivery at the site of injury should prolong the bioavailability of these molecules and, therefore, the duration of their therapeutic action. This constitutes an important challenge nowadays for the development of delivery systems for BMP-2. For instance, BMP-2 is currently administered clinically using collagen carriers requiring large amounts of BMP-2 (1.5 mg/mL) [33] to overcome the short half-life (about 8 days in the collagen carrier) [26, 49]. Therefore, it would be important to develop biodegradable microparticles capable of releasing bioactive BMP-2 in a sustained manner for a longer desired time. We developed a novel injectable drug delivery system consisting of starch-poly-ε-caprolactone (SPCL) microparticles to induce osteogenesis with smaller amounts of BMP-2 and with release over more sustained times.

Therefore, we investigated (1) whether biodegradable SPCL microparticles could serve as a carrier for controlled release of BMP-2 and DEX; and if this was possible, (2) whether the entrapped drugs, ie, DEX and BMP-2, remained bioactive during encapsulation and upon release; and (3) whether the released DEX and BMP-2 were equally active concerning osteogenesis in an osteo/myoblast cell line (C2C12).

Materials and Methods

We first encapsulated DEX and BMP-2 in SPCL microparticles. We investigated the loading efficiency and the release kinetics in vitro. The cytotoxicity of the microparticles per se was determined. Second, we investigated the bioactivity of the released substances in cell culture experiments. For this, we used a bone precursor cell line to see the inductive capacity. Finally, we tested the bioactive potential of both released substances. As DEX was not able to induce osteogenesis in the osteoprecursor cell line, we tested the osteogenic potential of the DEX in mesenchymal stem cells derived from adipose tissue (Fig. 1).

We produced biodegradable microparticles made of a polymeric blend of starch and poly-ε-caprolactone (SPCL; 30–70 wt %) [5] containing DEX as described previously [3, 4]. In this study, BMP-2 (R&D Systems, Minneapolis, MN) was loaded in the SPCL microparticles using an emulsion solvent extraction/evaporation technique [3, 29]. Briefly, 0.25 g SPCL was dissolved in 5 mL methylene chloride (Sigma-Aldrich Corp, St Louis, MO) under vigorous stirring. This solution was dropped into a 200-mL polyvinyl alcohol solution (0.5% v/v; molecular weight, 30,000–70,000 g/mol; Sigma-Aldrich) and emulsified at a speed of 600 rpm for 4 hours at room temperature to evaporate/extract the organic solvent, providing structural integrity to the microparticles by hardening them. For the microparticles to be loaded with BMP-2, a coencapsulation process was performed by using bovine serum albumin (BSA) (≥ 96%; Sigma-Aldrich). BSA protects bioactive molecules such as GFs and peptides from inactivation (eg, as a result of exposure to harsh organic solvents) by binding them [22, 34, 47]. BSA (200 mg) and BMP-2 (10 μg) were mixed and added to the SPCL solution just before addition to the stirring polyvinyl alcohol emulsification medium. At the end of BSA and BMP-2 loading, the 200-mL reaction medium was collected and stored at 4°C for quantification of unloaded proteins. The microparticles were thoroughly washed with distilled water and freeze-dried. The encapsulation efficiency was determined by establishing a relation between the amounts of the BMP-2 and BSA remaining in the reaction medium (unloaded) after loading and the initial amount available for incorporation [3, 51]. These measurements were performed after concentration of the 200 mL by using an ultraconcentrator (Amicon® Stirred Cell 8010; Millipore Corp, Bedford, MA). The BMP-2 was determined using an enzyme-linked

