Poly(lactic acid) microspheres for local delivery of bioinorganics for applications in bone regeneration

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Bioinorganics are a family of relatively simple and inexpensive inorganic compounds, which, although often present only in trace amounts, are known to play an important role in the normal functioning of organs and tissues, including bone. Bioinorganics can potentially be used for improving the clinical performance of synthetic bone graft substitutes, as an alternative to growth factors and other biologics. To this end, it is imperative to fully understand their mechanism of action. Currently, bioinorganics are usually incorporated into calcium phosphate and bioglasses. During degradation, these materials release a cocktail of ions, making the study of the effect of the bioinorganic of interest difficult, if not impossible. To overcome this issue, in the present study we attempted to develop a delivery system for bioinorganics based on polymeric microspheres. Calcium chloride, as a model for bioinorganics, was incorporated into poly(lactic acid) microspheres, using a single emulsion-solvent evaporation technique. These microspheres were used to study the effect of the released calcium ions on the proliferation and osteogenic differentiation of human mesenchymal stromal cells in a trans-well culture system. The results showed that it was possible to incorporate calcium into the microspheres and that the efficiency of incorporation could be increased by increasing the initial amount of poly(lactic acid), and by using sonication instead of mechanical force during the emulsification step. Furthermore, the release profile of calcium from the microspheres was also dependent on the emulsification method. Cell culture experiments revealed no effect on cell proliferation or alkaline phosphatase activity as a consequence of calcium release, however, the gene expression of bone morphogenetic protein 2 and osteopontin was increased when cells were cultured in the presence of calcium containing microspheres. In conclusion, this study demonstrates that it is possible to use the developed polymeric delivery system to study the specific chemical effects of bioinorganics on the cells behavior.

Keywords: bioinorganics, poly(lactic acid) microspheres, calcium release, single emulsion-solvent evaporation technique, osteogenic differentiation

1. Introduction

The search for successful synthetic alternatives to natural bone grafts has intensified in the past decade as a consequence of an increasing demand for bone regenerative strategies, resulting from a continuous ageing of the population in the developed countries. Synthetic bone graft substitutes, which are available as calcium-phosphate ceramics, bioglass, composites, etc. [1], can be produced in large quantities against acceptable cost, overcoming the most important issues related to the use of auto- or allograft [2]. Nevertheless, the clinical bone regenerative performance of these synthetic bone graft substitutes is in general considered inferior to that of their natural counterparts.

A widely investigated strategy to improve the biological performance of synthetic bone substitutes is the addition of biological compounds such as growth factors (e.g. osteoinductive bone morphogenetic protein 2 and 7) that affect processes related to bone formation directly or indirectly [3]. The use of growth factors is however associated with issues related to their limited stability, and high production and storage cost [4, 5]. Therefore less expensive alternatives are sought for.

The inorganic phase of natural bone consists of carbonated-hydroxyapatite [6]. Further elements such as sodium, fluoride, chloride, magnesium, strontium, zinc, copper and iron can also be found in the mineral portion of bone in lower or even trace amounts [7]. The biological roles and effects of these bioinorganics in the human body and, more specifically, in bone metabolism, have been described previously [8, 9]. However, the first interest in using bioinor-

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ganics as potential therapeutics was based on knowledge obtained from changes in systemic ion levels, such as deficiencies of essential micronutrients, epidemiological studies, nutritional studies of food, animal and tissue health during disease or pharmaceutical treatments [9]. Application of strontium ranelate as anti-osteoporotic agent is an example of a successful clinical application of bioinorganics. Strontium (Sr^{2+}) intake has shown an increase in bone matrix density and reduced bone fracture occurrence in osteoporotic women [10, 11]. The addition of fluoride as an anti-cariogenic agent (F^{-}) to toothpaste formulation is one of the well-known examples of the everyday use of bioinorganics [12]. These examples emphasize on the potential of bioinorganics as therapeutic agents in applications related to bone regeneration or bone-related diseases. Indeed, a number of *in vitro* and *in vivo* studies have demonstrated the effect of various inorganic ions on processes related to bone formation, such as osteogenesis, osteoclastogenesis and angiogenesis [13–15].

Currently, common approaches for using bioinorganics as therapeutic agents include their introduction into either calcium phosphate-based ceramics or bioactive glasses, which are two widely used classes of synthetic bone graft substitutes. In such a way, a carrier or scaffold material is combined with a bioinorganic, which is similar to growth factor based constructs [8, 13, 16]. Delivery of bioinorganic ions from ceramic or bioglass carriers usually relies on the carrier degradation. However, this process is complex, featuring release of a number of ions, and a change of other physical and chemical properties of the carrier, which makes it difficult to evaluate the effects of a single bioinorganic and understand its mechanisms of action.

Delivery systems based on polymers used in biomedical applications are potentially more suitable for studying biological effects of bioinorganics. Polymeric microspheres, for example, are used as carriers and delivery vehicles of growth factors [17] and antibiotics [18], allowing minimally invasive delivery. Polymeric microspheres have been tested for application as three-dimensional support for cell expansion and differentiation [19–22]. They have also been used for developing composite materials in order to tune the degradation and mechanical properties of bioceramics such as calcium phosphate ceramics [23]. To date, very few studies have explored the possibility of incorporating bioinorganics into polymeric matrices [24].

