PREPARATION, CHARACTERIZATION AND GENTAMICIN SULFATE RELEASE INVESTIGATION OF BIPHASIC INJECTABLE CALCIUM PHOSPHATE BONE CEMENT

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A calcium phosphate cement containing an antibiotic can be used for filling bone defects and to ensure local antibiotherapy. Therefore, in the present research, cement paste were prepared by combining cement liquids comprised of 4 wt.% Na2HPO4 with cement powders that consisted of β-tricalcium phosphate (β-TCP) and monocalcium phosphate monohydrate (MCPM). Gentamicin sulfate was also loaded on the cements and its in vitro release was evaluated over a period of time. The cement setting times were compared before and after drug addition. According to results, the initial and final setting times of samples came down after drug addition, reached to 5 and 15 min, respectively. Compressive strength of the drug-loaded samples aged in PBS measured about 30-40 MPa and showed it did not vary significantly with the period of aging until 36 days ($p<0.05$). pH values of the PBS solution containing samples descended gradually until reached to an equilibrium pH. Phase analysis of the samples with X-ray analysis (XRD) indicated the presence of monetite and β-TCP in all samples. Microstructure of the fracture surface showed that the cement particles tended to form a highly integrated microporous structure. Extrusion curves of cement paste indicating that it can be delivered through a surgical gun in small non-load bearing bone defects. Finally, the results showed it reached its maximum level (35% of the initial value of the drug) on day 15, suggesting no irreversible binding occurred between the cement paste and the antibiotic of Gentamicin sulfate.

INTRODUCTION

Few complications in reconstructive orthopedics pose a more challenging obstacle to clinical success than infection, and bacterial osteomyelitis remains an important and daunting orthopedic and clinical problem. A variety of prophylactic and therapeutic techniques have been designed to reduce the incidence and impact of infection. Conventional treatment using systemic antibiotics is expensive, prone to complications and often unsuccessful. Therefore, local therapy is desired to treat or prevent osteomyelitis [1,2].

Polymethylmethacrylate (PMMA) cements are currently used as local antibiotic delivery systems in clinic. However, PMMA is non-degradable and has to be removed through a secondary surgery [3]. Thus, biodegradable bone cements, like calcium sulfate or calcium phosphate, have been extensively studied as antibiotic carriers [4-9]. Calcium sulfate has been used as bone-void filler since 1928 and was developed as a commercially available antibiotic loading product (Osteoset BVF-Kit, USA) [7,10], but it cannot form a chemical bond with bone tissue at the early stage [11,12] and its resorption rate is too fast [10,13]. Calcium phosphate cements (CPC) with similar composition to the bone mineral phase are biocompatible and osteo-conductive and many studies have been carried out on CPC as drug carriers for different drugs and CPC formulations [8].

The possibility to obtain monolithic hydroxyapatite at body temperature via a cementitious reaction was put forward by LeGeros [14] and Brown and Chow [15] in the early eighties. This was a significant step forward in the field of bioceramics for bone regeneration, since it provided a material which, in addition to being bioactive, was mouldable and had the capacity of self-setting in vivo, within the bone cavity [16] and [17]. In addition, the development of injectable calcium phosphate cement formulations established good prospects for minimally invasive surgical techniques developed in recent years, less aggressive than the classical surgical methods.

Since then, calcium phosphate cements have attracted much attention and different formulations have been put forward [15-22]. In general, all CPC are formed by a combination of one or more calcium orthophosphates, which upon mixing with a liquid phase, usually water or an aqueous solution, form a paste which is able to set and harden after being implanted within the body.

Gentamicin is an aminoglycosidic antibiotic for bacterial infections caused by staphylococcus which are sensitive to Gram-negative bacteria [23] and of particular interest in orthopaedic surgery where it can be combined with bone graft materials to prevent from infection after surgery.

In this research, we prepared gentamicin sulfate loaded CaP bone cement and carried out an investigation of the cement injectability in a constant injection velocity; then, we investigated the effect of drug addition on the cement properties and its injectability; as a result, we obtained an injectable drug loaded calcium phosphate bone cement which did not contain any polymeric additives to improve injectability.