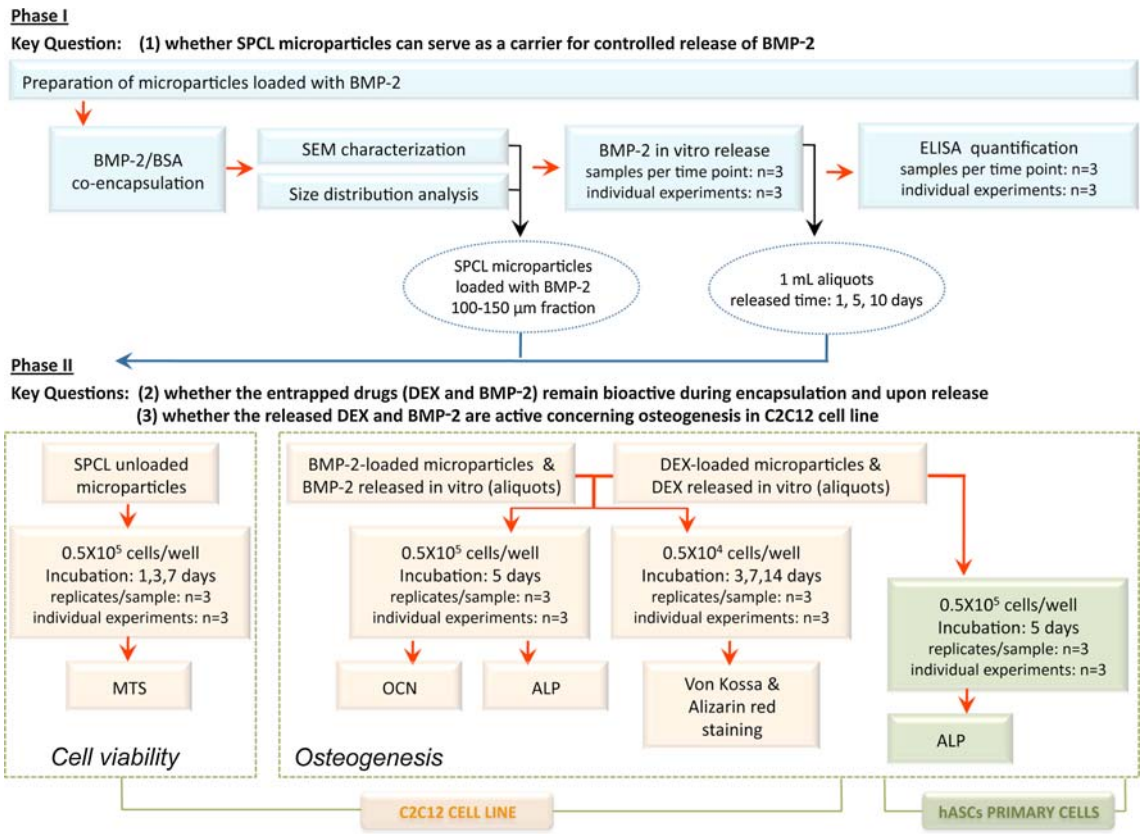

Fig. 1 A flowchart illustrates the experimental design of the study, including the procedures/assays employed and their scope and sequence. BMP-2-related samples were produced and characterized in Phase I (present study), whereas DEX-related samples were produced in previous studies [3, 4]. Phase II illustrates the in vitro studies using the different microparticles concerning cell viability and osteogenesis.

immunosorbent assay (ELISA) (R&D Systems) according to the manufacturer's instructions.

A fraction of BMP-2-loaded microparticles was collected and separated through a series of standard sieves (60–450 µm; Linker Industrie-Technik GmbH, Kassel, Germany) to determine their size distribution. Moreover, particles with a size in the range of 100 to 150 µm were chosen to study the in vitro release of BMP-2 and its effect on cells. Unloaded SPCL microparticles and those loaded with BSA alone were also produced to be used as controls. These unloaded SPCL microparticles had an average size distribution of 102.3 ± 4.1 µm [3]. The samples were sterilized by ethylene oxide using preoptimized conditions as described by Reis et al. [41].

To examine the surface morphology, shapes, and sizes of the obtained microparticles, scanning electron microscopy (SEM; Leica, Cambridge, UK) was performed. Dry samples were mounted onto aluminum stubs, gold sputter-coated (Fisons Instruments, Cambridge, UK), and viewed at magnifications of ×150 and ×400.

The release of BMP-2 from the loaded SPCL microparticles was assessed in vitro. All the experiments were independently performed three times and each experiment consisted of three separate measurements ($n = 9$). For that purpose, 150 mg BMP-2-loaded microparticles was suspended in 5 mL 0.01 mol/L phosphate-buffered saline (PBS; pH 7.4). All the samples were maintained at 37°C in an orbital shaker under constant agitation at 100 rpm. At predetermined time points, up to 10 days, 1-mL aliquots of the supernatant were taken and replaced with the same volume of fresh PBS solution. The supernatant was then stored at −80°C for BMP-2 quantification. Released BMP-2 was quantified by ELISA (R&D Systems) according to the manufacturer's instructions.

A mouse skeletal osteo/myoblast cell line (C2C12) obtained from the German Collection of Microorganisms and Cell Cultures DSMZ-ACC565 (Braunschweig, Germany) and human adipose-derived stem cells (ASC) of three different donors from the Blood Bank of the upper Austrian Red Cross (Linz, Austria) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Lonza, Basel, Switzerland), supplemented with 5% heat inactivated fetal calf serum (FCS) (Lonza) and 2 mmol/L L-glutamine (Lonza).

We tested the compatibility of the SPCL material (without growth factor) with C2C12 cells. All the experiments were independently performed three times and each experiment consisted each of three replicates (n = 9). A standard 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was used. The MTS test was performed according to the manufacturer's instructions (Promega, Madison, WI) and modified using protocols previously described [32, 45]. Briefly, C2C12 cells were plated in 24-well plates at 0.5×10^5 cells/mL (1 mL/well) and incubated at 37°C in a humidified environment containing 5% CO_2 for 24 hours. Subsequently, the culture medium was replaced by DMEM supplemented with a suspension of the SPCL microparticles (1 mg/mL). After 1, 3, and 7 days, the supernatants (containing the particles) were removed. Cells were incubated with 200 μL MTS reagent solution (5:1 ratio in serum-free DMEM culture medium without phenol red) per well at 37°C in a humidified environment containing 5% CO_2 for 3 hours. From each well, 100 μL medium was transferred to a 96-well plate and the absorbance at 490 nm was determined (BioTek Instruments, Winooski, VT). The results are expressed as the percentage of cell viability in comparison to the 100% viability attributed to the complete DMEM culture medium control.

