Application of Calcium Phosphate as a Controlled-Release Device

Tomoko Ito*† and Makoto Otsuka
Research Institute of Pharmaceutical Sciences, Musashino University; 1–1–20 Shin-machi, Nishitokyo, Tokyo 202–8585, Japan.
Received May 17, 2013

Calcium phosphate (CaP)-based compounds are biocompatible and have been accepted as promising candidates for novel drug-releasing devices. CaP is biodegradable and can be utilized as a durable drug release reservoir. We developed an injectable self-setting apatite cement. When a plasmid DNA complex containing CaP was injected into tumor-bearing mice, it solidified in the body and slowly released the DNA complex, inducing durable gene expression and high therapeutic effect on solid tumors. Encapsulation of a drug by CaP acts as a protective capsule for the unstable contents and improves biocompatibility. CaP nanocapsules encapsulating a plasmid DNA complex or drug-involved micelle were prepared, and they showed high stability against enzyme and protein degradation. CaP also showed high potential as a durable acid pH buffer. Aqueous alginate solution was found to form a soft gel in the body and was investigated as a drug-releasing device. However, degradation of the alginate gel is sometimes too rapid in an acidic environment such as the area around osteoporotic bones. We found that amorphous CaP powder added to the alginate gel could control the dissociation rate, buffering the pH inside the gel. Alginate gel including CaP powder and a drug for osteoporosis allowed sustained release of the drug under acidic conditions, and a good therapeutic effect was achieved in osteoporosis model rats. CaP could thus be a valuable material for drug-delivery systems as a slow-releasing drug reservoir, a protective coating, or a pH buffer.

Key words calcium phosphate; biodegradability; drug delivery; sustained release; injectable device

1. INTRODUCTION

Drug-delivery systems (DDS) have been widely investigated as a novel strategy for the treatment of intractable diseases. DDS provide the required dose at the required time to the targeted site, reducing undesirable side effects and achieving the long-term therapeutic concentration at a specific site. Among DDS technologies, the most widely studied are “slow-release devices” that gradually release the drug in the system over an extended period.1–3) Slow-release systems are often coupled with biodegradable solid devices, which gradually degrade in the body and continue to release the drug for a long period.4,5) Solid-type slow-release systems are mainly implanted surgically, which is often invasive and difficult depending on the site. Thus, an injectable sustained-release system is needed.6) Microspheres made of a biodegradable microrib have been explored as an injectable device,7) which allow the drug to be released over a period of several days. Injectable materials that self-assemble into a solid in the body would be another solution to avoid surgical implantation. A calcium phosphate (CaP) slurry that can be injected by a syringe to form a cement in the body was developed. CaP-based compounds have inorganic components similar to bone and teeth. They are biocompatible and biodegradable, making them suitable as implant devices.

This review describes the preparation of injectable self-setting apatite cement containing plasmid DNA complexes and its application in gene therapy in the first section.8) CaP nanocapsules would be other candidates for an injectable CaP device. A CaP protective coating was constructed on the surface of DNA complex particles9) or a micelle involving a drug,10) and high stability of the unstable contents was achieved. Another advantage of CaP is a durable pH-buffering effect. Amorphous CaP powder added to alginate gel controls the pH inside the gel, and the dissociation rate of the gel is slowed.11)

CaP DDS with high biocompatibility, biodegradability, and other advantageous properties is a promising candidate as material for novel drug-releasing devices.4,12) This review introduces our recent findings on CaP-based DDS.

2. PREPARATION OF INJECTABLE SELF-SETTING APATITE CEMENT AND APPLICATION TO GENE THERAPY

A variety of plasmid DNA complexes have been examined for nonviral gene therapy. Although certain plasmid delivery systems have achieved extragene expression in target tissues, the therapeutic effect is not yet satisfactory because of the short duration of gene expression. Gene-delivery systems that release the plasmid complex for long periods are thus required. A biodegradable device for the slow release of DNA complex was developed by Scherer et al., who prepared a collagen sponge incorporating a DNA complex13) and achieved durable expression of the therapeutic gene. These solid-type slow-release systems can only be implanted surgically, which is often problematic and, depending on the site, sometimes difficult. Injectable materials that self-set in the body in a

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: tito@shinyamanote.jp © 2013 The Pharmaceutical Society of Japan
biodegradable solid device are therefore needed for DNA complex particle-releasing systems.

