Injectable bone substitute composed of biodegradable polymer/hydroxyapatite composite microspheres containing antibiotics

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Injectable bone substitute composed of biodegradable polymer/hydroxyapatite composite microspheres containing antibiotics

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This certifies that the Master's Thesis
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ABSTRACT

Injectable bone substitute composed of biodegradable polymer/ hydroxyapatite composite microspheres containing antibiotics

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Many studies to control the absorption and release of drugs such as antibiotics and growth factors into and from the pores of inorganic microspheres have been conducted. The aim of this study was to confirm the possibility of methylcellulose aqueous solution containing poly(DL-lactide-co-glycolide)(PLGA)/hydroxyapatite (HA) composite microspheres and antibiotics, as a sustained drug release-bone substitute by estimating thermogelation temperature of methylcellulose aqueous solution by controlling salt added, injectability and drug-release behavior of the injecting system by controlling size and content of the microspheres.

HA nanoparticles which is a well-known key bioceramic material frequently used as a drug carrier, ocular implant, bone substitute for filling bone defects, scaffold matrix for tissue engineering and as a coating agent on biomedical implants due to its chemical and biological similarity with natural bone mineral were synthesized by coprecipitation method. PLGA/HA composite microspheres containing tetracycline (TC) were prepared by emulsion-solvent evaporation method. In this process, 2.5, 5, 10, and 20 % PLGA concentration in dichloromethane (DCM) were used in order to compare the effects of different PLGA concentration on size of the microspheres. By estimating thermogelation temperature of methylcellulose aqueous solution by controlling salt added, injectability and drug-release behavior of the injecting system by controlling size and content of the microspheres, we optimized the injectable bone substitute composed of methylcellulose (MC) aqueous solution and PLGA/HA composite microspheres containg TC. We prepared PLGA/HA microspheres

containing TC.

The PLGA/HA microspheres showed high drug loading efficiency of up to 70.3%. We could control the drug release behavior from the microspheres by controlling the size of microspheres from 17.8 ± 4.5 to 185.4 ± 29.5 μm. Especially, P20 with a particle diameter of 185.4 ± 29.5 μm showed high drug contents of over 70% and sustained drug release over 2 weeks without initial burst, a major problem of drug delivery systems.

Key words: injectable bone substitute, drug release, composite microsphere

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I. INTRODUCTION

1. Bone substitutes

A. Bone graft

The need for bone substitutes is rapidly increasing in the field of orthopaedic surgery, since advanced procedures are now being performed in reconstructive surgery after traumatic pathologies and iatrogenic bone losses secondary to bone resections for tumours, infections or pseudoarthroses. Moreover, the increasing number of elderly patients or individuals with various systemic pathologies and biological drawbacks related to bone healing processes, often requires the use of bone substitutes as an adjuvant therapy to be associated with prosthetic implants in order to improve biological fixation and osteointegration processes[1,2].

B. Bone graft characteristics

Osteogenesis, osteoinduction, and osteoconduction are the three essential elements of bone regeneration along with bonding between host bone and grafting material which is called osteointegration.

(A) Osteogenic potential

The osteogenic potential of a graft is derived from its cellular content. Osteogenic graft materials contain viable cells that possess the ability to form bone (determined osteogenic precursor cells) or the potential to differentiate into bone-forming cells (inducible osteogenic precursor cells). These cells participate in the early stages of the healing process to unite the graft to the host bone and must be protected during the grafting procedure to ensure viability. This potential to produce bone is characteristic only of fresh autogenous bone and bone marrow cells.

(B) Osteoconduction

Osteoconductivity is the physical property of a graft material that allows the ingrowth of neovasculature and infiltration of osteogenic precursor cells during the process known as creeping substitution. A graft material that is only osteoconductive transfers neither osteogenic cells nor inductive stimuli, but acts as a nonviable scaffold or trellis that supports the bone healing response. Materials that are osteoconductive include autogenous and allograft bone, demineralized bone matrix, collagen, and calcium phosphate ceramics.

(C) Osteoinduction

Bone induction is the process by which some factor or substance stimulates an undetermined osteoprogenitor stem cell to differentiate into an osteogenic cell and is mediated by numerous growth factors found within the bone matrix itself. This concept was introduced by Urist et al[3,4] in the 1960s after initial studies on the osteoinductive properties of demineralized bone matrix (DBM) and bone morphogenetic protein (BMP)[3,4]. In addition to these materials, autogenous bone and allograft are known to possess osteoinductive properties.

C. Bone graft materials

(A) Autograft

Autologous bone, the golden standard of bone grafting, provides optimal osteoconductive , osteoconductive, and osteogenic properties[5]. Iliac crest is the most frequently chosen donor site as it provides easy access to good quality and quantity cancellous autograft. Harvesting autologous bone from the iliac crest has, however, several downsides as it lengthens the overall surgical procedure and is usually complicated by residual pain and cosmetic disadvantages[6]. Furthermore, it may fail in clinical practice as most of the cellular (osteogenic) elements do not survive transplantion[7]. Other limitations include elderly or paediatric patients and patients with malignant disease[11-13]. In addition autograft harvesting is associated with a 8.5-20% of complications including haematoma formation, blood loss, nerve injury, hernia formation, infection, arterial injury, ureteral injury, fracture, pelvic instability, cosmetic defects, tumour transplantation, and sometimes chronic pain at the donor site [6,9-12].

(B) Allograft

Allograft is the most frequently chosen bone substitute and is regarded as the surgeon's second option[13]. Its use has increased 15-fold the past decade and accounts for about one-third of bone grafts performed in the United States[14]. The current increasing availability of allograft tissue has made it possible to manufacture customised types, such as dowels, strips, and chips[7]. Allograft bone has more limitations in the essential bone graft characteristics described earlier and yields more variable clinical results. In addition, allografts carry the risk of transferring viral diseases. The processing of allograft tissue lowers this risk but, that can significantly weaken the biologic and mechanical properties initially present in the bone tissue[15-17].

(C) Xenograft

Xenografting is the transfer of tissue, of whatever nature, from one species to another. It has been widely used in orthopaedic surgery, though with mixed success. Bovine bone has been used as an aid to spinal fusion, in the management of bone tumours, and the recreation of lost bone stock at revision joint replacement. Frequently such xenograft material is mixed with autograft in an attempt to overcome

the volume problem of autograft and yet the compatibility problem of xenograft.

Xenograft tissue can incorporate into a recipient. However, it does not always do so fully and may be associated with an inflammatory reaction following insertion. In recent years investigators have been looking at the use of transgenic bone from genetically-engineered pigs as a possible source of tissue for man. Laboratory studies using monkeys (if one ignores the animal rights issues for the moment) appear to be very promising. However, there are obviously major ethical issues with such a technique, so only time can tell whether it is likely to be used to any great extent in man. DBM can be produced through decalcification of cortical bone. Then, it is processed in order to reduce the potential for infection and immunogenic host response. The material produced, the DBM, retains the trabecular collagenous structure of the original tissue and can serve as a biologic osteoconductive scaffold despite the loss of structural strength once contributed by the preexisting bone mineral. Bone demineralisation does not eliminate all bone growth factors, which are now more bio-available with the mineral phase removed. Thus, DBM can be more osteoinductive than standard mineralised allograft[7,18]. Clinical results are not uniformly good, attributed partially to non-uniform processing methods[7,19,20]. DBM is supplied as a gel, malleable putty, flexible strips, mouldable paste with bone-chips or injectable bone paste. DBM, nonetheless, is widely used as a ‘‘bone graft extender’’ rather than as a bone graft substitute.

Extracellular bone matrix is very rich in collagen. Collagen contributes to mineral deposition, vascular ingrowth, and growth factor binding, providing a favourable environment to bone regeneration[21]. Nevertheless, collagen may carry potential immunogenicity and provides minimal structural support. So far, studies have demonstrated its primary use as a delivery system for other osteoconductive, osteoinductive, or osteogenic factors with mixed clinical results[18]. Collagen functions poorly as a graft material but once is coupled with bone morphogenetic proteins, osteoprogenitor precursors, or hydroxyapatite (HA), it enhances significantly graft incorporation. Collagen is usually used as composite with other bone substitutes (gel, granules, with biphasic ceramic of HA and tricalcium phosphate, bone marrow, etc.), and whilst they have shown inconsistent results, they can be used as effective autograft extenders[22].

(D) Synthetic materials

Ceramics are synthetic scaffolds made from calcium phosphate that have been used in dentistry and in orthopaedics since the 1980s[20,23,24]. Tricalcium phosphate ceramic has a stoichiometry similar to amorphous bone precursors, whereas HA has a stoichiometry similar to bone mineral. Ceramics do not exist naturally, but they have been shown to induce a biologic response similar to that of bone. Alone, synthetic ceramics posses no osteogenic or osteoinductive properties, and demonstrate minimal immediate structural support. When attached to healthy bone, osteoid is produced directly onto the surfaces of the ceramic in the absence of a soft tissue interface. Consequently, the osteoid mineralizes and the resulting new bone undergoes remodelling.