We then evaluated the effect of the DEX- and BMP-2-loaded microparticles on C2C12 cells for their potential to induce osteogenesis. For that, several assays were performed: osteocalcin (OCN) promoter activity, alkaline phosphatase (ALP) activity, and staining assays for the detection of mineralized matrix. To determine OCN (a well-known gene expressed during late osteogenesis) promoter activity, C2C12 cells were identically plated in 24-well plates following the same procedure described above for 24 hours. Subsequently, we transfected cells by a standard lipofectamine transient transfection, using a vector expressing the OCN promoter driving a luciferase reporter gene. Reporter vector DNA (1 μg) together with lipofectamine (2 μg; Invitrogen, Lofer, Germany) per well was used. The DNA was mixed with the lipofectamine transfection agent under serum-free conditions following the manufacturer's instructions. We incubated the cells with the transfection solution at 37°C in a humidified atmosphere containing 5% CO_2 in DMEM containing 5% FCS for 4 hours. After transfection, the FCS content was reduced to 1% and the medium was supplemented with 50 μmol/L ascorbic acid and 10 mmol/L β-glycerophosphate (osteogenic differentiation medium). We further cultivated the cells for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. Finally, the culture medium was changed with the different samples to be tested. Two different samples were used for this assay: Sample 1, collected supernatants from the BMP-2 and DEX in vitro release study (release time points: 1, 5, and 10 days), and Sample 2, suspensions of BMP-2- and DEX-loaded microparticles in osteogenic differentiation medium. For Sample 1, 1-mL aliquots (supernatants) were collected from the in vitro release studies and stored (−80°C) until analyzed. The collected aliquots were freeze-dried and resuspended for stimulating the cells using the same volume of osteogenic differentiation medium to maintain the released concentrations for each time point selected (DEX: 4, 9, and 10.5 mg/mL after 1, 5, and 10 days, respectively [3]). When the samples were placed in direct contact with the cells (Sample 2), the amounts of microparticles used ranged from 1 to 10 mg per mL DMEM culture medium. As controls, BMP-2 (100 ng/mL), BMP-2/BSA (at a ratio of 1:20 000; 100 ng BMP-2 and 2 mg BSA per mL), and DEX (10^{-8} mol/L; ie, 0.004 mg/L) solutions were used. Additionally, Transwell® experiments were carried out in parallel. This experiment aimed to evaluate any differences in cell behavior as a result of direct/indirect contact of the microparticles over the cells. In this case, 10 mg/mL loaded particles was added to the Transwell® inserts, ensuring the cells were only exposed to soluble factors released from the particles. For this purpose, 12-mm Transwell® inserts (0.4-μm pore size; Corning, Inc, Lowell, MA) were used. All the experiments were independently performed three times, and each experiment employed three replicates for each condition or sample tested (thus, n = 9 for each condition). The cells were then cultured for 5 days. During this culture time, the DMEM medium was not changed. At the end of the 5 days, the activation of the OCN promoter was measured using luminescence imaging (Xenogen Corp, Alameda, CA) upon administration of luciferin. The amount of photons was measured and established a relative measure of OCN promoter activity. The cell monolayer was washed twice with PBS without calcium (Lonza) and stored at −80°C with 100 μL PBS for further ALP assay.

We evaluated the effect of the released drug and loaded microparticles on the activity of ALP, a marker expressed by cells in a more osteoblast-differentiated stage in C2C12 after 5 days using a p-nitrophenol (pNP) assay [30]. This assay is based on the occurrence of hydrolysis of p-nitrophenyl phosphate (colorless compound) in the presence of ALP enzymes to yield free pNP product (yellow). This product can be spectrophotometrically measured at 405 nm. Briefly, cells were lysed by osmotic (0.25% Triton X-100™ solution in PBS) and heat shock. Subsequently, 50 μL substrate solution was added per well (containing p-nitrophenyl phosphate disodium salt) for 30 minutes at room temperature. We determined the optical density at 405 nm in a microplate reader and calculated the activity of ALP according to a standard curve. The standard curve was constructed using pNP solutions of known

concentrations. In addition to the use of C2C12 cells, the DEX samples (collected supernatants from the DEX release study and DEX-loaded microparticles) were tested in human ASCs for ALP activity to determine DEX bioactivity per se in primary stem cells.

We assessed mineralized matrix and calcium deposition as an indication of osteogenic differentiation. For each studied sample (DEX or BMP-2) and for each time point, the experiments were performed three times. Every experiment consisted of three replicates per tested sample and time point, resulting in a total of $n = 9$ per single condition. C2C12 cells were seeded at a density of 0.5×10^4 cell/mL (1 mL/well) using osteogenic differentiation medium supplemented with the DEX- and BMP-2-loaded microparticles up to 14 days. After different time points (3, 7, and 14 days), the cells were washed twice with PBS solution (without calcium and magnesium) and fixed with 4% formaldehyde solution in PBS. Von Kossa staining was performed to visualize the presence of mineralized depositions. Briefly, fixed cells were incubated with 5% silver nitrate solution for 30 minutes at room temperature. After cells were washed several times with distilled water, staining was developed using 5% sodium carbonate solution in 25% formaldehyde and 5% sodium thiosulfate solution in distilled water. After washing the cells with water, the staining was observed by phase-contrast microscopy (Zeiss, Oberkochen, Germany). Alizarin red staining for calcium deposition was performed by incubating fixed cells for 5 minutes in a 0.05% alizarin red solution in PBS at room temperature. The stained cells were washed with distilled water before microscopic observation.

All the obtained values are reported as mean ± standard deviation. The statistical analysis was performed with OriginPro® 7.0 (Microcal® software; OriginLab Corp, Northampton, MA). Normal distribution of the data was analyzed by applying the Shapiro-Wilk test implemented by Origin® for this purpose. We determined differences in average sizes of DEX- and BMP-2-loaded microparticles using Student's t test for two independent samples. The same statistical analysis was performed to determine differences in encapsulation efficiency between BSA and BMP-2 during the BSA/BMP-2 coencapsulation process and in OCN (quantified luminescence levels) and ALP activity between DEX- and BMP-2-loaded microparticles incubated with C2C12.

Results

SPCL microparticles appeared suitable as carriers for BMP-2 and DEX. SPCL particles were nontoxic and showed a sustained release of BMP-2 over a 10-day period in vitro. SPCL microparticles with spherical shapes and smooth surfaces, as confirmed by SEM, could be produced (Fig. 2A). DEX- and BMP-2-loaded SPCL microparticles exhibited a surface morphology similar to the unloaded ones (Fig. 2B–C). The microparticles loaded with DEX

