Project Completion Report

For the entire period 06/12/2016 to 05/12/2019

Section-A : Project Details

A1. Project Title:

Scaffolds Based on Self-assembling Peptide Dendrimers and Resorbable Calcium Phosphates for Endodontic Tissue Regeneration

A2. Project Sanction Order No.: No.BT/PR14704/MED/32/498/2015

(DBT Sanction No.: 102/IFD/SAN/3025/2016-2017,Date : 06.12.2016)

A3. Date of Project Initiation : 06.12.2016

(If this date is different than date mentioned in A2, please clarify the reason)

A4. Date of project completion:Ongoing

A5(a). Name of Principal Investigator : **Dr. Manoj Komath**

A5(b). Name of Co-PI/Co-Investigator : **Dr. V. Haridas**

A6 (a). Institute (Co-ordinating) : Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum.

A6 (b). Institute (Collaboration) :Indian Institute of Technology (IIT), Delhi.

A7 (a).PI Address with Contact Nos. (Landline & Mobile) &Email :

Name : Dr. Manoj Komath
Designation : Scientist G
Department : Biomedical Technology Wing
Institute : Sree Chitra Tirunal Institute for Medical Sciences and Technology
Address : Satelmond Palace, Poojappura, Trivandrum
PIN : 695012
Telephone : 0471 2520220 (O) Fax :0471-2340819,2341814
9387414142 (Mobile); E-mail : manoj@sctimst.ac.in

A7 (b).Co-PI Address with Contact Nos. (Landline & Mobile) &Email :

Name : Dr. V.Haridas
Designation : Associate Professor
Department : Dept of Chemistry
Institute : Indian Institute of Technology, Delhi
Address : Hauz Khas, New Delhi
PIN : 110016
Telephone : 011- 26591380 Fax : 011-26582037
9650327587(Mobile); E-mail : haridasv@iitd.ac.in

A8. Total Approved Cost : Rs.7599200/-

(Rupees Seventy-five Lakhs Ninety-nine Thousand and Two Hundred only)

(SCTIMST, Trivandrum : **Rs.3492400/-**; IIT Delhi : **Rs.4106800/-**)

A9. Approved Duration : **3** years (36 months)

Dates : 06.12.2016 to 05.12.2019; Extension obtained up to 07.03.2020.

(Vide DBT Order No.: No.BT/PR14704/MED/32/498/2015, Dated : 04.02.2020)

A10. Rationale and background information of project

Regenerative Endodontics is the upcoming area in dental research which explores tissue regeneration strategies to save tooth with necrotic pulp and defective dentine. In principle it is possible to regenerate dentine and pulp, making use of stem cell population available in the peri-apical region. However, tissue engineering concepts did not work out satisfactorily in Regenerative Endodontics because of the problems in degradation and compatibility of the scaffold materials in the root canal space. This project work is conceived to find a workable solution for this problem, by exploring the compatible, resorbable scaffolds for the regeneration of defective dental tissues.

The main focus of this study will be on the development and evaluation of peptide dendrimers for endodontic tissue regeneration. Dendrimers are self-assembling macromolecules, which can form micro-level and nano-level architectures, and their surface and bulk properties could be tuned according to need. The hypothesis in this project work is that a dendrimer scaffold with fine-tuned characteristics can influence the differentiation and proliferation of progenitor cells at the host site leading to the regeneration of endodontic tissues.

A11(i). Approved Objectives of the Project:

The general aim of this project is to develop and evaluate novel tissue engineering materials based on cross-linked self-assembling peptide dendrimers and resorbable calcium phosphate, which can enhance the differentiation and proliferation of dental progenitor cells, so as to be helpful in the endodontic treatment of teeth with pulp and dentine defects.

The specific objectives of this project are:

1. To synthesize peptide dendrimers with acetylene foci or diacetylene cores and to fine-tune the gelation chemistry so as to obtain freestanding porous structures having biodegradability and biocompatibility.
2. To isolate and culture human dental pulp progenitor cells in the lab for the *in vitro* testing of the material and to differentiate them into osteogenic and odontogenic lineages.

3. To conduct *in vitro* cell culture of the peptide dendrimer compositions and resorbable calcium phosphate material with the human pulp progenitor cells, and to study their effects on the differentiation potential.
4. To optimize the selected peptide dendrimer by tuning the surface chemistry so that maximized cell response is achieved, which can lead to the regeneration of pulpal and dentinal tissues.
5. To evaluate the optimised peptide dendrimer scaffold *in vivo* in animal model by implanting in the endodontic space, and to assess the ability of regenerating dentin and pulp histologically in comparison with resorbable calcium phosphate material.

A11(ii).Details of approved Work Plan and Milestone

(Work Plan as given in the Summary of the Project)

In the opening part of the project, a few candidate structures of peptide dendrimers with acetylene foci or diacetylene cores will be synthesized as freestanding porous structures. These should essentially have the required biodegradability and biocompatibility.

The material is expected to support progenitor cells and their differentiation. Hence, before proceeding for specific *in vitro* evaluation, human dental pulp progenitor cells will be isolated and characterized. The cell characteristics will be evaluated by analyzing colony forming efficiency, expression of specific stem cell markers and differentiated phenotypes.

The influence of the candidate peptide dendrimer compositions on the differentiation and proliferation of the human pulp progenitor cells will be studied in detail. For comparison, resorbable calcium phosphate material will be used which is approved for endodontic usage. The results will be compiled to select the composition having the most favourable cell response. The selected peptide dendrimer will be further optimized by tuning the surface chemistry, so as to have the maximized cell response.

The optimised peptide dendrimer scaffolds will be evaluated *in vivo* in an animal model (dog model proposed) by implanting in the endodontic space, as per standard procedures. The ability of the material for regenerating dentine and pulp will be studied through histology, in comparison with resorbable calcium phosphate material.

The final deliverable will be an injectable scaffold material which could be placed inside the open apex root after removing the necrotic pulp. This novel, resorbable, porous matrix will host the local stem cell populations needed for the regeneration and help in their natural differentiation to appropriate lineages. The scaffold will be of tremendous use in endodontics to save the necrotic open apex cases.

(Milestones/ Targets as given in the Project Proposal)

Period of study	*Achievable targets/ milestones*	*Status (at the completion)*
6 Months	Synthesis of basic compositions of peptide dendrimers; Isolation of human dental progenitor cells.	Done in time (both targets)
12 Months	Confirmation of differentiation of progenitor cells; Completion of material cell interaction study.	Done in time (both targets)
18 Months	Optimization of peptide dendrimercomposition and calcium phosphate for implantation.	Done in time
24 Months	Using dendrimer to modify the surface of barrier membranes. Isolation of pulp-derived cells.	Done in time (both targets)
30 Months	Animal implantation of optimized peptide dendrimerand calcium phosphate composition for implantation.	Finished. Some procedural delay occurred.
Further progress		
36 Months	Animal implantation completion (additional samples included). Histological analysis of the sections.	Done as planned
3 Months Extension	Compilation of the animal implantation data. Preparation of papers and the final report.	Done as planned

A12. Specific Recommendations made by the Task Force (if any):

- Nil -

Section-B : Scientific and Technical Progress

B1. Progress made against the Approved Objectives, Targets & Timelines during the Reporting Period (1000-1500 words for interim reports; 2500-3500 words for final report; data must be included in the form of up to 3 figures and/or tables for interim reports; up to 7 figures and/or tables for final reports).

Given below, in pages 5-52.

B2. Summary and Conclusions of the Progress made so far (minimum 100 words, maximum 200 words)

Given in pages 53-58.

B3. Details of New Leads Obtained, if any:

Given in page 59.

B4. Details of Publications & Patents, if any:

Given in page 60.

B1.1. THE WORK DONE IN THE FIRST YEAR (IIT Lab)

B1.1.1 Background of work :

The first part of the project work is developing a scaffold materials based on cross-linked self-assembling peptide dendrimers, which can inhabit dental progenitor cells and enhance their differentiation and proliferation, so as to be helpful in the dental tissue regeneration.

The role of extracellular matrix (ECM) is decisive in progenitor cell migration, homing and differentiation, during routine development of an organism. Current strategies are directed to design and engineer ECM mimetic scaffolds for guiding the stem cells to particular lineages and further regeneration. Provision of cell adhesive motifs like the Arginine-Glycine-Aspartate (RGD) peptides is an approach towards developing ECM mimetic scaffolds. In the present work, ECM mimetic dendritic peptide matrix using guanidine, appended to polydiacetylene (G-PDA), for periodontal tissue regeneration. Guanidine forms the functional end group of the amino acid arginine, imparting a positive charge to this cell adhesive peptide. Provision of multiple dendritic guanidine groups in a rigid poly diacetylene matrix can improve cell adhesion and migration due to the arginine mimetic effect. The

efficiency of the biomaterial was evaluated by adhesion, spreading and differentiation of human periodontal ligament fibroblasts.

B1.1.2 Molecular design scheme of G-PDA :

The compound has been designed and synthesized as Lys-based diacetylene monomers **M1-M2** (**Scheme 1, as in Figure 1 A**). **M1** is non-guanidinylated lysine-based diacetylene, whereas **M2** is guanidinylated diacetylene. For this, first bis(Boc) lysine **1** was coupled with propargylamine in the presence of dicyclohexyl-carbodiimide (DCC) and N-hydroxysuccinimide (NHS) to give monoalkyne**2**. Then

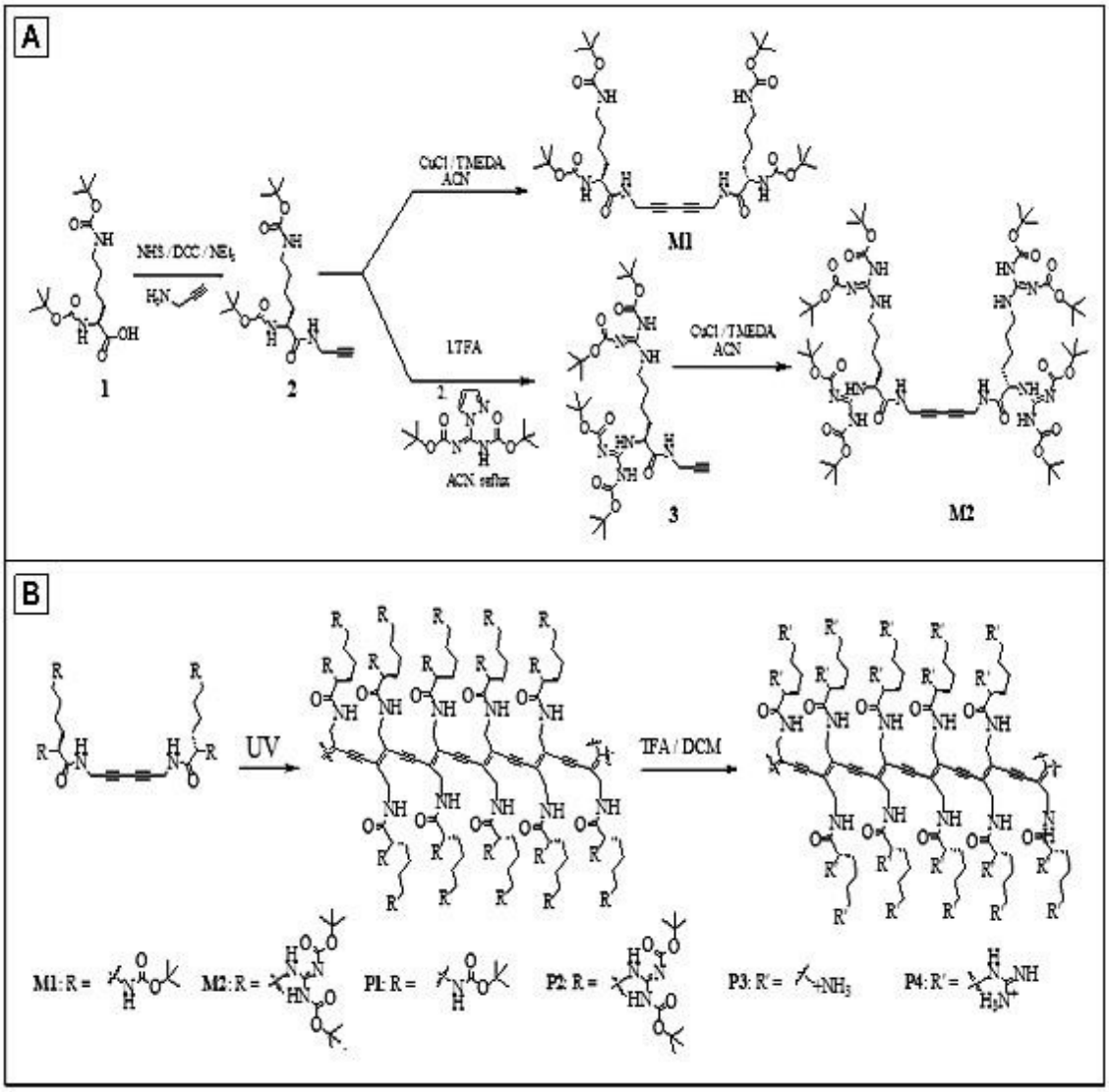


Figure 1. (A) Synthesis of monomers **M1-M2 (Scheme S1)**, (B) Synthesis of polydiacetylenes**P1-P4 (Scheme S2**).

the compound **2** underwent oxidation in the presence of *Hay catalyst* to give diacetylene**M1**.In order to synthesize the guanidinylateddiacetylene**M2**, the Boc-deprotection of **2** was carried out in the presence of trifluoroacetic acid (TFA). The subsequent coupling with 1-*H*-pyrazole-1-(*N*,*N*'-bis(*tert*-butyloxycarbonyl) carboxamidine of N-deprotected**2** yielded the diacetylene**M2** (**Scheme 1, as in Figure 1 A**).

Further the obtained monomers **M1-M2** were UV-irradiated to synthesize the corresponding PDA's **P1-P2** (**Scheme 2,as in Figure 1 B**). These polymers were water-insoluble due to the presence of hydrophobic Boc groups. Therefore, polymers **P1-P2** were deprotected using TFA to give water soluble PDA's **P3-P4** (**Scheme 2**).

B1.1.3 Synthesis and characterization

i) Synthesis of 2

To an ice-coldsolution of Bis(Boc)lysine**1** (1.000 g, 2.89 mmol) in 100 mL of dry dichloromethane was added N-hydroxysuccinimide (0.339 g, 3.47 mmol) and dicyclohexyl carbidimide (DCC) (0.716 g, 3.47 mmol) and stirred for 5 min. Propargylamine (0.22 mL, 3.47 mmol) and triethylamine was added to this and stirred for 24 h. The reaction mixture was filtered and washed with 0.2 N H_2SO_4, saturated aq. $NaHCO_3$, and water. The organic part was dried over anhydrous Na_2SO_4 and concentrated in vacuum to yield 0.980 g of the product**2**.

ii) Synthesis of M1

To a solution of **2** (0.100 g, 0.26 mmol) in 30 mL of acetonitrile was added *Hay catalyst* (a well-stirred solution of CuCl (10 mg, 0.10 mmol) and tetramethyl ethylene diamine (TMEDA) (0.033 mL, 0.22 mmol) in 5 mL of acetonitrile) and stirred under air. After 3 h, more *Hay catalyst* (5 mL) was added and left to stir for 12 h. Progress of the reaction was monitored through thin layer chromatography (TLC). After completion of reaction, the reaction mixture was evaporated, re-dissolved in dichloromethane, and washed sequentially with aqueous NH_4Cl + NH_4OH (9:1) solution, 0.2 N aq. H_2SO_4, saturated aq. $NaHCO_3$ solution and water. The organic part was separated, dried over anhydrous Na_2SO_4, filtered, and evaporated to yield 0.175 g of **M1**.

iii) Synthesis of 3

*(a) Deprotection of 2 :*To an ice-cold solution of **2** (0.224 g, 0.584 mmol) in dry CH_2Cl_2 (2 mL),2 mL TFAwas added and stirred at RT for 4 h. The reaction mixture was subjected to high vacuum to afford 0.106 g of the compound.

(b) Coupling of 1-H-pyrazole-1-(N,N'-bis(tert-butyloxycarbonyl) carboxamidine : To a well-stirred solution of deprotected**2**(0.100 g, 0.546 mmol) and *i*-Pr_2NEt (0.753 ml, 4.370mmol) in 10 ml acetonitrile, was added 1-*H*-pyrazole-1-(*N*,*N*'-bis(*tert*-butyloxy-carbonyl) carboxamidine (0.424 g,1.360mmol) and refluxed for 2h. After the completion of reaction as monitored by TLC, it was evaporated, re-dissolved in EtOAc and washed with water thrice. The organic layer was collected, dried over anhyd.Na_2SO_4 and evaporated to yield 0.186 g of the product**3**.

iv) Synthesis of M2

To a solution of**3** (0.700g, 1.048mmol) in 50 mL of acetonitrile was added *Hay catalyst* (a well-stirred solution of CuCl (51 mg, 0.524 mmol) and TMEDA (0.16 ml, 1.048 mmol) in 5 mL of acetonitrile for 5 min) and stirred under air. After 3h, more *Hay catalyst* was added and left to stir for 12h,monitoring the reaction in TLC. After completion, the reaction mixture was evaporated, re-dissolved in dichloromethane, and washed sequentially with NH_4Cl+NH_4OH solution (9:1), 0.2N aq.H_2SO_4, saturated aq.$NaHCO_3$ solution and water. The organic part was separated, dried over anhydrous Na_2SO_4, filtered and evaporated to yield 0.700g of **M2**.

v) Synthesis of P1 and P3

M1 was irradiated under UV lamp for 1-2 h to get the polydiacetylene**P1**.To a solution of **P1**(50 mg) in 1 mL dichloromethane was added 1 mL of trifluoroacetic acid (TFA) and stirred for 4h. Reaction mixture was evaporated under high vacuum toyield**P3**.

vi) Synthesis of P2 and P4

M2 was irradiated under UV lamp for 3 min to get the corresponding polydiacetylene**P2**.To a solution of **P2** (20 mg) in 1 mL dichloromethane was added 1 mL of TFA and stirred for 4h. Reaction mixture was evaporated under high vacuum to yield **P4**.

B1.2 THE WORK DONE IN THE FIRST YEAR (SCTIMST Lab)

B1.2.1 Background of the work :

The second part of the project work is testing the differentiation and proliferation of dental progenitor cells, so as to be helpful in the dental tissue regeneration. There are different sources for dental progenitor cells - human periodontal ligament is an easy and viable source. Primary cells isolated from periodontal ligament tissue consists of a heterogeneous population of fibroblasts, ligamental cells and a population of progenitor cells, each can be identified by characterisation with specific markers. The cell viability, adhesion and spreading on the **G-PDA** matrix will prove the cytocompatibility of the polymer, and its ability to sustain cellular activities. *In vitro* osteogenic marker expression is an indication of osteogenic differentiation of the cells, which ensures the local tissue regeneration including dentin and alveolar bone.

B1.2.2.Isolation of human dental progenitor cells :

i) Experimental plans

The approval from the Institutional Ethics Committee, Sree Chitra Tirunal Institute of Medical Science and Technology (SCTIMST), Thiruvananthapuram has been obtained for the study. Discarded extracted human permanent teeth for isolating cells were collected anonymously from Govt. Dental College, Thiruvananthapuram after obtaining the necessary approvals. The experimental plan consisted of isolation of human periodontal ligament cells (hPDL cells), their observation, the adhesion and spreading of the cells over the material surface and investigation of osteogenic differentiation on the material.

ii) Isolation and culture of human dental progenitor cells

The teeth were collected in Phosphate Buffered Saline (PBS) with 1000 IU Penicillin/Streptomycin and 25 µg Amphotericin B (Gibco). They were washed with PBS to remove the surface debris and then the periodontal ligament tissue was scrapped off and collected in a 35mm diameter tissue culture dish. These tissue fragments were treated with 0.25 % Trypsin for 5 min at 37 °C, washed with sterile

PBS and cultured in αMEM, with 10% FBS, 100 IU Penicillin/Streptomycin (Gibco) till cell outgrowths were noted. On achieving the confluent state, the cells were characterized and passaged. The confluent, adherent cells are detached using 0.25% trypsin (Gibco), at 37°C for 1 min and transferring the cells to fresh cell culture bottles, until sufficient number of cells is available for experiment.

iii) Characterisation of hPDLCs.