This review focuses on the development of biocompatible, biodegradable apatite cement with inorganic components similar to bone and teeth as novel drug-releasing devices. Apatite cement as a drug reservoir is commonly implanted surgically in the body. On the other hand, Brown and Chow were the first group to invent a self-setting CaP slurry. We found that it could be administered via a syringe into the living body and spontaneously solidified. The dissolution rate of the solidified apatite in the body can be controlled by its crystallinity. Another problem is the dispersion instability of DNA complexes. DNA/polycation (or cationic lipid) complex dispersion is generally unstable and rapidly aggregates, especially at high concentration, making it difficult to immobilize DNA complex fine particles in a solid in a redistributable state. On the other hand, we found that hyaluronic acid (HA) could be deposited onto DNA/polycation (or cationic lipid) complexes, markedly stabilizing their dispersion. We then prepared a highly durable gene-expression system composed of injectable self-setting apatite cement with finely dispersed DNA/polycation/HA complex particles.

The DNA/polyethyleneimine (PEI)/HA ternary complex particles can be freeze-dried without loss of gene activity, enabling the preparation of small DNA complex particles at sufficiently high concentration. The small DNA ternary complexes achieve high in vivo gene expression. A complex of plasmid DNA (pDNA)-GM-CSF/PEI/HA effectively suppressed tumor growth in tumor-bearing mice, and small tumors completely disappeared. However, multiple injections are required to achieve a satisfactory therapeutic effect, and a single dose rarely induced a high response, probably owing to the short duration of gene expression by such artificial vectors. Injectable self-setting apatite cement containing pDNA-GM-CSF/PEI/HA was prepared, and the efficacy of the durable DNA complex release system was examined.

An injectable apatite cement slurry was prepared as follows: Apatite cement bulk powder consisting of an equimolar mixture of tetracalcium phosphate and dicalcium phosphate dehydrate was prepared by grinding in an agate vibration mixer mill. The apatite/polymer composite cement bulk powder was ground with 5% or 20% atero collagen, dextran, or polyethylene glycol (PEG) in the vibration mixer mill. The apatite/polymer composite cement bulk powder was mixed with 25 mM phosphoric acid. A condensed suspension of DNA/PEI/HA complex small particles was prepared by mixing the components at highly diluted concentrations, followed by concentration in a lyophilizing and rehydration process. They were then mixed with the apatite cement slurry.

The apatite cement prepared with collagen was suspended in 25 mM phosphoric acid and allowed to set. It showed an X-ray diffraction (XRD) profile with a very low degree of crystallinity, similar to natural bone. The best accordant diffraction pattern of apatite cement was obtained with 20% collagen (the same content as natural bone). On the other hand, apatite cement with dextran or PEG did not completely lose crystallinity. This indicates that the meta-stable CaP transformed into apatite on collagen matrices, but did not completely transform without collagen.

When fluorescent-labeled DNA complex was added to the apatite cement slurry, fluorescence microscopy showed that the DNA complex particles were finely dispersed in the solidified apatite cement. Neither the size nor the ζ-potential of the DNA complex in the cement apparently changed from that of the original DNA complex particles.

It is known that osteoclasts dissolve and resorb bone by secreting an acidic liquid. Thus apatite cement implanted in body is expected to be degraded by osteoclasts to release the DNA complex. In order to simulate this, DNA-releasing ability was evaluated with MLC-6 cells, an osteoclast-like cell line with the same function as osteoclasts. B16 melanoma cells were used as nondegrading control cells. The cells were seeded on apatite cement, including YOYO-1-labeled DNA complex particles on the culture plate. The DNA release rate from the apatite was evaluated by measuring the fluorescence intensity of the medium. Release of the DNA complexes from the apatite cement was faster with MLC-6 cells than with B16 cells (Fig. 1a). The DNA complexes were continuously released from the apatite cement including collagen, which showed a n XRD pattern similar to that of natural bone. Higher affinity of the collagen-containing cement with MLC-6 cells than that with other polymers was also confirmed microscopically (Fig. 1b).

Osteoclasts also resorb excessive apatite, such as an ecto-

![Fig. 1](image-url) (a) Release Profile of DNA Complex from Solidified Apatite Cement Including 5% Collagen in the Presence of MLC-6 or B16 Cells (b) Microscopic images of apatite cements (with 5% collagen) including DNA/PEI/HA complexes after incubation for 28 d with MLC-6 cells.
Thus, the apatite cement inoculated into tumor-bearing mice was expected to be degraded by the cells. Apatite cement slurry including DNA/PEI/HA ternary complex particles was inoculated into the femur of mice using an injection syringe, and the volume of the inoculated apatite cement was evaluated by X-ray computed tomography (CT). The solidified cement gradually degraded, and the degradation rate was dependent on the type of polymer used as the additive. Apatite cements with collagen again showed much faster degradation (Fig. 2) than those with other polymers. The crystal structure of the cement similar to that of natural bone was readily recognized by osteoclasts.

