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Dual growth factor-releasing nanoparticle/hydrogel system for cartilage tissue engineering

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Abstract In order to induce the chondrogenesis of mesenchymal stem cells (MSCs) in tissue engineering, a variety of growth factors have been adapted and encouraging results have been demonstrated. In this study, we developed a delivery system for dual growth factors using a gelation rate controllable alginate solution (containing BMP-7) and polyion complex nanoparticles (containing TGF-β_2) to be applied for the chondrogenesis of MSCs. The dual growth factors (BMP-7/TGF-β_2)-loaded nanoparticle/hydrogel system showed a controlled release of both growth factors: a faster release of BMP-7 and a slower release of TGF-β_2, ca., approximately 80 and 30% release at the end of an incubation period (21 days), respectively, which may be highly desirable for chondrogenic differentiation of MSCs. On the contrary, the release of each growth factor from the dual growth factors-loaded hydrogel (without the nanoparticles) was much slower than that of the nanoparticle/hydrogel system, approximately 36% (BMP-7) and 16% (TGF-β_2) for 21 days, and this is more than likely attributed to the aggregation between growth factors during the hydrogel fabrication step. The nanoparticle/hydrogel system with separate growth factor loading may provide desirable growth factor delivery kinetics for cartilage regeneration, as well as the chondrogenesis of MSCs.

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1 Introduction

The repair of damaged articular cartilage, which rarely heals spontaneously and develops osteoarthritic changes, is a common clinical issue [1, 2]. Although various surgical techniques including subchondral drilling and joint arthroplasty have been attempted in order to restore the injured cartilage [3–5], their intrinsic healing capacity still remains to be a limitation. Autologous chondrocyte transplantation has been adapted as a promising technique for the treatment of articular cartilage defects [6, 7]. However, the long-term risk of developing osteoarthritis at the donor site [8] and the fibrous tissue formation instead of hyaline one at the chondrocyte implanted site [9, 10] have also been reported as critical problems for clinical application. Mesenchymal stem cells (MSCs), which have the potentials for self-regeneration and differentiation into certain cell types, have been given a great deal of attention as alternative cell sources for articular cartilage regeneration [1, 11–19]. The MSCs are isolated from various tissues including bone marrow, muscle, skin, vasculature, brain, liver, synovium and adipose tissue. It has been reported that the MSCs can bring about chondrogenic differentiation by various growth factors, including an insulin-like growth factor (IGF), a transforming growth factor-beta 1, 2, 3 (TGF-βs), and a bone morphogenic protein 2, 6, 7 (BMPs) [20–23]. In order to induce the chondrogenesis from the MSCs, high doses of growth factor or a combination of multiple growth factors (which more closely mimic the in vivo environment during the cell differentiation process) have been attempted [22, 24, 25].

Growth factors are highly sensitive to heat, pH and proteolytic degradation [26, 27]. Therefore, the use of delivery systems which can provide long-term stability and bioactivity of growth factors as well as the controlled

delivery of them should be considered for the enhanced chondrogenic differentiation of MSCs. In order to accomplish this, various hydrogels fabricated from natural polymers such as agarose, alginate, chitosan, fibrin, gelatin and hyaluronate as well as synthetic polymers such as poly(ethylene glycol), poly(vinyl alcohol), poly(acrylic acid) and their derivatives have been utilized for the delivery systems of growth factors [27]. Among these polymers, the alginate hydrogel has been widely used for the growth factor delivery because of its mild and easy gelation process that does not require any toxic chemicals that may inhibit the activity of growth factors. Furthermore, it has good binding affinity with growth factors, as well as a relatively slow release of them (especially positively charged growth factors in physiological condition) [28, 29]. The alginate, which is a monovalent salt form of alginic acid, is a linear block copolymer composed of β-D-mannuronate (M-block) and α-L-guluronate (G-block) linked by 1,4-glycoside linkage [30]. The G-block of alginate has correspondingly high affinities for divalent ions such as calcium (Ca^{2+}), strontium (Sr^{2+}), and barium (Ba^{2+}) at room temperature and when in an aqueous solution of divalent ions, the alginate chains are rapidly crosslinked via the staking of G-blocks to form an egg-box structure and subsequently become a gel. It was approved by the Food and Drug Administration (FDA) in the United States for human use as material for dressing wounds and also as a food additive. In order to introduce growth factors in alginate hydrogels for their sustained release, several strategies including direct loading [31], electrostatic interaction [32] and covalent binding [33] techniques have been attempted. The introduction of microparticles as a growth factor carrier in the hydrogel system was also done [34]. Although the microparticle/hydrogel systems were reported to have allowed for the sequential delivery of multiple growth factors, there was a loss of bioactivity of growth factors on account of being in direct contact with organic solvent during the fabrication process of microparticles (usually an emulsion method) and/or the chemical modification of microparticles as growth factor binding still remains as a limitation.

