Journal of Applied Pharmaceutical Science Vol. 7 (10), pp. 001-008, October, 2017
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2017.71001
ISSN 2231-3354

Synthesis, characterization and antimicrobial activity of nano-crystalline tricalcium silicate bio-cement

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ARTICLE INFO

Article history:
Received on: 10/08/2017
Accepted on: 06/10/2017
Available online: 30/10/2017

Key words:
Nano-tricalcium silicate, Solid state, Sol-gel, Antibacterial.

ABSTRACT

Tricalcium silicate phases were synthesized in nano-size particles by two different methods that are; the solid state reaction and the sol gel method. The solid state method is based on direct firing mixture of molar ratios from ultra pure calcium carbonate and silicon dioxide at about 1500°C, while the sol-gel process is depended on a reaction between calcium nitrate tetra hydrate and tetraethyl orthosilicate at pH = 4.5 the formed C_3S gel is calcined at 1500°C. A comparative study was carried out between the two synthesized powders in terms of setting times, micro-hardness test, pH values, calcium ion concentration, X-ray diffraction analysis, infrared spectroscopy and scanning electron microscopy (SEM). The evaluation of antibacterial activity of free released Ca^{2+} ions is performed using agar diffusion method. The data showed that C_3S powder synthesized by sol-gel method has more chemical reactivity with an increasing rate of hydration reaction than sample prepared by solid state reaction. The sol gel sample has shorter setting time, better hardness values, more alkaline hydration medium with higher pH and calcium ion concentration values. The test sol gel sample showed much better antimicrobial activity due to the superior influence of released Ca ions during the hydration process.

INTRODUCTION

Tricalcium silicate (C_3S) is the main component of mineral trioxide aggregate (MTA). It constitutes approximately 52% of the un-hydrated material (Formosa *et al*., 2012; Belio-Reyes *et al*., 2009). Both MTA and tricalcium silicate cements have confirmed bioactivity as a new phase of dental materials, namely the hydraulic silicate cements (Darvell and Wu, 2011), i.e. both form a bone-like hydroxyapatite layer on the surface when immersed in physiological solution (Sarkar *et al*., 2005; Reyes-Carmona *et al*., 2009; Coleman *et al*., 2007; Gandolfi *et al*., 2010). It is also contributory in maintaining the interface of bone–biomaterial if implanted in the body (Zhao *et al*., 2005). It was proved that silicon ion derived from calcium silicate-based biomaterials has an important role in stimulating proliferation, differentiation, osteogenic gene expression of osteo-related cells (Wenjuan *et al*., 2015). Furthermore, C_3S phases have advantageously a relatively low setting time compared with MTA, which is very suitable for replacement of MTA dental material as it has the same chemical composition and bioactivity (Chen *et al*., 2009). It also possesses excellent injectability, high bioactivity and moderate in vitro degradability and ultimately the body will be able to replace the implanted cement by natural tissue (Zhao *et al*., 2005). Hydration of Tricalcium silicate results in producing calcium silicate hydrate gel and free lime (calcium hydroxide, $Ca(OH)_2$) leaving a small portion of un-reacted material (Formosa *et al*., 2012; Zhao *et al*., 2005; Camilleri, 2011). The hydration of tricalcium silicate (C_3S) is shown in Eq. (1) (Taylor, 1997).

$$3CaO.SiO_2 + \sim 5.3H_2O \rightarrow \sim 1.7CaO.SiO_2.4H_2O + \sim 1.3Ca(OH)_2 \quad (1)$$

The $Ca(OH)_2$ released from the tricalcium silicate hydration possesses antibacterial and anti-inflammatory properties (Wang *et al*., 2008) because of the high (alkalinity) pH of the surrounding medium after it dissolves (Siqueira and Lopes, 1999).

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Also, the released $Ca(OH)_2$ may induce mineralization resulting in a repair of damaged dentin matrix (Estrela *et al.*, 1995). Pure tricalcium silicate can be prepared by solid state reaction or by the sol-gel method (Zhao and Chang, 2004).

In this work, a comparison study of tricalcium silicate phases prepared in the laboratory by two different methods; solid state reaction at elevated temperature and sol-gel method, was carried out by determining some physico-mechanical and hydration behavior such as setting times, hardness test, pH of curing medium, calcium ion determination, X-ray diffraction analysis, infrared spectroscopy and scanning electron microscopy. The evaluation antibacterial activity of both materials as influenced by the method of preparation.