The therapeutic effect of the slow-releasing apatite system was then examined in tumor model mice. DNA/PEI/HA complex particles were prepared with the pDNA encoding mouse granulocyte-macrophage colony-stimulating factor (GM-CSF). The DNA complex particles were mixed with an apatite cement slurry including 5% collagen. B16 cells were subcutaneously inoculated under the abdominal skin of mice. When the tumor size increased to 4 mm, apatite cement slurry including DNA complex was injected once around the tumor. The volume of apatite cement solidified in the body was also evaluated.

The tumors grew rapidly in the control mice injected with phosphate-buffered saline (PBS). In 1 of 5 mice that received a single dose of pDNA/PEI/HA complex particles without apatite cement, complete disappearance of the tumors was observed. An apparent therapeutic effect was also seen in the other 4 mice, in which tumor growth remained suppressed for 10 d after the injection, but tumor growth then restarted.

A significant effect was seen in mice injected with pDNA/PEI/HA complex particles in the apatite cement, and a complete response was seen in 3 of the 5 mice, without any tumor regrowth for up to 100 d (Fig. 3). X-Ray CT was performed
to evaluate the reduction rate of apatite cement volume in the mice. The volume reduction rate in the 3 mice in which the tumor disappeared was higher than that in the other 2 mice that showed tumor regrowth. This indicates the importance of successive release and potential of the injectable apatite cement as a durable gene-transfection device.

3. CaP NANOCAPSULES AS PROTECTIVE COATING FOR UNSTABLE DRUGS

3.1. Protection of DNA Complex by the CaP Envelope

As mentioned above, many types of polycations and cationic lipids have been developed for pDNA delivery. They can electrostatically bind to DNA molecules to form a complex. The negative charge of the DNA molecule, which interferes with the binding of the complex to the cells, is neutralized and condensed to a small, complex particle. Another important role of the cationic agents is to avoid enzymatic degradation. DNase is present inside and outside the cells in large amounts and lowers the efficiency of gene expression by the administered DNA. Polycations or cationic lipids can avoid the degradation by DNase to some extent but not completely. To sustain high-level, long-term gene expression, more effective protection is required.

Making use of the ability of CaP to be deposited on materials containing hydroxyl or carboxylic acid groups, we succeeded in encapsulation of the DNA/PEI/HA ternary complex. The HA molecule contains a large amount of hydroxyl and carboxylic acid groups, and a CaP envelope can be constructed by immersing the HA-coated DNA/PEI complex in simulated body fluid (SBF). Incubation in SBF overnight yielded highly stable nanocapsules including DNA complexes with a diameter of ca. 200 nm. The CaP envelope effectively protects the DNA complex against dissociation and degradation. CaP nanocapsules are taken up by cells and slowly degraded to release the DNA complexes. Gene expression with duration longer than 2 weeks was achieved in cultured cells (Fig. 4). This slow-release system including the pDNA-GM-CSF/PEI/HA complex also showed significant antitumor effects. A single injection of CaP nanocapsules into tumor-bearing mice induced complete tumor disappearance in all mice, without any tumor regrowth for up to 100 d (Fig. 5). CaP nanocapsules including a DNA complex thus seem promising as a sustained gene-expression device.

3.2. Simvastatin/Deoxycholic Acid Assembly Encapsulated by the CaP Envelope

The hypolipidemic agent simvastatin has recently shown promise as a therapeutic agent for osteoporosis. The effect of simvastatin in osteointegration of dental implants in osteoporotic patients was also reported. However, the statin drugs are insoluble in water. We found that simvastatin could be readily solubilized by deoxycholic acid, which is a low-toxic secondary bile acid produced by intestinal bacteria as a metabolic by-product. Hydrophobic simvastatin was solubilized in water by interacting with the

Fig. 4. In Vitro Green Fluorescence Protein Gene Expression Efficiency in B16 Melanoma Cells

Fig. 5. Therapeutic Effects of DNA Complexes with or without CaP Encapsulation in Tumor-Bearing Mice in Which B16 Cells Were Inoculated under the Abdominal Skin
deoxycholate micelle. However, such surfactant assemblies are known to be highly unstable in the body. Rapid collapse of the carrier micelle would cause a high local concentration of the drug, which may induce side effects such as rhabdomyolysis.