In this study, we fabricated a nanoparticle/hydrogel system for the controlled delivery of dual growth factors (faster BMP-7 and slower TGF-β_2 deliveries by TGF-β_2-immobilized polyion complex nanoparticles and the BMP-7-immobilized alginate hydrogel system). BMP-7 and TGF-β_2 are recognized as effective in bring about toward a chondrogenic differentiation, particularly by the combination of both growth factors (usually higher doses of BMP-7 than TGF-β_2) [25]. The fabrication of nanoparticles and the following growth factor immobilization does not involve any toxic organic solvents or chemical modification steps, and therefore can maintain the bioactivity of the growth factors. The release behavior of dual growth factors (BMP-7/TGF-β_2) from the nanoparticle-containing alginate hydrogel was compared with those of the dual growth factors from the alginate hydrogel without nanoparticles, as well as the single growth factors (BMP-7 or TGF-β_2) from the hydrogel.

2 Experimental

2.1 Materials

Sodium alginate (also called algin or alginic acid sodium salt; medium viscosity, Mw 80,000–120,000) was purchased from Sigma-Aldrich (St. Louis, MO) and washed several times with 65% methanol in order to remove impurities [35]. Calcium sulfate ($CaSO_4$; Oriental Chemical Industry, Korea) as a crosslinking agent of sodium alginate was used as received. Pluronic F68 (Mw 8,750) and heparin were purchased from BASF (Parsippany, NJ) and Celsus laboratories (Cincinnati, OH), respectively. Chitosan (low molecular weight, Mw 50,000–190,000) and poly(vinyl alcohol) (PVA; Mw 13,000–23,000; hydrolysis, 98%) were purchased from Sigma-Aldrich. Growth factors (TGF-β_2 and BMP-7) were purchased from R & D Systems (Minneapolis, MN). All other chemicals were analytical grades and were used as received. Water was purified (<18 mΩ) using a Milli-Q purification system (Millipore Co., Billerica, MA).

2.2 Fabrication of growth factor-immobilized nanoparticles

Growth factor (TGF-β_2)-immobilized nanoparticles were fabricated by the complexations of polymers and the growth factor (Fig. 1). Firstly, 100 μL of 20 wt% Pluronic F68 aqueous solution containing 7.52 μg (0.04 wt% based on Pluronic F68) of heparin (pH ~7.0) was gently shaken at room temperature in order to induce hydrogen bonding between Pluronic F68 and heparin [36]. Then, 1 μg of TGF-β_2 was added to 100 μL of Pluronic F68/heparin mixture [formation of ionic interaction between heparin (negative charge; pKa ~2.9 for the carboxylic acid and sulfate groups in heparin [37]) and TGF-β_2 (positive charge; pI ~7.4) without being shaken]. Subsequently, 100 μL of 0.5 wt% chitosan solution (in 0.1 M acetic acid) was introduced into the solution mixture (pH of the final solution, ~5.0) in order to form nanoparticles by an ionic interaction between heparin (negative charge) and chitosan (positive charge; pKa ~6.4 for the amine groups in chitosan) [37]. Then, 100 μL of 15 wt% PVA solution was added to an aqueous solution containing TGF-β_2-immobilized nanoparticles and was freeze-dried. The PVA was

