Annexure-IX

PROJECT COMPLETION REPORT

Notes:
1. 10 copies of the Project Completion Report (PCR) should be sent within one month of the completion or termination of the project.
2. The PCR should be in bound form.
3. Cover page should include the title of the project, file number, names and addresses of the investigation.

1. Title of the project:

DEVELOPMENT OF CALCIUM SULFATE BASED INJECTABLE BONE SUBSTITUTE

DST No:SR/S3/ME/0028/2009

2. Principal Investigator(s) and Co-Investigator(s):

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3. Implementing Institution(s) and other collaborating Institution(s):

Biomedical Technology Wing
Sree Chitra Tirunal Institute for
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Poojappura, Thiruvananthapuram
Kerala – 695 012

4. Date of commencement:

   24.06.2010

   (Date of crediting the cheque)

5. Planned date of completion:

   24.06.2012 (Scheduled 24 months period)

6. Actual date of completion:

   24.12.2012 (extension for 6 months approved)

7. Objectives as stated in the project proposal:

   The objective of the work is to develop a bone substitute product based on calcium sulfate for filling voids in bone surgeries. The product will be a self-setting, injectable material which is bioactive and safe for human use, with clinically acceptable setting time (12-20 minutes) and compressive strength (>12 MPa).

8. Deviation made from original objectives if any, while implementing the project and reasons thereof:

   No deviation.

   However, there is a delay in completing the biocompatibility tests. These tests require more than 6 months to complete. It was not possible to provide the samples because the second year funding was delayed. The work plan was re-activated after receiving the second year fund and extension approval (DST No:SR/S3/ME/0028/2009; Letter dated 4 Sep 2012). This document is prepared with the interim test reports of the biocompatibility tests. An addendum will be provided when we receive the full report later.

10. **Experimental work giving full details of experimental set up, methods adopted, data collected supported by necessary table, charts, diagrams & photographs:**

10.1. Methodology :

The work has been planned in three major steps which are described as below.

10.1.1 Design and Optimization of the Injectable Bone Substitute

The injectable bone substitute (IBS) proposed is basically a two component product, with a powder part and a liquid part. The powder part will contain a base compound (an inorganic material which can undergo self-setting reaction in presence of an aqueous medium), additives/fillers (compounds which can alter the bioactivity and resorption), gelling agents (compounds which can alter the rheological properties) and setting modifiers (compounds which can accelerate or retard the setting). The liquid part (or the 'wetting medium') will be deionised distilled water. The quantity of the wetting medium for obtaining optimised consistency of IBS is known as the "wetting ratio". The IBS could be prepared by mixing the powder part and liquid part in the prescribed ratio.

The base compound will be high purity (99%) calcium sulfate hemihydrate powder suitable for clinical use, prepared by heating calcium sulfate dihydrate (gypsum) to 120°C and then graded to different particle size ranges (46-75μ, 76-100μ and 101-125μ). Bioactive ceramic powders (hydroxyapatite and calcium phospho-silicate materials, prepared in-house) will be added as fillers in various particle size grades (46-75μ, 76-100μ and 101-125μ). Gelling agents (like alginates, chitosan and cellulose derivatives) will be added to impart injectability to the cement. As the gelling agent may alter the setting time of the IBS, setting modifiers are to be added in the formulation.

The optimization of the components of the powder will be done with respect to setting time and strength, as required for clinical applications. The setting time should be in between 12-20 minutes and compressive strength should be higher than 12 MPa. The setting time will be measured using Vicat's apparatus (as per the schematic shown in Figure 1) which records the resistance of the mixed cement to a piercing needle. The setting time will be the period at which the needle stops penetrating into the cement mass. Standard compressive strength test on pre-set pellets of prescribed sizes will be done using Universal Testing Machine.

The particle sizes and ratio of the filler materials (hydroxyapatite ceramic powders and bioactive calcium phospho-silicate powders) will decide the *in vivo* resorption and bioactivity. The particle size ranges of the fillers will be same as that of the base compound

particles. The filler ratio normally ranges from 15-30% by weight. More the filler, the higher will be the bioactivity. However, the filler may prolong the setting time and reduce the strength. The optimum quantity of the filler in the IBS will be identified through systematic testing of various combinations of base compound and filler.

The gelling agent, in powder form, will be added in the IBS powder mix in a weight ratio of 1% to 5%. The optimum ratio will be decided by the injectability parameter of the IBS paste, determined through extrusion rheometry (custom made, as per schematic shown in Figure 2).

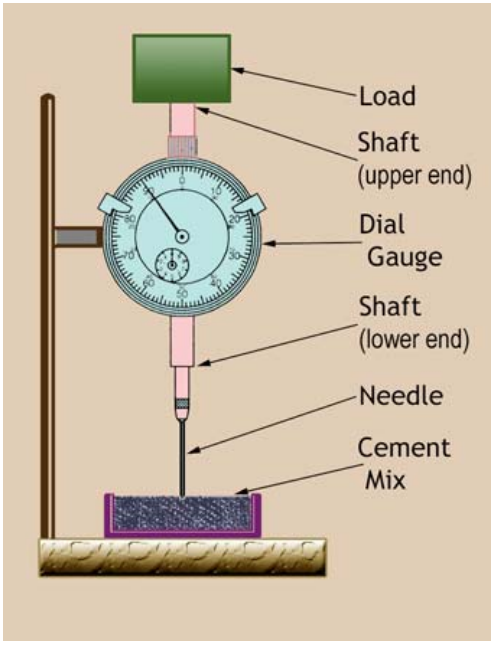

Figure 1: Schematic of the Vicat's Apparatus (Custom design).

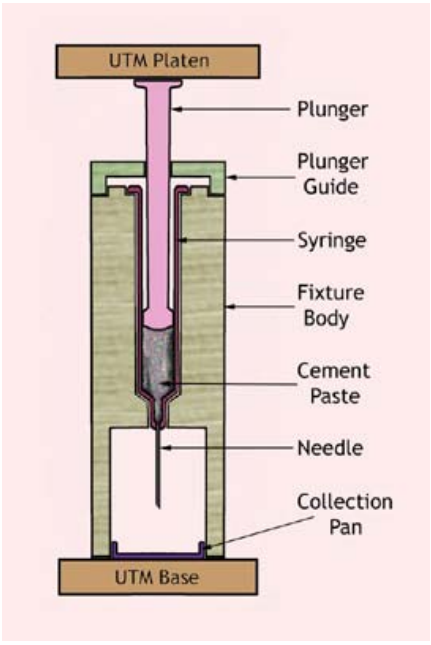

Figure 2: Schematic of the Extrusion Rheometer (Custom design)

The characterization of the IBS formulation is to be done in order to understand the material properties. This is also a part of the mandatory requirement for a medical product, prescribed by the International Standard ISO 10993.

The kinetics of cement setting and phase conversion will be analyzed using X-ray Diffractometry (XRD). Fourier Transform Infrared Spectroscopy (FTIR) and Energy Dispersive Electron Microprobe (EDS) will be used to confirm the nature of the final product. SEM will give information about the microstructure. Atomic Emission Spectroscopy (AES-ICP) will be used to determine the trace elements in the product. The dimensional changes during setting will be determined by setting in standard moulds and measuring the final dimensions. The porosity will be measured through mercury porosimetry.

10.1.2 Testing Biocompatibility of the IBS Formulation

The preliminary part in the biocompatibility evaluation is the screening tests of *cytotoxicity* and *haemolysis*. The cytotoxicity test (direct contact method) looks for the morphological changes in the *mouse fibroblast cells* cultured in contact with the material and the haemolysis test estimates the red blood cell lysis percentage.