It is important to identify the heterogeneous population of cells (including fibroblasts, ligamental cells and progenitor cells) in the primary cells isolated from periodontal ligament tissue. Fibroblasts form the predominant cell population, and this can be identified by spindle-like morphology and expression of Vimentin, a type III intermediate filament protein. The ligamentous nature of the cells can be identified by the positive expression of scleraxis. The periodontal ligament consists of progenitor cells at different stages of differentiation with varying lineage commitments. They can be identified by the Mesenchymal Stem Cell markers like CD90, CD 105 and Stro1.

The hPDL cells at the first passage were characterized by Immuno-flourescence imaging using the markers Vimentin (for fibroblast) Scleraxis (for ligament)and Mesenchymal Stem Cell Markers CD90, CD105 and Stro 1.The hPDLCs were seeded onto 1cm^2 glass coverslips at 5000 cells/cm^2 for characterization. The cells were fixed at 48 h using 4 % paraformaldehyde (PFA) for 1 h. The cells were washed thrice with Phosphate Buffered Saline (PBS) and permeabilised with 0.1 % triton X100. Non-specific antibodies were blocked by treating the cells with 1 % Bovine Serum Albumin (BSA) for 10 min. the cells were incubated with primary antibodies for Vimentin, scleraxis, CD 90, CD105 and Stro 1 (Abcam primary antibodies), at 4°C overnight. Next day, the cells were washed thrice with PBS and incubated with the corresponding secondary antibodies for 1h at room temperature (anti-Rabbit Alexaflour 546 for scleraxis and anti-Mouse Alexaflour 488 for the others). The nucleus was stained with Hoechst 33258 (5 µg/ml) and the samples were viewed under the fluorescence microscope (Leica DMI 6000 Germany).

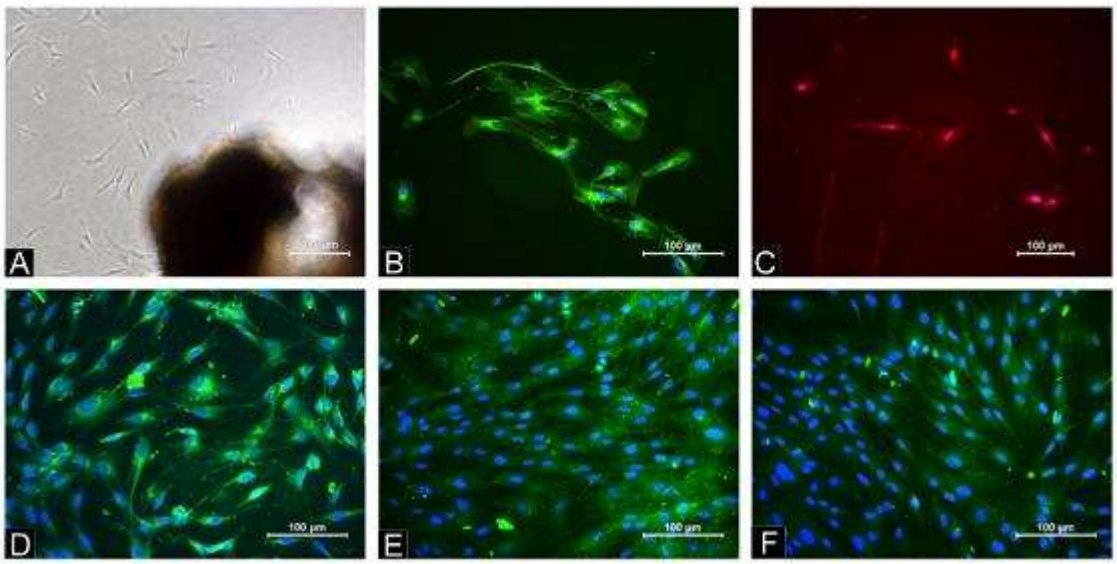


Figure 2: A) hPDL Cell outgrowth from tissue fragment kept as explant; B) Expression of intermediate filament Vimentin, characteristic of fibroblasts; C) Expression of ligament specific marker scleraxis; D) Expression of marker CD 90; E) Expression of marker CD105; and F) Expression of marker STRO 1.

The isolation procedure of using explant culture method to retrieve hPDL Cells resulted in cell outgrowth visible from 7 days onwards. Once confluent, these cells were sub cultured by normal trypsinization [Figure 2, picture A]. The Immunoflourescence staining of the first passage cells showed positive expression of the fibroblast marker vimentin, mesenchymal stem cell markers CD90, CD105 and Stro1 and the ligament specific marker Scleraxis. This confirms that periodontal ligament cells exhibiting fibroblast morphology consists of a population of progenitor cells similar to MSCs and also indicates the ligamental nature due to the presence of scleraxis. The presence of the type III intermediate filament protein Vimentin confirms the fibroblast nature of the cells with mesenchymal origin. Mesenchymal stem cells also exhibit a fibroblast morphology and were plastic adherent. CD 90 (Thy 1), CD 105 (Endoglin) and Stro 1 are specific cell surface proteins that are accepted Mesenchymal Stem Cell markers, the presence of which indicates a population of stem cells within the isolated hPDL Cells. [The results could be seen in Figure2, pictures B to F].

B1.2.3 Preparing G-PDA coated matrix for cell culture :

The prepared **G-PDA** dendrimer was dissolved in sterile deionised water at a concentration of 10 µg/ml. The solution was then drop casted onto sterile glass coverslips and 6 well tissue culture plates (Nunc, Thermofischer) as a thin matrix. Scanning Electron Microscopy (SEM) analysis and was done and images were taken (FEI quanta 200). Atomic force microscopy (AFM) evaluation was done using a scanner of 80 µm window (AGILENT SPM 500).

The samples were sterilized by ethylene oxide at 37°C before proceeding to cell culture. The uniform ECM mimetic matrix could facilitate cell viability, adhesion and spreading.

B1.2.4 Cell viability, adhesion and spreading on G-PDA :

The hPDLC response to G-PDA was analysed by a series of cytocompatibility tests. The hPDLCs at passage 2 were seeded onto the coated G-PDA matrix and cell adhesion and spreading was monitored at 24, 48, 72 and 96 h by phase contrast microscopy (Nikon) to identify any cell death or inhibition of growth. The hPDLCs on G-PDA matrix maintained their morphology and exhibited proliferation from day 1 to day 4 as seen by phase contrast images corresponding to 24, 48, 72, and 96 h. There is no evidence of cell death and a gradual increase in cell number can be seen, from 24 to 96 h, similar to the control. This revealed the absence of any cytotoxic dissolution products from the peptide coating. [Figure 3].

The viability of cells grown on G-PDA was assessed using 'live-dead imaging' with fluorescein diacetate (FDA) and propidium iodide (PI), at 24 h and 48 h. The culture medium was discarded and the cells were treated with FDA (5 µg/ml in serum free αMEM) for 10 min and PI (0.5 µg/ml) for 1 min and observed under fluorescence microscope (Leica). The live cells will uptake Fluorescein diacetate and convert it into green fluorescing fluorescein. PI is taken up by the nucleus of dead cells and appears red. The live/ dead imaging showed that the majority of cells were viable [Figure 4]. Very little dead cells were present in the G-PDA matrix, similar to the coverglass control. This confirms the absence of any toxicity or toxic products from the matrix.

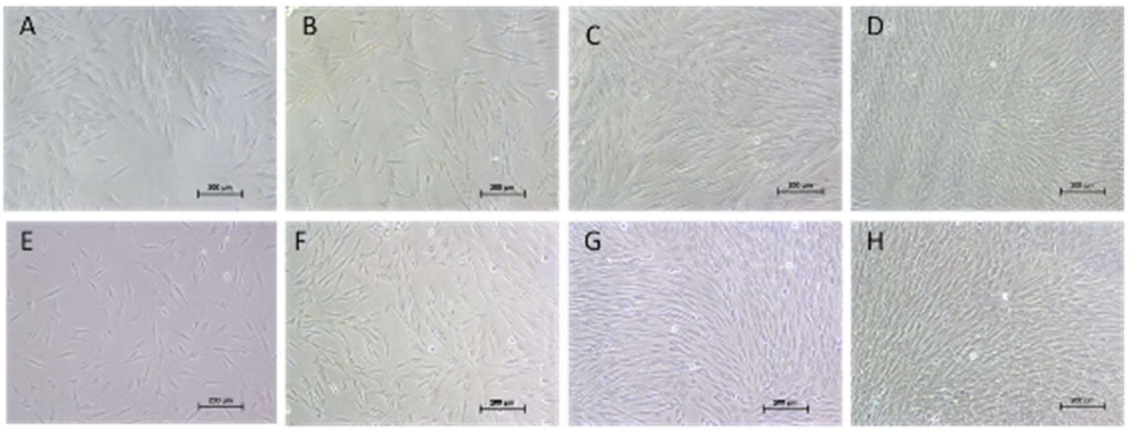


Figure 3: Pictures A,B,C and D : Phase contrast microscopic images of HPDL cells seeded onto G-PDA coated coverglass at 24h, 48 h, 72h and 96h respectively showing good cell spreading, the cells retain their morphology and there is no evidence of toxicity. Pictures E,F,G and H :hPDLCs in untreated glass coverslips at 24, 48, 72, and 96h (control).

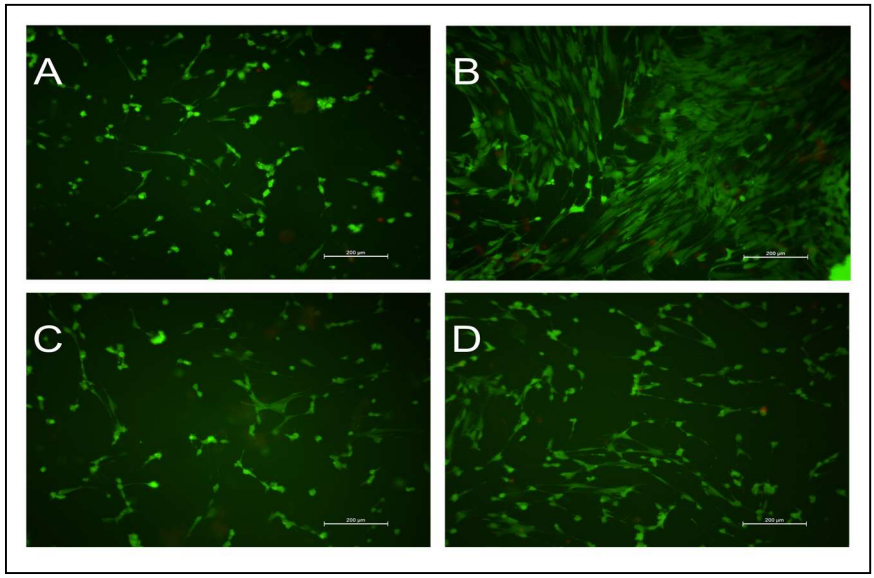


Figure 4 : PicturesA,B : FDA/PI live dead images of hPDLCs in G-PDA matrix at 24h and 48h respectively. Pictures C,D : FDA/PI live dead images of hPDLCs in glass coverslips (control) at 24h and 48h respectively.

The cytoskeletal organisation of the cells is an important indicator of maintenance of cell morphology and active cell spreading in the G-PDA matrix. This was analysed by staining actin cytoskeleton with Actin green 488 (Molecular probes, Thermo Fischer Scientific). The cells were fixed at 48 h with 4 % PFA for 1h, washed

thrice with PBS, and permeabilised with 0.1 % Triton X100 for 3min. The cells were incubated in the dark with Actin green 488 for 30min, after which, the cells were washed with PBS and nucleus was counterstained with Hoechst 33258 (5 µg/ml).

The cell spreading was further evaluated by SEM so as to visualize the lamellipodia and even minute filopodial extensions, which may not be evident from Actin cytoskeletal staining alone. The cells were cultured in G-PDA-coated and uncoated coverglass for 48 h, and fixed with 2.5 % gluteraldehyde for 1 h, washed five times with PB solution (0.2M disodium hydrogen phosphate and sodium dihydrogen phosphate in deionised water), and serial dehydration was done with ascending concentrations of ethanol and isoamyl acetate. The samples were dried in a critical point dryer and sputter coated with gold, before imaging.

The actin cytoskeleton staining showed a more elongated morphology of the cells on the G-PDA matrix, with cellular communications[Figure 5 A,B]. Cell spreading and migration is mediated through cytoskeletal extensions. SEM images confirm the presence of lamellipodia, and filopodial extensions, suggestive of active cell migration [Figure 5 C,D].

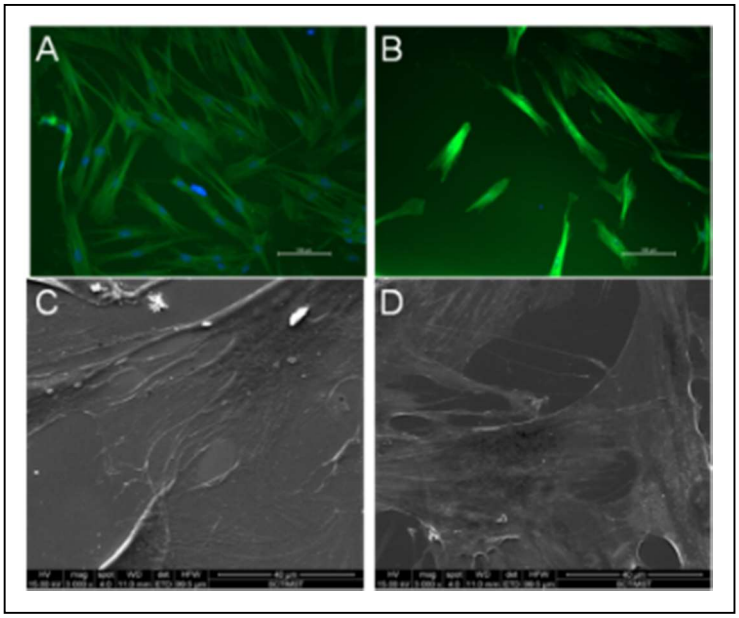


Figure 5 :PicturesA, B - Actin cytoskeleton staining of hPDLCs cultured in G-PDA and glass coverslips at 48 h respectively. Pictures C, D - SEM images of hPDLCs cultured in G-PDA and glass coverslips at 48 h respectively.

In two dimensional culture system, cells form lamellipodia, that attaches firmly to the substrate to provide traction for movement through filopodial extensions to the predetermined direction of movement. The polymer G-PDA was found to be cytocompatible, and support the cell adhesion and spreading. The guanidine groups at the ends and hydrophilic nature of G-PDA can enhance cell spreading. The presence of two guanidine groups makes G-PDA an electropositive matrix. The cell membrane of mammalian cells, being negatively charged, can electrostatically adhere to the G-PDA matrix. Also, it is plausible that the guanidine containing dendritic polymer might be mimicking the RGD peptide to some extent.

B1.2.5 Osteogenic differentiation of hPDLCs on G-PDA :

The osteogenic differentiation of the progenitor cells is an important parameter as far as dental tissue regeneration is concerned. The scaffold material should be capable of providing appropriate niche for osteogenic differentiation so that the regrowth of dentin, cementum and alveolar bone can occur. Therefore the steogenic differentiation of hPDLCs on G-PDA has been investigated.

The hPDLCs in passage 3 were used for the experiment. The cells were trypsinized and seeded onto glass coverslips and G-PDA matrix at a cell density of 10^4 cells/$mm^2$and was cultured in an osteogenic medium of αMEM containing 10 % FBS, 100 IU Penicillin Streptomycin; 50 µg /ml L Ascorbic acid, 10 mM sodium β Glycerophosphate and 10 nM Dexamethasone. The hPDLCs cultured in regular medium were used as control.

In order to identify osteogenic differentiation of the hPDLCs *in vitro*, histocytological evaluation was performed. Biomineralization, the deposition of calcium phosphates, is analyzed by staining using Alizarin red, a calcium chelating dye. The bound dye is solubilised and assessed colorimetrically, to quantify the mineralization. This is supplemented by Von Kossa staining with silver nitrate, which reacts with phosphate ions and get reduced to metallic silver under UV irradiation, giving a brown/black staining. The collagen matrix formation is also an important step in biomineralization, which is evaluated by the Masson's Trichrome staining that stains the collagen fibres turquoise blue.

i) Alizarin Red staining : The presence of calcium deposits, can be identified by staining the cell with Alizarin red. The mineralized nodules containing calcium will be stained red, retained after repeated washing. The hPDLCs in the osteogenic differentiation experiment were fixed at 7, 14, and 21 days with 4% PFA for 1 h, washed with PBS and then with deionised water, three times for 5 min each. Freshly prepared 2 % aqueous solution of alizarin red at pH of 4.2 was added to the cells and incubated for 20 min. After 20 min, the cells were washed with deionised water to remove the unbound dye. The cells were viewed and imaged in bright field mode of inverted phase contrast microscope (Nikon Digital Sight Fi2, Japan)[Figure 6].

Typical calcified nodules were evident at 14 and 21 days for periodontal ligament cells in osteogenic induction medium, in the presence and absence of **G-PDA**. However, the mineralization in peptide dendrimer coated samples was more, compared to the uncoated samples. The hPDLCs in regular medium did not show evidence of mineralization in the presence or absence of the dendrimer [Figure 6].

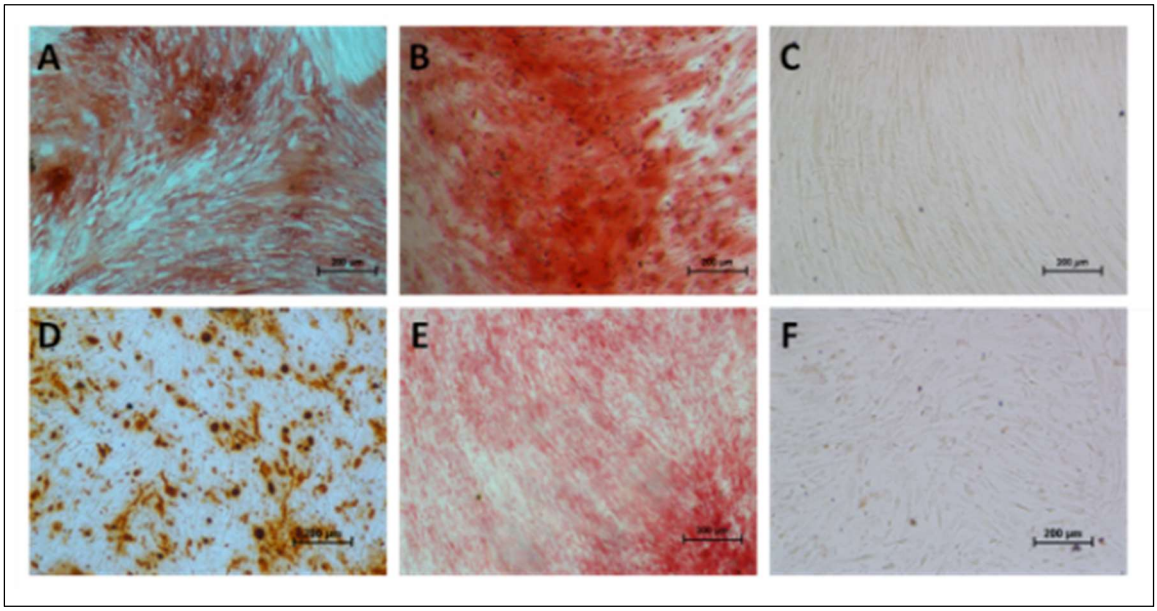


Figure 6: Images of Alizarin red staining : Pictures A,B – hPDLCs cultured in osteogenic medium on G-PDA at 14 and 21 days respectively; Picture C - The cells cultured in regular medium, in G-PDA. Picture D,E – hPDLCs cultured in osteogenic medium on glass cover slips at 14 and 21 days respectively (positive control), Picture F- hPDLCs in regular medium, glass cover slips (negative control).

ii) Von KossaStaining : The reaction of silver with phosphate ions in staining mark the presence of phosphate deposition. The hPDLCs in the osteogenic differentiation experiment were fixed at 7, 14, and 21 days with 4 % PFA for 1 h, washed first with PBS and with deionised water, three times for 5 min each. Freshly prepared 2 % aqueous solution of silver nitrate was added to the cells and UV irradiated for 30 min. After 30 min, the cells were washed with deionised water; the cells were viewed under bright field microscope, and images were taken [Figure 7].

The Von Kossa staining showed evidence of phosphate content as brown/black deposits, qualitatively higher than that in positive control. The observation is in complimentary with the Alizarin red data given in section 2.5.1, confirming the presence of calcium phosphate deposits in the G-PDA group. This may be attributed to the presence of basic guanidine groups, in the matrix, promoting the mineralization. The electropositive guanidine groups can also bind to anions like phosphate groups, resulting in mineralization [Figure 7].