In the present study, calcium salt was used as a model bioinorganic compound to develop a delivery system based on poly(lactic acid) (PLA) microspheres. Polylactides belong to poly(α-ester) family which is one of the widely studied classes of biodegradable polymers [25]. They are potentially suitable as delivery vehicles, as they degrade hydrolytically due to an ester bond in their backbone [25]. The degradation product of poly(lactic acid) is lactic acid which is further broken down into water and carbon dioxide in biological systems, hence, it is considered as bioresorbable [25, 26]. For microsphere preparation in the present study, a single emulsion-evaporation technique was used. Emulsion-solvent evaporation techniques are widely used for microsphere preparation [27, 28]. In both single and double emulsion techniques, microspheres are formed in a two-step process [29]. In the first step, the polymer is dispersed into microdroplets by applying mechanical or ultrasonic shear stress. The second step includes solvent evaporation and polymer precipitation, resulting in microsphere hardening. In this study, calcium salt was added to the polymer-solvent solution before emulsification. Calcium-containing microspheres were used to study proliferation and osteogenic differentiation of human mesenchymal stromal cells (hMSC) in vitro.

2. Materials and methods

2.1. Preparation of poly(lactic acid) microspheres

Poly(lactic acid) microspheres were prepared using a single (oil-in-water) emulsion-solvent evaporation technique. Calcium chloride dihydrate (Merck) was used as model salt to test the incorporation potential into poly(lactic acid) microspheres.

To optimize the process of microsphere preparation, varying amounts of amorphous poly(D,L-lactic acid) (poly(lactic acid), Purasorb PDL05, Purac, MW: 59000 g/mol) were dissolved in dichloromethane (DCM) (LiChrosolv®, Merck) to reach increasing concentrations of 4, 6, 8 and 10 % w/v poly(lactic acid) that served as the oil phase. Calcium chloride was added to the poly(lactic acid) solution to reach a concentration of 200 g/L and stirred magnetically for 30 min to prepare a slurry. 25 ml of this mixture with the poly(lactic acid) concentration of 4 or 6 % w/v or 20 ml of the mixture with poly(lactic acid) concentrations of for 8 or 10 % w/v were added to 25 ml of 1.5 % w/v poly(vinyl alcohol) (PVA) (Acros Organics) aqueous solution. Ultra-Turrax (T25 basic, IKA, Germany) was used to emulsify the mixture for 40 s at 8000 rpm before poring it into 125 ml 1.5 % w/v poly(vinyl alcohol) solution. In a parallel experiment, a formulation of 4 % w/v poly(lactic acid) was emulsified using an ultra-sonicator (Labsonic M, 50W, Braun) for 90 s. A formulation without addition of Ca^{2+} salt served as a control for every produced batch of microspheres.

Solvent evaporation and microsphere hardening occurred during magnetic stirring for 2 h at room temperature. Microspheres were then collected by centrifugation (3900 rpm, 10 min, 4 °C) and washed 3 times with cold Milli-Q water. Microspheres were then resuspended in 10 ml Milli-Q water, freeze-shocked in liquid nitrogen, and stored for at least 30 min at –20 °C before freeze-drying for at least 48 hours.

2.2. Characterization of poly(lactic acid) microspheres

The morphology of the microspheres was analyzed using an environmental scanning electron microscope (SEM; XL30, ESEM-FEG, Philips) in the secondary electron mode with an acceleration voltage of 10 KeV after gold-sputtering for 40 s at 30 mA (Cressington sputter coater). To determine the diameter of microspheres from various batches, both scanning electron microscope images and dynamic light scattering (DLS) equipment (Zetasizer Nano ZS, Malvern Instruments, Germany) was used. For the latter analysis, microspheres were redispersed in Milli-Q water in an ultrasonic water bath prior to the measurements.

2.3. Calcium content and release

10 ± 1 mg of poly(lactic acid) microspheres without or loaded with calcium chloride were completely dissolved in 750 µl DCM under shaking at 80 rpm, for 2 h at 37 °C. To extract the calcium ions, 150 µl Milli-Q water was added to the polymer solution and incubated overnight at 37 °C. The water phase was then collected and analyzed using a calcium assay kit (QuantiChrom^{TM}, BioAssay Systems, USA) to quantify the calcium content of the microspheres. A spectrophotometric plate reader (Thermo Scientific Multiskan GO) was used to read the optical density of calcium complexes at 612 nm.

Simulated physiological solution (SPS) buffered at pH 7.3 (137 mmol/l Na^+, 177 mmol/l Cl^-, 50 mmol/l HEPES in Milli-Q water) was used to study the release profile of calcium ions from the microspheres. 10 ± 1 mg of calcium-incorporated microspheres containing varying concentrations of poly(lactic acid) (4, 6, 8, and 10 % w/v) were precisely weighed and placed in 500 µl tubes. 150 µl of simulated physiological solution was added to each sample. Triplicates of each sample were then placed in a waterbath at 37 °C while shaking at 80 rpm. At dedicated time points between 4 hours to 15 days, three tubes of each condition were removed and centrifuged. The supernatant was collected and calcium content was quantified using the calcium assay kit as was described above.

2.4. In vitro characterization of poly(lactic acid) microspheres

Human mesenchymal stromal cells (hMSCs) were isolated from bone marrow after written consent as described previously [30, 31]. In short, bone marrow aspirates were resuspended in cell culture medium, plated at a density of $5 \cdot 10^5$ cells/cm^2 and cultured in proliferation medium (α-MEM (Gibco) supplemented with 10 % fetal bovine serum (Lonza), 2 mM L-glutamine (Gibco), 0.2 mM ascorbic acid (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) and 1 ng/m rhbFGF (AbDSerotec)). The medium was refreshed every 2–3 days. Cells were harvested at approximately 80 % confluency for subculture until passage 3.

