J Mater Sci: Mater Med (2009) 20:1357–1365
DOI 10.1007/s10856-008-3678-9

Stability of prostaglandin E_2 (PGE_2) embedded in poly-D,L-lactide-co-glycolide microspheres: a pre-conditioning approach for tissue engineering applications

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Received: 25 November 2008 / Accepted: 16 December 2008 / Published online: 22 January 2009

Abstract Prostaglandin E_2 (PGE_2) is involved in angiogenesis, bone repair and cartilage metabolism. Thus, PGE_2 might represent a suitable signaling molecule in different tissue engineering applications. PGE_2 also has a short half-life time. Its incorporation into poly-D,L-lactide-co-glycolide (PLGA) microspheres was demonstrated in a previous study. However, the stability of bioactive PGE_2 in these microspheres is unknown. With an adjusted mass spectrometry assay we investigated the amount of incorporated PGE_2 and the stability of PGE_2 in conventional cell culture medium and in PLGA microspheres. The stability of PGE_2 was closely pH dependent. Strong acidic or basic environments reduced the half-life from 300 h (pH 2.6–4.0) to below 50 h at pH 2.0 or pH 8.8. The half-life of PGE_2 incorporated into poly-D,L-lactide-co-glycolide increased drastically to 70 days at 37°C and to 300 days at 8°C. Analysis with scanning electron microscopy (SEM) and atomic force microscopy (AFM) demonstrated a distinct nanostructure of the polymeric phase and both nano- and microporosity.

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1 Introduction

Tissue engineering has become an important tool in the field of regenerative medicine, and is mainly based on three fundamental approaches. The first approach basically uses autologous cells for integration into a defect site. As a second approach, synthetic materials, so called scaffolds, are used, giving cells and regenerating tissue a preformed structure to allow the repair of critical size defects. Third, growth factors or signaling molecules are used to both modulate the biological performance of the tissue engineered construct and to stimulate the tissue response towards influencing the cellular response of the seeded cells with a view to proliferation and differentiation, but also to enable migration of cells into the repair site [1].

Growth factors and signaling molecules also have an increasingly important role in the next generation of tissue engineering, in which the functionalization of scaffold materials is expected to improve the quality and long-term performance of the regenerated tissue, e.g., by maintaining cellular differentiation and, thus, optimal tissue functions. Taken together, signaling molecules and growth factors are indispensable to implement biomimetic principles in the practice of tissue engineering for medical applications.

A defining question regarding the use of signaling molecules is whether to bind them chemically to a biomaterial or whether to deliver them in unbound form within a carrier material, which itself can further serve as a supporting structure for prior in vitro cell expansion and in vivo tissue regeneration. For this purpose, carrier systems with reliable biocompatibility and controllable release profiles are needed. In this respect, aliphatic polyesters of the poly (α-hydroxy)acids, especially poly-L-lactides (PLA) and polyglycolides (PGA) or their respective copolymers poly-L-lactide-co-glycolides (PLGA) represent well-established

and versatile materials for drug delivery and tissue engineering applications [2, 3]. Furthermore, these polymers are well established as implant materials and are approved by the U.S. Food and Drug Administration [4, 5]. Since their initial use as resorbable suture materials [6] the biocompatibility of PGA/PLA materials is well documented by numerous in vivo studies, especially in orthopaedic applications [7–10].

In the search for growth factors or signaling molecules that can act as tools for tissue regeneration it is helpful to focus on molecules that are involved in developmental processes, as these are often recapitulated in part during regeneration [11, 12]. Especially several transcription factors and members of the TGF-beta superfamily have been intensively investigated [13, 14].

Another promising group of molecules, which has not yet received much attention as a potential tool for tissue engineering applications, are substances known from the arachidonic acid metabolic pathway [15], mainly prostaglandin E_2 (PGE_2). There is an increasing body of evidence based on in vitro experiments and animal studies that implies an important role of PGE_2 in developmental processes and tissue regeneration [16, 17].

Regarding the role of prostaglandins in bone repair Li and coworkers demonstrated the functional role of prostaglandin receptors in osteoblasts [18]. Other studies with COX-inhibitors and COX knock-out mice demonstrated the osteoinductive effect of PGE_2 [19, 20], indicating its critical role in the orchestration of fracture healing. Consequently, PGE_2 has been recognized as an important target molecule in tissue engineering solutions for bone and cartilage regeneration [21]. In contrast to signaling molecules from the TGF-β superfamily, PGE_2 and prostaglandin receptor agonists are also available in pharmaceutical quality, but they are much less expensive than the relatively new TGF-β superfamily derived pharmaceuticals, from which only BMP-2 and BMP-7 are approved by the FDA. This makes PGE_2 even more interesting and potentially valuable for tissue engineering applications.

The limiting factor for the use of PGE_2 as a signaling molecule is its rapid turn-over under physiological conditions. The half-life of PGE_2 is approximately 30 s in the cardiovascular system and 35 min in amniotic fluid [22, 23]. Therefore, initial immobilization in a carrier system with the possibility of controlled PGE_2 release is required in order to achieve a sustained effect of prostaglandin in both in vivo and in vitro systems.

