PROJECT COMPLETION REPORT OF

APATITIC CALCIUM PHOSPHATE BONE CEMENTS

Principal Investigator

Dr. Manoj Komath

Co-investigator

Dr. Harikrishna Varma P.R.

BIOMEDICAL TECHNOLOGY WING
SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY
Satelmond Palace, Poojappura, Trivandrum -695012, INDIA

INDEX

	Page
Title of the Project	i
Abstract of the Project	.ii
PART 1 – The Proposed Work	
I. Broad Objectives	5
II. Precise Objectives	5
III. Work Plan	7
IV. Time Schedule	8
V. Budget Summary	9
PART 2 – Details of the Work Done	
I. General Introduction to the Area	10
II. Design of the Cement	11
2.1. The Powder Part	11
2.2. The Liquid Part	12
2.3. Formulating the Cement	13
III. Property Evaluation of the Cement	14
3.1. Testing of the Selected Formulation	14
3.2. Material Characteristics	15
3.2.1 *Setting Time*	15
3.2.2 *Compressive Strength*	16
3.2.3 *Precipitated Phase*	16
3.2.4 *Microstructure and Porosity*	18
3.2.5 *Shrinkage*	19
3.2.6 *Thermal Changes*	19
IV. Optimization of the Cement	20
4.1. The Effect of Various Parameters on the Quality	20
4.1.1 *Phase Conversion*	20
4.1.2 *Particle Size Effects on Strength*	21
4.1.3 *HA Particles Seeding Effects*	22
4.1.4 *Modification of the Wetting Solution*	23
4.1.5 *Porosity Control*	24
4.2. Optimization of the Cement formulation	26
V. Biocompatibility Evaluation	27
5.1. Selection of Tests	27
5.2. Screening Tests	28
5.2.1 Cytotoxicity	28
5.2.2 *Haemolysis*	29
5.2.3 Conclusion of the Biocompatibility Screening	29
5.3. Toxicological Studies	30
5.3.1 Acute Systemic Toxicity	30
5.3.2 Intracutaneous (Intradermal) Reactivity Test	30
5.3.3 Pyrogen Test	31
5.3.4 Maximization Sensitization Test	32
5.3.5 Implantation in Muscle	34
VI. Biofunctionality Studies	32
6.1. Human Osteoblast Cell Culture	32
VII. Summary and Conclusion	34
VIII. Overall Assessment and Future Outlook	35

Project Title :

APATITIC CALCIUM PHOSPHATE BONE CEMENTS

Principal Investigator :

Dr. Manoj Komath

Co-investigator :

Dr. Harikrishna Varma P.R.

Institution :

Biomedical Technology Wing,
Sree Chitra Tirunal Institute for
Medical Sciences and Technology,
Satelmond Palace, Poojappura
Thiruvananthapuram - 695 012

ABSTRACT OF THE PROJECT :

The project being proposed is on the development and evaluation of bone cements based on calcium phosphates. Calcium phosphate bone cements (CPBCs) are materials of special interest in the field of skeletal repair because they combine the properties of mouldability, biocompatibility and osteoconductivity. This uniqueness make them highly useful in filling and reconstructing bony defects arising out of surgery or loss during trauma.

The overall aim of the project is to design a self-setting, biocompatible bone cement for orthopedic filling, reconstruction and implant fixation applications. The formulation will contain calcium phosphate compound mixture, which can undergo a hydraulic reaction to set into defective and substituted apatites, represented by the general formula **$(Ca, Na, Mg, K)_{10}(PO_4, CO_3, HPO_4)_6(OH, Cl, F)_2$**. These phases resemble closely to the bone mineral in composition, and helps the healing of bone when implanted at the defect site. The work will be done in four major steps.

(1) Formulation of the cements : Exploration of the ingredients, additives and components (novel as well as conventional) which can form cements of desired phase composition. This step involves chemical synthesis and processing of materials. The formulations will be subjected to various physico-chemical analyses to identify the suitable ones.

(2) Optimization of the formulations : The most important requirement of a CPBC formulation is that the properties (setting time and strength) should satisfy the clinical needs. It is necessary to develop an understanding of the setting characteristics and mechanical properties of the cements. The setting time and strength will be regulated by suitable methods, and optimised so as to meet the clinical requirements.

(3) Biocompatibility evaluation : The optimised formulations will be evaluated under a protocol which include initial screening procedures like cytotoxicity test and haemolysis test, and detailed toxicological analyses (*in vitro* and *in vivo*). Modification of the cement formulation will be done if necessary, taking feedback from the evaluation results.

(4) Biofunctionality studies : Testing the efficacy of the cement through *in vitro* cell culture methods, with appropriate cell lines.

The work plan as a whole is the development and evaluation of a potential biomedical product. The studies will indicate the most useful formulation for bone graft application. However, making it a commercial product will require proof for *in vivo* efficacy through animal implantation and pre-clinical human trials. This will be done as the second phase of the project.

PART 1 – The Proposed Work

I. BROAD OBJECTIVES OF THE PROJECT

1. To test various calcium phosphate ingredients which can undergo cementing reaction and produce an *apatitic* precipitate belonging to the class **(Ca, Na, Mg, K)$_{10}$(PO$_4$, CO$_3$, HPO$_4$)$_6$(OH, Cl, F)$_2$** ,similar to the bone material.
2. To explore the setting characteristics and physico-chemical properties of the cement formulations and to select formulations with desirable features.
3. To optimise the structure and properties of the selected formulations for orthopedic filling and augmentation applications.
4. To conduct toxicological evaluation of the cements through *in vitro* and *in vivo* test methods following the international standards.
5. To investigate the biofunctionality of the toxicologically approved cement formulations, through *in vitro* cell culture methods.

II. PRECISE OBJECTIVES OF THE PROJECT

The project focuses on the synthesis and evaluation of a potential bio-medical product - **calcium phosphate bone cements**. The aim is to design and develop calcium phosphate cements which give *apatitic* (defective or substituted) mass similar to the biological apatites, intended for orthopedic filling and augmentation purposes. The precise objectives are discussed part by part as follows.

The initial part of the objective is to explore and find the cement formulations, which can form the desired phase composition. A calcium phosphate bone cement (CPBC) formulation essentially contains calcium and phosphorous based ingredients in powder form which, on mixing with an aqueous medium, form a workable and self-setting putty. The mechanism of setting is the dissolution of the ingredients in the medium and their re-precipitation as orthocalcium phosphates. At least two ingredients are necessary (one acidic and the other basic) to make a cement. The wetting medium consists of distilled water along with additives to regulate the setting properties.

Various combinations of ingredients, components and additives are to be investigated systematically to find the cement formulations which can produce phases of the class **$(Ca, Na, Mg, K)_{10}(PO_4, CO_3, HPO_4)_6(OH, Cl, F)_2$**. The work involves materials synthesis, processing and testing. A detailed analysis of the structure and properties of the cements is to be conducted. A vivid knowledge of the chemical phase formed and its microstructure, is essential because they influence the *in vivo* properties of the cement.

The next part is the optimisation of the formulations for the proposed application(s). A study of the setting characteristics and strength of the set mass is necessary in this regard. These properties are, in turn, controlled by factors like ingredient particle size, wetting ratio and ambient temperature. The various parameters are to be regulated to make the selected formulations satisfy the requirements of clinical implantation.

The subsequent part deals with the biocompatibility evaluation. The optimized formulations should satisfy the biocompatibility requirements prescribed for a biomedical product. Preliminary screenings include cytotoxicity (effect of the material on live cells cultured *in vitro*) and haemolysis (lysis of red blood cells due to the material) tests. After the screening, the cements have to be subjected to detailed toxicological analyses (mucous membrane irritation test, subcutaneous/muscle implantation, mutagenicity test etc.) following international protocols. Adverse results if any at any stage of evaluation are to be analysed in detail for the cause so that necessary modification can be done on the material.

The final part is to test the efficacy of the cement. The biofunctionality (related to bone) is to be investigated *in vitro*. The tests are done using osteoblast cell culture. The cell adhesion and proliferation will indicate the osteogenic potential of the material. This also enables a preliminary estimation of the *in vivo* performance of various formulations.

The study as a whole is expected to provide a clear idea as to, which of the apatitic cement formulation is most suitable for skeletal repair applications. It will indicate the ways of future upgradation of the cement as a commercial product.

