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## **PROJECT COMPLETION REPORT**

1. **Project Number** : 7431  
2. **Title of the Project** : **Shell nacre integrated bioactive composite materials for bone defect treatment**

3. **Funding Agency Name** : ICMR

4. **Project Reference Number provided by the Funding Agency:** ICMR SRF 2017-3562

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8. **Collaborating Institutions** : NA

9. **Date of Commencement** : 22.10.2018

10. **Duration** : 4 years

11. **Date of Completion** : 21.10.2022

12. **Objectives as approved :**

### **To develop bioactive composites based materials**

- Synthesis and characterization of novel bioactive inorganic -organic hybrid resin.
- Modification of fillers to achieve better mechanical properties and

bioactivity

- c. *In vitro* and *in vivo* biocompatibility evaluation
- d. Preclinical evaluation in animal model

13. Deviation made from original objectives if any, while implementing the project and reasons thereof : NA
14. Field/Experimental work giving full details of summary of methods adopted, data collected supported by necessary tables, charts, diagrams and photographs :

#### I. PREPARATION AND CHARACTERIZATION OF SHELL NACRE:

Collected marine pearl oyster shells (*Pinctada fucata*) shells and characterized the surface morphology of shells by scanning electron microscope (SEM) analysis. Different methods were tried to remove the prismatic layer and optimized the mild acid treatment and confirmed by SEM analysis. Analysed the trace elements (Cd, Hg, Pb, Al, Se, Cu, Zn, Mg, Mn, Fe) of shell nacre powder by inductively coupled plasma – optical emission spectrometry (ICP-OES) and further characterized by Fourier Transform Infrared Spectroscopy (FT-IR), FT-Raman spectroscopy, Micro Raman spectroscopy, X-ray Diffraction and thermogravimetric analysis.

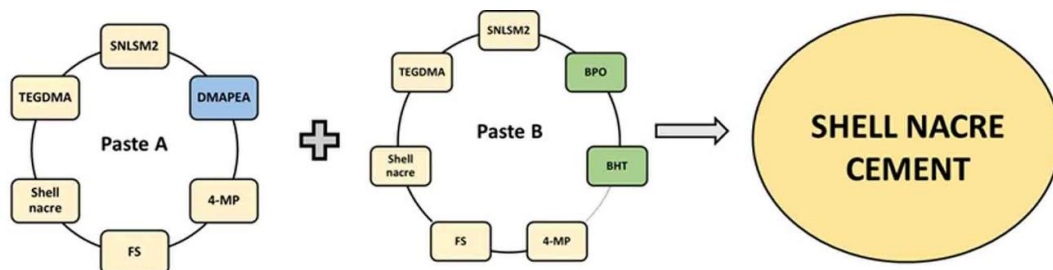


**Figure 1.** Processing of pearl oyster shells: (a) Shells of *Pinctada fucata*; (b) Soaking of shells in acetic acid and NaCl solution and (c) scrubbing the outer prismatic layer.

#### II. SYNTHESIS AND CHARACTERIZATION OF SNLSM RESIN:

Synthesized (0.1, 0.2, 0.5, 1, 2) shell nacre containing siloxane methacrylate resin by modified sol gel method. Characterized the resins by FT-IR, Gel permeation chromatography (GPC), High performance liquid chromatography (HPLC), Micro Raman, contact angle, differential scanning calorimetry, thermogravimetry, ICP-OES, X-ray photon spectroscopy (XPS),  $^{13}\text{C}$  and  $^1\text{H}$  NMR. Studied the bioactivity of cured shell nacre containing siloxane methacrylate resins cement of quartz filler by soaking in simulated body fluid for 0,1 and 7 days. The mineralization was confirmed by SEM-EDS analysis. Direct contact, MTT and cell adhesion of the photocured cement sample was studied.

#### III. PREPARATION AND OPTIMIZATION OF SHELL NACRE CEMENT:



**Figure 2.** Representation of the formulation of paste A and paste B which were mixed together to prepare the shell nacre cement.



**Figure 3** Preparation of shell nacre cement samples; Equal amount of paste A and paste B were mixed together and filled in the mold to get the cured samples of specific dimensions. Here the cured compressive strength samples were shown.

Bone void filling cements were prepared with different amount of shell nacre and studied radiopacity, porosity and compressive strength. Direct contact, MTT and cell adhesion of the cured cement samples were studied. Based on setting time, working time and compressive strength, optimized the formulation of chemical cure cement with different amount of DMAPEA, BPO, BHT and shell nacre powder. Studied the setting time and exotherm generation by isothermal DSC. Thermogravimetry analysis of the cured cement was carried out.. Studied cytotoxicity and cell viability of the cured shell nacre cement after 24 h, 48h and 72 h of curing with L929 cell line.

#### IV. BIOCOMPATIBILITY STUDIES

##### **Acute systemic toxicity:**

Sample SNC (100 nos.) of size 6X3 mm (diameter & height) (0.16g) were prepared, packed 2g per pack and sterilized by ethylene oxide. Sterile samples (16g) were submitted on to toxicology division, Sree Chitra Tirunal Institute for Medical Science and Technology for acute systemic toxicity evaluation based on ISO-10993-11: 2017(E) Annexure A 7 & 8 using the animal model mice.

##### **Animal intracutaneous (intra dermal) reactivity test**

Sample shell nacre cement (SNC) of size 6X3 mm (diameter & height) (0.16g) was prepared, packed 2g per pack, and sterilized by ethylene oxide. Sterile samples (16 g) were submitted to the toxicology division, Sree Chitra Tirunal Institute for Medical Science and

## Pyrogen test

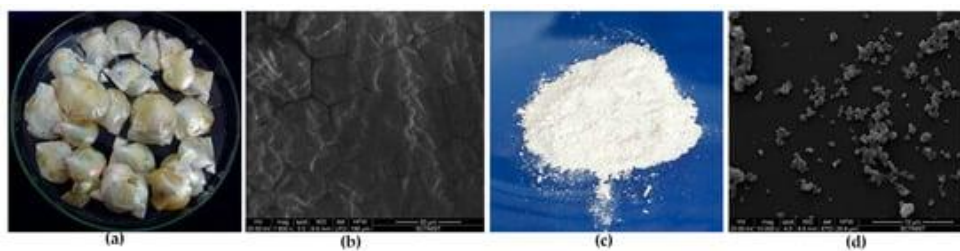
Sample SNC (200 nos.) of size 6X3 mm (diameter & height) (0.16g) was prepared, packed 2g per pack, and sterilized by ethylene oxide. Sterile samples (32 g) were submitted to the toxicology division for pyrogen test based on ISO 10993-11: 2017(E) Annexure G: Information on material mediated pyrogens using the animal model rabbit.