In previous studies we synthesized poly-D,L-lactide-co-glycolide microspheres, on which porcine chondrocytes were successfully cultivated [24]. Furthermore, we successfully incorporated PGE_2 into these microspheres and demonstrated a prolonged release of this molecule out of the microspheres [15]. However, for further investigations and possible clinical applications it is indispensable to analyze the stability of PGE_2 in its immobilized and, thus, mostly oxygen-, enzyme-, and solvent-free state. Furthermore, based on results from electron microscopy of degraded microspheres, a possible diffusion of water into the core of the microspheres could not be excluded [24]. Degradation of PLGA type polymers is usually associated with hydrolytic polymer chain cleavage. The ultimate degradation products are the monomers lactic acid and glycolic acid which are eliminated in vivo in the tricarboxylic acid cycle. Hence, water diffusing into these polymers initiates auto-catalytic cleavage of the co-polymer. Presumed osmotic effects might then accelerate internal hydrolytic degradation and osmotic pressure. As a consequence, immobilized PGE_2 would be exposed to increasingly acidic conditions.

Based on these considerations the stability of PGE_2 encapsulated in dry, solid poly-D,L-lactide-co-glycolide microspheres was analyzed under different thermal conditions and under increasing lactic acid concentrations with pH values of 8.8 (cell buffer) to 2.0 (1 M lactic acid).

2 Materials and methods

2.1 Chemicals

Prostaglandin E_2 (≥98%) and deuterated d_4-prostaglandin E_2 (≥98%) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Ethyl acetate (HPLC grade) was purchased from Promochem GmbH (Düren, Germany), hexane (HPLC grade) from Riedel-de Haën AG (Seelze, Germany). Methanol and acetonitrile of HPLC grade, acetone and diisopropyl ether (DIPE) of analytical grade were obtained from Merck (Darmstadt, Germany), N,N-diisopropylethylamine (DIPEA; sequanal grade) from Pierce (Oud Beijerland, Netherlands) and pentafluorobenzyl bromide from Lancaster (Eastgate, UK). Bis(trimethylsilyl) trifluoroacetamide (BSTFA) was purchased from Macherey and Nagel (Düren, Germany). Nitrogen, helium (99.996%) and methane (99.999%) were purchased from Messer Griesheim (Herborn, Germany). All other chemicals were of analytical grade. Silica TLC plates (LK6D, 5 × 20 cm) were obtained from Whatman (Maidstone, UK). PLGA for microsphere synthesis was received as gift from Boehringer Ingelheim (Germany; Resomer RG859SR).

As media Dulbecco's Modified Eagle's Medium was used both without any supplements and with supplement of fetal calf serum (10%), penicillin/streptomycin (10.000 units) and GI.

A 1 M lactic acid stock solution was diluted to obtain six different working solutions with concentrations in the range of 0–100 mM for measurements in cell culture media

and another set of six working solutions with concentrations in the range of 0–1 M for water-based experiments.

2.2 Apparatus

GC-MS analysis was carried out on a Finnigan MAT TSQ700 mass spectrometer equipped with a Varian 3400 gas chromatograph and a CTC A200S autosampler (Finnigan MAT, Bremen, Germany). The LC-MS analysis was carried out on a Applied Biosystem API3000 mass spectrometer equipped with two PE Series 200 liquid chromatography micro pumps (PerkinElmer, Waltham, MA, USA) and a CTC HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland).

SEM analysis was carried out using Philips XL 20 scanning electron microscope. AFM analysis was carried out using a Nanosurf easyScan 2 AFM system with non contact measurement mode cantilever.

2.3 Preparation of microspheres and incorporation of PGE_2

Microspheres were produced by dissolving 2 g PLGA (85:15 lactide–glycolide ratio) in 30 ml chloroform. Immobilization of PGE_2 and the desired PGE_2 content was adapted from a previous protocol [15] but instead of 1,1,1,3,3,3-hexafluoro-2-propanol as solvent for PGE_2, ethyl acetate was used. Briefly, 1 mg PGE_2 was dissolved in 1 ml ethyl acetate. From this stock solution, 70 µl were added to the above described solution of 2 g PLGA in 30 ml chloroform, resulting in a concentration of 35 µg/g PGE_2 in PLGA.

This mixture was added dropwise to a stirring 400 ml 0.5% polyvinyl alcohol solution (PVA, Fluka, Germany) as emulsifier in an oil-in-water setup, which was stirred overnight to allow for complete evaporation of the organic solvents.

The synthesized PGE_2-containing microspheres were washed three times with sterile deionized water to remove residual PVA, and were finally sterilized by UV irradiation for 20 min. The washing procedure resulted in a mean material loss of 8%.

2.4 Characterization of microspheres using SEM and AFM

SEM and AFM investigations were carried out on PGE_2 containing microspheres in their state after synthesis. Some microspheres were distributed on a graphite self-adhesive pad and mounted on a SEM sample holder while another set of microspheres were embedded in Resin (Epofix, Struers, Germany) and fine polished using diamond paste (1 µm, DP paste, Struers, Germany) after resin hardening, ensuring their cross-sectioning.