EXPERIMENTAL PROCEDURE

Materials

MCPM (Fluka, No. 21053), Na_2SO_4 (Merck No. 1.06643.2500) and β-TCP (synthesized in laboratory based on a solid state process [24]) were used as powder component in 1.3, 0.08 and 4 mole percent, respectively.

4 wt% Na_2HPO_4 (Merck No. 1.06585.1000) in deionized distilled water were used as the cement liquid. A commercial ampoule of Gentamicin sulfate [80 mg/2 ml (40 mg/ml) Gentamicin 80, Exir Pharmaceutical Co. IRAN] was added to the liquid component in drug loaded samples.

Cement preparation

The powder component fractions were mixed and dry milled for 1 hour to have a uniform particle size and even distribution. Then, the powder and the liquid components were mixed together with appropriate liquid to powder (L/P) ratios and kneaded with steel spatula on a glass plate to result a homogeneous paste. The cement pastes were molded under pressure (12 MPa) into a stainless steel die of 6 mm diameter and 12 mm height and allowed to set to create cylindrical samples. After the cement sample had been under the constant pressure (12 MPa), the mold assembly was removed from the pressure-loading device and placed in a punch press, which used a 5 mm diameter push rod centered on the plunger to push the plungers and the cement sample out of the mold body in one continuous motion. The push rod was not in direct contact with the cement specimen, thus reducing the risk of damaging the sample during this process.

Cement characterization

Particle size distribution analysis

Particle size distribution of the powder sample was determined by the Laser Particle Sizer Analysette 22 (Fritsch GmbH, Germany) recording the scattering of a He-Ne laser beam (wavelength 632.8 nm) caused by the solid material suspended in demineralized water. Disintegration of the solid powders was supported by an ultrasound bath with incorporated stirrer. The lowest particle size accessible is ca. 0.6 μm due to the wavelength used for measurement.

Surface area analysis

The BET method was employed to measure the surface area of samples with a Micromeritics Gemini 2360 surface area analyser, whereby nitrogen gas molecules are adsorbed onto a solid surface, which allows measurement of the surface area of a material. The samples were first prepared using a Gemini Vac Prep degasser. Dry and degassed samples were then analysed using the single/multipoint adsorption method for surface area assessment.

pH Measurement

The pH value of the environmental solution was monitored with a pH/Ion meter (Metrohm 692, Switzerland).

Setting time

To perform the setting time test it was put in molds and tested according to the Gillmore needle standard (ASTM C266-89). The initial setting time was defined as the time when a 113.5 g statistic pressure (needle diameter 2.1 mm) does not leave a visible print on the surface of the cement. Also, the final setting time was the corresponding time for a static pressure of 453.6 g (needle diameter 1.06 mm). Setting time of five samples of each composition was tested in this section.

Injectability

The injectability was defined as the ability of cement to move through a syringe–catheter device. This test was investigated through drawing extrusion curves of the cement paste. The cement paste was filled into an insulin syringe (without needle) which was placed between the compression plates of a computer-controlled Universal Testing Machine (Zwicki, Zwick/Roell, Ulm, Germany).

Mechanical properties

The mechanical strength of the scaffold was performed in a compression test (Zwicki, Zwick/Roell, Ulm, Germany) according to DIN EN ISO 3386. The cross-head speed of the compression was set to 1 mm/min.

X-ray diffraction

The phase composition of the set cement was analyzed using X-ray diffraction technique (XRD, Philips PW 3710), with voltage and current setting of 40kV and 40 mA, respectively and uses Cu-Kα radiation (1.5406Å). For qualitative analysis, XRD diagrams were recorded in the interval $5° \leq 2\theta \leq 40°$ at a scan speed of 2° /min giving a step size 0.02º and the step time 1 s.

Scanning electron microscopy

The samples were coated with a thin layer of gold (Au) by sputtering (EMITECH K450X, England) and then the shape and morphology of prepared cement were observed on a scanning electron microscope (SEM; Stereoscan S 360 Cambridge) that operated at the acceleration voltage of 25 kV.

High performance liquid chromatography (HPLC)

The amount of antibiotic released into distilled water was determined by HPLC (column: Symmetry™ C8 3.9 mm × 150 mm, mobile phase: 0.1 M sodium phosphate, pH 6.25, methanol 47/55, flow rate: 0.7 ml/min, detection: 280 nm) at different times.