The previous section showed the possibility of CaP nanocapsules forming around the carboxylic acid-containing molecular assembly. Deoxycholate also has a carboxyl group in its molecule. An attempt was therefore made to encapsulate a simvastatin/deoxycholate assembly in a CaP coating. Simvastatin was dissolved by the deoxycholate micelle and added to a 1.5-fold concentration of SBF. As in the case with the DNA complex, CaP was deposited on the micelle to maintain the stability of the nanocapsules. The simvastatin/deoxycholate assembly encapsulated with CaP showed much lower cytotoxicity in MLC-6 cells than the “naked” simvastatin/deoxycholate assembly without the CaP capsule (Fig. 6).

The therapeutic efficacy of the CaP nanocapsule including the simvastatin/deoxycholate assembly in osteoporosis model (OVX) mice was examined. When the naked simvastatin/deoxycholate assembly without the CaP coating was injected into OVX mice every week for 9 weeks, no effect was observed on bone strength, but evident muscle damage was observed including muscle deterioration or inflammation around the injection site. On the other hand, in OVX mice that received the CaP-coated simvastatin/deoxycholate assembly, whole-body therapeutic effects, such as improved body weight, bone mineral content, and bone mechanical strength (Fig. 7), were seen without any side effects. CaP encapsulation stabilized the simvastatin-containing assembly and prevented rapid release of the drug to decrease the side effects, resulting in high osteoporosis treatment efficacy. Biocompatible, biodegradable CaP nanocapsules are thus expected to become a novel device for effective drug delivery.

![Fig. 6. Cytotoxicity of Deoxycholic Acid/Simvastatin Complexes with or without CaP Encapsulation against Osteoclast-Like MLC-6 Cells](image)

![Fig. 7. Bone Mechanical Strength of Femurs 9 Weeks after the First Injection](image)

\*p<0.05, \***p<0.001 vs. control.
4. PREPARATION OF INJECTABLE AUTO-FORMING ALGINATE GEL AND CONTROL OF THE DEGRADATION RATE BY CaP

There are several drug-release devices utilizing CaP as biodegradable material, as mentioned above. One of the many potential advantages of CaP is its durable pH-buffering effect. Amorphous CaP (A-CaP) was obtained under alkaline conditions at a calcium and phosphorus ratio of 1.64. A moderate amount of alkaline hydroxyl groups is contained in the formulation. A-CaP is thus expected to be a biocompatible pH buffer, which neutralizes acid by absorbing protons and releasing hydroxyl anions. We employed an alginate acid gel as a biodegradable drug reservoir. Alginic acid is known to form a gel with divergent cations such as Ca$^{2+}$. Microspheres of calcium alginate encapsulating a drug can be prepared by dropping the alginate solution containing the drug into CaCl$_2$ solution, and alginate encapsulating a drug can be prepared by dropping the drug-release devices.31) We found that alginate gel could also be spontaneously formed by injection of alginate solution into the living body. Alginate soon forms a soft hydrogel with divergent cations such as Ca$^{2+}$ ion in the body fluid. However, bio-degradation of the resulting alginate gel is relatively rapid, and therefore long-term drug release could not be achieved. The rapid degradation of the alginate gel is due not only to the bio-degradation of the alginate chain but also to acidic dissociation of the ionic crosslinking.32) An injectable auto-forming alginate gel buffered with A-CaP powder was then prepared, and the effect of A-CaP on the dissociation rate of the gel and application to a drug-release device for osteoporosis was examined.13)