Initially, AFM analysis was performed on both surfaces (spherical outer surface and polished cross-sectioned inner surface) in non-contact mode with 3 s per line scan time on a 512 × 512 scan grid. Scan dimensions were set to either 67.2, 60 or 5 µm. Scan tilting was corrected to allow for mostly planar scanning.

After AFM measurements, both samples were gold sputter-coated in argon atmosphere at 1.0×10^{-2} mbar (180 s, 40 µA) using a Balzers SCD050 sputter coater previous to SEM analyzes.

2.5 PGE_2 determination in solid microspheres—sample preparation

Based on a previously published GC-MS/MS method for prostaglandin detection, a novel sample preparation procedure was developed to determine the incorporated PGE_2 amount in solid microspheres [25]. The optimized and finally chosen method was as follows: Aliquots of 5 mg microspheres were weighed before sample preparation. After adding of 10 µl of a deuterated internal standard (10 ng d_4-PGE_2) and 100 µl acetonitrile the samples were stored at 8°C overnight to allow the swelling of the PLGA and equilibration of the incorporated PGE_2 with the added internal standard.

Under vigorous shaking, a solution of 0.1 g methoxyamine hydrochloride in 1.5 M sodium acetate (pH 5.0) was added to form prostaglandin-methoxyamines and precipitate the main fraction of the dissolved PLGA polymer.

The polymer precipitates were separated via centrifugation and the supernatant was acidified with 260 µl of formic acid (50%, *v/v*) to pH 2.6 and extracted with a mixture of ethyl acetate and hexane (7:3, *v/v*). After evaporation the residues were esterified with a reaction mixture of acetone (80 µl), diisopropylethylamine (7 µl), and pentafluorobenzyl bromide (6 µl). The reaction was completed after 10 min at 40°C. The dried samples were applied to a thin layer chromatography plate, developed in a mixture of ethyl acetate and hexane (9:1, *v/v*). Finally the correspondent zone was scraped out (Rf: 0.17–0.39) and extracted with HPLC grade ethyl acetate. The extracts were evaporated and derivatized twice with 10 µl bis-trimethylsilyl-trifluoroacetamide (BSTFA), each at 60°C. These samples were analyzed using GC-MS/MS.

2.6 Stability analysis of PGE_2 in solution

Stability determination of PGE_2 in two different cell culture media (one with and one without any supplements), as well as in water with different concentrations of lactic acid was performed as follows:

To 1 ml of the samples (cell culture media or water) 100 ng PGE_2 and ascending concentrations of lactic acid

(0.001, 0.1, 10, 100, 1,000 mM) were added. Samples without added lactic acid served as control. Samples were incubated at 37°C and protected from light at all times at least for a period of 2 days. At defined time points, given as 0.25, 0.5, 1, 2, 8, 24 and 48 h, 100 µl aliquots of each solution were taken and frozen at −80°C. For a reference time point aliquots were taken immediately after adding lactic acid.

The frozen samples were brought to room temperature. 10 ng deuterated internal standard were added to each sample aliquot. After equilibration the samples were acidified with formic acid (2.5%, *v/v*) to pH 2.6 and extracted with a mixture of ethyl acetate and hexane (7/3, *v/v*). The extracts were dried under a gentle stream of nitrogen. After complete drying, the samples were dissolved in acetonitrile (20 µl), diluted in water (80 µl) and finally analyzed using LC-MS/MS methodology.

2.7 Stability analysis of PGE_2 in solid microspheres

Stability determination of PGE_2 in solid microspheres under different storage conditions was performed as follows: defined amounts of the solid microspheres (5 mg) containing about 10 ng PGE_2 were stored at 8°C and at 37°C using a heating block. Both experiments were carried out under exclusion of light and moisture. The samples were triplicated and frozen at −80°C at defined time points, given as 1, 2, 3, 6, 10, 17, 34 and 62 days. An aliquot was analyzed before temperature treatment as a reference.

The frozen aliquoted microsphere batches were thawed shortly before sample preparation. After addition of 10 µl deuterated internal standard (10 ng d_4-PGE_2) and 100 µl acetonitrile the samples were treated equally as described in Sect. 2.6. Finally these samples were analyzed with GC-MS/MS as well.

2.8 Determination of PGE_2 via GC-MS/MS

A 1.0 µl aliquot of the BSTFA solution was injected into the Finnigan MAT TSQ700 gas chromatograph/tandem mass spectrometer. Gas chromatography of prostaglandin derivatives was carried out on a J&W DB-1 capillary column (15 m, 0.25 mm inner diameter, 0.25 µm film thickness; Mascom, Bremen, Germany). An initial temperature of 100°C was held for 2 min, and then increased at 30°/min to 280°C and at 5°/min to 310°C. This temperature was held for 2 min. Monitoring and quantification of PGE_2 takes place in the negative ion chemical ionization (NICI) mode. Mass spectrometer conditions were: interface temperature 300°C, source temperature 130°C, methane CI gas pressure 50 Pa, argon collisions cell pressure 0.2 Pa, electron energy 70 eV, emission current 0.4 mA and electron multiplier 1,600 V. Precursor ions were $[M\text{-}PFB]^-$ ($[P]^-$) molecules (*m/z* 524 for PGE_2 and *m/z* 528 for IS), product ions were $[P\text{-}2(CH_3)_3SiOH]^-$ (*m/z* 344 for PGE_2 and *m/z* 348 for the internal standard).