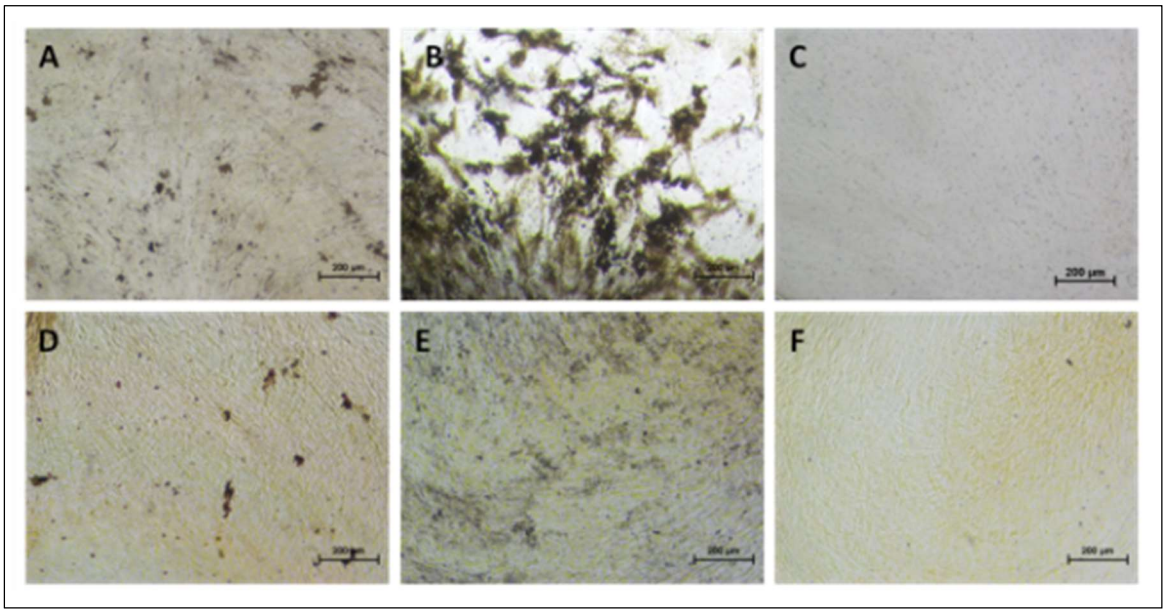


Figure 7 : Images of Von Kossa Staining of HPDL cells: Pictures A,B – hPDLCs cultured in osteogenic medium on G-PDA at 14 and 21 days respectively; Picture C - The cells cultured in regular medium, in G-PDA. Picture D,E – hPDLCs cultured in osteogenic medium on glass cover slips at 14 and 21 days respectively (positive control), Picture F- hPDLCs in regular medium, glass cover slips (negative control).

iii) Masson's Trichrome Staining **:** This staining technique helps in identifying collagen deposits during the culture of progenitor cells. The hPDLCs were fixed at 7, 14, and 21 days with 4% PFA for 1 h, washed first with PBS and then with deionised water, three times for 5 min each. The cells were treated with freshly prepared Bouin's solution and incubated for 1 h. The cells were washed with deionised water and the nucleus was stained with Hematoxylin for 5 min. After washing the cells were stained using Masson's Trichrome stain kit (Sigma), following manufacturer's instructions [Figure 8].

Typical bluish green stained collagen deposits were present in hPDLCs cultured in osteogenic medium on G-PDA at 7, 14 and 21 days, similar to the positive control. Deposition of a collagenous matrix is a natural function of periodontal ligament cells, especially, fibroblasts. Mineralization usually succeeds or occurs concurrently with collagen deposition. The presence of collagen deposition as well as the formation of calcium phosphate nodules indicate that the progenitor cells (hPDLCs) has the capacity to lay dentin and bone to repair defects [Figure 8].

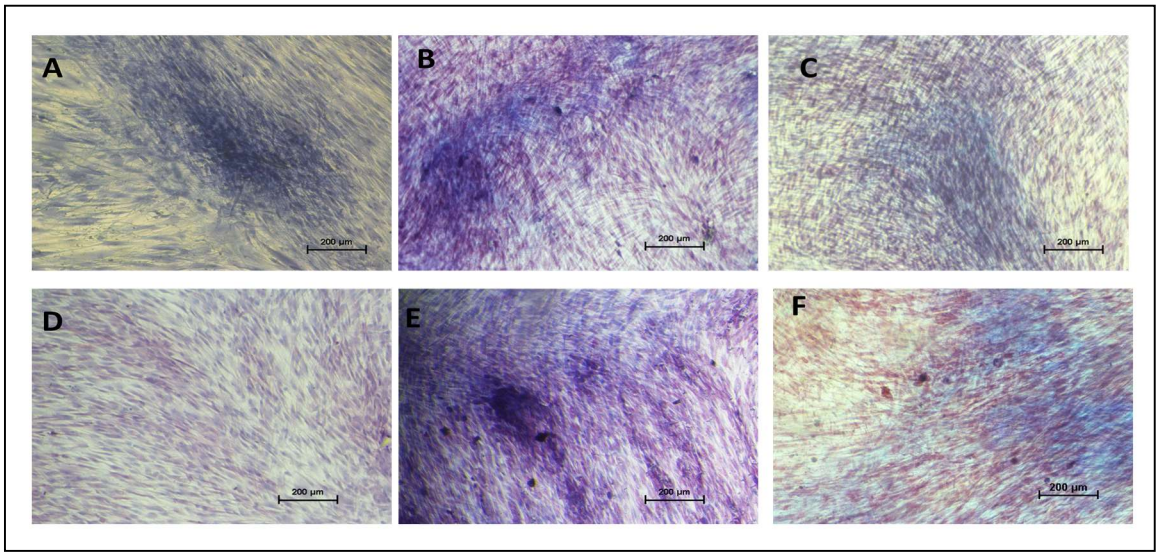


Figure 8 : Images of Masson's Trichrome staining of HPDL cells: Pictures A,B – hPDLCs cultured in osteogenic medium on G-PDA at 14 and 21 days respectively; Picture C - The cells cultured in regular medium, in G-PDA. Picture D,E – hPDLCs cultured in osteogenic medium on glass cover slips at 14 and 21 days respectively (positive control), Picture F- hPDLCs in regular medium, glass cover slips (negative control).

B1.2.6 Osteogenic marker expression :

This is done as a close confirmation step following the staining experiments done in section 2.5 to identify the osteogenic differentiation of the progenitor cells (i.e. hPDLCs). Cells undergoing osteogenic differentiation will secrete alkaline phosphatase enzymes and express osteonectin, the specific protein. The positive expression of these two markers will indicate theosteogenic differentiation of hPDLCs on the **G-PDA** matrix. In the experiment, the hPDLCs in passage 3 were seeded at a density of 10^4 cells/cm^2 onto **G-PDA** matrix and glass coverslip, and cultured in osteogenic medium. The cells were fixed at day 7 with 4 % PFA for 1 h, washed thrice with PBS, permeabilised with 0.1 % triton X 100 and washed. The non-specific antibodies were blocked by incubating with 1 % Bovine Serum Albumin for 10 min. The cells were incubated with mouse antihuman osteonectin antibody (QED bioscience) and rabbit-anti-human alkaline phosphatase antibody (Abcam) at 4 °C , overnight. The cells were then washed and incubated with corresponding secondary antibodies for 1 h in dark (anti-mouse Alexaflour 488 for osteonectin and anti-rabbit alexaflour 546 for alkaline phosphatase, Abcam antibodies). The nucleii of the cells were counterstained with Hoechst 33258 (5 µg/ml in PBS). The cells were viewed and imaged in an inverted fluorescence microscope (Leica) [Figure 9].

Alkaline phosphatase enzyme and the non-collagenous protein osteonectin, when expressed by hPDLCs in osteogenic medium, are specific markers for ECM maturation and matrix mineralization. In the present experiment the immunoflourescence staining of the cells with the osteonectin and alkaline phosphatase antibodies showed the presence of these markers at 7, 14 and 21 days. Representative images are presented in **Figure 9.** Alkaline phosphatase enzyme is the prerequisite for initiating mineral deposits. Osteonectin directs and regulates the sites of deposition of hydroxyapatite or the bone mineral. The presence of these markers indicates an osteogenic differentiation of the hPDLCs in presence of G-PDA.

This result confirms the staining experiments which indicate the osteogenic differentiation of the progenitor cells and the biomineralisation occurring during the culture.

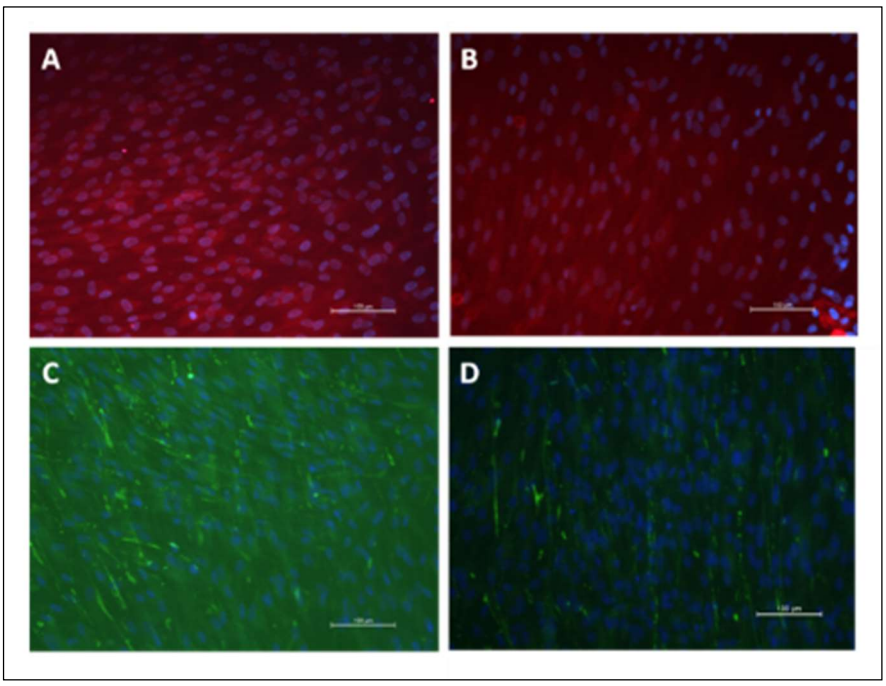


Figure 9 : A,B alkaline phosphate marker expression of hPDLCs cultured in PDAP1 coated and uncoated glass coverslips respectively. C,D – osteonectin expression of hPDLCs cultured in PDAP1 coated and uncoated glass coverslips respectively.

B1.2.7 Significance of this part of the work :

The aim of this study was to construct an ECM mimetic substrate to promote adhesion, spreading and differentiation of dental progenitor cells. A self-assembling dendrimer molecule based on polydiacetylene was designed for the purpose with, guanidine functional group (G-PDA) through original reaction steps.

For the cell interaction studies, progenitor cells from human periodontal ligament (hPDL cells) were isolated and cultured. The key finding is that the guanidine based polydiacetylene exhibited improved cell adhesion, spreading and differentiation. The cells showed osteogenic potential in presence of G-PDA which has been confirmed by staining and immunohistochemistry techniques.

It is clear that G-PDA provides appropriate niche for osteogenic differentiation of progenitor cells and hence could be used as the scaffold material for the regrowth of dentin, cementum and alveolar bone. It could be used as a biomimetic matrix for a wide range of potential applications in tissue engineering.

B1.3. THE WORK DONE IN THE SECOND YEAR (IIT Lab)

B1.3.1 Background of the work :

This part of the work explored the possibility of incorporating the amino acid **lysine** to the self-assembling poly-diacetylene matrix. The molecular designing has been done on the basis of the work done during the previous period, in which 'guanidine' appended matrix was successfully made and tested. It is hypothesised that lysine will be more effective than guanidine in cell homing, cell proliferation and cell differentiation. Lysine appended diacetylene matrix will have better regeneration ability and can act as an excellent scaffold material for periodontal tissue regeneration.

B1.3.2. Molecular design scheme of Lys-PDA :

Lysine amino acid used was of L-configuration, purchased from SRL India. Reagents were purchased from Sigma-Aldrich or Alfa Aesar. All reagents were used without further purification. Solvents employed in the reactions were distilled/ dried prior to use. Progress of reactions was monitored by silica gel thin layer chromatography (TLC). Compounds were purified by silica gel column chromatography.

Characterizations were done by the ^{1}H NMR, ^{13}C NMR, IR and High Resolution Mass Spectra (HRMS).Brucker-DPX-300 spectrometer was used for recoding ^{1}H NMR spectra. Tetramethylsilane (TMS) was used as an internal standard. Coupling constants are reported in Hz and the data are reported as **s** (singlet), **d** (doublet), **br** (broad), **t** (triplet), **m** (multiplet) and **dd** (double doublet). IR spectra were recorded on a Nicolet, Protégé 460 spectrometer as KBr pellets. Melting points were recorded on a Fisher-Scientific melting point apparatus. Rudolph Research Analytical Autopol® V Polarimeter was used to measure optical rotations; where concentrations are given in gram/100 mL. High Resolution mass spectra (HRMS) were recorded in BrukerMicrO-TOF-QII model using ESI technique. Raman spectra were acquired with 532 nm excitation using XploRA Confocal Raman (model no. X/01/220) spectrometer.

B1.3.3. Synthesis and characterization :

(i) Synthesis of monoalkyne2 :

To an ice-cold solution of Bis(Boc)lysine**1** (1.000 g, 2.89 mmol) (**Scheme 1**) in 100 mL of dry dichloromethane was added N-hydroxysuccinimide (NHS) (0.339 g, 3.47 mmol) and dicyclohexyl carboidiimide (DCC) (0.716 g, 3.47 mmol) sequentially and stirred for 5 min. To this stirring solution was added propargylamine (0.22 mL, 3.47 mmol) and triethylamine. The resulting mixture was stirred for 24 h. The reaction mixture was filtered and washed with 0.2 N H_2SO_4, saturated aq. $NaHCO_3$, and water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuum to yield 0.980 g of the product **2** (**Scheme 1**).[1]Yield: 88%, M.P.- 93-95 ^oC.

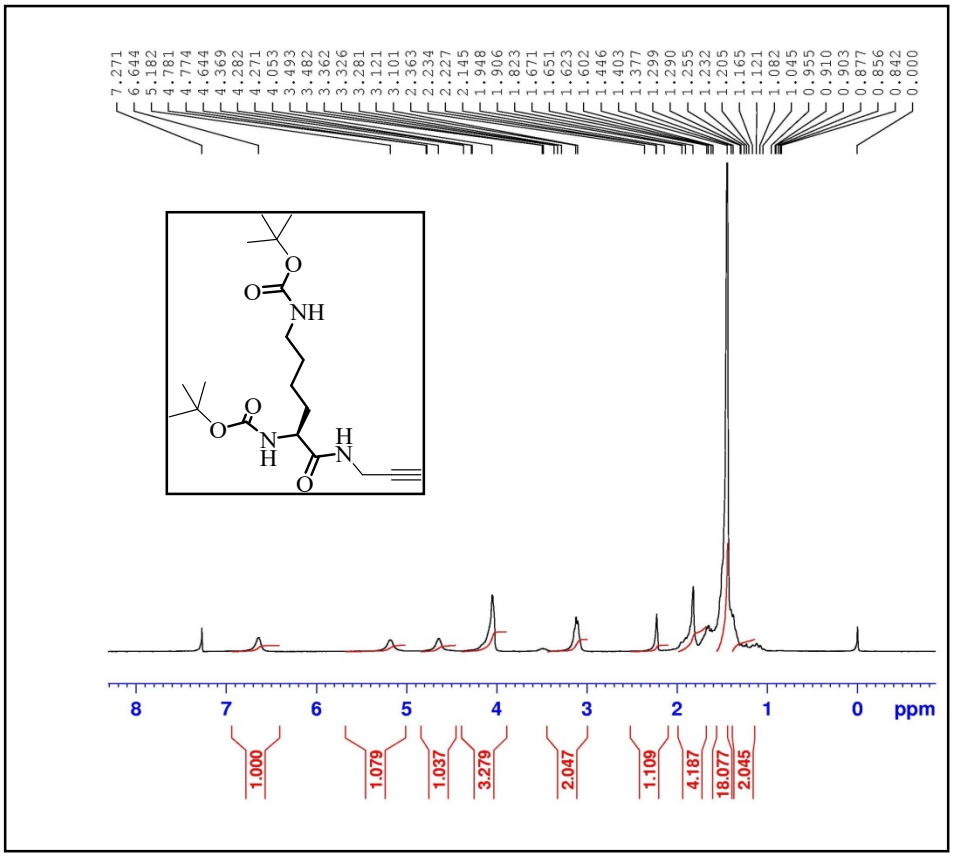


Figure 10:[1]H NMR ($CDCl_3$, 300MHz) of **2**.

[1]H NMR ($CDCl_3$, 300 MHz): δ 1.29 (m, 2H), 1.45 (s, 18H), 1.82 (m, 4H), 2.23 (s, 1H), 3.11 (br d, 2H), 4.05 (br s, 3H), 4.64 (br s, 1H), 5.18 (br s, 1H), 6.64 (br s, 1H). [13]C NMR ($CDCl_3$, 75MHz): δ 22.5, 28.3, 28.4, 29.0, 29.6, 32.0, 39.9, 54.1, 71.5, 79.1, 79.4, 80.1, 155.8, 156.2, 172.1. IR (KBr): 3332, 3270, 2977, 2936, 2869, 1684, 1657, 1519, 1455, 1369, 1389, 1339, 1255, 1170, 1067cm^{-1}. HRMS: calcd for $C_{19}H_{33}N_3NaO_5$ m/z 406.2312 found m/z 406.2311. $[\alpha]_D$:-1.8 (*c* = 0.38, $CHCl_3$).

(ii) Synthesis of diacetylene D1

To a solution of **2** (0.100 g, 0.26 mmol) in 30 mL of acetonitrile was added *Hay catalyst* (a well-stirred solution of CuCl (10 mg, 0.10 mmol) and tetramethyl ethylene diamine (TMEDA) (0.033 mL, 0.22 mmol) in 5 mL of acetonitrile) and stirred under air. After 3 h, more *Hay catalyst* (5 mL) was added and left to stir for 12 h. Progress of the reaction was monitored through thin layer chromatography (TLC). After completion of reaction, the reaction mixture was evaporated, re-dissolved in dichloromethane, and washed sequentially with aqueous NH_4Cl + NH_4OH (9:1) solution, 0.2 N aq. H_2SO_4, saturated aq. $NaHCO_3$ solution and water. The organic part was separated, dried over anhydrous Na_2SO_4, filtered, and evaporated to yield 0.175 g of **D1** (**Scheme 1**).[2]Yield: 85%, M.P.- 75-79 ^oC.

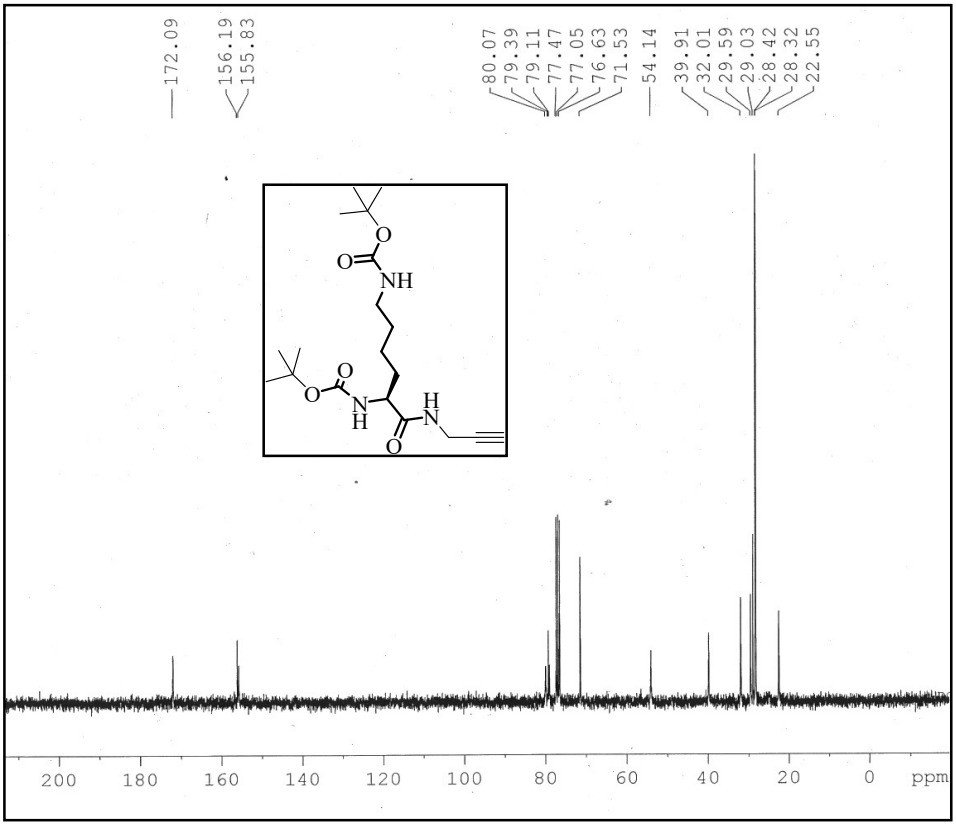


Figure 11:^{13}C NMR ($CDCl_3$, 75 MHz) of **2**.

