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Release of bioactive BMP from dextran-derived microspheres: A novel delivery concept

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Abstract

Recent developments of biotechnology have produced a great variety of protein and bioactive drugs. For these drugs to be used therapeutically, suitable drug delivery systems have become increasingly essential. Dextran-derived biomaterials have been considered to be compatible matrices for protein and bioactive drugs because of their hydrophilic properties and ability to control drug dissolution and permeability. A novel class of dextran–glycidylmethacrylate (Dex–GMA)/poly(ethylene glycol) (PEG) microspheres were designed and synthesized by polymerization of Dex–GMA emulsified in an aqueous PEG solution. Dex–GMA was prepared by substituting the hydroxyl groups in Dex by GMA. The drug loading and in vitro drug release was evaluated by routine procedure and the biological activity of BMP-loaded microspheres was studied by experimental cytology methods. Recombinant human bone morphogenetic protein-2 (rhBMP-2) were entrapped in dextran-derived microspheres quantitatively and with full preservation of their biological activity. In vitro release kinetics indicated that dextran-derived microspheres could retain rhBMP-2 in a variable manner depending on the preparation and degradation of the microspheres. The release profiles of rhBMP-2 from microspheres as a function of time showed that rhBMP-2 releasing kinetics in vitro fitted to first-order and Higuchi equations. The release profile in vitro was in accord with two phases kinetics law and more than 60% drug were released during 20 days. Cytology studies showed rhBMP-2 microspheres have good biological effects on cultured periodontal ligament cells, and could achieve a longer action time than concentration of rhBMP-2 solution. These properties make those microspheres interesting osteo-conductive BMP carriers, allowing to decrease the amount of implanted factor required for tissue regeneration.

Keywords: Bone morphogenetic proteins; Drug delivery system; Microspheres; In vitro release; Functionalized dextran; Poly(ethylene glycol); Periodontal ligament cells

1. Introduction

Bone morphogenetic proteins (BMPs) include a large number of proteins belonging to the TGF-β superfamily, which are characterized by their ability to induce bone, cartilage, and other multiplex tissues formation (Cowan et al., 2005; Vogelin et al., 2005). The therapeutic use of recombinant BMPs in the treatment of periodontal disease (destruction of the tooth ligaments, surrounding bone and tooth cementum, the latter of which anchors the ligaments to the tooth surface from the adjacent tooth socket) has attracted considerable interest due to their potent ability to stimulate intra-membranous bone formation

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without an endochondral intermediate. Their predictability in stimulating new bone may provide an alternative that has greater osteogenic potential than autogenous bone, other growth factors and bone substitutes (Kobayashi et al., 1999; King and Hughes, 2001a; King, 2001b; Selvig et al., 2002). Therefore, BMPs are likely candidates to stimulate periodontal regeneration because of their ability not only to promote osteogenesis but also to stimulate cementogenesis (new cementum formation) (Kobayashi et al., 1999; King and Hughes, 2001a; Selvig et al., 2002; Miranda et al., 2005; Takahashi et al., 2005; Wikesjo et al., 2005). However, understanding when to manipulate each of the various cells differentiation pathway with the application of single or multiple doses of BMPs at the appropriate concentration is dependent upon a suitable delivery system that can be modified in order to optimize its effect during periodontal wound healing. Furthermore, treatment of intrabony periodontal defects with BMPs are likely to not only require appropriate temporal release of the agent, but also adaptation of a carrier that is robust enough to maintain its integrity around the coronal aspect of the root in order to provide space maintenance and support the mucoperiosteal flap (King, 2001b). An optimized delivery system improve the osteopotency of the device while reducing as much as possible the amount of introduced BMP, which offers double advantage of being safer as well as less expensive (Maeda et al., 2004; Chen et al., 2005a, 2005b; Marion et al., 2005).

Modern biotechnology has resulted in the production of a great variety of pharmaceutically active proteins (Crommelin and Sindelar, 1997). Their unfavorable biopharmaceutical properties, however, have severely hampered the therapeutic and clinical applications of these proteinaceous drugs. A large number of delivery systems has been designed and evaluated for the release of proteins (Baldwin and Saltzman, 1998; Brannon-Peppas, 1995; Gombotz and Pettit, 1995). A series of organic and inorganic, natural and artificial substances have been used to deliver BMP in experimental or preclinical models (Maeda et al., 2004). Most of them were evaluated in different shape and pattern. Among all tested carriers, collagen sponge appeared to be the most intensively tested matrix in animal studies as well as in clinical trials (Boden et al., 2002; Burkus et al., 2003, 2004; Maeda et al., 2004; Kim et al., 2005). In clinical practice, absorbable collagen remains the only FDA approved recombinant human BMPs carrier (Burkus et al., 2003, 2004; Geiger et al., 2003). However, in spite of its good results as BMP carrier and important safety background, collagen sponge demonstrated some disadvantages owing to its xenogeneic origin (most from bovine and porcine skin). As with all protein-based therapies, an immune response might be induced and clinical trails demonstrated that around 18% of patients treated with rhBMP-2/collagen sponge developed anti-type I collagen antibodies (http://www.fda.gov/cdrh/pdf/P000058.html, FDA information on InFUSE™, 8 May 2003). Still the possibility of xenozoonoses from the animal tissue to the human recipient has to be carefully considered (Lee et al., 2001; Singla and Lee, 2002).