Table 1 Used LC-gradient with solvent A: water:acetonitrile (95:5) and solvent B: acetonitrile:water (95:5)

Step	Total time (min)	Flow rate (µl/min)	A (%)	B (%)
0	0.1	200.0	20.0	80.0
1	2.0	200.0	30.0	70.0
2	10.0	200.0	50.0	50.0
3	10.1	200.0	100.0	00.0
4	11.0	200.0	20.0	80.0
5	12.0	200.0	20.0	80.0

Both eluents containing 0.2% acetic acid

2.9 Determination of PGE_2 via LC-MS/MS

A 10 µl aliquot of the sample solution was injected into the Applied Biosystem API3000 liquid chromatograph/tandem mass spectrometer equipped with two PE200 Micropumps (Perkin Elmer), a CTC HTS PAL autosampler (CTC Analytics, Zingen, Switzerland) and a turbo-ion interface. A generic LC gradient of 12 min was used for sample separation (Table 1). Mass spectrometer conditions were: source type turbo spray, interface HSID (Ionics Mass Spectrom. Group Inc.; GSG Mess- und Analysengeräte GmbH), software Analyst 1.2, scan type MRM with negative polarity, ionspray voltage −4,200 V, temperature 400°C, collision gas 4 psi, potentials: declustering −10 V, focusing −15 V, entrance −9 V and exit −12 V, collision energy −25 eV, Q1 masses $[M\text{-}H]^-$ 351.3 (PGE_2) and 355.3 (d_4-PGE_2), Q1 masses: 271.1 (PGE_2) and 275.1 (d_4-PGE_2), dwell time 300 ms each.

3 Results

3.1 Incorporated PGE_2 concentrations in the microspheres

Prostaglandin E_2 levels of microspheres synthesized with water, chloroform, and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) as solvents, could be determined with 0.213 ± 0.03 µg/g poly-D,L-lactide-co-glycolide ($n = 4$) and were in line with our first experiments [15]. Using the alternative binary emulsion system of microsphere synthesized using ethyl acetate, PGE_2 concentrations of 1.67 ± 0.07 µg/g PLGA were measured ($n = 8$), which

represents an eightfold increase of the bioactive PGE_2 compared to the former co-solvent HFIP.

3.2 Microsphere morphology

PGE_2 containing microspheres were characterized for structural features using AFM and SEM. Figure 1a shows the superimposed AFM topography of a medium sized microsphere projected onto the corresponding SEM image. Figure 1b shows the flattened (derived data mode) surface in higher resolution, demonstrating a distinct microporosity which is apparent for most microspheres (Fig. 1c and d). Higher magnification of the surface structure further reveals a high degree of nano porosity.

Similar results are also characteristic for microspheres in cross section as well: Fig. 2a and c clearly show a homogeneous microporosity in SEM which can also be identified in AFM imaging (Fig. 2b). Interestingly, the cross-sectioned microspheres further show a nanoporosity with some secondary phase particles embedded inside the primary microsphere (Fig. 2d). On the nanoscale (Fig. 3), the polymer structure is apparently made up of very small (200–400 nm) sized particles, which are apparent on both the surface and inside the microspheres. In the 2D imaging, derived data mode, equally spaced lines are slightly visible which we interpret as vibration artifacts probably due to a non optimal damping of the AFM apparatus.

3.3 Stability of PGE_2 in different solvents

3.3.1 Stability of PGE_2 in water at 37°C

Determination of PGE_2 in pure water revealed at the beginning an evident dispersing problem and resulted in scattered low values, which could be solved by vigorously mixing the samples. After 8 h in ultrapure water, a concentration of 89.4 ng PGE_2 per sample was detected, followed by 85.0 ng/sample after 24 h and 81.5 ng/sample ($n = 3$) after 48 h. Supplementation with 1 M lactic acid gave 93.8 ng PGE_2 per sample after 8 h, 67.4 ng/sample after 24 h and 49.5 ng/sample ($n = 1$) after 48 h.

From these values a calculated half life of PGE_2 in the presence of 1 M lactic acid at 37°C could be estimated to be 44 h. By contrast, in the presence of 0–0.1 M lactic acid, PGE_2 had a half-life of about 300 h.

3.3.2 Stability of PGE_2 in cell culture media at 37°C

Cell culture media supplemented with PGE_2 and increasing lactic acid concentrations were analyzed initially (0 h), as well as after 0.25, 0.5, 1, 2, 8, 24 and 48 h at 37°C. The PGE_2 levels decreased with increasing time and increasing lactic acid concentration. The results were also independent of further addition of 10% FCS, P/S, and GI to the culture media. Figure 4 shows exemplarily the results of the FCS, P/S and GI supplemented culture media. The

