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Evaluation of ascorbic acid-loaded calcium phosphate bone cements: Physical properties and in vitro release behavior

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Abstract

In this study, different concentrations of ascorbic acid (50, 100 and 200 μg/mL) were added to the liquid phase of a calcium phosphate cement (CPC). The cements were immersed in simulated body fluid (SBF) for different intervals and physical, physicochemical and mechanical properties of them were evaluated. The release of added ascorbic acid from CPCs into the SBF solution was also studied. From the results, both setting time and injectability of CPC decreased by adding ascorbic acid, however the compressive strength was sharply increased before soaking in SBF solution. But, the compressive strength values of all cements (with or without ascorbic acid) soaked in SBF solution for more than 7 d duration were comparable. The X-ray diffractometry results showed that in vitro apatite formation ability of cement reactants did not change by adding ascorbic acid. The scanning electron microscopy images indicated that morphology of the formed apatite crystals was nano-needlelike and needle diameter was less than 100 nm. The loaded ascorbic acid was slowly released from CPC into the SBF solution so that about 10% and 20% of the loaded drug was released after 504 h for the cements containing 100 and 200 μg/mL ascorbic acid, respectively. The release rate was increased when the amount of added ascorbic acid decreased by 50 μg/mL.

Keywords: Drug delivery; Calcium phosphate; Bioactivity; Bone substitute; Nanoapatite

1. Introduction

A few millions sick people per year need bone graft or bone substitute to repair their bone defects resulted from a hurt or infections. Different types of bone replacements (grafts) have been known: unprocessed or processed allogeneic bone, animal-derived grafts and synthetic bone substitutes from bioceramics. Calcium phosphate cements (CPCs) are generally used for osteopathy treatment because of their excellent osteoconductivity and moldability [1]. They are also employed in medical surgeries and dentistry as filling materials for bone defect treatment [2]. Setting reaction of CPCs happened at low temperatures, originates from the quick formation of a solid apatite phase [3]. In other words, in CPCs, a powder phase is mixed with a liquid to form a paste that sets at room temperature [4–11]. Therefore, CPCs can be shaped or molded into defects before the completion of setting reaction (hardening) [12,13]. The biocompatibility of different types of CPCs has been shown in many clinical studies [14–18]. CPCs are also considered for bone tissue engineering scaffolds because of their porous structures and other distinct properties such as injectability and osteoconductivity [19,20].

CPCs are vastly used as carriers for local delivering of bone diseases drugs. In addition to bone revival, CPCs can simultaneously prevent the growth of pathogens in defected site by sustained release of loaded drugs. Subsequently, CPCs are used as carriers of various drugs such as growth factors [21], peptides [22] and antibiotics [4,23] which are biologically active after liberation.

Previously, an osteogenic supplement, i.e. a mixture of ascorbic acid, β-glycerophosphate and dexamethasone was incorporated into CPCs to enhance osteoinductivity [24]. Osteogenic differentiation of mesenchymal stem cells on these

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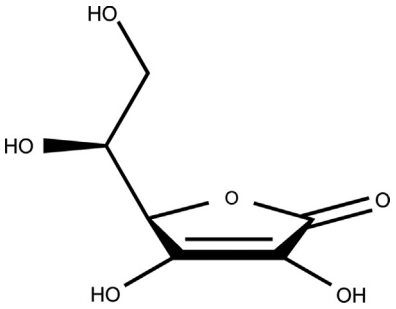

Fig. 1. Chemical structure of ascorbic acid.

cements was confirmed by gene expressions of some bone marker proteins. However the effect of this mixture on applied properties of CPCs was not evaluated, meanwhile each component has its own effect on cement characteristics. In our previous study, the release of dexamethasone from CPC matrix and its influence on differentiation of mesenchymal stem cells was studied [25].

Ascorbic acid (A.A) is another component of the above mentioned osteogenic compound which may individually affect the basic properties of CPCs. It is an organic compound with antioxidant properties. Ascorbic acid is one form of vitamin C that behaves as a vinylogous carboxylic acid (Fig. 1). Ascorbic acid is originally called L-hexuronic acid. It is derived from glucose and many animals are able to produce it, but humans require it as a part of their nutrition. In this study the effect of adding A.A on physical and physicochemical properties of CPC was determined. The releasing behavior of A.A from CPC was also studied.