III. WORK PLAN :

The first phase of the work, i.e. the formulation of the cements, involves synthesis of the ingredients. The essential compounds will be synthesized through wet chemical method or solid state reaction. In some cases, commercially available material will be used, taking cost effectiveness into consideration. The ingredients used will be checked for phase composition and chemical purity in batches, prior to the experiments. The formulations are prepared by mixing the graded powders in appropriate molar ratio.

The cementing process of the formulations will be tested through different techniques. The setting of the cement can be identified using Vicat's Needle method. The phase conversion can be analyzed using XRD. FTIR, EDAX and chemical estimation methods can be used to confirm the phase and chemical purity of the final product. SEM will give information about the microstructure. The dimensional changes and porosity can be assessed through size and weight measurements. The thermal changes during setting can be measured TGA-DTA technique.

The next phase is the optimization of the formulations. This will be done by considering the clinically relevant parameters, i.e. the consistency, setting time and mechanical strength. These are dependent on the particle size, wetting ratio and presence of accelerator or retarder. The various factors are to be optimised to obtain the formulations that meet the clinical requirements.

During the toxicological testing phase, initially, the various cement formulations will be subjected to screening tests of cytotoxicity and haemolysis. Cytotoxicity screening looks for the morphological changes in the fibroblast cells cultured over the material. The haemolysis test analyses the red blood cell lysis percentage. Only those materials which pass the cytotoxicity and haemolysis tests will be selected for further analysis. Thereafter, detailed analysis will be performed to deduce the toxicological profile. The proposed tests are subchronic oral toxicity test, intracutaneous irritation test, sensitization studies and intra-muscular implantation (these include the mandatory ones also). The feedback from the tests will be evaluated and modification in the formulations will be done, if necessary.

Toxicologically approved samples will be tested for efficacy *in vitro* by cell culture (using human osteoblast cell lines). The bioactivity of the samples can be estimated by observing the proliferation of cells (through MTT assay method and imaging techniques).

IV. TIME SCHEDULE

1-4 months

- Procurement of chemicals, consumables and arranging accessories for experiments.
- Recruitment of project assistant.
- Preliminary experiments (like the synthesis of essential compounds) to begin.

4 months onwards

- The cement formulation, properties studies and tests/analyses etc. Modifications of the formulation will be done form time to time, after taking feedback from other experiments, which may continue till the *in vivo* studies (i.e. till 14^{th} month)

13 – 20 months

- *In vitro* studies and compilation of results.
- Toxicological studies.

21 - 22 months

- Review and correlation of the results.

23 - 24 months

- Project report preparation and submission.

V. BUDGET SUMMARY :

Estimated expenditure during each financial year (April to March) covered by the duration of the project:

Items	***1st Year***	***2nd Year***	***Total (Rs)***
1.Wages	24,000.00	24,000.00	48,000.00
2.Consumables	13,500.00	8,500.00	22,000.00
3.Equipments	-	-	-
4.Travel	-	-	-
5.Research Literature	1,000.00	1,000.00	2,000.00
6.Other (Evaluation)	24,000.00	91,000.00	1,15,000.00
Subtotal	62,500.00	1,24,500.00	**1,87,000.00**

PART 2 – Details of the Work Done

I. GENERAL INTRODUCTION TO THE AREA:

The advent of calcium phosphate cements (CPCs) is considered as a remarkable development in the area of bone repair materials. The requirement for grafting bones arises quite often in clinical practice, in circumstances like bone loss due to trauma or tumor removal and surgical correction of skeletal parts. Grafting can be done with live bone, incised from the same individual (*autogenous*) or from a donor (*allogeneic*). However, live bone grafting techniques encountered problems, which limited their extensive application. The autogenous grafting involves more surgical procedures and related complications while the allogeneic grafts pose the risk of immunogenic response and infections. These are the factors, which prompted the search for synthetic materials suitable for bone grafts. Biocompatible and osteoconductive materials like calcium phosphate ceramics and bioactive glasses were developed for the purpose. These materials gained immediate popularity, and are now available commercially as granules intended for defect filling and reconstruction of bones.

The ceramic bone grafts, despite their wide use in bone repair, are of limited help in certain applications (e.g. fixation cases like hip arthroplasty, or filling of intricate cavities) where a cement consistency is required. Acrylic based (PMMA) cements are currently in use for the purpose. Even though acrylic cements offer good initial fixation, they ail from serious drawbacks like monomer toxicity, exothermic setting and shrinkage. Other similar materials like resin cements did not prove an alternative for fixation because of the presence of leachants and poor biodegradability.

A novel concept emerged in the field of bone cements, when it was shown in 1983 that calcium phosphates could undergo cementing reaction. It was considered as a breakthrough because the invention was not only a biocompatible cement, but also a material that gets converted into *apatitic calcium phosphate* which is similar to bone mineral in composition. Since then CPCs have become an important topic of research in the field of biomedical research. Several formulations have come up and the various

properties (material quality as well as biological performance) have been studied. A considerable wealth of publication has resulted.

The project work has been envisaged as an independent venture of design-development of a CPC formulation *ab initio*.

II. DESIGN OF THE CEMENT :

Generally, a CPC formulation contains calcium and phosphorous based ingredients in powder form which, on mixing with an aqueous medium, form a workable and self-setting putty or paste. The design is done by developing the powder part and liquid part and deciding the wetting ratio.

2.1. The Powder Part

The powder part is developed by exploring the various combinations of calcium and phosphate based compounds, which can undergo cementing reaction.

(i) The ingredients : At least two ingredients are necessary (one acidic and the other basic) to make the cement. The $^{Ca}/_{P}$ ratio is the deciding factor for the formation of apatitic phase. To form hydroxyapatite, a net Ca:P ratio of 10:6 is necessary in the mixture. A list of candidate compounds are listed in the table below.

TABLE I

Compound	***Abbreviation***	***Formula***	$^{Ca}/_{P}$ ***Ratio***
Phosphoric Acid, Anhydrous		H_3PO_4	0
Monocalcium Phosphate, Anhydrous	MCPA	$Ca(H_2PO_4)_2$	0.5
Dicalcium Phosphate, Anhydrous	DCPA	$CaHPO_4$	1
Dicalcium Phosphate, Dihydrate	DCPD	$CaHPO_4.2H_2O$	1
Tricalcium Phosphate (alpha & beta)	α/β - TCP	$Ca_3(PO_4)_2$	1.5
Tetracalcium Phosphate	TTCP	$Ca_4(PO_4)_2O$	2
Calcium Oxide/Hydroxide		$CaO/Ca(OH)_2$	$\propto$

These compounds were either bought from the market or synthesized in the lab (in the case of expensive or imported ones). Compounds like phosphoric acid, anhydrous dicalcium phosphate, dicalcium phosphate dihydrate, calcium oxide and calcium hydroxide were purchased (analytical grade). Monocalcium phosphate, tricalcium phosphate (both alpha and beta phases) and tetracalcium phosphate were synthesized.

(ii) Synthesis of compounds :

Monocalcium phosphate was precipitated by wet method by using $Ca(NO_3)_2$ and $(NH_4)_2HPO_4$ in a Ca/P ratio of 0.5. Calcium nitrate was added to vigorously stirred $(NH_4)_2HPO_4$ solution at pH 9.9 followed by immediate filtration. The product was washed in ammonium hydroxide and dried.

Tricalcium phosphate (TCP - alpha and beta phases) and tetracalcium phosphate (TTCP) were synthesized through high temperature method. For tricalcium phosphate, dicalcium phosphate and calcium oxide/carbonate were taken in a net Ca/P ratio of 1.5 and mixed thoroughly. The beta phase TCP was made by heating the mix to 1150°C for sufficient period and subsequent cooling. The mix has to undergo heating to 1200°C and fast quenching to obtain alpha phaseTCP. For tetra calcium phosphate, dicalcium phosphate and calcium oxide/carbonate were taken in net Ca/P ratio of 2, mixed thoroughly and heated to1350°C followed by a fast cooling.

All the phases were checked and the purity was confirmed with X-ray diffraction analysis. The masses obtained were dried properly, subjected to dry grinding and appropriately graded. Particles in the range (or less than) 100 micron were used to make cement.

(iii) Preparation of the mixtures :

The various combinations were prepared by selecting the compounds (given in the Table I) in the net Ca/P ratio of 1.67, that of hydroxyapatite. For mixing, the powder combination was filled in HDPE milling jars along with an equal weight of 0.6 mm alumina balls and rotated in a mixing machine for 30 min.