To engineer a fully synthetic carrier, free of potential contamination, could be of major interest. Additionally, synthetic polymers present other advantages. Firstly, they allow a better control of physicochemical properties than natural polymers, leading to more reproducible delivery kinetics of specific molecules (Freiberg and Zhu, 2004; Wang et al., 2005). Secondly, polymer can be easily chemically modified with functional groups making them, for example, bio-specific to a protein. Thus, the interaction of BMP with a polymeric matrix would provide a carrier able to retain the growth factor at the appropriate site, to locally deliver it in sustained fashion and to decrease the BMP amount used in clinical applications. Recent studies had showed that dextran-based hydrogels were endowed with the ability to retain bioactive growth factors within the polymer matrix and the capacity of those hydrogels to retain the bioactivity of rhBMP-2 or other growth factors was investigated both in vitro and in vivo (Kobayashi et al., 1999; Ferreira et al., 2005; Maire et al., 2005). For more widely therapeutic application of these protein-loaded gels, injectable dosage or new pharmaceutical forms are required. An attractive method to prepare polymeric microspheres in an all-aqueous system, avoiding the use of organic solvents was recently described (Stenekes et al., 1998, 1999), and is based on the phenomenon that in aqueous two-polymer system phase separation can occur. Microspheres as drug carriers have the advantages of sustained or controlled release, passive or active drug targeting to specific tissues and so on, which will notably reduce the side effects of drugs and improve their bioavailability. Therefore, microspheres as drug delivery system have drawn much attention in pharmaceutical field and have been successfully applied in some clinical trials.

Taking these factors into consideration, we had designed and tested this kind of microsphere carrier based on dextran-derived hydrogels, and prospected this polymer microspheres as new BMP carriers. Dextran contains primarily (1–6) linked α-D-glucopyranosyl residues with three hydroxyl groups per glucose ring. These hydroxyl groups in dextran (Dex) were reacted with glycidyl methacrylate (GMA) to form a dextran-based precursor (Dex–GMA), and this polysaccharide-based precursor was used to synthesize microspheres in an aqueous two phase system (Stenekes et al., 1998, 1999; Franssen et al., 1999), thereby avoiding the use of organic solvents and potential damage to the encapsulated protein structure (Manning et al., 1989).

2. Materials and methods

2.1. Materials

The following materials were obtained as gifts: Dextran T-70 (Dex, MW 69,800 with 5% branches) and poly(ethylene glycol) (PEG, MW 38,800) were provided by Sigma Chemical Co. (St. Louis, MO, USA) and dried at 60 °C in a vacuum oven for 2 days, Glycidylmethacrylate (GMA), triethylamine, 4-(*N*,*N*-dimethylamino)pyridine (DMAP), dimethyl sulfoxide (DMSO), *N*-methyl pyrrolidone (NMP), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium persulfate (APS) were obtained from Baotelai Chemicals (Xi'an, China) and dried at 70 °C in a vacuum oven overnight. All other reagents were of analytical grade.

2.2. Preparation of the dextran–glycidylmethacrylate (Dex–GMA)

Dex–GMA precursor was synthesized according to published procedure (Kim et al., 1999; Kim and Chu, 2000) with some modifications (Chen et al., 2005a, 2005b). Dextran was dissolved in a DMAP/DMSO solvent system (50 wt.%) at 60 °C under N_2 gas. After a complete dissolution, the solution was cooled down to 30 °C and triethylamine catalyst (6 mol% to GMA) was added to the dextran solution. The solution was stirred for 15 min at 30 °C, and GMA was then added to the dextran solution at a very slow rate. The reaction was conducted at 30 °C for 72 h under N_2 gas. The reaction product was precipitated with cold isopropyl alcohol, filtered, washed several times with isopropyl alcohol, and then dried in a vacuum oven at room temperature.

2.3. Preparation of microspheres

Taking the score of appearance and the score of drug embedding ratio as indexes, we investigated the influence of temperature, the proportion of Dex–GMA and PEG, and the stirring speed (in our preliminary studies, we identified these three are the most important factors affecting the performance of this multi-particulate drug delivery system coated with rhBMP-2) on the preparation of these microspheres. Several other factors, such as Dex–GMA and emulsion concentration, inlet temperature and curing time were found to have less influence on the properties the beads. Therefore, these factors were kept constant throughout the batches of the present study. A three-factor, three-level Box–Behnken design was used for the optimization process using a statistical software, Statgraphics® Plus, version 4.1 (Manugistics Inc., MD). The best preparation method of the microspheres was optimized and the finished products of which were given an initial determination namely reaction temperature 37 °C, PEG and Dex–GMA (1:4) 5 g, stirring speed 400 rpm. Microspheres were essentially prepared as the methods described by Niwa et al. (1994), Stenekes et al. (1998, 1999) and Franssen et al. (1999). In short, aqueous solutions of PEG and Dex–GMA in 0.22 M KCl were flushed for 10 min with N_2 gas and subsequently transferred into a scintillation vial. The total weight of PEG and Dex–GMA solution was 5 g. The two-phase system was vigorously mixed (vortex, type Scientific Industries, Vortex Genie 2, Model G-560-E, maximum intensity) for 60 s to create a water-in-water emulsion. Next, the emulsion was allowed to stabilize for 10–15 min at room temperature, followed by the addition of TEMED (100 μl, 20%, v/v, adjusted to pH 7 with 4 M HCl) and KPS (180 μl, 50 mg/ml). This system was incubated for 30 min at 37 °C to polymerize the methacryloyl groups coupled to the Dex–GMA chains. The Dex–GMA/PEG microspheres were then obtained by double-phase emulsified condensation polymerization and washed thrice with centrifugation steps. The collected microspheres were freeze-dried and preserved in a desiccator. The dried microspheres were sprinkled onto a piece of electric-glue paper, gold-sprinkled in a vacuum, then were examined by scanning electro-microscopes (SEM, S-2700, Hitachi, Tokyo, Japan). The dried microspheres were dispersed into anhydrous ethanol and agitated on a shaker table at 100 rpm for more than 10 min, and then added into the portal of a particle size analyzer (BI-90, Brookhaven Co., USA), the microspheres' size and size distribution could be obtained though the deferent equipment of the Brookhaven analyzer.