2. Materials and methods

2.1. Preparation of A.A-loaded CPCs and SBF solution

Tetracalcium phosphate (TTCP) was prepared by heating a 1:1 M ratio of calcium carbonate and anhydrous dicalcium phosphate, DCPA, (both from Merck, Germany) at 1500 °C for 6 h. The product was ground in a planetary mill until a fine powder (particle size lower than 10 μm) was reached. The solid phase of the cement was prepared by mixing TTCP and DCPA at molar ratio of 1:1. The liquid phase of the cement was also a solution of 3 wt% Na_2HPO_4. The CPC paste was prepared by mixing powder (*P*) and liquid (*L*) at a *P*/*L* ratio of 3 g mL^{-1}. To make A.A-containing CPCs, A.A (purchased from Sigma-Aldrich Company) was dissolved in the liquid phase of the cement. Table 1 represents the information of different CPCs containing various amounts of A.A along with the code of each specimen. The SBF solution was also prepared according to the method described elsewhere [25].

2.2. Characterization of CPCs

The effect of A.A on initial setting time (Ist), compressive strength (*CS*), injectability (*I*) and in vitro apatite formation of CPCs was evaluated. The initial setting time was recorded by using a Gillmor needle test with a needle weight of 113.5 g and tip diameter of 2.1 mm. The paste was considered to set when the needle did not create a visible effect onto the specimen.

Table 1
Composition of CPCs according to concentration of A.A in the liquid phase.

Code	Cement powder	Cement liquid
0a	$CaHPO_4$+TTCP	$NaHPO_4$ (3 wt%)
50a	$CaHPO_4$+TTCP	$NaHPO_4$ (3 wt%)+50 (μg/mL) A.A
100a	$CaHPO_4$+TTCP	$NaHPO_4$ (3 wt%)+100 (μg/mL) A.A
200a	$CaHPO_4$+TTCP	$NaHPO_4$ (3 wt%)+200 (μg/mL) A.A

For CS measurements, the powder phase and the liquid phase were mixed to make a paste. Then, the paste was shaped into a Teflon mold to form cylindrical samples with a diameter of 6 mm and a height of 12 mm. After setting, the specimen was removed from the mold, part of the samples were incubated at 37 °C and 100% relative humidity and part of them were immersed in 50 mL of SBF solution. The compressive strength of soaked samples was evaluated after different soaking periods by using a mechanical testing device (STM 120, Santam Co.) at a crosshead speed of 1 mm/min.

To specify injectability of CPCs, 6 g of each homogenized paste was transferred into a 3 mL syringe (internal tip diameter of 8.89 mm) and extruded by a compressive load vertically mounted on top of the plunger using a computerized universal testing device (STM 120, Santam Co., Iran). The crosshead speed of 15 mm/min and maximum load of 100 N were used. The weight of the extruded paste was recorded and the injectability was calculated according to the following expression:

$$I\ (\%) = 100(\text{weight of extruded cement})/(\text{initial weight of the paste}) \quad (1)$$

The phase composition of the samples was analyzed by an automated X-ray diffractometer (Phillips PW3710) after incubating for 24 h and soaking in SBF solution at 37 °C for 7, 14 and 21 d. After each indicated period, the sample was removed from the SBF, washed with distilled water, dried, ground to fine powder and weighed to evaluate and compare phase transitions occurred during the incubation and soaking procedures. Data were afforded from 10° to 50° 2θ with a scan rate of 0.02, 2θ/s.

The Fourier transform infrared (FTIR) spectroscopic measurements were performed on specimens with and without A.A. The tablet-shaped specimen was made by mixing and pressing each sample powders and spectroscopic grade of KBr. The Infrared spectra between 4000 and 400 cm^{-1} were measured at resolution of 2 cm^{-1} by using a Thermo Nicolet Nexus 870 spectrometer.

The microstructures of the specimens (before and after soaking in SBF) were examined by scanning electron microscopy (SEM, LEO 440i). For this aim, the surfaces of specimens were coated with a thin layer of gold and the analyzed.