In vitro cell culture experiments were performed using human mesenchymal stromal cells and microspheres containing 8 or 10 % w/v poly(lactic acid) prepared using mechanical emulsification and microspheres containing 4 % w/v poly(lactic acid) prepared using ultrasonic emulsification. Polymeric microspheres without the salt served as controls.

42.5 ± 0.5 mg microspheres were placed in 24-well plates (NUNC) and sterilized using isopropanol (Assink Chemie), followed by a three-hour evaporation step. The samples were subsequently washed twice with sterile phosphate buffered saline (PBS). Sterilized microspheres were then incubated overnight in 1 ml of basic medium (α-MEM (Gibco) supplemented with 10 % fetal bovine serum (Lonza), 2 mM L-glutamine (Gibco), 0.2 mM ascorbic acid (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco)) overnight at 37 °C in a humidified atmosphere with 5 % CO_2.

To study the effects of products released from the microspheres, cell culture was performed using trans-wells (24-well, Corning) with two compartments. The cells were seeded in the bottom compartment of the trans-wells at a density of 20 000 cells/cm^2 and the microspheres were placed in the top compartment. Cells were cultured for 7 days in either basic or osteogenic medium (basic medium supplemented with 10 nM dexamethasone (Sigma)) with refreshment every 2-3 days. Human mesenchymal stromal cells cultured on tissue culture plate, without introducing the microspheres, served as controls. After 7 days, the medium was aspirated, cells were washed with phosphate buffered saline, and DNA content of the cells was quantified using CyQuant Cell Proliferation Assay kit (Invitrogen) according to the manufacturer's protocol. Alkaline phosphatase (ALP) activity, as an early marker of ostegenic differentiation, was assessed and normalized for DNA amounts using CDP-star kit (Roche Applied Science) according to the manufacturer's protocol.

Expression of markers of osteogenesis at the mRNA level was determined using quantitative real-time PCR (QPCR). Total RNA of the cells was isolated by using a combination of TRIzol® (Life Technologies) method and NucleoSpin® RNA isolation and purification kit (Macherey-Nagel) according to the manufacturer's protocol. Total RNA content was then measured using a NanoDrop® ND-1000 spectrophotometer. The cDNA of the cultures were then prepared using iScript kit (Bio-Rad) according to the manufacturer's protocol and diluted 10 times in RNase-free water. The quantitative real-time PCR measurements were performed on a Bio-Rad equipment using SYBR Green I Master Mix (Invitrogen). The primer (Sigma) sequence of the selected genes (bone morphogenetic protein 2, osteopontin and osteocalcin) is listed in Table 1. Expression of all genes was normalized for the levels of the housekeeping gene GAPDH and corrected for gene expression of human mesenchymal stromal cells cultured on tissue culture plas-

Table 1. Primer sequences of osteogenic markers and housekeeping gene used for quantitative real-time PCR.

Gene	Primer sequences
GAPDH (housekeeping gene)	5'-CCATGGTGTCTGAGCGATGT 5'-CGCTCTCTGCTCCTCCTGTT
Bone morphogenetic protein 2 (BMP2)	5'-GCATCTGTTCTCGGAAAACCT 5'-ACTACCAGAAACGAGTGGGAA
Osteocalcin (OC)	5'-CGCCTGGGTCTCTTCACTAC 5'-TGAGAGCCCTCACACTCCTC
Osteopontin (OP)	5'-CCAAGTAAGTCCAACGAAAG 5'-GGTGATGTCCTCGTCTGTA

tic in basic medium. Induction folds of the target genes were calculated using ΔΔCT method.

During cell culture, medium was collected at every medium refreshments (2, 4 and 6 days) and its calcium content was measured using calcium assay kit as described above in order to determine the calcium release profile in cell culture medium.

2.5. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc test with a significance level of $p < 0.05$. For all figures the following applies: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

3.1. Microsphere characterization

Scanning electron microscope imaging (Fig. 1a–f) demonstrated formation of microspheres in all conditions tested. No morphological differences were observed between microspheres with and without calcium salt incorporation. Microspheres containing 4 % w/v poly(lactic acid) prepared by either mechanical or ultrasonic emulsification presented small pores with approximate diameter of 0.5–1.0 μm on the surface, whereas microspheres with higher poly(lactic acid) concentrations showed a smooth surface structure.

Average particle size and size distribution of microspheres (data not shown) with different poly(lactic acid) concentrations (4–10 % w/v), without and with calcium chloride incorporation were analyzed using dynamic light scattering technique. The particles, however, appeared to be polydisperse (which is in accordance with scanning electron microscope results) and the particle size exceeded the maximum measurable size of 2 μm of the available dynamic light scattering equipment. In order to get an approximation of the microsphere size, the average particle size of the microspheres obtained from dynamic light scattering analysis was compared with measurements obtained from scanning electron microscope images. As shown in Fig. 2, poly(lactic acid) microspheres had an average diameter of 4–6 μm. Increasing the polymer concentration led to a slight increase in average microsphere size. No effect of the emulsification technique was observed on the average microsphere diameter, as both mechanically and ultrasonically