2.4. Microparticle loading

For release studies, microspheres were loaded with rhBMP-2 by two approaches according to established methods (Stenekes et al., 2000, 2001). In the first approach, rhBMP-2 was added to the phase separated PEG/Dex–GMA system prior to vortexing. Alternatively, rhBMP-2 was dispersed in the Dex–GMA solution before adding PEG. The subsequent steps in the microsphere preparation procedure were unchanged. Solutions of rhBMP-2 were composed of ^{125}I–ubiquitin recombinant human bone morphogenetic protein 2 (^{125}I–rhBMP-2) (Perkin-Elmer Life Sciences, Boston, MA) and unlabeled-rhBMP-2(R&D Systems, Minneapolis, MN) in a mass ratio of 0.06 to allow for detection of drug release. The rhBMP-2 concentration in the reaction solutions was 200 ng/ml. This concentration of rhBMP-2 has been shown to be the therapeutic in the treatment of full and partial thickness bone or cartilage defects (Driesang and Hunziker, 2000; Hunziker et al., 2001; Hunziker and Driesang, 2003; Li et al., 2004). The encapsulation efficiency of rhBMP-2 is defined as the amount if rhBMP-2 in the microspheres divided by the amount of rhBMP-2 added to the two phase ×100%.

2.5. In vitro release of rhBMP-2 from microspheres

Finally, the in vitro release of rhBMP-2 from microspheres was examined over the course of 28 days. Five milligrams of microspheres were loaded, as previously described, and incubated in 3 ml phosphate buffer solution (PBS), agitated on a shaker table (70 rpm) at 37 °C. After 0.5, 1, 2, 3, 6, 10, 14, 18, 21, and 28 days, the supernatant of each specimen was collected and analysed for radioactivity using a gamma counter (Cobra II Autogramma, Packard, Meridian, CT). The amount of rhBMP-2 in the supernatant was determined by correlation to a standard curve. Cumulative release was determined by normalizing the total rhBMP-2 release at each time point with the sum of the total rhBMP-2 release over the course of 28 days and the rhBMP-2 remaining in the microspheres at day 28. Release rate was determined by taking the slope of the percent cumulative release curve for the sample over the stated range and averaging the resultant slopes for each formulation. Accordingly, rate is stated in terms of the change in the percent cumulative release per day. For all formulations, n was initially 6. However, some samples were damaged in the collection of supernatant, resulting in an n of 4–6. Cumulative release values and release rates for microspheres in each buffer (PBS with or without dextranase) were statistically compared using F test and Tukey's multiple comparison test ($P < 0.05$). Likewise, values for three series microspheres (synthesized by Dex–GMA with different DS of 4.7, 6.3, or 7.8) were statistically compared. Values are reported as average ± S.D. Accordingly, since dextran-based materials are enzymatically degraded, so in vitro release of rhBMP-2 in PBS containing

dextranase (final enzyme concentration 0.1 U/ml) was also studied.

2.6. Bioactivity of rhBMP-2 loaded microspheres

Periodontal ligament (PDL) cells are believed to play an important role in the periodontal regeneration; that is, they may differentiate into special cells, which make cementum, bone, and attachment apparatus. RhBMP-2 has been found to promote the osteoblastic differentiation and decrease cell proliferation of human PDL cells (Kobayashi et al., 1999; King and Hughes, 2001a; King, 2001b; Selvig et al., 2002; Si and Liu, 2002; Pitaru et al., 2002; Markopoulou et al., 2003). To examine the bioactivity of rhBMP-2 loaded microspheres, we detected the biological effects of microspheres on cell metabolism, proliferation, and osteoblastic differentiation in human PDL cells. As reported by Kobayashi et al. (1999), rhBMP-2 at concentrations about 200 ng/ml significantly stimulated alkaline phosphatase (ALPase) activity and parathyroid hormone (PTH)-dependent 3′,5′-cyclic adenosine monophosphate accumulation, which are early markers of osteoblast differentiation in the human PDL cells, the rhBMP-2 concentration we investigated in this study was 200 ng/ml.

2.6.1. Isolation and culture of human PDL cells

Human PDL cells were obtained from premolars extracted for orthodontic reasons from a 14-year-old patient, using explant cultures as previously described by Somerman et al. (1988). Briefly, premolars were extracted, washing twice with saline, and the PDL fragments from the middle third of the root cureted. The fragments were washed and cultured in Dulbecco's modified Eagle medium (DMEM, obtained from Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum (FBS), 50 μg/ml penicillin and 50 ng/ml amphotericin B at 37 °C in a 5% CO_2 air atmosphere. When the cells growing out from explants reached confluence, they were trypsinized with 0.2% trypsin in PBS for the second culture. Human PDL cells used in this study were between the third and the ninth passage. All experiments were performed using cells between the second and the tenth passages. The protocol was approved by the Ethical Committee in Research from the Fourth Military Medical University.

2.6.2. Cell metabolism of PDL cells

Human PDL cells were detached from the culture flasks by Trypsin–EDTA and the cell suspension was adjusted at 5×10^4 cells/ml in culture medium. One hundred microliters of this cell suspension containing 5×10^3 cells were seed in each well of a 96-well tissue culture plate (Nunc A/S, Roskilde, Denmark). Cells were cultivated in the DMEM containing 4500 mg/l D-glucose, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, and 2% FCS in the absence (control) or presence of 200 ng/ml rhBMP-2 (BMP), 50 mg/ml rhBMP-2 loaded microspheres (BMP-MPs, equal to 200 ng/ml rhBMP-2) at 37 °C in humidified air containing 5% CO_2 for 2, 3, 5, 7, and 10 days. After cultured period, the metabolic cell activity was measured by tetrazolium salt assay. Cells were incubated for 4 h with tetrazolium salt (MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-2,5-tetrazolium bromide, final concentration 0.5 mg/ml) at 37 °C. Formed formazan crystals were solubilized and absorbance was measured at 540 nm wavelength by a microtiter plate reader (Titertek, Helsinki, Finland).