## IN VIVO OSSEOINTEGRATION

All experimental procedures and protocols were conducted as per the guidelines and recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India, and with the approval of the Institutional Animal Ethics Committee (IAEC), B Form No. SCT/IAEC-285/Sep/2018/97 extension dated 15.05.2021. Male Sprague-Dawley rats (390–510 g) of age 15-18 weeks were used for the implantation studies. After 6 and 12 weeks post-cortical femoral surgery, rats were euthanized and samples were collected for further analysis. The gross morphology analysis did not show any abnormalities. X-ray and microcomputed tomography analysis proved the osseointegration of the *in situ* set shell nacre cement samples when compared to the PMMA surgical simplex P samples.

### Processing of Shells

The first and foremost task of the present work was to process the shells of *Pinctada fucata* and to remove the outer prismatic layer. Since the objective of the present study was to obtain shell nacre powder with both an inorganic matrix and an organic matrix together, use of a mild acetic acid along with NaCl was attempted. This method enabled the faster removal of calcite with brisk effervescence, and further, manual scrubbing easily removed the outer prismatic layer. Subsequently, the lustrous nacreous shells (**Figure 4a**) were washed and dried. SEM observation of lustrous nacreous shells exhibited the characteristic brick-and-mortar structure of shell nacre (**Figure 4b**) where the aragonite crystals were glued together with the organic matrix, as seen by Sun and Bhushan [55]. The lustrous nacreous shells were ball milled and sieved to obtain shell nacre powder (**Figure 4c**). SEM analysis of the shell powder revealed an irregular morphology (**Figure 4d**). The obtained shell nacre powder was heterogenous with particle size smaller than 0.5  $\mu\text{m}$  (ImageJ analysis). In contrast, other studies have reported and used shell nacre powder of larger sizes, such as 50–100  $\mu\text{m}$  [50] and 42.5  $\mu\text{m}$  [51], for implantation studies.

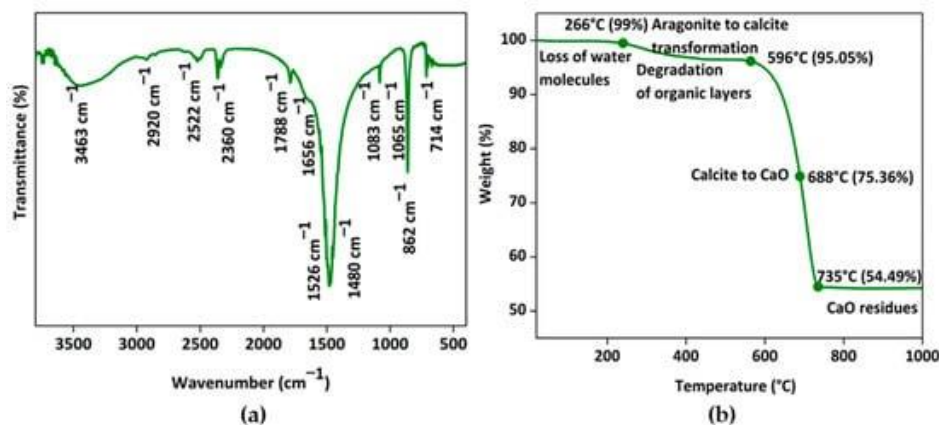


**Figure 4.** Processing of shells: (a) nacreous shells after prismatic layer removal; (b) SEM observation of nacreous shells; (c) macroscopic appearance; and (d) SEM observation of

shell nacre powder.

### 3.2. Characterization of Shell Nacre Powder

The obtained shell nacre powder was characterized by FTIR, TGA, Raman, XRD, and ICP-OES analysis. FTIR analysis of shell nacre powder is shown in **Figure 5a**. The presence of peaks for the internal vibration modes of  $\text{CO}_3^{2-}$  ions of calcium at  $714\text{ cm}^{-1}$ ,  $862\text{ cm}^{-1}$ ,  $1083\text{ cm}^{-1}$ , and  $1480\text{ cm}^{-1}$  and a splitting peak at  $714\text{ cm}^{-1}$  confirmed the aragonite form. The strongest peak of the spectrum was at  $1480\text{ cm}^{-1}$ , which was due to the overlap of peaks of the organic matrix and carbonate ions. The broad peak at  $3463\text{ cm}^{-1}$  was the stretching modes of OH/NH of the organic matrix or the adsorbed water molecules as reported [56,57,58]. A strong peak at  $2920\text{ cm}^{-1}$  was attributed to the CH stretching modes of the organic matrix. The peaks of OH of  $\text{HCO}_3^-$ -groups in the crystal lattice or at the mineral/organic interface groups of carboxylic groups were observed at  $2522\text{ cm}^{-1}$ , as well as stretching of carbonyl groups of acidic proteins at  $1788\text{ cm}^{-1}$ . Peaks at  $1656$  and  $1526\text{ cm}^{-1}$  were attributed to the amide I/amide II bonds of proteins of the organic matrix. A shoulder at  $1065\text{ cm}^{-1}$  was assigned to the C-O stretching of carbohydrates present in the organic matrix; further, the peak also indicated the  $\text{SO}_3^-$ -vibration of the sulphated glycosaminoglycans. Similar observations were recorded during the investigation of organic polymers of Venus clams [59]. It was reported that the organic matrix of shell nacre is comprised of the water-soluble matrix and the water-insoluble matrix. The water-soluble matrix contains the acidic proteins and polysaccharides, whereas the water-insoluble matrix contains the chitin, lipid, and the alanine and glycine rich silk-like proteins [60]. In the present study, the peaks of aragonite, proteins, carbohydrates, and water were observed, and the results were consistent with the findings of Balmain et al. and Zouari et al. during investigations of the organic content of the shell nacre of *Pinctada maxima* shells [56] and *Pinctada radiata* shells [61]. Thus, the FTIR study confirmed the presence of both organic and inorganic constituents in shell nacre powder.