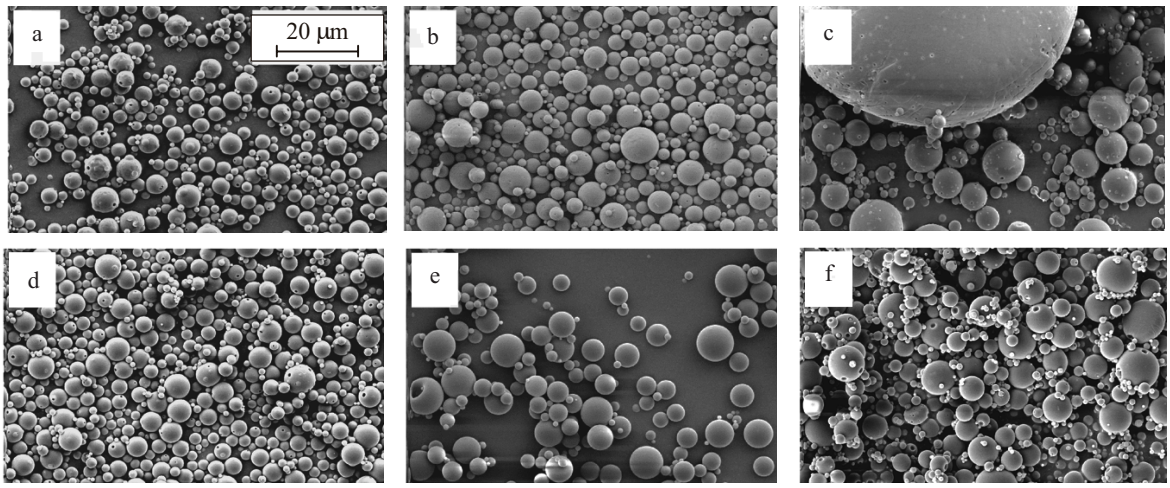

Fig. 1. Microspheres prepared by single emulsion technique and mechanical emulsification PLA 4 % (a), PLA 10 % (b), PLA 4 % + ultrasound (c), PLA 4 % + Ca (d), PLA 10 % + Ca (e), PLA 4 % + Ca + ultrasound (f).

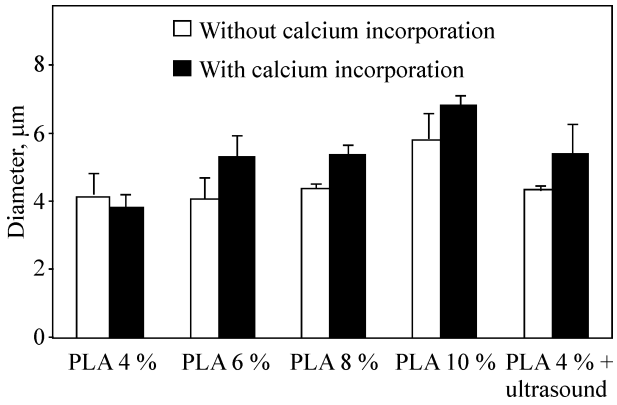

Fig. 2. Average diameter of microspheres prepared with various poly (lactic acid) concentrations and emulsification technique.

prepared microspheres containing 4 % poly(lactic acid) possessed a comparable size.

3.2. Efficiency of calcium incorporation into and release profile from poly(lactic acid) microspheres

The amount of calcium chloride salt incorporated into poly(lactic acid) microspheres was in the range of 0.5 to 2.2 µg/mg of microspheres (Fig. 3a). No calcium was detected in microspheres without salt incorporation. A direct positive correlation between poly(lactic acid) concentration and level of calcium was found in the sample prepared by mechanical emulsification; the higher the poly(lactic acid) content the higher the amount of calcium. Interestingly, method of emulsification had a strong effect of the incorporation efficiency. Calcium content of microspheres containing 4 % w/v poly(lactic acid) prepared using ultrasonic emulsification was more than four times higher than that of microspheres with the same poly(lactic acid) content prepared using mechanical emulsification.

The calcium release profile of microspheres was investigated in simulated physiological solution for 15 days. The results are presented as a ratio between Ca^{2+} released and the total calcium content (M_t/M_∞) (Fig. 3b). A burst release was observed for all samples prepared using mechanical emulsification, with approximately 80 % of the ions released within 4 hours. After 2–4 days the calcium release profile reached a plateau in these conditions, which corresponded to approximately 90 % of the total calcium content. However, the release profile of 4 % poly(lactic acid) microsphere prepared using ultrasonic emulsification showed a more sustained release with approximately 5 % released after 1 day, increasing to approximately 17 % after 10 days in simulated physiological solution.

3.3. In vitro characterization of poly(lactic acid) microspheres

A trans-well cell culture system (Fig. 4a) was used to study the effect of compounds released from the microspheres on the proliferation and osteogenic differentiation of hMSC. As is depicted in Fig. 4b, no significant differences in DNA content were observed among cells cultured with different batches of microspheres, independent of the medium used. Similarly, there were no differences between cells cultured in presence or in absence of the microspheres. Also alkaline phosphatase activity was not significantly different among different conditions; only the osteogenic medium seemed to mildly increase the alkaline phosphatase activity (Fig. 4c).