2.6.3. Determination of ALP activity

Human PDL cells were detached from the culture flasks, washed twice in complete medium, and sedimented by centrifugation at 800 rpm for 8 min. Ten microliters of the human PDL cells suspension containing 2×10^6 cells were seeded to each well of a 24-well tissue culture plate (Nunc A/S, Roskilde, Denmark). Cells were cultivated in the DMEM containing 4500 mg/l D-glucose, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, and 2% FCS in the absence (control) or presence of 200 ng/ml rhBMP-2 (BMP), 50 mg/ml rhBMP-2 loaded microspheres (BMP-MPs, equal to 200 ng/ml rhBMP-2) at 37 °C in humidified air containing 5% CO_2. At the same time intervals, ALP activity was assayed in the supernatant of each well using 4-nitrophenyl-phosphate as substrate (10 mmol/l, ALP kit, Roche Diagnostics, Mannheim, Germany). The measurement of absorbance at 405 nm. ALP activity (mU/culture) was calculated according to the assay instruction.

2.6.4. Assay for detection of osteoblast character

Cells were cultivated and groups as described in ALP activity assay. Osteopontin (OPN) and osteocalcin (OCN) production were measured using an OPN ELISA kit (Biosource, Nivelles, Belgium) and an OCN ELISA kit (Biosource, Nivelles, Belgium). The supernatant of homogenates of the 5-, 7-, and 10 days cultivated in the culture medium absence or presence of 200 ng/ml rhBMP-2, 50 mg/ml rhBMP-2 loaded microspheres, or standard solution (OPN: 0–80 ng/ml; OCN: 0–35 ng/ml) were pipetted into the wells of 96-well tissue culture plates coated with antibody specific for human OPN and incubated for 2 h. After incubation, liquid in each well was removed and were rinsed thrice by PBS. This was followed by incubation with horseradish peroxidase (HRP)-conjugated antibodies against each protein for 2 h at room temperature. For OCN detection, supernatant or standard solutions and HRP-conjugated antibody were pipetted into the wells and incubated for 2 h. After incubation, liquid was removed and rinsed in each well thrice by PBS. Then the chromogenic solution, consisting of tetramethylbenzidine, was added into the wells and incubated for 30 min. After stopping the reaction, absorbance (OPN for 450 nm and OCN for 405–690 nm) was measured by an ELISA microtiter plate reader (Titertek, Helsinki, Finland). Concentration of each protein was calculated (ng or pg/culture) according to the standard value.

2.6.5. Statistical analysis

Non-parametric one-way analysis of variance (ANOVA) and multiple comparisons were used to the test for the assays of multiple comparisons to compare with the control by the software program (Abacus Concept Inc., Berkley, CA). For the cell growth assay, a parametric analysis of variance based on the Tukey test was used. All assays were performed at least thrice and a confidence interval of 95% was adopted ($P < 0.05$).

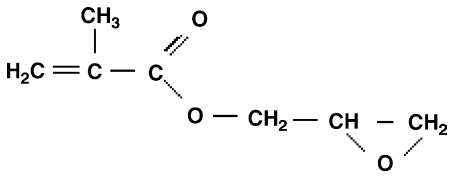

Fig. 1. Chemical structure of glycidylmethacrylate (GMA).

3. Results and discussion

3.1. Synthesis of Dex–GMA

The chemical structure of GMA is showed in Fig. 1, and the chemosynthesis equation is showed in Fig. 2. The degree of substitution (DS), the amount of hydroxyl groups per dextran glucose ring were substituted by GMA of Dex–GMA precursor, was estimated by published ^{1}H-NMR method (Kim et al., 1999; Kim and Chu, 2000; Chen et al., 2005b). There is an anomeric proton attached to the C1 position of dextran glucose ring and appears at 4.5–5.5 ppm in NMR spectrum, where the protons of hydroxyl groups appear. This proton does not react during the GMA substitution reaction, while some of the other protons of other hydroxyl groups are substituted by GMA. So, we can use the ratio of the normalized, integrated intensities of the sum of the hydroxyl group peaks to the normalized, integrated intensities of the anomeric proton peak to estimate the DS. For unsubstituted pure dextran, the ratio should be 3; while for GMA-substituted dextran, this ratio should be more than 3 and the magnitude would depend on the number of substitution. Thus, the DS could be calculated from the ratio: 3 × (difference of the NMR proton intensity between dextran and dextran derivatives)/dextran. We have prepared a series of Dex–GMA having a wide range from 4.7 to 7.8. It was found that the solubility of Dex–GMA was greatly improved even with very low DS. And the Dex–GMA precursor synthesized in this study had a DS of 4.7, 6.3, and 7.8.

3.2. Microspheres

The microspheres prepared after polymerization of Dex–GMA in the discontinuous phase, and have a smooth and uniform surface, typically have a particle size of 31.12 ± 12.66 μm and the DS of Dex–GMA have no obvious influence on particle size distribution. The morphology and particle size analysis showed no difference between loaded and unloaded freeze-dried microspheres, but adsorbed drugs in the surfaces of dried microspheres could be seen (Fig. 3). The particle sizes and size distribution of the microspheres were as measured by a Brookhaven analyzer. Fig. 4 shows the size distribution of microspheres prepared using the optimization process. Almost all microspheres had sizes ranging from 10 to 60 μm in diameter and about 68% of the microspheres were in an even narrower size range of 20–40 μm. These sizes can easily pass through a 23-gauge needle for injection use. RhBMP-2 could be encapsulated with a very high efficiency using both approaches studied. When rhBMP-2 was added to the phase separated system, the encapsulation efficiency was $(86.6 \pm 0.8)\%$ (mean ± S.D. for five independently prepared microsphere batches). Amount of rhBMP-2 loaded in the microspheres are accurately 1‰ (w/w). Interestingly, almost quantitative encapsulation was observed when rhBMP-2 dispersed in the Dex–GMA solution prior to addition to the PEG solution $(87.8 \pm 1.2)\%$, so in this study, rhBMP-2 were encapsulated in the microspheres using the second approach.