Statistical analysis

All experiments were performed in fifth replicate**.** The results were given as means ± standard error (SE). Statistical analysis was performed by using One-way ANOVA and Tukey test with significance reported when P<0.05. Also for investigation of group normalizing, Kolmogorov-Smirnov test was used.

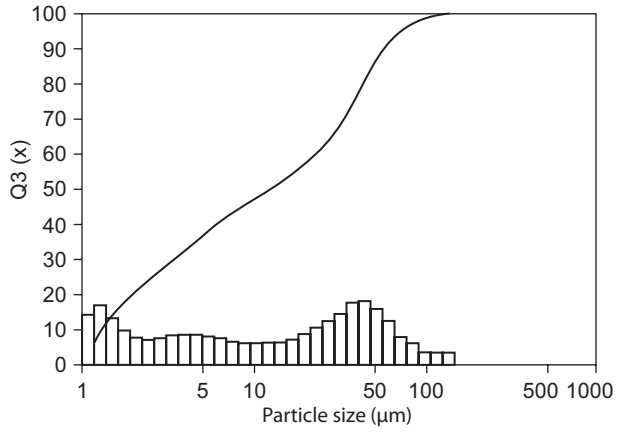


Figure 1. The weight percent and cumulative volume on the Y axis is plotted versus the particle diameter in microns on the X axis.

RESULTS

Particle Size Distribution

Particle size distribution results showed a broad particle size distribution and the mean particle diameter (d_{50}) of 12 μm, which is shown in Figure 1. According to BET technique, Specific surface area was approximately 6 m^2/g. As that is illustrated in Figure 1, the powder component had a broad particle size distribution and moderate particle diameter. In fact, the particle size of the powder plays an important role in the extent of powder reactivity. Specific surface area of powder increases with decreasing particle size; as a result, increases cement hydration and reactivity. Also, broad particle size distribution will help to better cement injectability [25].

Setting Time

According to the results given in Figure 2, the setting times of the cements prepared with different concentrations of Na_2HPO_4 did vary significantly with the liquid component concentration ($p < 0.05$). As we expected, our results showed that increasing of the Na_2HPO_4 concentration would led to an increase of the setting time. However, the initial setting time of 8.5 min and the final setting time of 23.5 min were obtained in 4wt% Na_2HPO_4.

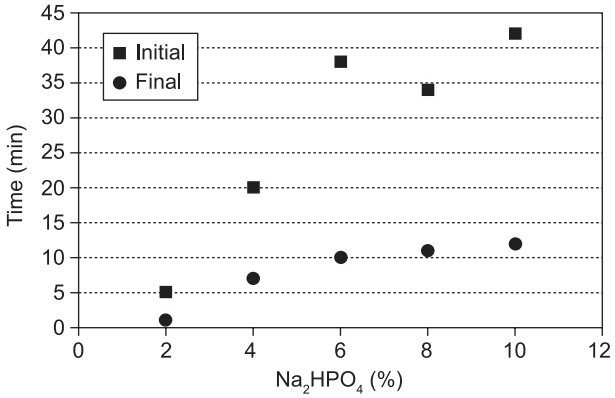


Figure 2. The initial and final setting times of samples with different concentrations of Na_2HPO_4.

In the next step, the drug-loaded samples prepared by adding different amounts of gentamicin sulfate to the liquid component (L/P= 0.4 ml/g). The initial and final setting times of samples containing 0.2 ml drug came down to 5 and 15 min, respectively. With increasing the drug dose to more than 0.2 ml, the setting times increased which could be attributed to the change of sulfate ion concentration [26] in calcium phosphate bone cements (Figure 3).