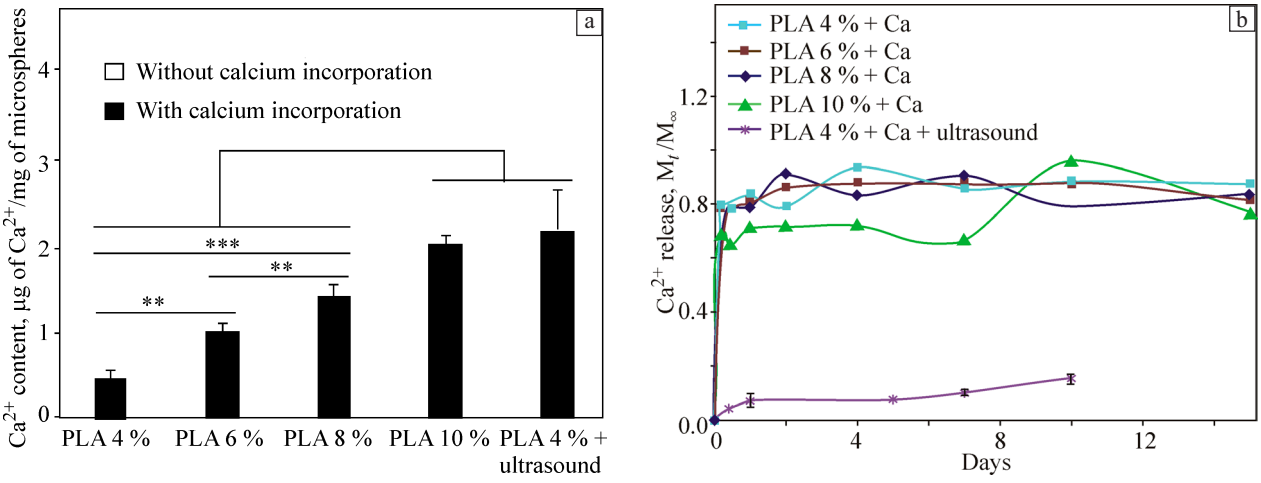

Fig. 3. Calcium content of microspheres prepared with either mechanical or ultrasonic emulsification, and with varying PLA concentrations (a) and calcium release profile of 4–10 % w/v PLA microspheres in simulated physiological solution during 15 days (b). In mechanically emulsified microspheres, increasing PLA concentrations correlated with increased calcium incorporation. Ultrasonic preparation of microspheres with 4 % w/v PLA resulted in the highest calcium content. All mechanically prepared microspheres showed a burst release of approximately 80 % within the first 4 hours while ultrasonically prepared microspheres showed a more sustained release with 17 % calcium release after 10 days of immersion.

Quantitative real-time PCR data showed that both bone morphogenetic protein 2 and osteopontin genes were upregulated in cells cultured in the presence of calcium chloride containing microspheres as compared to their unloaded counterparts and controls, both in basic and in osteogenic medium (Fig. 5a, b). A significant difference was observed in the expression of bone morphogenetic protein 2 when human mesenchymal stromal cells where cultured with calcium containing 10 % w/v poly(lactic acid) microspheres in basic medium compared to other conditions. In osteogenic medium, the expression of bone morphogenetic protein 2 was significantly higher in Ca^{2+} containing 4 and 10 w/v poly(lactic acid) microspheres compared to the control. Ca^{2+} containing 8 and 10 % w/v poly(lactic acid) microspheres resulted in higher expression of osteopontin in basic medium in comparison with non-loaded microspheres (4 and 10 % w/v poly(lactic acid)). Osteopontin gene was also upregulated in osteogenic medium when human mesenchymal stromal cells were cultured with 10 % w/v poly(lactic acid) microspheres compared to non-loaded microspheres (8 and 4 % w/v) and control. In contrast to the expression of bone morphogenetic protein 2 and osteopontin, no significant differences were observed in the expression of osteocalcin among different conditions (Fig. 5c)

The calcium ion release profile of calcium chloride-loaded microspheres in basic culture medium after 2, 4, and 6 days is shown in Fig. 6. α-MEM contains 1.8 mM Ca^{2+} ions and the fetal bovine serum added to the medium also contains additional ions. The level of Ca^{2+} in the medium was measured using the calcium kit and used as the basic Ca^{2+} levels of all the conditions (day 0). After two days of culture in basic medium, Ca^{2+} content of the medium increased by 0.5 mM and 0.2 mM in the 8 and 10 % w/v poly(lactic acid) conditions, respectively. After 4 and 6 days, a slight decrease of approximately 0.2 mM was observed. A continuous increase of the calcium ion concen-

