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Self Setting Bone Cement Formulations Based on Egg shell Derived TetraCalcium Phosphate BioCeramics

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Abstract

Egg shells have been used as a calcium source for synthesis of tetra calcium phosphate (ETTCP) by solid state reaction method. The cell parameters and cell volume of ETTCP measured by X-ray powder diffraction method were lower than the tetra calcium phosphate prepared using synthetic $Ca(CO)_3$ (pure TTCP) for comparison. The vibration bands of ETTCP were also slightly different from the vibration bands of pure TTCP characterized by Fourier transformed infrared spectroscopy. ETTCP has been tried as a main component in a self setting bone cement to evaluate the advantages of the presence of the biologically relevant ions such as Mg^{2+}, Sr^{2+}, SiO^{2-}_4, F^-, K^+ and Na^+ ions in the cement properties. The setting time of the ETTCP derived cement was ~ 11 min compared to ~ 16 min of the pure TTCP derived cement. The amount of hydroxyapatite formed as the end product was about 12% higher for ETTCP derived cement than pure TTCP derived cement after 28 days of immersion in phosphate buffer solution as confirmed by phase analysis. Elemental analysis also indicates the presence of trace elements in minor concentration in ETTCP derived cement. Although both the cements showed similar compressive strength after 28 days, the initial strength of the ETTCP derived cement was remarkably higher during initial stages of the hardening reaction (24 h–7 days) compared to TTCP derived cement. Cell viability of L6 cells was higher and cell spreading was more for the ETTCP derived cement than pure TTCP derived cement. The present study has demonstrated the advantages of eggshell derived TTCP in bone cement formulations due to the presence of biologically relevant ions. This may help the clinician with brief surgical procedure by using faster setting cement as well as the patient to have quick recovery with a higher initial strength of cement.

Keywords: Tetra calcium phosphate; Egg shells; Bone cements; Hydroxyapatite; Biologically relevant ions

Introduction

Bioceramics derived from natural sources like coral [1], fish bone [2], oyster shell [3] and bovine bone, etc. [4,5] inherit some of the properties of the raw materials such as pore structure, carbonate content and minor concentration of trace elements etc. [6]. Egg shell consists of calcium carbonate (94%), calcium phosphate (1%), organic matter (4%) and magnesium carbonate (1%). Egg shell waste has been value engineered to various products for biomaterial applications such as calcium carbonate, hydroxyapatite (HA), β-tricalcium phosphate (β-TCP) etc. [6,7] and the various calcium phosphates derived from egg shell wastes were listed in Table 1. Human bone contains ions such as Mg^{2+}, Sr^{2+}, SiO^{2-}_4, F^-, K^+ and Na^+ in minor concentration and these ions play an important role in the bone regeneration. For example, Sr^{2+} stimulates bone formation by a dual mode of action a stimulatory role on bone-forming osteoblast cells and an inhibitory role on bone resorbing osteoclast cells. Doping calcium phosphates with magnesium has resulted in improved densification, cell attachment, proliferation and alkaline phosphatase production. It has also been reported that calcium phosphate doped with two or more trace elements has shown better biologic performance such as accelerated mineralization and significantly higher bone formation [8]. The egg shell derived HA, β-TCP and biphasic calcium phosphates derived from waste has already been used as a bone graft substitute in *in vivo* models [9]. Due to the presence of various biologically relevant ions such as Mg^{2+}, Sr^{2+}, SiO^{2-}_4, F^-, K^+ and Na^+ in an optimal composition, the egg shell derived bioceramics exhibits enhanced biological properties [6].

Calcium phosphates	Synthesis route	References
Hydroxyapatite	Microwave processing Wet chemical process Solid state reaction Wet precipitation method Mechanochemical synthesis Combustion method Hydrothermal method	[6,7,14,15] [9] [16] [17] [18-20] [21] [22,23]
Calcium deficient hydroxyapatite	Microwave processing	[6]
β-Tricalcium phophate	Microwave processing Solid state reaction Mechanochemical synthesis	[6] [16] [24]
Biphasic Calcium Phosphate	Hydrothermal method	[7]

Table 1: List of calcium phosphates derived from egg shells.

