BIOLOGICAL EVALUATION OF BIOMATERIALS FOR REGENERATION OF DENTAL TISSUES USING TOOTH-DERIVED CELLS

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A THESIS PRESENTED BY

EVA C DAS

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DOCTOR OF PHILOSOPHY

2021

DECLARATION

I, **Eva C Das**, hereby declare that I have personally carried out the work depicted in the thesis entitled ***"Biological Evaluation of Biomaterials for Regeneration of Dental Tissues using Tooth-Derived Cells"*** except where due acknowledgment has been made in the text. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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Reg. No. PhD/03/2015

22 /04/ 2021

TRIVANDRUM

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The thesis entitled, ***"Biological Evaluation of Biomaterials for the Regeneration of Dental Tissues using Tooth Derived Cells"*** was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

Clearance was obtained from the Institutional Ethics Committee/ Institutional Animal Ethics for carrying out the study.

Dr. Manoj Komath

Date: 22 /04/ 2021
Place: TRIVANDRUM

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Dr. Eva C Das

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LIST OF ABBREVIATIONS

AFM	–	Atomic Force Spectroscopy
ALP	–	Alkaline Phosphatase
βTCP	–	β Tricalcium Phosphate
BioCaS	–	Bioactive Calcium Sulfate
BSA	–	Bovine serum albumin
CaS	–	Calcium Sulfate Cements
CEJ	–	Cemento Enamel Junction
CEMP	–	Cementum Membrane Protein
CPC	–	Calcium Phosphate Cement,
DAPCs	–	human Dental Apical Papilla Cells
DFDBA	–	Demineralized freeze-Dried Bone Allografts
DMP1	–	Dentin Matrix Protein 1
ECM	–	Extra Cellular Matrix
ePTFE	–	expanded Polytetrafluoroethylene)
EtO	–	Ethylene Oxide (Sterilization)
FBS	–	Fetal bovine serum
FDA	–	Fluorescein diacetate
FTIR	–	Fourier Transform Infrared Spectroscopy
G-PDA	–	Guanidine polydiacetylene
G-PDA/PCL	–	Guanidine – Polydiacetylene / Polycaprolactone
GTR/GBR	–	Guided tissue/bone regeneration
HA	–	Hydroxyapatite
h.dec.DCs	–	human Deciduous Dental cells
hDPCs	–	human Dental Pulp Cells
hPDLCs	–	human Periodontal Ligament Cells
ICC/IF	–	Immuno Cyto Chemistry/ Immuno Fluorescence
Lys-PDA	–	Lysine polydiacetylene
Lys-PDA/PCL	–	Lysine – Polydiacetylene / Polycaprolactone
MEM	–	Minimal essential medium
MSC	–	Mesenchymal Stem Cell
MTA	–	Mineral Trioxide Aggregate
MTT	–	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	–	Phosphate buffered saline

PCL	–	Poly Caprolactone
PI	–	Propidium Iodide
PRF	–	Platelet Rich Fibrin
PRP	–	Platelet Rich Plasma
SEM	–	Scanning Electron Microscopy
TCM	–	Tissue Collection Medium

LIST OF NOTATIONS

%	Percentage
µg	Microgram
µL	Micro liter
µm	Micrometer
cm	Centimeter
cm^2	Centimeter square
g	Gram
h	Hour
M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
°C	Degree Celsius

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SYNOPSIS

The thesis entitled “Biological Evaluation of Biomaterials for Regeneration of Dental Tissues using Tooth-derived Cells” submitted by Dr Eva C Das, PhD Student (PhD/03/2015), Division of Bioceramics, SCTIMST, deals with a study in the field of Regenerative Dentistry. The work explores the rational methods of evaluating new biomaterials and scaffolds intended for the regeneration of the tooth support structures, using human derived dental progenitor cells. The work is presented in the thesis in 6 chapters.

The first chapter is an introduction to the diseases and treatment strategies of dental tissues, with an emphasis on the biomaterial based regenerative approaches in dentistry. This chapter briefly describes the challenges in understanding the biomaterial based regenerative approaches in dentistry and specific cellular responses to regenerative dental biomaterials. Dental caries, traumatic injuries and periodontitis constitute a global dental health burden. Regenerative Dentistry aims to apply the principles of ‘regenerative medicine’ to regrow dental tissues to its original structure and functions. The tissue engineering approach towards regeneration calls for a healthy coordination of progenitor cells, biomimetic scaffolds and adequate growth factors to promote tissue regeneration. However, in the case of dental tissues, the presence of progenitor cells within the dental pulp and periodontal ligament are reported to aid regeneration. The growth factors are also provided by the host environment. Therefore, attention can be focused on biomaterial and scaffolds for tooth regeneration.