2.2 The Liquid Part

The cement chemistry operates with distilled water as wetting medium. However, it is observed that the conversion rates are very slow and the cement mix may not set at required time period. Therefore, the use of phosphate solutions was suggested to accelerate the setting. The wetting medium is generally made by dissolving biocompatible phosphates (the accelerator) in distilled water.

Sodium phosphates were observed to satisfy the requirements of an accelerator. Analar grade sodium di-hydrogen phosphate and di-sodium hydrogen phosphate were made into solutions at different concentrations. These were stored and used as wetting solutions.

2.3. Formulating the cement

In formulating a calcium phosphate cement, the requirement is that, when the powder part and the liquid part are mixed, it should give a self-setting mass. The various powder combinations were tested for setting by wetting with the solutions of different concentrations. The wetting ratio, i.e. the amount of the medium to be added to the cement, was adjusted so that a putty-like consistency is obtained. The setting time and strength of the putty were kept as markers for identifying suitable formulation. Best combination was decided empirically through systematic trials.

The simplest of the compositions was phosphoric acid and calcium hydroxide, but there is a lack of control in setting with these reactants. Other compositions involving monocalcium phosphate, dicalcium phosphate (DCPA and DCPD), tricalcium phosphate (alpha & beta phases) and tetracalcium phosphate (TTCP), gave varying ranges of setting time and strength. Among these, TTCP-DCPD mixture was apparently found the best. Disodium hydrogen phosphate was found to be an effective setting accelerator (Na_2HPO_4). The cement was optimized with this combination.

The cement powder was prepared by mixing the TTCP and DCPD particles in equimolar ratio (i.e. the net Ca/P ratio is 1.67). The wetting ratio was varied according to the workability requirements. The cement was observed to form a workable putty only in a narrow range of wetting ratio. Insufficient wetting will make the cement powder an inhomogeneous mass, with which shaping is difficult. Excessive amount of medium, on the other hand, will give a loose paste. For the proposed application, a putty that can be shaped with fingers will be ideal. The optimum wetting to obtain such a form was found to be 0.5ml of the medium per 1g of cement powder. This wetting ratio was followed throughout the study, for setting the cement.

III. PROPERTY EVALUATION OF THE CEMENT

3.1 Testing of the selected formulation:

The following materials testing techniques/methods were used to evaluate the material properties of the cement.

The setting times of the cement were assessed using a custom-made Vicat-type apparatus (Fig.1). It consisted of a steel needle (diameter 1mm) 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 plastic well (of 5mm depth), which is meant to hold 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. As the mass solidifies, it will resist the penetration of the needle. The setting time can be identified as a steep decrease in the value.

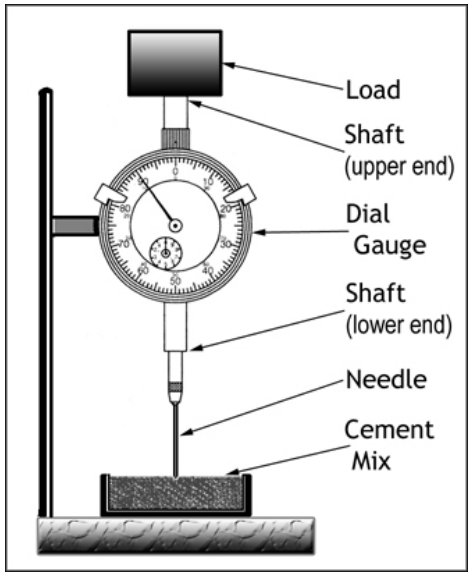


Fig.1. *The Vicat type apparatus used for the measurement of setting time of the cement mix.*

There are two stages identified in the setting of CPBCs - the *initial setting time* which denotes the end of workability of the putty after wetting, and the *final setting time* which indicates the hardening of the set mass. The initial setting time was measured by monitoring the penetration of a blunt needle. The needle can no more penetrate after the initial setting. Thereafter, a needle with a sharp tip was used on the set mass to see the final setting. The tip cracks the putty and enters into the surface region till the mass hardens.

The compressive strength of the set cement was measured in a Universal Testing Machine (Instron model 1193) as per ASTM Standard F 451-95. The cement samples were molded in the form of cylindrical pellets (6mm dia and 12mm height), incubated in 100% humidity for 24 h and dried before the analysis. The pellets were compressed along their height 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 pellets.

The phase analysis of the set cement was carried out using X-ray diffraction technique (XRD - Siemens D 5005). The set cement mass was kept in 100% humidity at physiological conditions for 24 h and then dried and powdered for analysis. The structural analysis was done in an FTIR spectrometer (Nicolet model - Impact 410) by KBr pellet technique, after powdering the set cement. The $^{Ca}/_{P}$ ratio was determined using electron microprobe energy dispersive analysis (EDAX-Oxford EDS system), where Wollastonite and GaP standards were used for Ca and P, respectively.

The microstructure of the cement was observed in scanning electron microscope (Hitachi SEM, model S2400), after cutting the set mass across and coating with gold. The porosity was found by measuring the density of the cement mass and comparing it with the theoretical density of the phase. The change in dimensions during the setting was assessed using standard moulds. The setting temperature was monitored in a differential thermal analyzer (DTA- TA Instruments, model SDT 2960) in isothermal mode with alumina as the standard.

3.2. Material Characteristics

The following characteristics were identified for selected formulation (unoptimised), in the tests done.

3.2.1 *Setting time* : A known quantity of the cement powder was taken and wetted with the medium in a ratio 0.5 ml/g. After mixing for 30sec, the putty was loaded in the apparatus for measurements.

Distilled water as the wetting medium made the setting slow – it took more than 60 min to set. Addition of disodium hydrogen phosphate (Na_2HPO_4) as

accelerator at the concentration of 0.1M in the medium, showed a marked effect that the initial setting time came down to the range of 15 min. At higher concentrations, the initial and final setting times decreased as given in the plot (figure 2).

3.2.2 *Compressive strength* : Among the mechanical properties of bone repair materials, compressive strength (CS) is the most widely explored one. The strength of cement depends on various factors like particle size of the cement powder, nature of additives and wetting ratio. The effect of concentration of accelerator in the medium was investigated in order to optimize the cement.

The range of CS obtained (10-12 MPa) was found comparable to reported values for calcium phosphate cements. The effect of the accelerator (Na_2HPO_4) concentration on the CS was studied by keeping the wetting ratio constant. The results are shown in figure 3. A linear decrease in CS with the increase in concentration can e observed.

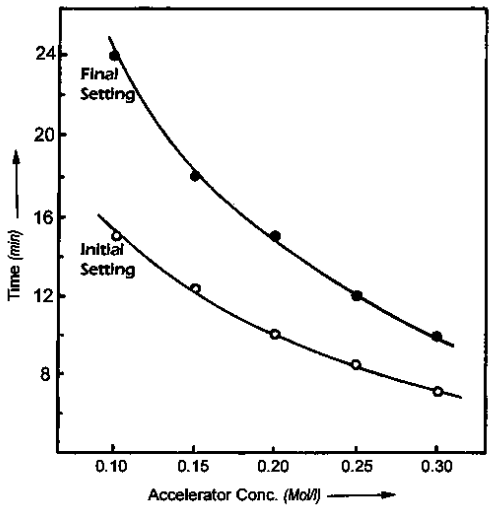


Fig.2. *The setting times of the cement mix measured using Vicat's technique, as a function of accelerator concentration*

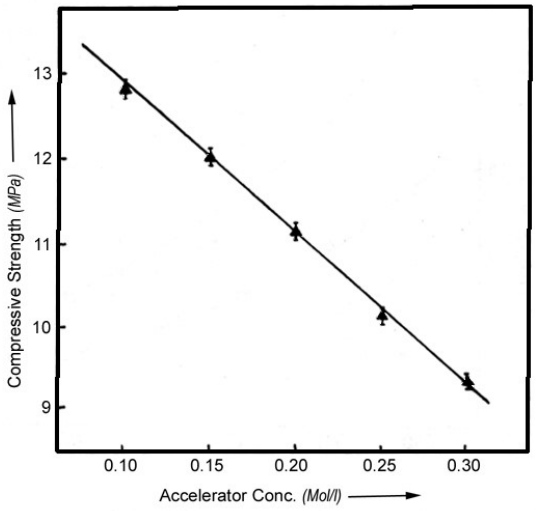


Fig.3. *The compressive strength of the set cement as a function of accelerator concentration in the wetting solution.*

3.2.3 *Precipitated phase* : The calcium phosphate phase formed in the cement (set with 0.2M Na_2HPO_4 in the medium at a wetting ratio 0.5ml/g) was analyzed in XRD. The diffraction pattern of the raw powder and that of the set mass (kept for 24h in 100% humidity) are shown in figure 4.