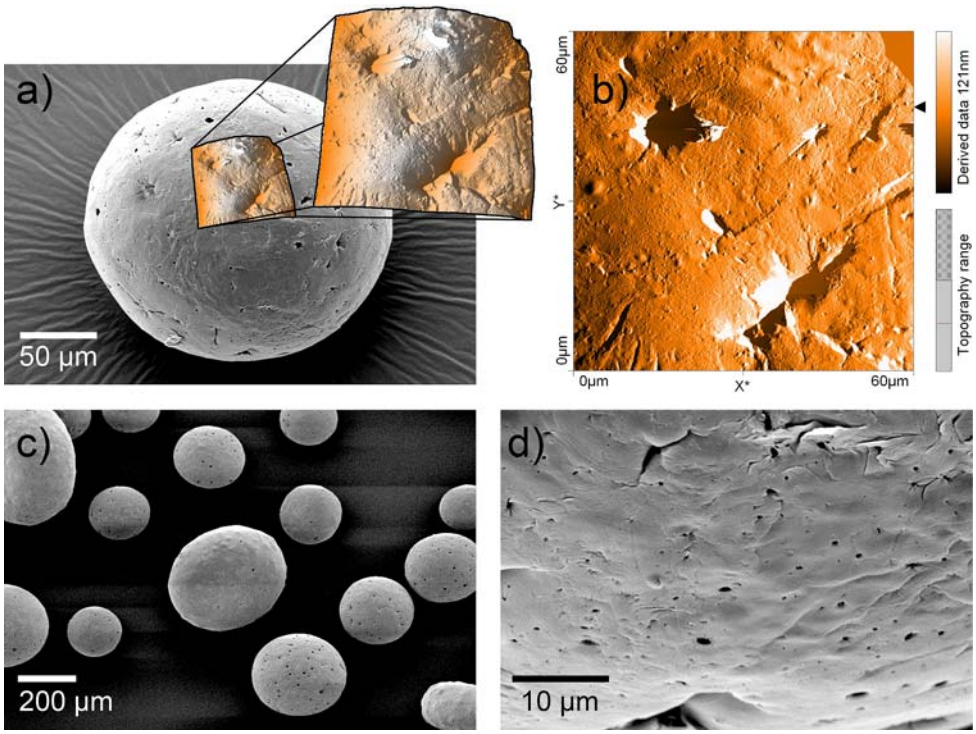

Fig. 1 SEM and AFM characterizations of PGE_2 containing microspheres. **a** SEM image of a medium sized microsphere with superimposed AFM surface scanning. **b** AFM topographic scan as shown in (**a**) in "derived data" mode. **c** Overview of multiple microspheres with diameters in the range of 200–500 µm. **d** Higher resolution SEM imaging showing a distinct micro porosity

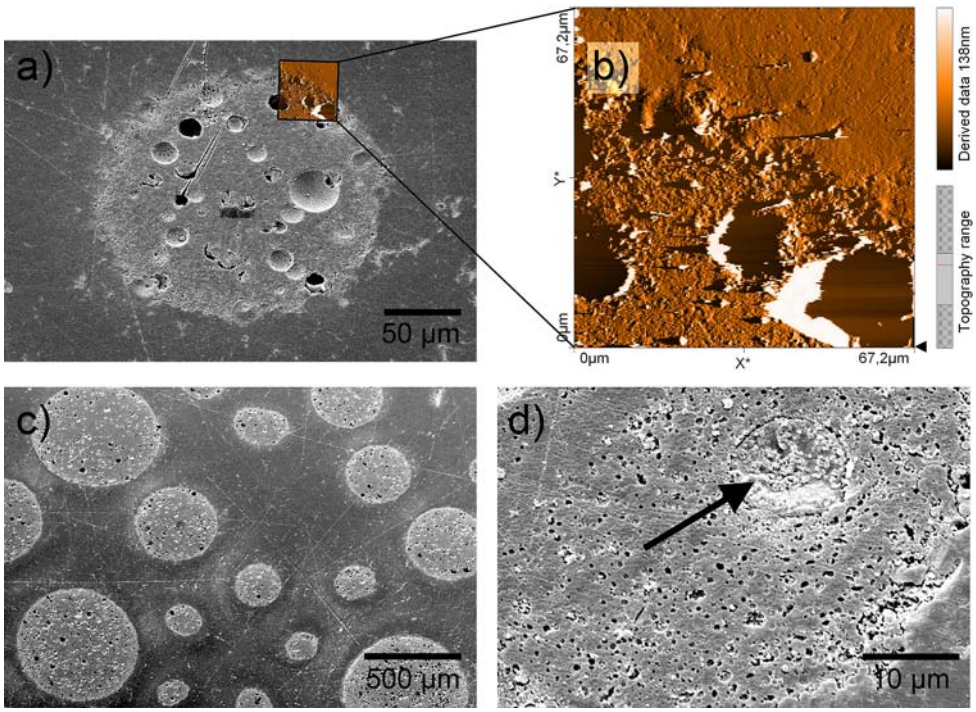

Fig. 2 SEM and AFM characterizations of PGE_2 containing microspheres in cross section (embedded and polished in resin). **a** SEM image of a medium sized microsphere with superimposed AFM surface scanning showing distinct microporosity. **b** AFM topographic scan as shown in (**a**) in "derived data" mode. **c** Overview of multiple microspheres with diameters in the range of 200–500 µm. **d** Higher resolution SEM imaging showing a distinct nano porosity and a secondary phase globular particle (arrow)