Tetra calcium phosphate [TTCP, $Ca_4(PO_4)_2O$] also known as Hilgenstokite is formed at temperatures above 1300°C in the CaO–P_2O_5 system [10]. TTCP is the most basic calcium phosphate with a Ca/P ratio greater than HA and has the highest solubility at pH<4. But it is metastable, hence the synthesis of pure monophasic TTCP requires rapid quenching to room temperature prevent decomposition into other phases [11]. TTCP on hydrolysis slowly transforms to HA as the final product with the release of $Ca(OH)_2$ (Eq. 1) [12] and also reacts with acidic calcium phosphates such as dicalcium phosphate anhydrous or dicalcium phosphate dihydrate to form HA as the only product (Eq. 2) [13].

$$3Ca_4(PO_4)_2O + 3H_2O \rightarrow Ca_{10}(PO)_4(OH)_2 + 2Ca(OH)_2 \quad (1)$$

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$$2Ca_4(PO_4)_2O + 2CaHPO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 \quad (2)$$

Hence it is used as a main component in injectable self-setting calcium phosphate bone cements. To the best of the available literature, TTCP has not been synthesized from the natural resources. In the present study, we have synthesized phase-pure TTCP from egg shell waste (ETTCP) and also evaluated self- setting apatite forming cement formulations using ETTCP for bone tissue engineering applications.

Materials and Methods

TTCP synthesis from egg shells

Hen's eggshells were collected and their surface was mechanically cleaned by stripping the membrane, rinsing with water followed by oven drying at 100°C overnight and finally hand powdered in an agate mortar. From the earlier literature, eggshells were found to contain 94% calcium carbonate [7,14,16] and organic impurities make up the rest. Hence while calculating the molar concentration for synthesis of ETTCP, this egg shell powder is assumed as 94% pure $CaCO_3$ and accordingly the raw egg shell powder was weighed. ETTCP was prepared by heating a mixture consisting equimolar concentration of dicalcium phosphate anhydrous, DA (Central drug house, India) and calcium carbonate (as egg shell powder) at 1500°C for 6 h (Eq. 3) in a furnace followed by quenching at room temperature. TTCP was also prepared from the synthetic $CaCO_3$ (Merck, India) for comparison and it is coded as pure TTCP.

$$2CaHPO_4 + 2CaCO_3 \rightarrow Ca_4P_2O_9 + 2CO_2 + H_2O \quad (3)$$

Characterization

The phase and crystalline nature of ETTCP was studied by X-ray powder diffraction method (XRD, D8 DISCOVER, Bruker) with CuKα radiation (k=1.54 A). Scanning rate of 1 step/s with step size of 0.1°/step was used for recording the diffraction pattern. The functional groups of the ETTCP were analyzed using Fourier transformed infrared spectroscopy (FT-IR) (Spectrum Two, Perkin Elmer, Germany). The spectra were collected in the spectral range of 4000-500 cm^{-1} using KBr pellet technique with spectral resolution of 4 cm^{-1}. The particle size of the synthesized ETTCP was measured by Microtrac S3500 laser diffraction particle size analysis (USA).

Formulation of bone cement

The powder component of the egg shell derived TTCP cement (ETTCP/DA) contained ETTCP and dicalcium phosphate anhydrous, $CaHPO_4$ in equimolar ratio. Citric acid anhydrous (CA) and disodium hydrogen phosphate (Na_2HPO_4) was purchased from Merck, India. The liquid component contained 1M disodium hydrogen phosphate as an accelerator [25-27] and 15% CA as hardener [28]. The liquid and powder was taken in the ratio of 0.5 ml/g in a glass slab and it was mixed with a spatula to a paste like consistency. The paste was then packed in a polypropylene cylindrical mould of required dimension and allowed to harden at room temperature and at 100% humidity. Cement samples prepared with pure TTCP was taken for comparison in all the studies and it is coded as pure TTCP/DA.

Setting time

ASTM C191-13 standard test method was used to measure the initial and final setting time [29]. The setting time of the cement samples (n=5) were measured using a fabricated Vicat type apparatus (Indian standard 5513: 1996). The Vicat initial setting time is the time elapsed between the initial contact of cement with liquid and the time when the penetration of needle is measured to be 25 mm or less. The Vicat final setting time is the time elapsed between initial contact of cement with liquid and the time when the needle does not leave a complete circular impression in the paste surface.

Quantification of HA Phase in Cement

Cement samples of dimension 10×2 mm were prepared as mentioned above and allowed to harden in room temperature. After fabrication, the cement samples were stored in phosphate buffered solution (PBS) of pH 7.4 at 37°C. Samples were collected at specified time intervals of 3, 6, 12, 24, 48 h, 7 days, 28 days and 3 months. The samples were dried in an oven at 70°C for 30 min, powdered and XRD analysis was done. The peaks were indexed by means of Joint Committee on Powder Diffraction Standards, JCPDS 25-1137 for TTCP, JCPDS 9-432 for HA and JCPDS 9-0080 for DCPA.