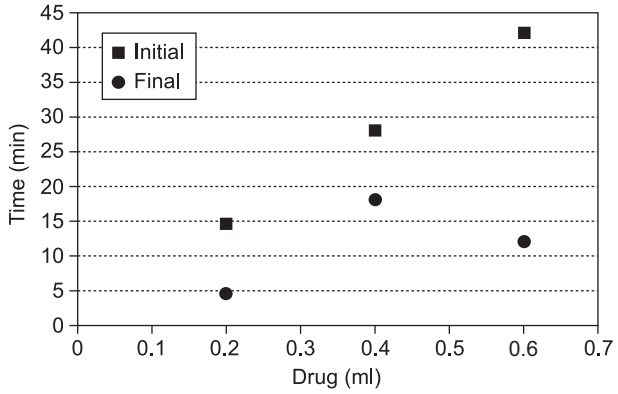


Figure 3. The initial and final setting time for drug-loaded samples containing various drug amounts.

Compressive Strength

After 24 hours aging in an incubator of 37°C and 100% humidity, samples were incubated in Phosphate-Buffered Saline solution (PBS) which was prepared according to British Pharmacopoeia (2.38 g/L Na_2HPO_4, 0.19 g/L KH_2PO_4 and 8 g/L NaCl) with the same conditions. Compressive strength of drug-loaded samples was measured following 36 days aging. Five parallel experiments were carried out for each group of data. According to the results illustrated in Figure 4, the compressive strength of the cement samples was about 30-40 MPa and it did not vary significantly with the period of aging upon 36 days ($p < 0.05$).

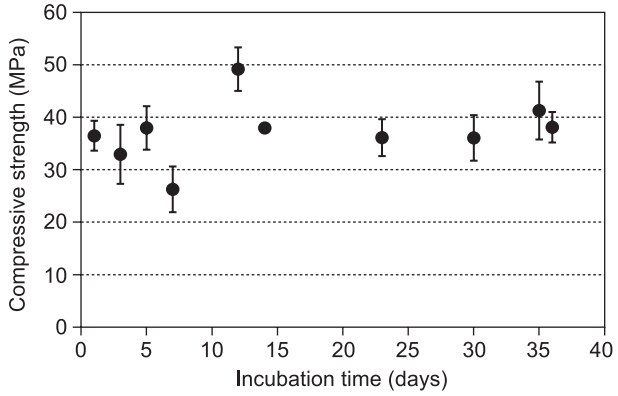


Figure 4. Compressive strength of drug loaded samples following 36 days aging in PBS.

pH Measurements

In order to get an idea about the equilibrium pH of the cement paste after hardening, cylindrical cement samples were incubated in 25 ml PBS. pH values of the PBS solution containing samples measured following 30 days aging. The beginning of the pH profile was probably determined by the dissolution of fast-dissolving MCPM resulting in a sharp pH decrease. Then, the pH profile decreased until reached to an equilibrium pH of the existing phases [27] which is given in Figure 5.

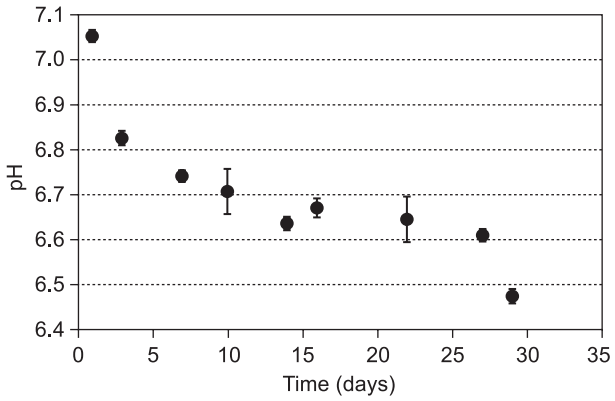


Figure 5. pH measurement after 30 days aging in PBS solution.

Phase Analysis

The set cement mass was kept in 100% humidity in PBS at physiological conditions about 30 days, and then dried and powdered. The crystallographic phases were determined by XRD. According to JCPDS # 9-80, there were peaks corresponding to monetite at d= 2.95, 3.37, 3.35 and 2.72. Also, according to JCPDS # 9-169, there were peaks corresponding to β-TCP at d= 2.88, 2.61, 3.21, 3.45 and 1.93 (Fig. 6). The results indicated that there existed two phases of monetite and β-Tricalcium phosphate.