^1H NMR ($CDCl_3$, 300 MHz): δ 1.44 (s+m, 40H), 2.10 (m, 8H), 3.11 (br s, 4H), 4.15 (m, 6H), 4.77 (br s, 2H), 5.50 (br s, 2H), 7.41 (br s, 2H); ^{13}C NMR ($CDCl_3$, 75MHz): δ 22.7, 28.3, 28.4, 29.5, 29.8, 32.3, 40.1, 54.1, 67.8, 74.3, 79.1, 79.9, 155.9, 156.2, 172.5. IR (KBr): 3333, 2976, 2932, 2866, 1695, 1527, 1453, 1367, 1249, 1171cm^{-1}. HRMS: calcd for $C_{38}H_{64}N_6O_{10}Na$ m/z 787.4576 found m/z 787.4571; $[\alpha]^D$: -20.18 (*c* 0.22, MeOH).

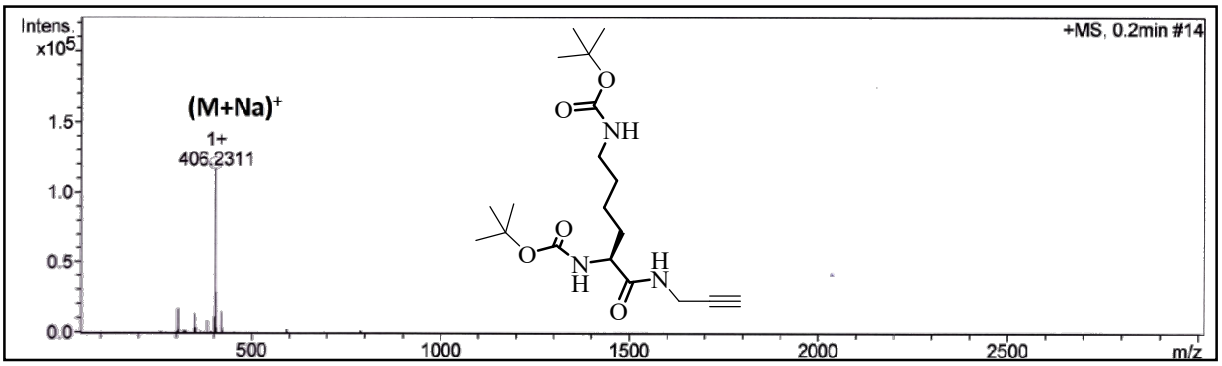


Figure 12: HRMS of compound**2**.

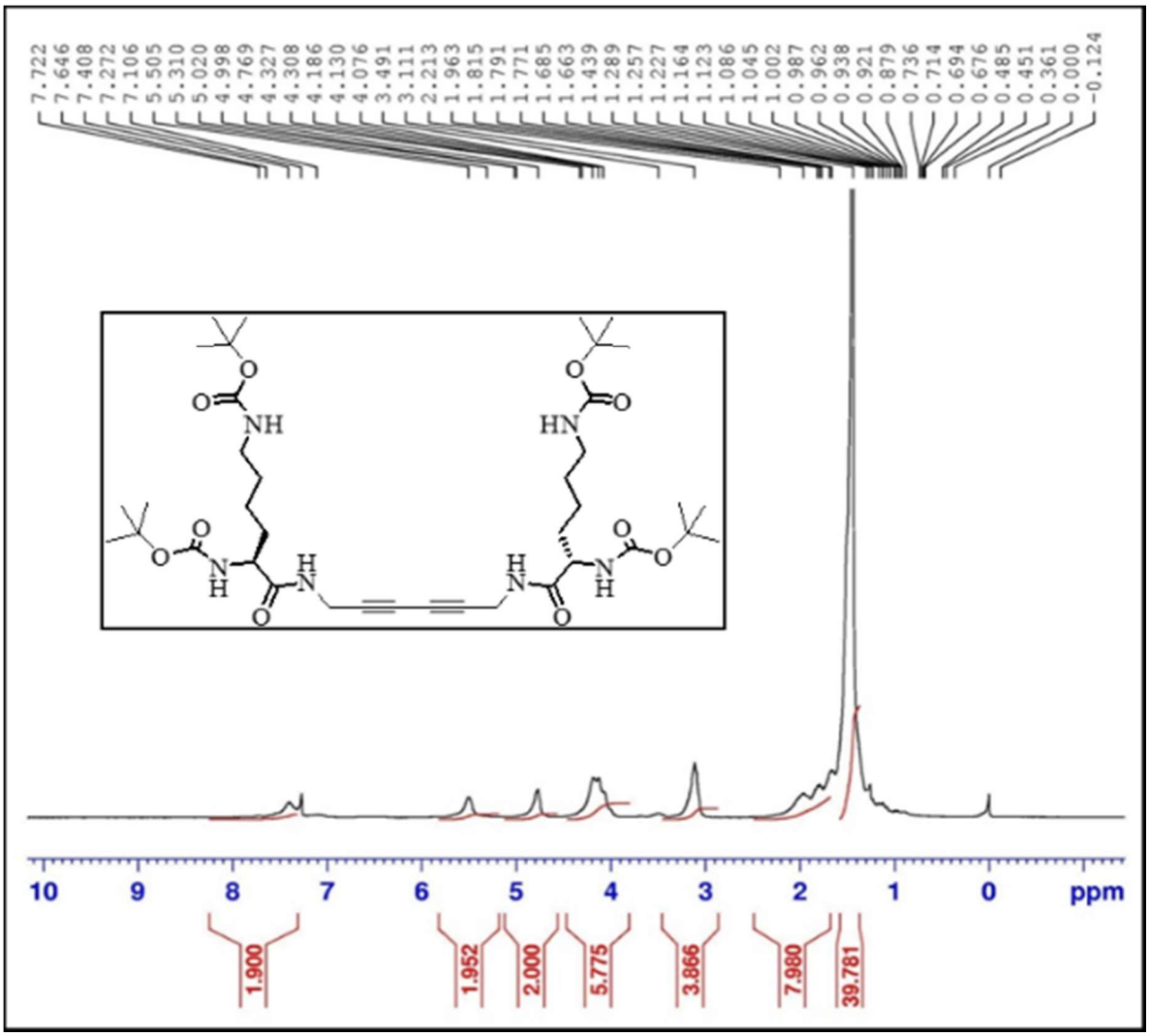


Figure 13:^{1}H NMR ($CDCl_3$, 300MHz) of **D1**.

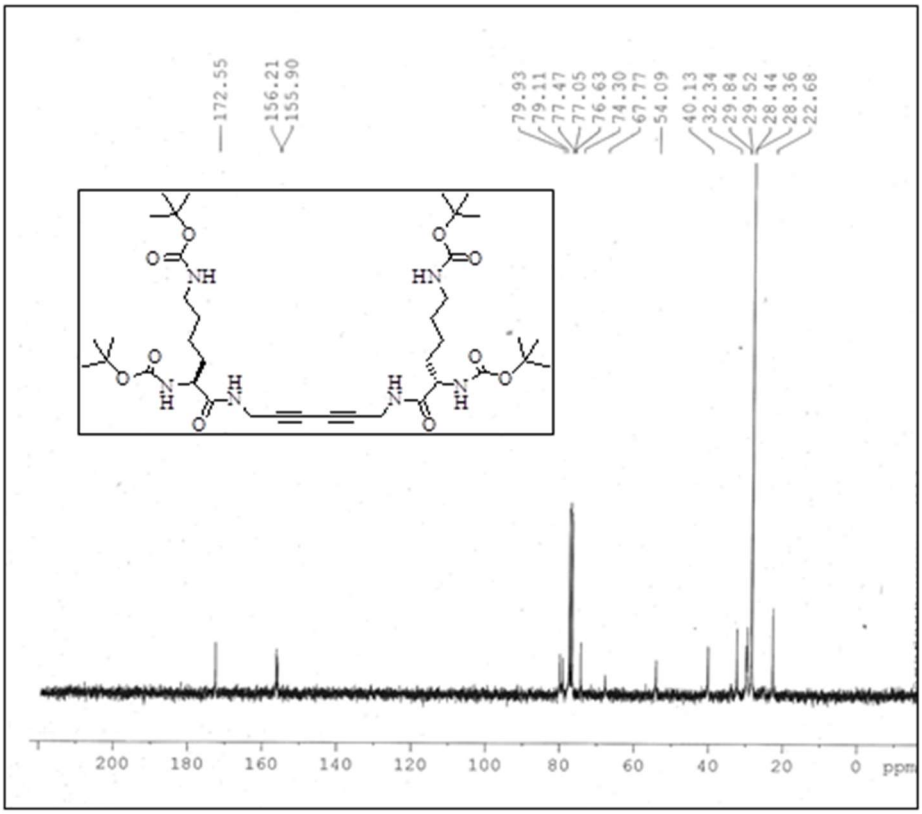


Figure 14 :^{13}C NMR ($CDCl_3$, 75 MHz) of **D1**.

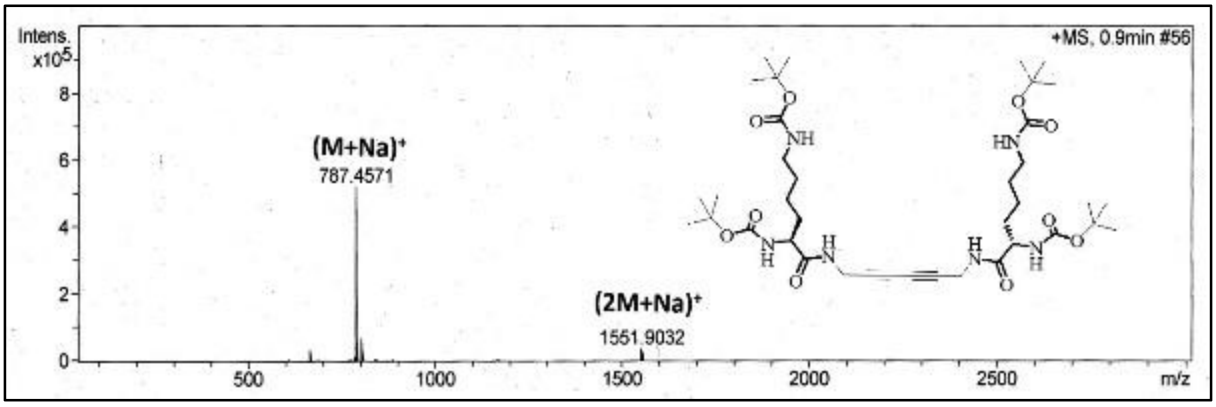
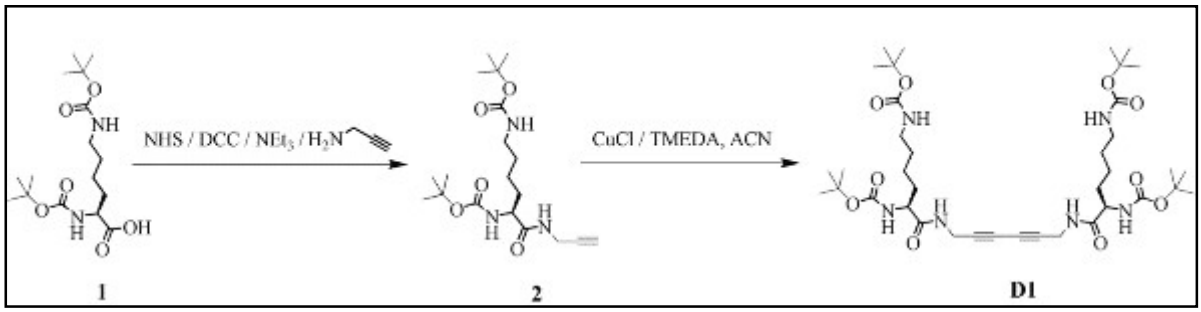


Figure 15 : HRMS of monomer **D1**.

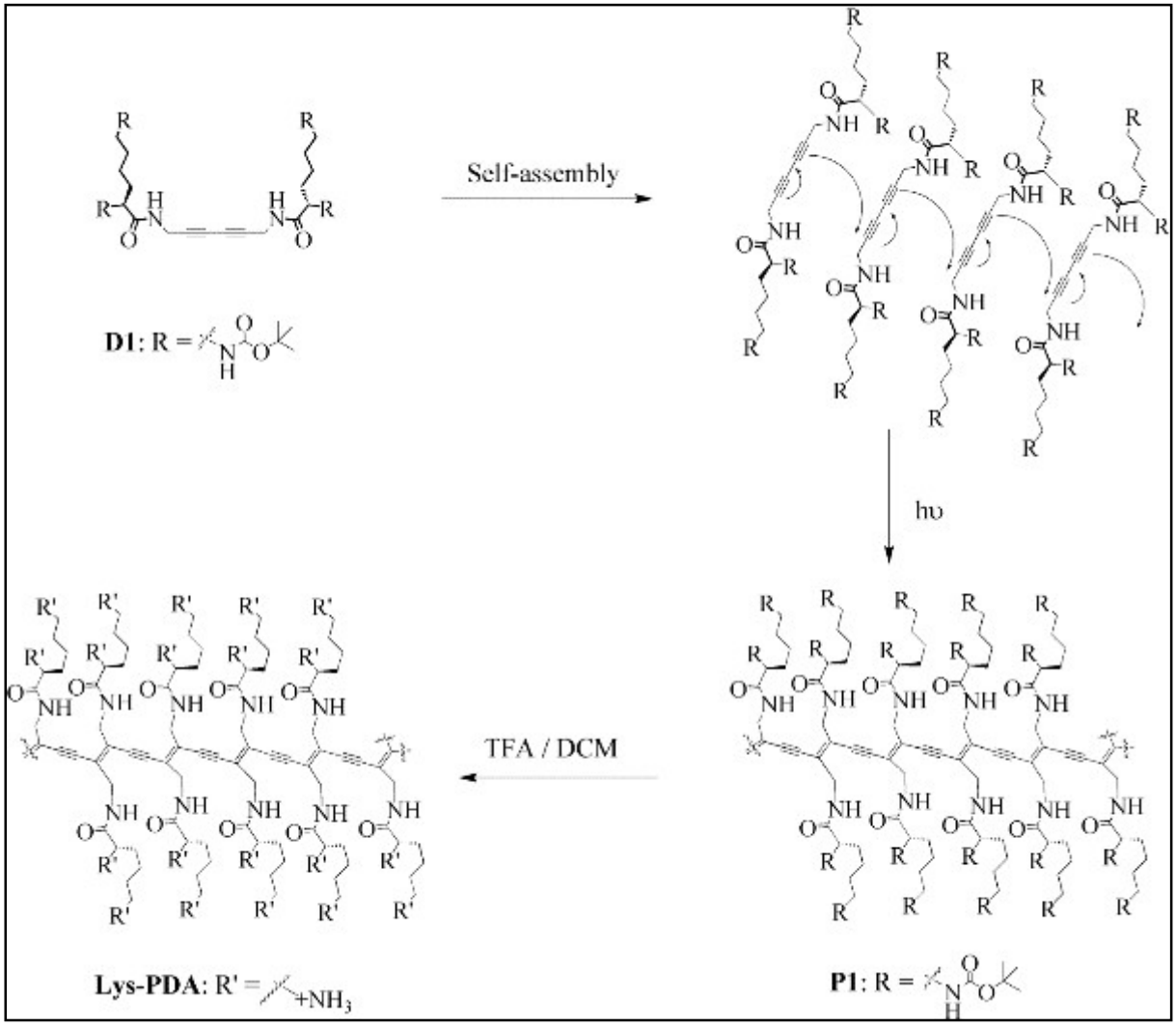
(iii) Synthesis of lysine appended polydiacetylene (Lys-PDA)

Diacetylene **D1** was irradiated under UV lamp for 1-2 hrs to get the corresponding polydiacetylene **P1** (**Scheme 2**). Then to a solution of **P1**(50 mg) in 1 mL dichloromethane was added 1 mL of trifluoroacetic acid (TFA) and stirred for 4h. Reaction mixture was evaporated under high vacuum toyield the final lysine appended polydiacetylene **Lys-PDA**.[3]

(iv) ExperimentalSchemes of Lys-PDA



Scheme 1: Synthesis of diacetylene monomer **D1**.**1:** Basic Lysine unit ;
2:LysineMonoalkyne ; **D1:** Lysine Diacetylene



Scheme 2 : Synthesis of Lysine appended polydiacetylene **Lys-PDA.**
D1: Lysine diacetylene ; **P1:** Lysine polydiacetylene (Boc Protected) ;
Lys-PDA: Lysine polydiacetylene (Deprotected).

B1.4 THE WORK DONE IN THE SECOND YEAR (SCTIMST Lab)

B1.4.1 Background of the work :

The present work comprises the *in vitro* functional evaluation of the synthesized Lys-PDA material and the scope of its use as a synthetic osteogenic matrix to biofunctionalise periodontal barrier membranes. The final application is the regeneration of periodontal ligament and supporting alveolar bone surrounding the teeth.

B1.4.2 PreparingLys-PDA matrix for cell culture :

(i) Lys-PDA matrix :

The Lys-PDA, procured was dissolved in sterile deionised water at a concentration of 1mg/ml and coated onto cell culture wells and glass coverslips for *in vitro* cell–material interaction studies. The polymerisation of Lys-PDA was carried out by UV irradiation at 254 nm for 1 h. The presence of the polymerised coating was confirmed by phase contrast imaging, fluorescence microscopy (since Lys-PDA is capable of emitting autoflourescence) and SEM [Figure 16]. The samples were sterilised for cell culture using ETO sterilisation at 37^{o}C.The cell–material interaction studies were done using human dental periodontal ligament cells (hPDLCs) [The detailed protocol of the isolation, characterisation and culture of the hPDLCs was submitted in the previous progress report].

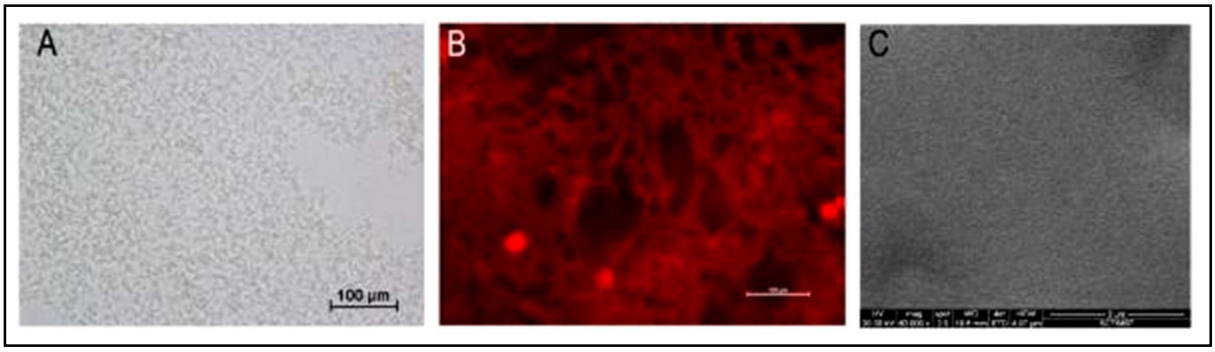


Figure 16. A,B) **Lys-PDA** matrix – phase contrast and fluorescence microscopic image respectively. Auto fluorescence was noted on excitation at 550 nm. C) SEM – **Lys-PDA** matrix. Scale bar: A,B: 100µm; C: 2µm

(ii) Biofunctionalisation of electrospun PCL mats with Lys-PDA matrix :

The Lys-PDA was also coated onto electrospun polycaprolactone (PCL) membranes (which can be utilised as functional periodontal ligament barrier membranes) to enhance the hydrophilicity and bioactivity, thereby improving hPDL cell adhesion and differentiation.