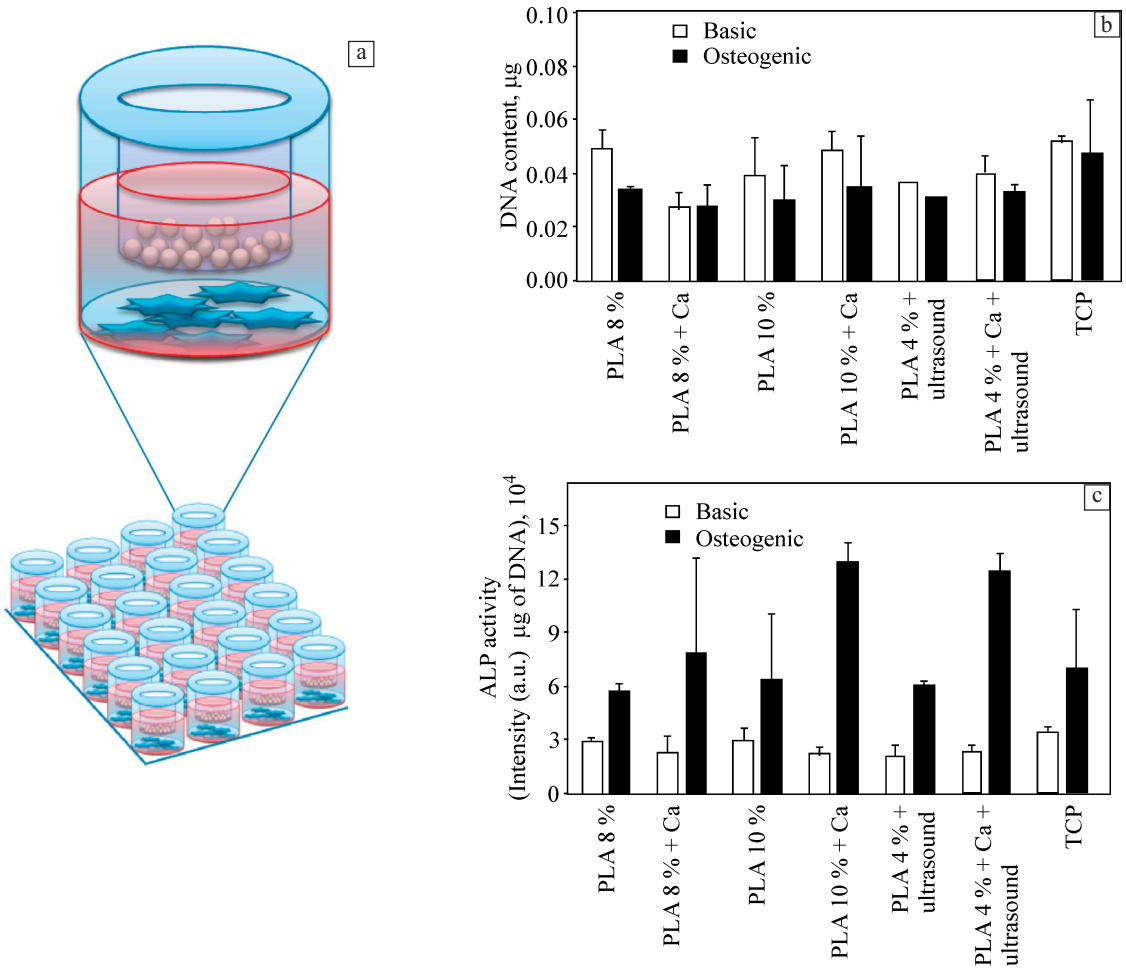

Fig. 4. A schematic of cell culture trans-well set up in which the microspheres and the cells were placed in top and bottom compartments, respectively (a), DNA content (b) and alkaline phosphatase activity normalized for DNA (c) of human mesenchymal stromal cells cultured in presence of microspheres for 7 days. No significant differences were observed in either DNA content or alkaline phosphatase activity among different conditions. TCP—tissue culture plastic.

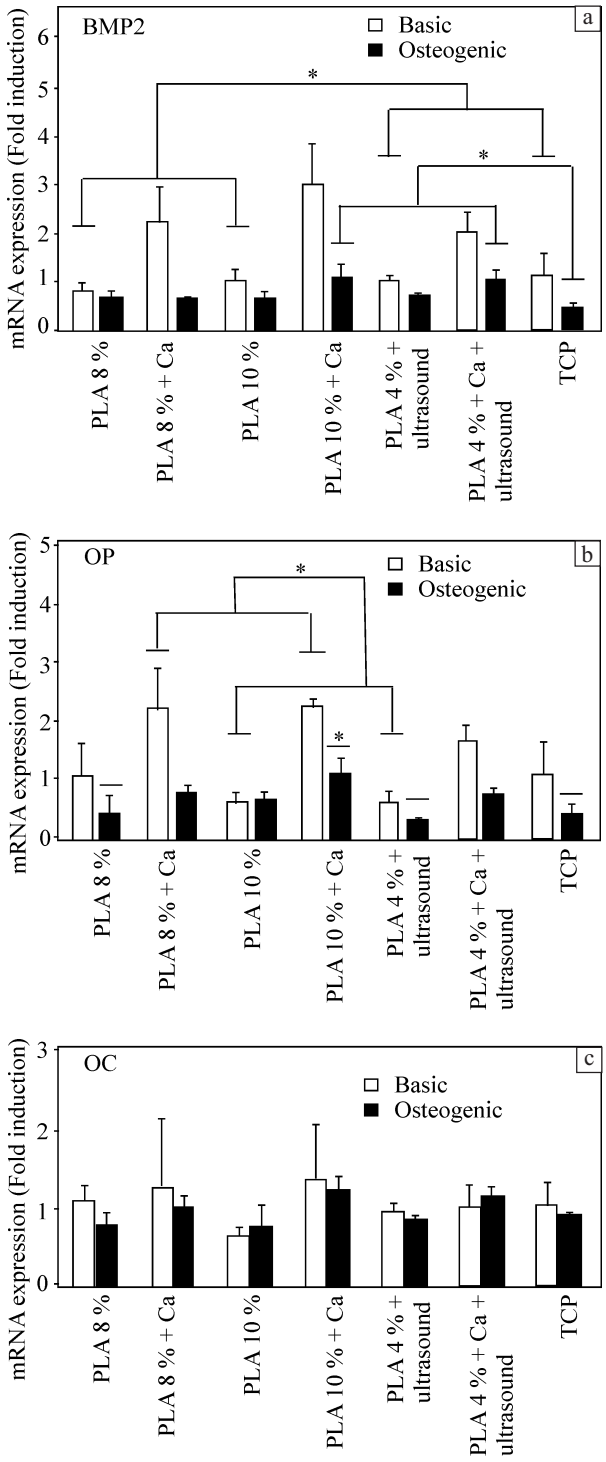

Fig. 5. Expression of bone morphogenetic protein 2 (a), osteopontin (b) and osteocalcin (c) genes by human mesenchymal stromal cells cultured in presence of different microspheres in transwells for 7 days. Higher expression of bone morphogenetic protein 2 and osteopontin was observed in calcium salt-loaded microspheres compared to non-loaded spheres and control in basic culture medium. Expression of osteocalcin gene was similar in all the conditions.

tration of approximately 0.2 mM at each time point was observed for microspheres with 4 % w/v poly(lactic acid) produced using ultrasonic emulsification.