An acidic environment is found not only in tumor tissue but also in the vicinity of osteoporotic bone. Osteoporosis is caused by excessive bone resorption and inadequate formation of new bone during remodeling. Bone resorption is carried out by osteoclasts, which dissolve bone by secreting an acidic substance,24) and therefore the vicinity of osteoporotic bones is acidic. An alginate gel containing simvastatin, a therapeutic agent for osteoporosis, and highly soluble A-CaP as a pH buffer was prepared. The buffering effect of A-CaP in the alginate gel was evaluated by immersing the gels with or without A-CaP in acetic buffer (pH 4.5) and measuring the pH inside the gels. Change in pH was very slow during the first hour in all the alginate gels regardless of the presence of A-CaP. During the period, protons infiltrating into the gel were neutralized by alkaline materials such as sodium carboxylate contained in the gels. Then the pH value rapidly decreased to 4.5 in the gels without A-CaP (Fig. 8). They also showed relatively rapid degradation and weight loss in the acidic buffer, although they were stable in Tris–HCl buffer at pH 7.4 (Fig. 9). On the other hand, A-CaP-containing gel maintained higher pH (Fig. 8) and showed much slower degradation (Fig. 9). A-CaP inside the gel appeared to exert a buffering effect to prevent the rapid decrease in pH and gel dissociation. Simvastatin-containing A-CaP/alginate gel was prepared, and the release profile of the drug in acetic buffer at pH 4.5 or in Tris–HCl buffer at pH 7.4 was evaluated. Only small amounts of simvastatin were released from the alginate gels regardless of the A-CaP content at pH 7.4. In acetic buffer, the release rate from the gels without A-CaP increased, while gels with A-CaP still showed gradual release of the drug.

The therapeutic effect of the alginate/A-CaP gel including simvastatin on O VX rats was examined. The acidic environment around osteoporotic bones was expected to facilitate the release of the drug. Alginate solutions containing simvastatin and A-CaP were injected intramuscularly into the right femur of O VX rats every 2 weeks, and the bone mineral contents (BMC) of the femora were measured every week by X-ray CT. The alginate solution injected into rats formed a soft hydrated gel in the injection site. The gels without A-CaP nearly disappeared within 2 weeks, while those with A-CaP remained detectable by palpation. Rats that received simvastatin in the A-CaP-buffered alginate gel showed significant improvement of BMC compared with those treated with the drug in the gel without A-CaP (Fig. 10). The importance of A-CaP as buffering agent to control the dissociation rate to achieve sustained release and high therapeutic efficacy was confirmed. Alginate gel containing A-CaP is thus a promising drug-release device considering the biocompatibility, injectability, and control of the degradation rate with the addition of A-CaP.

CaP could be applied to drug-delivery devices in various formulations. They can play a role as a slow-release drug reservoir, protective coating, or pH buffer. Coupling of this valu-
able inorganic material with organic systems will offer new methods to create highly effective therapeutic systems.

REFERENCES

1) Drachman DE, Edelman ER, Seifert P, Groothuis AR, Bornstein DA, Kamath KR, Palassis M, Yang D, Nott SH, Rogers C. Neointimal thickening after stent delivery of paclitaxel: change in composition and arrest of growth over six months. J. Am. Coll. Cardiol., 36, 2325–2332 (2000).

2) Uchida A, Shinto Y, Araki N, Ono K. Slow release of anticancer drugs from porous calcium hydroxyapatite ceramic. J. Orthop. Res., 10, 440–445 (1992).

3) Langer R. Invited review: polymeric delivery systems for controlled drug release. Chem. Eng. Commun., 6, 1–48 (1980).

4) Habraken WJ, Tipton AJ. Ceramic composites as matrices and scaffolds for drug delivery in tissue engineering. Adv. Drug Deliv. Rev., 59, 234–248 (2007).

5) Middleton JC, Tipton AJ. Synthetic biodegradable polymers as orthopedic devices. Biomaterials, 21, 2333–2346 (2000).

6) Jeong B, Bae YH, Lee DS, Kim SW. Biodegradable block copolymers as injectable drug-delivery system. Nature, 388, 860–862 (1997).

7) Lin CC, Metters AT. Hydrosolts in controlled release formulations: network design and mathematical modeling. Adv. Drug Deliv. Rev., 58, 1379–1408 (2006).

8) Ito T, Koyama Y, Otsuka M. DNA complex-releasing system by injectable self-setting apatite cement. J. Gene Med., 14, 251–261 (2012).

9) Ito T, Otsuka M. Preparation of hydroxyapatite nanocapsule including DNA/PEI/hyaluronic acid ternary complex for durable gene delivery. Mol. Ther., 19, S60 (2011).

10) Ito T, Takemasa M, Makino K, Otsuka M. Preparation of calcium phosphate nanocapsule including simvastatin/deoxycholic acid assembly, and their therapeutic effect in osteoporosis model mice. J. Pharm. Pharmacol., 65, 494–502 (2013).

11) Ito T, Satoo M, Uchino T, Senna M, Iafisco M, Prat M, Rimondini L, Otsuka M. Preparation of injectable auto-forming alginate gel containing simvastatin with amorphous calcium phosphate as a controlled release medium and their therapeutic effect in osteoporosis model rat. J. Mater. Sci. Mater. Med., 23, 1291–1297 (2012).