2.3. Drug release study

The release test was performed over 504 h. The cylindrical samples (similar to samples used for *CS* test) were weighted

and submerged in closed dark-glassy bottles containing 50 mL of SBF solution. The bottles including the specimens were placed in an orbital shaker and rotated at 20 rpm at 37 °C. At defined time intervals (0.5, 1, 2, 4, 7, 24, 48, 96, 168, 288 and 504 h), the whole volume of the solution was removed to recognize the released A.A and the bottles were nourished with fresh solution. The drug released from the cement matrix into SBF was recorded by UV visible spectroscopy (UV–vis spectrophotometer T60) through measuring the absorbance number at 243 nm. A calibration curve was plotted by measuring UV absorption of different known concentrations of A.A solutions. Consequently, the cumulative concentration of A.A was plotted against time.

To recognize the mechanisms of A.A release, the power law (Peppas) equation [6] was fitted on experimental data:

$$M_t/M_0 = Kt^n \quad (2)$$

where M_t is the cumulative amount of released drug at time t, M_0 is the initial amount of loaded drug, K is the kinetic constant and n is release exponent.

2.4. Calculations and statistical analysis

Data were processed using Microsoft Excel 2010 software and the results were presented as average value ± standard deviation of at least 4 experiments. Significance between the mean values was calculated using standard software program (SPSS GmbH, Munich, Germany) and the $p \leq 0.05$ was considered significant.

3. Results

3.1. Physical properties and phase composition of CPCs

Table 2 lists the initial setting time, compressive strength and injectability of various CPCs (with and without A.A). The setting time decreased by adding A.A to the cement composition. In more details, CPC without A.A (0a) was initially set at about 35 min whereas addition of 50 μg/mL A.A to the cement liquid causes a reduction of initial setting time by about 20%. The initial setting times of 100a and 200a specimens are nearly equal (difference is not statistically significant) but significantly lower than that of 0a.

The injectability of the cements is not good. In 0a specimen, about 72% of the paste could be extruded and filter pressing phenomenon was observed during injection process. Injectability falls when A.A is added to the cement composition and just about 57–63% of the cement paste is extruded. Overall, the results showed that the injectability of cements (with or without A.A) is in the range of 58–75%. It is a usual phenomenon in CPCs and may be resulted from the relatively high powder to liquid ratio of the cements.

As shown in Table 2, before soaking, the CS value of A.A-free CPC (0a) is lower than that of A.A-containing ones.

Table 2
Initial setting time, injectability and compressive strength of CPCs.

	Ist (min)	*I* (%)	*CS* (MPa)				
			(0 d)	(1 d)	(7 d)	(14 d)	(21 d)
0a	34.8 ± 0.8	75.2 ± 1.73	4.0 ± 1.4	9.7 ± 2.2	14.2 ± 4.6	16.2 ± 3.1	13.9 ± 3.1
50a	29.6 ± 1.1	57.6 ± 2.11	21.0 ± 2.6	18.6 ± 2.2	15.2 ± 4.5	27.5 ± 10.7	18.8 ± 3.1
100a	22.2 ± 2.7	63.4 ± 0.7	30.5 ± 2.8	14.5 ± 3.2	11.1 ± 2.9	36.7 ± 2.0	16.3 ± 3.3
200a	25.1 ± 0.8	60.5 ± 0.9	25.6 ± 6.3	14.4 ± 5.9	13.0 ± 4.1	32.1 ± 5.8	13.7 ± 7.2

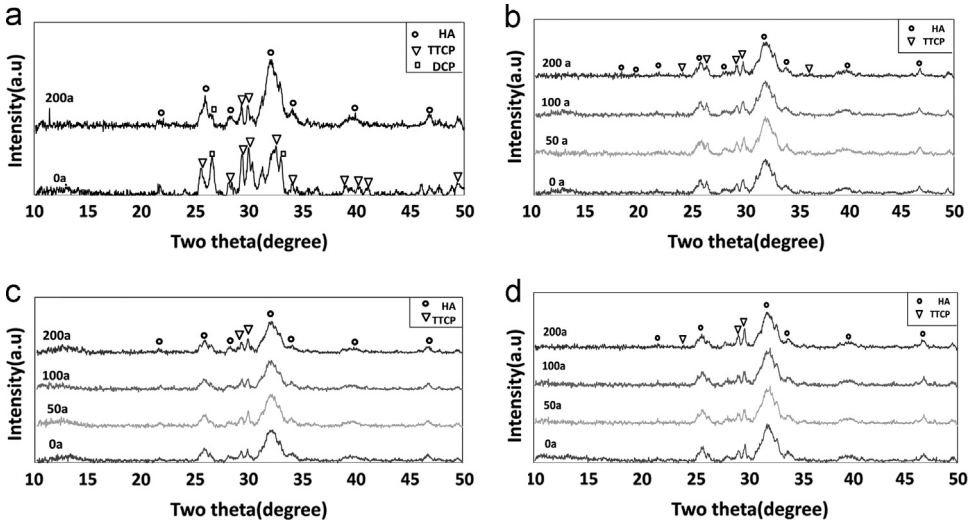

Fig. 2. The XRD patterns of different CPCs soaked in SBF solution for different periods: (a) 0 d (unsoaked samples), (b) 7 d, (c) 14 d and (d) 21 d.