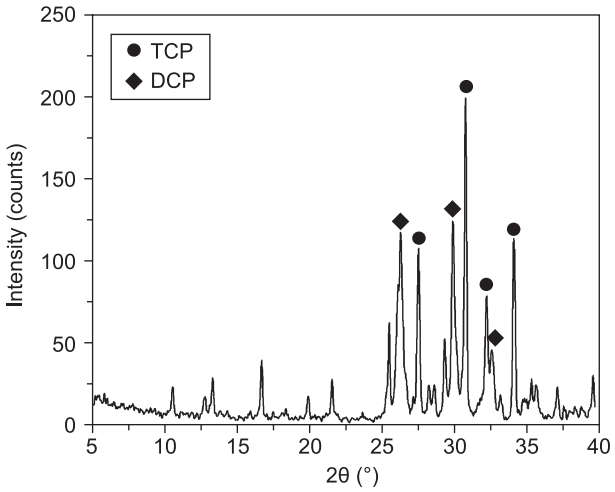


Figure 6. XRD patterns following 29 days incubation in PBS solution.

Microstructure analysis

After the reaction was stopped at a specific time in each sample, the fracture surfaces of the samples following compression test was gold sputtered, and observed by scanning electron microscopy (SEM).

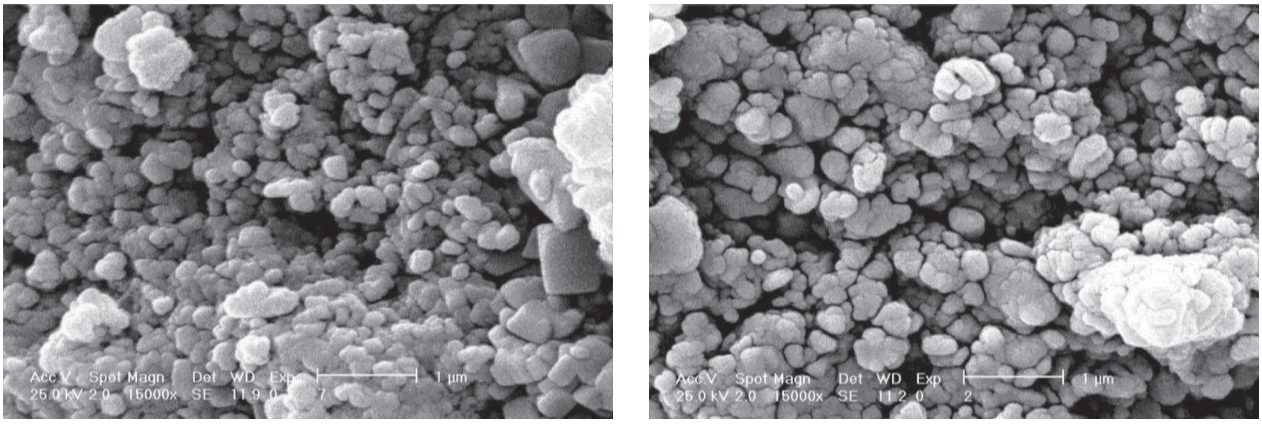
The results showed similar microstructures of crystals in the range of about 100-200 nm and the mass was found porous (microporous structure). By increasing the incubation time, from 15 days to 29 days, such microstructures formed a larger agglomerated structure in the micrometer porous structure (Figure 7).

Injectability Tests

A 1 ml, Insulin syringe, with 0.91 mm inner diameter and 0.2 mm opening was used for injectability tests. The cement powder was mixed with liquid component (L/P = 0.4 ml/g). To obtain a homogeneous paste, the paste was kneaded and filled into the syringe which was fixed between two compression plates of a computer-controlled Universal Testing Machine. After 3 min (half of the initial setting time) mixing, force was applied on the syringe piston. Figure 8 shows the syringe force profiles required to keep constant the velocity displacement of the plunger. The extrusion force was recorded while the plunger was moving the syringe length until there was no remaining cement in the syringe. The cement paste extrusion curves of two velocity displacements (0.5 or 2 mm/min) were compared. Next, this process was repeated and compared in drug loaded samples. Extrusion curves characterized a slight increase in force continuing by a final dramatic increase at a maximum force of about 300-350 N.