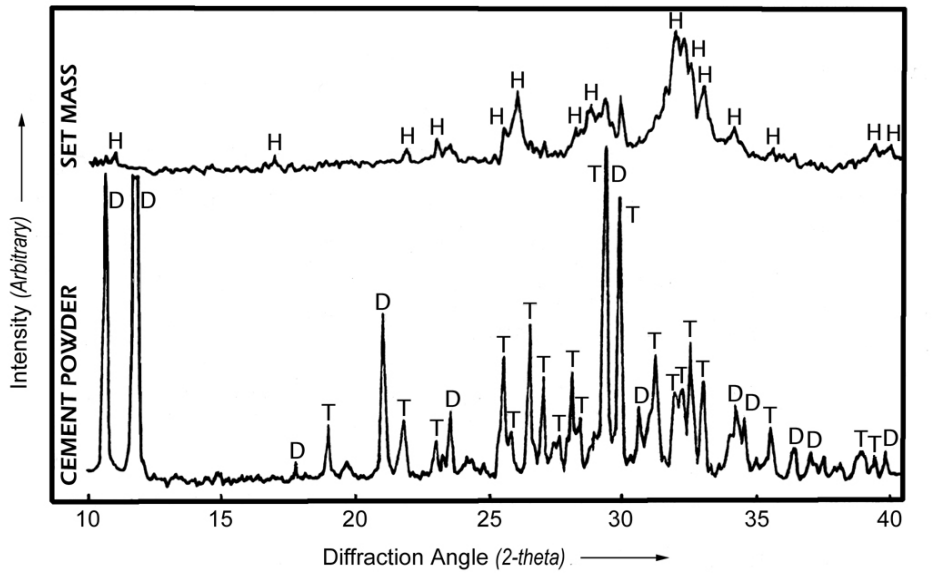


Fig.4. *The diffraction patterns of the raw cement powder and the set mass. The phase conversion is evident. The notations on the peaks correspond to the following compounds :* ***D*** *- Dicalcium phosphate dihydrate,* ***T****–Tetra calcium phosphate and* ***H*** *– Hydroxyapatite.*

The peaks corresponding to hydroxyapatite (HA) can be seen prominently in the cement (JCPDS Card No: 9-432). The broadness of the HA peaks indicates that the precipitated material is not well-crystallized. The peaks of DCPD were totally absent in the set cement. Traces of TTCP, however, were still present. This is due to the incomplete conversion of the compound owing to the rate limiting nature of dissolution.

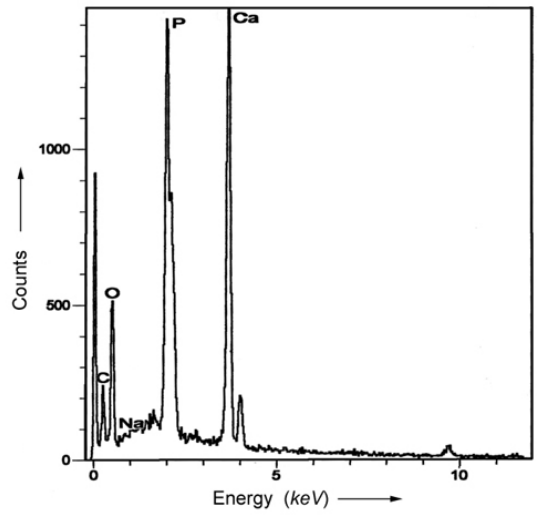


Fig.5. *The EDAX spectrum of the set cement.*

The result of the EDAX analysis is given in figure.5. Ca/P ratio (atomic percentages) determined is 1.69. This is close to the theoretical value for HA (1.67). The presence of sodium was found only in traces.

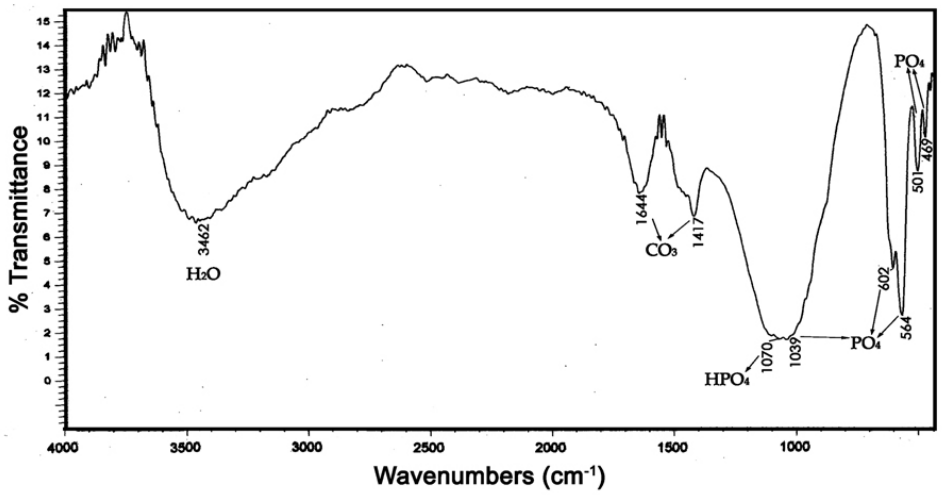


Fig.6. *The FTIR spectrum of the set cement.*

The FTIR of the above sample (KBr pellet method) is shown in figure.6, in which typical absorption bands corresponding to hydroxyapatite are seen. Trace amounts of carbonate appeared in the spectrum, which may be a result of atmospheric CO_2 contamination. (Carbonate group in CPCs is not considered as an impurity because the bone material is basically a -CO_3 substituted hydroxyapatite).

3.2.4 *Microstructure and porosity* : The scanning electron micrograph of the cut face of set cement (kept in 100% humidity for 24h at 37°C) is shown in figure.7a.

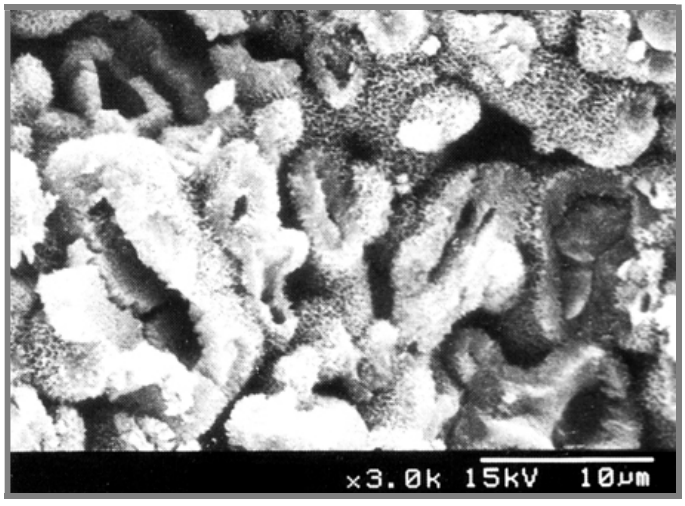


Fig.7a. *SEM picture of the set cement mass. A lump of particles, inter-connected in the form of porous structure, is seen.*

The micromorphology shows lumps of particles, interconnected in the form of porous structure. Apatitic nanocrystallites can be seen grown all over the surfaces of the matrix. This crystallites growth is clearer in a magnified view of a single particle (figure.7b). The growth morphology is similar to the reported nucleated growth of apatitic calcium phosphates. These appear to be heterogeneously nucleated over the surfaces of the reactant particles.

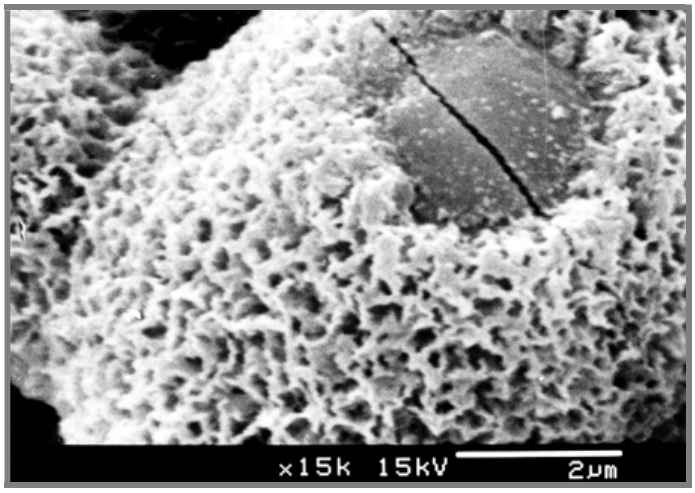


Fig.7b. *A magnified view of one of the particles. The growth of apatitic nanocrystallites is clearly seen.*

The density of the cement material was found as to be 1.52g/cc from the mass-volume relationship. A comparison with the theoretical density (3.16g/cc) of HA, gave ~ 52% gross porosity value.