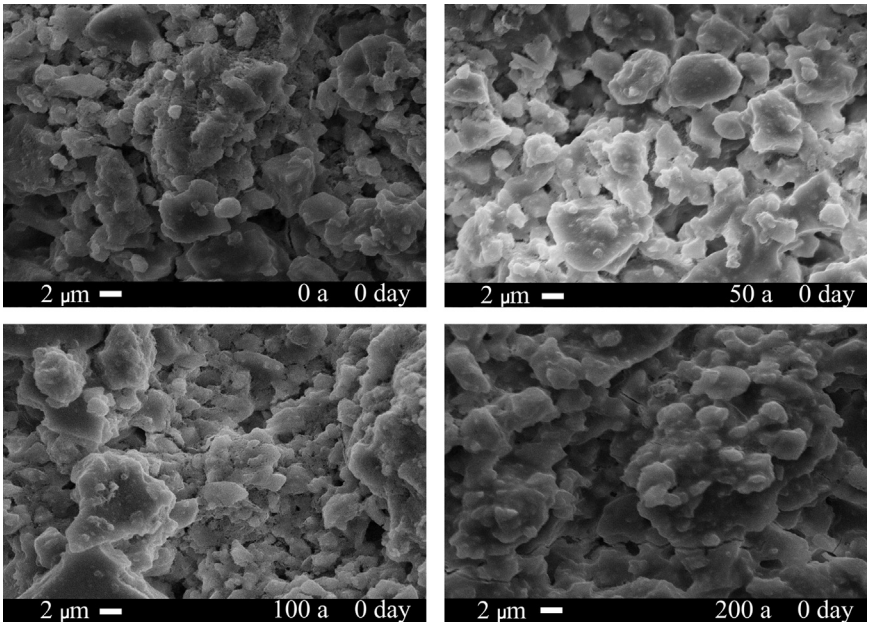

Fig. 3. The SEM images of A.A-loaded CPCs before soaking in SBF solution.

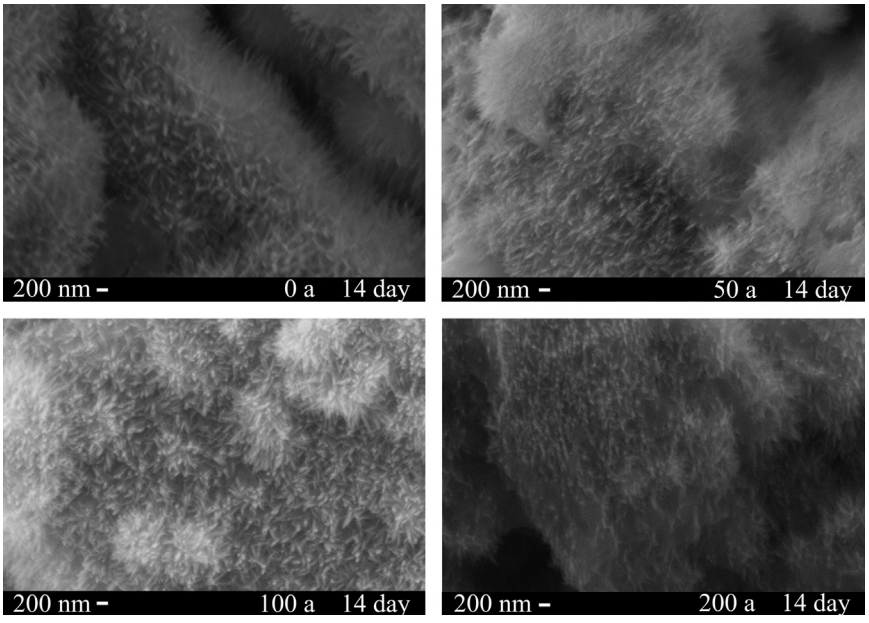

Fig. 4. Morphology of CPCs containing different amounts of A.A after soaking in SBF solution for 14 d. Nanosized needlelike crystals with tight entanglement is observed for all cements.