Percentage of conversion of HA in the cement samples was calculated as reported by Ishikawa in 1995 [26-27]. The intensity of peak at 29.8° (0 4 0) for TTCP, 26.4° (0 2 0) for DA and 25.9° (0 0 2) peak of HA was considered for quantifying the phases. 'X' pert Highscore plus software was used to measure the intensity of these peaks and the values were used to estimate the extent of formation to HA in the cement. The equation used for quantifying the percentage conversion of various cement samples to HA is shown below.

$$\%conversion = \left\{ \frac{A_t}{A_\infty} + \frac{\left[1 - \left(\frac{D_t}{D_0}\right) + 1 - \left(\frac{T_t}{T_0}\right)\right]}{2} \right\} / 2 \times 100 \quad (4)$$

Where, peak intensities of DCPA and TTCP in the unreacted cement samples were denoted as D_0 and T_0 respectively. A_∞ is the peak intensity of the HA in the reacted cement after ~ 100% conversion which occurs nearly at the end of 3 months. The A_∞ values were found to be different for pure TTCP/DA and ETTCP/DA cement and hence, the respective values were used in calculating percentage conversion to HA in various cements. The D_t, T_t and A_t were the peak intensities of DCPA, TTCP and HA phases in the reacted cements at various time 't' (12 h, 24 h, 2 day, 7 day and 28 day) after mixing. The percentage of HA formed in various cement samples were quantified and plotted.

Field emission scanning electron microscopy

Cylindrical samples of dimension 5×5 mm were prepared as mentioned above and allowed to harden in PBS of pH 7.4. After 24 hours the samples were air dried and fractured. The surface morphology of the fractured cement samples was studied by Field emission scanning electron microscopy (FESEM, FEI Quant 400, Netherlands). Elemental composition of the surface was investigated by energy dispersive spectroscopy (EDS) analysis.

Compressive strength

Samples (n=5) of dimension 12×6 mm were prepared as mentioned above and allowed to harden in phosphate buffer solution (PBS) of pH 7.4. Compressive testing was then carried out at time intervals of 1, 3, 7 and 28 days. The compressive load was applied at a cross-head speed of 0.5 mm/min according to the specified ASTM F-451-08 standard [30]. The compressive testing of the samples was performed using a screw-driven mechanical testing machine (Model 4467, Instron Corp., MA). The compressive strength (MPa) was calculated using the equation

$$CS = 4F / \pi dia^2 \quad (5)$$

Where, F is the max load until fracture (N) and dia is the diameter (mm) of cylindrical pellet.

Cell viability assay

Rat skeletal muscle (L6) cells were purchased from National Centre for Cell Sciences, Pune, India). Cells were grown in Dulbecco`s modified Eagle`s medium, DMEM (Himedia) containing 10% fetal bovine serum, FBS (Invitrogen) and 1% Antibiotic-antimycotic solution (Invitrogen) at 5% CO_2 at 37°C. The cement samples were prepared in cylindrical shape of dimension of 6×2 mm and allowed to harden for 1 h. The samples were sterilized in an autoclave. The samples were transferred to growth medium and allowed to incubate for 24 h. After 24 h, the growth medium was extracted and methylthiazolyl diphenyl- tetrazolium bromide (MTT) assay was conducted using the same.

For MTT assay, L6 cells were seeded in a 24 well plate at a density of 30000 cells per well. Cells were allowed to grow for 18 h to achieve about 50% confluency. The growth medium was then replaced with extract collected from the cement samples and cells were allowed to grow for 24, 48 and 72 h. Wells without the addition of extract are set as control. After specified period, extracts were replaced with growth medium containing 0.5 mg/ml MTT (Sisco research laboratory chemicals, Mumbai) and allowed to incubate for 4 h. The purple colored crystals of formazan were dissolved by adding dimethyl sulfoxide, DMSO (Sisco research laboratory chemicals, Mumbai) to each well. The optical density (OD) of the solution was measured at a wavelength of 570 nm using a Microplate reader (Bio-Rad, Richmond, California). The % cell viability was calculated using following formula,

$$\%\,\text{Cellviability} = \frac{\text{O.D.of Sample}}{\text{O.D.of Control}} * 100 \qquad (6)$$

Cell adhesion

L6 cells were seeded on the surface of the samples with a concentration of 1×10^5/ml. Then the culture media of 200 µl was added along the sides of the wall and incubated at 37°C in humidified atmosphere at 5% CO_2 for 24 h. The culture media was removed and the samples were rinsed with phosphate buffered solution (PBS). Then the cells were fixed on samples by 2.5% glutaraldehyde followed by rinsing with PBS. The samples were then dehydrated using various grades of alcohol and dried. The dried samples were gold sputter coated and observed in SEM to study cell attachment.