After passing the screening tests, the IBS formulation will be subjected to detailed toxicological analysis. All the mandatory tests (*systemic toxicity test, intramuscular implantation test, intracutaneous irritation test, sensitization test, pyrogen test, mutagenicity test and sterility test*) prescribed for a bone substitute material by the International Standard ISO 10993 are to be carried out.

10.1.3 Testing Bioactivity of the IBS Formulation

Assessing the bioactivity (i.e. potential for hard tissue regeneration) of the IBS formulation is crucial in the product development. This could be done *in vitro* by cell culture using *human osteoblast cell lines*. The adhesion and proliferation of cells will be assessed through MTT assay method and imaging techniques. The cell adhesion and proliferation will define the bioactivity of the material.

10.2 Results

10.2.1 Designing calcium sulfate cement

Making a calcium sulfate cement formulation is the initial part of designing an injectable bone substitute (IBS). Calcium sulfate hemihydrate (Plaster of Paris) constitutes the main ingredient, which gets converted to gypsum on wetting with water. High purity calcium sulfate dihydrate (Aldrich chemical, 99.9% purity) was procured, crushed, and heated to 125°C to obtain fully pure calcium sulfate hemihydrate phase.

The conversion of the power has been confirmed through X-Ray Diffractometry (Bruker D8 Advance) and the diffraction peaks were compared with the standard diffraction files (PDF) of calcium sulfate compounds to identify the phases. The XRD of the calcium sulfate dihydrate powder heated at 100ºC, 125ºC and 800ºC are given in Figure 3 (in the 2-theta diffraction angle range 28.5 to 33.5).

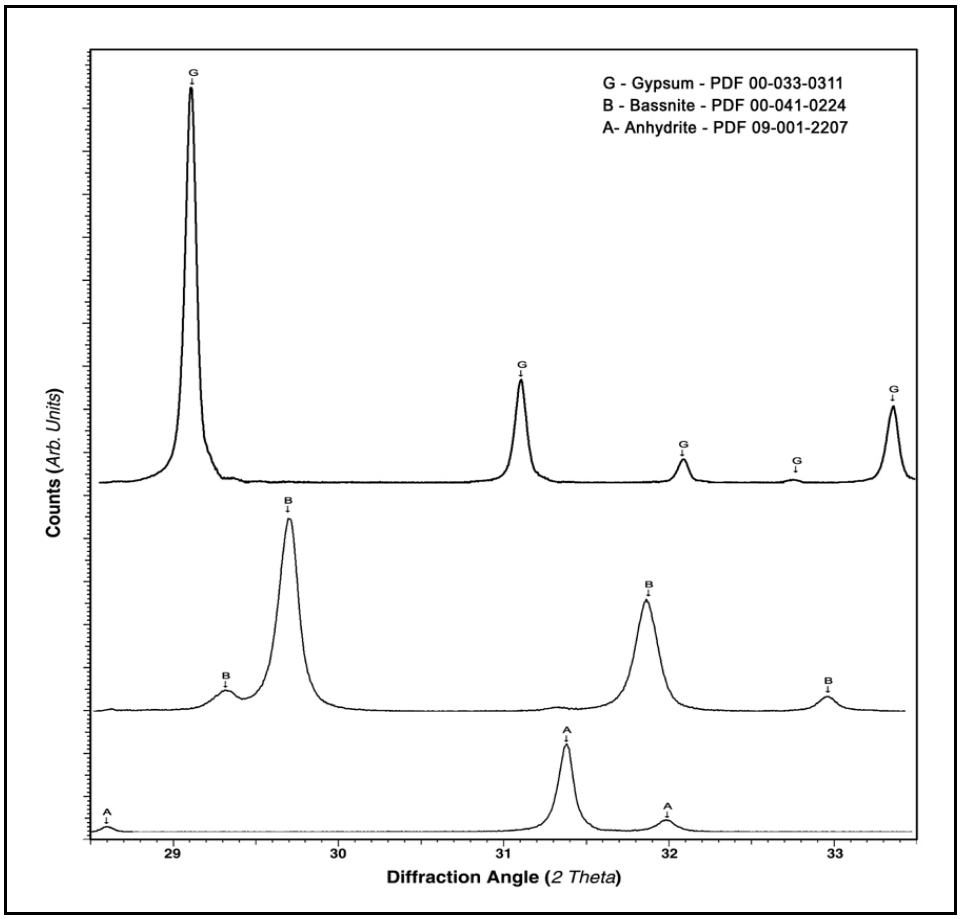

Figure 3. Study of the phase conversion of calcium sulfate dihydrate (Gypsum) during heating, through XRD. The spectra in the 2-theta diffraction angle range 28.5 to 33.5 are compiled. The top trace corresponds to a heating of 100ºC, the middle trace, to 125ºC and the bottom trace, to 800ºC. The diffraction peaks from the standard diffraction files of related compounds are marked.

It could be seen that the powder heated at 100ºC is pure calcium sulfate dihydrate (gypsum, PDF 00-033-0311), which gets converted to calcium sulfate hemihydrate (bassnite, PDF 00-041-0224) after 120ºC. At 125ºC, phase-pure material is obtained. Dehydration of the sample occurs on escalating the temperature, which gets completed by 800ºC, converting it to anhydrous calcium sulfate (09-001-2207).

Phase-pure calcium sulfate hemihydrate powder was prepared in required quantities and graded to different particle size ranges using standard test sieves. The powder grades of 46-75μ, 76-100μ and 101-125μ were used for further experiments. The properties of the cement were found to depend primarily on the grade of the powder and the wetting ratio.

The wetting medium used was distilled water. Wetting ratio (i.e. the amount of wetting medium needed to make cement) is an important parameter in the cement design. Ideal wetting ratio for the IBS is the minimum quantity of water needed to make a thick paste. This was determined by adding distilled water from a graduated pipette to a pre-weighed quantity of calcium sulfate hemihydrate powder. The wetting ratio was found to be 0.6 ml for 1 gram of the powder.

10.2.2 Setting time measurement:

The setting times of the cement compositions were assessed using a custom-made Vicat-type apparatus (Figure 4). It consisted of a 1mm dia steel needle fitted to the edge of a shaft attached to a dial gauge. The shaft was suspended vertically in a stand under a constant load of 100gf. The needle goes into a well (of 5mm depth, made on a metal block) meant for holding the cement putty. The cement was mixed and filled in the well and the needle was allowed to penetrate into it. The distance traveled by the tip can be measured to 0.01mm accuracy in the gauge. The needle penetration was repeated at time intervals of 30s and the penetration depth was recorded. In the case of unset putty, the needle will easily go through the mass to the lowest position. When the putty starts solidifying it resists the penetration. The time elapsed from the starting of the mixing, to the full resistance towards needle penetration is taken as setting time [Ref: Komath M and Varma HK,

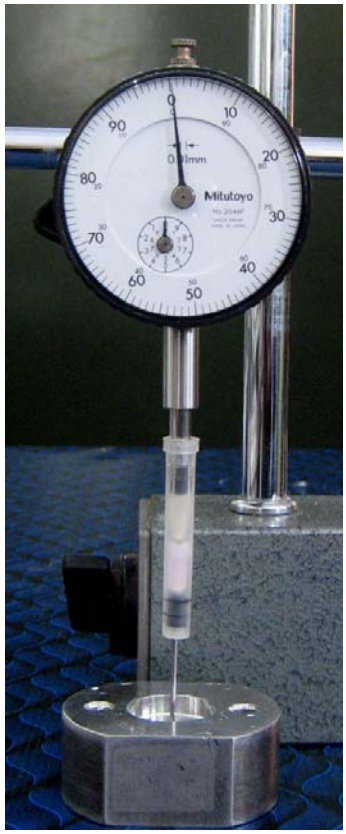

Figure 4. The Vicat's Apparatus (modified).