Two approaches can be taken towards biomaterial based regeneration. First approach is the use of biomimetic molecules and polymeric matrices for promoting endogenous cell homing and differentiation. Second approach is to use bioactive materials with regenerative potential, preferably resorbable calcium phosphate and sulfate based cements to achieve regeneration. Since these biomaterials directly come to contact with the cells of dental pulp and periodontal ligament, it would be ideal to study the biological properties of suitable prototype bioactive dental materials *in vitro* using isolated, cultured tooth-derived cells.

The second chapter constitutes a categorized, thorough review of literature to understand the most appropriate and relevant study designs that highlights the isolation and characterization of tooth-derived cells and their characterization and utility in dental biomaterial evaluations. An extensive literature survey is also carried out on the possible

biomimetic matrices based on self-assembling peptide dendrimers towards their use in dentistry. A substantial literature review was also done on the modifications and applications of calcium-phosphate formulations with optimal phosphate ion concentrations, with an emphasis on the role of calcium and phosphate ions on the induction of osteogenic differentiation (applicable to all hard tissues except enamel) by the tooth-derived cells. The availability of suitable small animal models and surgical techniques with minimal trauma to the animals were also studied upon. Based on the extensive literature review, a suitable research hypothesis is formulated, the objectives through which the hypothesis can be proved are enumerated and the methodology of the study was developed, with adequate modifications applied wherever necessary.

The research hypothesis for the study is "Biomimetic matrices and bioactive cements can be evaluated using appropriate *in vitro* model systems for their ability to regenerate damaged dental tissue structures."

To prove this research hypothesis, the major objectives of the study was set as:

1. Isolation and characterization of tooth-derived cells for *in vitro* analysis of biomaterials
2. Identification and optimization of suitable biomaterials for regeneration of Dental Pulp and Periodontium.
    i. Biomimetic matrices based on self-assembling dendritic peptides
    ii. Calcium Phosphates, Calcium Sulfate cement combinations.
3. Evaluation of the selected biomaterials using tooth-derived cell culture system.
4. Biomaterial modifications to suit clinical applications and relevant *in vivo* evaluation.

Chapter 3 describes in detail, the study plan and the experiments designed to achieve the objectives set forth for the thesis. A detailed description of each and every protocols, the reagents and consumables used, and the equipments used for the experiments is narrated in this chapter. The chapter is further divided into four subsections. **Section 1** describes the procedures carried out to establish a tooth-derived cell culture system utilizing the human periodontal ligament cells (hPDLCs) and human Dental Pulp Cells (hDPCs). The cell isolation procedures, culture conditions, characterization and differentiation studies are described in detail. **Section**

2 describes in detail, the procurement and modification of lysine appended polydiacetylene and guanidine appended polydiacetylene; two self-assembling dendritic peptides with potential biomimetic properties; and their cellular interactions with hPDLCs. The cytocompatibility and osteogenic differentiation potential with hPDLCs is described in detail. **Section 3** describes in detail, the cytocompatibility of calcium-sulfate/ phosphate cement combinations for potential dental applications. The experiments for cellular interactions with hPDLCs and hDPCs are described in detail. **Section 4** describes the modifications of the dendritic peptide molecules for potential barrier membrane applications by coating inert electrospun PCL mats with the biomolecules and the cytocompatibility evaluation using hPDLCs. The section also describes in detail, the *in vivo* evaluation of the lysine appended polydiacetylene coated electrospun PCL mats as barrier membrane in a rat maxillary periodontal defect model. The *in vivo* evaluation of the resorbable calcium-sulfate/phosphate cement system as alveolar bone graft substitute in a rat maxillary alveolar bone defect model is also described in detail.