Results and Discussion

XRD pattern of pure TTCP and ETTCP were shown in Figure 1a. Both the patterns correspond to the JCPDS No. 25-1137 of the Hilgenstokite phase [11]. The absence of peaks other than TTCP confirms the formation of monophasic TTCP powder. TTCP has a monoclinic crystal system and P21 space group. The cell parameters were calculated by least-squares fit method using the program "UnitCellWin" were listed in Table 2. There is a decrease in the cell parameter and cell volume of ETTCP compared to pure TTCP, which may be attributed to the presence of ions such as Mg^{2+}, F^-, K^+, Na^+ derived from the egg shells which has smaller ionic radii compared to the Ca^{2+} into the crystal structure of the ETTCP. FT-IR spectra of both the pure TTCP and ETTCP (Figure 1b) consists of bands due to the vibration modes of phosphate ions (v1–v4) as listed in Table 3. Typically it consist of a broad band around 1005 cm^{-1} due to v3 (three fold degenerate stretching mode), weak band arising from v1 (symmetric stretching mode) between 959 and 942 cm^{-1} and v4 threefold degenerate deformational mode giving rise to two sharp isolated band between 620 and 562 cm^{-1} [31]. The vibration bands of ETTCP were found to be slightly different from pure TTCP bands, due to the substitution of Mg^{2+}, Na^+, Sr^{2+} ions for the Ca^{2+} ion in ETTCP sample [6].

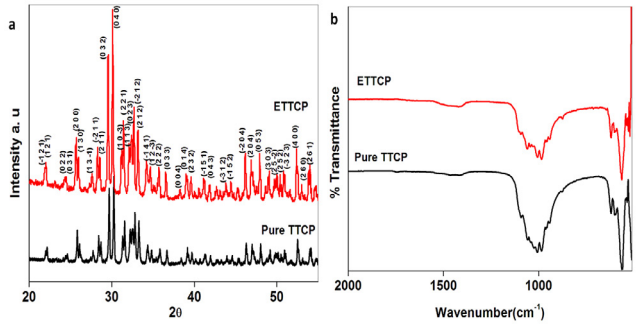

Figure 1: a) XRD pattern and **(b**) FT-IR spectra of pure TTCP and ETTCP.

Parameters		Pure TTCP	ETTCP
Cell parameters	a (A°)	7.02	6.95
	b (A°)	11.98	11.86
	c (A°)	9.47	9.39
	β (°)	90.9	91.0
	Cell volume $(A°)^3$	797	773
Particle size (µm)		1.68 ± 0.6	1.18 ± 0.4

Table 2: Average crystal lattice parameters and particle size of ETTCP.

Pure TTCP (cm^{-1})	ETTCP (cm^{-1})	Assignment
1095	1105	v3, threefold degenerate stretching mode of PO_4^{3-}
1063	1094	
-	1063	
1042	1045	
1024	-	
1008	1005	
992	983	
960	959	v1, symmetric stretching mode
-	955	
940	942	
620	620	v4, threefold degenerate deformational mode
599	598	
564	562	

Table 3: Vibrational bands of Pure TTCP and ETTCP.

Samples	Initial setting time (min)	Final setting time (min)
Pure TTCP/DA	9 ± 0.7	16 ± 0.5
ETTCP/DA	6 ± 0.5	11 ± 0.6

Table 4: Initial and final setting time of various cements.

The setting time measured for various cements using Vicat needle apparatus were listed in Table 4. The initial and the final setting time of the ETTCP/DA cement was found to be decreased compared to the pure TTCP/DA. This should be attributed to the various ion substitutions in the ETTCP crystal lattice making the crystal structure to be less stable leading to the faster setting reaction than the pure TTCP/DA cement. As faster setting time is beneficial for the clinician during surgical procedures, use of ETTCP/DA seems to give added advantage as bone cement.