Fully-injectable calcium phosphate cement - A promise to dentistry, Indian J. Dent. Res., 15 (2004) 89].

The setting time of the calcium sulfate cement mix was found to have strong dependency on particle sizes. With increasing particle size, the setting time increased. For the range 45-75 μ the setting time was 4 minutes.

10.2.3 Strength measurements:

The compressive strength of the set cement was measured in a Universal Testing Machine (Instron model 1193) as per ASTM Standard F 451. The cement was mixed in the prescribed ratio and filled in split-moulds (Figure 5) so as to make cylindrical pellets with diameter 6mm and height 12mm on complete drying. The pellets were then incubated in 100% humidity for 24 h and dried prior to the analysis. Each pellet was compressed along the axis in between the platens of the machine at a cross-head speed of 1mm/min. The compressive strength was calculated from the break load and the dimensions of the pellet.

The effect of particle size of cement powder on the compressive strength of set mass was studied. The strength of set mass decreased with increasing particle size. The value for the range 45-75 μ was 14.2 MPa.

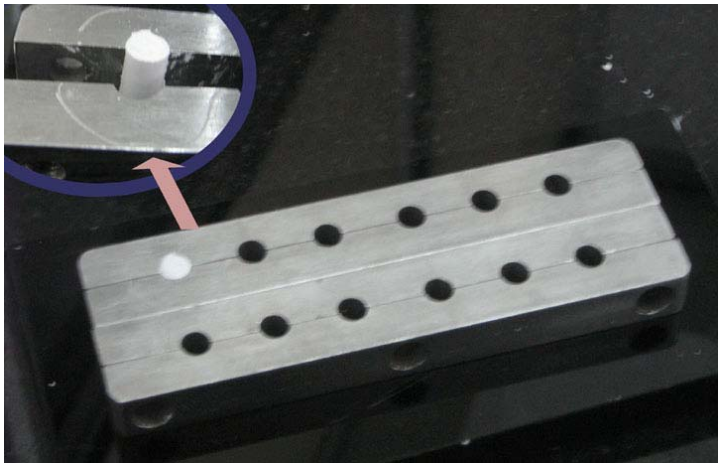

Figure 5. The split mould used for the preparation of the pellets for compressive strength test.

10.2.4 Phase formation in the set cement:

The set cement was analysed for phase in XRD (as described earlier, in subsection 10.2.1). It showed a complete conversion to Bassnite to Gypsum, identified from the standard peak positions as given in Figure 3. The kinetics of conversion was also studied, by fast scanning of the mixed cement putty in XRD at 2 minutes interval

after mixing. It was interesting to identify that the full conversion to Gypsum occurs within the first 2 minutes. There is no significant phase change at the setting time. This means, the phase conversion in the mixed cement mass occurs quickly and the setting happens while the crystals grow inside the mass and entangle to form a stable structure.

10.2.5 Microstructure of the set cement:

The microstructure of the different particle size compositions of the cement was analysed using scanning electron microscopy (Hitachi S2400 model SEM). The dried cement pellets were coated with gold before loading into SEM. The images (Figure 6) showed calcium sulfate dihydrate crystals grown into needle-like morphology, as specified in literature [Lewry AJ, The setting of gypsum plaster, J Mater Sci 29 (1994) 5279]. It is clear that the growing crystals interlock and entangle in the setting cement mass, making a solid structure with mechanical strength. The crystals are randomly oriented with micropores in between the crystallites. Voids also are present, possibly may be the spaces where the liquid part dried off.

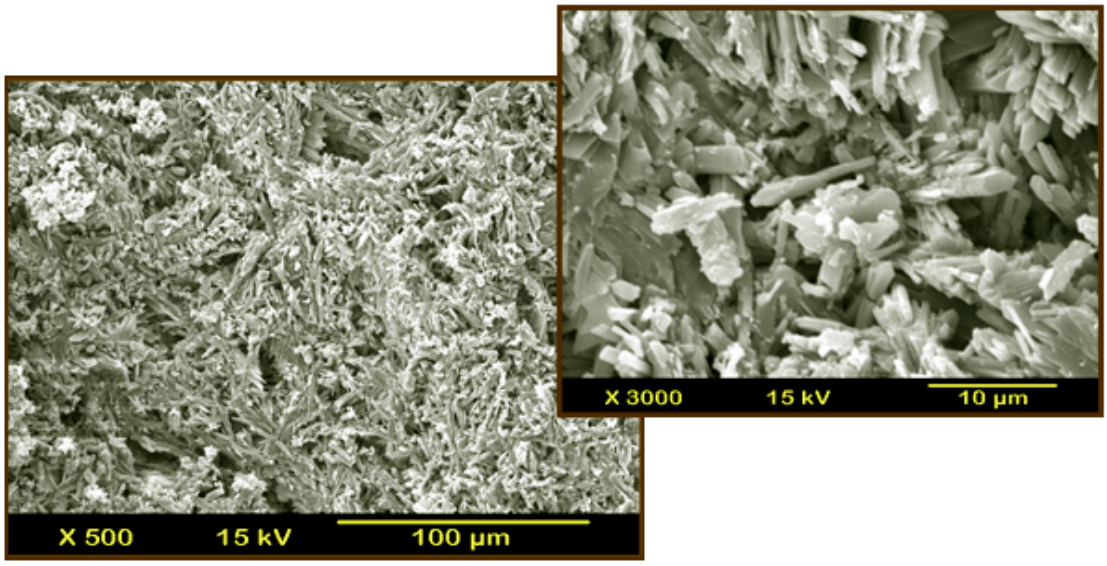

Figure 6. Scanning electron micrographs of set cement mass, given in two magnifications

10.2.6 Density and porosity

The densities of pellets of the cement compositions of different particle size grades were determined from the dimensions and weight of each pellet. The values obtained were much lower than the theoretical density of calcium sulfate dihydrate, 2.32g/cm^3. It is noticed that the set cement densified more when the particle sizes were decreased. However, the maximum obtainable density was about 55% of the theoretical value.

The porosity analysis of calcium sulfate cement was carried out in a Mercury Intrusion Porosimeter (Quantachrome Poremaster). Large numbers of smaller pores (100 microns to 0.01 microns) were found in the cement. There was a consistent porosity distribution in the range 5-100 microns. The percentage porosity increased as the particle sizes increased. These results are in good agreement with microstructural variations observed in SEM analysis.

10.2.7 Effect of addition of fillers in the cement

The second step in arriving to the composition of the injectable bone substitute (IBS) is to choose bioactive fillers and optimize their ratios in the cement mix. Two bioactive and resorbable materials in fine powder form were used as additives - calcium phosphate (hydroxyapatite) ceramics and bioactive calcium phospho-silicate glass.

The filler powders were synthesized in the lab through conventional bioceramic/bioglass making methods, and crushed and sieved to the required particle size. Resorption (after implantation) of the cement mass will depend upon the ratio of the fillers. The hydroxyapatite ceramic and bioactive glass powders were added in the optimized calcium sulfate cement in definite weight ratios.

Setting time estimations were repeated on the cement modified with the fillers for different ratios. Same quantity of the cement was weighed out and wetted with same quantity of water (0.6 ml per gram) to make the cement. It is observed that the setting time increased with the filler content. Figure 7 shows the variation of setting time with the percentage filler ratio. The setting time increased from 4 minutes to 11 minutes, when the filler ratio was increased from 0 to 50%.