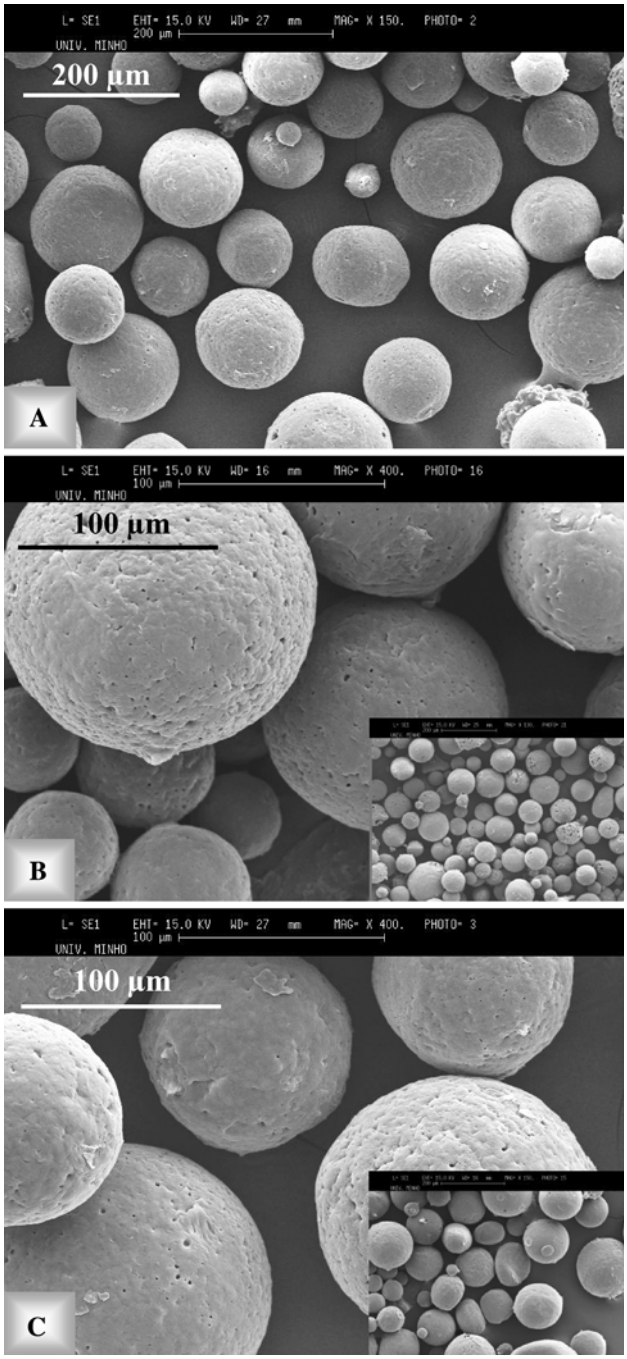

Fig. 2A–C SPCL microparticles were characterized by spherical shapes and smooth surfaces, as shown by SEM micrographs of the obtained particles: (**A**) unloaded SPCL microparticles (magnification, ×150), (**B**) DEX-loaded SPCL microparticles (magnification, ×400), and (**C**) BMP-2-loaded SPCL microparticles (magnification, ×400) (Insets: magnification, ×150).

($p = 0.0070$) and BMP-2 ($p = 0.0007$) were larger in size than the unloaded SPLC microparticles. Furthermore, the DEX-loaded microparticles had a larger ($p = 0.0113$) average size than the BMP-2-loaded ones (Fig. 3). The encapsulation efficiency values for BSA and BMP-2 during the coencapsulation process were relatively low (27.02% ± 3.4% and 24.86% ± 0.7%, respectively) and similar ($p = 0.3418$). The in vitro release studies indicated the presence of a burst release during the first 12 hours, which continued up to 2 days (Fig. 4). After this period, a plateau was reached and maintained until the end of the release period. After 10 days, more than 60% of the entrapped BMP-2 was released. Incubating C2C12 cells with the SPCL microparticles maintained cell viability up to 87% (Fig. 5).

Released BMP-2 was bioactive as shown by activation of the OCN promoter in C2C12 cells. Administration of BMP-2-loaded particles increased ($p = 0.0112$) expression of OCN promoter activity as determined by luminescence measurements (Fig. 6A). The levels of luminescence for the supernatants collected after 10 days of BMP-2 in vitro release (85.4 ± 1.6 ng/mL BMP-2 released) were similar ($p = 0.2771$) to those obtained for the BMP-2 control solution (100 ng/mL). These values were lower ($p = 0.0019$) than those obtained for the cells incubated directly with the BMP-2-loaded microparticles. However, increasing the concentration of incubated BMP-2-loaded microparticles did not further enhance ($p = 0.5552$) OCN promoter activity. On the other hand, neither DEX samples nor DEX control solution induced the expression of the OCN promoter in the C2C12 cells (Fig. 6B). The luminescence levels of DEX-related samples were even lower ($p = 0.0104$) than the values for DMEM culture media used as negative control for this assay. In addition, the levels of luminescence quantified for DEX-loaded microparticles were very similar ($p = 0.1231$) to those obtained when Transwell® experiments were performed, thus, indicating no influence on the DEX action as result of indirect/direct contact of the loaded microparticles over the cells.

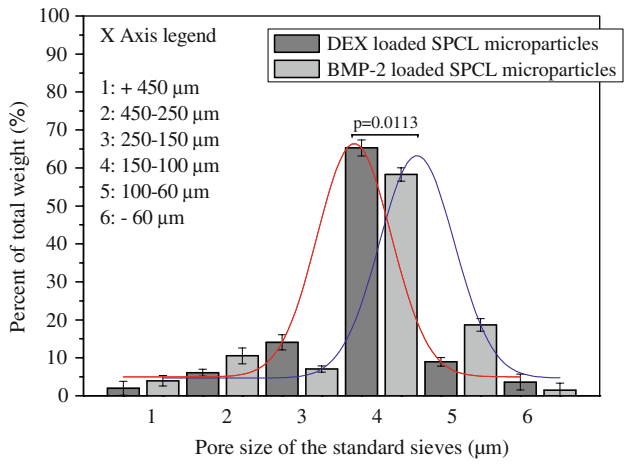

Fig. 3 A graph shows the size distribution of the DEX- and BMP-2-loaded SPCL microparticles, as determined using standard sieves. The DEX-loaded microparticles had a larger ($p = 0.0113$) average size than the BMP-2-loaded ones.