HA is a well-known key bioceramic material frequently used as a drug carrier, ocular implant, bone substitute for filling bone defects, scaffold matrix for tissue engineering and as a coating agent on biomedical implants due to its chemical and biological similarity with natural bone mineral[25]. The stoichiometric HA has chemical composition of $Ca_{10}(PO_4)_6(OH)_2$ with atomic calcium to phosphorus (Ca/P) ratio of 1.67[26]. It is known to be biocompatible, bioactive i.e. ability to form a direct chemical bond with living tissues, osteoconductive, non-toxic, non-inflammatory and non-immunogenic agent[25,26]. As per literature survey, use of conventional HA in the form of thin film, powder, dense or porous blocks are abundance at microscale level[27-29]. But microscale HA is typically highly stable phase i.e. less bioresorbable, which is an undesirable characteristic because it impedes the rate of bone regeneration when used as a bone substitute in alveolar ridge augmentation related orthopedic surgery[30]. It is desirable that the HA for use in implants be bioresorbable so that it can be replaced, over a period of time, with regenerated bone upon implantation. The resorbability of HA can be improved with help of some ceramic oxides and ionic doping agents[31,32] or reducing its grain size to nano-level. Engineering HA at nano-level[33] would have amazing functional properties due to its grain size, large surface area to volume ratio and ultra fine structure similar to biological apatite, which would have a great impact on implant-cell interaction in body environment. Further, osteoconductivity, solubility, sinterability and mechanical reliability of the HA can be promoted by controlling its particle size and structural morphology in the order of nanoscale[34]. Keeping the above

points in view, present study was aimed to synthesise nanocrystalline HA by wet chemical method with superior bioresorbtion. HA is the main mineral component of bones and teeth, and it has been widely used in the orthopedic and dental fields as a paste, granules, or porous blocks for implants[35]. It is biodegradable, but the process is very slow. HA is also utilized as an adsorbent during chromatography for purification and separation because of its excellent adsorption of many molecules[36]. Blocks of porous HA and HA ceramics have also been investigated for use in sustained-release drug delivery systems (DDS) for various therapeutic agents[37-39]. It has already been reported that implanted HA preparations of antibiotics and anticancer drugs showed slow release in animals[20].

2. Injectable bone substitutes

In recent years, there have been reports of successful in vivo bone formation in experimental animals using bone marrow mesenchymal stem cells, a solid matrix, and bone morphogenetic proteins[39,40]. If this bone formation can be achieved using injectable gels, this may offer several advantages over the preformed solid scaffold approach. A fluid material can fill any shape of defect, may incorporate various therapeutic agents (e.g., growth factors), need not contain residual solvents such as those present in a preformed scaffold, and finally they do not require an open surgical procedure for placement. For these reasons, several injectable biomaterials such as collagen gel[41], polyethylene oxide[42], calcium alginate[43] and fibrin glue[44,45] have been developed. Recently, with the increasing popularity of minimally invasive techniques, one of the major thrusts in orthopedics has been to develop injectable systems that can mould to the shape of a bone cavity and polymerize when injected in situ. Such devices should shorten the surgical operation time, minimize the damaging effects of large muscle retraction, reduce the size of the scars and lessen post-operative pain, allowing patients to achieve rapid recovery in a cost-effective manner.

Two types of injectable bone substitutes (IBS) are presently under development in laboratories. The first consists of ionic hydraulic cements that set in vivo after injection. Their components need to be mixed before injection, and hardening is achieved by recrystallization of the mineral phase. For instance, Norian SRS[46], a new ionic cement that hardens in physiological conditions, is a blend of monocalcium

phosphate monohydrate (MCPM) with α-tricalcium phosphate (α-TCP) and calcium carbonate (CC). The hardening reaction occurs when a sodium phosphate solution is added and mixed with the powder. The main limitations with these materials are the need to mix the components before use and the existence of a dense structure after hardening that cannot be readily colonized by cells. The most commonly used injectable bone cement is poly(methyl methacrylate) (PMMA), but it suffers from the fact that it does not degrade and that its high curing temperatures can cause necrosis of the surrounding tissue[47]. Therefore, further development of alternative injectable materials is necessary.

The second type consists of CaP ceramic suspensions in carrier phase, which are ready to use. Injectable biomaterial has recently been developed that combines a polymeric water solution viscous phase (nonionic cellulose ether) with bioactive CaP ceramic granules[48]. Ionic polymers are not used because of their spontaneous ionic reticulation in the presence of CaP in water, causing a volumetric contraction of the composite mixture. Instead, cellulosic nonionic hydrophilic derivatives have been used. This formulation can be modified to provide ready to-use sterilized injectable material that decreases the risk of infection since the product does not need to be prepared during surgery. The fillers are maintained in viscous phase at the surgical site. The mechanical strength of this material results from early osteoconduction and bone substitution[49]. Cellulose ethers like methylcellulose (MC) and hydroxypropylmethylcellulose (HPMC) posse the unique property of reversible thermogelation. In solution form these polymers are completely hydrated and there is little polymer–polymer interaction other than simple entanglement. As the temperature is increased, an initial drop in viscosity is observed due to the decrease in the hydration water. When critical temperature is reached, sufficient dehydration occurs to promote polymer–polymer interactions instead of polymer–solvent interactions. As a consequence, these cellulose ether solutions start to gel. Upon cooling, the gelation process is completely reversed and the gel formed will revert to the sol state, recovering its original consistency. The temperature at which the gelation process starts and the strength of the gel formed depend on the type and degree of substitution of the gum, molecular weight, concentration and presence of electrolytes[50].

The thermogelation property of MC and HPMC has meant that they have a wide

range of multidisciplinary applications. In the food industry, one well-known application of the thermogelation properties of MC and HPMC, along with their film forming ability, is their use in batter formulas or as edible coatings[51] for moisture retention and oil reduction in fried food. Their use as coatings in marinated chicken strips also has performed successfully to avoid the migration of acetic acid from the product to the oil, thus prolonging the useful life of the frying oil[52]. Another application is found in bone tissue engineering. Thermogelation of MC and HPMC also make them useful as carrier material of bone substitute such as calcium phosphate granules. According to the 'Hofmeister series', an order of ions ranked in terms of how strongly they affect hydrophobicity of a solute, ions may either stabilize or weaken the structure of water due to their different interactions with water molecules. Anion in a salt, believed to deliver stronger effect than cation, is the deciding factor for the salt effect. A typical Hofmeister order for anions was reported as $SO_4^{2-}>F^->Cl^->Br^->NO_3^->ClO_4^->I^->SCN^-$. Ions on the left can be strongly hydrated, exhibiting strong interactions with water molecules. As a result, they tend to cause 'salt-out' or to enhance hydrophobicity of a solute in water. In contrast, ions on the right can be weakly hydrated, tending to cause 'salt-in', which increases the solubility of a non-polar solute.

3. Bone substitutes containing therapeutic agents

Many studies have shown the benefit of in vitro and in vivo associations of therapeutic agents such as antibiotics, growth factors and steroid hormones with bone substitute[53]. Conventional treatment of acute and chronic bacterial osteomyelitis includes surgical removal of necrotic bone tissue and repeated irrigations combined with high systemic doses of antibiotic drug substances for prolonged periods of time. This treatment frequently causes unwanted side effects and often fails to cure bacterial bone infections. Therefore, drug delivery systems providing controlled release of antibiotics were studied for local therapy as an alternative[54]. All of these experiments were performed with adsorption of the drug in mineral phases. However, with these formulations the amount of drug really adsorbed is difficult to control and release kinetics is often quite variable. Microencapsulation of drugs can be proposed to solve these problems. More recently drug delivery systems were developed from

biodegradable materials such as degradable polymers[55].

PLGA-based microparticles offer various advantages compared to other controlled DDSs, including: (i) the possibility to accurately control the resulting drug release kinetics over periods of days to months; (ii) complete biodegradability (avoiding the removal of empty remnants upon drug exhaust); (iii) good biocompatibility, even if directly administered into brain tissue[56]; (iv) easy administration using standard syringes and needles. Thus, this type of controlled drug delivery system can be very helpful to optimize the therapeutic efficiency of medical treatments and to reduce serious side effects[57,58]. There are other factors that may have an effect on the drug release mechanism like the type/ properties of the carrier microshere, the structure and properties of the drug molecule and thus the type of interactions established between the drug and the polymeric matrix. The size of the microsphere, a key factor in the rate of release, may have a significant effect on product performance as well as safety. Large particles can origin capillary obstruction when injected intravenously. Beyond that, particle size can also influence the syringability of the product. In order to understand the effect of these properties on the drug release profile, microspheres containing antibiotics with different sizes were produced studies were performed.