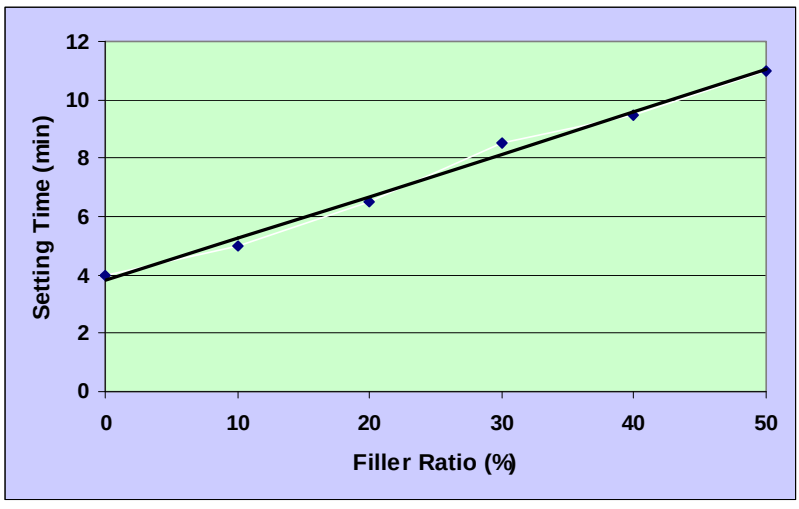

Figure 7. Variation of setting time with hydroxyapatite particle addition.

The variation of the mean compressive strength values with the addition of filler particles was also determined, as per the method described earlier. The graph is shown in Figure 8. The compressive strength of the set cement is observed to decrease with the increase in the filler percentage. The compressive strength decreased to below 9 MPa when the filler ratio was increased to 50%.

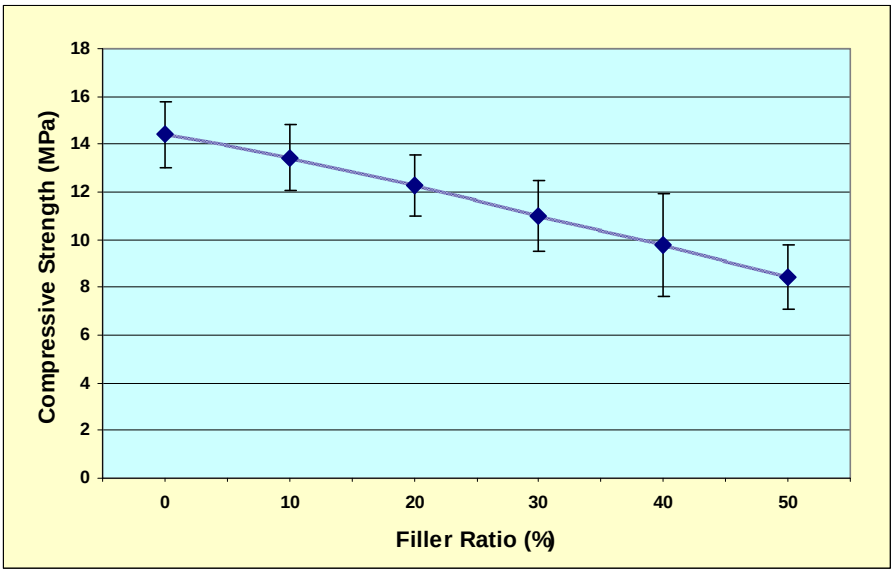

Figure 8. Variation of compressive strength with hydroxyapatite particle addition.

The optimization point is the quantity of the filler which gives clinically acceptable values of setting time and strength. It has already been specified in the literature that a bone filler cement should have setting time above 10 minutes so as to allow sufficient working time. Also, the compressive strength should be higher than 12 MPa, which is the average value for cancellous bone [Komath M. and Varma HK, Development of a Fully-Injectable Calcium Phosphate Cement for Orthopaedic and Dental Applications, Bull. Mater Sci. 26 (2003) 415]. Considering these requirements, the ideal filler ratio was fixed to 25%. This composition gave a setting time just above 7 minutes and a mean compressive strength around 12 MPa.

10.2.8 Development of injectable composition

It is the property of 'injectability' of the mixed cement (before it sets) that makes an IBS attractive. Injectability is related to the ability of a slurry or a paste to extrude through a hollow needle or cannula when pressed out from a syringe or applicator [Bohner M and Baroud G, Injectability of calcium phosphate pastes, Biomaterials 26 (2005)1553–1563]. Generally, this is quantified correlating to the force required to extrude the full quantity before the setting time.

As an initial test, the optimized cement formulation made was mixed at different powder-to-liquid ratios and tested for flow through an 18 gauge needle by back-filling in a syringe. It was seen that the mix, even in the form of loose slurry, does not flow smoothly through the needle. The paste in the syringe was observed to undergo 'filter pressing' wherein the liquid content get expelled first. The use of biocompatible gelling agents (like sodium alginate, chitosan derivatives or cellulose derivatives) induced better rheological properties to the mixed paste, but observed to prolong the setting time. In most cases, 1-2% content of the gelling agent increased the setting time beyond the useful limit.

Biocompatible setting modifiers (like phosphates and sulfates of sodium) were added along with gelling agents, to reduce the effect of prolonging setting time. It was observed that phosphate ions (contained in compounds like disodium hydrogen phosphate and trisodium phosphate) increased the flow properties of the mixed cement paste drastically, without significantly affecting the setting time. A demonstration of the extrusion of the mixed cement paste (containing 1.5% disodium hydrogen phosphate), from a 2ml syringe fitted with 18 gauge needle, is shown in Figure 9.