Chapter 4 categorically presents the results of the experiments described in chapter 3, substantiated with adequate and relevant figures, tables and graphs. The methodology followed for the isolation of tooth-derived cells was successful in establishing a healthy population of hPDLCs, hDPCs, deciduous teeth cells and dental apical papilla cells which showed a potential to differentiate to osteogenic (hard tissue formation) lineage upon induction. The hPDLCs and hDPCs were characterized to confirm the presence of progenitor cell population and were selected for further studies. The biomimetic molecules lysine and guanidine appended polydiacetylenes exhibited good cytocompatibility and osteogenic differentiation *potential in vitro*. The bioactive calcium-sulfate/phosphate cements also exhibited good cytocompatibility and osteoinductive properties *in vitro* with the periodontal ligament cells as well as the dental pulp cells. The dendritic peptide molecules were successfully used to biofunctionalize inert PCL barrier membranes and good cytocompatibility was found *in vitro*. The identification of a suitable small animal model and the optimization of surgical techniques to reduce surgical trauma has resulted in better post-operative survival of the laboratory rats in the current *in vivo* system. The *in vivo* studies confirmed the barrier function of the PCL and lysine appended polydiacetylene biofunctionalized PCL barrier membranes by preventing the epithelial overgrowth. The bioactive calcium-sulfate/phosphate cement combinations showed evidence of new bone formation and resorption of the cements in rat maxillary alveolar bone defect model.

In chapter 5, a thorough discussion about the results obtained is carried out in relation to the existing literature. The establishment of a standardized tooth-derived cell culture system is a pioneering step towards appropriate *in vitro* biological evaluation platform for the prospective dental biomaterials. The tooth-derived cell culture system with hPDLCs and hDPCs was successful in elucidating the specific biological responses of biomimetic molecules and bioactive cements, reiterating their translational potential in regenerative dentistry. The *in vitro* results were correlated with the *in vivo* results to select the most appropriate biomaterials for dental tissue regeneration.

Chapter 6 briefly summarizes the results of the study and the interpretations. The results obtained are analyzed to draw appropriate conclusions and guidance to future perspectives regarding clinical translation of the biomaterials. The biomimetic matrices performed well in the *in vitro* system, indicating further studies are required to establish their bioactivity *in vivo.* The bioactive cements showed good potential for periodontal regeneration *in vitro* and *in vivo,* and dental pulp regeneration *in vitro*. The resorbable, bioactive calcium-sulfate/phosphate combinations are a potential biomaterial for tooth regeneration.

The overall work confirms that the objectives to prove the research hypotheses are achieved through the planned methodology. The experiments done and their outcomes are successful, evidenced by the peer-reviewed publications.

The study outcome has notable achievements which could contribute to the field of Regenerative Dentistry in the following aspects-

- Development of cell culture models based on human dental progenitor cells for biomaterial evaluation.

- Devising rational methods of evaluating and screening new-generation biomaterials *in vitro* for their efficacy.

Publications

1. Das EC, Dhawan S, Babu J, et al. Self-assembling polymeric dendritic peptide as functional osteogenic matrix for periodontal regeneration scaffolds—an in vitro study. J Periodont Res. 2019;00:1–13. https://doi.org/10.1111/jre.12647 (Impact factor :2.926)

2. Eva C. Das, Jisha Babu, Sameer Dhawan, Anil Kumar PR, T.V. Kumary, V. Haridas, Manoj Komath, Synthetic Osteogenic Matrix using Polymeric Dendritic Peptides for treating Human Periodontal defects – design and in vitro evaluation, Materials Today: Proceedings, Volume 15, Part 2,2019,Pages 199-216, https://doi.org/10.1016/j.matpr. 2019.04.192. (Impact factor : 1.3)

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Chapter 1

INTRODUCTION

Dentistry or stomatology deals with the diseases, disorders, and conditions of the oral cavity including the dentition, oral mucosa, the alveolar bones, the temporomandibular joints and craniofacial structures; and the diagnosis, prevention, and treatment of the same. The field of dentistry includes other aspects of the craniofacial complex like the supporting, muscular, lymphatic, nervous, vascular, and anatomical structures. However, the main concern to dentistry is the tooth as an organ of mastication. Tooth structure enables an organism to chew the food appropriately to aid proper digestion and absorption of food for the growth and development of the organism.

The tooth consists of three different unique hard tissue components –enamel, dentin and cementum. The dental pulp and the periodontal ligament form the soft tissue components that maintain nutritive, sensory and regenerative functions to preserve the vitality and health of the teeth. There is always a rich supply of progenitor cells from dental pulp and periodontal ligament. Each of this tissue part is prone to degradation or damage.