4. Objective of this study

The aim of this study was to confirm the possibility of MC aqueous solution containing PLGA/HA composite microspheres and antibiotics, as a sustained drug release-bone substitute by estimating thermogelation temperature of MC aqueous solution by controlling salt added, injectability and drug-release behavior of the injecting system by controlling size and content of the microspheres.

Ⅱ. MATERIALS AND METHODS

1. Synthesis of the hydroxyapatite nanoparticles

The HA nanoparticles was prepared by coprecipitation method using $Ca(NO_3)_2 \cdot 4H_2O$ (Sigma, USA) and $(NH_4)_2HPO_4$ (Sigma, USA) as Ca and P precursors, respectively. Figure 1 shows the schematic diagram of the formation mechanism of HA nanoparticles. 0.3M aqueous solution of $(NH_4)_2HPO_4$ was added drop wise to 0.5M aqueous solution of $Ca(NO_3)_2 \cdot 4H_2O$ under a stirring condition of 10000 rpm to form a reaction solution (Figure 2). The pH of the reaction solution was adjusted to 10 by adding concentrated NH_4OH solution using a syringe and it was maintained at a constant temperature of 60 ℃. A suspension of HA seed particles precipitated from the reaction solution and they were aged for 24 h. The suspension, after aging, was filtered, washed until there is complete removal of water soluble ammonium chloride (NH_4Cl_2) and freeze-dried.

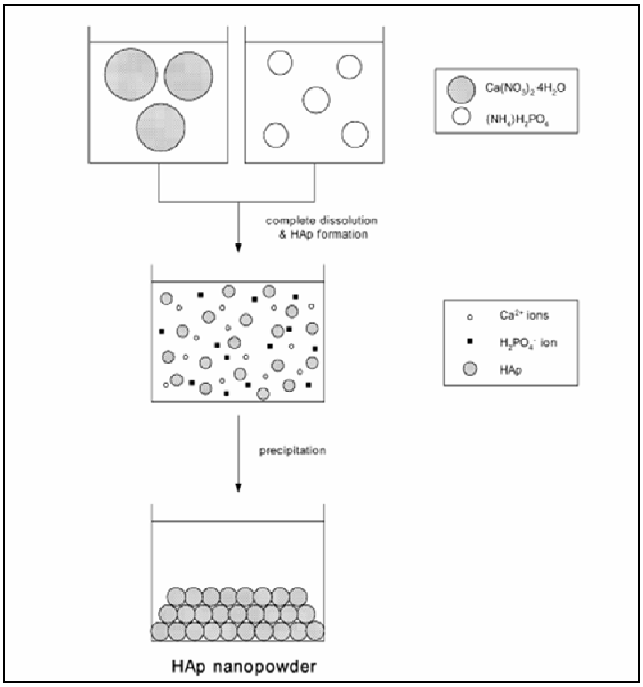


Figure 1. Schematic diagram showing the formation mechanism of HA nanoparticles.

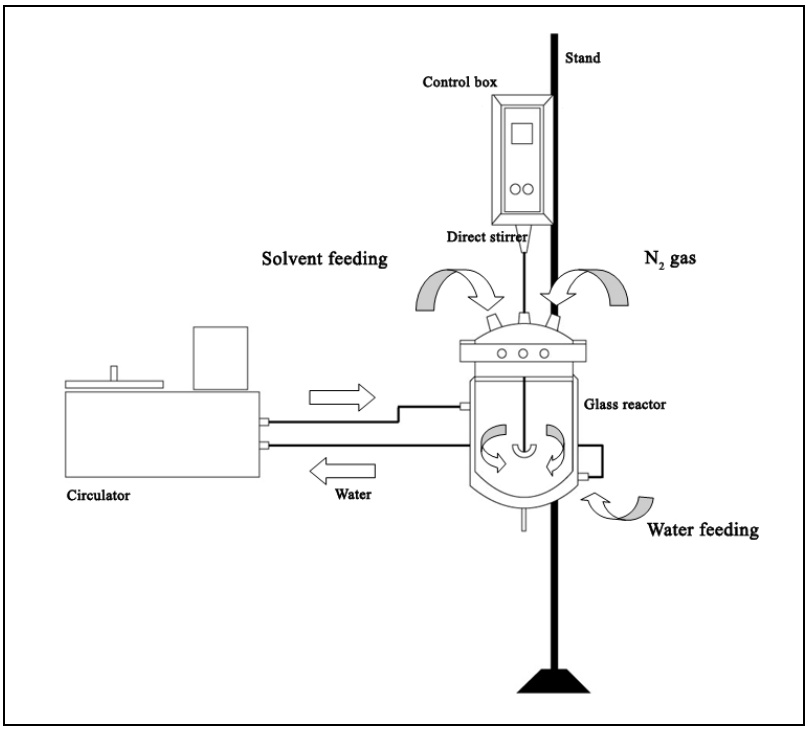


Figure 2. Schematic diagram of coprecipitation apparatus.

2. Characterization of the hydroxyapatite nanoparticles

A. Crystalline analysis

The structures of the synthesized HA particles were investigated using X-ray diffractometer (XRD; X'pert PW1830, Philips, Japan) with Ni-filtered Cu-kα ray (λ=1.5406 nm). Diffraction data were collected between 20° and 80° at a scan rate of 4°/min. The crystalline structures were identified according to JCPDS software (PCPDFWIN1.30, JCPDS-ICDD).

B. Morphological characterization

The average size and morphology were estimated using a transmission electron microscope (TEM; JEM4010, JEOL, Japan). For the TEM measurement, the aqueous dispersion of the particles was drop-cast onto a carbon coated copper grid and grid was air dried at room temperature before loading into the microscope.

3. Preparation of poly(DL-lactide-co-glycolide)/hydroxyapatite microspheres

PLGA (75:25), dichloromethane (DCM) and poly(vinyl alcohol) (PVA) were purchased from Sigma (Sigma-Aldrich Korea LTD). Tetracycline (TC) was obtained from Chong Kun Dang, Korea. PLGA/HA microspheres loaded with TC were prepared by an oil-in-water (O/W) emulsion/solvent evaporation protocol. Briefly, PLGA was dissolved in DCM at a concentration of 2.5-20%. The TC and HA powders were added in the PLGA solution. In this study, the amounts of TC and HA were fixed with a rate of 10:1:1 (PLGA:TC:HA (w:w:w)).

The PLGA solution containing TC and HA was mixed by magnetic stirrer and sonicated. The mixed organic solution was added to an aqueous solution containing 2% PVA. This mixture was homogenized with 3000 rpm and the organic phase was evaporated using a rotative evaporator under partial vacuum. The PLGA/HA microspheres were washed three times with distilled water in order to remove the PVA residue and the final product was freeze-dried. Four microsphere samples of different recipes were prepared (Table 1). In order to compare the effects of different PLGA concentration on size of the microspheres, 2.5, 5, 10, and 20% PLGA concentration in DCM were used and expressed as P2.5, P5, P10, and P20 respectively.

Table 1. Four microsphere samples of different recipes with 2.5, 5, 10, and 20% PLGA concentration in DCM with fixed amount of HA and TC

Sample number	PLGA concentration in DCM (% w/v)	PLGA/HA/TC ratio (w/w/w)
P2.5	2.5% PLGA in DCM	10 : 1 : 1
P5	5% PLGA in DCM	10 : 1 : 1
P10	10% PLGA in DCM	10 : 1 : 1
P20	20% PLGA in DCM	10 : 1 : 1

4. Characterization of the poly(DL-lactide-co-glycolide)/hydroxyapatite microspheres

A. Morphological characterization and particle size determination

The average size and morphology were estimated using an optical microscope (Olympus CK2, Olympus optical, Japan). For each sample the diameter of at least 100 microspheres was measured and averaged.

B. Drug content of the microspheres

The drug content of microspheres was determined spectrophotometrically after extraction from the microspheres. Briefly, 200 mg of microspheres loaded TC were dissolved in 5 mL DCM and subsequently an excess of methyl alcohol was added. This mixture was stirred to precipitate the PLGA and to remove DCM. After filtering the PLGA, 5 mL of aqueous solution dissolving TC was prepared. This solution was analyzed spectrophotometrically using calibration curves on a UV-visible spectrophotometer (UVD-3200, LABOMED, USA) at absorption maxima 275 nm. Loading efficiency was expressed as follow:

* Loading efficiency = (actual drug loading / theoretical drug loading) × 100

5. The phase transition temperature

MC (with a viscosity of 400 cP for a 2% (w/v) aqueous solution at 25 ℃) was obtained from Sigma (Sigma-Aldrich Korea LTD). The rheological tests of 2% MC aqueous solution were conducted on a fluid rheometer (RVDV-III Rheometer, Brookfield engineering laboratories, USA) equipped with temperature controller. The viscosities of 2% MC aqueous solutions blended with salt were measured through heating from 10 to 60 ℃ at a scanning rate of 2 ℃/ min. A ‘gel’ criterion was defined as the temperature at which the viscosity of the solution was suddenly raised. In this test, salt contents were varied from 2 to 6% to adjust the gelation temperature to 37 ℃, body temperature.