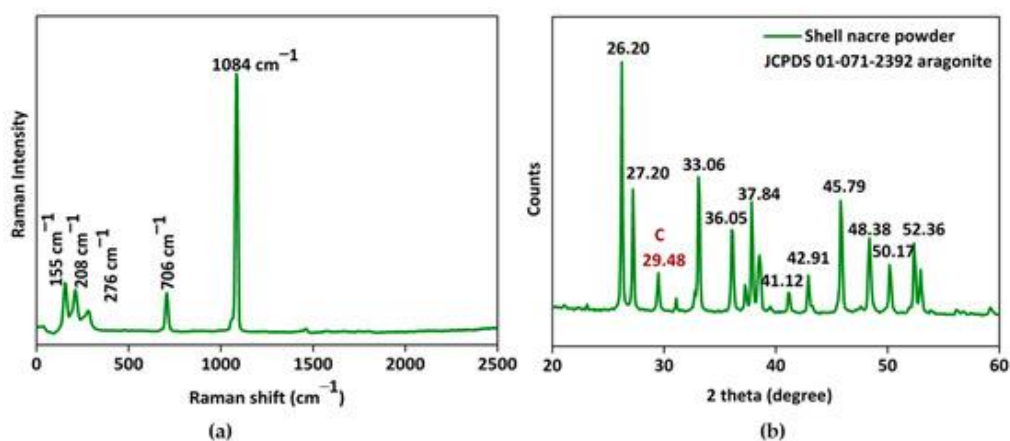


**Figure 5.** Characterization of shell nacre powder: (a) FTIR spectrum from 400 to  $3750\text{ cm}^{-1}$  and (b) thermogram from RT to  $1000\text{ }^{\circ}\text{C}$  confirming the presence of both organic and inorganic constituents.

The thermogram of shell nacre powder is shown in **Figure 5b**. The initial loss of water content was observed at  $266\text{ }^{\circ}\text{C}$ , which was followed by the degradation of organic content and the transformation of the aragonite to calcite at  $596\text{ }^{\circ}\text{C}$ . The gradual major loss (41%) occurred between  $596\text{ }^{\circ}\text{C}$  and  $735\text{ }^{\circ}\text{C}$  during the conversion of calcite to calcium oxide with

the release of CO<sub>2</sub>, leaving a final residue of calcium oxide at 1000 °C. As organic molecules are complexed with aragonite, the degradation of organic molecules occurred only at 596 °C. Once the organic content was lost, the aragonite crystal was transformed into calcite, and gradually the entire calcite was transformed into calcium oxide. Thus, the thermogravimetry changes proved that shell nacre powder was comprised of 5% organic and 95% inorganic content. Similar TGA results were reported by Balmain et al. and Zouari et al. during investigations of the organic content of the shell nacre of *Pinctada maxima* shells [56] and *Pinctada radiata* shells [61].

The micro-Raman spectrum (Figure 6a) exhibited the characteristic carbonate stretching vibrations of aragonite at 1084 cm<sup>-1</sup> and lattice vibration modes of aragonite at 155 cm<sup>-1</sup>, 208 cm<sup>-1</sup>, and 706 cm<sup>-1</sup>. The X-ray diffraction pattern (Figure 6b) of shell nacre powder matched with the aragonite crystals of JCPDS 01-071-2392 and had characteristic sharp diffraction lines of well-crystallized aragonite of single mineral phase. Both the Raman and XRD studies proved that the shell nacre powder contained an aragonite form of calcium carbonate. However, a calcite peak at 276 cm<sup>-1</sup> in the Raman spectrum and a calcite diffraction line at 29.48° in the XRD spectrum were seen, which may be due to the heat generated during the milling of shell nacre powder, due to traces of the external prismatic layer, or due to the presence of calcite in shell nacre itself [56,61,62].



**Figure 6.** Characterization of shell nacre powder: (a) micro-Raman spectrum and (b) XRD pattern confirming the aragonite nature of shell nacre powder.

**Table 1** presents the result of the trace element analysis of shell nacre powder. Copper was not detected, while manganese and zinc were present at very low levels (0.772 and 0.4827 ppm, respectively). Iron was detected at 5.889 ppm, and magnesium was found at a concentration of 102.87 ppm. Among the deleterious heavy metals, cadmium and selenium were below the detection limit, while lead and mercury were present at the levels of 1.4 ppm and 0.772 ppm, respectively. Further, the trace element analysis was concluded based on ASTM F2103-18 [63] and ASTM F1609-08 [64], which specify that the permissible limits of mercury and lead should be less than 5 and 30 ppm, respectively, and the total content of harmful heavy metals should be less than 50 ppm. In the present study, shell nacre powder contained mercury and lead, which were below the permissible limits, and the other heavy metals cadmium, selenium, and copper were below the detection limits. The total content of harmful heavy metals in the shell nacre powder was below 50 ppm. Therefore, the shell nacre powder obtained through the acetic acid-based method is suitable for use in biomedical applications, as it contains both organic and inorganic contents and is free of harmful heavy metals.

**Table 1.** ICP OES analysis of shell nacre powder

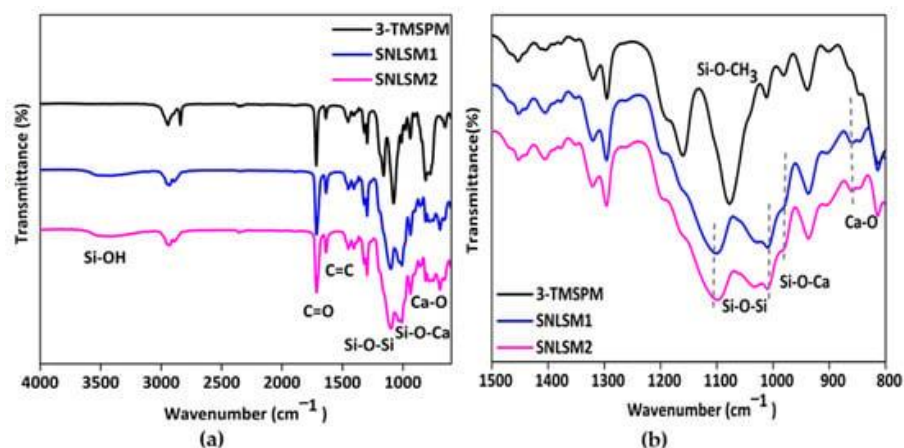
Elements analyzed	Total amount (PPM)
Cu	BDL <sup>1</sup>
Fe	5.889
Mg	102.87
Mn	0.772
Zn	0.4827
Cd	BDL <sup>1</sup>
Pb	0.772
Hg	1.4
Se	BDL <sup>1</sup>