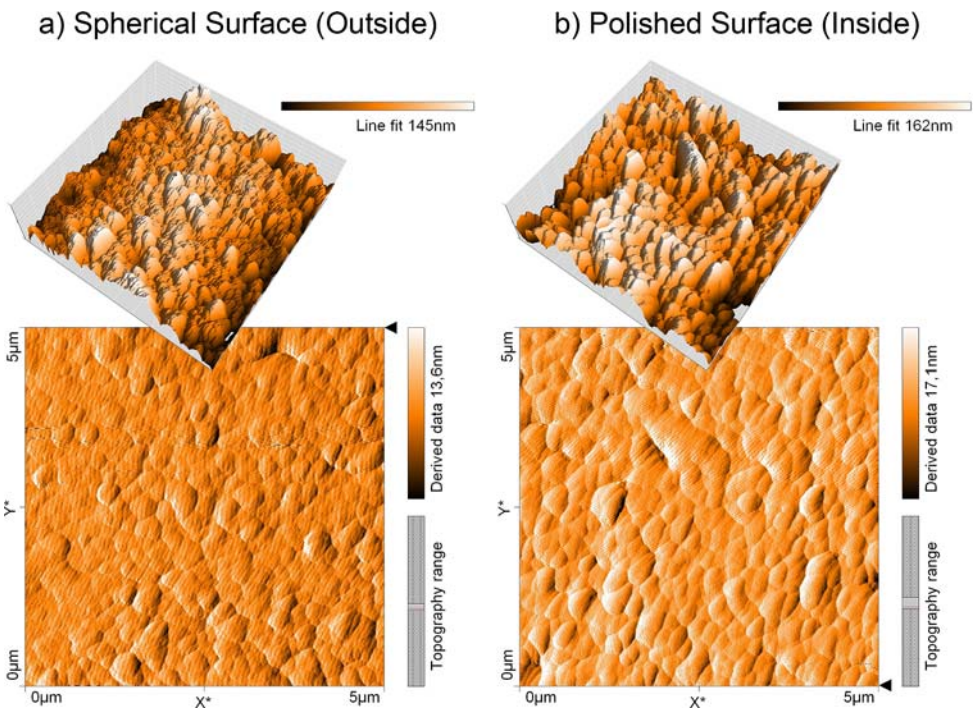

Fig. 3 Comparison of the superficial (**a**) and internal (**b**) nanotopography by AFM analysis. Both surfaces show a polymer structure made up of approximately 200–400 nm particles

calculated half-lives of PGE_2 in these media were 102 h for 0.1 M lactic acid (pH 3.2) and 30 h for 0–0.01 M (pH 8.5–8.7), respectively. This set of graphs demonstrates that the concentration of lactic acid is not the main factor determining stability of prostaglandin E_2 in solution, but the resulting pH-value in the used buffer system. The used culture media were buffered to a slightly alkaline pH (pH 8.8), and minor lactic acid concentrations did not change the pH to a sufficiently high acidic value to result in a significant decomposition of PGE_2.

Table 2 shows the calculated and approximated PGE_2 half-lifes of the different solutions. The PGE_2-stability as a function of the measured pH-values is summarized in Fig. 5.

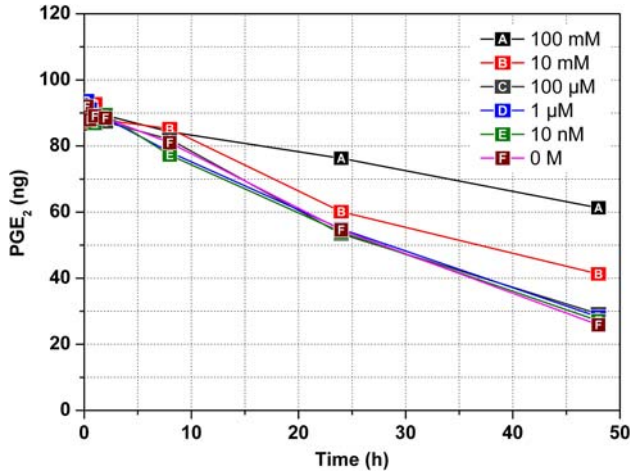

Fig. 4 Time dependent PGE_2 levels in standard cell culture media (plus FCS, P/S and GI) under increasing lactic acid concentrations (0–0.1 M) at 37°C ($n = 1$)

Table 2 Calculated and pH-dependent half-lives of the measured test solutions

Lactic acid concentration	pH-value	Half-life period	Solvent
Saturated solution	1.0	Not measured	Water
1 M	2.0	44 h	Water
0–0.1 M	2.6–4.4	300 h	Water
0.1 M	3.2	102 h	Cell media I
0–0.01 M	8.5–8.7	30 h	Cell media I
0.1 M	3.2	86 h	Cell media II
0.01 M	8.4	39 h	Cell media II
0–0.001 M	8.7–8.8	26 h	Cell media II