The higher compressive strength of unsoaked A.A-loaded cement may relate to the formation of ascorbate complexes from the reaction of ascorbic acid and calcium ions. The authors suggest that formation of these complexes is the main reason of decreased setting time and inadequate injectability of A.A-loaded cements. The results of XRD (Fig. 2) also show that apatite phase has been well produced in A.A-loaded cement even before soaking in SBF. It increases the compressive strength of loaded samples. After soaking, the mechanical strength of 0a specimen is also increased due to the formation of apatite phase (Fig. 2). It is suggested that decrease in compressive strength of A.A-loaded cement at days 1 and 7 is due to the drug liberation and dissolution of A.A and ascorbate complexes in SBF while CS decrease at day 21 relates to dissolution of apatite crystals. Moreover, increase in compressive strength of A.A-loaded cement at day 14 (compared to day 1 and 7) is probably assigned to the growth of apatite phase by time elapsing. It should be noted that the differences between CS values of A.A-containing cement at days 1, 7 and 21 are not statistically significant ($p > 0.05$).

Fig. 2 shows the XRD diagrams of CPCs soaked for different time intervals. The patterns of all incubated A.A-loaded CPCs were similar to that of 200a specimen (data are not shown). As shown in Fig. 2a there are some differences between the unsoaked samples with and without A.A. No diffraction pattern corresponding to apatite phase is detected in

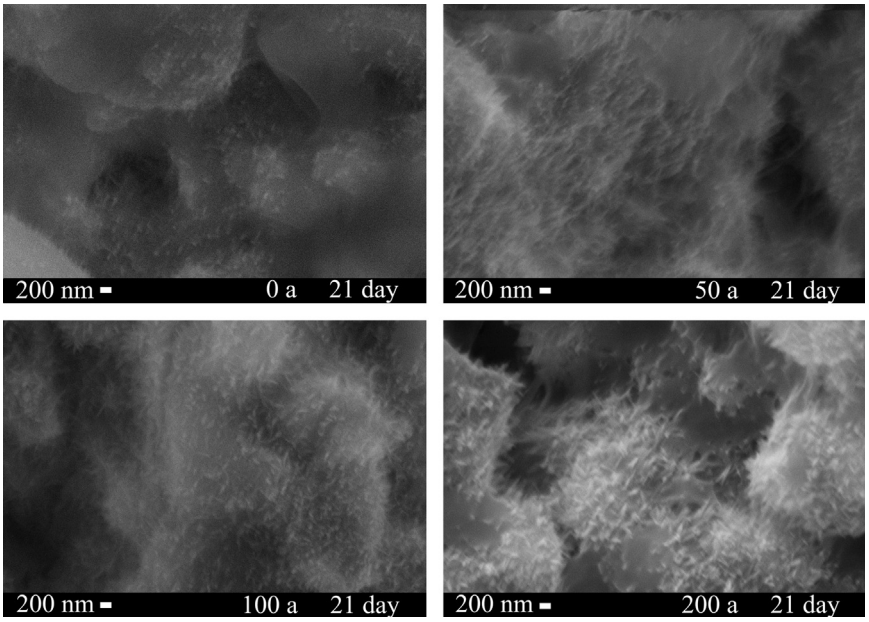

Fig. 5. The morphology of CPCs containing different amounts of A.A after soaking in SBF solution for 21 d.

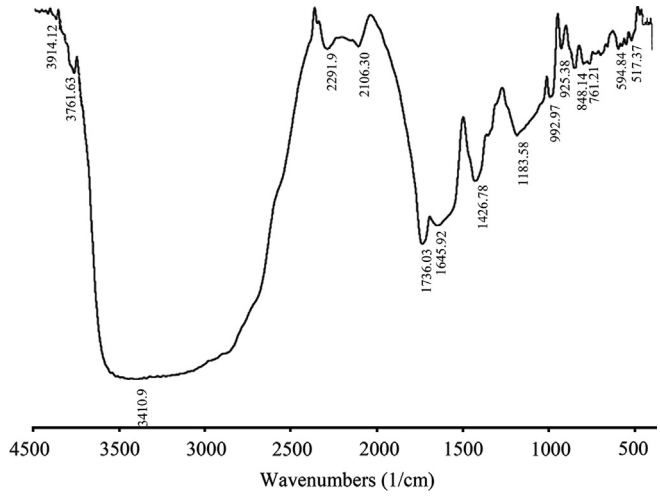

Fig. 6. The FTIR spectra of ascorbic acid.