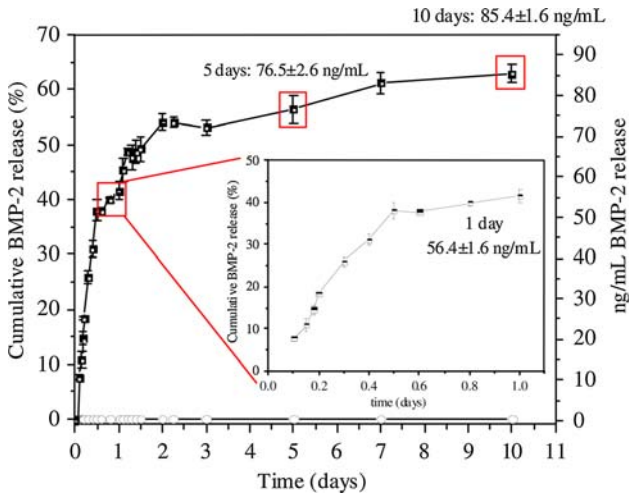

Fig. 4 A graph shows the in vitro release profiles of BMP-2 after incubation of the loaded particles in PBS medium for a period of 10 days, as quantified by ELISA. The open circles correspond to the unloaded SPCL microparticles incubated in PBS used as control sample for this experiment. BMP-2 shows a burst release during the first 12 hours (inset graph) and a sustained release for the 10-day period, which is longer than for the commercially available BMP-2 product.

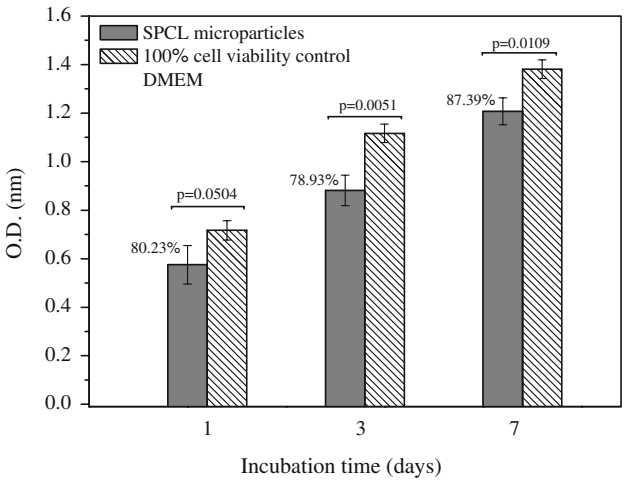

Fig. 5 A graph shows cell viability of C2C12 cells upon exposure to SPCL microparticles, as determined by the MTS test. DMEM without SPCL microparticles was used as control. The indicated values refer to the percentage of cell viability compared to the values obtained for the cells cultivated in DMEM, which were set at 100% at each respective time point. Values of cell viability higher than 75% were obtained for each time point tested. Therefore, these microparticles can serve as a carrier for BMP-2. Values are mean ± standard deviation ($n = 3$). Pairs of values statistically compared are indicated in the graph with the corresponding p values. O.D. = optical density.

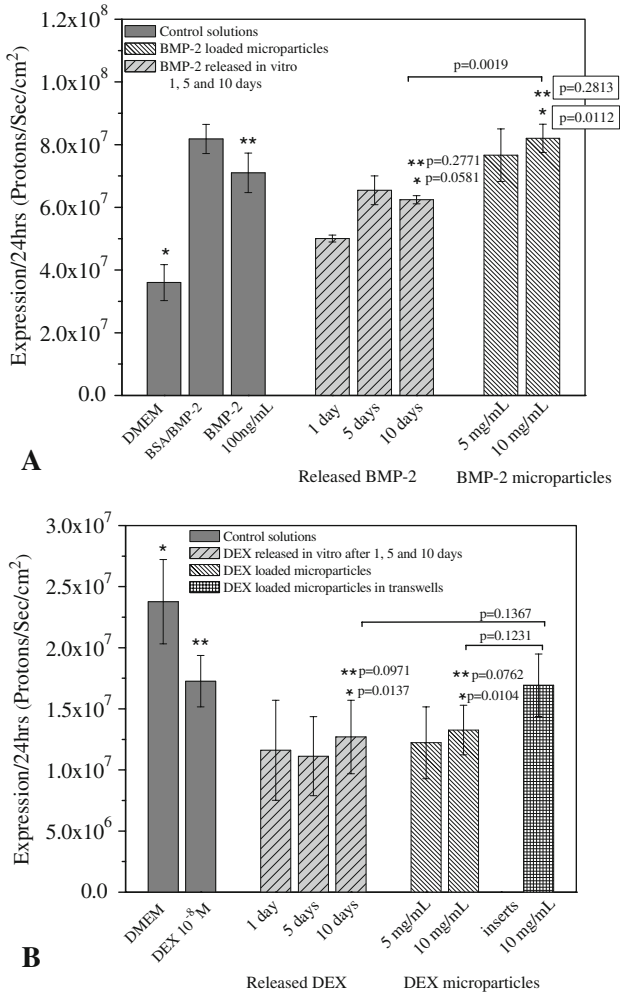

Fig. 6A–B Graphs show OCN promoter activity, as determined by luciferase activity, in C2C12 cells after administration of (**A**) BMP-2 or (**B**) DEX. The luciferase gene in the transfected vector is driven by the OCN promoter. Luciferase activity is measured after administration of luciferin by photon emission intensity. Controls include culture medium only (DMEM), BSA/BMP-2 (2 mg/100 ng per mL), BMP-2 (100 ng/mL), or DEX (10^{-8} mol/L) solutions. Samples include supernatants from the in vitro release of BMP-2 and DEX after 1, 5, and 10 days in PBS and BMP-2- and DEX-loaded microparticles incubated directly with the cells (5 and 10 mg/mL). In addition, Transwell® experiments using DEX-loaded microparticles (10 mg/mL) are presented for comparison, representing the released fraction without direct contact. These graphs show bioactivity of the drugs is maintained. Values are mean ± standard deviation. * Indicates the comparison to the negative control (DMEM), ** indicates the comparison to the positive control (BMP-2 or DEX solutions). Comparisons with p values between the tested samples are indicated by horizontal bars and vertical ticks.