Electrospun PCL membranes were made using an in-house standardised protocol. The PCL membranes were washed thoroughly, dried and coated with 1mg/ml Lys-PDA solution in sterile deionised water. The polymerisation of Lys-PDA was carried out by UV irradiation at 254 nm for 1 h. The hydrophilicity of Lys-PDA/PCL mat was evaluated by water contact angle measurement and the presence of Lys-PDA on PCL mats was confirmed by SEM and FTIR spectroscopic evaluations. FTIR spectroscopy was carried out to identify the presence of characteristic amine (NH_2) and C=O peaks in the Lys-PDA/PCL mat [Figure 17].

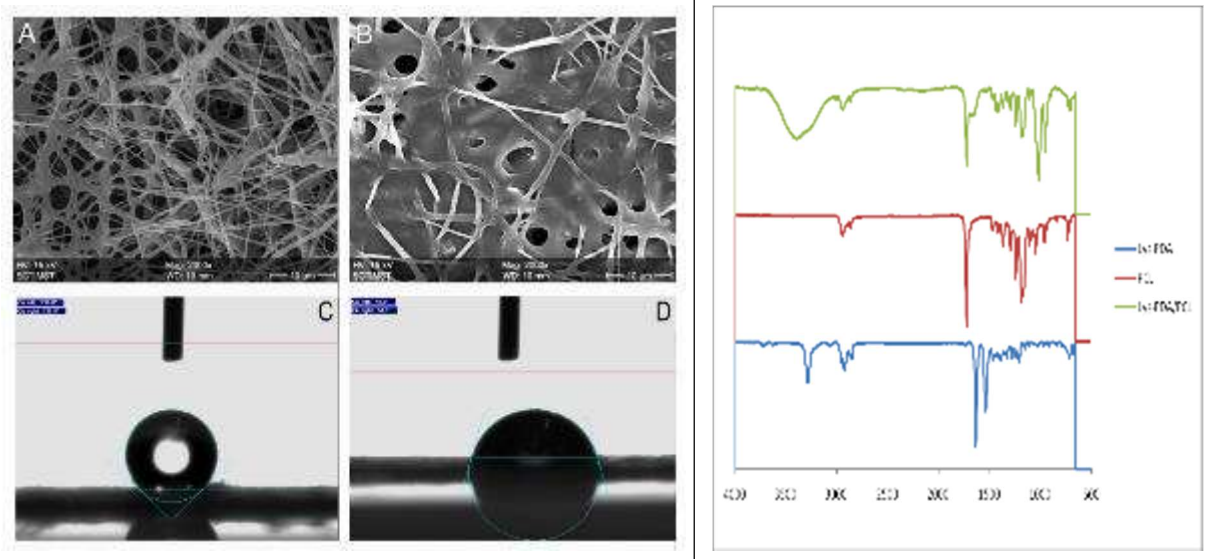


Figure 17. A) Electrospun PCL mat; B) Lys-PDA-biofunctionalized PCL mat (Lys-PDA/PCL) – wherein the scale bar: 10µm. C,D) Sessile drop water contact angle on PCL and Lys-PDA/PCL respectively. Significant reduction in water contact angle for Lys-PDA/PCL is evident. The FTIR data shows the presence of characteristic peaks for primary amine at 3400-3300cm^{-1} region, both in Lys-PDA and Lys-PDA/PCL, which is absent in PCL. Also, the presence of C=O peak of PCL at 1722 cm^{-1} is present in PCL and Lys-PDA/PCL, but absent in Lys-PDA.

B1.4.3 Isolation of human dental progenitor cells

(i) ***Experimental plans :***

The approval from the Institutional Ethics Committee (of SCTIMST) has been obtained for the study. Discarded extracted human permanent teeth for isolating cells were collected anonymously from Govt. Dental College, Thiruvananthapuram after obtaining the necessary approvals. The experimental plan consisted of isolation of human periodontal ligament cells (hPDL cells), their observation, the adhesion and spreading of the cells over the material surface and investigation of osteogenic differentiation on the material.

(ii) Isolation and culture of human dental progenitor cells :

The teeth were collected in Phosphate Buffered Saline (PBS) with 1000 IU Penicillin/Streptomycin and 25 µg Amphotericin B (Gibco). They were washed with PBS and then the periodontal ligament tissue was scrapped off. These tissue fragments were treated with 0.25 % Trypsin and cultured in αMEM, with 10% FBS, 100 IU Penicillin/Streptomycin (Gibco) till cell outgrowths were noted. On achieving the confluent state, the cells were characterized and passaged.

(iii) Characterisation of hPDLCs :

It is important to identify the heterogeneous population of cells (including fibroblasts, ligamental cells and progenitor cells) in the primary cells isolated from periodontal ligament tissue. The periodontal ligament consists of progenitor cells at different stages of differentiation with varying lineage commitments. They can be identified by the Mesenchymal Stem Cell markers like CD90, CD 105 and Stro1.

The hPDL cells at the first passage were characterized by Immuno-flourescence imaging using the markers Vimentin (for fibroblast), Scleraxis (for ligament)and Mesenchymal Stem Cell Markers CD90, CD105 and Stro 1. The hPDLCs were seeded onto 1cm^2 glass coverslips at 5000cells/cm^2 for characterization. The cells were fixed at 48h using 4% paraformaldehyde (PFA) for 1h. The cells were washed thrice with Phosphate Buffered Saline (PBS) and permeabilised with 0.1% triton X100. The cells were incubated with primary

antibodies for Vimentin, scleraxis, CD 90, CD105 and Stro 1 (Abcam primary antibodies), at 4°C overnight. Then washed thrice with PBS and incubated with the corresponding secondary antibodies for 1h at room temperature (anti-Rabbit Alexaflour 546 for scleraxis and anti-Mouse Alexaflour 488 for the others). The nucleus was stained with Hoechst 33258 (5 µg/ml) and the samples were viewed under the fluorescence microscope (Leica DMI 6000 Germany).

The isolation procedure of using explant culture method to retrieve hPDL Cells resulted in cell outgrowth visible from 7 days onwards. The Immunoflourescence staining of the first passage cells showed positive expression of the fibroblast marker vimentin, mesenchymal stem cell markers CD90, CD105 and Stro1 and the ligament specific marker Scleraxis. This confirms that periodontal ligament cells exhibiting fibroblast morphology consists of a population of progenitor cells similar to MSCs and also indicates the ligamental nature due to the presence of scleraxis [Figures not included].

B1.4.4 Cell viability, adhesion and spreading on Lys-PDA matrix

(i). ***Phase contrast imaging***: The hPDLC response to Lys-PDA was analysed by a series of cytocompatibility tests. The hPDLCs at passage 2 were seeded onto the coated Lys-PDA matrix and cell adhesion and spreading was monitored at 24, 48, 72 and 96h by phase-contrast microscopy (Nikon) to identify any cell death or inhibition of growth. The hPDLCs on Lys-PDA matrix maintained their morphology and exhibited proliferation from day 1 to day 4 as seen by phase contrast images corresponding to 24, 48, 72, and 96 h. There is no evidence of cell death, and on the other hand, a gradual increase in cell number can be seen, from 24 to 96 h, similar to the control. This revealed the absence of any cytotoxic dissolution products from the peptide coating [Figure 18].

(ii). FDA/PI live /dead assay: The viability of cells grown on Lys-PDA was assessed using 'live-dead imaging' with fluorescein diacetate (FDA) and propidium iodide (PI), at 24 h and 48 h. The culture medium was discarded and the cells were treated with FDA (5 µg/ml in serum free αMEM) for 10 min and PI (0.5 µg/ml) for 1 min and observed under fluorescence microscope (Leica). The live cells will uptake

Fluorescein diacetate and convert it into green fluorescing fluorescein. PI is taken up by the nucleus of dead cells and appears red. The live/ dead imaging showed that the majority of cells were viable [Figure 18]. Very little dead cells were present in the Lys-PDA matrix, similar to the coverglass control. This confirms the absence of any toxicity or toxic products from the matrix.

(iii) MTT Assay: The hPDL cell metabolic activity was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. hPDL cells were seeded onto the wells of 24 well cell culture plate at a density of 3X105 cells/well and cultured for 24 h, after which, the test materials – Lys-PDA (10µl/well), Lys-PDA/PCL discs (4mm diameter) and PCL discs (4mm diameter) were carefully placed on the cell layer and cultured for 24 h. The cells cultured in 0.13% phenol was taken as the toxic control and the cells alone were taken as the non-toxic control. After 24 h, the medium was discarded and the cells were incubated with 100µl of 1mg/ml MTT solution for 2 h in the dark. The formation of formazan crystals (MTT reduction product) was visualised under microscope. The formazan crystals were dissolved in 100% isopropanol and colourimetrically assessed in a microplate reader at 570nm. The percentage metabolic activity of the cells was calculated from the optical density values [Figure 18].

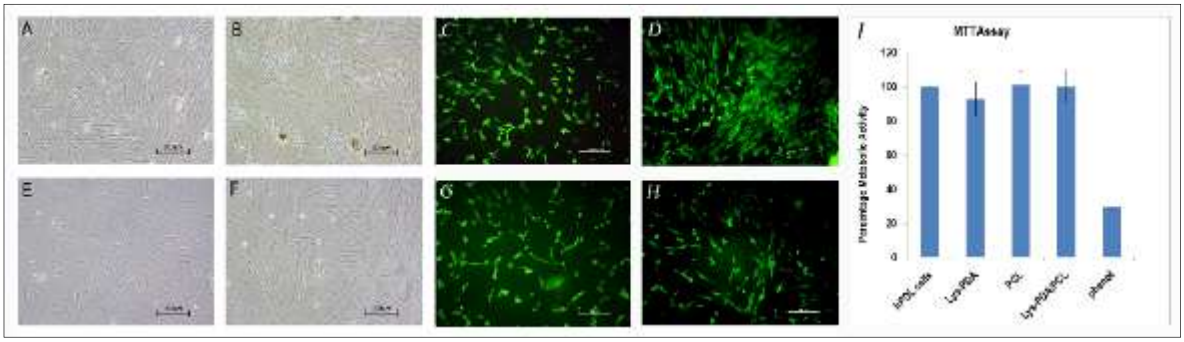


Figure 18 : A,B) hPDL cells on Lys-PDA matrix at 24 and 48h respectively. The cells show good morphology and are adherent, without signs of cytotoxicity. C,D) FDA/PI live/dead images of hPDLcells on Lys-PDA matrix at 24 and 48h, respectively. E,F) Phase contrast images of hPDL cells on coverglass at 24h and 48h respectively. G,H) FDA/PI live/dead images of hPDLcells on coverglass at 24 and 48 h respectively. Scale bar: 200µm.The results of the MTT Assay for the corresponding samples are shown in the right panel.

(iv) Cell morphology and adhesion and spreading : The cytoskeletal organisation of the cells is an important indicator of maintenance of cell morphology and active cell spreading in the Lys-PDA matrix. This was analysed by staining actin cytoskeleton with Actin green 488 (Molecular probes, Thermo Fischer Scientific). The cells were fixed at 48h with 4% PFA for 1h, washed thrice with PBS, and permeabilised with 0.1% Triton X100 for 3min. The cells were incubated in the dark with Actin green 488 for 30min, after which, the cells were washed with PBS and nucleus was counterstained with Hoechst 33258 (5µg/ml).

The cell spreading was further evaluated by SEM so as to visualize the lamellipodia and even minute filopodial extensions, which may not be evident from Actin cytoskeletal staining alone. The cells were cultured in Lys-PDA-coated and uncoated cover glass for 48h, and fixed with 2.5% gluteraldehyde for 1h. Afterwards, washed five times with PB solution (0.2M disodium hydrogen phosphate and sodium dihydrogen phosphate in deionised water), followed by serial dehydration with ascending concentrations of ethanol and isoamyl acetate. The samples were dried in a critical point dryer and sputter coated with gold, before imaging.

The actin cytoskeleton staining showed a more elongated morphology of the cells on the Lys-PDA matrix, with cellular communications [Figure 19]. Cell spreading and migration is mediated through cytoskeletal extensions. SEM images confirm the presence of lamellipodia and filopodial extensions, suggestive of active cell migration [Figure 19].

In two dimensional culture systems, cells form lamellipodia that attaches firmly to the substrate to provide traction for movement through filopodial extensions to the predetermined direction of movement.The polymer Lys-PDA was found to be cytocompatible, and support the cell adhesion and spreading. The positive amine groups at the ends of poly diacetylene matrix can interact with the negatively charged cell membrane and thus improve the cell adhesion and spreading on the Lys-PDA matrix. The cytoskeletal organisation of the hPDL cells is well maintained on the Lys-PDA matrix [Figure 19].

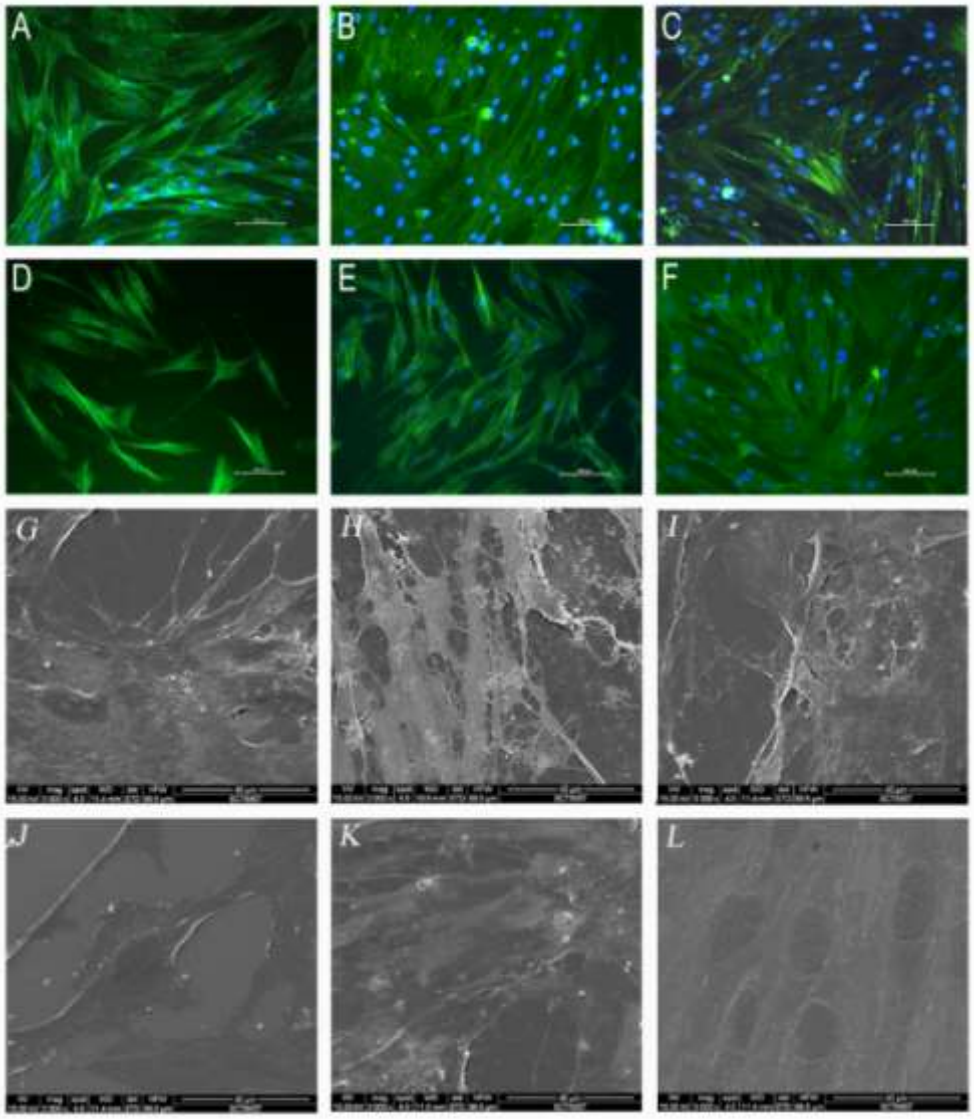


Figure 19 : A, B, C) Actin cytoskeletal staining of hPDL cells on Lys-PDA matrix after 24, 48 and 72 h respectively. D,E,F) Actin cytoskeletal staining of hPDL cells on coverglass after 24, 48 and 72 h respectively. G,H,I) SEM images of hPDL cells on Lys-PDA matrix at 24, 48 and 72h, respectively. J,K,L) SEM images of hPDL cells on coverglass at 24, 48 and 72h respectively. Scale bar: A-F: 100µm, G-L : 40µm

B1.4.5 Osteogenic differentiation of hPDLCs on Lys-PDA

The Lys-PDA matrix should, ideally, be capable of providing appropriate niche for osteogenic differentiation of hPDLCs so that the periodontal ligament regeneration with newly formed cementum and alveolar bone can occur. Therefore the osteogenic differentiation of hPDLCs on Lys-PDA matrix has been investigated.

The hPDLCs in passage 3 were used for the experiment. The cells were trypsinized and seeded onto glass coverslips and Lys-PDA matrix at a cell density of 10^4 cells/$mm^2$and was cultured in an osteogenic medium of αMEM containing 10% FBS, 100 IU Penicillin Streptomycin; 50 μg/ml L-Ascorbic acid, 10 mM sodium β Glycerophosphate and 10 nM Dexamethasone. The hPDLCs cultured in regular medium were used as control.

In order to identify osteogenic differentiation of the hPDLCs *in vitro*, histological evaluation was performed. Biomineralization, the deposition of calcium phosphates, is analyzed by staining using Alizarin red, a calcium chelating dye. The bound dye is solubilised and assessed colorimetrically, to quantify the mineralization. This is supplemented by Von Kossa staining with silver nitrate, which reacts with phosphate ions and get reduced to metallic silver under UV irradiation, giving a brown/black staining.

The presence of osteogenic markers was evaluated by immunocytochemistry/ immunofluorescence (ICC/IF) staining of the hPDLCs for alkaline phosphatase enzyme and osteonectin.

(i). Alizarin Red staining and assay: The presence of calcium deposits can be identified by staining the cell with Alizarin red. The mineralized nodules containing calcium will be stained red, retained after repeated washing. The hPDLCs in the osteogenic differentiation experiment were fixed at 7, 14 and 21 days with 4% PFA for 1h, washed with PBS and then with deionised water, three times for 5 min each. Freshly prepared 2% aqueous solution of alizarin red at pH of 4.2 was added to the cells and incubated for 20min. Thereafter, the cells were washed with deionised water to remove the unbound dye. The cells were viewed and imaged in bright field mode of inverted phase contrast microscope (Nikon Digital Sight Fi2, Japan) [Figure 20]. The dye was solubilised in isopropanol and colourimetrically assessed at 505 nm to quantify the mineral deposits.

Typical calcified nodules were evident at 14 and 21 days for periodontal ligament cells in osteogenic induction medium, in the presence and absence of Lys-PDA. However, the mineralization in peptide dendrimer coated samples was more,

compared to the uncoated samples. The hPDLCs in regular medium did not show evidence of mineralization in the presence or absence of the dendrimer [Figure 20].

(ii).Von KossaStaining : The reaction of silver with phosphate ions during staining, mark the presence of phosphate deposition. The hPDLCs in the osteogenic differentiation experiment were fixed at 7, 14 and 21 days with 4 % PFA for 1h, washed first with PBS and with deionised water, three times for 5min each. Freshly prepared 2% aqueous solution of silver nitrate was added to the cells and UV irradiated for 30 min. After 30min, the cells were washed with deionised water; the cells were viewed under bright field microscope, and images were taken [Figure 21].

The Von Kossa staining showed evidence of phosphate content as brown/black deposits, qualitatively higher than that in positive control. The observation is in complimentary with the Alizarin red data given earlier, confirming the presence of calcium phosphate deposits in the Lys-PDA group. This may be attributed to the presence of basic guanidine groups in the matrix, promoting the mineralization. The electropositive guanidine groups can also bind to anions like phosphate groups, resulting in mineralization [Figure 21].

(iii). Osteogenic marker expression: The presence of osteogenic marker protein osteonectin and alkaline phosphatase enzyme was evaluated. The hPDL cells in passage 3 were seeded at a density of 10^4 cells/cm^2 onto Lys-PDA matrix and glass coverslips, and were cultured in osteogenic medium. The cells were fixed at day 7 with 4% PFA for 1h, washed thrice with PBS, permeabilized with 0.1% Triton X-100 and washed. The non-specific antibodies were blocked by incubating with 1% Bovine Serum Albumin for 10 min. The cells were incubated with mouse anti-human osteonectin antibody (QED bioscience) and rabbit anti-human alkaline phosphatase (Abcam) at 4°C, overnight. The cells were then washed and incubated with corresponding secondary antibodies [anti mouse AlexaFluor 488 for osteonectin and anti rabbitAlexaFluor 546 (Abcam) for alkaline phosphatase] for 1 h in dark. The cell nuclei were counterstained with Hoechst 33258 (5µg/ml) and imaged in the inverted fluorescence microscope (Leica). The presence of osteogenic marker protein osteonectin and alkaline phosphatase enzyme was evaluated.