4. Discussion

In this study, we have investigated the possibility of incorporation of bioinorganics into and their release from polymeric microparticles. Microparticles were made from poly(lactic acid), and calcium chloride served as model bioinorganic. Lactic acid, the degradation product of poly(lactic acid), is a natural metabolite and therefore does not elicit toxic effects [25]. Furthermore, due to the synthetic nature of poly(lactic acid), batch-to-batch uniformity of the polymer is high [25]. Calcium was selected as it is one of the constituents of calcium phosphates, a widely used synthetic bone graft substitute. The microsphere based delivery system as developed here would in the first place allow for studying biological effects of a single bioinorganic on cell behavior since they exclusively release the ions incorporated in them. In contrast, current carriers of bioinorganics, including calcium phosphate and bioactive glass, release a cocktail of ions, making analysis of the effect of individual ions difficult, if not impossible.

The surface morphology of microspheres prepared in this study did not vary upon incorporation of calcium salt. In poly(lactic acid) microspheres containing 4 % PLA, a porous surface was observed to be independent of salt incorporation and emulsification technique. Both, spheres with and without salt exhibited a smooth surface structure at higher poly(lactic acid) concentrations. Increasing PLA concentrations from 4 to 10 w/v % increased the average diameter of microspheres from 4 to 6 μm in a relatively linear manner. Nevertheless, the results of scanning electron microscope imaging and dynamic light scattering measure-

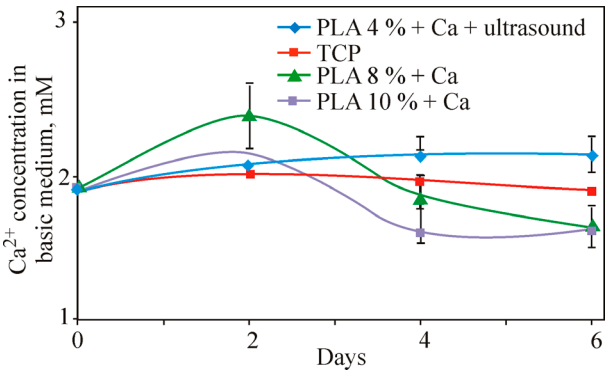

Fig. 6. Calcium levels in basic medium during human mesenchymal stromal cells cultured in the presence of microspheres in transwell inserts. In the presence of calcium salt-loaded microspheres prepared by mechanical homogenization, Ca^{2+} levels were elevated after 2 days, decreasing at the later time points. Ca^{2+} levels of cultures containing microsphere prepared by ultrasound showed a gradual increase over time.

ments showed polydispersity for all the microsphere batches. Controlling the physical properties of microspheres including particle size and surface roughness was not within the scope of this study, but are obviously properties that can be further modified in order to better control the efficiency of incorporation and release profiles.

Various strategies exist for incorporating inorganic materials into polymer matrices. Inorganics-incorporated interpenetrating polymer networks, incorporation of bioinorganic groups via chemical bonds to the polymer backbone and dual inorganic-organic hybrid polymers are examples of these different strategies [32]. However, different physical and chemical polymer-salt interactions are involved in each of these strategies. In the present study, the inorganic salt is expected to be either adsorbed on the surface, or physically embedded into polymer matrix.

Incorporation of 0.5–2.2 μg calcium chloride per mg was achieved in microspheres prepared with 4–10 % w/v poly(lactic acid) and mechanical homogenization. It was shown that the calcium incorporation was directly related to the poly(lactic acid) concentration. Polymer concentration is known to critically influence the inner porosity and consequently the density of microspheres when a single emulsion technique is used for preparation [33], which correlated to the results of the present study where calcium incorporation increased with increasing poly(lactic acid) content. An absence of micro- and nanopores on the surface of microspheres prepared with higher concentrations of poly(lactic acid) is also due to an increase in the density of the polymer matrix. Density and inner pore structure of microspheres are considered important parameters for achieving a higher incorporation efficiency [33, 34]. Indeed, a maximal salt incorporation and release over longer periods is expected to be achieved by dense microspheres with poorly interconnected pores. Other parameters that play a critical role in incorporation of drugs into microspheres are comprehensively studied elsewhere [35–37]. The highest efficiency of calcium incorporation into microspheres achieved in this study was approximately 0.2 wt % for microspheres prepared with 10 % w/v poly(lactic acid) using mechanical emulsification and for microspheres containing 4 % w/v poly(lactic acid) and ultrasonically emulsified. Although these amounts are relatively low, certainly in comparison with calcium levels found in body fluid, they may be sufficient to mimic the amounts of other trace elements in, for example, bone tissue.

Efficiency of incorporation was strongly affected by the method of emulsification, as was demonstrated by microspheres containing 4 % of poly(lactic acid), which were produced using either mechanical or ultrasonic emulsification methods. Calcium content of 4 % w/v poly(lactic acid) microspheres was approximately four times higher when emulsification was achieved by sonication compared to mechanical homogenization. The effects of different emulsification techniques on incorporation of bovine serum albumin (BSA) in poly(lactic-co-glycolic acid) (PLGA) matrix using a double emulsion technique was previously studied [38], however, no significant differences between different techniques were observed. The fact that bovine serum albumin molecules are much larger compared to calcium chloride salt used here, possibly resulting in a higher viscosity of the bovine serum albumin–polymer mixture, may explain opposing observations in the two studies. An increase in viscosity may limit the shear forces applied to the emulsion in the homogenization step.