MATERIALS AND METHODS

Materials preparation and characterization

Tricalcium silicate bio-ceramic has been prepared by two different methods as follows:

a. Solid state reaction

C_3S phase (S1) was synthesized by firing molded cubes of 3:1 CaO: SiO_2 molar ratio, using ultra pure $CaCO_3$ and quartz (99.6% SiO_2), in the presence of 0.5% boric acid at 1000º C for 2- hours (Lea, 2004). The product was ground, remolded using carbon tetrachloride and fired at 1450º C for 2-hours. The firing process was repeated until completion of the reaction. The end product was checked for the presence of free lime.

b. Sol-gel method

Tricalcium silicate powder (C_3S - S2) was synthesized by sol-gel method as described by H. Zhen *et al*. (Zhen *et al*., 2010). Formulation of C_3S phase by this method is based on the reaction between calcium nitrate tetra hydrate $Ca(NO_3)_2.4H_2O$ and tetraethyl orthosilicate (TEOS), $(Si(OC_2H_5)_4)$. 1.5 mol of $Ca(NO_3)_2.4H_2O$ was added drop wise to a solution of 0.5 mol TEOS in 200 ml water during continuous stirring. The pH of the solution should be adjusted at 4.5 with the aid of nitric acid. The mixture was heated to 50-55 ℃ for 2 h and the solution temperature was maintained at 75 ℃ for 24 h. The prepared gel has been dried at 120 ℃ over night and calcined at 1500 °C at a firing rate of 10°C/min for 9h.

The two synthesized powders were investigated by X-ray diffraction (XRD) to verify the identity of the synthesized compound, using a copper target with radiation; wavelength=1.54-nm, X-ray was generated at 40-KV with a current of 2-5-Ma. The scanning speed was 1^o/minute (Fig. 1(a, b)). The final materials were finely ground in an agate mill until the desired particle size, which was evaluated by Transmission Electron Microscope (TEM) (JEM-1230) at 100Kv (Fig. 2 (a, b)).

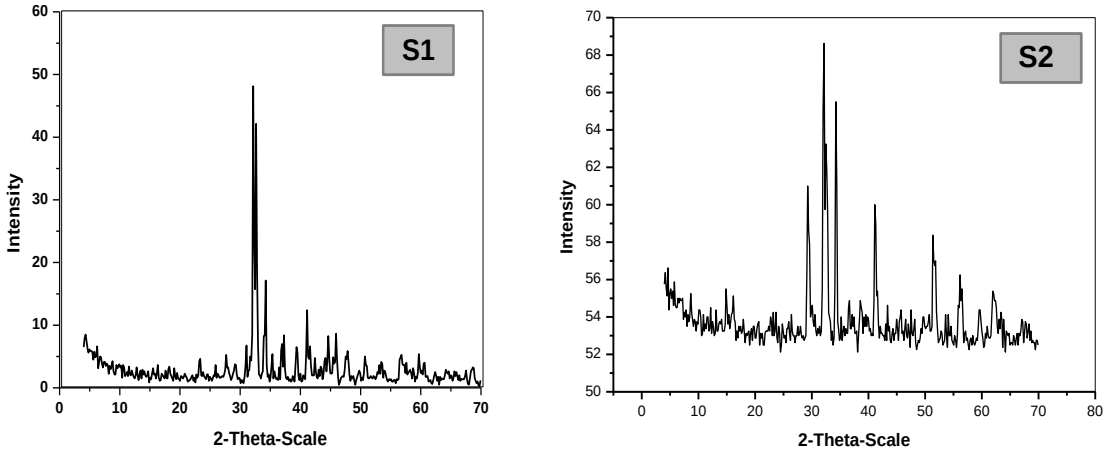
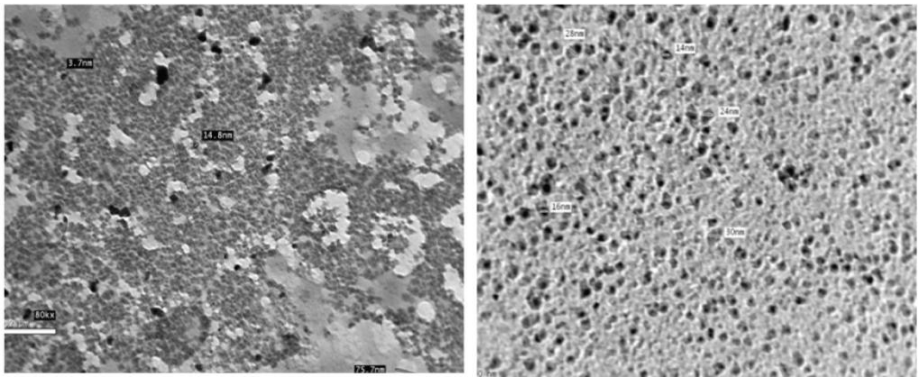


Fig. 1: XRD patterns of tricalcium silicate synthesized by two different methods.



A B

Fig. 2: Transmission electron photomicrograph of both tricalcium silicate synthesized by two different methods, (a) S1 particle size ranged from 3-75 nm and (b) S2 particle size ranged from 14-30 nm.