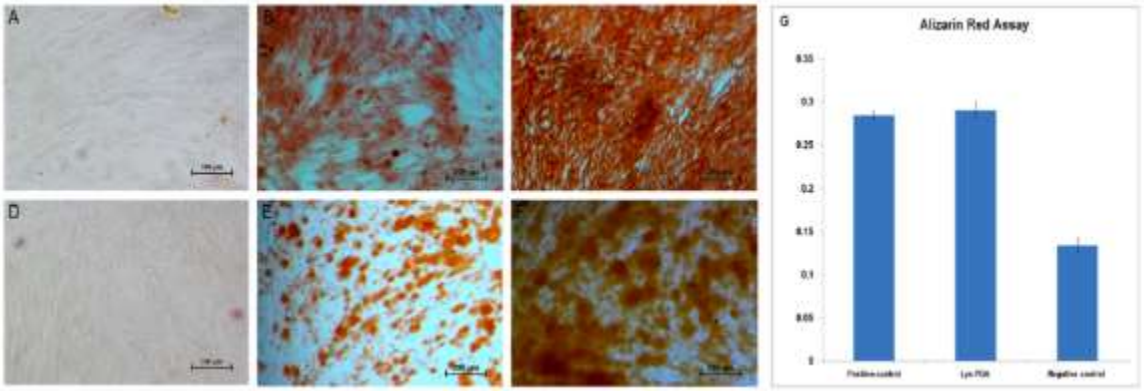


Figure 20 : A,B,C) alizarin red staining of hPDL Cells on Lys-PDA matrix after 7,14 and 21 days respectively. D,E,F) alizarin red staining of hPDL Cells on coverglass after 7, 14 and 21 days respectively. G) Alizarin red assay showing quantity of mineralization after 21days.

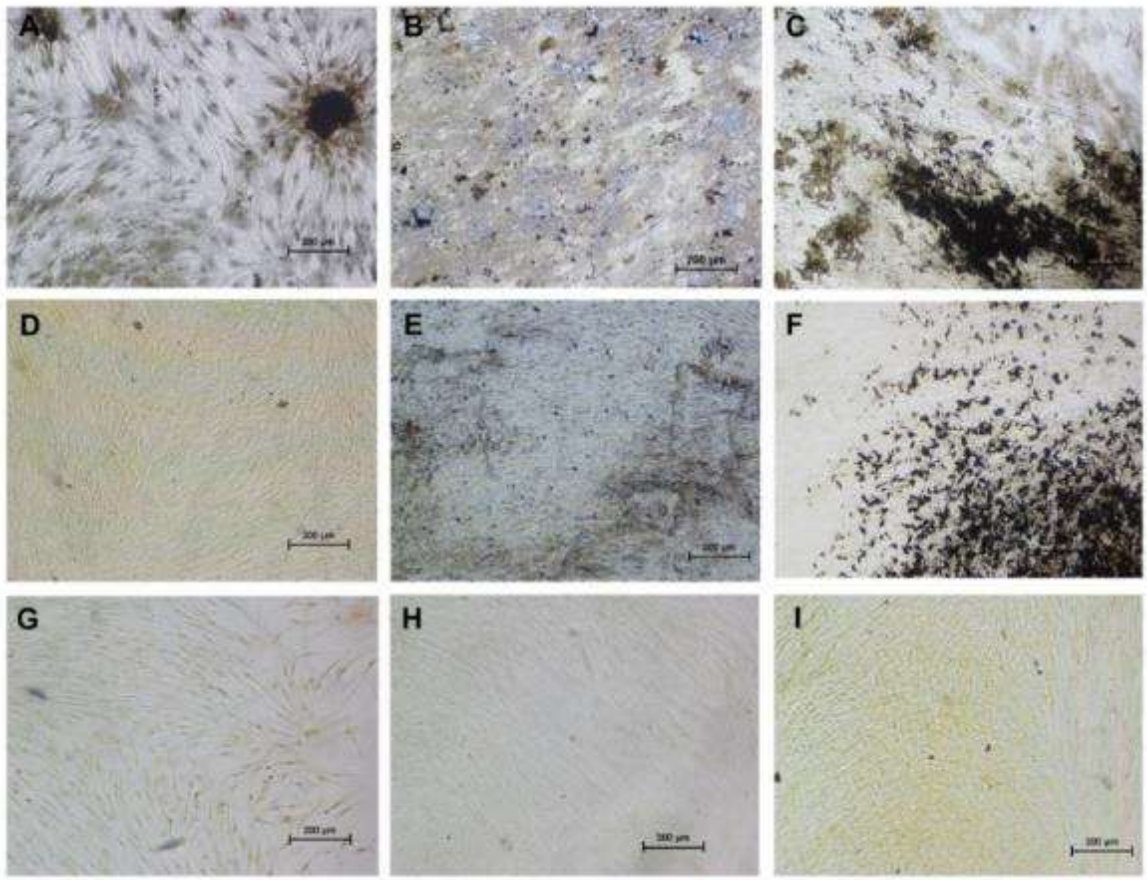


Figure 21 : A,B,C) Von Kossa staining for mineralisation of hPDL Cells on Lys-PDA matrix at 7,14 and 21 days respectively; D, E, F)Von Kossa staining for mineralisation of hPDL Cells on coverglass (positive control) at 7,14 and 21 days respectively. G,H,I)Von Kossa staining for mineralisation of hPDL Cells on coverglass (negative control) at 7,14 and 21 days respectively. Scale bar: 200µm

The hPDL cells in passage 3 were seeded at a density of 10^4 cells/cm^2 onto Lys-PDA matrix and glass coverslips, and were cultured in osteogenic medium. The cells were fixed at day 7 with 4% PFA for 1 h, washed thrice with PBS, permeabilized with 0.1% Triton X-100 and washed. The non-specific antibodies were blocked by incubating with 1% Bovine Serum Albumin for 10min. The cells were incubated with mouse antihuman osteonectin antibody (QED bioscience) and rabbit anti-human alkaline phosphatase (Abcam) at 4°C, overnight. The cells were then washed and incubated with corresponding secondary antibodies [anti-mouse AlexaFluor 488 for osteonectin and anti-rabbitAlexaFluor 546 (Abcam) for alkaline phosphatase] for 1h in dark. The cell nuclei were counterstained with Hoechst 33258 (5µg/ml) and imaged in the Leica inverted fluorescence microscope [Figure 22].

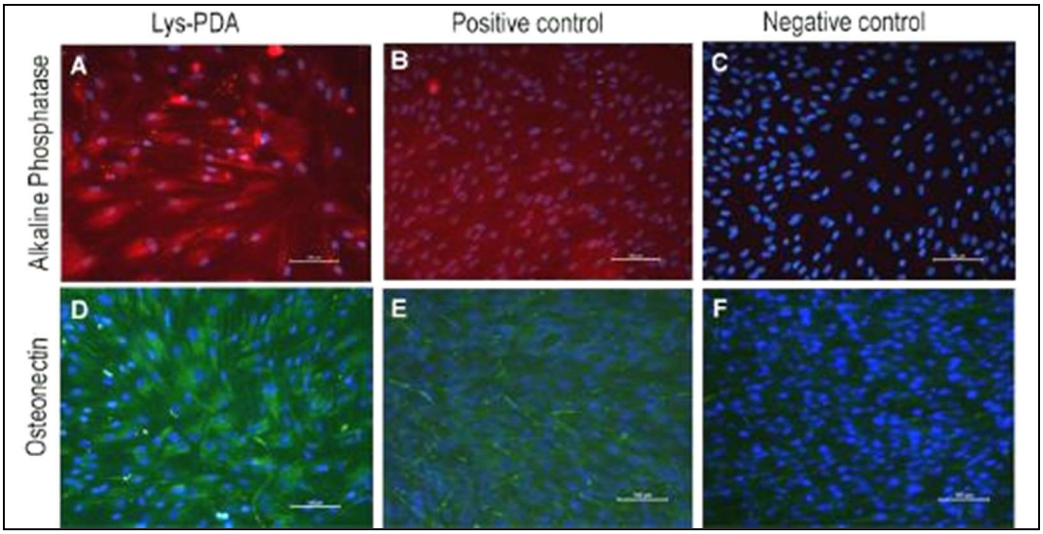


Figure 22 : A,B,C) Alkaline phosphatase enzyme expression of hPDLCells on Lys-PDA matrix, positive control and negative control respectively, at 7 days. D,E,F) Osteonectin expression of hPDLCells on Lys-PDA matrix, positive control and negative control respectively, at 7 days. Scale bar : 100µm

B1.4.6 hPDL cell viability, adhesion and spreading on PCL and Lys-PDA/PCL mat

The *in vitro* functional evaluation of Lys-PDA/PCL mats was done using the hPDLCs. Uncoated PCL mats were taken as control samples. The hPDLCs were seeded onto PCL and Lys-PDA/PCL mats and evaluated for hPDLCs viability and spreading by FDA/PI, actin cytoskeleton staining and SEM analysis. For FDA/PI,

the cell culture medium was discarded at 24h, and 48h, and the cells were treated with 5 µg/ml FDA in αMEM for 10 min and 5 µg/ml PI for 1 min and observed under fluorescence microscope (Leica). For actin cytoskeletal staining, the cells at 24 h were fixed in 4% PFA for 1 h, washed thrice with PBS, permeabilized with Triton X-100 for 5 min and incubated with ActinGreen 488 for 30 min in the dark, after which, the cells were washed with PBS and nucleus was counterstained with Hoechst 33258 (5 µg/ml). For SEM evaluation, the cells were fixed in 2.5% gluteral dehyde at 24 and 48 h and processed through previously described protocol, gold sputter coated and viewed and imaged in Scanning electron microscope [Figure 23].

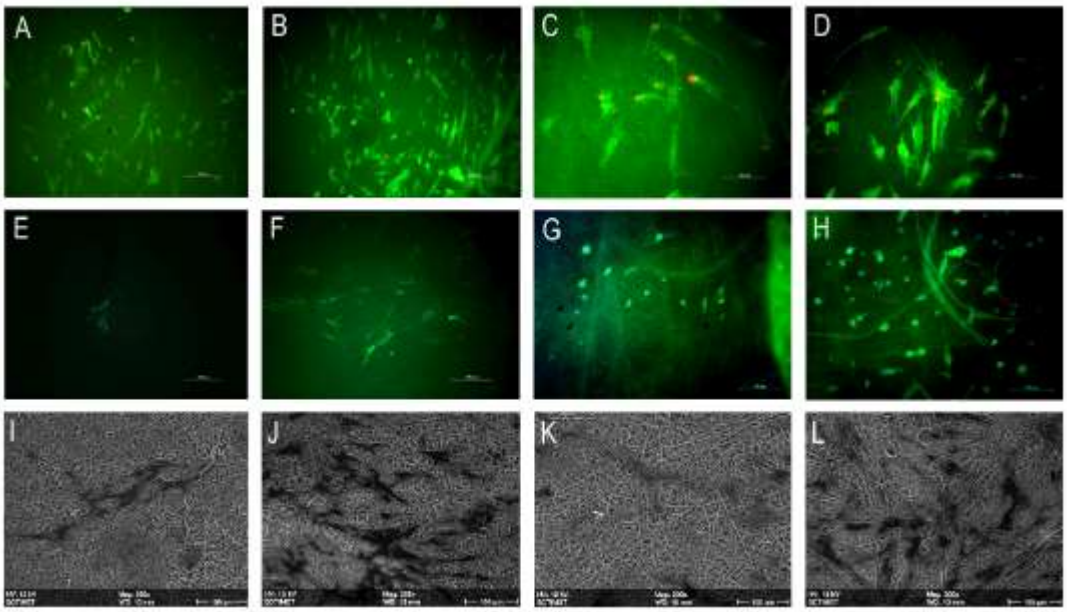


Figure 23: A,B) FDA/PI live/dead images of hPDLcells on Lys-PDA/PCL mat at 24 and 48 h respectively.C,D) Actin cytoskeleton staining of hPDLcells on Lys-PDA/PCL at 24 h and 48 h respectively. E, F). FDA/PI live/dead images of hPDLcells on PCL mat at 24 and 48 h. G,H)Actin cytoskeleton staining of hPDL cells on PCL mat at 24h and 48 h respectively. Osteonectin expression of hPDLCells on Lys-PDA matrix, positive control and negative control respectively, at 7 days. Scale bar : 100µm. I,J)SEM images of hPDL cells on PCL nd Lys-PDA/PCL mats at 24 h respectively. K,L))SEM images of hPDL cells on PCL and Lys-PDA/PCL mats at 48 h respectively.

The hPDLCs on Lys-PDA/PCL mats exhibited good viability, adhesion and spreading, when compared to that on bare PCL mats, indicating the positive biological effects of Lys-PDA on improving the cell responses. Therefore, Lys-PDA can be used as a bioactive coating to functionalize inert barrier membranes like PCL,

to improve selective repopulation of periodontal defect areas and promote regeneration of periodontium.

B1.4.7 Significance of this part of the work :

This part of the project work introduces Lysine appended polydiacetylene (abbreviated as Lys-PDA), as candidate 'extra-cellular matrix (ECM) mimicking' molecular structure for dental tissue regeneration application. In this study, the material has been synthesized in the form of a self-assembling nano-structured matrix. Dendritic peptide molecule with lysine amino acid incorporated onto a photo polymerisable diacetylene was used as a starting unit. The polydiacetylene core can provide a rigid back bone to attach the functional dendritic peptide lysine, which forms a bioactive terminal of the polymer.

A coating of the Lys-PDA dendrimer was prepared and the surface interactions with the cultured progenitor (hPDL) cells were assessed by a series of cytocompatibility tests. The cells cultured on Lys-PDA showed high compatibility and viability ('live-dead imaging' and actin staining). The cell spreading, evaluated by SEM, confirmed the presence of lamellipodia and filopodial extensions, suggestive of active cell migration. The osteogenic differentiation of hPDLCs on Lys-PDA was studied through histocytological evaluation. Evidence of calcium phosphate nodule formation was confirmed by staining using Alizarin red and Von Kossa. The presence of osteonectin and alkaline phosphatase markers, identified by immunoflourescence staining, established the osteogenic differentiation of the hPDLCs in presence of Lys-PDA.

Electrospun PCL barrier membranes were functionalised with Lys-PDA and characterised. The cell-material interaction studies showed improved hPDL cell adhesion and spreading on the Lys-PDA/PCL membranes, confirming its utility as a bioactive barrier membrane for periodontal regeneration.

The studies done during this period, establishes that Lysine appended polydiacetylene (Lys-PDA) could be helpful for the regrowth of dentin, cementum and alveolar bone. It is a promising material for regenerative dentistry.

B1.5. THE WORK DONE IN THE THIRD YEAR (Both Labs, Material-Cell Interaction Studies)

B1.5.1 Background of the work.

The basic aim of this part of work was to investigate the dental pulp cell response to calcium sulfate, calcium phosphate and calcium sulfate phosphate cements. Biomaterials mediated dental pulp regeneration have been reported to be carried out using calcium hydroxide, MTA, and other bioactive cements, with varying success. It has been identified that certain novel, in-house developed phosphate modified calcium sulfate (BioCaS), calcium sulfate (CaS) and calcium phosphate cements (CPC) had shown promising results as alveolar bone grafts. Hence it was decided to proceed with the evaluation of dental pulp cell response to these bioactive cements, which will provide valuable information towards the use of the bioactive, phosphate containing cements as cost effective dental pulp capping agents.

B1.5.2. Isolation of human Dental Pulp cells

Two previously reported cell isolation techniques were carried out to isolate the dental pulp cells from discarded extracted human permanent teeth, as follows.

i) Tissue explant culture method:

Extracted discarded human permanent teeth were collected through ethical route, after taking necessary permissions from institutions. They were first kept in sterile phosphate buffered solution (PBS) with 1000IU penicillin/streptomycin and 25 µg Amphotericin B. Coronal access opening were prepared in the collected teeth and dental pulps were extirpated using H files. The pulp tissues were collected in sterile phosphate buffered solution (PBS) with 1000IU penicillin/streptomycin and 25 µg Amphotericin B. The samples were transferred to class II biosafety cabinet (Esco, Singapore) for sterile handling. The pulp tissues were transferred to a 35mm cell culture petri dish and minced to 2mmX3mm tissue pieces. These tissue sections were treated with 0.25% trypsin for 5 min at 37^{o}C. The tissue fragments were then cultured in 60mm cell culture petri dishes (Nunc, Thermofischer) in αMEM, 10% FBS, and 100 IU pen/strep (Gibco), till cell outgrowths were noted in phase contrast microscopy.

ii) Tissue digestion method:

Extracted discarded human permanent teeth were collected in sterile phosphate buffered solution (PBS) with 1000IU penicillin/streptomycin and 25µg Amphotericin B. Coronal access opening were prepared in the collected teeth and dental pulps were extirpated using H files. The pulp tissues were collected in sterile phosphate buffered solution (PBS) with 1000IU penicillin/streptomycin and 25 µg Amphotericin B. The samples were transferred to class II biosafety cabinet (Esco, Singapore) for sterile handling. The pulp tissues were transferred to a 35mm cell culture petri dish and minced to 2mm X 3mm tissue pieces. The tissue fragments were digested in 4%collagenase, 3% dispase, in serum free αMEM for 30 min inside the CO_2 incubator at 37°C, 5% CO_2, 95% O_2. The digested tissue fragments along with the medium was centrifuged at 2200rpm for 5 min. the supernatant was discarded and the cell pellet was dispersed in αMEM, 10% FBS, and 100 IU pen/strep and cultured in 60mm cell culture petri dishes, till a healthy population of cells were observed in phase contrast microscopy.

B1.5.3.Characterisation of human Dental Pulp cells

The hDPCsin passage 2 was seeded onto 1cm^2 glass coverslips at a cell density of 5X10^3 cells/ cm^2 and cultured for 48 h. After 48 h, the culture medium was discarded; the cells attached to coverglass was washed in sterile PBS to remove residual serum, and fixed by adding 4% Paraformaldehyde (PFA) solution, and kept at room temperature for 1 h. The cells were then washed with PBS, permeabilised using 0.1% triton X100 for 1 min and washed with PBS. The non-specific antibody binding was blocked by incubating the cells in 10% Bovine Serum Albumin (BSA) for 30 min. the cells were then incubated overnight at 4°C, with the corresponding primary antibodies [rabbit antihuman CD73 and DMP1; mouse antihuman Vimentin, CD90, CD105 and Stro1]. The next day, the cells were washed thrice with PBS and incubated with the corresponding secondary antibodies (anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 546, Abcam), for 1 h in dark, at room temperature. The cells were then washed thrice in PBS and the cell nuclei was stained using Hoechst 33258 (0.5µg/ml in PBS) for 1 min. the cells were then given a final PBS wash, viewed and images were taken using inverted fluorescence

microscope (Leica DMI 6000, Germany) equipped with filters suitable for green (I3) , blue (A) and red (N21) emission.

The hDPCs showed positive expression of Vimentin and the MSC markers CD 73, CD 90 and Stro 1 [Figure 24]. In addition the Dentin Matrix Protein (DMP1) expression was positive. The actin cytoskeletal staining revealed the fibroblast morphology.

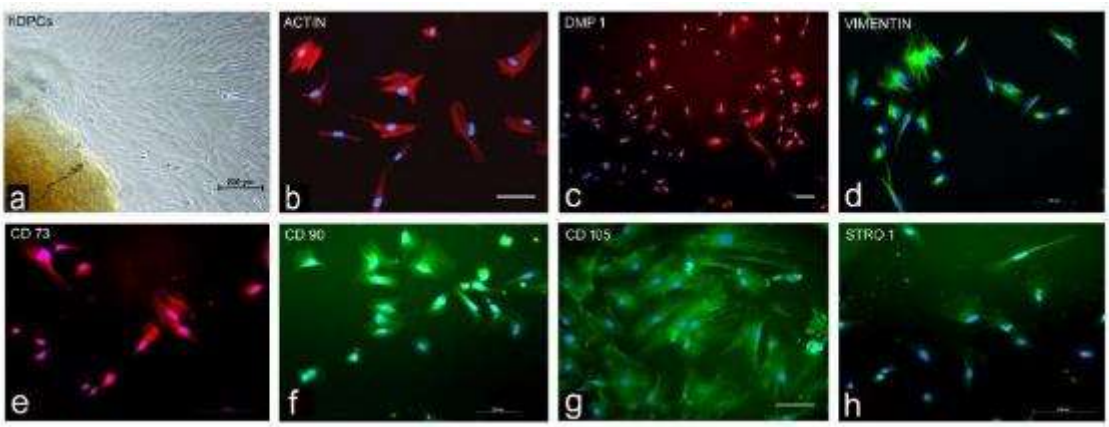


Figure 24: a) hDPC cell outgrowth from the pulp tissue explant. b) Actin cytoskeletal staining of hDPCs. c-h) expression of Dentin Matrix protein (DMP1), Vimentin, CD 73, CD 90 , 105 and Stro 1 by hDPCs.