<sup>1</sup> BDL Below the lower detection limit for Cu - 0.0097 PPM, Cd - 0.0027 PPM Se - 0.0750 PPM

### 3.3. Characterization of Resins

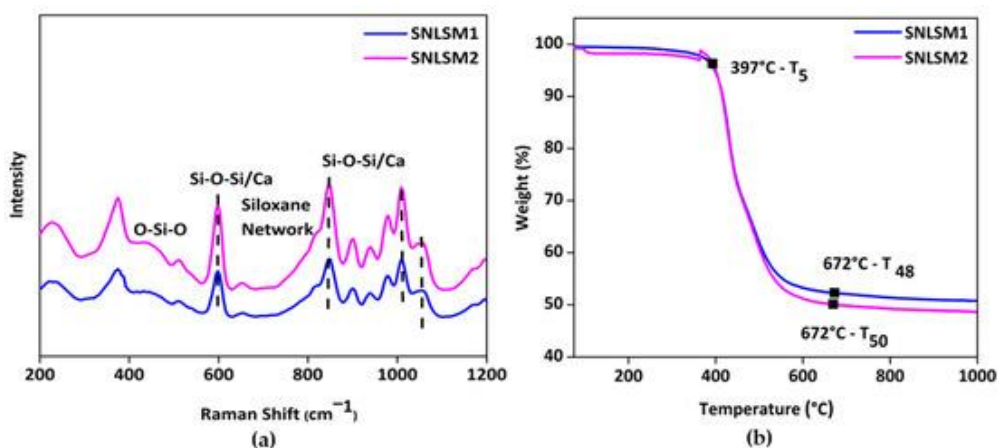
The next objective was synthesis and characterization of the shell nacre-containing siloxane methacrylate resins. During the synthesis, alkoxy silane underwent hydrolysis, NaOH favoured the condensation of the hydrolysed precursor, and a ladder-structured inorganic siloxane network with methacrylate side chain was formed. The synthesized resins showed no change in refractive index  $\sim 1.47$  after the addition of 1% and 2% shell nacre powder. Similar refractive index values were recorded for ormoresins [65], zirconium-containing resin [21], and strontium-containing resin [20].

The molecular architecture of the siloxane was identified by FTIR analysis, which reveals distinct peaks in the  $1000\text{--}1200\text{ cm}^{-1}$  region. A random structure is characterized by multiple peaks, a cage structure by a single sharp peak, and a ladder structure by bimodal peaks [66]. FTIR analysis (Figure 7a) of the resins proved the presence of bimodal peaks at  $1101\text{ cm}^{-1}$  and  $1033\text{ cm}^{-1}$ . This confirmed the polycondensation and formation of a ladder-structured inorganic siloxane backbone. It was reported that an alkali catalyst favoured the formation of ladder-structured siloxane, and the results were in agreement with previous studies [22,67,68]. A shift in the peak from  $980\text{ cm}^{-1}$  to  $987\text{ cm}^{-1}$  in both the resins confirmed the presence of Si-O-Ca in the network. Further, the presence of a peak at  $862\text{ cm}^{-1}$  (Figure 7b) corresponded to Ca-O in the siloxane network of SNLSM1 and SNLSM2, and the findings were consistent with previous studies [69]. Both the resins retained the characteristic acrylate groups C=C at  $1637\text{ cm}^{-1}$  and C = O at  $1716\text{ cm}^{-1}$ . Thus, the FTIR analysis confirmed that the formation of shell nacre integrated a ladder-structured siloxane skeleton with methacrylate side chain.



**Figure 7.** FTIR analysis of resins: (a) FTIR spectrum from 600 to 4000  $\text{cm}^{-1}$  and (b) FTIR spectrum from 800 to 1500  $\text{cm}^{-1}$  showing the presence of bimodal shaped peaks.

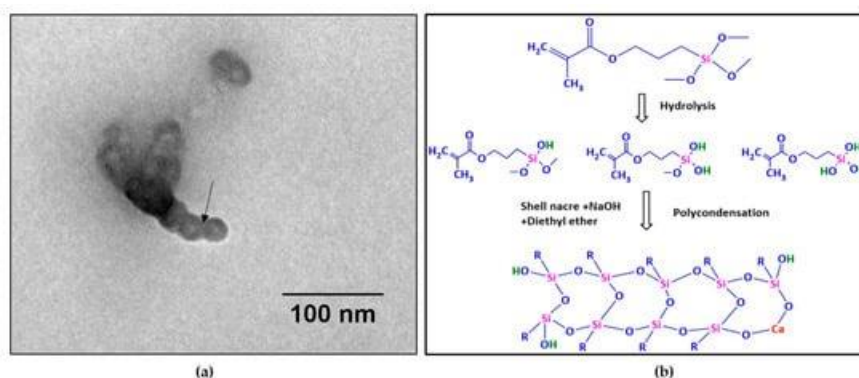
Raman analysis of the resins (**Figure 8a**) proved that the siloxane unit was four-membered, which was confirmed by the presence of a peak at 598  $\text{cm}^{-1}$ , and similar results were documented in a previous study [70]. Further, siloxane network formation was understood by a peak around 829  $\text{cm}^{-1}$  as reported by the Artaki group [71]. Again, the integration of shell nacre was confirmed by the shift in siloxane peaks at 598, 846, 1008, and 1058  $\text{cm}^{-1}$  in both the resins, and these findings were consistent with previous research [72].



**Figure 8.** Characterization of resins: (a) micro-Raman spectrum showing the formation of four-membered siloxane units and (b) thermogravimetric analysis confirming the intense siloxane backbone with shell nacre integration.

**Figure 8b** shows the thermogravimetric analysis of the resins from 25  $^{\circ}\text{C}$  to 1000  $^{\circ}\text{C}$ .  $T_5$  of both SNLSM1 and SNLSM2 was recorded at around 397  $^{\circ}\text{C}$ . SNLSM2 exhibited  $T_{50}$  (temperature at which 50% degradation occurred) at 672  $^{\circ}\text{C}$ , whereas no  $T_{50}$  was noted for SNLSM1, and it was left with  $\sim 52\%$  residue. Previous studies proved that ladder-structured siloxane methacrylate has an intense siloxane network [68] and high thermal stability [73]. TGA results of SNLSM1 were similar to an earlier study of ladder-structured siloxane methacrylate, which was also left with  $\sim 52\%$  residue [22]. This revealed that the addition of shell nacre had not modified the siloxane network of SNLSM1, whereas in the case of SNLSM2, shell nacre modified the siloxane network. Being a divalent, calcium modified the organic silica network, and the growth of siloxane was prevented after the integration of shell nacre [69].