Physical characteristics of the two synthesized materials

Setting time

The setting time for this work was carried out according to ANSI/ADA specification no. 57 for root canal sealers and cements. Five Scoops of the powder were mixed with the required drops of distilled water that give the best workable paste to fill a mold of dimensions; 10 mm ± 0.1 mm height. Mixing was done on a clean glass slab with a clean spatula, for thirty seconds. Each mold was placed on a glass plate 1-mm thick X 25 mm wide X 75 mm long and filled with the tested S1 and S2 pastes to a level surface.

After 120 ± 5 seconds from the beginning of mixing, the assembly was placed in a cabinet maintained at 37± 1°C temperature and 95% relative humidity. The Gilmore needle (having a flat end diameter of 2 mm ± 01mm) was carefully lowered on to the horizontal surface of the material. Indentations were repeated at 30-second intervals until the indenter fails to make a complete circular indentation in the cement. The indenter was wiped clean between indentations**.**

Hardness test

The prepared pastes of both synthesized materials were poured into a cylindrical brass mold (diameter = 10 mm, height = 2 mm). The paste was placed in the mold into two approximately equal layers. Each layer was compacted and pressed along the surface of the mold until homogenous specimen was obtained. Immediately after molding, the samples were cured in a humidity chamber at 100% relative humidity at a constant temperature of 37 °C for 24 h. At the end of the moist curing period the samples were demoulded and then cured under distillated water until the time of testing at a constant temperature of 37 °C such as 1, 3, 7and 28 days.

The hardness test (Eid *et al*., 2012) was carried out on three samples of each case of the hardened cement pastes. The test was performed with a Vickers indentation hardness tester at five points on one side of each sample. A load of 1 Kg was applied for 15 seconds and the depth of indentation was then recorded.

pH of immersion solution and calcium ion concentration

To test in vitro pH variation and calcium ion released of the two types cement pastes (S1&S2) in distilled water. 1/2 inch cylinder of each type of cements was directly poured into a 25 ml beaker and then 15 ml of distilled water was injected into the container to cover the paste (Nurit *et al*., 1993). The test samples were stored in a 37°C, 100% humidity water bath. After 3, 7 and 28 days of immersion all solutions were filtrated and the pH value was measured using an electrolyte-type pH meter and the concentration of calcium ion was measured by atomic absorption spectra (Savant AA, GBC, Australia)..

X-ray diffraction of the prepared cement pastes

Selected hydrated samples were investigated by X-ray diffraction (XRD) to verify the identity of the hydrated compounds.

ATR/FTIR Spectroscopy

The hydrated specimens of the two synthesized materials were investigated using IR spectra. IR spectra were recorded on JASCO 4600 model FTIR spectrometer.

Scanning electron microscopy (SEM)

The morphology of the hydrated dry samples of the two specimens was investigated using scanning electron microscopy (SEM), with the aid of (Jeol JSM-T_{20}) after coating of the samples by a thin layer of gold using sputter coater S_{150}A.

Antibacterial Activity

The antimicrobial activity of both synthesized materials (S1&S2) has been done at one concentration by the agar diffusion method against reference strain of *staphaurogenosa*. Bacteria were diluted to obtain a suspension of approximately 5×10^8 colony-forming units/ml (0.5in a McFarland ephelometer) in sterile TSB (Trypticase Soy Broth) (Merck, Germany) (McFarland, 1907). Microbial strain was confirmed by both Gram staining and colony forming and growth characteristics. *Staphauggenosa* was inoculated with sterile cotton swabs onto TSA agar (Merck). Wells 4 mm in diameter and 4 mm deep were prepared on plates with a copper puncher, and immediately filled with freshly manipulated test materials. Positive controls antibiotic Gentamycin incubated for the same period under identical conditions. After prediffusion of the test materials for 2 h at room temperature, all the plates were incubated at 37°C and evaluated at 24, 48 and 72 h. Microbial inhibition zones were measured with a 0.5-mm precision ruler.

RESULTS

Setting time

Fig. (3) shows the setting times of the two synthesized tricalcium silicate samples (S1 and S2). The data reveal that the setting time of the C_3S sample prepared by the sol-gel method (S2) is lower than that of S1 sample by about 4-5 min, but both samples showed their setting times within a time range of 30-35 min that is very important for dental and medical applications as it is suitable time range to manipulate such material that has good mechanical properties comparing with calcium phosphate cements (Radwan *et al*., 2016).