12) Ginebra MP, Traykova T, Planell JA. Calcium phosphate cements as bone drug delivery systems: A review. J. Control. Release, 113, 102–110 (2006).

13) Scherer F, Schillinger U, Putz U, Stemberger A, Plank C. Nonviral vector loaded collagen sponges for sustained gene delivery in vitro and in vivo. J. Gene Med., 4, 634–643 (2002).

14) Brown WE, Chow CL. A new calcium phosphate water-setting cement. Cements Research Progress (Brown PW ed.), American Ceramic Society, Westerville, OH, pp. 352–379 (1986).

15) Ogries M, Brunner S, Schuller S, Kircheis R, Wagner E. PEI-gylated DNA/transfer-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther., 6, 595–605 (1999).

16) Ito T, Iida-Tanaka N, Koyama Y. Efficient in vivo gene transfection by stable DNA/PEI complexes coated by hyaluronic acid. J. Drug Target., 16, 276–281 (2008).

17) Ito T, Iida-Tanaka N, Nidome T, Kawano T, Kubo K, Yoshikawa K, Sato I, Yang Z, Koyama Y. Hyaluronic acid and its derivative as a multi-functional gene expression enhancer: protection from non-specific interactions, adhesion to targeted cells, and transcriptional activation. J. Control. Release, 112, 382–388 (2006).

18) Ito T, Yoshihara C, Hamada K, Koyama Y. DNA/polyethylene imine/hyaluronic acid small complex particles and tumor suppressor gene transfer in mice. Biomaterials, 31, 2912–2918 (2010).

19) Otsuka M, Kuninaga T, Otsuka K, Higuchi WJ. Effect of nanostructure on biodegradation behaviors of self-setting apatite/collagen composite cements containing vitamin K2 in rats. J. Biomed. Mater. Res. B Appl. Biomater., 79, 176–184 (2006).

20) Park JB, Lakes RS. Biomaterials. An Introduction, 2nd edition, Plenum Press, New York, pp. 193–196 (1992).

21) Baron R. Molecular mechanisms of bone resorption by the osteoclast. Anat. Rec., 224, 317–324 (1989).

22) Teitelbaum SL. Bone resorption by osteoclasts. Science, 289, 1504–1508 (2000).

23) Sakiyama H, Masuda R, Inoue N, Yamamoto K, Kuriwai K, Nakagawa K, Yoshida K. Establishment and characterization of macrophage-like cell lines expressing osteoclast-specific markers. J. Bone Miner. Metab., 19, 220–227 (2001).

24) Bagi CM, Miller SC. Osteoclast features of cells that resorb demineralized and mineral-containing bone implants in rats. Scanning Microsc., 3, 963–968, discussion, 969–970 (1989).

25) Asou Y, Ritting SR, Yoshitake H, Tsuji K, Shinomiya K, Nifujii A, Denhardt DT, Noda M. Osteopontin facilitates angiogenesis, accumulation of osteoclasts, and resorption in ectopic bone. Endocrinol., 142, 1325–1332 (2001).

26) Kokubo T, Takada H. How useful is SBF in predicting in vivo bone bioactivity? Biomaterials, 27, 2907–2915 (2006).

27) Tanahashi M, Matsuda T. Surface functional group dependence on apatite formation on self-assembled monolayers in a simulated body fluid. J. Biomed. Mater. Res., 34, 385–395 (1997).

28) Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G. Stimulation of bone formation in vitro and in rodents by statins. Science, 286, 1946–1949 (1999).

29) Du Z, Chen J, Yan F, Xiao Y. Effects of simvastatin on bone healing around titanium implants in osteoporotic rats. Clin. Oral Implants Res., 20, 145–150 (2009).

30) Du Z, Chen J, Yan F, Doan N, Ivanovski S, Xiao Y. Serum bone formation marker correlation with improved osteointegration in osteoporotic rats treated with simvastatin. Clin. Oral Implants Res., 24, 422–427 (2013).

31) Elnashar MM, Yassin MA, Moniem AEFA, Bary EMA. Surprising performance of alginate beads for the release of low-molecular-weight drugs. J. Appl. Polym. Sci., 116, 3021–3026 (2010).

32) Donati I, Paolelli S. Material properties of alginates. Microbiology Monographs 13: Alginites: Biology and Applications (Steinbüchel A, Rehm BHA, eds.) Springer-Verlag, Berlin, Heidelberg, pp. 1–53 (2009).