B1.5.4 *In vitro* osteogenic differentiation of human Dental Pulp cells

The dental pulp cells in passage 3 were used for the *in vitro* osteogenic differentiation of the cells. The cells, upon confluency, were trypsinised using 0.25% trypsin for 1 min at 37°C, counted and seeded onto the 1 cm^2 sterile, glass coverslips at a cell density of 10^4 cells/cm^2. The control cells were cultured in regular medium (MEMα containing 10% FBS and 100 IU Penicillin/streptomycin) and the test cells were cultured in an osteogenic medium (αMEM containing 10% FBS, 100 IU Penicillin-Streptomycin; 50µg/ml L-ascorbic acid, 10mM sodium β-glycero phosphate and 10 nM dexamethasone). The differentiation potential of the cells was evaluated by the expression of osteogenic markers Alkaline phosphatase, Osteonectin, collagen 1 and DMP 1 was evaluated for hDPCs [Figure 25].

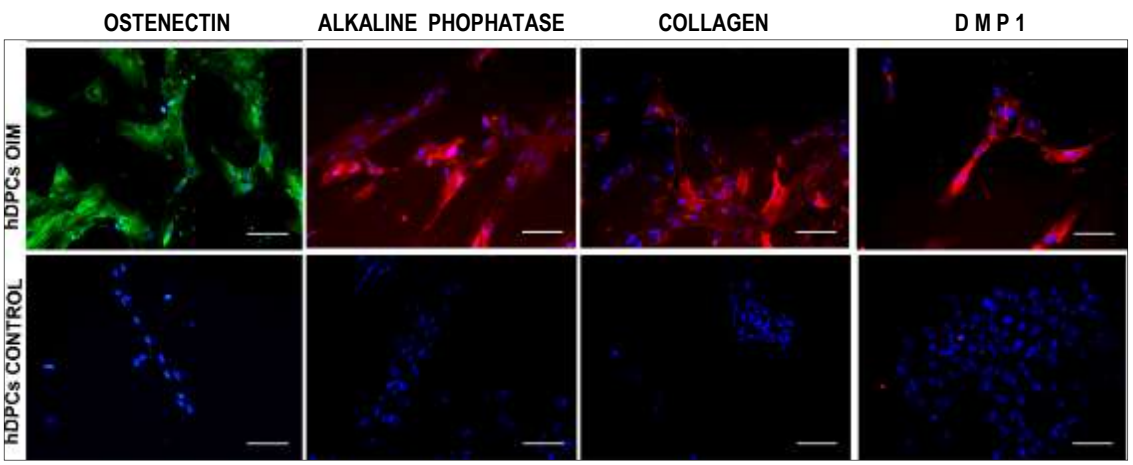


Figure 25. The *in vitro* osteogenic differentiation of human Dental Pulp cells with and without Osteogenic Induction Medium (top row and bottom row, respectively).

B1.5.5 *In vitro* compatibility of human Dental Pulp cells to the test samples

The isolated and characterised hDPCs in passage 3, were seeded onto 1cm^2 glass coverslips coated with a thin CAS, CPC and BioCaS cement layers, dried and sterilised using ETO. The cells were cultured for 24 h, after which, the cells were fixed in 4% paraformaldehyde for 1 h, washed thrice with PBS, permeabilised with triton X100, washed again, and incubated with Phalloidin Cytopainter 555 (Abcam) for 1 h, in darkness. After incubation, the cells were washed with PBS, and nuclei were stained using Hoechst 33258 (5 µg/ml) for 1 min. the cells were then washed with PBS and viewed under fluorescence microscope using suitable filters.

The hDPCs exhibited good cytoskeletal organisation on control cover glass as well as on the cements at 24 h, indicating the compatibility with calcium cements.

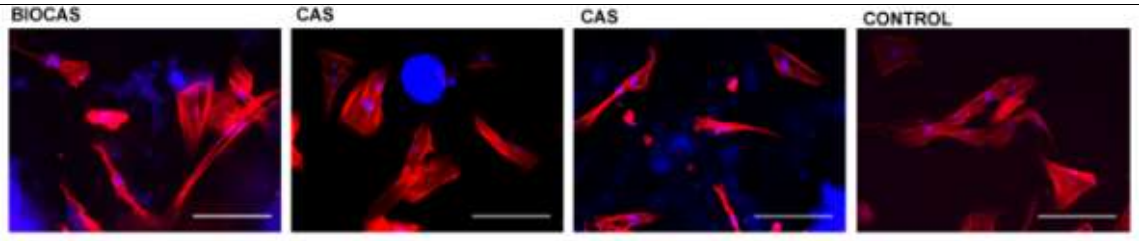


Figure 26. Actin cytoskeleton staining of hDPCs on CAS, CPC and BIOCAS coated coverglass and on coverglass (control)**.**

B1.5.6 Osteogenic differentiation of hDPCs in the presence of CaS, CPC and BioCaS cements.

The osteogenic differentiation of hDPCs in the presence of the cements was evaluated using ICC/IF staining of the hDPCs cultured on CaS, CPC and BioCaS cement, at day 7. The cells were seeded onto 1cm^2 glass cover slips smeared with a thin layer of the respective cement, which was allowed to set and then sterilised using ETO. The cells were fixed with 4% PFA for 1 h at 7, 14 and 21 days. They were washed thrice with PBS, permeabilised with triton X100, washed again with PBS, treated with 10%BSA for 30min to block non-specific antibodies, and were incubated with the corresponding primary antibodies – Alkaline Phosphatase enzyme, Osteonectin, DMP 1 and Collagen Type I, overnight at 4^{o}C. The next day, the cells were washed thrice with PBS, and incubated with the corresponding secondary antibodies – Anti-mouse AlexaFluor 488 and Anti-rabbit AlexaFluor 545, for 1 h in dark. The cells were washed thrice with PBS, the nuclei were stained using Hoechst 33258 (5µg/ml), washed and viewed under Fluorescence microscope.

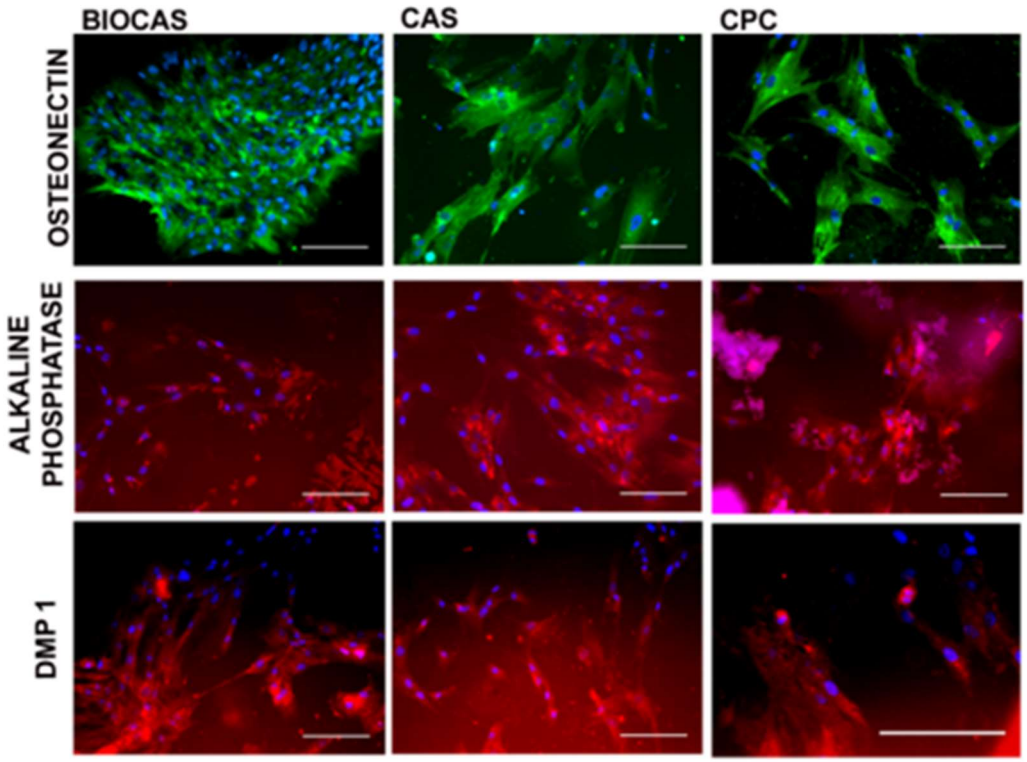


Figure 27. Osteogenic expression of the cements in human Dental Pulp cells.

The hDPCs showed positive expression of the osteogenic markers alkaline phosphatase, osteonectin and DMP 1 at day 7, when cultured onto the cements. However, the cells did not show evidence of collagen deposition at day 7, which may be due to the absence of L Ascorbic acid, which is needed for the collagen formation, since no induction medium was used with hDPCs cultured in the presence of the cements.

B1.5.7 Significance of the work

Phosphate modified calcium sulfate (BioCaS), and calcium phosphate cements (CPC) are new generation calcium based tissue regeneration materials. Dental pulp cell responses to these bioactive cements were evaluated so as to identify a material suitable for making cost effective dental pulp capping agents. For the purpose, methods for isolating and culturing dental pulp progenitor cells (Dental Pulp Cells - hDPCs) were developed.

Human Dental Pulp Cells (hDPCs) were isolated from discarded teeth and cultured, and then characterised. The hDPCs showed positive expression of Vimentin and the MSC markers CD 73, CD 90 and Stro 1. In addition the Dentin Matrix Protein (DMP1) expression was positive. The actin cytoskeletal staining revealed the fibroblast morphology. They exhibited good cytoskeletal organisation on control cover glass as well as on the cements at 24 h, upon actin cytoskeletal staining. This proved the success of the cell culture model adopted.

The osteogenic differentiation of hDPCs in the presence of the cements was evaluated using ICC/IF staining of the cells cultured on CaS, CPC and BioCaS. The hDPCs showed positive expression of the osteogenic markers alkaline phosphatase, osteonectin and DMP 1 at day 7, when cultured onto the cements. However, the cells did not show evidence of collagen deposition at day 7, which may be due to the absence of L Ascorbic acid, which is needed for the collagen formation, since no induction medium was used with hDPCs cultured in the presence of the cements. The cement samples were found osteogenic when cultured using Human Dental Pulp Cells (hDPCs).

B1.6. THE WORK DONE IN THE FINAL PHASE (SCTIMST Lab, Animal Experiments)

B1.6.1 Plans for the animal experiments.

In order to do clinical translation, preclinical usage tests using animal models need to be carried out. Lys-PDA/PCL mat was selected for preclinical evaluation because lysine is a component of collagen, the main protein component in the periodontal extra-cellular matrix. In another series of tests, the calcium mineral based cementing materials – calcium sulfate or gypsum cement (CaS), calcium phosphate or apatitic cement (CPC) and Bioactive calcium sulfate-phosphate cementing composition (BioCaS) - were evaluated for their potential as alveolar bone grafts/barrier grafts using a rat maxillary alveolar bone defect model.

B1.6.2 Preparatory part of animal surgery.

i) Animal selection and permissions :

The study was conducted after obtaining the necessary Institutional Animal Ethics Committee clearance (IAEC), and all the procedures were carried out ethically. Sprague Dawley rats of either sex were selected for the *in vivo* studies. Animal weighing above 350g were selected for the studies. Total 6 defect sites were dedicated to the test groups and sham control. Since bilateral defects were created, a total of nine (9) animals were used for the study. Each defect site was taken as a single unit and the time duration was fixed at 3 months (12 weeks).

ii) Surgery part :

Bilateral maxillary alveolar bone defects were created buccal to the first molars, using surgical micro motor handpiece and tungsten carbide burs of 0.8mm diameter at a speed of 2300rpm, under ambient cooling or using cold saline irrigation. Briefly, the selected animals were weighed and anaesthetized using individually calculated dose/weight of Ketamine and xylasine administered intramuscularly on either thighs. Once anaesthetized, the animals were placed on their backs securely on a special surgical platform and the jaws were kept open using elastic bands. The positions of the maxillary teeth are shown in Figure 28.

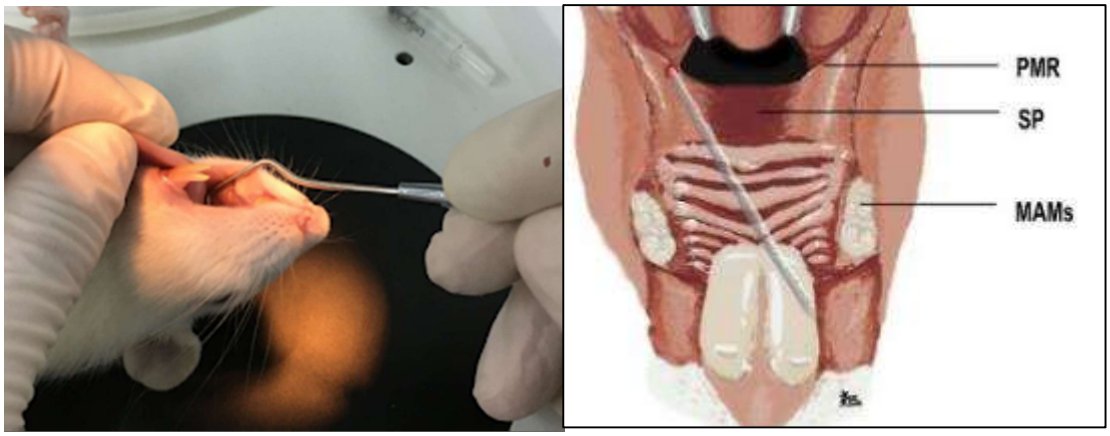


Figure 28. Schematic of the surgery in rat jaw

B1.6.3 Pre-clinical studies on Lys-PDA/ PCL mats as GTR Barrier Membranes.

i) Surgical placement of the graft :

The mucosa adjacent to the mesiobuccal root of the first molar was reflected along with the periosteum [Figure 29]. Approximately 2 mm of alveolar bone surrounding the tooth root was removed carefully using WC burs, and the samples (PCL or Lys-PDA-coated PCL mats) were placed carefully over the defects such that they were in contact with the tooth at the gingival level, covering the defects {Figure 30]. The bone defects without the mats were considered as the sham control.

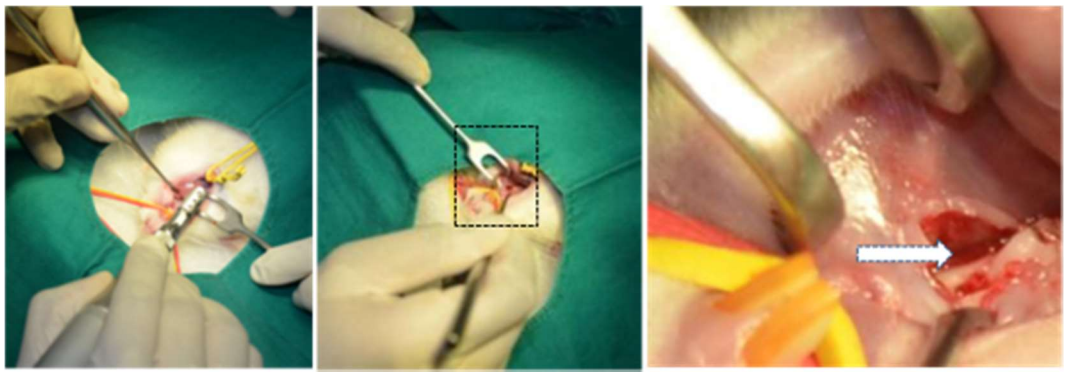


Figure 29. Periodontal defect creation using low speed micro motor hand piece and tungsten carbide bur.

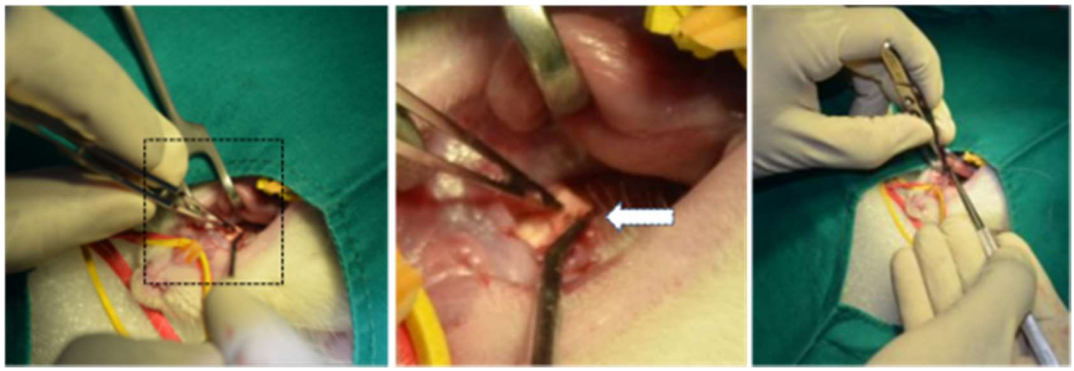


Figure 30. Placement of Lys-PDA coated PCL Barrier membranes onto the defect

The sample distribution is as follows :

Test material – Lys-PDA/PCL mat; n = 6

Control material – PCL mat; n = 6

Sham control – No materials; n = 6

ii) Tissue harvesting and histopathology evaluation : The animals were euthanized after 3 months by CO_2 inhalation in closed chambers. The animals were decapitated and the entire head containing the alveolar bone was collected in 10 % formalin as fixative, in sealed containers and transferred to the histopathology lab. The fixed samples were processed through standardised procedures and embedded in PMMA blocks for sectioning and histopathology. Micro CT analysis of the defects was carried out in select samples.

The PMMA embedded tissue specimens were sectioned using a hard tissue microtome, with average width of the sections being 100µm. The specimens were viewed under microscope. Van Gieson's staining and Stevenel's blue staining was carried out to visualise the new bone formation and the osteoblasts cells respectively.

iii) Results : The histopathology evaluation shows the presence of gingival overgrowth in the sham control, which is absent in PCL and Lys-PDA coated PCL group. From the results it can be inferred that the barrier function of the PCL mat and Lys-PDA/PCL mats is maintained. However, the osteogenic activity manifested by the new bone formation was minimal in both the PCL and Lys-PDA/PCL groups.

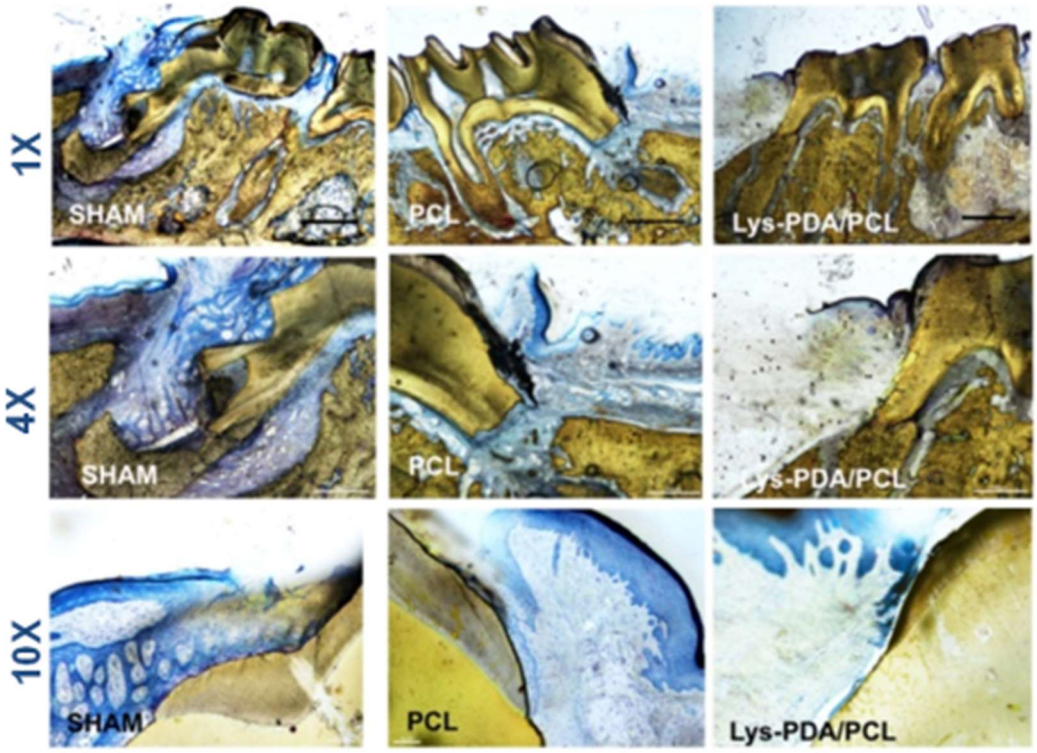


Figure 31. The histopathology images showing new bone formation in PCL and Lys-PDA coated PCL groups, compared to the sham group.