3.2.5 *Shrinkage* : Bone cement shrinkage during setting is one of the problems encountered in bone repair when acrylic cements are used. The shrinkage of the TTCP-DCPD cement was assessed by setting the cement inside dies of regular geometry and known dimension. The set cement, preserved in 100% humidity did not show any linear shrinkage at an accuracy level of ±0.05mm. The set cement, on drying up, showed an average linear shrinkage of 0.2%.

3.2.6 *Thermal changes* : Observations on the setting temperature of the cement in DTA showed a decrease of ~1°C with respect to the reference sample of same weight, at isothermal conditions (figure 8). This is a clear advantage over acrylic bone

cements, for which the exothermic setting reaction elevates the local temperature to about 100°C, causing tissue necrosis in application.

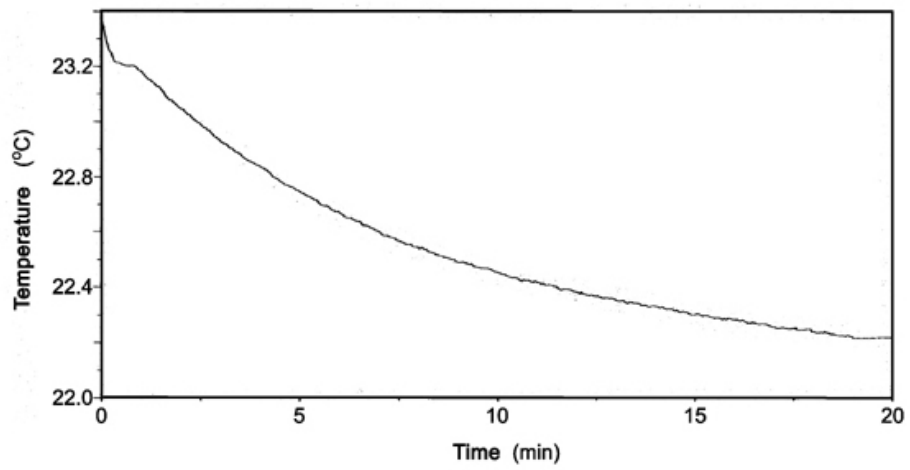


Fig.8. *The setting temperature changes of the cement, identified through differential thermal analysis.*

IV. OPTIMIZATION OF THE CEMENT

4.1. The effect of various parameters on the quality of the cement.

The effect of various parameters has been investigated to find the optimum design of the cement.

4.1.1 *Phase conversion* : The cement putty hardens because the entangled growth of hydroxyapatite crystallites. The phase formation is rate limiting and the accelerator is incorporated to enhance the kinetics. The formation of hydroxyapatite phase from the ingredients at various concentrations of the accelerator in the wetting medium, is observed using X-ray diffractometry.

The samples were prepared by mixing 2g of cement powder and 1ml of wetting solution. For each test, the concentration of the accelerator was varied from 0 to 1N at various steps. The solidified cement was crushed to fine powder and analysed in X-ray diffractometer. All spectra were recorded at the same parameters, in the range 10 – 40°. To track the phase conversion, the prominent peak of DCPD at the d-value

7.57Å, corresponding to (0 2 0) plane, is used. Hydroxyapatite peaks were not used for the purpose because of two reasons. First, newly formed hydroxyapatite has low crystallinity and hence it is difficult to observe the peaks and quantify the conversion. Second, the DCPD (0 2 0) peak is very strong and it is highly sensitive to the phase changes. The peak intensities are compared with the strongest peak of quartz (Analar grade, added 1%w/w to the powder) at the d-value 3.34Å, corresponding to (1 0 1) plane. Each test is repeated 3 times to see the spread in the values.

The relative peak intensities against the accelerator concentration are shown in figure 9. This is an indication of the quantity of DCPD taking part in the initial reaction with TTCP, to get converted to hydroxyapatite. It is clear that the accelerator (Na_2HPO_4) has a significant effect on phase conversion. Concentrations up to 0.1 M reduces the remnant DCPD (i.e. increases the conversion) drastically, which comes to a saturation after 0.2 M.

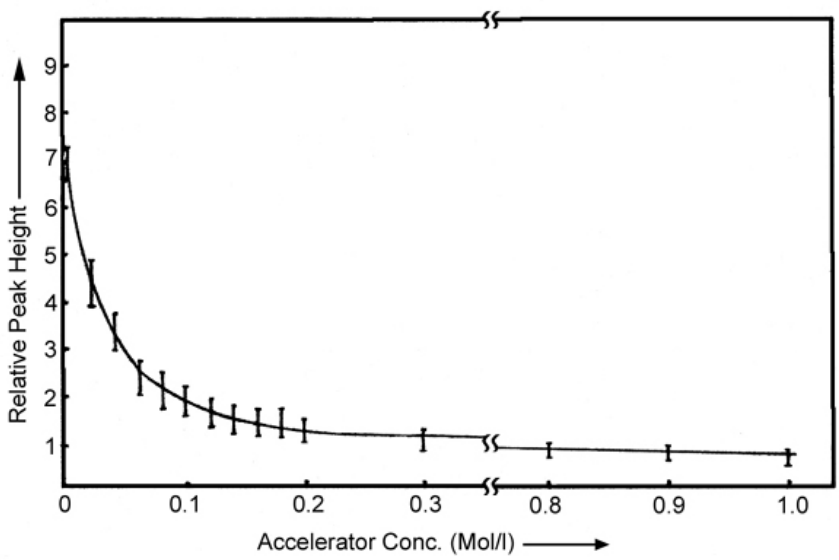


Fig.9. *The phase conversion in the cement observed in XRD, on increasing the accelerator concentration. The data points are relative intensities of the DCPD (0 2 0) peak in comparison with quartz (1 0 1) peak.*

4.1.2 *Particle size effects on strength*: Particle size is one of the deciding parameters of the properties of the cement. The effect of the particle sizes of the ingredient powder on the compressive strength of the set cement is investigated. TTCP and DCPD powders were milled down to 100 micron sizes. They were independently

sieved and graded and then mixed to form the powder part of the cement. Three different particle sizes were selected – 100μ-less (as milled powder with >100μ particles sieved out), 75-50μ and 50μ-less. Cement powder is prepared in each case, wetted with 0.2M Na_2HPO_4 solution (in the wetting ratio of 0.5ml/g) and moulded into pellets for compressive strength studies. Cylindrical pellets of 6mm dia and 12mm height were made, incubated in 100% humidity for 24 h and dried. The break-load along the axis was measured in Universal Testing Machine at a cross-head speed of 1mm/min. The break loads observed and the mean compressive strengths are given in Table II. The decreasing particle sizes seemed to degrade the mechanical strength of the cement.

TABLE II

	100μ-less	**75-50μ**	**50μ-less**
1	404	309	172
2	314	310	211
3	357	311	325
4	313	222	219
5	326	190	290
Mean	342.8	262.2	243.4
Compressive Strength*(MPa)*	12.13	9.52	8.61

4.1.3 *HA particles seeding effects* : The cement setting is observed to be affected by additives. There are reports of adding hydroxyapatite to the cement powder to enhance the nucleation while setting reaction is progressing ('seeding technique'). Investigations are done to observe the seeding effect of hydroxyapatite particles (HA ceramic powder) in the cement. The effects on compressive strength and setting time were assessed. The setting time was not affected much by the HA addition. The effects both the concentration of particles in the cement powder and their particle size on compressive strength were investigated.

In the first part of the study, HA powder with particle sizes 150-250μ was added to the cement (75-50μ powder) in various percentages from 20 – 60 % w/w. Table III shows the corresponding break-load values of the samples in the compression test and average compressive strength. A decrease in strength is observed with the increase of HA content.

In the second part of the study, HA powder with various particle size ranges was added to the cement (75-50μ powder) in a fixed percentages of 20%. This is to see whether there is any significance to seeding particle size. Table IV shows the break-load values of the samples in the compression test and average compressive strength. The trend is not very clear, except the fact that the presence of HA decreases the strength of the cement.