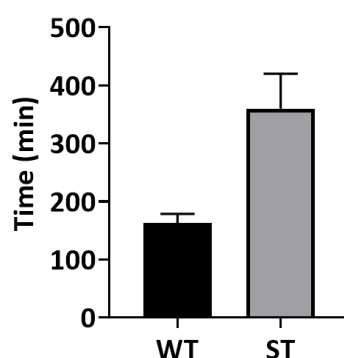
Further, TEM observation of SNLSM2 (**Figure 9a**) revealed the ladder-structured siloxane backbone of the resin. The four-membered siloxane unit was seen as small dots with interconnections between them. The interconnection was labelled with an arrow mark. The size of each siloxane unit was  $\sim 10$  nm (ImageJ). The ladder structure of SNLSM2 was smaller when compared with the previously reported ladder structure of LSM [22]. This reflected the change in the structure of SNLSM2 (**Figure 9b**) after the addition of shell nacre. As SNLSM2 evidenced the presence of shell nacre in all the above characterization studies, it was further taken for the cement formulation.



**Figure 9.** (a) TEM image of SNLSM2 resin (scale bar represents 100 nm); (b) scheme of synthesis of SNLSM2 resin.

### 3.4. Formulation of Shell Nacre Cement

Control of setting time, faster mixing, and enhanced mechanical properties are the advantages of the two-paste system. SNLSM2 (12%) was diluted with an equivalent amount of triethylene glycol dimethacrylate (12%) and formulated as a two-paste system with initiator, activator, stabilizers, fumed silica, and shell nacre powder. During the mixing of Paste A and Paste B, BPO along with DMAPEA promoted the polymerization of the organic network by breaking the C=C bond of the methacrylate moieties of the resin and TEGDMA, and the chain growth was continued. The amounts of BPO, DMAPEA, and BHT were optimized, and the working and setting times of the cement were 3 min and 6 min (Figure 10), respectively. The two pastes were mixed easily, and samples of specific dimensions were prepared according to the needs of experiments.



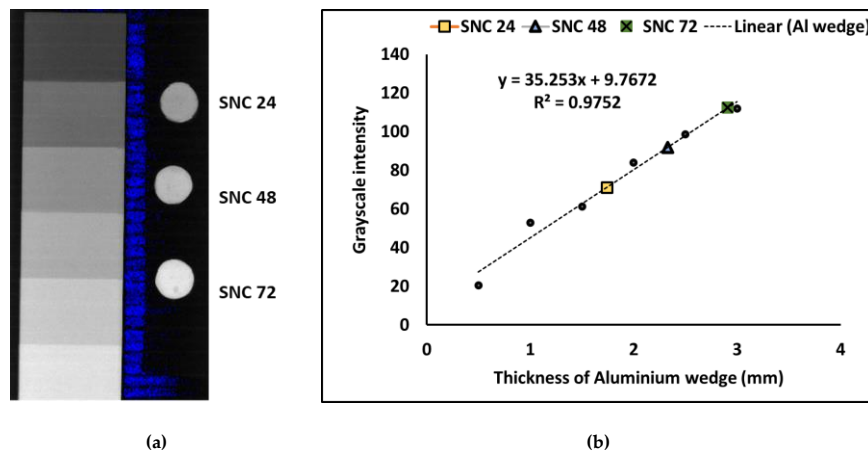
**Figure 10.** Working and setting time of the formulated cement

### 3.5. Characterization of Shell Nacre Cement

There is a need for a bone void filling cement with synergistic properties such as radiopacity, mechanical properties, non-cytotoxicity, low shrinkage, and minimal exotherm. To achieve this synergism, either the organic resin matrix or the filler part can be modified. In the case of our SNC cement, both strategies were employed by modifying the organic resin matrix with the ormocer SNLSM2 and introducing shell nacre powder in the filler part. Different cements were prepared by varying the amount of shell nacre powder (24%, 48%, and 72%), and the effect on the physicochemical properties such as radiopacity and LPS (%) and the mechanical properties of the cement was studied.

#### 3.5.1. Radiopacity Evaluation

Radiopacity is essential for the successful clinical monitoring of the material. Barium sulphate and zirconium dioxide are used as radiopacifier in the clinically available PMMA cement. However, their toxicity and effects on mechanical properties demand an alternative [74]. In the present study, shell nacre powder acted as a radiopacifier. The radiopacity values of SNC 24, SNC 48, and SNC 72 were 1.7, 2.3, and 2.9 mm equivalent to the thickness of the Al wedge (**Figure 11**). The radiopacity of the cement composition was increased with a higher concentration of shell nacre, and the inherent radiopacity of shell nacre conferred the cement compositions with radiopacity. Previous studies have reported that many other radiopacifier, such as triphenyl bismuth, tantalum powder, bismuth salicylate, iodine-containing co-polymers, bromine-containing co-polymer [74], and gold [75], were investigated as radiopacifiers of bone cement. However, none of them have been proved to be as osteogenic and multi-functional as shell nacre powder.