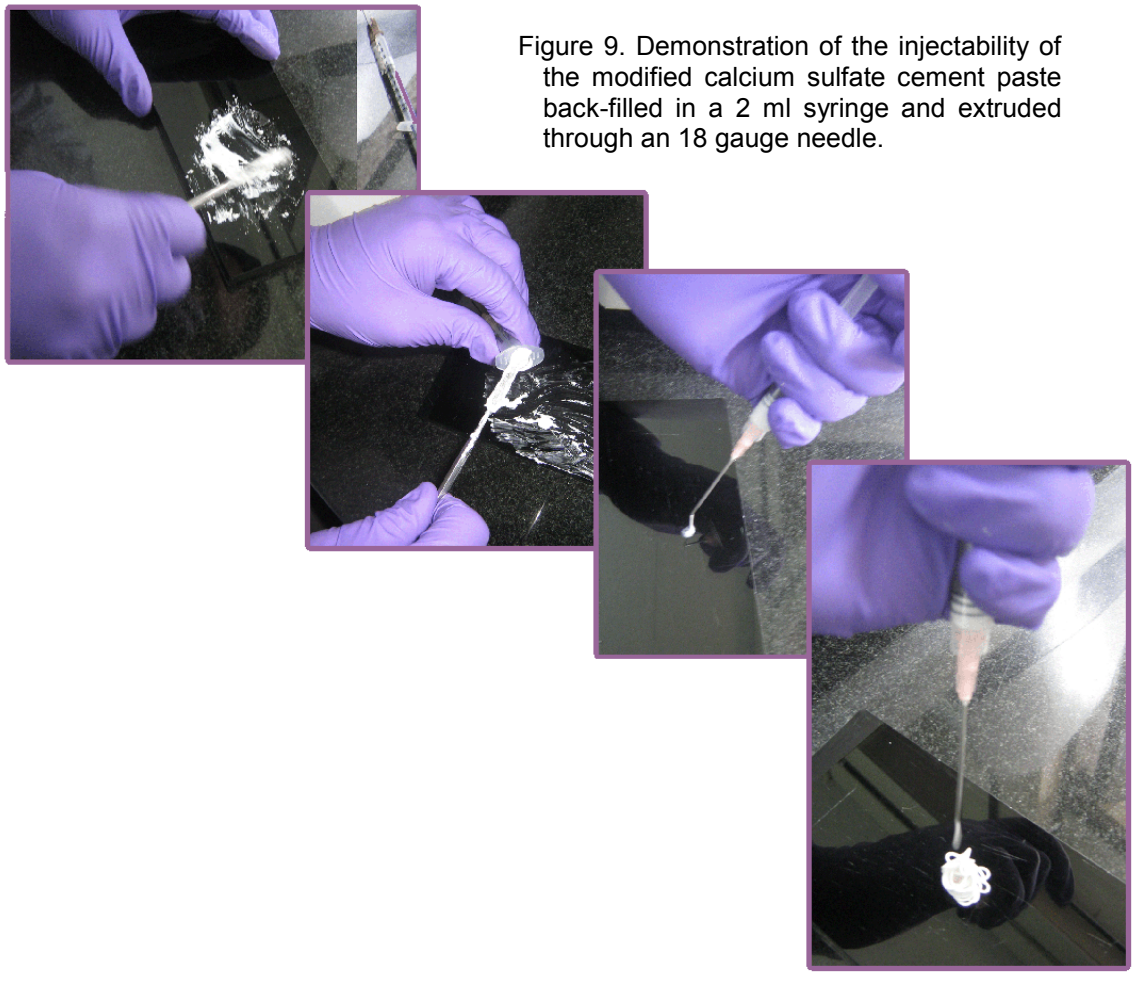

Figure 9. Demonstration of the injectability of the modified calcium sulfate cement paste back-filled in a 2 ml syringe and extruded through an 18 gauge needle.

Figure 10. Attachment for the extrusion rheometry experiment

The injectability of the cement was tested through extrusion rheometry [Martin PJ, Wilson DI, Bonnett PE, Rheological study of a talc-based paste for extrusion-granulation, J European Ceramic Society, 24 (2004) 3155]. A capillary type rheometer was custom fabricated, in which a plastic syringe acts as the main body of the rheometer. Needles of appropriate inner diameter were used as extruding capillary. This was snugly inserted vertically into a cavity made in an acrylic cylinder (Figure 10). The cylinder has a detachable bottom chamber designed to collect the extruded material. The set up was placed under the platen of a Universal Testing Machine with its axis coinciding with that of the machine so as to apply an axial force on the cement mix at certain rate, making it extrude.

The cement was mixed and back filled in the syringe (rheometer) and plunger was placed squeezing the paste till the first drop appears at the tip of the needle. The rheometer set up was then subjected to extrusion in the UTM at a speed of 1mm per minute. The extrusion distance (more correctly, the distance the plunger traveled) versus the force measured was recorded.

Figure 11 shows the behavior of the conventional calcium sulfate cement mix when extruded from the rheometer. The force increases continuously as the plunger is pressed down. Filter pressing occurs, leading to the expulsion of the liquid part, leaving the particulate part inside the rheometer. Further pressing of the plunger will lead to drastic increase in the force and the final rupture of the rheometer. This occurs within a distance of travel of 3mm.

The result of the experiment repeated with the modified calcium sulfate cement mix (containing 1.5% disodium hydrogen phosphate) is shown in figure 12. It could be seen that the mix extrudes from the rheometer fully. After the initial increase, the force remains at an average value of 25 N, which is below the force a human thumb can exert on a plunger. The whole quantity of the mix could be extruded from the syringe.

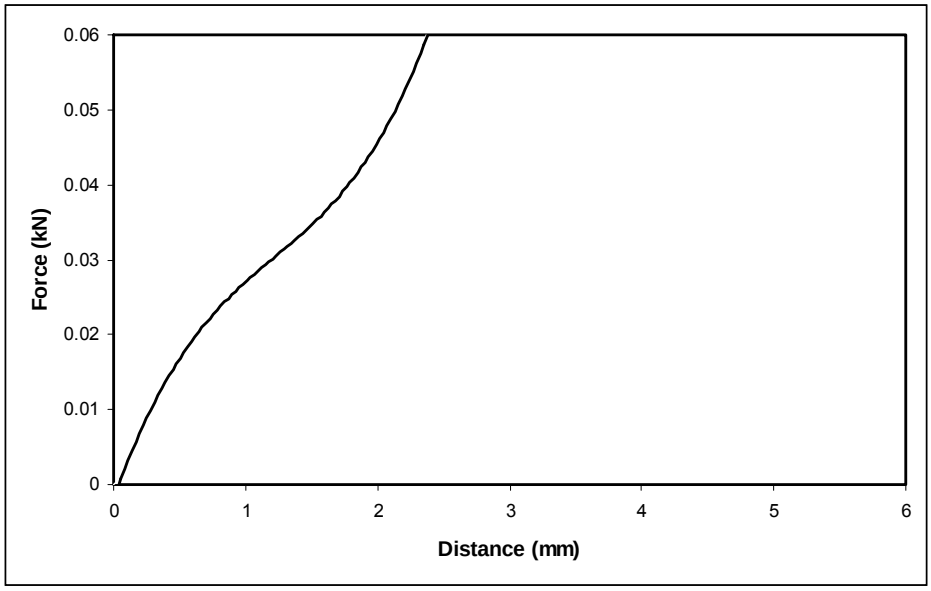

Figure 11. Extrusion force on the conventional calcium sulfate cement mix against the distance traveled by the plunger in the in the capillary rheometer shown in Figure 10 when tested in universal testing machine.

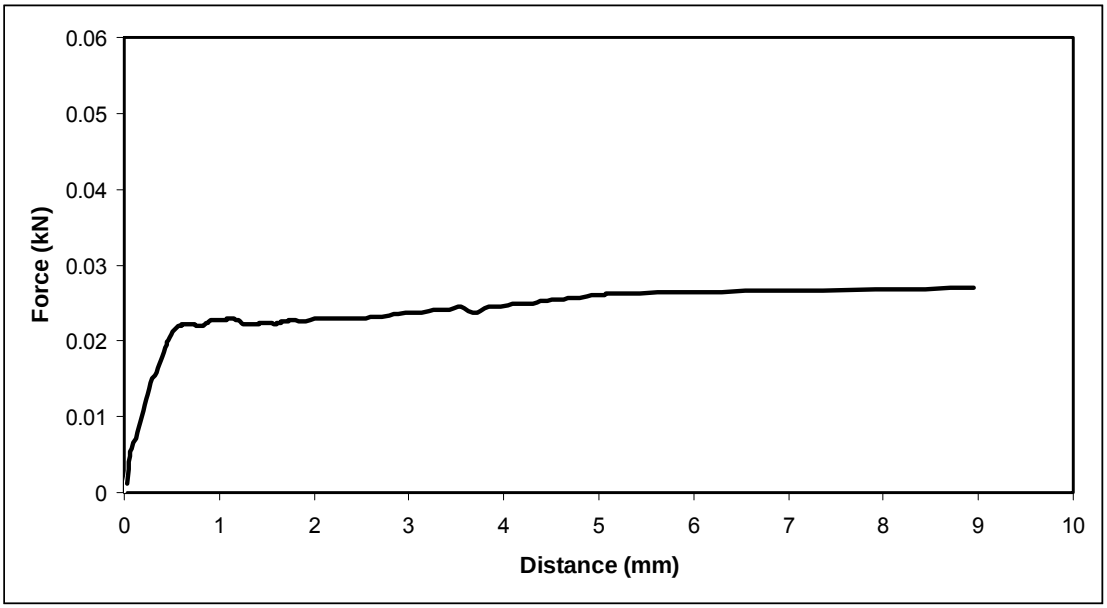

Figure 12. Extrusion force on the modified calcium sulfate cement mix (containing 1.5% disodium hydrogen phosphate) against the distance traveled by the plunger in the capillary rheometer shown in Figure 10 when tested in universal testing machine.