the composition of control group. It is well known that, the setting phenomenon in CPCs occurs due to the apatite precipitation. Regarding to XRD of 0a specimen, it can be stated that the concentration of the formed apatite phase is negligible and it is not detected by the X-ray diffractometer. In contrast, for the cements containing A.A, considerable amounts of the reactants (TTCP and DCPA) have been converted into apatite phase even before soaking. It reveals that A.A encourages formation of apatite phase in the CPCs. After soaking in SBF solution, the reactants are considerably converted to apatite phase though some remained TTCP is still found.

Fig. 3 shows the morphology of CPCs before soaking in SBF solution. For 0a specimen, a relatively porous structure is observed with micropores size of 1–10 μm. As seen in the pictures, microstructure of the cements becomes granular and more compacted when A.A is used in cement composition.

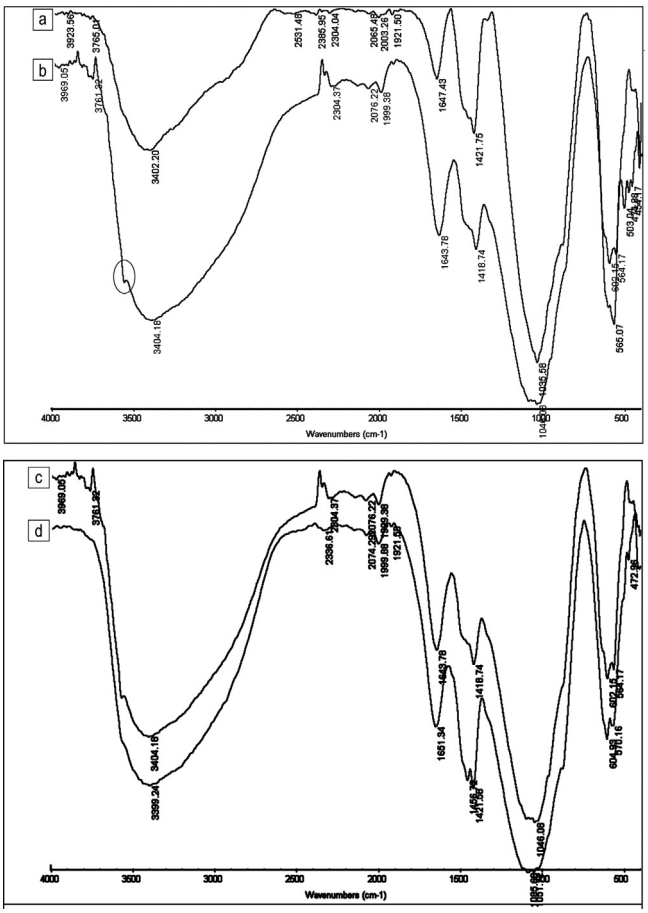

Fig. 7. The FTIR spectra of unsoaked samples: (a) unsoaked 0a, (b) and (c) unsoaked 200a, and (d) 200a soaked for 14 d.

Fig. 4 illustrates the surface morphology of the samples after soaking in SBF solution for 14 days. The results of SEM determine formation of nanosized needle-shaped crystals of

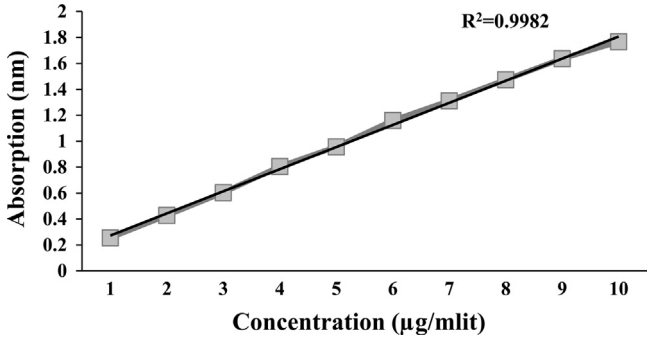

Fig. 8. Calibration curve of A.A: correlation between adsorption intensity and concentration of A.A in SBF.