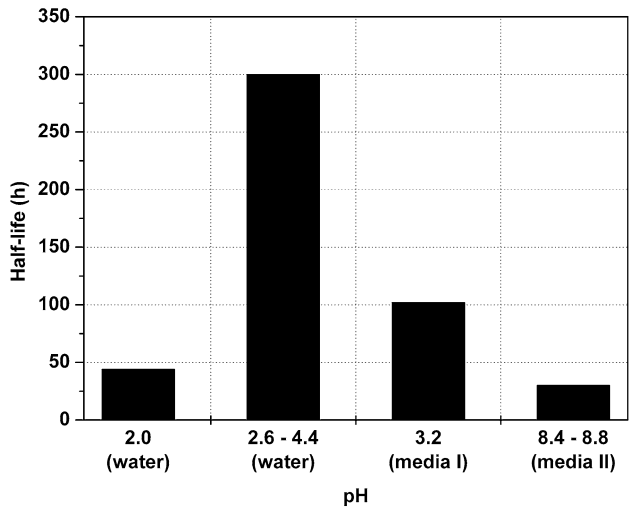

Fig. 5 Calculated half-life of PGE_2 in water and cell culture media (±FCS, P/S and GI) under increasing pH values at 37°C

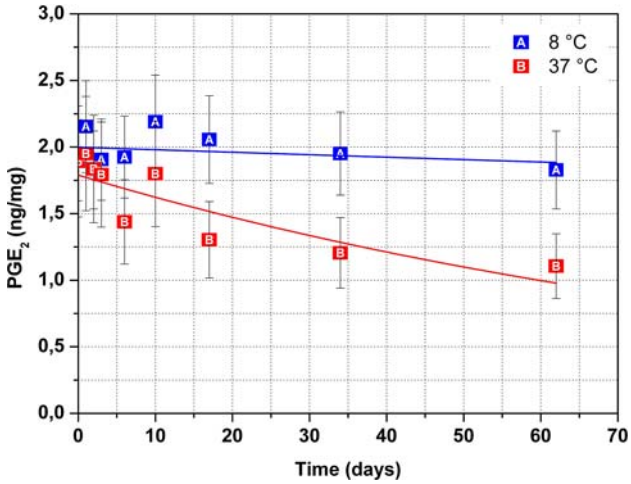

Fig. 6 Stability of PGE_2 in dry solid PGLA polymer matrix at a storage temperature at 8°C ($n = 3$, A) and at 37°C ($n = 3$, B)

3.4 Stability of PGE_2 in solid microspheres at different temperatures

PGE_2 levels at 8°C and 37°C were determined via gas-chromatography mass-spectrometry. At 8°C a half life of approximately 760 days for PGE_2 inside the microspheres was estimated, while for 37°C a half-life of 73 days has been estimated. Both graphs are presented in Fig. 6.

4 Discussion

Growth factors and signaling molecules have become more and more important for tissue engineering applications. In the concept of the so-called "guided tissue engineering" they play a crucial role in functionalizing biomaterials. In the search for an innovative and cost-efficient signaling molecule prostaglandin E_2 represents an interesting candidate, especially in light of its potential to enhance angiogenesis [26, 27], bone regeneration as well as osteoblast and chondrocyte proliferation [17, 18, 28]. In this context, in vivo and in vitro experiments revealed the angiogenic effects of low doses of prostaglandin in the microgram range [26, 27] comparable to the amounts which were reached in the present study. Furthermore, in previous studies our own group could demonstrate a dose dependent proliferating effect of PGE_2 on rat growth plate chondrocytes with optimal proliferative effects also in a low dose range (10^{-8} mol/l) [28, 29]. These findings indicate that low doses of PGE_2 are recognized as effective for several tissue engineering applications.

However, its short half-life under physiological conditions is the limiting factor for its extended use in tissue engineering applications. In this context, we were able to

show in a previous study the possibility of incorporating PGE_2 into (85:15) PLGA microspheres [15], anticipating a distinct increase of PGE_2's chemical and biochemical stability, due to the supposed exclusion of oxygen, moisture and enzymes. Nevertheless, the stability of PGE_2 under these new conditions was unknown. In the present report we demonstrated the long term stability of incorporated PGE_2 under different conditions.

For this purpose we established adapted mass spectrometric methods for the determination of PGE_2 concentration in solid PLGA microspheres, and for stability measurements of PGE_2 in liquids. We also investigated the stability of prostaglandin E_2 in cell culture media with added lactic acid, and the thermal stability of PGE_2 in solid PLGA microspheres. Furthermore, PGE_2 containing microspheres were characterized morphologically using both SEM and AFM imaging techniques, demonstrating an intricate structure made up of micropores and nanopores, while also showing a clustered particulate make-up of the polymer itself.

The microsphere preparation was performed according to our previously published methods [15] but now allowed for the comparison of PGE_2 immobilization efficiency in two different emulsion systems. In the previous study we used PLGA-$CHCl_3$ and PGE_2-HFIP as organic phase in 0.5% PVA as outer watery phase, while in the present experiments HFIP was replaced by ethyl acetate. Under these conditions the immobilization efficacy for PGE_2 could be significantly increased from 0.213 μg/g PLGA in the HFIP based to 1.67 μg/g PLGA in the ethyl acetate system. The main difference between HFIP and ethyl acetate is their different solubility in water. While HFIP is completely miscible, ethyl acetate is only miscible up to 9% which might result in a higher loss of PGE_2 to the outer watery phase of the HFIP emulsion system during microsphere synthesis.