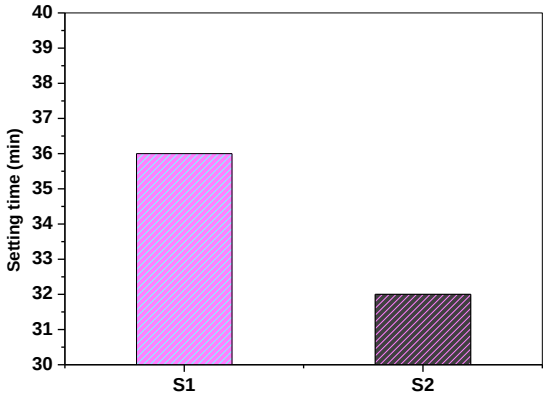


Fig. 3: Setting time of the two synthesized materials (S1 and S2).

Micro hardness

The hardness test for the two investigated materials (S1&S2) was considered in this study as a guide for the mechanical properties of the prepared C_3S phase. Fig.4. shows the hardness Vickers values in relation with the curing periods of the two materials (S1&S2). The data indicate that for both samples the hardness Vickers values showed a steady increase with curing period up to 7 days and from 7 to 28 days a little increase or almost same hardness values were detected. The tricalcium silicate phase S2 that is synthesized by sol-gel method showed higher hardness values at all curing periods.

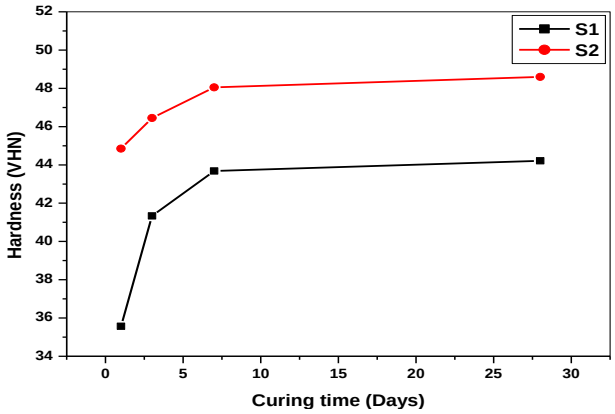


Fig. 4: Micro hardness of the two synthesized materials (S1 and S2).

pH variations of the curing medium

The pH values given in Fig. 5 reveal that the curing medium of S2 sample is more alkaline than that of S1 as it showed higher pH values at all curing ages. These values were found to be almost constant in the range between pH 11-11.2 up to 28 days curing periods. For sample prepared by solid state reaction (S1), the pH values slightly decrease from 3 to 7 curing age, followed by a strong decrease for the 28 days sample.

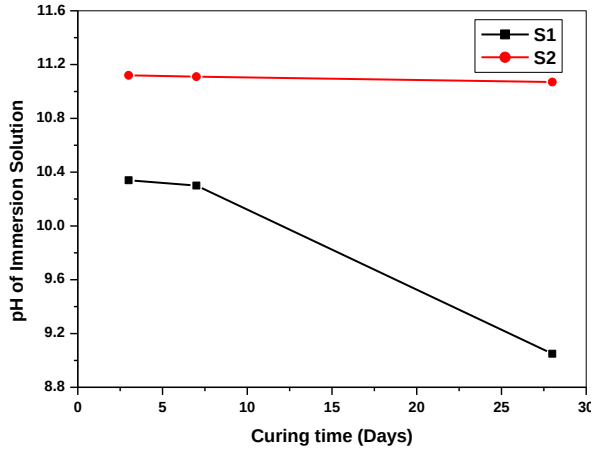


Fig. 5: pH Values of immersion liquid of the two synthesized materials (S1 and S2).

Calcium ion concentration

The concentration of calcium ion in the immersion liquid for the two synthesized samples is given in Fig. 6. The data indicate that the Ca^{++} ion concentrations for sample S2 are higher than those of S1 except a very little increase in Ca^{++} ions for S1 sample after 7 days curing age. The presence of Ca^{++} ions in the hydration medium of C_3S phase is mainly due to the liberation of $Ca(OH)_2$ during the hydration process as given in eq. 1. Liberation of $Ca(OH)_2$ increases the pH of the immersion liquid as given in Fig. 5.

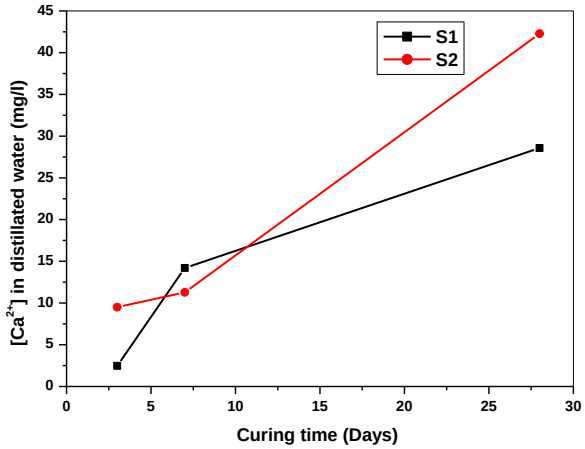


Fig. 6: Calcium ion concentration of the curing liquid of pastes of S1 and S2.