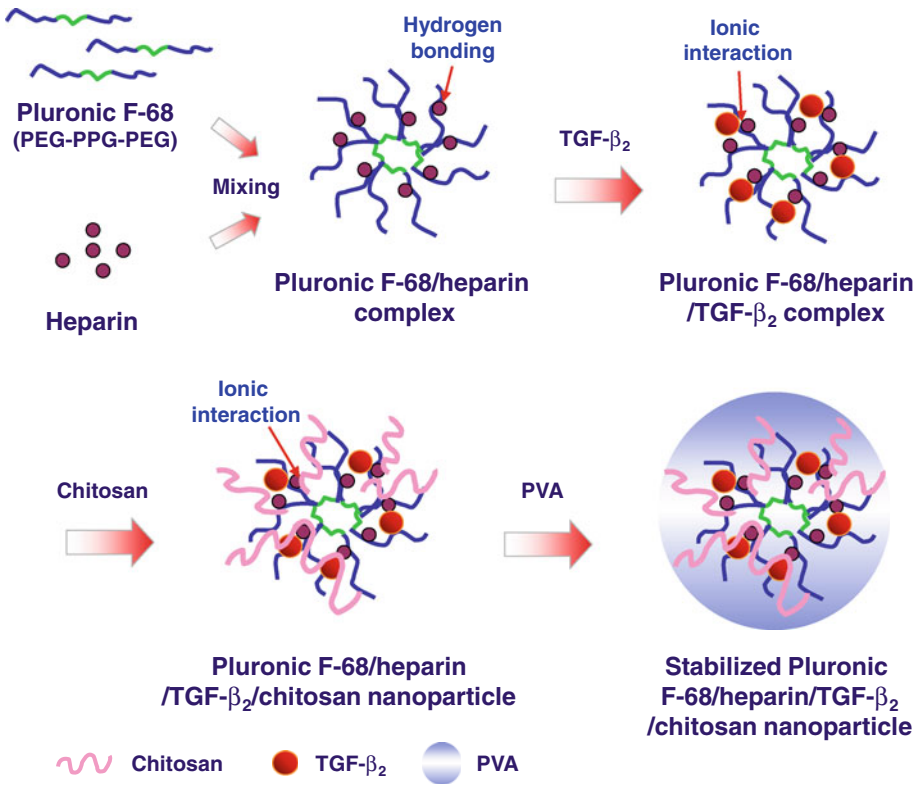

Fig. 1 The schematic diagrams showing the fabrication steps of the growth factor-immobilized nanoparticles

used for surface stabilization of the nanoparticles and the prevention of aggregation between them in an aqueous condition [38]. The size and morphology of TGF-β_2-immobilized nanoparticles in a dry state were observed by a field emission-scanning electron microscope (FE-SEM; JSM-6700F, JEOL, Japan). The size distribution of TGF-β_2-immobilized nanoparticles which were re-suspended in an aqueous solution was analyzed using an electrophoretic light scattering photometer (ELS-8000, Otsuka electronics, Japan).

2.3 Fabrication of growth factor-immobilized alginate hydrogel

Sodium alginate powder was dissolved in phosphate buffered saline (PBS, pH ~7.4) to be a polymer concentration of 2 wt% (0.5 mL). After $CaSO_4$ powders, which are used as a crosslinker of sodium alginate, were homogeneously dispersed in PBS containing growth factors (0.5 mL) [single BMP-7 (2 μg/mL); single TGF-β_2 (2 μg/mL); dual BMP-7/TGF-β_2 (each 2 μg/mL); dual BMP-7/TGF-β_2 (in nanoparticle) (each 2 μg/mL), respectively] to be a concentration of 0.8 wt%. Then, the alginate solution was directly mixed with the growth factor-containing $CaSO_4$ solution having the same volume (final alginate concentration, 1 wt%; final each growth factor concentration, 1 μg/mL) in the polypropylene (PP) mold (ø ~12.5 mm). The growth factor loading amount in the (nanoparticles/) hydrogel system was assumed as same as the initial growth factor content. The alginate/growth factor-containing $CaSO_4$ mixture solutions changed into a gel state over time. The gelation rate of alginate/$CaSO_4$ system was controlled by adjusting the concentration of $CaSO_4$ [39].