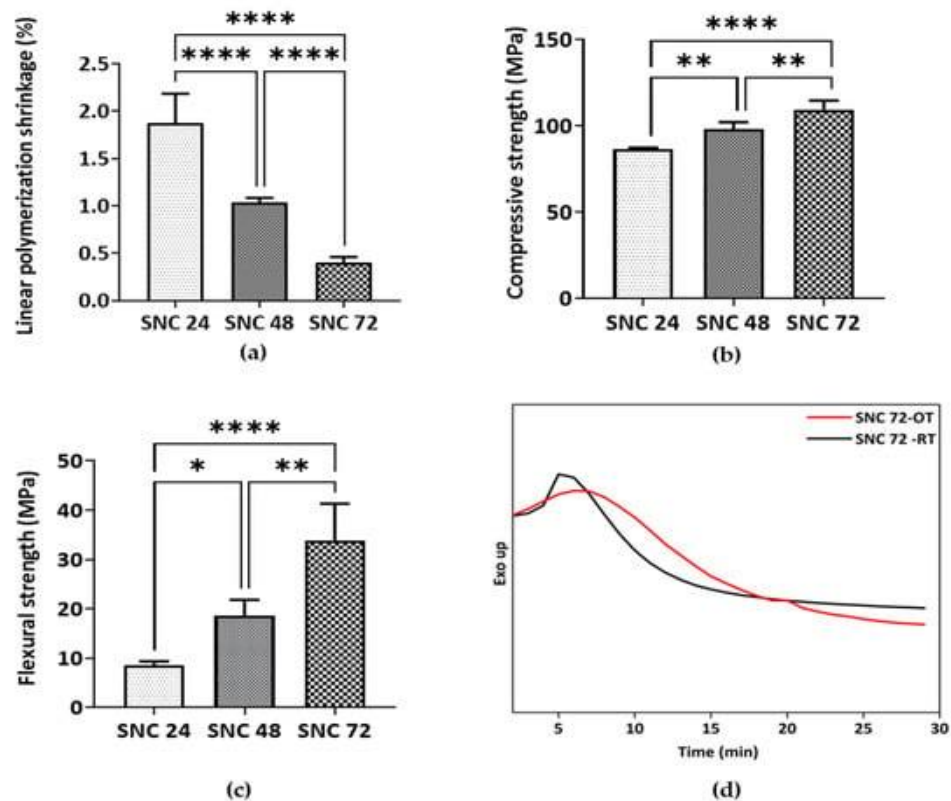


**Figure 11.** Radiopacity evaluation: (a) Scout images of shell nacre cement SNC 24, 48 and 72 with Aluminum wedge obtained using micro-computed tomography; (b) A standard curve was plotted with the thickness of each step of the Al wedge against the mean grayscale intensity of the Al step wedge. The radiopacity of the SNC samples equivalent to the thickness of the Al wedge was determined from the standard curve.

#### 3.5.2. Evaluation of LPS

The LPS (%) value of the compositions SNC 24, SNC 48, and SNC 72 was 1.8, 1.03, and 0.4% (**Figure 12a**), respectively. This indicated a reduction in the shrinkage with an increasing amount of shell nacre powder. Shrinkage of cement after polymerization is a major concern, and commercially available bone cements typically have a range of 5–10%

shrinkage [76]. In contrast, the shrinkage of SNC 72 was only 0.4%, which was significantly lower than the other cements. However, even the minimal shell nacre powder-containing cement SNC 24 had a value of 1.8%, which reflected the multi-functionality of SNLSM2. Therefore, the synergistic action of both SNLSM2 and shell nacre reduced the linear polymerization shrinkage of the SNC compositions.



**Figure 12.** Characterization of shell nacre cement: (a) Evaluation of linear polymerization shrinkage ( $n = 6$ ); (b) compressive strength ( $n = 4$ ); and (c) flexural strength ( $n = 4$ ) analysis of cured SNC 24, SNC 48, and SNC 72 samples. Ordinary one-way ANOVA. Tukey's multiple comparisons test. \*— $p < 0.05$ , \*\*— $p < 0.01$ , \*\*\*\*— $p < 0.0001$ . Results are shown with mean and standard deviation of the mean. (d) Isothermal DSC of SNC 72 at 24 °C (SNC 72-OT) and 37 °C (SNC 72-RT) showing the exotherm generated during the curing of SNC 72.

### 3.5.3. Evaluation of Mechanical Properties

SNC 72 exhibited average compressive and flexural strength of ~110 MPa (Figure 12b) and ~35 MPa (Figure 12c), respectively, which is significantly higher than the other cement compositions. Being an inorganic–organic hybrid filler, shell nacre was incorporated into the cement compositions without any silanization. Both the ormocer SNLSM2 and shell nacre contributed to the mechanical properties, which was understood by the increase in both compressive and flexural strength with increasing shell nacre content. Previous studies by Shen et al. and Du et al. reported that the compressive strength of calcium sulphate composites of oyster powder and abalone shell powder was only ~11 MPa [37] and ~5 MPa [40], respectively. Another study with calcium phosphate cement showed that an increasing concentration of nacre reduced the compressive strength [38]. In contrast, the compressive strength of the current SNC 72 composite was ~110 MPa, which was 10–20 times higher than the reported calcium sulphate composites, and the mechanical properties were enhanced

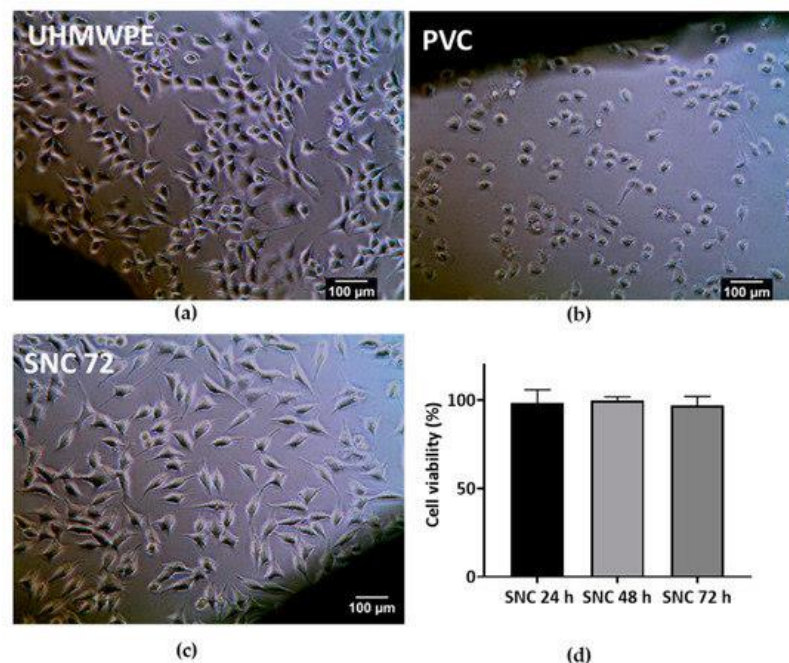
with increasing shell nacre content. A previous study by Wu et al. reported that addition of amphiphilic raspberry particles to PMMA reduced the compressive strength [77], and similarly, addition of magnesium oxide also reduced the mechanical properties of cement [78]. However, in the shell nacre cement compositions, the mechanical properties were improved by the bonding of shell nacre powder with the SNLSM2 resin. A study by Yang et al. found that use of acrylic acid and styrene instead of methyl methacrylate in PMMA cement made the compressive strength lower than that of PMMA cement [16]. In contrast, SNLSM2 imparted good compressive strength even to the cement with a low concentration of shell nacre powder (24 wt.%).