X-Ray Diffraction

Figs. 7& 8 represent the XRD data of the two C_3S samples (S1 and S2) cured for 1, 3, 7 and 28 days. Investigation of XRD patterns given in Figs. 7& 8, indicates that by increasing curing periods, there is a diminishing trend in peak height of all characteristics peaks of the anhydrous phases. The XRD data of C_3S phase prepared by sol-gel method S2 (Fig.8) reveals a little more peak diminishing than S1 prepared by solid state reaction. Overlapping of the characteristic peak of CSH over those of the anhydrous phase could be detected in the two figures. The main XRD peak height of $Ca(OH)_2$ at $2\theta=18$ is much more clear in XRD pattern of S2 than S1 and its intensity increase with curing period in S2 more than S1.

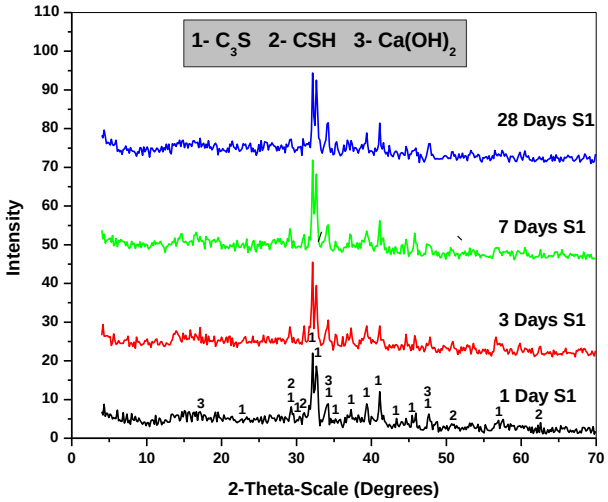


Fig. 7: XRD patterns of pastes of the investigated material S1 cured for 1, 3, 7 and 28 days

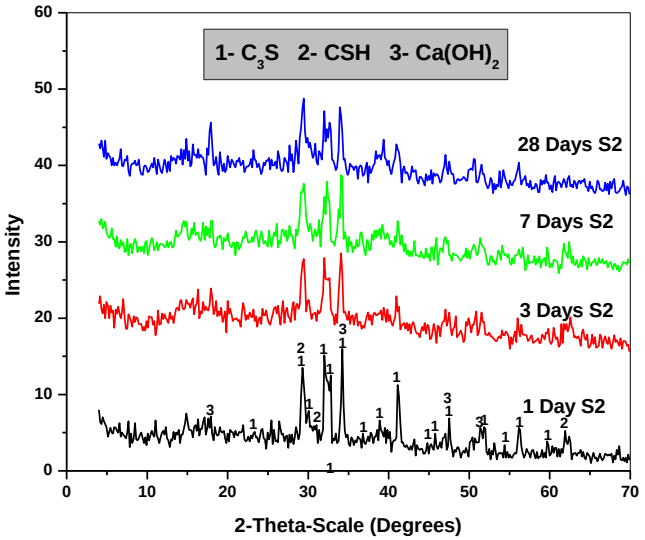


Fig. 8: XRD patterns of pastes of the investigated material S2 cured for 1, 3, 7 and 28 days

FT-IR Spectra

The infrared spectra of pastes of S1 and S2 samples cured for 1, 3, 7 and 28 days are given in Figs. 9&10. The IR spectra of calcium silicate phase S1 (Fig. 9), indicates an increasing trend in almost all band intensities of the remaining anhydrous phase at ≈ 500 (cm^{-1}) and Si-O stretching vibration with hydration period because of overlapping of the IR bands of the hydrated compounds at:- 445,815,950 cm^{-1},and incorporated ν_2H_2O and OH^- at 1630&3365 cm^{-1} and 2500&3700 cm^{-1}, respectively, characteristic to the main hydration product C-S-H compound of C_3S cement.

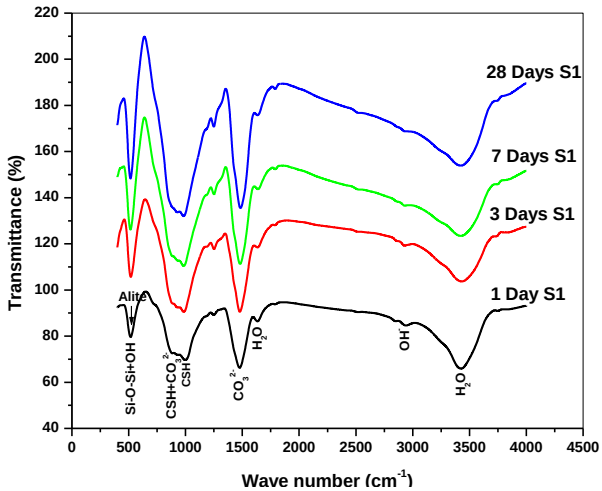


Fig. 9: IR spectra of pastes of the investigated material S1 cured for 1, 3, 7 and 28 days

The main IR bands of Aragonite (ν_2,ύ$_3CO_3^{2-}$ antisymmetric stretching) at 850, 1490 and 1460 cm^{-1} is mainly due to the partial carbonation reaction of the hydrated phases by the action of atmospheric carbon dioxide (Gadsden, 1975). Fig. 10 which represents the IR spectra of S2 samples indicates a decrease of the above mentioned bands from 1 to 3 days of curing and then increase again up to 28 days.