TABLE III

	Cement alone	***Cement with 20% HA***	***Cement with 40% HA***	***Cement with 60% HA***
1	262	243	181	159
2	207	264	227	157
3	264	199	148	197
4	203	160	200	131
5	213	185	193	140
Mean	229.8	210.2	189.8	156.8
Compressive Strength*(MPa)*	8.10	7.43	6.71	5.55

TABLE IV

	Cement alone	***Cement with*** 150-250μ ***HA***	***Cement with*** 250-355μ ***HA***	***Cement with*** 355-500μ ***HA***
1	325	256	211	230
2	290	258	204	232
3	290	252	234	238
4	305	247	223	217
5	284	235	245	219
Mean	298.8	249.6	223.4	227.2
Compressive Strength*(MPa)*	10.57	8.83	7.91	8.04

The phase conversion was analysed in these samples using X-ray diffractometry, as done earlier. The reaction rate is observed to change only slightly.

4.1.4 *Modification of the wetting solution* : The wetting solution composition also affects the properties of the cement directly. In fact, there is not much choice in the wetting medium because it should be biocompatible, devoid of metal ions and must contain phosphate group. An attempt to modify the wetting medium was done, by preparing it in the simulated body fluid (SBF-K9 solution). Here also, the effects on

compressive strength and setting time were assessed. There observed a slight decrease in the setting time, but the effects were not drastic.

For the compressive strength measurements, the samples were prepared by using wetting medium in SBF. The accelerator (Na_2HPO_4) was dissolved in SBF at 0.1M and 0.2M concentrations, as well as in double-distilled deionised (DDI) water at 0.2M concentration and used as wetting media. The break-load of the samples along the axis was measured in Universal Testing Machine at a cross-head speed of 1mm/min. The values in each case are given in Table V, along with average compressive strength. A notable increase in the compressive strength was observed on using SBF.

TABLE V

	0.2M Na_2HPO_4 in DDI water	*0.1M Na_2HPO_4 in SBF*	*0.2M Na_2HPO_4 in SBF*
1	187	328	351
2	185	284	291
3	257	274	212
4	213	242	335
5	115	336	282
Mean	191.4	292.8	294.2
Compressive Strength*(MPa)*	6.77	10.36	10.40

4.1.5 *Porosity control* : One of the features which decides the *in vivo* performance of the cement, is the porosity. The micro-morphological studies did not reveal any connected porosity (Section 2.5). However, inter-particulate closed porosities are observed (Fig. 7a). It is reported that introduction of larger porosities (even closed porosities) will enhance the apposition with newly growing bone, *in vivo*.

An attempt is done to increase the porosity of the cement mass. This should be done without affecting the biocompatibility and without hampering the setting properties. Only way is to incorporate biocompatible, water-soluble particles in the powder part. Glucose grains were found a suitable candidate for the purpose. The rationale is that the particles will dissolve during setting, leaving gap at their positions.

The glucose grains were crushed, sieved and graded in various ranges from 100 to 355µ sizes. These were dry-mixed in the cement powder to a ratio of 50% by weight. The cement was mixed and solidified in the form of cylindrical pellets. They were washed in water in a shaker for 10h, cleaned ultrasonically and dried in oven overnight. The weight and dimensions of each pellet were measured, from which the bulk density is determined. The percentage porosity was calculated by comparing with the theoretical density of hydroxyapatite.

The values are given in Table VI. On the addition of 50% w/w glucose powder, the percentage porosity increases to 62% from the normal value of 52%. On increasing the glucose particle size to 300-350µ, the value went up to 67.5%.

TABLE VI

Particle Size	***Trial No***	***Pellet Length***	***Pellet Volume***	***Mass***	***Density***	***Mean Density***	***% Porosity***
Cement alone	i	11.20	0.3165	0.489	1.545	1.523	51.74
	ii	11.10	0.3137	0.472	1.505		
	iii	11.00	0.3108	0.472	1.519		
<100µ	i	10.72	0.3049	0.370	1.214	1.197	62.07
	ii	10.45	0.2953	0.354	1.199		
	iii	10.51	0.2970	0.350	1.179		
100 - 150µ	i	9.49	0.2597	0.295	1.136	1.136	64.00
	ii	9.95	0.2811	0.312	1.110		
	iii	10.55	0.2981	0.346	1.161		
150 - 180µ	i	8.34	0.2357	0.266	1.129	1.153	63.46
	ii	7.69	0.2173	0.255	1.174		
	iii	3.15	0.0890	0.103	1.157		
180 - 250µ	i	7.47	0.2111	0.244	1.156	1.196	62.10
	ii	8.66	0.2447	0.303	1.239		
	iii	8.84	0.2498	0.298	1.193		
250 - 300µ	i	9.23	0.2608	0.271	1.039	1.063	66.32
	ii	10.35	0.2925	0.319	1.091		
	iii	9.96	0.2815	0.298	1.059		
300 - 350µ	i	8.86	0.2504	0.260	1.038	1.025	67.52
	ii	3.60	0.1017	0.103	1.013		
	iii	5.60	0.1582	0.162	1.024		

4.2. Optimization of the cement formulation for orthopedic application

The studies establish a route to obtain a self-setting calcium phosphate cement of apatitic nature from TTCP+DCPD formulation. The characteristics of the cement (handling properties, setting time, strength, microstructure etc) were investigated and the influence of factors like particle size, wetting ratio and wetting medium composition were assessed. The next step is to optimize the design of the cement for clinical application. The cement is primarily designed as a bone filler in orthopaedic procedures. The main optimization parameters are workability, setting times and strength.

The handling properties of an aqueous cement are mainly decided by the particle sizes and the wetting ratio. The particle sizes of the ingredients (TTCP+DCPD) are already fixed to 100μ-less range, to have a putty consistency on wetting. The wetting ratio is selected to be 0.5ml/g considering the workability, as described earlier.

The setting characteristics and strength of the cement are controlled by the amount of accelerator added. The accelerator becomes inevitable because the inherent setting time of TTCP+DCPD combination is unacceptably long. The slow precipitation is due to the rate limiting nature of dissolution of the reactants, which prevents the supersaturation of PO_4 ions. The function of the accelerator is to impart PO_4 ions to the medium to create sufficient supersaturation. This speeds up the precipitation reaction, thereby bringing down the setting time. The effect of disodium hydrogen phosphate (Na_2HPO_4) addition on the initial and final setting times of the TTCP+DCPD cement can be seen in figure 2.

The manipulation of the setting times of CPCs is significant, as they should meet the requirements of surgical procedures. The initial setting time should be adjusted so as to allow sufficient time gap for shaping and filling. After the filling, it is not advisable to disturb the set cement till its hardening because any mechanical strain during this period will produce cracks and adversely affect the strength. Therefore it requires a shortest possible final setting time so that the wound closure is not delayed.

An initial setting time of about 8 min and a final setting time less than 15 min are recommended for orthopedic applications. However, bringing down the setting times by the addition of the accelerator affects the mechanical strength of the cement adversely, as evident from the compressive strength data (figure 3). The mechanical requirement of the cement intended for filling non-load-bearing areas is such that the compressive strength should be comparable to that of human trabecular bone, i.e. not less than 10 MPa.

Thus, for optimizing the cement, the accelerator concentration has to be selected judiciously, taking the setting time and mechanical strength into consideration. Following the data represented in figures 2 and 3, it was found that Na_2HPO_4 concentration of 0.2M in the medium would make the cement ideal for orthopedic filling applications. The phase conversion data presented in section 2.6 (i) (figure 9) also support the selection. The initial and final setting times of the cement at this concentration are 10 min and 15 min, respectively and the corresponding compressive strength is 11.15 MPa.

V. BIOCOMPATIBILITY EVALUATION

5.1 Selection of Tests

Critical biocompatibility evaluation is an essential step in the development of any material intended for biomedical applications. In essence, the biocompatibility tests should ensure, insofar as possible, the safety of the material. The material, when used in patients should not impose any unnecessary adverse or toxic stress to the patient. The overall aim is to provide the patient maximum benefit with minimum risk.

The biocompatibility evaluation of CPC has been done by a battery of tests recognized internationally, based on ISO Standard 10993 “Biological evaluation of medical devices”. All the tests involving animals were performed by taking prior permission from the Institutional Animal Ethics Committee, SCTIMST. The animal husbandry was in accordance with ISO 10993-2 and National regulatory requirements for laboratory animals, under the Quality Platform ISO 17025.