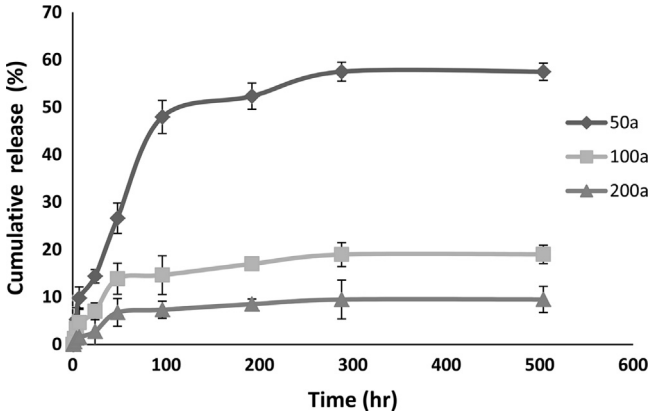

Fig. 9. Cumulative of ascorbic acid released from specimens 50a, 100a and 200a.

Table 3
Components of power low equation fitted on experimental release data.

CPC	*K*	*n*	R^2
50a	9.667	0.308	0.873
100a	7.571	0.153	0.936
200a	3.585	0.162	0.948

apatite phase (needle width < 100 nm) which are tightly entangled to each other. It seems that the size of needles and coarseness of microstructure increase in the presence of A.A.

Fig. 5 shows the morphology of CPCs after 21 d soaking in SBF solution. Needlelike crystals of apatite phase are still observed but diameter of them decreased compared to samples soaked for 7 and 14 d. It seems that the hydroxyapatite phase is going to disappear.

Fig. 6 shows the FTIR spectrum of ascorbic acid with broad absorption bands of OH at 3000–3500 cm^{-1}. Four O–H bands of ascorbic acid molecules are seen at 3529, 3420, 3359, 3215 cm^{-1} [26,27]. The C–O and C=O bands are also found at 1426 and 1736 cm^{-1}. Fig. 7 shows the FTIR spectra of 0a and 200a specimens before soaking (a and b) in SBF solution. The patterns of 200a specimen before and after soaking are also compared in Fig. 7c and d. From the location of the peaks, the following points can be pointed out: absorption band at 1035 cm^{-1} relates to the PO_4^{3-} while absorption band at around 1421 cm^{-1} is ascribed to carbonate groups. The band at 1644 cm^{-1} is appointed to adsorbed water. Also, the FTIR spectra show a broad band at 3100–3600 cm^{-1} which belongs to the OH^- group of adsorbed water molecule [28,29]. In unsoaked 200a specimen (Fig. 7b), some absorption bands of A.A molecules are seen (at around) 2000–2300 cm^{-1}.

3.2. A.A release

Release of A.A from the CPC substrates was studied by UV spectroscopy. The release of A.A has been calculated based on the intensity of the maximum absorbance of the collected SBF at 243 nm and its correlation with the prepared calibration curve (Fig. 8). Fig. 9 shows the cumulative release of A.A from various CPCs as function of time. A burst release of about 2–5% is observed for specimens 100a and 200a whereas it is about 12% for 50a. The A.A release from 50a was strongly increased during the first 96 h whereas sharp increase in amount of released A.A from 100a and 200a specimens continues up to 48 h. In other words, for the first 48 h of liberation, about 57.49%, 18.99% and 9.49% of the A.A was released from specimens 50a, 100a and 200a, respectively. It indicates that the specimen 50a has more release rate than 100a and 200a.

4. Discussion

The important properties of CPCs are setting time and compressive strength. The setting reaction of the TTCP/DCPD-based cements is a dissolution-controlled process during the initial step of reaction. In this step, dissolution of the reactant in the liquid phase leads to formation of a medium around the particles that is supersaturated with respect to hydroxyapatite. This leads to precipitation of apatite phase over the surfaces of the reactants. At the second step, further growth of apatite crystals occurs through a diffusion-controlled mechanism, which progresses the mechanical strength of CPCs [30].