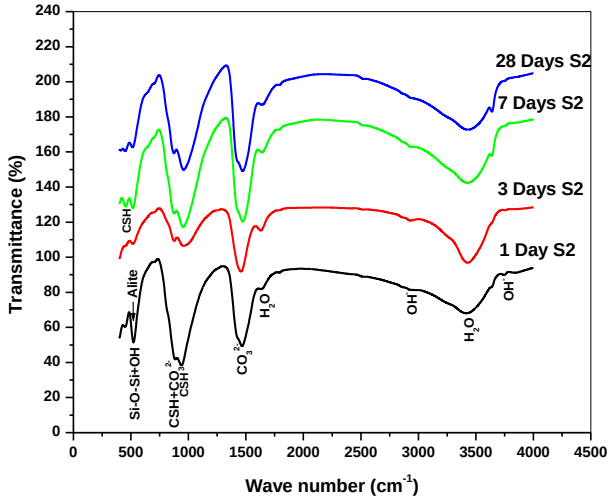


Fig. 10: IR spectra of pastes of the investigated material S2 cured for 1, 3, 7 and 28 days

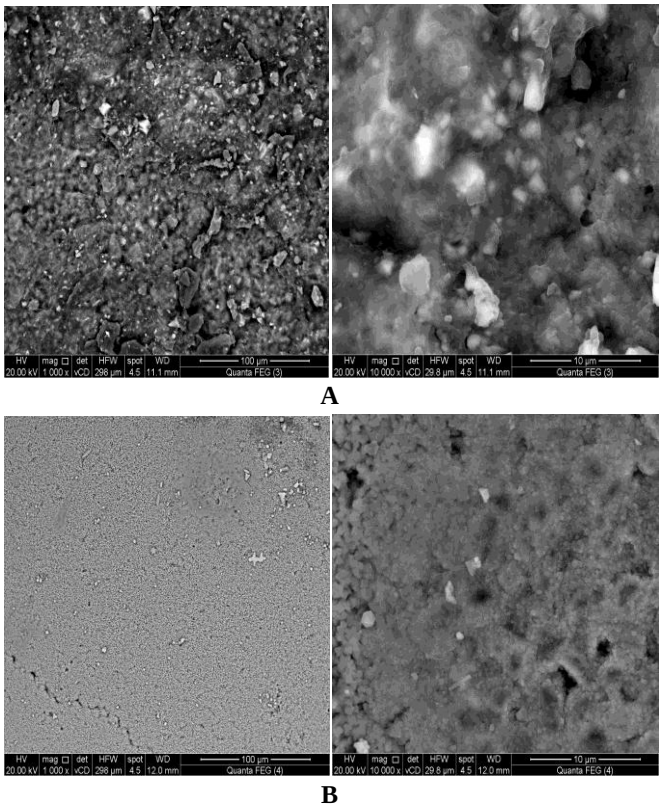


Fig. 11: SEM micrographs of pastes of the two formulated C_3S materials cured at 28 days, (a) S1 sample and (b) S2 sample

Scanning Electron Microscopy (SEM)

Fig. 11 represents the SEM micrographs of 28 days cured pastes of S1 (plates a) and S2 (plates b). Investigation of the micrographs given in Fig. (11) reveals that there is a clear difference in the morphology between the prepared sample S1 and S2.The S2 micrograph (Fig. 11. b) showed more dense structure, the anhydrous C_3S grains were found to be covered by a huge gel

(fibrous) crystals of calcium silicate hydrate (C-S-H). The less dense structure of S1 sample can be seen in the micrographs plate a, in which the coarse anhydrous particles are also embedded in the fibrous crystals of calcium silicate hydrate in more porous system. $Ca(OH)_2$ plates are partially found in a small amounts in the two micrographs.

Antibacterial Test

Data recorded in Fig. 12 revealed that the release of Ca ions from test sample S2 showed a better antibacterial activity compared to that of sample S1. Gentamycin effect as an antibacterial agent showed a superior effect than that of releasing Ca ions from test materials. The clear zone induced post treatment with S1 showed a clear zone in the order of 3.12 mm (26.15%), while the Gentamycin as a positive control recorded a clear zone of 6.5 mm, while S2 showed a more potential; as antibacterial recording 4.80 mm (52%) as shown in Table 1.