2.4 Growth factor release study

Growth factors [BMP-7, TGF-β_2, BMP-7/TGF-β_2 or BMP-7/TGF-β_2 (in nanoparticles)]-immobilized cylindrical alginate hydrogels (diameter, ~12.5 mm; thickness, ~8 mm; volume, 1 mL) were incubated in a 2 mL PBS supplemented with 1% BSA (Sigma-Aldrich) at 37°C for a period of up to 3 weeks while undergoing mild shaking (~50 rpm) in order to perform the release study. At preset time intervals, the total incubation solutions were collected and replaced with fresh PBS. The amount of released growth factors in the collected medium was determined using an ELISA kit.

3 Results

3.1 Characterization of nanoparticle/hydrogel system

Growth factor (TGF-β_2)-immobilized nanoparticles as a secondary delivery carrier in order to provide a more sustained growth factor release in the hydrogel system were

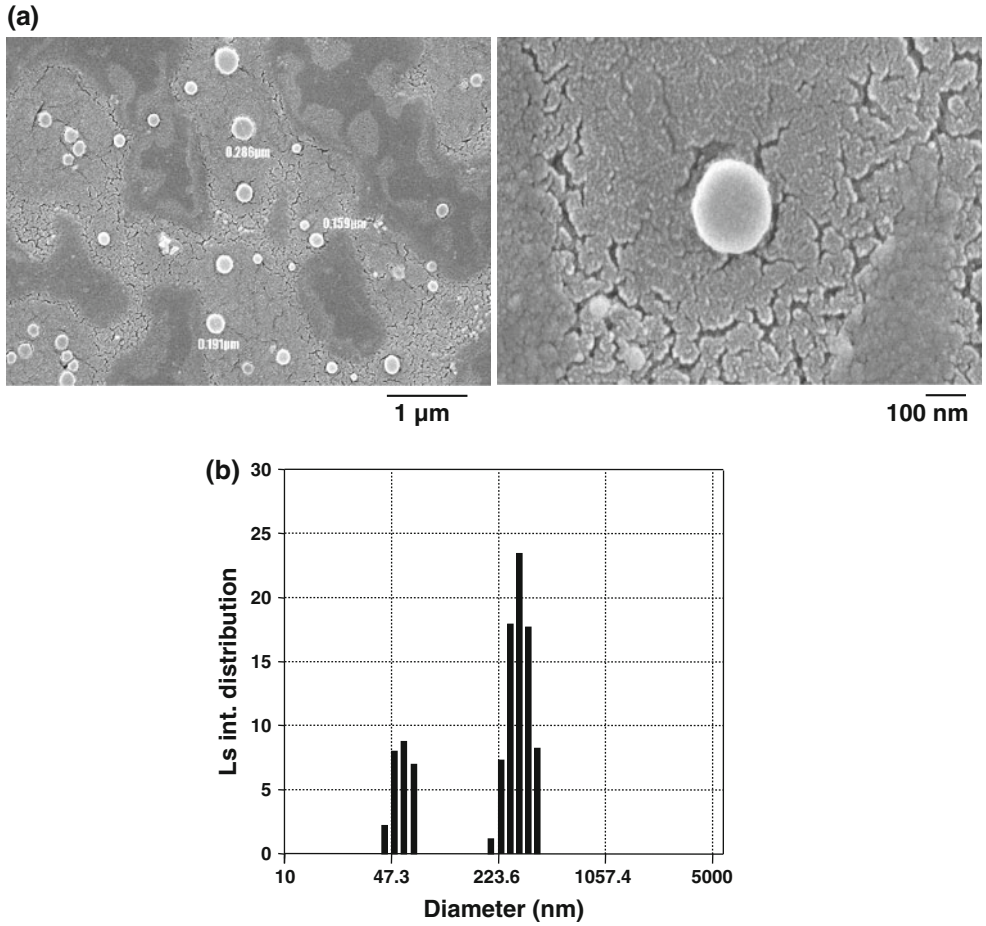

Fig. 2 (**a**) FE-SEM images and (**b**) size distribution of the growth factor-immobilized nanoparticles

fabricated by a complexation between the polymers (Pluronic F68/heparin) and TGF-β_2, and the following chitosan complexation, as well as PVA stabilization (refer to Fig. 1). Figure 2a shows the FE-SEM image of the prepared TGF-β_2-immobilized nanoparticles in a dry state. The average diameter of nanoparticles was approximately 100–200 nm with spherical shape. It was observed that the dried nanoparticles were re-suspended in an aqueous solution in a stable and uniform manner without the collapse of the nanoparticles, which can allow for homogeneous dispersion in the hydrogel. The nanoparticles have an average diameter of 426.3 ± 15.7 nm in the re-suspended state, as analyzed by ELS (Fig. 2b).