6. In vitro drug release test

200 mg of dried TC-loaded microspheres were immersed in 20 mL of saline and continuously shaken at 37 ℃. At preset time intervals, the samples were centrifuged and 3 mL of the upper clear solution was extracted. Meanwhile, 3 mL of the saline was renewed. The TC content of the release solution was analyzed by UV-visible spectrophotometer (UVD-3200, LABOMED, USA) at absorption maxima 275 nm and room temperature. All release experiments were carried out in duplicate or triplicate, and the average data were used for drawing figures.

7. Injectability test

The injectabilities of MC aqueous solution dispersed with PLGA/HA microspheres were measured to determine optimum injection condition through a needle, by the modification of the method of Bohner and Baroud[39]. For this, MC aqueous solution dispersed with PLGA/HA microspheres were filled in 1 mL with needles (18G). The syringe was placed in a cylinder-shaped equipment, which was attached in an universal test machine (AG-5000G, Shimadzu, Japan). A stainless rod (diameter, 4 mm) was hammered vertically at a crosshead speed of 10 mm/min on the gasket and the load–displacement curves were obtained. In this test, the powder/solution ratios were varied from 40/60 to 60/40 (w/v).

III. RESULTS

1. Characterization of the hydroxyapatite nanoparticles

A. Crystalline analysis

HA nanoparticles were synthesized by precipitation process. The phase of the dried samples was analyzed using an XRD. The prepared HA having broad diffracted peaks with low crystallinity indicates a microcrystalline nature (Figure 3). The XRD peaks exhibit no extraneous phase corresponding to HA, which suggests that the reaction has been completed.

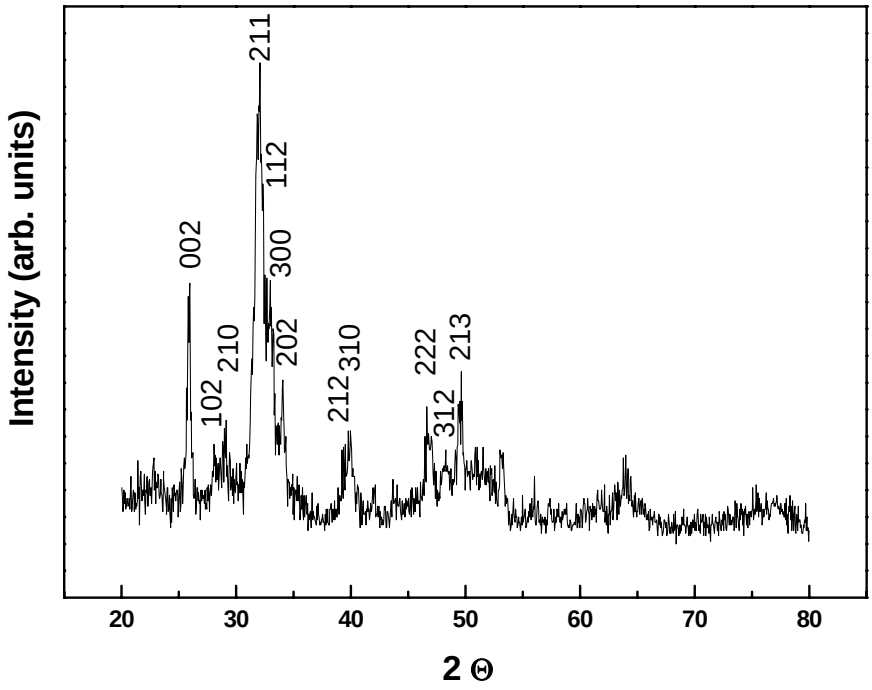


Figure 3. XRD patterns of HA nanoparticles.

B. Morphological characterization

The particles size and morphology were examined using TEM for the HA nanoparticles synthesized by coprecipitation method. Figure 4 shows the TEM image of the HA nanoparticles with needle-shape. The crystallite size was about 20 nm in width and 100 nm in length.

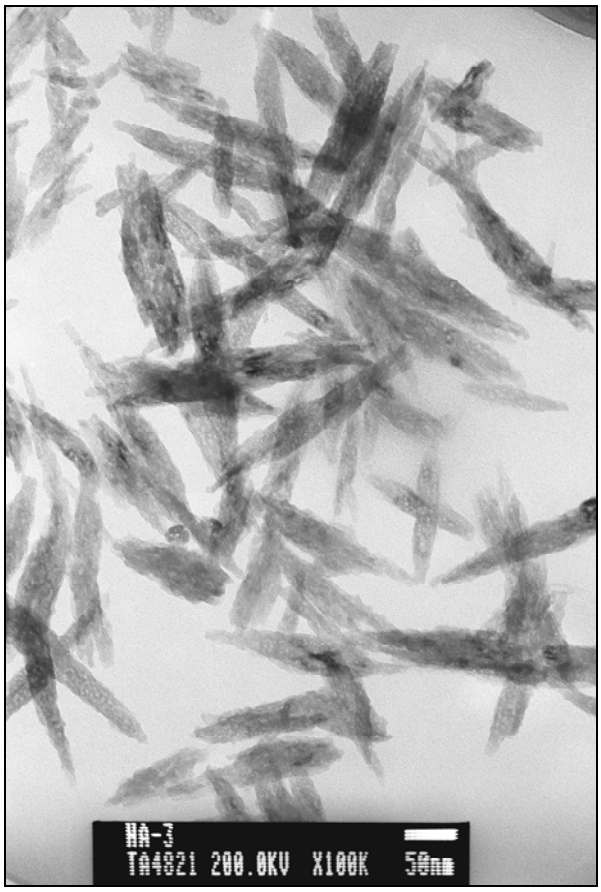
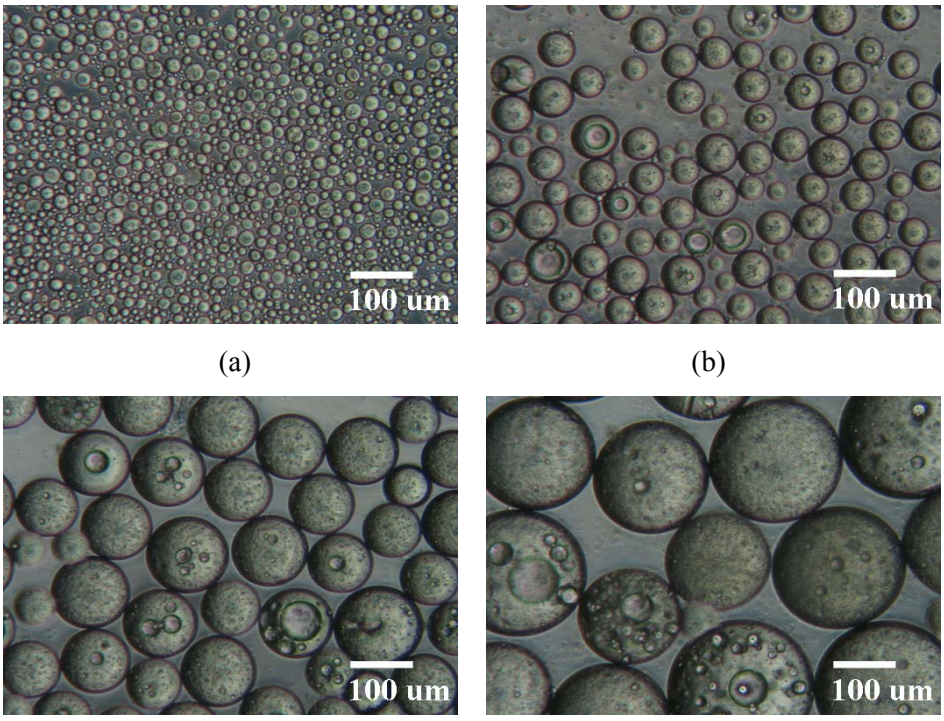


Figure 4. TEM image of HA nanoparticles.