B1.6.4 Bioactive cements as barrier grafts for periodontal regeneration.

i) Surgical placement of the graft :

The mucosa adjacent to palatal aspect of the root of the first molar was reflected along with the periosteum. Approximately 2 mm of alveolar bone surrounding the tooth root was removed carefully using WC burs (as shown in figure 29].

The cement samples were pr-epacked aseptically - Bioactive calcium sulfate-phosphate cementing composition (BioCaS) calcium sulfate or gypsum cement (CaS) and calcium phosphate or apatitic cement (CPC). Each of there were mixed in sterile conditions and placed carefully in defects. After ensuring a complete fill of the defects, the mucosa was sutured back using 6.0 vicryl sutures and animals were kept under observation. The study period was decided to be 1 month (4 weeks). The bone defects without the cements were considered as the sham control. The sample distribution is as follows :

Test material – BioCaS cement, n = 3

Control material – CPC cement, n = 3

Control material – CaS cement, n = 3

Sham control – No materials, n = 3

ii) Tissue harvesting and histopathology evaluation **:** The animals were euthanized after 3 months (12 weeks) by CO_2 inhalation in closed chambers. The animals were decapitated and the entire head containing the alveolar bone was collected in 10 % formalin as fixative, in sealed containers and transferred to the histopathology lab. The fixed samples were processed through standardised procedures and embedded in PMMA blocks for sectioning and histopathology. Micro CT analysis of the defects was carried out in selected samples.

The PMMA embedded tissue specimens were sectioned using a hard tissue microtome, with an average thickness of 100µm sections. The specimens were viewed under microscope. VanGieson's staining and Stevenel's blue staining were done to visualise the new bone formation and the osteoblasts cells respectively.

iii) Results

The histopathology evaluation shows the absence of new bone formation in the sham group. New bone formation was observed in all three groups with cement samples – CPC, CaS and BioCaS groups. In CPC and BioCaS groups, the presence of residual cement particles was noted, with evidence of active resorption and new bone formation at the cement–cell interfaces. In CaS group, complete resorption of cement was noted, and the new bone formed was having a trabecular appearance. This observation on CaS is in accordance with the existing literature, which reports a fast resorption of calcium sulfate material *in vivo*. BioCaS cement, though a modified form of CaS material, has reduced the resorption rate than that of the CaS. The nature of histological appearance confirms the osteotransductivity of the cement. The results of CPC group is also in accordance with the published literature.

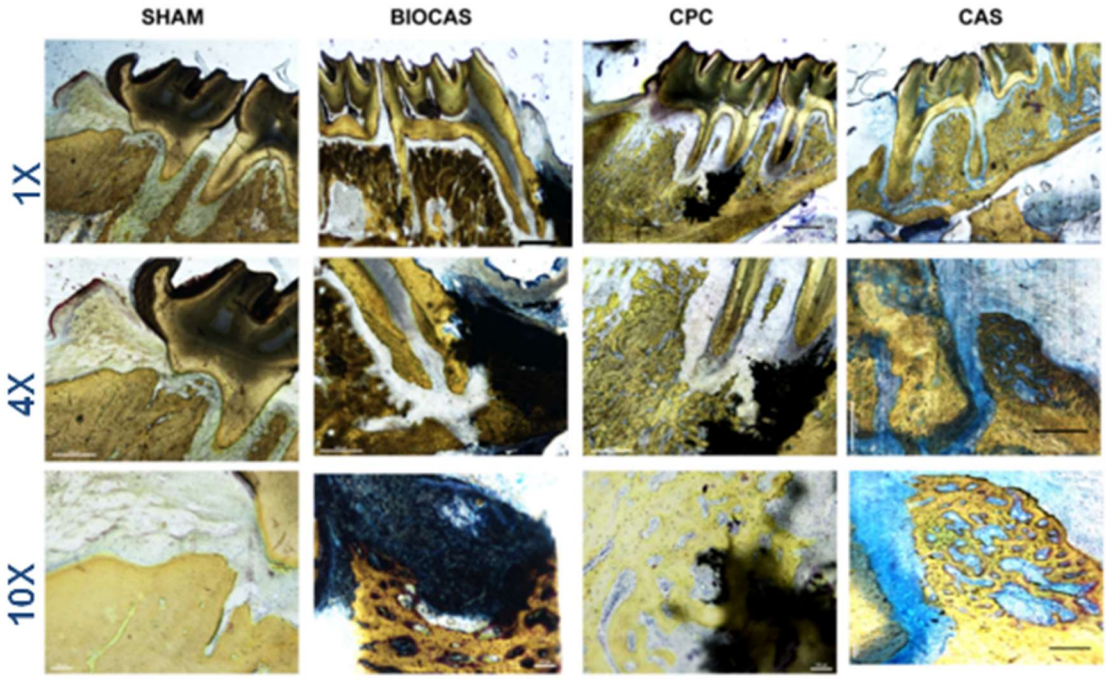


Figure 32. The histopathology images of the different calcium based cement materials, BioCaS, CPC and CaS, along with the sham defect. New bone formation is seen at the cement – tissue interface in CPC and BioCaS group. The cement material was absent in CaS group but new trabecular bone formation is present.

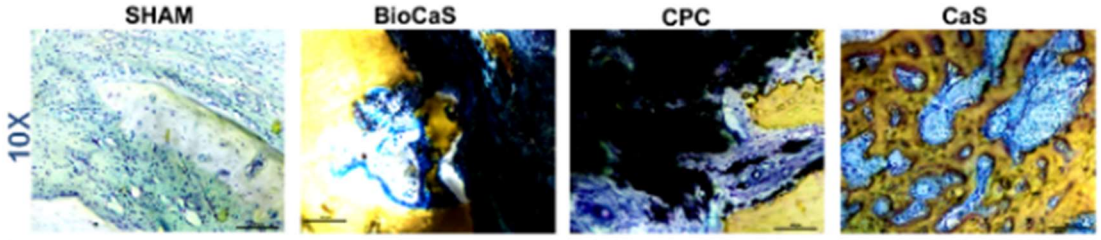


Figure 33. Magnified view of material- tissue interface. The inferences of the Figure 32 is clearly seen in these images.

B1.6.5 Significance of the work.

The animal experiments were planned and conducted to check the periodontal defect healing using the materials designed under the project work. In one set of implantation, Lys-PDA coated PCL membrane was tested along with the

control (uncoated PCL membrane) for its efficacy as a GTR material, in periodontal defects in rats.

Histopathology evaluation showed the presence of gingival overgrowth in the sham control, which is absent in PCL and Lys-PDA coated PCL group. From the results it can be inferred that the barrier function of the PCL mat and Lys-PDA/PCL mats is maintained. However, the osteogenic activity manifested by the new bone formation was minimal in both the PCL and Lys-PDA/PCL groups. It has to be confirmed with further experiments.

In the other set of implantation, the calcium mineral based cementing materials – calcium sulfate or gypsum cement (CaS), calcium phosphate or apatitic cement (CPC) and Bioactive calcium sulfate-phosphate cementing composition (BioCaS) - were evaluated for their potential as alveolar bone grafts/barrier grafts using a rat maxillary alveolar bone defect model.

The histopathology evaluation showed new bone formation in all three cement groups – (CPC, CaS and BioCaS) where as in the sham group, the absence of new bone formation was seen. CaS material completely resorbed within the period. In CPC and BioCaS groups, the presence of residual cement particles was noted, with evidence of active resorption and new bone formation at the cement–cell interfaces. In BioCaS samples, there was new bone formation having a trabecular appearance. BioCaS cement has reduced the resorption rate of the CaS, confirming the osteotransductivity of the cement.

B2. Summary and Conclusions of the Progress made so far

B2.1 Summary of the progress

The prime aim of this project is to develop and evaluate novel tissue engineering materials based on cross-linked self-assembling peptide dendrimers and resorbable calcium phosphate, which can enhance the differentiation and proliferation of dental progenitor cells.

In the first part of the work, a self-assembling dendrimer molecule based on polydiacetylene was designed for the purpose with, guanidine functional group (G-PDA) through original reaction steps. This is a candidate 'extra-cellular material mimicking' molecular structure for dental tissue regeneration application. Dendritic peptide molecule with lysine amino acid incorporated onto a photo polymerisable diacetylene was used as a starting unit. The polydiacetylene core can provide a rigid back bone to attach the functional dendritic peptide guanidine which forms a bioactive terminal of the polymer.

Progenitor cells from human periodontal ligament (hPDL cells) were isolated, cultured and characterized for the cell interaction studies. Human dental tissues were collected ethically from discarded human teeth. The progenitor cells from periodontal ligament (PDL) isolated and cultured successfully. They were characterized by immuno-flourescence imaging using the markers Vimentin (for fibroblast) Scleraxis (for ligament) and Mesenchymal Stem Cell Markers CD90, CD105 and Stro 1 and ensured the 'stemness'.

A flat coating of the G-PDA dendrimer was prepared and the interaction with the cultured progenitor (hPDL) cells were assessed by a series of cytocompatibility tests. The viability of cells grown on G-PDA was assessed using 'live-dead imaging'. The cytoskeletal organisation of the cells was analysed by staining with actin stain. The cells cultured on G-PDA showed high compatibility and viability. The cell spreading was further evaluated by SEM which confirmed the presence of lamellipodia and filopodial extensions, suggestive of active cell migration.

Thereafter the osteogenic differentiation of hPDLCs on G-PDA was studied through histocytological evaluation. Biomineralization, the deposition of calcium

phosphates, was analyzed by staining using Alizarin red and Von Kossa staining. Evidence of calcium phosphate nodule formation was confirmed. Masson's Trichrome Staining showed deposition of collagenous matrix by the cells, showing the capability of remodelling of bone/dentin. As a confirmation step for biomineralisation, immunoflourescence staining of the cells with the osteonectin and alkaline phosphatase antibodies were done. The presence of these markers established the osteogenic differentiation of the hPDLCs in presence of G-PDA.

This part of the work establish that guanidine appended polydiacetylene (G-PDA) can successfully be used as a scaffold for osteogenic differentiation of progenitor cells. It could be helpful for the regrowth of the mineral part of the periodontal area - dentin, cementum and alveolar bone.

The second part of the project work introduces Lysine appended polydiacetylene (abbreviated as Lys-PDA), as candidate 'extra-cellular matrix (ECM) mimicking' molecular structure for dental tissue regeneration application. The material has been synthesized in the form of a self-assembling nano-structured matrix. Dendritic peptide molecule with lysine amino acid incorporated onto a photo polymerisable diacetylene was used as a starting unit. The polydiacetylene core can provide a rigid back bone to attach the functional dendritic peptide lysine, which forms a bioactive terminal of the polymer.

A coating of the Lys-PDA dendrimer was prepared and the surface interactions with the cultured progenitor (hPDL) cells were assessed by a series of cytocompatibility tests. The cells cultured on Lys-PDA showed high compatibility and viability ('live-dead imaging' and actin staining). The cell spreading, evaluated by SEM, confirmed the presence of lamellipodia and filopodial extensions, suggestive of active cell migration. The osteogenic differentiation of hPDLCs on Lys-PDA was studied through histocytological evaluation. Evidence of calcium phosphate nodule formation was confirmed by staining using Alizarin red and Von Kossa. The presence of osteonectin and alkaline phosphatase markers, identified by immunoflourescence staining, established the osteogenic differentiation of the hPDLCs in presence of Lys-PDA.

Continuing with the novel self-assembling dendrimer molecules based on polydiacetylene, Lysine appended polydiacetylene (Lys-PDA), was selected as the candidate 'extra-cellular matrix (ECM) mimicking' material for practical applications. This was identified to be helpful to functionalise the Guided Tissue Regeneration (GTR) membranes.

Electrospun PCL barrier membranes were made and functionalised with Lys-PDA. The cell-material interaction studies showed improved hPDL cell adhesion and spreading on the Lys-PDA/PCL membranes, confirming its utility as a bioactive barrier membrane for periodontal regeneration.

The third part of the project was aimed to make cost effective dental pulp capping agents based on injectable calcium based formulations. Phosphate modified calcium sulfate (BioCaS) and calcium phosphate cements (CPC) are new generation calcium based tissue regeneration materials. Dental pulp cell response to these bioactive cements were studied to define their efficacy. For the purpose, methods for isolating and culturing dental pulp progenitor cells, were developed.

Dental Pulp Cells (hDPCs) were isolated from discarded teeth and cultured, and then characterised. The hDPCs showed positive expression of Vimentin, of the MSC markers CD 73, CD 90 and Stro 1, and of the Dentin Matrix Protein (DMP1). The actin cytoskeletal staining revealed fibroblast morphology and good cytoskeletal organisation.

The osteogenic differentiation of hDPCs in the presence of the cements was evaluated using ICC/IF staining of the cells cultured on CaS, CPC and BioCaS. The hDPCs showed positive expression of the osteogenic markers alkaline phosphatase, osteonectin and DMP 1 at day 7, when cultured onto the cements.

The next part was animal experiments to check the periodontal defect healing using the materials designed under the project work. In one set of implantation, Lys-PDA coated PCL membrane was tested along with the control (uncoated PCL membrane) for its efficacy as a GTR material, in periodontal defects in rats.

Histopathology evaluation showed the presence of gingival overgrowth in the sham control, which is absent in PCL and Lys-PDA coated PCL group. From the results it can be inferred that the barrier function of the PCL mat and Lys-PDA/PCL mats is maintained. However, the osteogenic activity manifested by the new bone formation was minimal in both the PCL and Lys-PDA/PCL groups. It has to be confirmed with further experiments.

In the other set of implantation, the calcium mineral based cementing materials – calcium sulfate or gypsum cement (CaS), calcium phosphate or apatitic cement (CPC) and Bioactive calcium sulfate-phosphate cementing composition (BioCaS) - were evaluated for their potential as alveolar bone grafts/barrier grafts using a rat maxillary alveolar bone defect model.

The histopathology evaluation showed new bone formation in all three cement groups – (CPC, CaS and BioCaS) where as in the sham group, the absence of new bone formation was seen. CaS material completely resorbed within the period. In CPC and BioCaS groups, the presence of residual cement particles was noted, with evidence of active resorption and new bone formation at the cement–cell interfaces. In BioCaS samples, there was new bone formation having a trabecular appearance. BioCaS cement has reduced the resorption rate of the CaS, confirming the osteotransductivity of the cement.

B2.2 Objectives achieved

The specific objectives of this project are numbered one by one and the comments of the status of each are given.

1. To synthesize peptide dendrimers with acetylene foci or diacetylene cores and to fine-tune the gelation chemistry so as to obtain freestanding porous structures having biodegradability and biocompatibility.

→ Two such molecules were designed and synthesized – (i) Guanidine appended polydiacetylene (G-PDA) and (ii) Lysine appended polydiacetylene (Lys-PDA). These were generated as UV-polymerisable form and supplied for the experiments.

2. To isolate and culture human dental pulp progenitor cells in the lab for the in vitro testing of the material and to differentiate them into osteogenic and odontogenic lineages.

→ Dental pulp cells (hDPCs) were isolated from discarded teeth and cultured, and then characterised.

→ As an extended work, human periodontal ligament (hPDL cells) were isolated, cultured and characterized.

3. To conduct *in vitro* cell culture of the peptide dendrimer compositions and resorbable calcium phosphate material with the human pulp progenitor cells, and to study their effects on the differentiation potential.

→ The cell-interactions and osteogenic potential of the Guanidine appended polydiacetylene (G-PDA) were extensively studied using human periodontal ligament (hPDL cells).

→ The cell-interactions and osteogenic potential of the Lysine appended polydiacetylene (Lys-PDA) were extensively studied using human periodontal ligament (hPDL cells).

→ The cell-interactions and osteogenic potential of the calcium mineral based cementing materials were evaluated using human dental pulp cells (hDPCs). The materials include (i) calcium phosphate or apatitic cement (CPC) and (ii) Bioactive calcium sulfate-phosphate cementing composition (BioCaS).

4. To optimize the selected peptide dendrimer by tuning the surface chemistry so that maximized cell response is achieved, which can lead to the regeneration of pulpal and dentinal tissues.

→ This was done and Lysine appended polydiacetylene (Lys-PDA) was selected for tissue regeneration applications. (It has been tested for periodontal tissue regeneration, as an extended objective).

5. To evaluate the optimised peptide dendrimer scaffold in vivo in animal model by implanting in the endodontic space, and to assess the ability of regenerating dentin and pulp histologically in comparison with resorbable calcium phosphate material.

→ The animal experiments were conducted with the ECM-mimicking material, Lysine appended polydiacetylene (Lys-PDA), coated onto a PCL electrospun mat, for periodontal regeneration.
(The objective was shifted to periodontal tissue regeneration, because it is more challenging and useful).

→ The animal experiments were conducted with the calcium mineral based cementing materials for periodontal regeneration. The materials include (i) calcium phosphate or apatitic cement (CPC) and (ii) Bioactive calcium sulfate-phosphate cementing composition (BioCaS). Periodontal tissue regeneration was focussed because it is more challenging and useful.

→ (The animal experiments to study the ability of the materials for regenerating dentin and pulp is kept as a future work. The priority is to design and develop a product for periodontal tissue regeneration based on the above studies).

B3. Details of New Leads Obtained, if any:

- In the work, guanidine appended polydiacetylene has been synthesised through original methods developed in house. As per the literature survey, guanidine-appended polydiacetylene molecular structure is being designed for the first time.
- In the work done during the period, Lysine appended polydiacetylenehas been synthesised through original methods developed in-house. As per the literature survey, Lysine appended polydiacetylene molecular structure is being designed for periodontal tissue regeneration for the first time.
- It was possible to isolate and culture important dental progenitor cells from human periodontal ligament and human dental pulp. This is a notable achievement considering Indian situation.
- A cell culture model has been created using hPDL progenitor cells to test the candidate scaffolds for their potential in regenerating dental tissues.
- A cell culture model has been created using Dental Pulp Cells (hDPCs) to test regenerative potential of endodontic biomaterials.
- A GTR membrane of PCL electrospun material with ECM-mimicking coating of lysine appended polydiacetylene (Lys-PDA), was generated. It exhibited improved cell adhesion, spreading and enhanced the *in vitro* differentiation potential of the hPDLCs.

B4. Details of Publications & Patents, if any:

(a) Paper Published :

1. Eva C. Das, Sameer Dhawan, Jisha Babu, PR Anil Kumar, TV Kumary, V Haridas and Manoj Komath, Self-assembling polymeric dendritic peptide as functional osteogenic matrix for periodontal regeneration scaffolds—an *in vitro* study. J Periodontal Res, 54 (2019) 468

2. Eva C Das, Jisha Babu, Sameer Dhawan, Anil Kumar PR, TV Kumary and V Haridas, Manoj Komath. Synthetic Osteogenic Matrix using Polymeric Dendritic Peptides for treating Human Periodontal defects – design and *in vitro* evaluation. Materials Today: Proceedings 15 (2019) 199.

3. Eva C Das, T V Kumary, P R Anil Kumar and Manoj Komath, Calcium sulfate-based bioactive cement for periodontal regeneration: An in vitro study. Indian J Dent Res 30 (2019) 558.

(b) Conference presentation :

A paper was presented at the International conference on BioMaterials, BioEngineering and BioTheranostics (BioMET 2018, 24th-28th July, 2018, Vellore Institute of Technology, Vellore) organised by Society for Biomaterials and Artifical Organs (India) and Society for Tissue Engineering and Regenerative Medicine (India).

Title: "Synthetic Osteogenic Matrix using Polymeric Dendritic Peptides for treating Human Periodontal defects – design and in vitro evaluation".

Authors :Eva C Das, JishaB abu, Sameer Dhawan, Anil Kumar PR, TV Kumary, V Haridas*, Manoj Komath*.