In the present study we demonstrated the applicability of an adapted analytical mass spectrometric procedure to determine incorporated PGE_2 in synthesized (85:15) PLGA microspheres. This modified analytical method opens up the possibility to study the effectivity of different PGE_2 incorporation routes especially due to chemical binding of PGE_2.

The common usage of PGLA-type polymers in drug release systems is based on their well-known physicochemical properties [2, 3, 30]. In this context, it can not be excluded that as a result of the chosen strategy of superficial solvent evaporation the microspheres developed a spherical capsule in which the dissolved polymer finally depleted in the center of the microspheres. The formation of hollow cavities demonstrated in our former results ([24]) might have been a result of this effect (Figs. 1 and 2, [24]). In water the 85:15 polymer showed insignificant superficial erosion over 80 days, but internal degradation as a consequence of water diffusion into these cavities [24]. Erosion is combined with polymer hydrolysis which results in an increase of lactic and glycolic acid.

Therefore, in one part of our present study we were interested in the stability of PGE_2 as a function of an increasing lactic acidic environment. Comparing the results of our cell culture media experiments it became clear that the added amounts of lactic acid are not primarily responsible for changing PGE_2 half-life. Increased or decreased pH values are the main determining factors for chemical PGE_2 decomposition. Thus, our results demonstrated a decreased half-live time under extreme pH conditions. Acidic or basic properties lead to intramolecular elimination of water and drastically reduced half-lives of 44 h at pH 2.0 and 26–39 h at pH 8.4–8.8 with pure culture medium and buffered lactic acid, respectively. In water (supplemented with lactic acid) at pH 2.6–4.4 a maximal half-life of 300 h was observed. Added cell culture media may increase the solubility of hydrophobic, fatty acid-like PGE_2 and thereby decreased its chemical stability (half-life reduced to 86–102 h at pH 3.2). These results demonstrate the importance of preventing water influx into microsphere cavities by synthesis of compact solid microspheres.

Since PGE_2 has a very short half-live time under physiological conditions from approximately 30 s in the cardiovascular system and 35 min in amniotic fluid [22, 23] due to environmental influences the half-live time should be dramatically increased for its possible application as a suitable signaling molecule in tissue engineering applications. In this context, polymer-embedded prostaglandins should have prolonged half-lives due to effective exclusion of moisture and oxygen. As in micro security capsules, enclosed biological substances should be protected against influences from the outside environmental conditions. Only elevated temperatures or powerful light beams might result in thermal decomposition or solid-state photochemical reactions, respectively. However, these conditions have not been investigated so far. For this reason our experimental investigations were focused on two different temperatures namely experiments at the most likely storage condition of 8°C for later short-time storage as well as experiments at a dry pseudo-in vivo condition of 37°C. Freezer experiments and experiments under light exposure were not carried out due to the well known high stability of solid or dissolved PGE_2 under these conditions [24] and the assumed exclusion of light under in vitro or in vivo conditions.

In contrast to the stability determination of PGE_2 in different solvents via LC-MS/MS we used our modified GC-MS/MS methodology in all solid microsphere determinations. The performed derivatizations and further cleaning steps (e.g., TLC) ensured that no dissolved polymer, oligomer, or monomer of the lactide-glycolide matrix could pollute the mass spectrometer. A single extraction

step as in the case of our liquid samples was insufficient due to the good solubility of the PLGA polymer in the used extraction solvents. As expected, the PGE_2-modified solid microspheres in our study guaranteed a prolonged stability of PGE_2. Exclusion of moisture, light, and probably oxygen resulted in a drastically prolonged half-life of PGE_2 compared to experiments with PGE_2 in solution. The approximate half-life was 760 days at 8°C, which shows an adequate stability for short-time storage in a refrigerator. For prolonged storage the use of a freezer remains essential.

Solid microspheres kept under quasi "in vivo"-conditions showed a distinctly shorter half-life of about 73 days. Nevertheless, this enhanced stability of polymer-embedded PGE_2 compared to free PGE_2 is absolutely sufficient for long-term release in various tissue engineering applications under real in vivo conditions.

According to van't Hoff's rule of thumb a temperature decrease of 3×10°C will result in a half-life of approximately 600 days, which is in the same range as the experimentally observed stability at 8°C.

5 Conclusions

In conclusion, our experiments demonstrates a dramatically prolonged half life time of PGE_2 embedded in PLGA microspheres compared to its stability in solution or under physiological conditions. Based on the cell-stimulating effects of PGE_2 known from in vivo and in vitro studies, we propose the drug-release system presented in this study as an interesting tool for several tissue engineering applications. So far, incorporation of small amounts of PGE_2 has been achieved according to the initial aim of our studies. In ongoing experiments we analyse the incorporation of varying amounts of PGE_2 in PLGA microspheres. These tests are a crucial prerequisite for future studies on bioavailability with different cell types. In this context, several in vitro and in vivo experiments indicate that low doses of PGE_2 may have stimulatory effects in different tissue engineering applications. Finally, the present new analytical tool allows the analysis of incorporated PGE_2 in different synthesis routes of PLGA microspheres, which is of special interest for the further functionalization of PLGA-based microspheres.

Acknowledgment This work was supported by grant from the Deutsche Forschungsgemeinschaft (Se 263/17-1).

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