2. Characterization of the poly(DL-lactide-co-glycolide)/hydroxyapatite microshperes

A. Morphological characterization and particle size determination

Figure 5 shows the morphological characterization of PLGA/HA microspheres by optical microscope. All PLGA/HA microspheres prepared have spherical shapes with some pores. Figure 6 shows the effect of polymer concentration on the particle size of microspheres. For these evaluations, PVA as stabilizer was used at a constant concentration of 2% (w/v). PLGA concentrations were varied from 2.5 to 20% (w/v). The particle size of microspheres was increased linealy by increasing PLGA concentration (Table 2).



(a) (b)

(c) (d)

Figure 5. The optical images of PLGA/HA microspheres with PLGA concentration of (a) 2.5%, (b) 5%, (c) 10%, and (d) 20%.

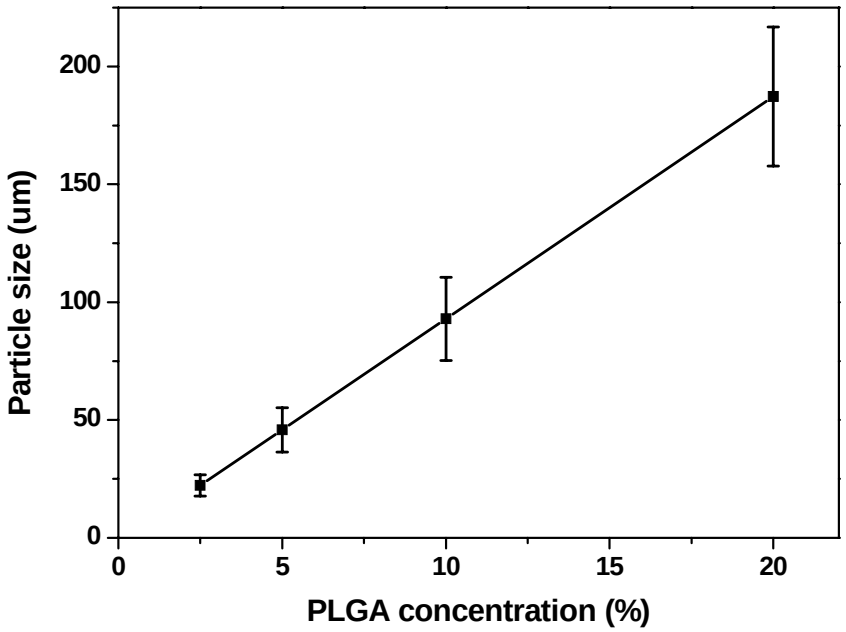


Figure 6. Effect of PLGA concentration on the mean particle size of PLGA/HA microspheres (at the concentration of 2% (w/v) of PVA).

B. Drug content of the microspheres

Drug contents of each sample of microspheres were recorded in Table 2. The drug contents varied from 0.8 to 70.3% as the particles size increases. The larger the particle size was, the higher the drug content was.

Table 2. Particle size and drug loading efficiency of TC-loaded PLGA/HA microspheres with different recipes

Sample number	PLGA concentration in DCM (% w/v)	Particle size (μm)	Loading efficiency (%)
P2.5	2.5% PLGA in DCM	17.8 ± 4.5	0.8
P5	5% PLGA in DCM	48.7 ± 9.4	10.6
P10	10% PLGA in DCM	96.3 ± 17.7	21.8

P20	20% PLGA in DCM	185.4 ± 29.5	70.3

3. The phase transition temperature

Five MC aqueous solution samples of different recipes were prepared (Table 3). In order to compare the effects of different salt concentration on gelation temperature of the 2% MC, pure 2% MC and 2% MC blended with 2, 4, 5, and 6% salt were used in 2MC, 2MC2, 2MC4, 2MC5, and 2MC6 respectively.

The viscosities of 2 % MC blended with salt were measured through heating from 10 to 60 ℃ at a scanning rate of 2 ℃/ min. A 'gel' criterion was defined as the temperature at which the viscosity of the solution was suddenly raised. The viscosity of each sample was increased as temperature increased and increased suddenly at specific temperature for each samples. The gelation temperature of 2% MC blended with salt was decreased by increasing the amount of salt blened from 54 to 32.5 ℃ in the prepared samples (Figure 7). The critical concentration of salt added in 2% MC to lower the gelation temperature below the body temperature (37 ℃) was over 5%. The corresponding photos of the sample with different amount of salt were described in Figure 8. 2% MC blended with over 5% salt at 37 ℃ shows high turbidity representing complete gelation (Figure 8(a)). The fluidity of 2% MC blended with over 5% salt at 37 ℃ was low, which represent complete gelation too (Figure 8(b)).

Table 3. Gelation temperature and fluidity of 2% MC aqueous solution blended with different amount of salt

Sample number	Concentration of MC and NaCl	Gelation temperature (℃)	Fluidity
2MC	2% MC in distilled water	54.0	O
2MC2	2% MC, 2% NaCl in distilled water	44.4	O
2MC4	2% MC, 4% NaCl in distilled water	38.4	O
2MC5	2% MC, 5% NaCl in distilled water	35.7	×
2MC6	2% MC, 6% NaCl in distilled water	32.5	×

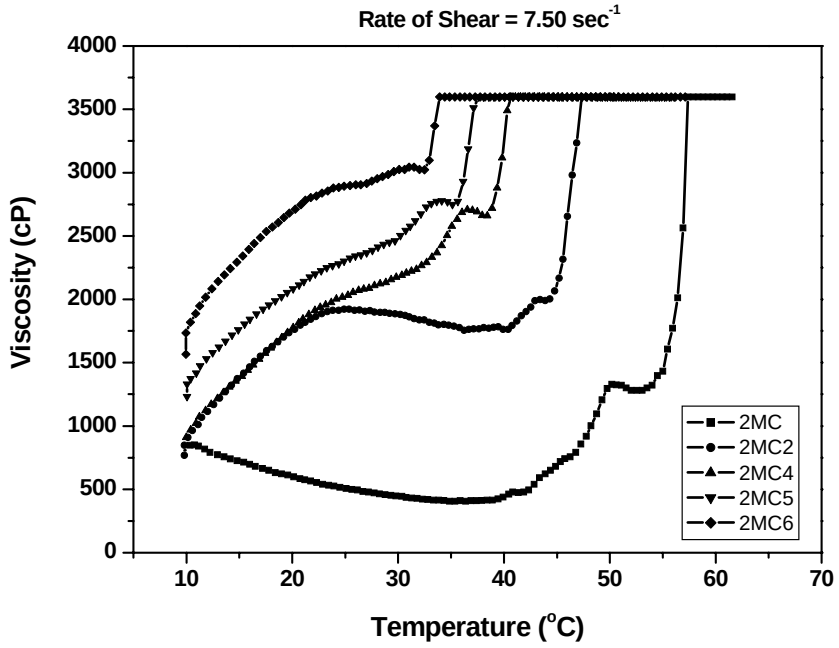
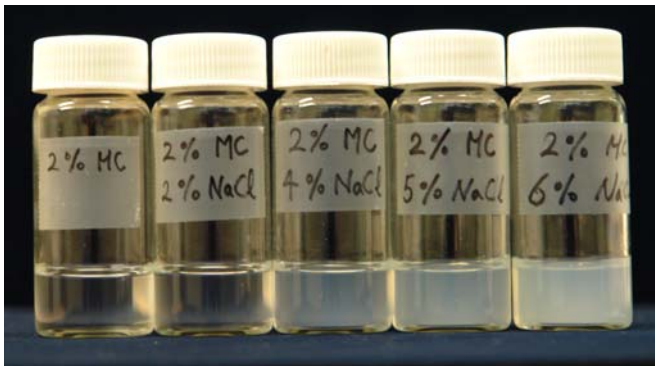
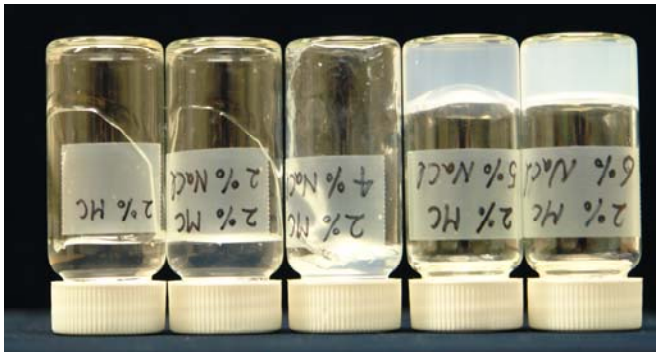


Figure 7. Gelation temperatures of 2% methylcellulose aqueous solution blended with different amounts of salt (pure 2% MC, 2% MC blended with 2, 4, 5, and 6% NaCl) through heating from 10 to 60 ℃ at a scanning rate of 2 ℃/ min.



(a)



(b)

Figure 8. (a) Turbidity and (b) fluidity of 2% methylcellulose aqueous solution blended with different amount of salt (pure 2% MC, 2% MC blended with 2, 4, 5, and 6% NaCl at 37 ℃).