10.2.9 Biocompatibility evaluation – Screening tests

Three samples were selected for the screening tests – bare calcium sulfate (CAS), modified cement with disodium hydrogen phosphate content (CASP) and modified cement with the binder carboxymethyl chitosan (CASPC). The samples were set in the form of pellets and preserved at 100% humidity for 24h at physiological conditions, prior to the analyses. Sterilization was done using ETO (Ethylene trioxide).

(i) In vitro Cell Culture Cytotoxicity : The cytotoxicity studies were carried out with mouse fibroblast cells (L929, ATCC strain) in 'direct-contact' method along with MTT Assay. The medium used was Eagle's MEM with 10% fetal bovine serum. High density Polyethylene discs were used as the negative control and stabilized PVC Discs as positive control. Test samples were preconditioned with culture medium containing serum for 30 minutes at 37ºC. Brisk effervescence was observed, however with no shift in pH during conditioning of the material. Approximately $3x10^4$ cells were seeded per well to a 24 well plate, supplied with the medium. Test samples, negative controls and positive controls in six replicates were placed on the cells. After incubation at 37± 10ºC for 24 to 26h, cell monolayer was examined microscopically for the response around the test samples. The cells in contact with the test samples showed the normal morphology by and large, indicating that the test materials are not toxic to cells.

Test materials and control samples were removed and MTT assay was performed on the cells remaining in the wells. The significance of MTT assay is that it enables to measure the metabolic activity of cells as live cells reduce yellow colored tetrazolium salt [3-(4, 5-Dimethyl thiazol -2-yl)-2, 5diphenyl tetrazolium bromide] to purple colored formazan. After removing test materials from the wells, 200µl of MTT solution (1mg/ml MEM without supplements) was added in each and incubated for 2h. Afterwards MTT solution was removed, 400 µl of isopropanol was added to all wells and swayed the plate. The color developed was quantified by measuring the absorbance at 570nm using spectrophotometer. The percentage metabolic activity data obtained for test samples were compared with those of positive with negative controls, the graphical form of which is given in figure 13.

(ii) In vitro Haemocompatibility: This test was done to check the interaction of materials with blood, as prescribed by ISO 10993-4. The prepared (dry and sterile) samples of CAS, CASP and CASPC, in triplicate, were exposed to 1ml of human blood which was collected into the anticoagulant. They were incubated for 30 min under agitation at 70 rpm using an Environ shaker kept at 35 ± 2ºC. Empty polystyrene culture dishes were used as reference material. The hemoglobin counts in the whole blood samples were measured

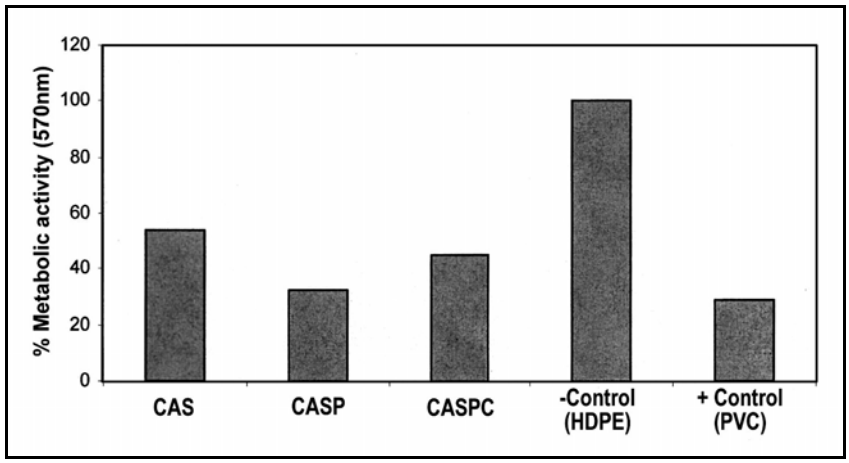

Figure 13. The MTT Assay data obtained after direct contact cell culture using mouse L292 cell lines.

using an automatic haematology analyzer (Sysmex-K 4500). The free hemoglobin liberated in to the plasma after exposure to materials was measured using Diode array Spectrophotometer and the percentage hemolysis was calculated using the formula (Free Hb /Total Hb) x 100. The results are given in the following table.

Sample ID.	Percentage hemolysis
CAS a	0.16
CAS b	0.19
CAS c	0.13
CASP a	0.12
CASP b	0.11
CASP c	0.18
CASPC a	0.19
CASPC b	0.07
CASPC c	0.09

Average % hemolysis in the 3 references after 30 min exposure was 0.07± 0.01. Normal hemolysis is taken as <0.1 %. Only 2 samples of CASPC were compatible with this requirement.

10.2.10 Biocompatibility evaluation – Toxicological Tests

Toxicological evaluation was done as prescribed by ISO 10993. The modified cement (IBS) sample containing bioactive fillers and binders were subjected to the analysis in order to gather the toxicological profile of the material.

(i) Acute Systemic Toxicity : The systemic response of the extracts of the material were tested in mice, under protocol ISO 10993-11. The normal saline extract and cotton seed oil extracts were used, prepared in aseptic conditions. The extraction was done by treating 4 grams of the set cement (in pellet form) with normal saline and cotton seed oil, for 1 hour at 121º C. The extraction media alone was used as control. The test animal used was Swiss Albino Mice, in the weight range 17-23g. 10 mice (5 males and 5 female) were randomly selected for the test with each extract and a similar set was used as control. The test extracts and their controls were injected to the respective groups of mice intraperitonially, at a dose of 50ml/kg body weight. The observations were done immediately after injection and thereafter at 24h, 48h and 72h.

The preliminary result showed that the test sample did not elicit any systemic toxicity.

(ii) Intracutaneous (Intradermal) Reactivity Test : This was done to evaluate local responses to the extracts of the material (in normal saline and cotton seed oil) following intracutaneous injection into rabbits, under ISO 10993-10. The extraction was done as in the previous test. The test animal used was New Zealand white rabbit, adults with weight below 2kg. 3 healthy and smooth skinned animals were selected and prepared by closely clipping the fur on the back. The test extracts were aseptically injected subcutaneously on the back at a dose of 2ml per site. Total 8 sites were selected on each side, with test extracts at upper 5 sites (normal saline extract on the left and cotton seed oil extract on the right) and their controls at 3 sites below to them. The observations were done for erythema and oedema, immediately after injection and thereafter at 24h, 48h and 72h and graded as specified in the standard. For each animal, the scores of all the observations were added for each test extract and each control, and divided by the total number of observations. The *primary irritation score* for both erythema and oedema in each animal were obtained by subtracting the average scores of the controls from that of the test. The primary irritation scores from all the animals for each test extracts were added and divided by the number of animals to obtain the *primary irritation index* for each case. The value is then compared with the primary irritation categories.

The preliminary result showed that the test sample did not cause erythema and oedema. The detailed score chart is awaited.

(iii) Maximization Sensitization Test : This test is done according to ISO 10993-10, to determine the potential for a substance or material under test to produce skin sensitization in the guinea pig. The extraction was done by treating 2 grams of the set cement (in pellet form) with 10ml of physiological saline, for 24 hours at 70ºC. The liquid

was then filtered with sterilized Whatman filter paper. Same quantity of physiological saline alone was used as control. The equal volume mixture of these with Freund's complete adjuvant was also used in the experiment.

The test animal used was Hartley strain Guinea pigs, in the weight range 300g – 500g. 15 healthy adults (males and female) were randomly selected for the test, 10 in the test group and 5 in the control group. The dorsal intra scapular region (for intradermal or topical application) and flank region (for challenge dose) of each animal were clipped before experiment. The course of experiment is executed in three phases – intradermal induction phase, topical induction phase and challenge phase.