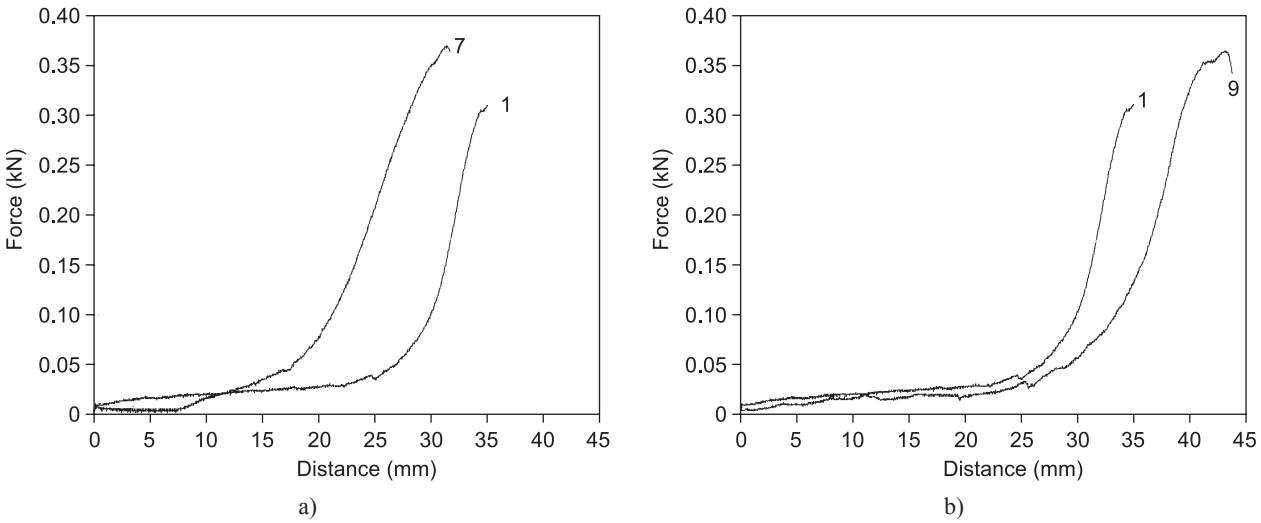
Drug Release

Drug release measurement performed by HPLC following 15 days aging of cylindrical samples in 25 ml Ringer's solution (Figure 9). The results showed two



a) b)

Figure 7. SEM image of samples following (a) 15 days and (b) 29 days incubation in PBS (30 000×).



a) b)

Figure 8. Extrusion curves of samples; a) the comparison between the sample 1 without drug and the sample 7 which was drug loaded with the same injection rate of 0.5 mm/min; b) the sample 7 and 9 which both were drug loaded with different injection rates of 0.5 and 2 mm.min 1, respectively.

stages: the first stage occurred during the first 10 days which was corresponded to the fast drug release from the surface of matrix. Afterwards in the second stage, the release rate markedly slowed down to zero between days 10 and 15 due to drug release from pores and cracks presenting in the cement structure. In this long term release, the drug release reached up to 35% of the total amount of gentamicin sulfate during 15 days.

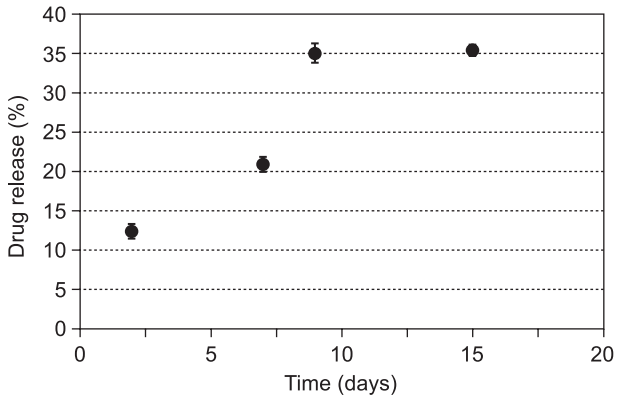


Figure 9. Drug release profile of samples following aging in Ringer's solution.