4. In vitro drug release test

As it can be seen in Figure 5, spherical drug-loaded microspheres of different size were obtained. In vitro drug release was performed at 37 ℃ using a saline solution in order to achieve physiological condition. TC loaded PLGA/HA microspheres were used to investigate the drug release profiles as function of microsphere size. The effects of the size of the PLGA-based microspheres on the resulting drug release kinetics in saline solution are illustrated in Figure 9. It shows the percentage of TC released over time for P2.5, P5, P10, and P20. Clearly, the relative TC release rate decreased with increasing system dimension. P2.5, P5, and P10 showed the perfect release of TC within 1 day with high initial bust. Whereas, the accumulated release of

TC in P20 was only 17% within 1 day without high initial bust. P20 showed the sustained release of TC until 2 weeks (Figure 10). Faster release rates were observed from formulations containing smaller microspheres than those having larger size. Release data showed that formulations containing the smallest microspheres (P2.5) displayed the faster and higher release rates than those formulations containing the larger microspheres.

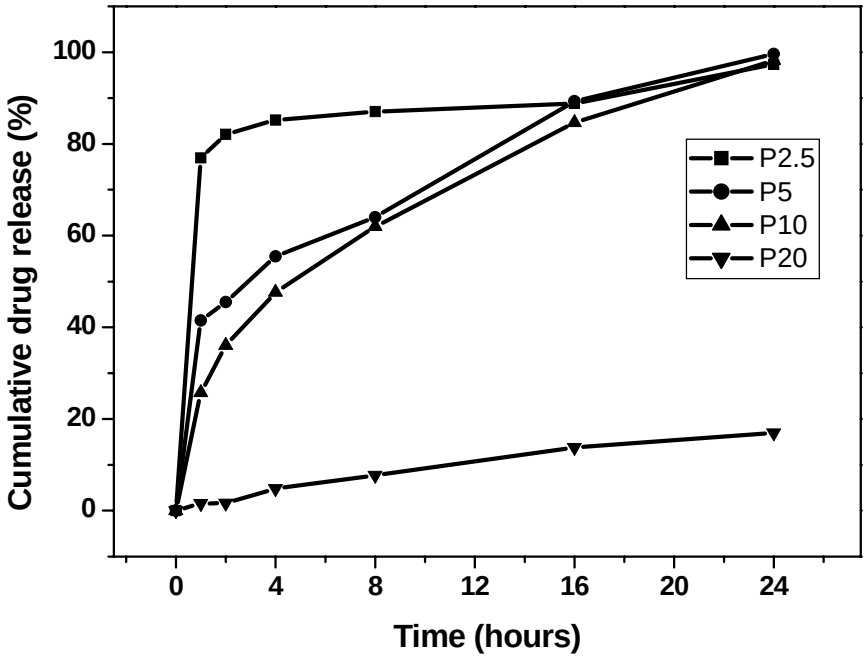


Figure 9. Cumulative drug release aspects from different size of microspheres in saline at 37 ℃.

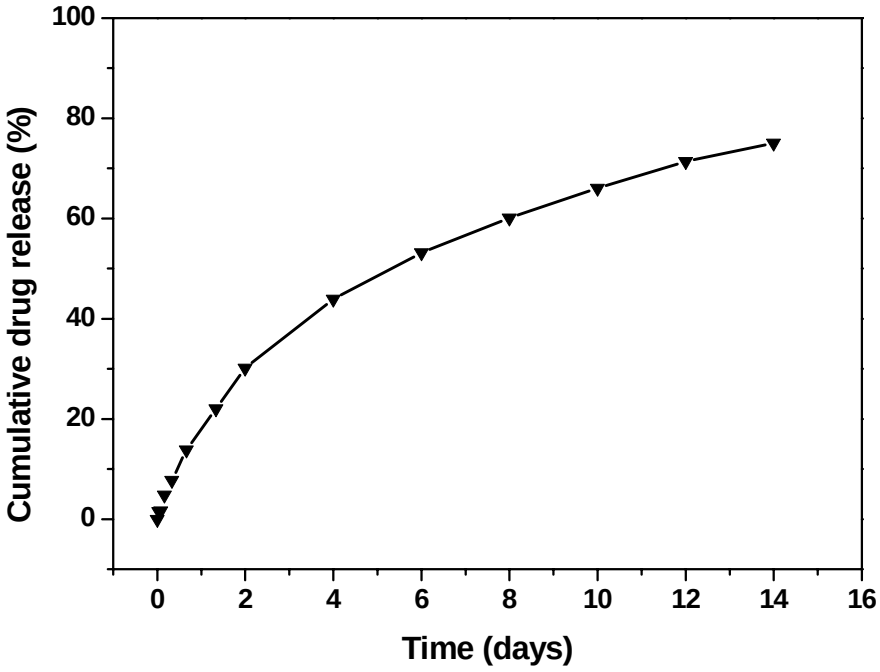


Figure 10. Cumulative drug release aspects from P20 in saline at 37 ℃.

5. Injectability test

To investigate the injectability of the PLGA/HA microparticle-dispersed MC solutions, load–displacement curves through a needle sizes of 18G were obtained (Figure 11). The powder/solution ratios were varied from 40/60 to 60/40 (w/v) to evaluate the effect of particle content on injectability. For these evaluations, the particle size was fixed as P5 (48.7 ± 9.4 μm). The PLGA/HA microparticle dispersed MC solutions with particle/solution ratio of 40/60 (w/v) was easily ejected through 18G needle with little load until the syringe was empty without remaining the microparticles inside. Whereas, as the particle/solution ratio increased, the PLGA/HA microparticle dispersed MC solutions were required much stronger loads for the ejection of the mixture solutions and the amount of the microparticles remained uninjected until maximul load (120 N) were more.

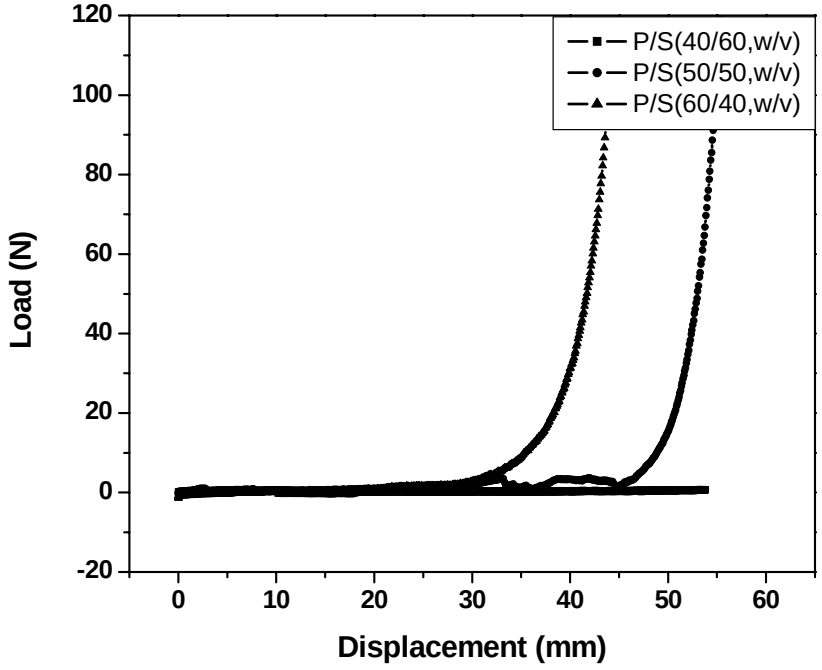


Figure 11. Load-displacement curves of methylcellulose aqueous solution dispersed with PLGA/HA microspheres (P5) with different particle/solution ratios through a syringe with needle (18G).

Ⅲ. DISCUSSION

The purpose of this study was to confirm the possibility of MC aqueous solution containing PLGA/HA composite microspheres and antibiotics, as a sustained drug release-bone substitute by estimating thermogelation temperature of MC aqueous solution by controlling salt added, injectability and drug-release behavior of the injecting system by controlling size and content of the microspheres.