In the intradermal induction phase, the test solutions (physiological saline and its mixture with Freund's complete adjuvant) were intradermally injected to the intrascapular regions to the test group at a dose of 0.1ml. The same dose of the control solution was given to the control group also. In the topical induction phase, seven days after the intradermal injection, the test and control materials were topically applied to the respective groups. The application is done (after pre-treating with 10% sodium lauryl sulfate) onto the intrascapular region of each pig using a 2X4 cm filter paper patch and covered with an occlusive dressing. The dressings and patches were removed after 48h. Challenge phase was done 14 days after the topical application. The test and control animals were challenged with test material extract using filter paper patches and were covered with an occlusive dressing, which were removed after 24h for observation. The appearance of the challenge skin sites of test and control animals were observed at 24h, 48h and 72h after removal of dressings and patches. The skin reactions for erythema and edema were scored and recorded the numerical grading as per the standard.

The preliminary result showed that the test sample did not cause any sensitization. The final score report is awaited.

(iv) Pyrogen Test : This is to identify the presence of any pyrogenic substances of either endotoxin or non-endotoxin origin, in the sample as per ISO 10993-11. The method is to measure the rise in temperature following the intravenous injection of the test solution. The test extract was prepared by exposing a definite surface area of the cement to 0.9% NaCl preparation at 37ºC for 1 hour.

The animals selected were New Zealand white rabbits, healthy adults with weight below 2kg. They were climatised for 7 days at a controlled temperature 22±3ºC. The rectal temperature was measured daily, using a thermometer, with the help of rabbit restrainer. 3 rabbits whose body temperature is below 39.8ºC and the daily variation within 1ºC, were selected. The rectal temperature recorded 30 minutes prior to the test

was taken as the reference value. The extract was then injected intravenously into the marginal ear vein of each rabbit, at a dose of 10 ml/kg body weight. Rectal temperatures were recorded at 30 minutes interval starting from 1hr to 3hrs after the injection.

The preliminary result showed that the test sample is not pyrogenic.

(v) Implantation In Muscle.: This is done to assess the biological response of muscle tissue to the material through implantation at time periods 1 week, 4 weeks and 12 weeks. Teflon tubes (2mm diameter and 5mm length, ETO sterilized) were used for implantation. The test samples were prepared by filling the cement into these tubes. Bare Teflon tubes were used as controls. 4 test samples and 3 controls were used per animal.

The animals selected were New Zealand white rabbits of either sex with weight below 2.5kg. Healthy adults whose paravertebral muscles are sufficiently large in size, were used for the experiment. Total number of animals was 9 (3 animals per period, 3 periods). The Implantation procedure was carried out under clean and aseptic conditions. Prior to the test, each animal was prepared by clipping the fur on either side of spine. They were then anaesthetized and the skin was lightly swabbed using 70% alcohol followed by air-drying. Incisions were made into the paravertebral muscle (4 sites, 25mm apart) to insert the test material intramuscularly along one side of the spine. Similarly, the control materials (3 each) were implanted intramuscularly in the contra lateral muscle. The incision was then closed using sterilized sutures.

At the end of each implantation period the respective animals (3 nos/period) were euthanised by an overdose of anaesthetic agent. The test and control implant material along with the surrounding tissues were collected. The sites of implantations were macroscopically examined for haemorrhage, necrosis, discoloration and infection. The collected materials and tissues were then fixed in 10% buffered formalin and were subjected to histopathological evaluation.

The histological analysis of the test samples is ongoing and the results are awaited.

10.2.11 Bioactivity study

In the bioactivity study, the human osteoblast cell viability study (through In-vitro MTT Assay) has been conducted. Bare calcium sulfate (CAS) and optimized IBS cement were tested for bioactivity, after preparing in pellet form. The MTT assay following to cell culture (using human osteoblast cell line) is a reliable method to measure the metabolic activity of cells. The metabolically active cells reduce yellow colored tetrazolium salt [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to purple colored formazan.

Test samples were preconditioned with culture medium containing serum for 30 minutes at 37ºC. They were placed a 24 well plate, supplied with the medium. Human osteoblast (HOS) cells were subcultured and seeded at a density of 1×10^4 /cm^2 onto the test materials and incubated at 37±2ºC for 48h. After that, the culture medium was replaced with 400 µl fresh culture medium to which 50 µl MTT (5mg/ml in serum free medium) was added and incubated overnight at 37°C. 400 µl of isopropanol was added to all wells and kept for 20 min with orbital shaking of 40-60 rpm. The color developed was quantified by measuring absorbance at 570 nm using a microplate reader (Biotek).

The modified cement (IBS) showed 83 % activity, whereas bare calcium sulfate showed only 36%.

Scanning electron microscopy (FEI Quanta 200 Environmental Scanning Electron Microscope) was done on the samples before and after the human osteoblast cell culture to see the morphological changes of the crystals. The surface of the dry sample was observed at first. The same sample was subjected to human osteoblast cell culturing as above and observed again. Though the cells were not identified, the surface structure was seen significantly modified (figure 14). The formation of new crystallites in leaf-like structure in the presence of osteoblast cells indicates the bioactivity of the material.

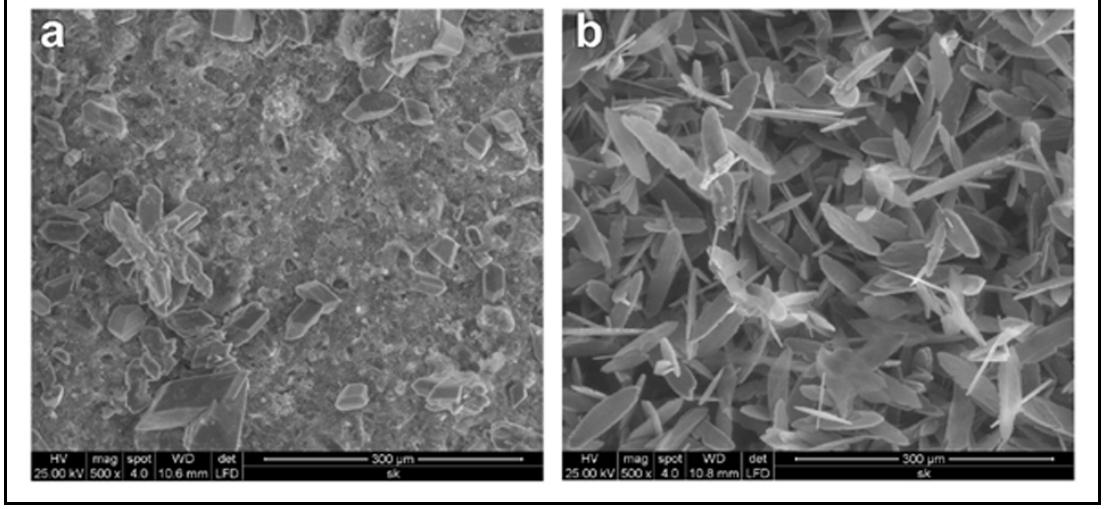

Figure 14. The SEM pictures of cement pellet surface before (picture a) and after (picture b) osteoblast cell culture.

11. Detailed analysis of results indicating contributions made towards increasing the state of knowledge in the subject:

The present project is aimed at the designing of an injectable bone substitute (IBS) and its biological evaluation. To start with, calcium sulfate cement was designed by converting high purity calcium sulfate dihydrate to calcium sulfate hemihydrate (Plaster of Paris) by heating to 125°C. Phase-pure calcium sulfate hemihydrate powder, thus prepared, was graded to size ranges 46-75μ, 76-100μ and 101-125μ for further studies. The wetting medium for the cement was distilled water, in an appropriate ratio, roughly 0.6 ml for 1 gram of the powder.