3.3. In vitro release of rhBMP-2 in PBS

As shown in Fig. 5, both microspheres exhibited similar rhBMP-2 release profiles in standard PBS. Burst release values from DS 4.7 microspheres, DS 6.3 microspheres DS 7.8 microspheres were $(50.7 \pm 1.5)\%$, $(46.7 \pm 1.4)\%$, and $(44.2 \pm 1.7)\%$, respectively (Table 1). Relative little further release was observed over the 28-day period. Both microspheres exhibited a burst release, a phase 2-release rate of approximately 5.0% per day from days 1–3, and a phase 3-release rate of approximately 0.2% per day from days 6–21. In fact, final cumulative release values for both microspheres were less than 65%. Accordingly, in vitro release of rhBMP-2 showed the release rate more faster when presence of dextranase in PBS ($P < 0.05$). The release profiles of rhBMP-2 from microspheres as a function of time showed that rhBMP-2 releasing kinetics in vitro fitted to first-order and Higuchi equations. The release profile in vitro was in accord with two phases kinetics law and more than 60% drug were released during 20 days. Further more, changing the DS

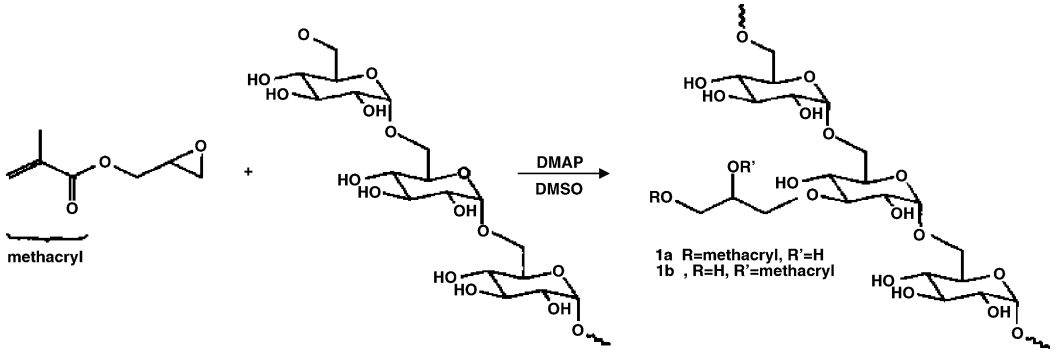

Fig. 2. The chemosynthesis equation of Dex–GMA synthesis.

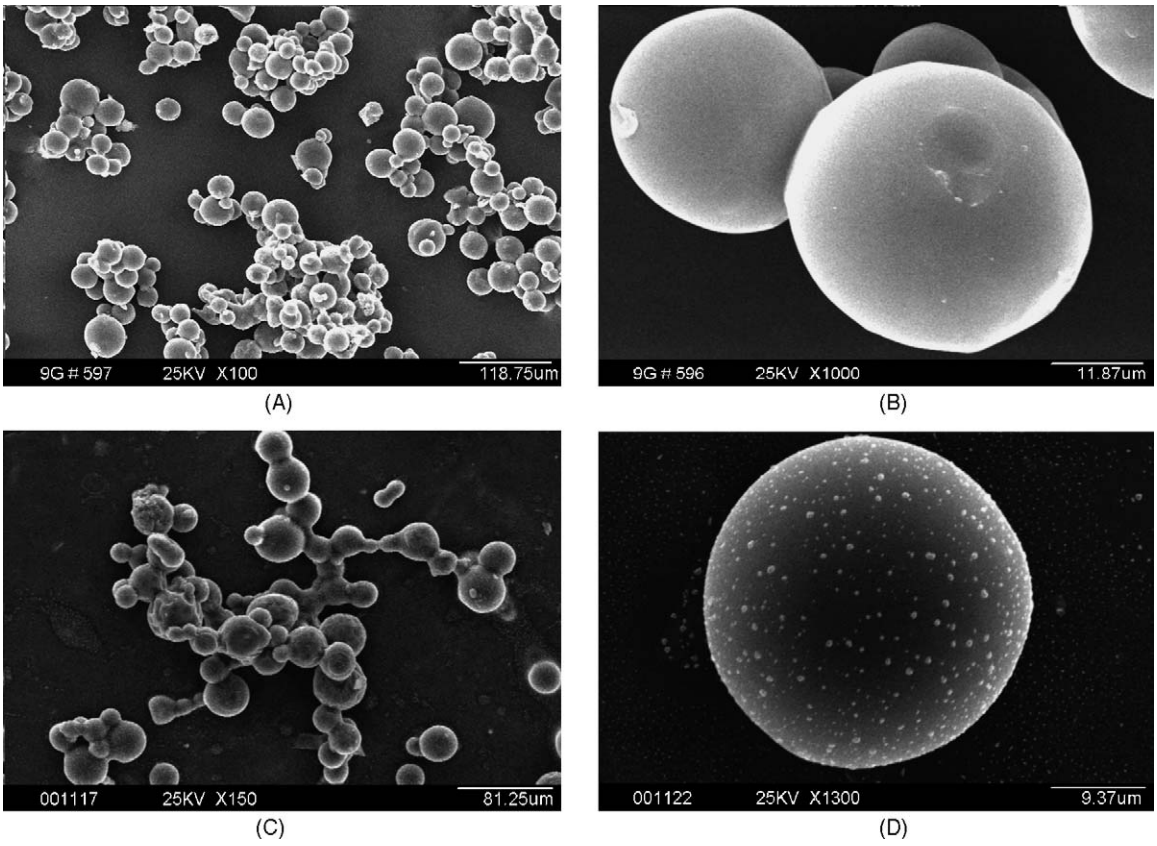

Fig. 3. SEM photographs of dextran-derived microspheres, unloaded microspheres (A and B), and loaded microspheres (C and D).