The XRD pattern of the cement samples taken at different time periods of immersion in PBS were shown in Figure 2a. Although peaks corresponding to HA is present in both pure TTCP/DA and ETTCP/DA, the intensity of the HA peaks is found to be higher in ETTCP/DA compared to pure TTCP/DA at all the time period studied. The amount of HA phase formed in the cement at different periods was calculated and plotted in the graph shown in Figure 2b. The percentage of HA formed in ETTCP/DA was found to be higher than the pure TTCP/DA cement. 97% of HA formation was observed at the end of 28 days for ETTCP/DA whereas 85% was observed for pure TTCP/DA cement. The setting reaction of the cement can be explained in two stages. Initial hardening was due to chelation reaction between CA in liquid component and calcium from the powder component causing rapid setting and the second reaction was the continuous transformation of the components of the cements to HA [32]. As ETTCP will have a less stable crystal structure compared to pure TTCP due to ion substitutions, the solubility of the ETTCP during the cement reaction will also be enhanced. The chelation reaction with Ca ion released from dissolved ETTCP in cement liquid would be high causing an accelerated setting time. The continuous transformation of the components of the

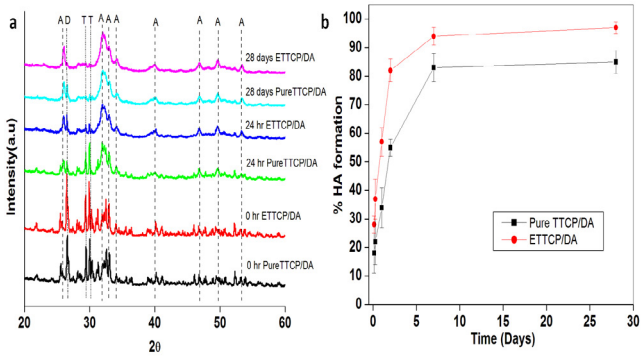

Figure 2: (a) XRD patterns of the cement and **(b)** percentage HA formation in the cement immersed in PBS at various time intervals (A=HA, T=TTCP and D=DA).

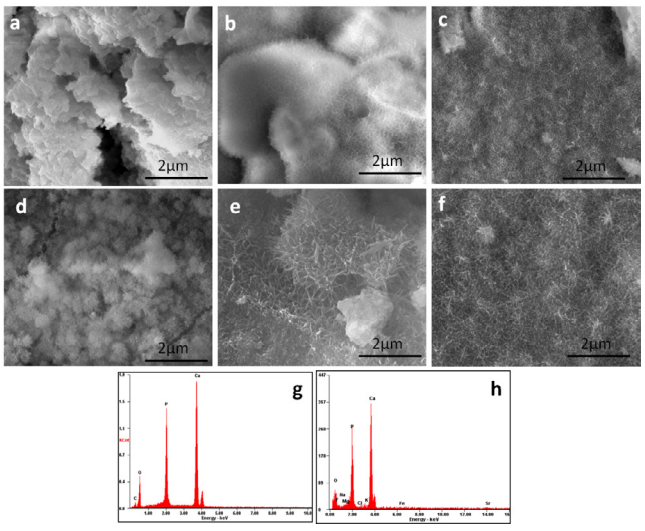

Figure 3: SEM image of the fractured surface of cement sample immersed in PBS. **a, b** and **c** correspond to the pure TTCP/DA and **d, e** and **f** correspond to the ETTCP/DA at 24 h, 72 h and 1 week respectively.
g and **h** corresponds to the EDS spectrum of c and f respectively.

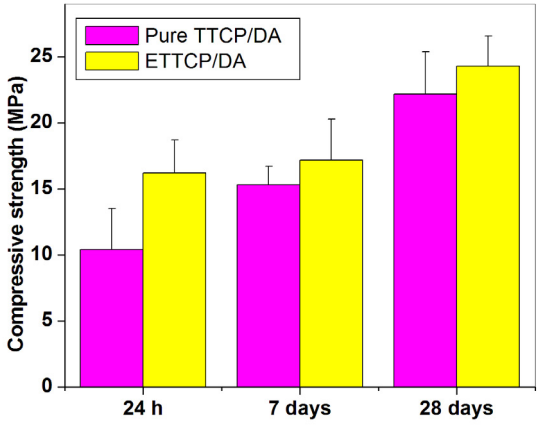

Figure 4: Compressive strength of the cement samples at different time period.

ETTCP/DA cement to form HA would also be high as solubility of the ETTCP in the formulation was enhanced.