TCP has a stoichiometry similar to amorphous bone precursors, whereas HA has a stoichiometry similar to bone mineral[25]. HA is a well-known key bioceramic material frequently used as a drug carrier, ocular implant, bone substitute for filling bone defects, scaffold matrix for tissue engineering and as a coating agent on biomedical implants due to its chemical and biological similarity with natural bone mineral. The stoichiometric HA has chemical composition of $Ca_{10}(PO_4)_6(OH)_2$ with atomic calcium to phosphorus (Ca/P) ratio of 1.67[26]. It is known to be biocompatible, bioactive i.e. ability to form a direct chemical bond with living tissues, osteoconductive, non-toxic, noninflammatory and non-immunogenic agent[25,26]. As per literature survey, use of conventional HA in the form of thin film, powder, dense or porous blocks are abundance at microscale level[27-29]. HA is the main mineral component of bones and teeth, and it has been widely used in the orthopedic and dental fields as a paste, granules, or porous blocks for implants[35]. It is biodegradable, but the process is very slow. HA is also utilized as an adsorbent during chromatography for purification and separation because of its excellent adsorption of many molecules. Blocks of porous HA and HA ceramics have also been investigated for use in sustained-release DDS for various therapeutic agents. It has already been reported that implanted HA preparations of antibiotics and anticancer drugs showed slow release in animals. But microscale HA is typically highly stable phase i.e. less bioresorbable, which is an undesirable characteristic because it impedes the rate of bone regeneration when used as a bone substitute in alveolar ridge augmentation related orthopedic surgery[30]. It is desirable that the HA for use in implants be bioresorbable so that it can be replaced, over a period of time, with regenerated bone upon implantation. The resorbability of HA can be improved with help of some ceramic oxides and ionic doping agents[31,32] or reducing its grain size to nano-level[33]. Engineering HA at nano-level would have amazing functional properties due to its

grain size, large surface area to volume ratio and ultra fine structure similar to biological apatite, which would have a great impact on implant-cell interaction in body environment. Further, osteoconductivity, solubility, sinterability and mechanical reliability of the HA can be promoted by controlling its particle size and structural morphology in the order of nanoscale[34]. Keeping the above points in view, present study was aimed to synthesise nanocrystalline HA by coprecipitation method with superior bioresorption. The phase of the dried samples was analyzed using XRD. The prepared HA having broad diffracted peaks with low crystallinity indicates a microcrystalline nature. The XRD peaks exhibit no extraneous phase corresponding to HA, which suggests that the reaction has been completed. The particles size and morphology were examined using TEM for the HA nanoparticles synthesized by coprecipitation method. Figure 4 shows the TEM image of the HA nanoparticles with needle-shape. The crystallite size was about 20 nm in width and 100 nm in length.

PLGA-based microparticles offer various advantages compared to other controlled DDSs, including: (i) the possibility to accurately control the resulting drug release kinetics over periods of days to months; (ii) complete biodegradability (avoiding the removal of empty remnants upon drug exhaust); (iii) good biocompatibility, even if directly administered into brain tissue[56]; (iv) easy administration using standard syringes and needles. Thus, this type of controlled drug delivery system can be very helpful to optimize the therapeutic efficiency of medical treatments and to reduce serious side effects[57,58]. There are other factors that may have an effect on the drug release mechanism like the type/ properties of the carrier microshere, the structure and properties of the drug molecule and thus the type of interactions established between the drug and the polymeric matrix. The size of the microsphere, a key factor in the rate of release, may have a significant effect on product performance as well as safety. Large particles can origin capillary obstruction when injected intravenously. Beyond that, particle size can also influence the injectability of the product. In order to understand the effect of these properties on the drug release profile, microspheres containing antibiotics with different sizes were produced studies were performed.

Figure 5 shows the morphological characterization of PLGA/HA microspheres by optical microscope. All PLGA/HA microspheres prepared have spherical shapes with some pores. Figure 6 shows the effect of polymer concentration on the particle size of microspheres. For these evaluations, PVA as stabilizer was used at a constant

concentration of 2% (w/v). PLGA concentrations were varied from 2.5 to 20% (w/v). The particle size of microspheres was increased linealy by increasing PLGA concentration. Polymer concentration in the internal phase was a crucial factor in increasing the size of microspheres, as its concentration was increased. This is in agreement with the findings of E.J.A.M Schlicher et al. DCM diffuses from the solution into the water carrying some PLGA molecules with it. So as the PLGA concentration increase, the amount of PLGA based on DCM increase. Therefore particle size increases at a fixed other conditions. Also as polymer concentration increases, viscosity of organic solution increases. High viscous resistance to the shear forces hinders the microsphere formation. It was demonstrated that the size of emulsion droplets depends on the balance between stirring shear force and droplet cohesion. It was considered that the higher concentration of PLGA, at a fixed stirring shear force, results in a higher viscosity of the oil phase, which makes it difficult for small droplets to form. As can be seen in Figure 5, we could control the size of PLGA/HA microspheres by changing concentration of PLGA solution linealy ranging from 17.8 ± 4.5 to 185.4 ± 29.5 μm.

Drug contents of each sample of microspheres were recorded in Table 2. The drug contents varied from 0.8 to 70.3% as the particles size increases. The larger the size of particles was, the higher the drug content of it was. It is considered that the larger microspheres have relatively less surface area faced with solvents during cleaning process. It is corresponded to the fact that an increase in system size is a priority expected to result in reduced relative release rates due to the increased length of the diffusion pathways and, thus, decreased drug concentration gradients.

The viscosity of each sample was increased as temperature increased and increased suddenly at specific temperature for each samples. The gelation temperature of 2% MC blended with salt was decreased by increasing the amount of salt blended from 54 to 32.5 ℃ in the prepared samples. The critical concentration of salt added in 2% MC to lower the gelation temperature below the body temperature (37 ℃) was over 5%. The corresponding photos of the sample with different amount of salt were described in Figure 8. 2% MC blended with over 5% salt at 37 ℃ shows high turbidity representing complete gelation (Figure 8(a)). The fluidity of 2% MC blended with over 5% salt at 37 ℃ was low, which represent complete gelation too (Figure 8(b)).

As we know, Cl^- tends to have a stronger interaction with water molecules than the interaction between water molecules. Thus, some of the original hydrogen-bonding network formed by water is destroyed by the salt and this effect is similar to increasing temperature. The competition for water molecules from NaCl, and the salt-induced destruction of the hydrogen bonds between the MC chains and water cause the decrease of MC solubility in water. As a result, at the same temperature, there are more hydrophobic aggregates of MC in a salted MC solution than a salt-free one, leading to stronger light scattering and lower transmittance as compared at a given temperature (Figure 8). Therefore, the clouding point of a salted sample appears at a lower temperature. The increased salt content results in fewer free water molecules available around MC chains and a stronger hydrophobic environment for MC, which causes the turbidity of MC with more salt higher than that of MC with less salt at a given temperature (37 ℃) as shown in Figure 8.

As it can be seen in Figure 5, spherical drug-loaded microspheres of different size were obtained. In vitro drug release was performed at 37 ℃ using a saline solution in order to achieve physiological condition. TC loaded PLGA/HA microspheres were used to investigate the drug release profiles as function of microsphere size. Clearly, the relative TC release rate decreased with increasing system dimension. P2.5, P5, and P10 showed the perfect release of TC within 1 day with high initial bust. Whereas, the accumulated release of TC in P20 was only 17% within 1 day without high initial bust. P20 showed the sustained release of TC until 2 weeks (Figure 10). Faster release rates were observed from formulations containing smaller microspheres than those having larger size. Release data showed that formulations containing the smallest microspheres (P2.5) displayed the faster and higher release rates than those formulations containing the larger microspheres. The release rate becomes quite slower at the larger microspheres in this system.

As diffusion is known to play a major role in the control of drug release from PLGA-based microparticles[59], an increase in system size is a priority expected to result in reduced relative release rates (due to the increased length of the diffusion pathways and, thus, decreased drug concentration gradients). The absence of this ‘increased diffusion pathway length effect’ in the case of non-porous, PLGA-based microparticles can be attributed to accelerated polymer degradation[60]. Upon water imbibition the polyester is hydrolytically cleaved into shorter chain alcohols and acids

throughout the system ("bulk erosion"). Due to concentration gradients, the latter diffuse into the surrounding bulk fluid, where they are neutralized. In addition, bases from the release medium diffuse into the microparticles, neutralizing the generated acids. However, diffusional processes in polymeric systems are relatively slow and the rate at which the acids are generated can be higher than the rate at which they are neutralized. Consequently, the pH within the microparticles can significantly drop[60]. As the ester bond cleavage is catalyzed by protons, significant decreases in micro pH lead to accelerated polymer degradation (autocatalysis) and, thus, increased drug mobilities and release rates.

The PLGA degradation rate increased with increasing microparticle dimension, irrespective of the presence/absence of the drug. This might be attributable to the following phenomena: (1) Autocatalytic effects. With increasing microparticle dimension the length of the diffusion pathways for the generated acids (and bases from the release medium) increases. Thus, the decrease in micro-pH becomes more pronounced and ester bond cleavage is accelerated. (2) Altered release rates of shorter chain PLGA fractions. With increasing microparticle dimension the length of the diffusion pathways for shorter chain degradation products increases. Thus, the release rate of these compounds from the microparticles decreases. Consequently, the average polymer molecular weight in large devices decreases more rapidly than in small ones (from which short chain degradation products are rapidly released).