Fig. 12: Evaluation of antibacterial activity of free released Ca ion against *Staph aureaus* strain using gel diffusion assay Gentamycin [G] as positive control.

Table 1: Antibacterial activity data for the two formulated C_3S phases, S1 and S2.

Sample	CZA mm	RA. mm	% inhibition
Gentamycin Control	6.50	---	---
S1	3.12	1.7	26.15
S2	4.80	3.38	52

Where: CZA: clear zone.
RA: Reduced area.

DISCUSSION

In general, preparation of high temperature phases in the laboratory is done by firing the appropriate molar ratios of the reactant oxides to temperature at which the phase equilibrium gives the most stable composition. Due to the low temperature stability limit of C_3S, a rapid cooling rate should be applied to a temperature at which the most kinetically stable phase is maintained. Also, the cooling rate may affect the crystal size of the prepared phase, i.e. the rapid cooling results in a smaller crystal size that is ground more easily. The chemical reactivity of the synthesized phase may be changed if the cooling rate is very slow as the C_3S phase may decompose in the temperature range between 1250 °C – 1100 °C (Lea, 2004). In this study, the tri-calcium silicate phase (C_3S) was prepared by sol-gel method to compare its chemical reactivity during hydration process and the antibacterial activity with that prepared by solid state reaction. The sol-gel method is a wet-chemical preparation reaction between highly pure starting reactants resulting in a well formed final C_3S product in the reaction medium before firing process (Zhen *et al*., 2010). By this way the phase decomposition is prevented and the final product has much more fine nano-size grains (Fig. 2b). The C_3S phase (S2) formulated by sol-gel method showed shorter setting time as given in Fig. 3. which is may be attributed to large specific surface area of S2 sample. The presence of large amounts of nano-size grains in the hydration medium may act as nucleating centers that enhances the hydration reaction (Lea, 2004). As was mentioned above, the hydration reaction of C_3S phase results in formation C-S-H as a hydration compound and $Ca(OH)_2$ (free lime) as by-product, Eq. 1. this is responsible for high pH values (alkalinity) of the hydration medium (Taylor, 1997). The pH data given in Fig. 5, the sol-gel sample (S2) showed higher pH values than S1 sample prepared by solid state reaction as it may have a relatively more chemical reactivity toward hydration reaction (Camilleri, 2011). The concentration of Ca^{++} ions given in Fig. 6 emphasizes the higher alkalinity of the hydration medium of pastes of sample S2 than S1. The hardness Vickers values represented in Fig. 4.of S2 samples are higher at all curing ages than S1 that is also attributed to the higher hydration reaction rate and more fine grains of S2 sample as mentioned above. By comparing the XRD patterns of pastes of S1 and S2 at different hydration periods, Fig. 7 & 8, it can be seen that the characteristic peaks of the anhydrous S2 particles are diminishing more clear than those of S1 with the curing age and the XRD peak of $Ca(OH)_2$ at $2\theta = 18°$ could be detected clearly and it increases with curing periods for S2 more than S1 that may be related to better chemical reactivity and enhanced hydration rate of S2 sample. Investigation of the IR spectra of the prepared samples (Figs. 9 & 10) can explain the aforementioned findings.

The IR spectra of S1 sample, Fig. 9, showed a slight increase of the characteristics band of C-S-H compound, (445,815,950 cm^{-1},and incorporated ν_2H_2O and OH^- at 1630&3365 cm^{-1} and 2500&3700 cm^{-1}, respectively), while in case of S2 sample these bands showed a little decrease in their intensity due to the fast hydration rate of this sample at the first curing day which results in formation of increasing amounts of hydrated products that may encapsulate the anhydrous particle preventing their further hydration. At later hydration ages (7 and 28 days), this covering layer will be distorted and the water molecules will come in contact with the anhydrous C_3S grains (Camilleri, 2008). A continuous increase in the Aragonite bands (ν_2,ύ$_3CO_3^{2-}$ antisymmetric stretching) at 850, 1490 and 1460 cm^{-1}as a result of carbonation process by the action of atmospheric carbon dioxide (Gadsden, 1975).

Data recorded that realize Ca ions showed an antibacterial potentials, where growth of *staph aureus*compare with Gentamycinas a positive control. Data recorded by BystroÈm *et al*. (1985) demonstrated that the liberated $Ca(OH)_2$ will get rid of all microorganisms if the therapy lasted for one month. Reit and DaÂhlen (1988) found that infection persisted in 26% of the canals

after 2 weeks of dressing with calcium hydroxide. SjoÈgren *et al.* (SjoÈgren *et al*., 1991) stated that the elimination of the bacteria in root canal could medicate with calcium hydroxide during 7 days in 100% of the cases. Also, we can conclude that bacterial strain may vary in its reaction to Ca^{2+} ions and that is time dependent as was stated by Ørstavik and Haapasalo (1990). They found that the use of $Ca(OH)_2$ for 10 days may disinfect the facultative bacteria in dentinal tubules.