The setting time, however, should not exceed 30 min for reasons of clinical applications. Fast setting is desirable because it enables the graft to attain geometric integrity and good mechanical strength within a short period of time. Generally, the clinical requirements were 5 < Ist < 15 min in bone surgery, orthopaedy, and dentistry. In this study the initial setting time of original CPC was about 34 min however it may change by modifying its characteristics such as particle size of reactants. Adding vitamin C (A.A) to CPCs decreased the setting time. The XRD results demonstrated that presence of A.A in cement composition increased apatite precipitation, so that after setting, apatite is dominant phase of cement composition (unlike the sample without A.A). This could be the main reason for decreased setting time of CPCs at the presence of A.A. Better apatite formation ability of A.A-containing CPCS can be attributed to the reduced pH of medium (by adding A.A) resulting in increased solubility of reactants. Hence, supersaturation with respect to hydroxyapatite is reached faster by introducing Ca and PO_4 ions.

The reduced setting time of CPCs by adding A.A molecules can also be attributed to the formation of calcium ascorbate complexes produced by the reaction of calcium cations and COO^- anions to form organic–inorganic compounds.

CPCs are promising materials for wide ranges of clinical applications, because of their excellent osteoconductivity and bone replacement capability. However, the relatively low strength and susceptibility to brittle terrible fracture of the CPCs have seriously limited their uses to non-stress-bearing applications. In this study, the compressive strength was about 25–30 MPa for A.A-loaded samples. It is significantly higher than the CPC without A.A. These results can be easily described by the higher content of apatite phase in A.A-containing CPCs (and interlocking of their crystals) as shown in XRD figures. Another characteristic of CPC was injectability which changed by adding A.A. Injectability is an important necessity of CPCs when using them as bone filler in defected sites with difficult accessibility. It can be improved by decreasing in setting process or by changing the reactant particle interactions. It is also decreased with increasing the solid content of the paste. The reduced injectability of cements 50a, 100a and 200a compared to 0a is assigned to the faster hardening process of these cements by the processes discussed elsewhere. Conversion of reactants to apatite phase continues during soaking the cements in SBF solution. Although no apparent differences are observed between the XRD patterns of soaked 0a and other A.A-containing cements, the SEM images suggest coarser crystals of apatite for A.A-added CPCs.

The most important limitation of many localized drug delivery systems is a fast release of the loaded drug happening within the initial hours of implantation. For supporting an effective therapeutic range, controlled release of drug should be managed; otherwise an ineffective therapy obtained by fast absorption of the released drug results in initial high-peak plasma level. CPCs are potential substrates for loading different types of loaded drug from these matrixes. This study determined the release of A.A which is water-soluble vitamin (C) and cannot be synthesized in the body. It has variety of biological, pharmaceutical and dermatological actions. Vitamin C advances collagen biosynthesis, supplies photoprotection, causes melanin reduction, scavenges free radical, and increases the immunity (anti-virus effect) [31]. All cements show good potential for sustained release of A.A. The results settle that the rate of release decreases by using higher A.A dosage, because at defined intervals, differences between concentrations of A.A-loaded samples are statistically significant ($p < 0.05$).

Table 3 shows the fit parameters of Peppas equation with each corresponding correlation coefficient (R^2) [32]. A relatively good regression ($R^2 = 0.936$ and 0.948) is observed for 100a and 200a specimens. In power law model, "K" is experimentally settled parameters which relies on the structural and geometrical distinctive of the dosage matrix and "n", the release exponent, relies on the drug release mechanism. As shown in Table 3, the K parameter decreases with increasing A.A concentration. It may be assigned to more compacted microstructure of 100a and 200a cements and better capability of them for maintaining A.A. The n values of 100a and 200a specimens are nearly equal but they are much lower than that of 50a. It reflects the higher controlled release of A.A from specimens 100a and 200a compared to 50a. The poor release of A.A from 100a and 200a specimens may relate to the strong interactions of A.A molecules with calcium phosphate particles (due to the high concentration of these molecules in 100a and 200a samples) and formation of more compacted microstructure compared to 50a (as confirmed by SEM in Fig.3). Furthermore, since the n values are lower than 0.5, a diffusion-controlled mechanism is suggested for the release of A.A from CPCs. The compacted microstructure can retard the release of A.A by restriction of diffusion process.

5. Conclusions

The following conclusions can be derived from this study:

- Adding A.A to CPC, decreased initial setting time, depending on the amount of the additive.
- The presence of A.A encourages formation of apatite phase in the CPCs, but after soaking in SBF solution, increasing A.A concentrations did not influence on rate of apatite formation.
- The mechanical strength of CPCs increases with increasing A.A concentration even for unsoaked samples.
- CPCs are favorable matrices for A.A loading, because a sustained release is found during 504 h.

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