Calcium-containing microspheres emulsified mechanically showed a burst Ca^{2+} release with approximately 80 wt % of total calcium content released after 4 h of immersion in simulated physiological solution, independent of the initial poly(lactic acid) concentration. However, microspheres prepared by probe sonication showed a Ca^{2+} release of only 5 wt % of the total amount within the first day, increasing to 17 % after 10 days. Comparable results regarding release of bovine serum albumin were observed in an earlier study [34], where the mechanically homogenized samples released approximately 60 % w/v bovine serum albumin within 24 h, an amount that was decreased to 14 % w/v when sonication of the first emulsion was used [38].

Different effects of processing technique including sonication and mechanical homogenization on microsphere characteristics observed here can be explained by differences in efficiency of homogenization, stability of the formed emulsion and polymer density. Microsphere preparation using emulsification techniques is mainly a two-step process [29]. In the first step the polymer solution is dispersed into microdroplets by either mechanically or ultrasonically applied shear stress. The second step is characterized by hardening of the microspheres through evaporation of the solvent and precipitation of the polymer. Homogenization by probe sonication is more effective in microdroplet formation which consequently leads to a higher compound distribution throughout the microsphere [38]. The process of solvent elimination and shrinkage of the microsphere critically determines the morphology, compound incorporation and release profile. A homogenous compound distribution results in less salt leaching during harvesting and washing steps of microspheres [29, 38], resulting in a higher inorganic content.

Emulsions prepared by probe sonication are more stable than mechanically homogenized or magnetically stirred ones, as was previously observed [38]. It is critical to limit phase separation of microdroplets after the emulsification step. This phase separation may occur during the time between homogenization of the sample and pouring and diluting the emulsion in poly(vinyl alcohol). Separation of microdroplets in the emulsion decreases the efficiency of mechanical homogenization even more, and consequently the compound distribution in the spheres becomes less homogeneous [38]. Similar to polymer concentration, the emulsification technique can influence the density of the

formed microspheres based on the shear forces employed to the bioinorganic-polymer mixture.

Based on the facts mentioned above, adsorption of bioinorganics on the polymer surface is expected to be dominant polymer-bioinorganic interaction when mechanical emulsification is employed. In the case of sonication, the homogenous and stable salt distribution into poly(lactic acid) may result in improved embedding of the bioinorganic into the bulk of the microsphere, leading to a more sustained release of the ions which is controlled by the degradation of the polymer.

The *in vitro* cell culture study was used to investigate whether the delivery system presented indeed can be used to study the biological effects of a sole ion. Ca^{2+} ions were selected as one of the constituents of calcium phosphate ceramics, and for their earlier demonstrated effects on the osteogenic differentiation of human mesenchymal stromal cells [13, 39]. A trans-well system was selected to study the effect of the released ions, independent of the physical contact with the microspheres. Microspheres with the highest calcium content were selected for cell culture experiments.

DNA quantification after 7 days of culture revealed no effect of microspheres, with or without calcium, on cell proliferation. The calcium concentration of the cell culture medium for calcium-loaded microspheres prepared using mechanical homogenization was approximately 2.0–2.4 mM after 2 days, which is in accordance with the burst release observed upon immersion of microspheres in simulated physiological solution. At later time points, a plateau was reached at approximately 1.5 mM. The slight decrease in Ca^{2+} level in cell medium at 4 and 6 days is plausibly due to (local) supersaturation of the medium with calcium, that may lead to precipitation of, for example a calcium-phosphate phase on the surface of microspheres, as was previously observed by Barradas et al. [40]. The ultrasonically emulsified samples showed a gradual release of Ca^{2+} from polymer matrix, similar to the observation upon immersion in simulated physiological solution. It was previously shown that a Ca^{2+} concentration of 7.8 mM led to a significantly higher cell proliferation [39], which was not observed in the present study, plausibly because of the lower calcium ion levels in the medium. Quantification of alkaline phosphatase activity did not show significant effects of any of the conditions tested, which is in accordance with an earlier study suggesting that calcium is not involved in the pathway that regulates alkaline phosphatase gene expression and protein activity [39].

In an earlier study using human mesenchymal stromal cells, in which standard cell culture was performed with medium containing elevated Ca^{2+} levels (7.8 mM), increased levels of bone morphogenetic protein 2, osteopontin and osteocalcin were observed as compared to control medium condition [39]. Human mesenchymal stromal cells cultured in the presence of calcium-containing microspheres generally showed an upregulation of bone morphogenetic protein 2 and osteopontin in the present study, in comparison to microspheres without the salt. This was not the case for osteocalcin, which could be explained by the fact that osteocalcin is a late osteogenic marker [41], possibly not yet expressed after 7 days of culture.

It should be emphasized that this study was meant as the development step of a system to study the effects of individual ions on cell behavior. Additional experiments, with microspheres offering distinct release profiles, and extended cell culture study over a longer time period, and including other assays of proliferation, differentiation and mineralization are required to obtain a complete picture of the effects of calcium ions released from poly(lactic acid) microspheres.

5. Conclusion

The results of the present study demonstrated that the bioinorganics-containing polymeric microspheres can be used for studying biological effects of individual ions. In conventional cell culture setups using ceramics or bioglass, a combination of a number of parameters, including chemical and topographical features is studied simultaneously, making it difficult to study the effect of a single parameter. However, a combination of bioinorganics-containing polymer microspheres and a trans-well cell culture set up offers a platform in which studying the chemical effects of a single bioinorganic independent of other physical and chemical parameters is possible. Further optimization of the system is needed in terms of incorporation efficiency and release profile, as well as validation of the platform using other bioinorganics.

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