The alginate hydrogel with controllable gelation rate, which is done by adjusting the $CaSO_4$ concentration [39], was selected as a carrier for growth factors and nanoparticles. The hydrogel may also be a good carrier for MSCs. For the growth factor immobilization, we prepared the alginate solution in PBS containing 0.4 wt% $CaSO_4$ (the gelation time, approximately 4 min; refer to Fig. 3), as the gelation time was sufficient to handle the solutions for the experiment. The growth factors can easily be dispersed in the alginate solution prior to gel formation. Two different growth factors, BMP-7 and TGF-β_2, which are recognized as effective inducers toward a chondrogenic differentiation, were incorporated into the alginate hydrogel, separately and both of them together. The TGF-β_2-immobilized nanoparticles were also incorporated into the alginate hydrogel containing BMP-7, by dispersing the nanoparticles in the alginate solution prior to the gel formation.

3.2 Growth factor release behavior

The release profiles of growth factors from the growth factors-immobilized alginate hydrogels (single BMP-7 or TGF-β_2; and dual BMP-7/TGF-β_2) are shown in Fig. 4. The growth factors were continuously released from the single growth factor-loaded hydrogels for 21 days (up to 70% for BMP-7 and 50% for TGF-β_2), as shown in Fig. 4a. The slower release of TGF-β_2 than BMP-7 may be explained by their respective molecular weights, TGF-β_2 (25.0 kDa) has relatively higher molecular weight of than BMP-7 (15.4 kDa) and this may account for the slower release. It was reported that the proteins including growth

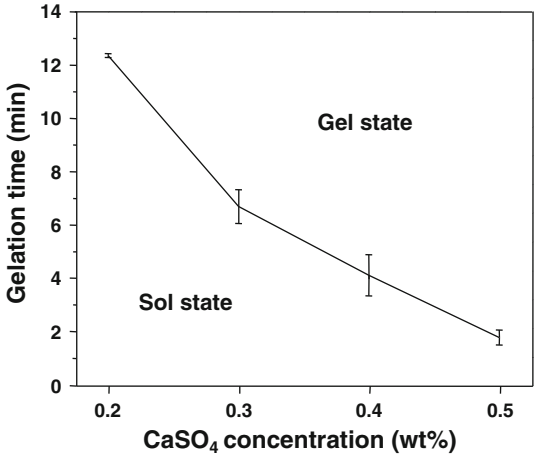

Fig. 3 Gelation rates of alginate solution in PBS as a function of $CaSO_4$ concentration ($n = 5$)

factors in alginate hydrogel were released by the diffusion of them through the pores in the hydrogel network (pore size 5–200 nm, depending of crosslink density) [40, 41]. The release of each growth factor from the dual growth factors (BMP-7/TGF-β_2)-loaded hydrogel was much slower as compared to the single growth factor-loaded one, approximately 36% (BMP-7) and 16% (TGF-β_2) for 21 days, as shown in Fig. 4b, even though the same amount of growth factors were loaded. This phenomenon may be explained by the aggregation between growth factors with different isoelectric points (TGF-β_2, pI $\sim$7.4; BMP-7, pI $\sim$8.9) during the hydrogel fabrication step (dispersing of the growth factors in alginate solution), that can also reduce the bioactivity of the growth factors [42, 43].

The release profiles for each growth factor from the nanoparticle/hydrogel system [TGF-β_2 (in nanoparticle)/BMP-7 (in hydrogel) are shown in Fig. 5. The dual growth factors-loaded nanoparticle/hydrogel system showed a much higher BMP-7 release behavior than the dual growth factors-loaded hydrogel (without nanoparticles), more similarly to the single BMP-7-loaded hydrogel (refer to Fig. 4a), indicating the growth factor release without aggregation. The TGF-β_2 immobilized in the nanoparticles showed a much slower release than the BMP-7, indicating the individual and sequential release of dual growth factors from the nanoparticle/hydrogel system. The possible mechanisms for the sequential release of both growth factors in the nanoparticle/hydrogel system are shown in Fig. 6.