Also, direct contact of our extract to test bacterial bay be the cause of antibacterial potentials as released Ca ions from the formulated Ca source may depend on the bioactivity, low water solubility and diffusibility. At the same time our data may be on the contrary, to those reported the insistence of the root canal bacteria as reported by Ørstavik *et al.* (1991), they observed that after one week, the stability of bacteria is about one third of the root canal and Barbosa *et al.* (1997) stated that after 7 days, a positive cultures may be yielded in some cases, if about 25% dressed with calcium hydroxide yielded positive cultures. Even cases that yield negative cultures may result in failure (SjoĔgren, 1996; SjoĔgren *et al*., 1997).

Also, our data may be on the contrary of the other as they explained the bacterial behavior against Ca ions could be due to the bacterial type tested and pH value that may cause severe pressure by which some microorganisms will be able to proliferate with the aid of special adaptation mechanisms. Some especial enzymatic and buffering systems may influence the tolerance of the bacteria to pH variations and help to maintain a constant pH medium (Padan *et al*., 1981).

Also, several studies have tested the passivity of free lime in eliminating bacterial cells inside dentinal tubules, where Haapasalo and Ørstavik (1987) stated that a calcium hydroxide paste could not eliminate, even ostensibly, Enterococcus faecalis in the tubules. Safavi *et al.* (1990) demonstrated that after a long treatment period some bacterial system in dentinal tubules with calcium hydroxide/saline solution as the upper layer bacteria can cover and in turn protect the remaining bacteria deeply allocated in the dental tubules. The OH^- ions must diffuse inside the dentine in order to have a deep effect against the bacteria in the dentinal tubules. At the same time it was found that there is a buffering effect for the dentine due to the proton donors, such as H_2PO_4, H_2CO_3, and HCO_3, in hydroxyapatite layer, that may keep the pH constant (Wang and Hume, 1988; Nerwich *et al*., 1993). So, to increase the efficiency of Ca ions to inhibit bacteria in tubules, it is a must for OH^- ions diffusion ability to exceed the dentine buffer ability to a pH level enough to destroy bacteria. The bacterial cell arrangement in root canal may decrease the antimicrobial effects of free lime (Siqueira *et al*., 1996; Siqueira and Uzeda, 1996). Bacteria colonizing necrotic tissue in ramifications, is thmuses and irregularities are also, probably, protected from the action of calcium hydroxide due to pH neutralization. Therefore, a short-term dressing with OH^- ions appear to get rid of mainly bacterial cells in this substance as in the main root canal or in the circum pulpaldentine. These areas are also commonly affected by the chemo mechanical procedures. For any medicament, it is a must to diffuse into the root canal to have a successful action (Ørstavik, 1997). A suspension solution of free lime or sustained release Ca^{++} and OH^- ions have great cytotoxic potential due to high pH values.

CONCLUSION

The ultra-pure nano-size tricalcium silicate bio-ceramics can be formulated in the laboratory by two different methods, which are solid state reaction (S1 sample) and sol gel method (S2 sample). The results indicate that the S2 C_3S sample has shorter setting time and exhibits better hardness values than S1 sample prepared by the solid state method. It also showed more alkaline hydration medium with higher pH values, so, the C_3S prepared by sol-gel method could be more reactive than that synthesized by solid state reaction directly at elevated temperatures and slow cooling rate. The data of XRD and IR emphasized these findings. The antimicrobial test of S1 and S2 showed that the increasing amount of Ca^{++} ions liberated for S2 (52% inhibition) sample resulting in a better antibacterial activity compared to S1 sample (26.15% inhibition). The antimicrobial results clearly indicate the superior effect of free lime liberated during the hydration reaction of tricalcium silicate phase, which might be used as one of the excellent bioactive materials for both dental applications and bioactive repair material for bone tissue.

ACKNOWLEDGMENTS

The authors would like to acknowledge and thank Dr. Aly Fahmy Mohamed El-Sayed, Head of research and development sector-Holding Company for Production of Vaccines, Sera and Drugs (EGYVAC) for assistance in testing and interpretation of antimicrobial data.

Financial support and sponsorship: Nil.

Conflict of Interests: There are no conflicts of interest.

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How to cite this article:

El-Hamid HK, Abo-Almaged HH, Radwan MM. Synthesis, characterization and antimicrobial activity of nano-crystalline tricalcium silicate bio-cement. J App Pharm Sci, 2017; 7 (10): 001-008.