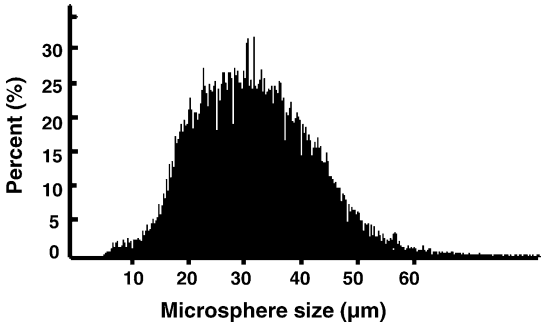

Fig. 4. Size distribution (in diameter) of the microspheres.

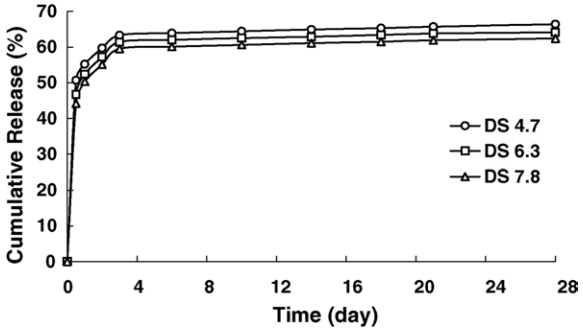

Fig. 5. RhBMP-2 release profiles in standard PBS.

of Dex–GMA may influence drug release, rhBMP-2 release rate from microspheres was slower when the DS of Dex–GMA was increased, which could be due to DS increased so as to increase the DS of the microspheres. During the initial stages, the microspheres sopped up and sphere enlarged rapidly, the drug rapidly released from microspheres through the exoteric micro-aperture; when swelling was counterpoise, the drug release would slow down and be determined by drug pervasion and microspheres biodegradation. So we can speculate that rhBMP-2 release from mirospheres may be controlled by some preparation technique change. This might have important clinical meanings. In periodontal tissue or bone defect, the need concentration and interval of rhBMP-2 may be different. It could be hypothesized that when topically applied, microspheres present different demand of tissue regeneration, even at high concentration, can prevent rapid release by increase the DS of Dex–GMA as well as promote secondary release by decrease DS. The functionary mechanism need further studies.

3.4. In vitro biological activity detection

3.4.1. PDL cells cultured with rhBMP-2 microspheres

PDL cells cultured with rhBMP-2 microspheres were in shuttle or polygon shape with multiple inter-connected umbos in different size, lengths, and extended adequately (Fig. 6A–C). But when cells were incubated in the culture medium absence of rhBMP-2, the cells became decrepit, more granule-like substance in the surface of cells (Fig. 6D).

Table 1
Burst release, Phase 2 and 3 release rates, and final cumulative rhBMP-2 release from microspheres in buffers of PBS and PBS with dextranase

	Microspheres (DS of Dex-GMA)	Buffer	
		PBS	PBS with dextranase
Burst release (%)	4.7	50.7 ± 1.5*	39.7 ± 2.4*
	6.3	46.7 ± 1.4	36.3 ± 1.4
	7.8	44.2 ± 1.7	35.6 ± 1.8
Phase 2 release rates (%/day)	4.7	4.3 ± 0.9	4.1 ± 1.2
(days 1–3)	6.3	4.9 ± 1.0	4.7 ± 1.0
	7.8	5.1 ± 1.9	5.3 ± 1.4*
Phase 3 release rates (%/day)	4.7	0.2 ± 0.1	2.3 ± 0.4*
(days 6–21)	6.3	0.2 ± 0.0	2.0 ± 0.3
	7.8	0.2 ± 0.1	1.7 ± 0.7
Final cumulative release (%)	4.7	66.4 ± 1.7*	89.7 ± 2.5*
	6.3	64.1 ± 1.5	84.3 ± 1.8
	7.8	62.4 ± 0.9	81.3 ± 1.5

* Indicates statistically greater values ($P < 0.05$) between microspheres synthesized by different DS of Dex-GMA in each buffer.

3.4.2. Effects of rhBMP-2 microspheres on cell metabolism

To investigate the effect of rhBMP-2 microspheres on cell metabolism in human PDL cells, cells were incubated in the culture medium presence or absence of 50 ng/ml rhBMP-2, 50 mg/ml rhBMP-2 loaded microspheres, and significant differences were found at the control group and BMP or BMP-MPs group in 3 days ($P < 0.05$), but there were no statistically differences between BMP and BMP-MPs group ($P > 0.05$). On the contrary, after 5 days difference became significant between BMP and BMP-MPs group ($P < 0.05$) and the differences were significantly increased ($P < 0.01$) (Fig. 7).