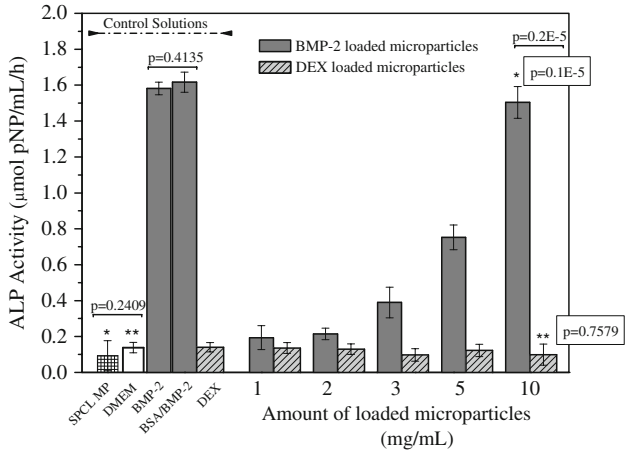

Fig. 7 A graph shows ALP activity in C2C12 cells, as determined using pNP assay. Controls include unloaded SPCL microparticles (SPCL MP), BSA/BMP-2 (2 mg/100 ng per mL), and BMP-2 (100 ng/mL) or DEX (10^{-8} mol/L) solution. Samples include BMP-2- and DEX-loaded microparticles incubated directly with the cells (1–10 mg/mL). The graph suggests bioactivity is maintained and there is a difference in osteogenetic potential between DEX and BMP-2. Values are mean ± standard deviation (n = 3). Each pair of values compared is indicated when compared to unloaded SPCL microparticles, to negative control (DMEM) (**), and between a pair of controls or samples (horizontal bars).

BMP-2 was more bioactive than DEX in C2C12 cells. DEX was bioactive as shown by the possibility to induce osteogenic differentiation in ASCs. ALP activity of C2C12 cells increased upon incubation with the BMP-2-loaded microparticles (Fig. 7). We observed higher levels of ALP activity for cells treated with BMP-2-loaded microparticles than for cells treated with the DEX-loaded microparticles ($p = 0.000002$) and unloaded microparticles ($p = 0.000001$). We found the ALP activity of C2C12 cells treated with unloaded SPCL microparticles similar ($p = 0.2409$) to the levels in DMEM culture media. In addition, no change in ALP activity was obtained when the cells were treated with BMP-2 compared to BSA/BMP-2 control solutions ($p = 0.4135$). Concomitantly, von Kossa staining demonstrated the presence of mineralization nodules in C2C12 cells treated with BMP-2-loaded microparticles (Fig. 8A–D). Similarly, alizarin red staining for calcium depositions revealed a higher staining intensity of C2C12 cells incubated with BMP-2-loaded microparticles than with both unloaded SPCL microparticles and DMEM alone (Fig. 8E–G). Furthermore, the intensity of von Kossa and alizarin red stained zones increased with culture time. Moreover, staining was more intense with increasing concentrations of BMP-2 microparticles. In contrast, no mineralization nodules or calcium depositions were detected for C2C12 cells treated with DEX-loaded microparticles. DEX-loaded microparticles and DEX control solutions did not exhibit activation of the OCN promoter (Fig. 6B) or ALP activity (Fig. 7) in C2C12 cells. However, when ASCs were used, DEX was bioactive as shown by a high increase of ALP activity ($p = 0.000004$) upon incubation of DEX-loaded microparticles with these cells (Fig. 9).