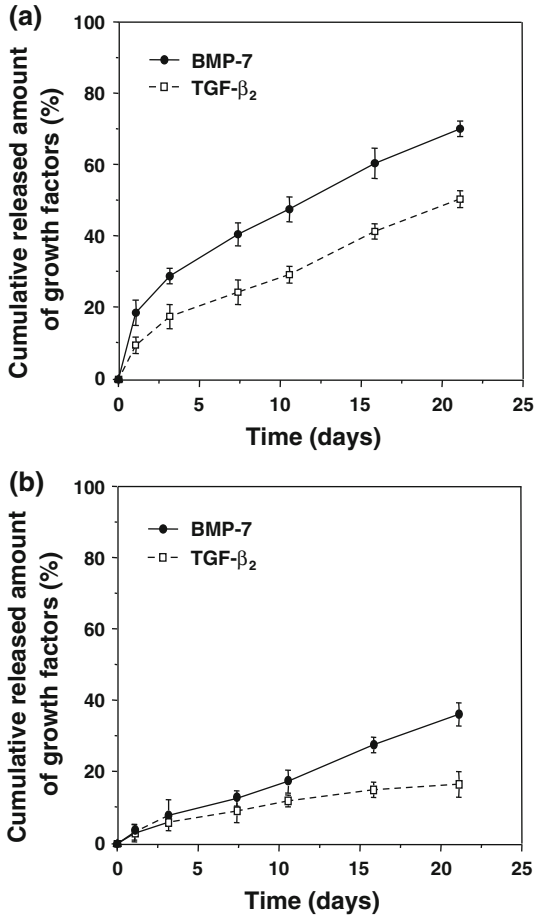

Fig. 4 Cumulative released amount of growth factors from (**a**) the single growth factor (TGF-β_2 or BMP-7)-immobilized hydrogels and (**b**) the dual growth factor (TGF-β_2 and BMP-7)- immobilized hydrogel ($n = 3$)

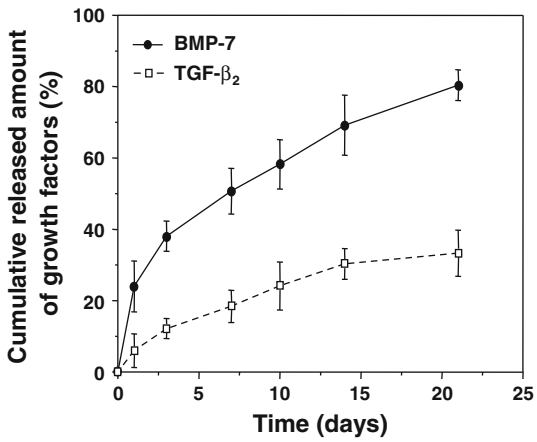

Fig. 5 Cumulative released amount of growth factors from the nanoparticle/hydrogel system (TGF-β_2 in nanoparticle/BMP-7 in hydrogel) ($n = 3$)

4 Discussion

MSCs have been referred to as attractive cell sources for cartilage regeneration, and this is attributed to their capacity of differentiation into multi-lineages, self-renewal, abundance of sources, and accessibility [11–19]. It is well understood that bioactive molecules such as