According to device categorization of ISO-10993; Section 4, CPC comes under "permanent contact bone implant device". The following specific tests have been selected as per section 5.2 of ISO-10993.

(a) Screening tests

1. Cytotoxicity
2. Haemlolysis

(b) Toxicological tests

3. Acute Systemic Toxicity
4. Intracutaneous (Intradermal) Reactivity Test
5. Pyrogen Test
6. Maximization Sensitization Test
7. Implantation in Muscle.

The screening tests checks the preliminary safety with respect to cells and blood. When the material passes these tests, further tests are done to assess the toxicological profile.

5.2 Screening Tests

The optimized cement was subjected to cytotoxicity and haemolysis screenings following the ISO/ASTM protocols, in order to qualify it for clinical application. 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 by autoclaving at 393K for 20min.

5.2.1 Cytotoxicity :

The cytotoxicity studies were carried out with mouse fibroblast cells (L929) in 'direct-contact method'. The medium used was Eagle's MEM with 10% fetal bovine serum. The cells were subcultured into monolayers and the samples were placed (in duplicate) over the cells. After an incubation of 24h at 310K under 5% CO_2, the cell morphology was analyzed under a phase contrast microscope (figure.10).

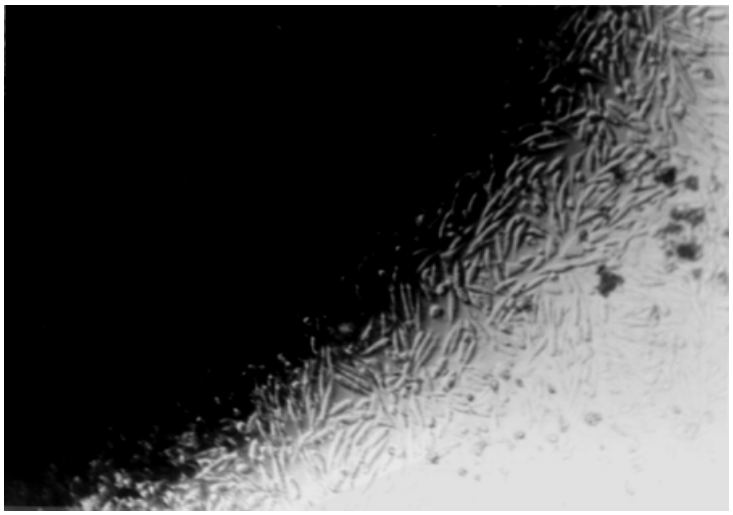


Fig.10. *Image of the cell-culture cyto-toxicity experiments. The mouse fibroblast cells in contact with the cement pellet are seen. Note the intact morphology of the cells .*

The cells in contact with the pellets showed the normal morphology as the cells cultured without any sample, proving that the cement is not toxic to cells.

5.2.2 *Haemolysis* :

The haemolytic potential of the cement was tested using fresh rabbit blood with potassium oxalate as anticoagulant. The sterilized cement pellets (5 numbers, each weighing 0.5g) were prepared in 5ml normal saline and incubated with 0.1ml blood for 90min at 37°C. The haemolysis was assessed after centrifuging, using a spectrophotometer, in comparison with the controls.

The absorbance (Optical Density) values at 545nm were measured and the percentage haemolysis was calculated as :

$$\% \text{ haemolysis} = \frac{\text{Mean of test OD} - \text{OD of negative control}}{\text{OD of positive control} - \text{OD of negative control}} \times 100$$

The percentage of haemolysis due to the cement is found to be 0.78, which is well below the toxicity range.

5.2.3 Conclusion of the Biocompatibility screening

CPC samples passed the screening tests of cell culture cytotoxicity and haemolysis. Thus the product qualifies for toxicological and pre-clinical evaluation.

5.3. Toxicological Studies:

5.3.1 Acute Systemic Toxicity

(i) Aim : To assess the systemic response of the extracts (normal saline and cotton seed oil) in mice, under protocol ISO 10993-11 (1993E).

(ii) Method : The extraction was done by treating 4 grams of the set cement (in pellet form) with normal saline and cottonseed oil, for 1 hour at 121º C. Then the test samples were removed and an aliquot of both were taken to check the pH. The extraction media alone was used as control. The procedures were done in aseptic conditions.

The test animal used was Swiss Albino Mice, in the weight range 17g – 23g, healthy and active. 10 mice (5 males and 5 female) were randomly selected for the test with each extract and a similar set was used as control. They were caged in polypropylene cages having steel top and paddy husk bedding, with 5 animals per cage. The mice were fed with potable water and commercial mice feed. They were put in fasting overnight prior to test and 3-4 hours post medication.

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.

(iii) Results : During the test observation period, all the animals were normal. No adverse effects or loss in body weight or mortality were observed. The test sample met the requirements specified in the protocol to pass the test.

5.3.2 Intracutaneous (Intradermal) Reactivity Test

(i) Aim : 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 : 1995E.

(ii) Method : The extraction was done as in the previous test. 4 grams of the set cement (in pellet form) were treated with normal saline and cotton seed oil, for 1 hour at 121º C, in aseptic conditions.

The test animal used was New Zealand white rabbit, adults with weight below 2kg. 3 healthy and smooth skinned animals were selected in order to apply both the test extracts and their controls to each of them. The rabbits were housed in anodized aluminium cages and were fed with potable water and commercial rabbit feed.

The animals were prepared by closely clipping the fur on the back. The skin was ensured free of mechanical trauma or signs of irritation. 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.

The observations were done for erythema and oedema, immediately after injection and at 24h, 48h and 72h and graded with scores specified in the standard. For each animal, all the scores were added for each test and 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 scores from all the animals for each 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.

(iii) Results : The material under test did not elicit any erythemic or edematous intracutaneous reactivity for normal saline extract. The cotton seed oil extract of the material is seen to have elicited a negligible erythemic and edematous response.

5.3.3 Pyrogen Test

(i) Aim : To test the presence of any pyrogenic substances of either endotoxin or non-endotoxin origin, in the sample. This was done by measuring the rise in temperature following the intravenous injection of the test solution, as per ISO 10993-11 (1993 E).

(ii) Method : The test extract was prepared by exposing a surface area of 480 cm^2 of the cement (by casting in sheet form) to a quantity of 160ml of sodium chloride injection (containing 0.9% NaCl). The treatment is done for 1 hour at a temperature of 37ºC. The liquid was then filtered with sterilized Whatman filter paper, into a pyrogen-free beaker.

The animals selected were New Zealand white rabbits, healthy adults with weight below 2kg. They were housed in anodized aluminium cages and were fed with potable water and commercial rabbit feed. The test animals were kept in isolated cages in an area of uniform temperature, free from disturbances, which is likely to excite them. They were climatised for 7 days at a controlled temperature 22±3ºC. The rectal temperature was measured daily, using a thermometer. Rabbit restrainer was used during the measurements. The criteria for the selection of the test animals were (i) the body temperature should not exceed 39.8ºC, and (ii) the daily variation in temperature should not be higher than 1ºC. 3 rabbits satisfying the criteria were selected.

The test animals were kept restrained during the experiment. The rectal temperature was recorded 30 minutes prior to the test, which is 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.

(iii) Results : None of the animals did show any abnormality during the experimental period. No rise in temperature was observed following the intravenous injection of the material extract. The material meets the requirements for the absence of pyrogens.

5.3.4. Maximization Sensitization Test

(i) Aim : To determine the potential for a substance or material under test to produce skin sensitization in the guinea pig, according to ISO 10993-10 :1995 (E).

(ii) Method : The extraction was done by treating 2 grams of the set cement (in pellet form) with 10 ml of physiological saline, for 24 hours at 70º C. 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 were 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. They were caged individually in

aluminium cages. The animals were fed with commercial feed and potable water supplemented with vitamin-C.

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 clipped intrascapular regions to the test group at a dose of 0.1 ml. The same dose of the control solution is 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. The dressings 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.

(iii) Results : The test and control animals did not show any abnormalities during the experimental period. The numerical grading for erythema and edema were found zero. Result of the study cleared that the physiological saline extract of the material does not elicit any skin sensitization potential in guinea pigs and suggest that the test material meets the requirements of the test.

5.3.5. Implantation In Muscle.

(i) Aim : To assess the biological response of muscle tissue to the material through implantation at time periods 1 week, 4 weeks and 12 weeks, as per The test is standard ISO 10993-6, 1994(E) Clause 5.