Dental caries and Periodontitis, the two detrimental diseases affecting the teeth, and traumatic dental injuries can cause inflammation, infections, necrosis causing severe pain and discomfort, ultimately leading to tooth loss if left untreated. The cost of inert replacements to the structure and function of lost tissues using dental restorations, root canal therapies and dental implants is overwhelming. Loss of teeth results in masticatory insufficiency, reduced nutritional uptake, malalignment and migration of opposing and adjacent teeth, reduced sensory inputs and corresponding alveolar bone resorption, along with aesthetic concerns [Jepsen *et al.*, 2017].

Dental restorative approaches are often used as damage control approaches to limit tooth structure loss due to dental caries or traumatic injuries with artificial replacements to resolve aesthetic problems due to lost tooth structure. Earlier metallic restorations including dental amalgam, direct filling gold or metallic inlays are unaesthetic and chances of

secondary caries are high. The emergence of tooth colored restorations including dental composites and ceramic crowns have alleviated the aesthetic concerns with provisions for repair of existing restorations to prevent secondary caries [Blum *et al*., 2018]. Complete loss of tooth requires tooth replacement by artificial means, of which dental implants have a much better biological approach towards rehabilitation than the conventional removable dentures or fixed partial dentures attached to the adjacent teeth. However, the choice of treatment is dependent on the patient's overall health, alveolar bone health and affordability [Kutkut *et al.*, 2018].

Currently, there is a paradigm shift in dentistry to focus on regenerative approaches rather than restoration and/or replacement. Novel biologics and biomaterials are being developed, modified and improvised to suit regenerative approaches. Technological advances have rendered the use of biomolecules like extracellular matrix (ECM) mimetic molecules, bioactive cements, synthetic barrier membranes, injectable hydrogels, natural and synthetic polymers etc. towards tooth regeneration.

This chapter encompasses a brief introduction to Regenerative Dentistry, the role of dental tissues and their embryology towards regenerative approaches, and the presence and role of the resident dental progenitor cells. The chapter also describes the role of various biomaterials and the need for their biological evaluation prior to clinical translation. An effort is made to the use of appropriate *in vivo* animal models and their relevance towards pre-clinical evaluation of potential dental biomaterials.

1.1 Regenerative dentistry

Regenerative dentistry is an emerging field that continually challenges the traditional and modern concepts in dental research. It adopts the principles of regenerative medicine and translates the scientific knowledge into novel clinical treatment strategies. The regenerative approaches are based on the understanding of the basic mechanisms of tooth development, the biological processes of healing and repair, and the potential for regeneration. It applies the biologic principles to harness the innate healing and regenerative potential of dental tissues, and/or engineering the damaged tissue or organ [Volponi *et al.*, 2018].

Regenerative Medicine is an interdisciplinary treatment strategy that applies the biologic principles of growth and development and advances in bioengineering to modulate the cellular events during tissue damage to promote regeneration. This strategy evolved, owing to the need to find an alternative to organ replacement and maintenance therapies following organ/tissue damage, thereby reducing the need for organ donors, and the associated ethical issues. Regenerative therapies utilize therapeutics, tissue engineered scaffolds, growth factors, mediators, engineered biomolecules and matrices to enhance the intrinsic regenerative capacity of the host tissues, by immunomodulation, or favorably altering host tissue environment to achieve regeneration [Mao *et al.,* 2015].

Regenerative Dentistry emerged through application of the principles of Regenerative Medicine that attends to the regeneration of dental tissues through the use of biologics (e.g. Platelet Rich Plasma - PRP, Platelet Rich Fibrin – PRF, induced blood clots), therapeutics (antibiotics, growth factors etc.), biomaterials, and regenerative treatment strategies that modulate and/or recapitulate the natural wound healing and regenerative events within the dental tissues, to promote tooth regeneration [Volponi *et al.,* 2018]. The evolution of Regenerative Dentistry is brought about by the knowledge on the cellular and molecular processes concerned with tooth development and regeneration [Muller *et al*., 2017]. Regenerative Dentistry utilizes technological advancements in bioengineering, 3D bioprinting, electrospinning of natural and synthetic polymers, directed self-assembly of biomolecules, injectable hydrogels loaded with therapeutics or bioactive molecules, advanced functionally and structurally graded scaffolds with variable porosities and angiogenic properties, biomaterials with tunable bioactivity and resorption etc. [Yelick *et al*., 2019]. Since human tooth is considered as an organ, any damage to tooth associated tissues can be benefitted from the principles of Regenerative Dentistry. This approach attempts to bring together the collective treatment approaches to restore the structure and function of the tooth to a near perfect state comparable to the healthy natural teeth.