The setting times of the cement compositions were assessed using a custom-made Vicat-type apparatus. The compressive strength of the set cement, moulded in pellet shape, was measured in a Universal Testing Machine (Instron model 1193) as per ASTM Standard F 451 for each composition. The particle size range 45-75 μ was found ideal with setting time of 4 min and compressive strength of 14.2 MPa.

The phase formation in the set cement was analysed in XRD, in which a complete conversion to Bassnite (hemihydrate form) to Gypsum (dihydrate form) was identified. It was observed that the phase conversion occurs quickly and the setting happens while the crystals grow inside the mass and entangle to form a stable structure.

The microstructure of the different compositions of the cement showed needle-like morphology. The crystals were observed to interlock and entangle in the cement mass, making the solid structure. The crystals were randomly oriented and micro-pores were seen in between the crystallites. Maximum obtainable density was about 55% of the theoretical value. The porosity was assessed by Mercury Intrusion Porosimetry.

The effects of bioactive fillers (hydroxyapatite ceramics and bioactive calcium phospho-silicate glass) on the cement properties were studied. The hydroxyapatite ceramic and bioactive glass powders of appropriate grades were added in the optimized calcium sulfate cement in definite weight ratios. The optimization point is the quantity of the filler which gives clinically acceptable values of setting time and strength, i.e. setting time above 10 minutes and compressive strength higher than 12 MPa. The ideal filler ratio was fixed to 25%.

The next step was to induce ‘injectability’, assessed by extruding through an 18 gauge needle by back-filling in a syringe. It was seen that the bare cement mix, even in the form of loose slurry, does not flow smoothly through the needle. The use of biocompatible gelling agents (like sodium alginate, chitosan and cellulose derivatives)

improved the rheological properties to the mixed paste, but they caused prolonging of the setting time. It was observed that biocompatible sodium phosphate based compounds increased the flow properties of the mixed cement paste drastically. The injectability of the cement was tested through extrusion rheometry in a custom fabricated rheometer loaded in a UTM. It was not possible to extrude the bare cement, where as the modified calcium sulfate cement mix (containing 1.5% disodium hydrogen phosphate) extruded from the rheometer fully at a force lower than the human thumb force.

The final optimized formulation of the IBS has been subjected to biocompatibility and bioactivity studies. Biocompatibility studies include initial in vitro screening and toxicological animal experiments.In the Biocompatibility Screening of the material, In vitro Cell Culture Cytotoxicity and Haemocompatibility were done.

The response of mouse fibroblast (L929) cells to IBS samples with sodium phosphate and carboxymethyl chitosan were compared with bare calcium sulfate (CAS), through *in vitro cell culture cytotoxicity.* The cells in contact with the test samples showed the normal morphology by and large, indicating that the test materials are not toxic to cells. The cells after the interaction with the material in direct contact have been subjected MTT Assay study. The cement with the binder carboxymethyl chitosan was found to inhabit more viable cells, in the study.

In the in vitro Haemocompatibility study using human blood, 2 samples with the binder carboxymethyl chitosan were found compatible.

The Toxicological Tests prescribed by ISO 10993 were done on the material. Being an implantable material, the following tests were selected : Acute Systemic Toxicity, Intracutaneous (Intradermal) Reactivity Test , Maximization Sensitization Test and Pyrogen Test. The preliminary results of these tests showed satisfactory resuts, showing the safety and biocompatibility of the material. Implantation In Muscle.has been done to see the local effectsto tissues. The histological analysis of the test samples is ongoing and the results are awaited.

In the bioactivity study using human osteoblast cells The modified cement (IBS) showed 83 % activity, whereas bare calcium sulfate showed only 36%. The effect of osteoblast on the material was evident from the SEM study. The formation of new crystallites growth in leaf-like structure due to osteoblast cells indicates the bioactivity of the material.

As an overall comment, the basic objective proposed in the project is met.

12. Conclusions summarising the achievements and indication of scope for future work:

The work in the project covers the development of a bone substitute product, including the biocompatibility evaluation and bioactivity testing. An injectable, self-setting cement has been developed based on calcium sulfate material, composited with bioactive ceramic particles. This is a biocompatible and osteoconductive bone graft product which meets the clinical needs, as the outcome of this project shows. (This statement is based on the initial results of the biocompatibility and bioactivity studies; the final reports are awaited).

This is a new generation bone graft material having the unique combination of properties of osteoconductivity, resorbability and injectability. It could be used for routine clinical application, as a filler or binder or graft extender, in orthopedics. In dentistry, it finds applications in alveolar ridge augmentation, for the filling of extraction socket and periapical lesions (or cysts in maxillary and mandibular bone) and for supporting oral implants. The attractive feature of the product is injectability, which makes it more useful than the solid bone graft materials like calcium phosphate ceramics and bioactive glasses. The product could be delivered into the bone defect sites through a narrow cannula or needle, which makes it possible to fill the sites which are not directly accessible. Also, the bone grafting procedures could be made minimally invasive.

The future scope of the proposed work is the development of 'drug-loaded' injectable bone substitute (IBS). The IBS material is aqueous based and therefore water-soluble derivatives of the drugs could be loaded easily. This will be useful in three clinical areas, namely osteomyelitis treatment, osteoporosis management and bone cancer treatment. Antibiotics could be delivered locally in infections leading to osteomyelitis. In osteoporosis, IBS loaded with hormones/medicines could be injected into the affected area so as to reverse the effect of degeneration. IBS is applicable as well in delivering cancer chemotherapy drugs at tumor removal sites. These proposed scopes could be perfected by conducting preclinical and clinical studies.

13. S&T benefits accrued:

i. List of Research publications

(One paper in peer reviewed journal)

1. S. Sandhya, S. Sureshbabu, H. K. Varma, and Manoj Komath, Nucleation kinetics of the formation of low dimensional calcium sulfate dihydrate crystals in isopropyl alcohol medium, Cryst. Res. Technol. 47 (2012) 780–792.

ii. Manpower trained on the project

a) Research Scientists or Research Associates

- Nil

b) No. of Ph.D. produced

- Nil

c) Other Technical Personnel trained

- Two (Project Assistant), M.Sc. qualification.

iii. Patents taken, if any

- Nil

14. Financial Position:

No	Financial Position/ Budget Head	Funds Sanctioned (1st Year)	Funds Sanctioned (2nd Year)	Expenditure	
I	Salaries/ Manpower costs	96,000	2,00,000	Statement of expenditure will be submitted at the end of the financial year 2012-13	
II	Equipment	3,27,621			
III	Supplies & Materials	2,74,000			
IV	Contingencies	20,000			
V	Travel	30,000			
VI	Overhead Expenses	1,00,000			
VII	Others, if any				
	Total	8,47,621	2,00,000		

15. Procurement/ Usage of Equipment

a)

S No	Name of Equipment	Make/Model	Cost (FE/ Rs)	Date of Installation	Utilisation Rate (%)	Remarks regarding maintenance/ breakdown
1	Vacuum furnace with accessories	OKAY 40T 7Y	Rs.2,97,383 /-	March 2011	60%	No breakdown till date
2	Ultrasonic cleaner	MAXSELL MX100SH-4LQ	Rs.19,800 /-	February 2011	80%	

b) Plans for utilising the equipment facilities in future

These equipments will remain part of the Investigators' Laboratory. They had already been entered in the Stock Register of the Institution. The remaining part of the present project is continuing, and the equipments are currently in use for the purpose. In addition, these will be used for the Bioceramics sample production/processing in the laboratory.

Name and Signature with Date

Place : Trivandrum

a._____
(Principal Investigator)

Date : 07 January 2013

b._____
(Co-Investigator)