As PLGA degradation is known to follow pseudo-first-order kinetics[61], the following equation was fitted to the experimentally determined results: $Mw\ (t) = Mw\ (t = 0) \cdot \exp(-k\mathrm{degr} \cdot t)$ (3) where Mw (t) and Mw ($t = 0$) are the average polymer molecular weights at time t and $t = 0$ (before exposure to the release medium), respectively; $k$degr denotes the degradation rate constant of the polymer.

Encapsulation can now be considered as a means of controlling the release of different drugs[62]. At the present time, most therapeutic agents, such as antibiotics, growth factors[53], steroid hormones[63] or anticancer drugs, are associated with bone substitutes by drug adsorption. These DDSs do not allow release over more than a few days. In this study, PLGA microparticles allow a TC release extending over several weeks as in other soon to be published applications with other drugs[64]. Like these, drug release rate from the microspheres is influenced by many factor such as diffusion, degradation, and solubility of drugs. We evaluated the drug release kinetics

for at most 2 weeks, especially 1 day in P2.5, P5, and P10. In this study, diffusion differences according to the particle sizes are considered crucial factor because degradation time of PLGA microsphere is far longer than 1 day and the prepared PLGA spheres have many open pores. Future research should also be aimed towards effect of degradation rate of spheres on the drug release kinetics.

The PLGA/HA microparticle-dispersed MC solutions with particle/solution ratio of 40/60 (w/v) was easily ejected through 18G needle with little load until the syringe was empty without remaining the microparticles inside by load under 50 N[65]. Whereas, as the particle/solution ratio increased, the PLGA/HA microparticle-dispersed MC solutions were required much stronger loads for the ejection of the mixture solutions and in case of particle/solution ratio of 50/50 and 60/40, the microparticles were still remained in the syringe even after maximum load (120 N) was applied and the amount of the microparticles remained uninjected until maximul load (120 N) were more. From this result, we can see that the content of particles in MC solution is crucial factor of injectability. The injection of PLGA/HA microparticle-dispersed MC solution with particle to solution ratio of 40/60 (w/v) using 18G needle was possible perfectly. The size and content of particles in MC solution enable to be injected can be different by needle gauges. If the larger needle guage was used, the larger size and more content of particles in MC solution could be possible to be injected.

Ⅴ. CONCLUSION

The aim of this study was to confirm the possibility of MC aqueous solution containing PLGA/HA composite microspheres and antibiotics, as a sustained drug release bone substitute. HA is a well-known key bioceramic material frequently used as a drug carrier, ocular implant, bone substitute for filling bone defects, scaffold matrix for tissue engineering and as a coating agent on biomedical implants due to its chemical and biological similarity with natural bone mineral. PLGA-based microparticles offer various advantages compared to other controlled drug delivery systems due to its possibility to accurately control the resulting drug release kinetics over periods of days to months. Cellulose ethers like MC and HPMC possess the unique property of reversible thermogelation. We prepared injectable bone substitute for these advantages. By estimating thermogelation temperature of MC aqueous solution by controlling salt added, injectability and drug-release behavior of the injecting system by controlling size and content of the microspheres, we optimized the injectable bone substitute composed of MC aqueous solution and PLGA/HA composite microspheres containg antibiotic, TC. The PLGA/HA microspheres showed high drug loading efficiency of up to 70.3%. We could control the drug release behavior from the microspheres by controlling the size of microspheres from 17.8 ± 4.5 to 185.4 ± 29.5 μm. Especially, P20 with a particle diameter of 185.4 ± 29.5 μm showed high drug contents of over 70% and sustained drug release over 2 weeks without initial burst, a major problem of drug delivery systems.

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ABSTRACT (IN KOREAN)

항생제를 함유한 생분해성 고분자/수산화아파타이트 복합 마이크로 입자로 구성된 주입형 골 대체재

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이 세 호

이제까지 주입형 골대체재에 항생제와 같은 약물을 무기 입자의 기공에 넣어 흡수 및 방출을 조절하고자 하는 연구가 많이 진행되어 왔다. 그러나 이보다는 생분해성 고분자에 약물을 함유시켜 방출 속도를 제어하는 것이 효과적일 것으로 기대된다. 본 연구에서는 생분해성 고분자와 수산화아파타이트 (HA)로 구성된 복합 마이크로 입자를 제조한 후 methylcellulose (MC) 수용액에 도입하여, 복합 마이크로 입자의 주입성과 약물 방출 거동을 평가하였다. 또한 입자 제조 시 poly(DL-lactide-co-glycolide) (PLGA)의 농도를 변화시켜 항생제를 함유한 다양한 크기의 PLGA/HA 마이크로 입자를 제조한 후, 주입성 및 *in vitro*에서 시간에 따른 약물 방출 농도를 측정하여 마이크로 입자의 입도와 약물 방출 거동에 관한 상관 관계를 규명함으로써 생분해성 고분자/HA 복합 마이크로 입자와 MC 수용액 시스템의 서방성 주입형 골 대체재로의 가능성을 타진하였다.

골조직 재생재료로 뼈를 구성하는 성분과 가장 유사한 조성을 가지고 있고, 생체적합성, 생체활성 및 골과 직접적인 결합 등 우수한 성질을 가진 HA 나노 입자를 침전법을 이용하여 제조하였다.

약물방출 조절을 위해 PLGA/HA/tetracycline (TC)으로 구성된 복합 마이크로 입자를 유화 용매 증발법으로 제조하였으며, 입자 크기를 조절하기 위해 유화과정에서 PLGA 농도를 2.5~20%로 변화시켰다. 모든 입자에서 HA와 TC의 함량은 각각 PLGA의 질량의 10%로 고정하였다. 온도가역적인 2% MC 수용액에 NaCl을 2~6%로 첨가한 후, 점도계를 이용하여 점도를 측정함으로써 겔화 온도가 체온(37 ℃)에 맞는 조건을

확립하였다. 주입성은 PLGA/HA/TC 마이크로 입자를 제조한 후 입자/MC (w/v) 비율을 40/60~60/40로 변화시키면서 주입거리에 따른 하중을 만능시험기로 측정하였다. 항생제 방출 거동은 입자의 크기를 4개의 군으로 하여 37 ℃에서 60 rpm으로 교반하면서, 시간에 따라 방출된 TC의 양을 자외-가시광선 분광광도계를 이용하여 정량 분석하였다.

침전법으로 제조한 HA는 TEM과 XRD를 이용하여 100 nm의 침상 구조를 갖는 HA임을 확인하였다. 마이크로 입자는 PLGA의 농도에 따라 입자 크기가 직선적으로 증가하여 (17.8±4.5)~(185.4±29.5) μm의 크기를 나타내었다. 약물의 함유효율도 입자 크기가 증가함에 따라 증가하여 0.8~70.3%의 범위를 나타내었다. 2% MC 수용액에 NaCl을 5%이상 첨가하면 겔화 온도가 체온인 37 ℃ 이하로 감소함을 알 수 있었다. 주입성 평가는 입자크기가 약 50 μm인 시편에서 입자/MC(w/v) 함량이 40:60일 때 주입거리 끝까지 5 N 이하의 균일한 하중으로 주입이 용이함을 나타내었고, 입자의 함량이 증가할수록 하중이 증가함을 알 수 있었다. 약물 방출은 입자크기가 17.8~96.3 μm 인 시편에서 모두 1일 후에 거의 100% 가까운 약물이 급격하게 방출되는 양상을 나타내었으나, 입자크기가 185.4 μm인 시편에서는 약물이 2주 후에 80% 정도의 약물이 방출되어 2주 후까지도 서서히 약물이 방출되는 서방형임을 확인할 수 있었다. 이는 입자의 크기에 따른 확산 속도의 차이에서 기인하는 것으로 생각되며, 입자의 크기가 작아지면 확산 경로가 짧아지고, 약물의 농도차가 커지면서 방출 속도가 빨라지게 된다. 입자크기가 17.8~96.3 μm인 시편의 경우, 1일 동안의 방출 경향 또한 입자의 크기가 작을수록 약물 방출속도가 증가하였다.

유화 용매 증발법을 이용하여 PLGA/HA/TC 복합 마이크로 입자를 제조할 수 있었고, 2% MC 수용액에 혼합하여 주입 가능성을 확인하였다. 또한 입자의 크기를 조절함으로써 약물의 방출 속도를 제어할 수 있었으며, 특히 입자의 크기가 (185.4 ± 29.5) μm인 시편의 경우 70% 이상의 약물 함유효율과 2주 이상의 약물서방성을 보여줌으로써 서방형 골대체재로의 가능성을 보여주었다.

핵심되는 말: 주입형 골 대체재, 약물방출, 복합 마이크로입자