3.4.3. Effects of rhBMP-2 microspheres on ALP activity of PDL cells

To evaluate the effects of rhBMP-2 microspheres on ALP activity of PDL cells, we measured the ALP activity of the homogenate's supernatant of different cultures. The same phenomena were found as MTT assay. Significant differences were

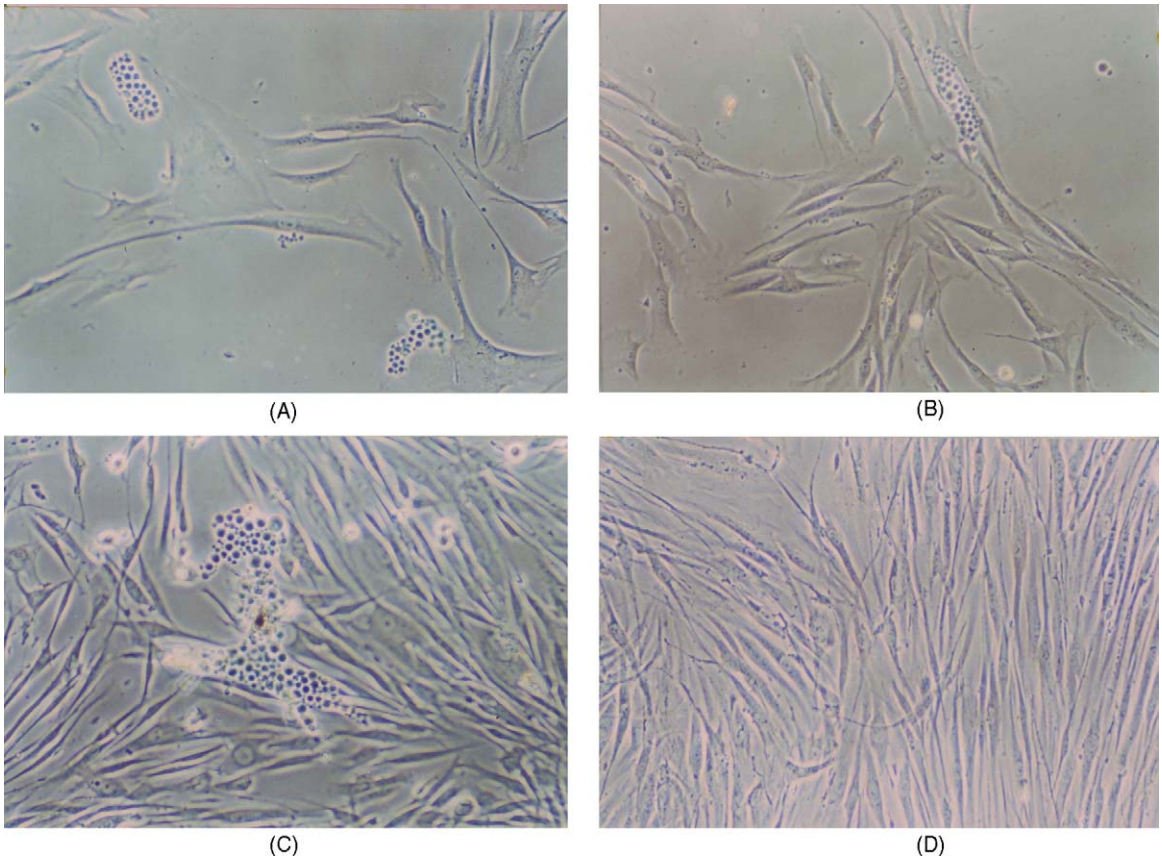

Fig. 6. PDL cells were incubated in the culture medium presence of rhBMP-2 loaded microspheres, 2 days (A) ×40, 5 days (B) ×40, 10 days (C) ×20 or absence of rhBMP-2 loaded microspheres, 10 days (D) ×20.

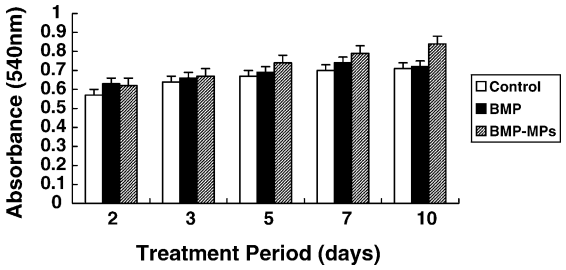

Fig. 7. Effects of rhBMP-2 microspheres on cell metabolism (MTT assay) in human PDL cells, which were cultured in different culture for 2, 3, 5, 7, and 10 days in the presence (BMP or BMP-MPs) or absence of 200 ng/ml rhBMP-2 (values are expressed as mean ± S.D. of six determinations).

found at the control group and BMP or BMP-MPs group in 5 days ($P < 0.05$), but there were no statistically difference between BMP and BMP-MPs group ($P > 0.05$). On the contrary, after 7 days difference became significant between BMP and BMP-MPs group ($P < 0.01$) and the values were more significantly ($P < 0.001$) (Fig. 8).

3.4.4. *Effects of rhBMP-2 microspheres on osteoblast differentiation markers*

Amounts of OCN in the culture could not be measured accurately in the supernatants due to values lower than the detection levels. OPN concentration in the three groups was not different during the experimental period from days 2 to 5. After 7 days, the increase of OPN concentration in BMP-MPs cultures was significant (Fig. 9).

Periodontal regeneration is expected to reconstitute the lost or injured tissues to restore the architecture and function of the periodontal tissues, including cementum, periodontal ligament and alveolar bone. And the basis of periodontal regeneration is PDL cells, which are the main responsive cells to form the new attachment. Periodontal disease is a chronic infective disease of the periodontal tissues caused by bacteria present in dental plaque. This condition induces the breakdown of the tooth supporting apparatus until teeth are lost. It has been widely acknowledged that there is hardly any effective method in the prevention and treatment of periodontal tissue defects. Surgery may be indicated to arrest disease progression and regenerate lost tissues and

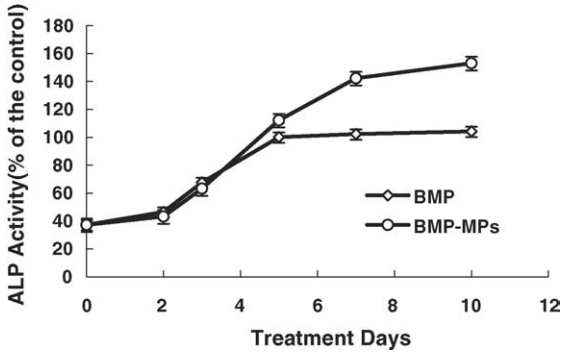

Fig. 8. Effects of rhBMP-2 microspheres on ALP activity of PDL cells. Human PDL cells were cultured in different culture for 2, 3, 5, 7, and 10 days in the presence (BMP or BMP-MPs) or absence of 200 ng/ml rhBMP-2 (values are expressed as mean ± S.D. of four determinations).