The SEM images of the fractured surface of cement samples are shown in Figure 3. The formation of needle shaped recrystallized HA was found to increase with time in the cements. The precipitation and recrystallization of the cement precursors leading to the formation of HA on the surface of the unreacted powder is evident from the SEM images. ETTCP/DA cement showed a better crystallization of HA compared to pure TTCP/DA cement. Figure 3c showed the presence of unreacted precursors till 1 week in pure TTCP/DA whereas uniform formation of HA with no evidence of unreacted precursor in ETTCP/DA is observed. The presence of trace ions from egg shells were found in the ETTCP/DA samples from the EDS analysis (Figure 3h). As these trace ions play specific roles in bone remodeling process, the biological efficacy of the bone cement will be enhanced [24,7].

Compressive strength measured for the cement samples after immersion in PBS for different time intervals is shown in Figure 4. The compressive strength was also found to increase with increasing time duration in both the samples. Compressive strength was markedly high in ETTCP/DA sample compared to pure TTCP/DA sample at 24 h. But with increase in time, the difference in strength between the cements is decreased. Compressive strength has been reported to increase proportionally with the increasing precipitation of HA crystals in the bone cement [27,33]. The entanglement of newly precipitated HA crystals in the cement is responsible for the mechanical properties of the sample. As ETTCP/DA cement has an accelerated formation of HA than the pure TTCP/DA, its compressive strength in the initial time period was considerably higher.

The percentage viability of L6 cells on the cement samples at different time period is shown in Figure 5. Both the samples were found to be non toxic with percentage cell viability of above 80% at all time intervals. Percentage of viable cells present in ETTCP/DA cement at 24 h was found to be higher than that of pure TTCP/DA cement. This attributes to the better adhesion on to the ETTCP/DA cement compared to the pure TTCP/DA cement. The morphologies of L6 cells observed by SEM are shown in Figure 6. Although cells were found adhered on the surface of both the samples, the spreading of cells was more pronounced in ETTCP/DA compared to pure TTCP/DA after 24 h of incubation. Previous studies with egg shell derived HA [7] has also

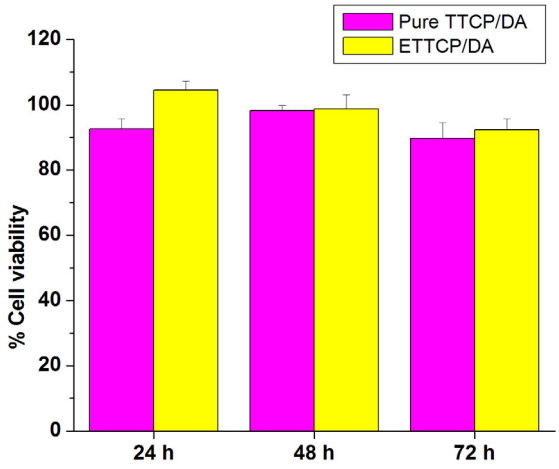

Figure 5: Cell viability assay of cement samples with L6 cells for 72hrs.

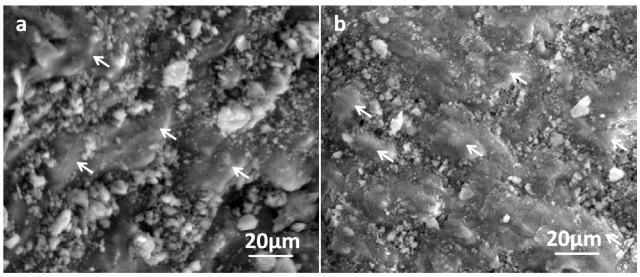

Figure 6: SEM image of the Pure TTCP/DA **a)** and ETTCP/DA **b)** cement samples after cell adhesion on the surface for 24 hrs.

shown immense enhancement in the cell behaviour due to presence of these trace elements derived from egg shells.

Conclusion

In the current study phase-pure TTCP has been successfully synthesized from egg shell waste. ETTCP was used as a component in apatite based bone cement and the role of these trace ions in various properties of the cement such as setting time, compressive strength and biologic properties were evaluated. The setting reaction was found to be accelerated with increased recrystallization of HA in the ETTCP/DA cement. The substitution of biologically relevant ions such as Mg^{2+}, Sr^{2+}, SiO^{2-}_4, F^-, K^+ and Na^+ in ETTCP/DA has greatly improved the cell behavior on the surface of the cement samples. These results clearly suggest that egg shell derived TTCP as a precursor could immensely improve the material and biological properties of the self setting bone cement.

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