#### 3.5.4. Investigation of Exotherm Generated

SNC 72 exhibited better mechanical properties, radiopacity, and low linear polymerization shrinkage, and so SNC 72 was selected for further studies (isothermal DSC and cytotoxicity). As curing is affected by temperature, both the room temperature of the surgery room and the human body temperature were considered, and isothermal DSC was carried out at both 24 °C and 37 °C. The exotherm generated by SNC 72 was 2.227 J/g and 0.5781 J/g at 37 °C and 24 °C, respectively (**Figure 12d**). Although the heating rate and other conditions were similar, variations in enthalpy change  $\delta H$  were found owing to curing of SNC 72 at two different temperatures. A previous study reported that the exotherm generated by PMMA cement was 52 kJ/mole of PMMA, which was very high and was another major drawback of PMMA cement [10]. Another study reported no significant change in maximum polymerization temperature of Palacos cement after modification with 2-hydroxyethyl methacrylate, calcium chloride, and sodium carbonate [78]. Similarly, addition of graphene and graphene oxide powders to PMMA showed no changes in exotherm generation [79]. In contrast, the combination of shell nacre powder and SNLSM2 resulted in a reduction in the exotherm in the present study, which was very minimal when compared with the commercially available cements and other experimental cements.

#### 3.6. Cytotoxicity Studies

The cytotoxicity evaluation of SNC 72 was conducted in accordance with ISO 10993-5 using mouse fibroblast cell line L929. L929 cells exhibited no features of cytotoxicity, after contact negative control UHMWPE (**Figure 13a**), whereas the positive control PVC, showed severe cytotoxicity (**Figure 13b**) as expected. During contact with the cured sterile SNC 72 cement sample, L929 cells maintained the spindle morphology and no cytotoxicity features were observed as shown in **Figure 13c**. Both SNC 72 and the negative control were graded 0, and the positive control was graded 4. Cell viability of SNC 72 samples after 24 h, 48 h, and 72 h of curing was studied with L929 cells. After 24 h contact with samples, L929 cells showed more than 95% cell viability (**Figure 13d**). This again confirmed the non-cytotoxic nature of the SNC 72 samples. A previous study found that addition of increasing amounts of calcium sulphate (40% by weight) to PMMA cement resulted in a reduction in cell viability [80]. On the other hand, a higher addition of shell nacre (72% by weight) to SNC 72 did not cause any toxicity issues.



**Figure 13.** Cytotoxicity studies: Direct contact of (a) SNC 72; (b) negative control UHMWPE; and (c) positive control PVC with L929 cells for 24 h. Magnification 20 X. Scale bar represents 100 µm. (d) Cell viability assay of SNC 72 cement samples after 24, 48, and 72 h of curing with L929 cells.

In summary, *Pinctada fucata* shells were processed to obtain shell nacre powder, which consisted of 5% organic component and 95% inorganic aragonite. Further, the synthesized SNLSM2, which featured shell nacre, integrated a ladder-structured siloxane backbone and methacrylate side chain. Shell nacre cements were then formulated using different concentrations of shell nacre powder (24%, 48%, and 72%) and SNLSM2 (12%). Among the formulated cements, SNC 72 was selected for its low linear polymerization shrinkage (%), high radiopacity, and good mechanical properties. Importantly, SNC 72 was non-cytotoxic and exhibited a minimal exotherm. Overall, this study demonstrates the successful development of shell nacre cement and its advantageous properties.

The biocompatibility of SNC 72 was confirmed through acute systemic toxicity, animal intracutaneous (intra-dermal) reactivity test, and pyrogen test, which proved that the material satisfied the requirements of the test as per ISO 10993-parts10 and 11 standards. SNC 72 exhibited osseointegration during *in situ* setting in 2mm femoral cortical defect in rats. SNC 72 adhered firmly to the bone without any signs of inflammation and infection, whereas the clinical control PMMA cement showed inadequate adhesion. Overall, this study demonstrated the successful development of shell nacre cement and its advantageous properties

**15. Detailed analysis of results :** It is discussed above in 14

**16. Summary sheet of not more than 2 pages under following heads :**  
**(Title, Introduction, Rationale, Objectives, Methodology, Results, Translational Potential)**

## **Title**

Shell nacre integrated bioactive composite materials for bone defect treatment

## **Introduction**

The project's main aim is to develop an *in situ* setting bioactive bone cement based on shell nacre and inorganic-organic hybrid resin. This bioactive cement system can be used to fill any irregular bone voids of load-bearing sites. Generally, calcium phosphate cement and calcium sulphate cement are used for filling defects but the mechanical mismatch leads to the failure of this cement. In many clinical situations, inert PMMA bone cement is utilized for bone defect management due to the lack of suitable material for load-bearing sites. Reports of revision surgeries and amputations insisted on the dire need for bioactive osteoconductive biocompatible cement.

## **Rationale**

Shell nacre is an emerging bone substitute. It is the inner nacreous layer of pearl oyster shell. Shell nacre is composed of aragonite crystals with an organic layer inter-tiled between them. It is osteogenic, angiogenic, and biocompatible. Shell nacre or water-soluble extract of shell nacre is used directly as a bone substitute/bone void filler or mixed with blood. No processing method is reported to set and mold shell nacre powder into different anatomical forms.

## **Objectives**

- To develop a processing method to get shell nacre powder and characterization
2. Synthesis and characterization of shell nacre containing inorganic-organic hybrid resin.
- 3) formulation of shell nacre cement using the synthesized resin and shell nacre powder and its characterization
- 4) biocompatibility evaluation and 5) preclinical evaluation in an animal model

## **Methodology**

Shell nacre powder was processed from the shells of *Pinctada fucata* and characterized. Shell nacre containing ladder structured siloxane methacrylate resin was synthesized by modified sol gel method and then characterized. Shell nacre cements were then formulated using different concentrations of shell nacre powder (24%, 48%, and 72%) and SNLSM2 (12%). Among the formulated cements, SNC 72 was selected for its low linear polymerization shrinkage (%), high radiopacity, and good mechanical properties. Following the *in vitro* (direct contact, MTT assay and cell adhesion) and *in vivo* biocompatibility evaluation (acute systemic toxicity, irritation test and pyrogen test), osseointegration was studied in a 2mm femoral defect in Sprague Dawley rats.