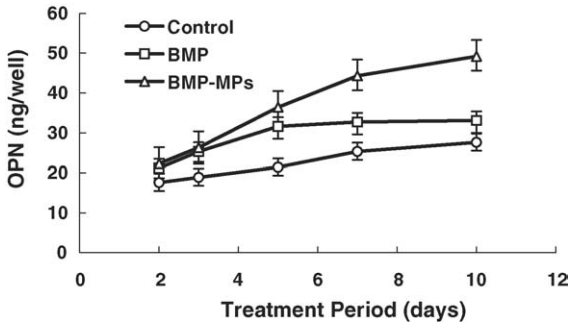

Fig. 9. Effects of rhBMP-2 microspheres on OPN production in human PDL cells, human PDL cells were cultured in different culture for 2, 3, 5, 7, and 10 days in the presence (BMP or BMP-MPs) or absence of 200 ng/ml rhBMP-2 (values are expressed as mean ± S.D. of four determinations).

several surgical techniques have been developed to regenerate periodontal tissues including guided tissue regeneration (GTR), bone grafting (BG). There is evidence that GTR has provided a tangible effect and that continuous presence of some growth factors at the periodontal tissue interface is essential, which can accelerate the soft and hard tissue regeneration. Polypeptide growth factors have been shown to modulate the wound healing response in both hard and soft tissues. During the past decade, many investigators have demonstrated the anabolic effects of these wound-healing molecules on the promotion of periodontal attachment structures, namely alveolar bone, periodontal ligament and tooth root cementum. The molecular cloning and large-scale purification of growth factors has allowed expanded in vivo studies on periodontal tissue regeneration (Kobayashi et al., 1999; King and Hughes, 2001a; King, 2001b; Selvig et al., 2002). However, one thing is certain no matter what the genuine mechanism may be, growth factor therapy must resolve the drawbacks such as short-term growth factors retention and their biological activity can be loosed in a very short time in vivo. Using agents containing large concentrations of growth factors for sustained release may be effective to resolve the question, but a large number of topical growth factor agents are not yet ideal. Indeed, the currently doctor-used GTR film in GTR treatments, or scaffolds used in bone and periodontal tissue engineering containing low or little amount of growth factors, give only mechanical obstructive or support process because GTR film and scaffolds themselves cannot maintain high level enough of growth factors to induce tissue regeneration. Therefore, it is rather inviting to search for new pharmaceutical forms that can sustain elevated growth factors levels and increase or improve tissue regeneration in periodontal diseases treatment or bone and cartilage defect repair (Hench et al., 2004; Schmokel et al., 2004; Holland et al., 2004). BMPs on mineralised tissue formation indicate that BMPs are good candidates for use in stimulating periodontal regeneration. Relatively, little is known about the mechanisms of actions of BMPs during periodontal regeneration, although recent evidence suggests that the effects of BMPs may be profoundly influenced by various factors including root surface conditioning, delivery systems and masticatory forces (King and Hughes, 2001a). So we used rhBMP-2, one of the most potent growth factors that stimulates osteoblast differentiation

and bone formation, as the experimental drug in this study.

PDL cells are thought to relate to several cells like fibroblast and also hard tissue cells, such as osteoblast and cementoblast. Due to the importance of regeneration therapy in periodontal management, we try to search a sound way for growth factor application in periodontal tissue regeneration. In this present study, we measured the cell metabolism by MTT assay. This assay determines the activity of mitochondrial dehydrogenase and correlates with cell number and cell viability, and significant differences were found after 5-day culture. At the same time, we used ALP activity and OPN production to analyze osteoblastic differentiation and OCN as parameters for mineralization and osteoblastic maturation in vitro. The results showed rhBMP-2 microspheres have the same biological effects during the experimental period of 2–5, or 7 days as the same concentration of rhBMP-2 solution, and could achieve a longer action time. The results indicated that with a small or same amount of rhBMP-2 could achieve equivalent effect to the concentrated rhBMP-2 solution when enveloped it into dextran-based microspheres, at the same time, could prolong bioactive rhBMP-2 retention in a long-term.

4. Conclusion

The main objective of this study was to evaluate in vitro drug delivery microspheres based on dextran-derived biomaterials as BMP carriers. These microspheres were manufactured in an all-aqueous system, avoiding the use of organic solvents so as to avoid the bioactivity loss of BMP during preparation. The microsphere diameter was about 20–40 μm, namely micro-carriers or micro-gels, which present a double advantage. First, they provide a carrier with high exchange surface, which would potentially enhance the growth factor availability. Second, microsphere carriers provide a surgically convenient injectable material suitable for implantation into irregularly shaped skeletal defects, which would become widely used in the treatment of periodontal diseases and articular cartilage defects. The present in vitro study evaluated dextran-derived microspheres as potential BMP or other bioactive drug carrier. The in vitro drug release can be adjusted and the bioactivity of encapsulated drug could be protected very well. While the combined mechanisms of drug and microsphere are still not fully understood. On the other hand, this drug carrier still needs more animal experiment and in vivo studies to approve its validity and safety.

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