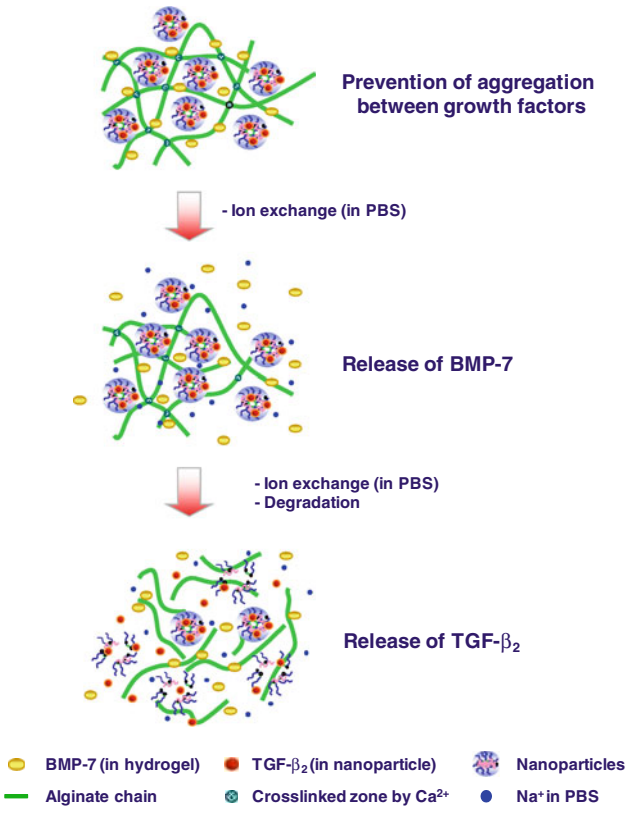

Fig. 6 Possible mechanisms for the sequential release of growth factors in the nanoparticle/hydrogel system

growth factors and cytokines play an important role for cell adhesion, proliferation and differentiation. The high doses of growth factors, such as IGF, TGF-βs and BMPs, have demonstrated effective induction of chondrogenesis from the MSCs. However, concerns regarding the cost and safety of growth factors remain as practical limitations [44]. In order to have efficient and economical induction of the chondrogenesis from MSCs, multiple growth factor systems as a soluble form in cell culture mediums have been adapted [22, 24, 25]. Recently, it was generally agreed upon that the use of delivery systems which can provide long-term stability and bioactivity as well as the controlled delivery of growth factors may become a more advanced system for chondrogenic differentiation. The sequential delivery of growth factors have also been applied to prevent dedifferentiation of MSCs [45].

This study focused on a delivery system with individual and controlled release of dual BMP-7 and TGF-β_2 (faster release of BMP-7 and slower release of TGF-β_2), which can more efficiently enhance the chondrogenesis of MSCs, as well as cartilage regeneration, based on a previous report [25]. In order to illustrate this, we prepared a dual growth factors-loaded nanoparticle/hydrogel system (TGF-β_2 immobilized in Pluronic F68/heparin/chitosan complex nanoparticles and BMP-7 immobilized in gelation rate-controllable alginate). Heparin has been widely used for the binding of various growth factors because of their controlled release [46, 47]. TGF-β_2 was immobilized in the nanoparticle by ion complexations between both *O*-sulfate and *N*-sulfate groups of heparin molecules in the nanoparticle and certain lysine, as well as arginine residues in the growth factor. The immobilized growth factor can be released from the nanoparticles by their degradation as well as ion exchange in PBS [48]. The loading of growth factors into alginate hydrogel is commonly performed in an excess $CaCl_2$ solution for crosslinking. This gelation process may cause the loss of the growth factor by the diffusion of the growth factor from the concentrated gel to a less concentrated large volume crosslinking solution [28]. In order to prevent the loss of growth factor during the process, we utilized the alginate/$CaSO_4$ mixture solution so that gelation can occur in one phase without the use of an excess crosslinking solution. The gelation rate can be controlled by adjusting the $CaSO_4$ concentration. The gelation time of alginate solution in PBS decreased when $CaSO_4$ concentrations were increased from approximately 12 min with 0.2 wt% $CaSO_4$ to 2 min with 0.5 wt% $CaSO_4$ [39]. The control of gelation rates is particularly advantageous as an injectable cell or growth factor carrier system for the direct injection into damaged tissues, such as cartilage, bone, etc. The growth factors in the alginate hydrogel can be continuously released by the diffusion through pores in the hydrogel network as well as ion exchange in PBS [40]. From the individual and controlled release of both growth factors in nanoparticle/hydrogel system (refer to Fig. 5), we can recognize that the nanoparticle/hydrogel system may provide desirable growth factor delivery kinetics for cartilage regeneration, as well as the chondrogenesis of MSCs, i.e., fast release of BMP-7 but slow release of TGF-β_2. In order to verify this, the studies on the in vitro chondrogenesis of MSCs (bone marrow and adipose stem cells) and in vivo cartilage regeneration (rat model) using the nanoparticle/hydrogel system with dual growth factors continues.

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