## **Results**

Shell nacre powder was processed from the shells of *Pinctada fucata* using the acetic acid and NaCl method. Shell nacre powder comprised of both organic and inorganic constituents and it was found to be free of harmful heavy metals. Furthermore, it lacks cytotoxicity and exhibited cyto-compatible properties. shell nacre containing siloxane methacrylate resin was synthesized. FTIR, Raman, DSC, TGA, NMR of the resins proved that SNLSM2 comprised of ladder structured siloxane backbone and methacrylate side chain. TEM images exhibited the ladder structured siloxane backbone. SNLSM2 was transparent with medium viscosity of 144 Pa s and it was selected for bone void filling cement formulation. Shell nacre cements were then formulated using different concentrations of shell nacre powder (24%, 48%, and 72%) and SNLSM2 (12%). Among the formulated cements,

SNC 72 was selected for its low linear polymerization shrinkage (%), high radiopacity, and good mechanical properties. Importantly, SNC 72 was non-cytotoxic and exhibited a minimal exotherm. The biocompatibility of SNC 72 was confirmed through acute systemic toxicity, animal intracutaneous (intradermal) reactivity test, and pyrogen test, which proved that the material satisfied the requirements of the test as per ISO 10993-parts 10 and 11 standards. SNC 72 exhibited osseointegration during *in situ* setting in 2mm femoral cortical defect in rats.

### **Translational Potential**

- SNLSM2 holds tremendous potential for hard tissue restorative applications.
- Shell nacre cement has the potential to become a leading bone void filling cement for bone defect management.

### **17. Contributions made towards increasing the state of knowledge in the subject :**

- Acetic acid-based processing method was developed to obtain shell nacre powder from the shells of *Pinctada fucata* while preserving its inherent properties. The obtained shell nacre powder comprised of both organic (5%) and inorganic (aragonite) (95%) constituents and free of harmful heavy metals. Shell nacre powder lacked cytotoxicity and exhibited cyto-compatible properties.
- Shell nacre containing ladder structured siloxane methacrylate resin were synthesized. Spectroscopy and Thermal Spectroscopy and thermal studies revealed the formation of ladder structured siloxane backbone and intact methacrylate side chain. SNLSM2 played a significant role in achieving low shrinkage, improved mechanical properties and minimal exotherm generation of the shell nacre cement.
- Shell nacre cement is a novel bone void filling cement made of shell nacre powder and ladder structured siloxane methacrylate resin. It possessed both the desired physico-chemical and biological properties. SNC 72 exhibited low linear polymerization shrinkage, minimal exotherm generation, higher mechanical properties and non-cytotoxic nature. SNC 72 is biocompatible and satisfied the acute systemic toxicity, animal intracutaneous (intradermal) reactivity test, and pyrogen test as per the ISO 10993 part 1, 10 and 11. The *in situ* cured SNC 72 demonstrated osseointegration in the femoral defect of Sprague Dawley rats after 6 weeks and 12 weeks.

### **18. Conclusions summarising the achievements and indication of scope for future work :**

The study explored the potential of shell nacre, whose origin as a bone substitute date back to the Mayan age. Through careful research, a processing method was developed to obtain shell nacre powder while preserving its inherent properties. The obtained shell nacre powder comprised of both organic and inorganic constituents and free of harmful heavy metals. Moreover, it exhibited non-cytotoxic and cyto-compatible properties. SNLSM2 resin shows great promise for hard tissue restorative applications. Its unique properties of ladder structured siloxane backbone and intact methacrylate side chain provide added advantage of the resin. SNLSM2 played a significant role in achieving low shrinkage, improved mechanical properties and minimal exotherm of the shell nacre cement. SNC 72 is a pioneering bone void filling cement made of shell nacre powder and ladder structured

siloxane methacrylate resin. It possessed both the desired physico-chemical and biological properties. It surpasses the commercially available options and the experimental cements, due to its superior properties like low linear polymerization shrinkage, minimal exotherm generation, higher mechanical properties, non-cytotoxic nature, biocompatibility, osteogenesis and osseointegration.

Further research into the 3 D printability of the resin may enhance the versatility of the SNLSM2 resin. Further research and testing will be necessary to fully explore the potential and refine the formula of SNC 72 for clinical use. Additionally, examining the flow properties and formulating as an injectable cement will enhance the versatility of the cement. Future investigations into *in vivo* long term degradation studies, *in vivo* critical sized defects in osteoporotic models will provide even more evidence for the superiority of shell nacre scaffolds and cement.

**19. Science and Technology benefits accrued :**

**a. List of research publications with complete details :**

- 1. Jeyatha, W. B., Paul, W., Mani, S., & Lizymol, P. P. (2022).** Synthesis and characterization of ladder structured ormocer resin of siloxane backbone and methacrylate side chain. *Materials Letters*, 310, 131192.
- 2. Wilson, Bridget Jeyatha, and Lizymol Philipose Pampadykandathil.** "Novel Bone Void Filling Cement Compositions Based on Shell Nacre and Siloxane Methacrylate Resin: Development and Characterization." *Bioengineering* 10, no. 7 (2023): 752.

**b. Manpower trained on the project :**

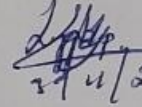
- |                                                   |            |
|---------------------------------------------------|------------|
| <b>i. Research Scientists or Research Fellows</b> | <b>1</b>   |
| <b>ii. No. of PhD's produced</b>                  | <b>1</b>   |
| <b>iii. Other Technical Personnel trained</b>     | <b>Nil</b> |
| <b>c. Patents taken, if any</b>                   | <b>Nil</b> |
| <b>d. Products developed, if any</b>              | <b>Nil</b> |

**20. Abstract: (In 300 words for possible publication in ..... Bulletin) NA**

**21. Procurement/Usage of Equipment:NA**

b. Suggestions for disposal of equipment(s): Not Applicable

Dr. Lizymol P.P

  
27/11/2025

(Name and Signature of PIs with date)

**Routing:** Signed copy of "Project completion Report" by PI → [root@sctimst.ac.in](mailto:root@sctimst.ac.in), [rpc@sctimst.ac.in](mailto:rpc@sctimst.ac.in)