DISCUSSION

The setting reaction of brushite cement can be represented by the following equation [28]:

$$\beta\text{-}Ca_3(PO_4)_2 + Ca(H_2PO_4)_2{\cdot}H_2O + 7\,H_2O \rightarrow \rightarrow 4CaHPO_4{\cdot}2H_2O \quad (1)$$

As could be found from the above reaction scheme, equimolar mixture of β-TCP and MCPM forms brushite. In order to all the MCPM react, we used extra amounts of β-TCP in the cement samples. As a result, there was not remaining unsolved MCPM (which is highly acidic calcium phosphate and could decrease the pH of the cement paste). Moreover, the hardened cement samples containing residual β-TCP (which is a basic calcium phosphate) could increase the pH of the cement paste.

Setting time comparison of the drug-loaded and without drug samples suggested that the drug addition could result a decrease in setting time. However, the setting time increased with increasing the drug dose more than 0.2 ml which is contributed to presence of sulfate ion concentrations in gentamicin sulfate of the cement. In this study, we adjusted the amount of drug in cement with its setting time; then we kept it constant in other steps.

The compressive strengths of the drug-loaded samples following 36 days aging in incubator did not vary significantly. These results showed that the cement could maintain its strength (about 30-40 MPa) during immersion in PBS, which could be explained with the stability of the cement final products following aging.

The XRD spectra of all samples indicated no significant crystallographic differences between samples aged for different days and revealed the presence of monetite and β-TCP, but no brushite crystals were observed. Since water is a reactant in cement systems, its ejection during compaction may reduce the degree of conversion to brushite; in addition, there are only minor differences in calculations between monetite and brushite, because the solubility isotherm of monetite is the same as brushite approximately.

As the incubation time increased, SEM results showed an increase in porous structure with larger agglomerates. It revealed that the cement formed porous integrated structure as the aging time increased.

pH values tended to decrease with increasing the incubatiion time. However, it is in the range of neutral pH that is probably because of using excess amounts of β-TCP to reduce the cement acidity. Finally, it reached to an equilibrium point in the solution phase diagram of the two existing final products, monetite and β-TCP, that was at about 6.4.

To draw a release profile, in this research, we investigated the drug release of the constant amount of drug in different incubation times. The drug release results revealed two release steps. In the first step, the profile has linear relationship with the time. This high initial drug concentration release could provide a good barrier against infection in the adjacent bone which it continued slowly until second step (like other antibiotic releasing from bone cements). Compared with other drug loaded CPCs in which the drug release was completed during 7 days, there only released 35% of drug after 15 days. Different factors such as difference in cement composition and microstructure, different doses of drug and different method of measurement could change the results significantly.

Obviously, the injectability of CPC is important in clinical applications that involve defects with limited accessibility or narrow cavities, when there is a need for precise placement of the paste to conform to a defect area, or when using minimally invasive surgical techniques. It is worth mentioning that, injectable calcium phosphate cements may widen the applications of CPC in areas like filling narrow defects, *in situ* fracture fixations, and small non-load bearing orthopedic applications [29-34]. It has been reported by surgeons that calcium phosphate cements are poorly injectable, thus, some researchers added polymeric additives to overcome this problem. In this research authors tried to obtain injectable calcium phosphate cement without adding polymers which could avoid contradicting drug properties. In extrusion curves, a maximum force of about 300-350 N was achieved which is in agreement with other authors. However, the increase of injection rate (from 0.5 to 2 $mm.min^{-1}$) would led to an increase in plunger movement and a decrease in injectability. Hence, the lower rate of injection caused an increase in injectabilty. Also, drug addition could shift

the extrusion curves towards less plunger movement. As a result, it is found that drug addition not only did not disturb cement injectability, but also could help to improve it. Our results showed that this cement has the potential to be delivered through a syringe or an applicator of practices which involve minimally invasive methods, and filling in limited acce

CONCLUSIONS

In conclusion, we prepared biphasic drug loaded calcium phosphate bone cement consisting of β-TCP and DCP phases which level off the acidity of brushite cement. Moreover this cement could release gentamicin sulfate within the time in the surgical site and could be injected through a syringe or a surgical gun. Furthermore, its compressive strength was more than that of human trabecular bone (10 MPa) and comparable to other reports; besides it did not vary significantly with the incubation times. This cement will be investigated in an animal study experiments in near future.

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