Ideally, for dental pulp regeneration to be complete, the tissue formed should be vascularized, with dentinal walls lined by newly differentiated odontoblasts that are capable of continued dentin deposition onto the existing dentin matrix if a part of healthy pulp tissue remains. This can be enhanced by proper biomaterials that can bring about regeneration and are able to resorb at par with the new tissue formation [Volponi *et al*., 2018]. Similarly, the criterion for periodontal regeneration is healthy ligamental attachment between supporting

alveolar bone and cementum through Sharpey's fibers, without the formation of long junctional epithelium. Alveolar bone regeneration criteria are the clinical restoration of bone height and width in a treated periodontal defect [Position Paper: Periodontal Regeneration, 2005].

In order to apply the principles of Regenerative Dentistry, a clear understanding is needed, related to the tooth structure, the development and embryology of the dental tissues, the resident progenitor cell population and their role in routine maintenance, and the responses, repair and regeneration following diseases or injuries.

1.1.1 Tooth structure and components.

The tooth is a complex organ with adequate strength, flexibility and sensory responses. It is firmly attached to the jaw bones to serve the purpose of mastication. Apart from mastication, proper dentition also aids in phonation and contributes to the aesthetics. Healthy teeth and supporting structures contribute to the proper growth and development of the face, by promoting the growth of jaw bones. Any diseases affecting the teeth and supporting structures can cause discomfort, pain and considerable difficulty in mastication of food, depending on the severity of the conditions. The tooth consists of a hard, inert, acellular outer covering called enamel, supported by dentin, the less mineralized, more resilient and vital hard tissue. Dentin is formed and maintained by the dental pulp, the soft connective tissue component that forms the nutritive, sensory and regenerative element to the dentin. Together, the dentin and pulp is known as the pulp-dentin complex, with dental pulp having the ability to lay down primary, secondary, reactionary or reparative dentin, depending on the needs and external stimuli [Nanci and Ten Cate, 2017].

Enamel is highly mineralized (96 %) with calcium-fluorapatite crystals and traces of organic components - mainly proteins in between, arranged in a well organized manner. Enamel, once lost, cannot be regenerated, due to the loss of progenitor cells during tooth eruption. The dentin consists of an organic layer composed of dense collagen fibers arranged as closely packed tubules traversing the entire thickness of dentin, and retains the extensions of the odontoblasts within the tubules, and hence is vital. The cell bodies of the corresponding odontoblasts line the inner boundary of dentinal tubules and form the outermost boundary of dental pulp. The dense collagen matrix of dentin is 70 % mineralized with calcium hydroxyapatite crystals, providing the much needed resilience to the tooth. The dentin is

capable of regeneration and repair, as long as the odontoblasts or odontoblast progenitor cells from the dental pulp are available (Figure 1). The dentin encloses the dental pulp and retains the formative, nutritive, sensory and reparative functions, as long as it is vital [Nanci and Ten Cate, 2017].

The tooth is attached to the maxilla and mandible through a specialized supporting structure composed of the cementum, the alveolar bone and the periodontal ligament, which are protected by the gingiva, and the structure together is called the periodontium. The tooth attachment is achieved by means of the periodontal ligament, connected on either side to the alveolar bone and the cementum, through Sharpey's fibers. These collagen fibers act as individual units, remodeled and repaired individually, without affecting the whole system, to withstand the heavy masticatory forces. The cementum covers the root dentine and is composed of mineralized collagen matrix similar to bone, but contains 5% more mineral concentration. It is divided into acellular primary cementum to which PDL fibers are attached and cellular or secondary cementum that has an adaptive role. The cementum, PDL, and alveolar bone function as a single functional attachment unit. (Figure 1) [Nanci and Ten Cate, 2017].