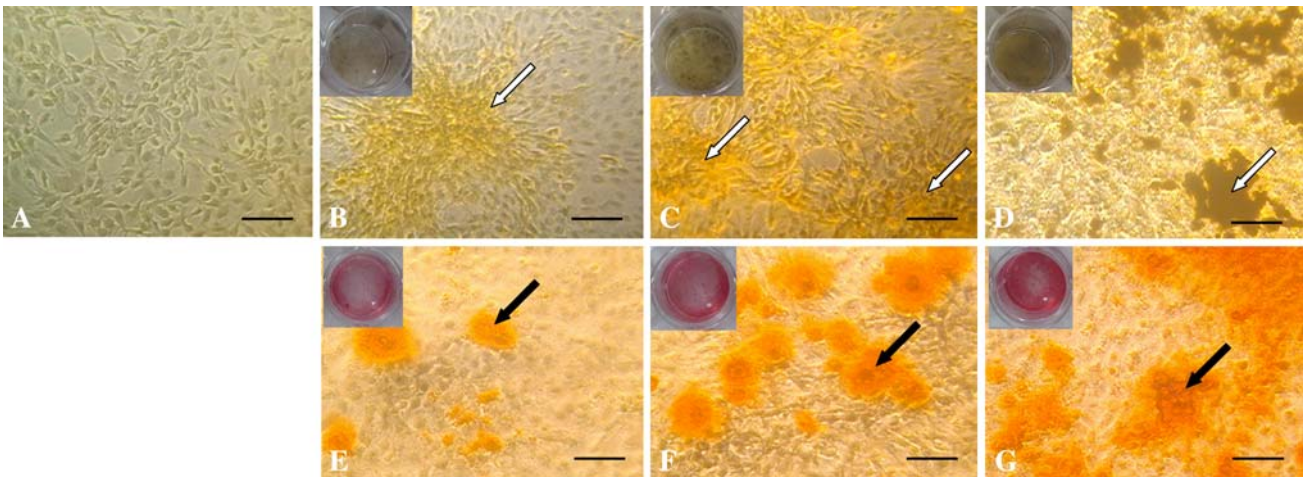

Fig. 8A–G Photomicrographs illustrate mineralization and calcium deposition in C2C12 cells upon incubation with BMP-2-loaded microparticles stained with von Kossa [(**A**) control cells; and after (**B**) 3 days, (**C**) 7 days, (**D**) 14 days of culture] and alizarin red [after (**E**) 3 days, (**F**) 7 days, and (**G**) 14 days of culture]. Bar = 50 μm. Staining zones of mineralization nodules (after 3 days of culture) and mineral deposition (from 14 days of culture) are indicated with white arrows. Similarly, the presence of calcium, indicated with black arrows, can be observed as early as 3 days of culture. The insets show overviews of a representative well as a whole.

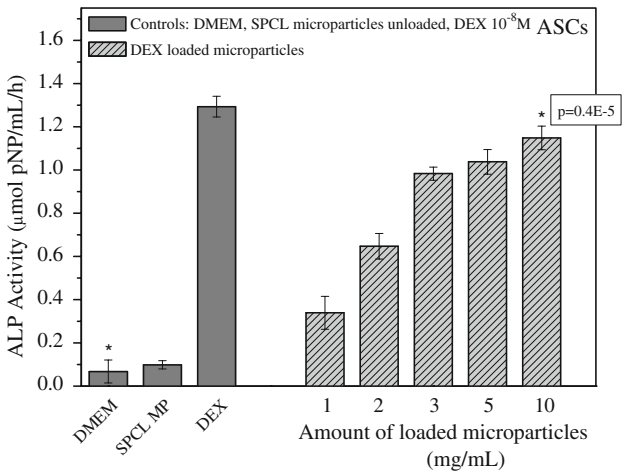

Fig. 9 A graph shows ALP activity in human ASCs incubated with DEX-loaded microparticles, as determined using pNP assay. Controls include unloaded SPCL microparticles (SPCL MP) and DEX (10^{-8} mol/L) solution. Samples include DEX-loaded microparticles incubated directly with the cells (1–10 mg/mL). This assay shows DEX indeed was bioactive, as shown by a high increase of ALP activity ($p = 0.000004$) upon incubation of DEX-loaded microparticles with these cells compared with the DMEM control. Values are mean ± standard deviation ($n = 3$).

Discussion

Clinically, high doses of BMP-2 are needed to exert its effects in nonunion tibial fractures or anterior fusion of the lumbar spine, which can be detrimental by inducing hyperostosis or heterotopic ossification. BMP-2 is applied using a bovine collagen sponge that can only be applied using open surgery and may evoke immune reactions. Our study aimed to develop and characterize a novel injectable drug release system for using SPCL microparticles. Therefore, we investigated (1) whether biodegradable SPCL microparticles could serve as a carrier for controlled release of BMP-2 and DEX; and if this was possible; (2) whether the entrapped drugs, ie, DEX and BMP-2, remained bioactive during encapsulation and upon release; and (3) whether the released DEX and BMP-2 were equally active concerning osteogenesis in an osteo/myoblast cell line (C2C12).

We emphasize several limitations. First, while we succeeded in encapsulating BMP-2 in the SPCL microparticles, the resulting encapsulation efficiency was low when compared with the theoretical 100%. Nevertheless, the amounts of BMP-2 released showed satisfactory bioactivity and did not jeopardize the functionality of the drug delivery system. Second, we used an in vitro rather than an in vivo model. This allowed us to show the effectiveness of our system per se. Cell lines are commonly used for these purposes. It diminishes biodiversity, and therefore, the obtained results are focused on the investigated actions. However, when applying these microparticles in vivo, the activity must be determined, as the surroundings are not standardized media but tissue and tissue fluids. Furthermore, under in vivo conditions, immune reactions might occur. Third, we employed $n = 9$ samples in all assays. Increasing the number of samples may increase the certainty of results. However, statistical analysis revealed adequate power. Finally, we used limited, if standard, assays; others for osteogenesis are available. However, the tests we employed are commonly used and give sufficient evidence that osteogenesis occurred and to what extent.

Indeed, our encapsulation efficiency for BMP-2 is lower than some other values reported in the literature for GFs