(ii) Method : 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 were 9 (3 animals per period, 3 periods). They were grouped into 3, housed in anodized aluminium cages and fed with potable water and commercial rabbit feed.

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 site 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.

(iii) Results : The important observations are given below.

At 1 week period : The general physical condition of all the experimental animals was normal. The increase in body weight and feed intake was normal during the

experimental period. None of the animal showed any abnormality or behavioral changes during the period.

12 test and 8 control implanted material was retrieved at the end of one week. There was no encapsulation, infection or necrosis around the implanted materials. Localized vascularization observed around the implanted (both test and control) material.

At 4 weeks period : The general physical condition of all the experimental animals was normal. The increase in body weight and feed intake was normal during the experimental period. None of the animal showed any abnormality or behavioral changes during these period.

All the 11 test and 8 control implanted material was retrieved at the end of 4 weeks. No encapsulation, hemorrhage or necrosis observed around the implanted material. Slight vascularization observed around the test samples.

At 12 weeks period : None of the experimental animals show any abnormality or behavioral changes during the 12 week implantation period. The increase in body weight and feed intake was normal during the implantation period.

11 test and 9 control implanted material was retrieved at the end of 12 week. There was no encapsulation, hemorrhage, infection or necrosis around the implanted material. The response of the tissue to the test implants was similar to that of control implants.

Summary : The histopathological reports indicated that there is a severe chronic inflammation present around the implant site in most samples at *one week* post implantation period. The chronic inflammation persists around the implant site at *four weeks* post implantation in some samples. There is a mild chronic inflammation and evidence of repair observed at *twelve weeks* post implantation period.

VI. BIOFUNCTIONALITY STUDIES

The biofunctionality tests are necessary to evaluate the efficacy of any biomaterial. For a bone cement, the major aspects to be investigated are the ability to bond with bone, the osteoconductive properties and the rate of *in vivo* resorption. As complete biofunctionality tests are elaborate and long, a full-fledged testing is beyond the scope of the project. Therefore, only *in vitro* cell culture studies are taken up in the present investigation. The rationale is to check the response of the human oseoblast (bone forming) cells to the material. This gives a preliminary estimate of the *in vivo* performance of the material.

6.1. Human Osteoblast Cell Culture :

(i) Aim : To conduct *in vitro* cell response and cell viability studies using human osteoblast cells in 'test on extract' method based on ISO 10993-5.

(ii) Method : Source of cell line used was NCCS Strain Human Osteoblast (HOS). This cell-line is an established and well characterised human osteoblast cell-line that has demonstrated reproducible results.

The culture medium used was MEM supplemented with foetal bovine serum. Ultra high molecular weight polyethylene was used as negative control and dilute phenol as positive control.

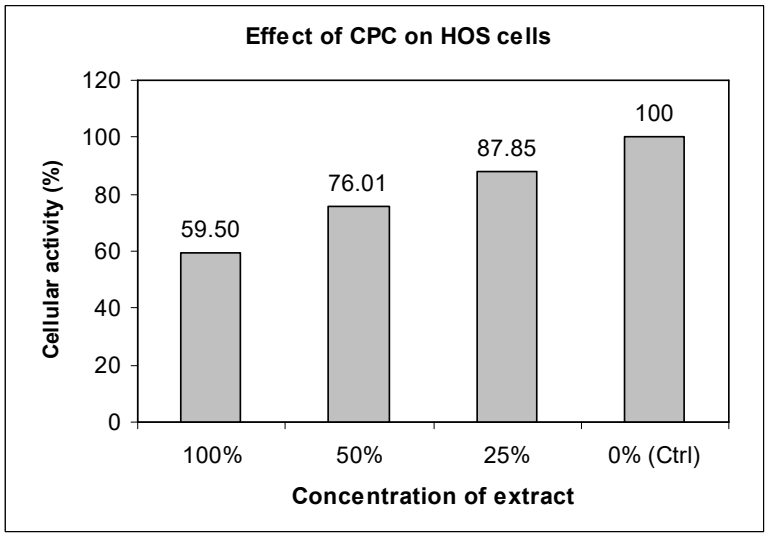
The cement samples were prepared by casting the mixed cement in the form of discs of 10mm diameter. Extract was taken by incubating test sample with media containing serum at 37°C for 24h at a ratio of 1.25cm^2/ml. 100% Extracts were diluted to get concentrations of 50% and 25% with media. Different dilutions of extracts of the test sample, negative control and positive control were added to subconfluent monolayer of osteoblast cells, in triplicate. After the incubation at 37 ± 2 °C for 24 ± 1 h, cell culture was examined microscopically for cellular response. Cellular responses were scored as 0, 1, 2, 3 and 4 corresponding to none, slight, mild, moderate and severe. Subsequently, in each case, the cell viability is assessed through MTT Assay. The average cell activity and percentage cell activity were calculated in comparison with a control cell culture, in MTT Assay method.

(iii) Results : The 100% extract of test sample CPC gave a none cytotoxic response to osteoblast cells. Extracts of negative control gave none cytotoxic response and positive control gave severe cytotoxic response, as expected.

Sample	***Cytotoxicity scale***	***Interpretation***
Negative Control	0	None
Positive Control	4	Severely Cytotoxic
CPC	0	None

On MTT assay, cells after contact with 100% extract showed a metabolic activity of 60% and 50% extract showed 76% activity, with respect to the control cells. The values are given as a table and bar-chart, below.

	Dilution of extract			***0 % (Control cells)***
	100%	***50%***	***25%***	
Trial - 1	0.059	0.057	0.046	0.067
Trial - 2	0.036	0.047	0.075	0.085
Trial - 3	0.05	0.081	0.102	0.104
Trial - 4	0.046	0.059	0.059	0.065
Avg cell activity	0.048	0.061	0.071	0.080
% Activity	59.50	76.01	87.85	100



VII. SUMMARY AND CONCLUSION

The studies on the development of apatitic calcium phosphate cement have been conducted as per the work plan submitted. A clinically relevant calcium phosphate cement formulation has been designed and developed.

Systematic investigation using various candidate ingredients provided the most suitable formulation, with TTCP and DCPD in the powder part and Na_2HPO_4 as the accelerator in the liquid part. The material properties of this cement (setting time, compressive strength, phase composition, micro-morphology etc.) were studied in detail. The various tests proved that the cement is ideally suitable for bone substitution applications. The basic formulation is optimized according to the clinical requirements.

The biocompatibility screening revealed that the cement material is non-cytotoxic and non-haemolytic. After passing the screening tests, the material has been subjected to systematic toxicological evaluation. A battery of tests has been done on the material, following the International Standard ISO-10993 (Acute Systemic Toxicity, Intracutaneous /Intradermal Reactivity Test, Pyrogen Test, Maximization Sensitization Test and Implantation in Muscle). The cement formulation was found to have no systemic toxic effects. None of the components contain any pyrogenic or allergic substance. The material was safe in intracutaneous implantation and it was found compatible with muscle tissue. Thus, the calcium phosphate cement formulation passes all the essential toxicological tests and qualifies for pre-clinical evaluation.

The efficacy of the cement formulation is checked in vitro using human osteoblast (bone forming) cells. The cells were found fully compatible with the cells. The reasonable cell viability seen in MTT Assay method ensures the success of the cement when implanted in live bone.

VIII. OVERALL ASSESSMENT AND FUTURE OUTLOOK

Calcium phosphate cements are considered as new-generation materials among synthetic bone substitutes, with notable advantages. In global perspective, only a few groups are involved in the development of this product. Some of the early versions of the CPCs are now available as commercial products in the West, but these are not being marketed in India. The use synthetic bone substitutes itself is very minimal in India, because of the cost factor of imported products. The calcium phosphate cement formulation developed in the project has technological, social and economical relevance.

The work done in the project provides the know-how of developing an apatitic calcium phosphate cement. The material satisfies the basic requirements of a mouldable and self-setting bone substitute and, obviously, is a candidate for several potential applications in the areas of orthopedics and dentistry. The biological evaluation shows the material has excellent biocompatibility. The biofunctionality studies ensure the efficacy of the material in clinical use.

A new-generation bone substitute is obtained, which will be a useful and viable biomedical product. However, this calcium phosphate cement formulation has to undergo few more steps before it's marketing. There are two major phases ahead – the pre-clinical studies in animals (long term bone-implantation) and the human clinical trials. The necessary steps are being taken to subject the cement formulation for further tests so that it could be brought from the laboratory to the patient.