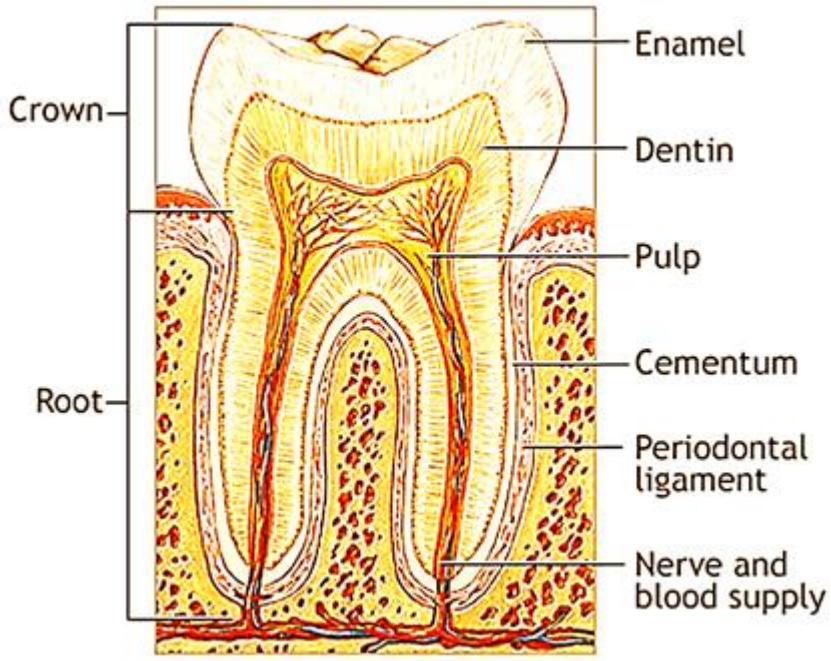


Figure 1: Structure and Anatomy of human tooth.

In the case of humans, a limited succession in dentition occurs by means of the deciduous and primary dentition, to accommodate the growth and development of facial structures. Anatomically, the tooth is divided into a crown and root, demarcated by a thin line called the cemento enamel junction (CEJ). The dentin part above the CEJ is covered by enamel and that below is covered by the cementum. The crown is responsible for pulverizing the food, by means of the sharp and strong cusps and ridges, while the root anchors the tooth to the supporting alveolar bones firmly, to allow mastication.

1.1.2 Tooth embryology and development.

The development of tooth organ is a long and complicated biological process, regulated by the sequential and reciprocal interactions between the oral ectoderm and the underlying neural crest cell derived mesenchyme (ectomesenchyme). A series of epithelial thickenings are formed, which proliferates and grows into the sites of future primary teeth, these structures being termed as dental lamina and the tooth buds forms inside the dental mesenchyme as condensations and the odontogenic signals begin to fire [Amrollahi *et al.*, 2016]. Next, in the cap stage, the primary enamel the epithelial cells are compartmentalized to three distinct regions - outer epithelium, inner epithelium, and central cell layers stratum intermediate and stellate reticulum. The inner enamel epithelium of ectodermal origin differentiates to ameloblasts during the bell stage. This triggers the differentiation of odontoblasts from the underlying dental papilla to lay down the dentin matrix, while the ameloblasts lay down enamel. These specialized cells called ameloblasts are lost once the tooth erupts; making the regeneration of enamel impossible once it is formed. After tooth formation, dental papilla is covered in a layer of dentin and resides as the dental pulp, incorporating the odontoblasts. A portion of the dental papilla is retained in the tooth root apex for continued root end formation and maturation, i.e., for 2-3 years after tooth eruption. Once tooth root formation is complete, the apical papilla is lost [Nanci and Ten Cate, 2017]. Routine dentin deposition by odontoblasts continues throughout life, and when stimulated, can lay down reactionary dentin. The tissue known as reparative dentin is formed by odontoblast-like cells differentiating from the dental pulp.

Root maturation and the development of cementum, alveolar bone, and periodontal ligament occur after the crown part followed by tooth eruption. The dental follicle cells, which form the investing cell layers around the whole tooth germ during tooth development,

give rise to the cementoblasts and periodontal tissues including periodontal ligament, supporting alveolar bone and gingiva [Ten Cate,1997; Honda *et al*., 2010]. Continual remodeling of periodontal ligament happens throughout the life, as the periodontal ligament fibroblasts can lay down collagen, and differentiate to osteoblasts or cementoblasts, upon need.

1.1.3 The resident dental progenitor cell population

Stem cells or progenitor cells are population of cells that retain the characteristics of self-renewal and differentiation that is essential for the regeneration of tissues. The tooth is a treasure house of progenitor/stem cells [Saito *et al.,* 2015]. Their function is regeneration and regular remodeling of dental tissues following damages due to sub-acute trauma or disease conditions. Different human dental stem/progenitor cells have been isolated and characterized including dental pulp stem cells, stem cells from exfoliated deciduous teeth, periodontal ligament stem cells, stem cells from apical papilla, and dental follicle progenitor cells. These tooth-derived stem cells exhibit mesenchymal stem cell properties like self-renewal, multilineage differentiation potential and sharing certain common stem cell markers like CD 90, CD 73, CD 105 and STRO-1. These dental stem cells are reported to differentiate into osteo/odontogenic, chondrogenic, neurogenic and myogenic lineages.