[9, 24, 34]. However, many of the reported encapsulation methods include the use of high temperatures and considerable amounts of toxic organic solvents without using a carrier protein to protect the GFs. As a result, high values of encapsulation efficiency are obtained, but the bioactivity of the GFs is in many cases compromised. Therefore, we used coencapsulation with BSA as carrier protein. Moreover, all the processing and encapsulation were performed at room temperature. This resulted in less encapsulation efficiency, but the bioactivity of the GFs was preserved. Thus, ours appeared a more efficient method, needing less GF for encapsulation purposes. We chose SPCL blend materials to encapsulate DEX and BMP-2 because of their known biocompatibility [32, 35, 47] and proven biodegradability [1, 2, 4]. Moreover, the combination of a hydrophilic natural material (starch) with a hydrophobic synthetic polymer (polycaprolactone) in a single blended material constitutes the major advantage of these microparticles. Thus, the resulting system is characterized by a desired biodegradability, biocompatibility, good processing versatility, and tailored hydrophilicity. Therefore, these loaded SPCL microparticles should result in a satisfactory biodegradable carrier to induce osteogenesis for bone TE. In a previous study [4], we tested the release of DEX from the SPCL microparticles in PBS and enzymatic controlled environments, as well as their capability to degrade in these conditions. The release mechanism was characterized by diffusion phenomena and at late stages by degradation of the microparticles. As a consequence, the biodegradation of the polymeric matrix controlled the overall DEX release process. The initial burst release observed for BMP-2 in the present work and previously for DEX [3, 4] can be explained as a result of drug entrapment in the proximity of the microparticles' surface. Subsequently, the BMP-2-loaded SPCL microparticles start to take up water from the aqueous environment, with the consequent swelling of the polymeric matrix and diffusion of the BMP-2 through the polymer into the medium [11, 40]. Because of the hydrophobicity of poly-ε-caprolactone, the release of BMP-2 from the SPCL microparticles is sustained from 2 to up to 10 days. This is an evident advantage of this carrier system, being able to maintain bioactivity and BMP-2 being released up to 10 days. The pronounced burst release has been considered a drawback for several control delivery systems [17, 56]. However, for osteogenesis, early high concentrations of BMP-2 are needed. Subsequently, more constant and sustained release is desired. The possibility of more sustained release of BMP-2 for longer time periods, as result of some of the mentioned properties, represents the most important benefit when compared with other delivery systems proposed for BMP-2 [9, 37]. Thus, in this particular case, we believe this system may be advantageous for bone TE.

We found the BMP-2-loaded microparticles induced osteogenesis in C2C12 cells and did not show any cytotoxicity. Moreover, the bioactivity of the released BMP-2 was confirmed since transfected C2C12 cells showed activation of the OCN promoter as measured by luminescence intensity. Activation of the OCN promoter is indicative of late-stage osteogenic differentiation [55]. This induction was maximal, as increasing concentration of BMP-2-loaded microparticles did not change activity, showing a plateau in luminescence intensity. This means the BMP-2 induced a maximal cellular response. The BMP-2 receptor is maximally activated due to binding of the BMP-2, and thus no further increase in OCN activity can be found. BMP-2 receptor I and II activity is mainly regulated by downstream signal transduction molecules, such as Smad1, 5, and 8, all of which phosphorylate Smad4. Interactions with RunX2 are possible [8]. This indicates the concentration of BMP-2 is enough to exert the maximal biologic effect. The data suggest the occurrence of osteogenesis as result of a highly bioactive BMP-2 released from the microparticles in C2C12 cells. Furthermore, DEX was not suitable as an osteogenic agent for C2C12 cells but could induce osteogenesis in ASCs. Both encapsulated drugs (DEX and BMP-2) remained bioactive upon encapsulation in and after release from the SPCL microparticles.

These observations were corroborated by determinations of ALP, an early osteogenic marker. The increase in ALP levels was a result, solely, of the release of bioactive BMP-2 from the SPCL microparticles loaded with this GF. Furthermore, this is an indication that BSA did not interfere with the osteogenic potential of BMP-2. It is well known BMP-2 can upregulate ALP activity [23]. Similar differentiation behavior was observed by the two different assays. Histology results confirmed highly bioactive BMP-2 was released from the SPCL microparticles, inducing mineralization including calcium deposition. As this activity is exerted via an ALP-independent pathway, we conclude our released BMP-2 exerts maximal effects, as mineralization was also induced [31]. Thus, the BMP-2-loaded microparticles and the BMP-2 released from the microparticles were bioactive and able to induce osteogenesis and maturation. These two distinct processes of BMP-2 action also occur in primary myoblasts using BMP-2 gene therapy [13]. In contrast, DEX-loaded microparticles were not able to induce osteogenesis, as indicated by absent ALP activity and OCN promoter activation. Interestingly, the same results were obtained with control solutions of plain DEX (without being encapsulated). Thus, the inactivity of the DEX samples was not a result of the encapsulation or defective in vitro release, but rather dependent on the cell type used, ie, C2C12 cells. Glucocorticoids, including DEX, inhibit proliferation and differentiation capacity in C2C12 cells [44, 48]. This was

confirmed by the ability of DEX-loaded microparticles to induce osteogenesis in ASCs in contrast to C2C12 cells, as observed by increased values of ALP. This suggests the DEX-loaded SPCL microparticles were capable of stimulating the development of an osteogenic phenotype in human ASCs by releasing bioactive DEX. Thus, DEX may not be suitable to induce osteogenesis in the C2C12 cell line, as has been described previously [44, 48]. However, ASCs are more clinically relevant in light of bone TE. Therefore, this line of investigation will be of continued interest in the future. Especially as DEX is not a protein, it will elicit fewer immunologic reactions. Furthermore, the production of DEX is cheaper than that of BMP-2. However, DEX also contains the drawbacks of glucocorticoids. Therefore, careful investigations are necessary to evaluate its clinical suitability.

In summary, starch-based materials are able to enhance cell attachment and proliferation of several cell types [32, 35, 45, 47]. Therefore, we tested the suitability of the combination of a microparticle system made of SPCL with bioactive molecules to induce osteogenesis. Although the encapsulation efficiency was low for BMP-2 incorporation, the bioactivity was well preserved upon its release using the encapsulation method proposed in this study. DEX was encapsulated with high encapsulation efficiency values and was bioactive in ASCs while inducing osteogenesis. Our data suggest the biodegradability and biocompatibility of starch-based materials, as well as defined release profiles of encapsulated drugs, may allow their successful use as devices for controlled/sustained delivery of bioactive molecules in bone TE. Our approach has the advantage of lower concentrations of BMP-2 with a more sustained release time than currently available with commercial products. This system could be introduced to the defect using fibrin sealant. Fibrin sealant is a natural product that keeps the encapsulated drug local and does not interfere with natural bone healing.

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