The periodontal ligament harbors a heterogeneous cell population capable of differentiation to cementoblasts (cementum forming cells), osteoblasts (bone-forming cells) and fibroblasts that lays down and remodels collagen [Ozer *et al.*, 2013]. The dental pulp cells can be induced to differentiate into osteogenic, odontogenic, and dentinogenic lineages, along with potential to differentiate to chondrogenic, adipogenic, neurogenic and myogenic lineages [Gronthos *et al.*, 2000]. The tooth-derived cells may exhibit variations in their osteogenic differentiation potential, and similar histological and marker expressions are reported to be used for osteo/cemento/odontogenic differentiation since differentiation to all three lineages depends on type 1 collagen deposition, and its mineralization [Winning *et al*., 2019].Currently, no specific biomarkers that uniquely define dental pulp cells or periodontal ligament cells are reported, but they do express MSC-like phenotypic markers such as CD73, CD90, CD105, and STRO-1, as well as fibroblast marker Vimentin. The isolated, characterized tooth-derived cells are invaluable tools in *in vitro* cell culture systems to study cellular responses to biomaterials, biomolecules and antimicrobial agents.

1.2 The diseases of dental pulp and periodontium

Dental caries and periodontitis are two major dental diseases that can result in tooth loss [Frencken *et al.*, 2017]. The localized dissolution of enamel through the acids produced by biofilms adhering to enamel surface in the constant presence of sugars lead to dental caries, which can progress to damage dentin and cause pulpal inflammation and infection, if left untreated [Fejerskov *et al*.,2015]. Diseases of periodontium (gingiva and periodontal ligament) are primarily inflammatory in response to bacterial toxins released from dental plaque micro biota, and can lead to tooth loss, if left untreated [Chapple *et al.*,2015]. In addition, traumatic injuries can do considerable damage to tooth structure, dental pulp and periodontium [Peti *et al*., 2018].

Understanding the epidemiology of the dental diseases, the differences between the conventional and regenerative treatment strategies and the role of biomaterials in the treatment approaches is important to bring about cost effective and most ideal biomaterials for the treatment of dental disease conditions.

1.2.1 The epidemiology of diseases of dental pulp and periodontium

The Global Burden of Disease Study 2016 estimated that oral diseases affected half of the world's population (3.58 billion people) with dental caries (tooth decay) in permanent teeth being the most prevalent condition assessed. Periodontal (gum) disease, which may result in tooth loss, was estimated to be the 11th most prevalent disease globally [Kassebaum *et al*., 2017]. Periodontitis, or the inflammation of the periodontium, affects the attachment apparatus that anchors the tooth to the supporting alveolar bone, often damaging the bone, resulting in tooth loss. More than 80 % of adult population suffers from varying intensity of periodontitis. Dental treatment is costly, averaging 5 % of total health expenditure and 20 % of out-of-pocket health expenditure in most high-income countries. The direct cost of treatment increases from that of minimally invasive procedures to prevent caries or periodontitis, to aesthetic restorations, endodontic and periodontal managements, and replacement of teeth with dentures and implants. There are indirect burdens of physical extortion, pain, psychological stress and time loss [Frencken *et al.*, 2017]. The replacement of lost tooth by means of dental implants is also prone to conditions like peri-implantitis, which may require removal of the implants, resulting in further trauma and economic burden [Tonetti *et al.*, 2017].

1.2.2 Conventional treatment strategies for diseases of tooth

The conventional treatment strategies for management of dental carries involves placement of appropriate restorations, currently, tooth colored aesthetic restorations, to restore the lost tooth structure, so that the tooth function is maintained. Since enamel cannot be regenerated, replacement with a most suitable substitute is the only solution so far. Currently, the restorative materials are classified into resin based (consisting of dental composites), and cement based (consisting of glass ionomer cements). The dental composites are retained by micro-mechanical retention through resin tags into etched enamel and conditioned dentin, whereas the glass ionomer cements form weak chemical bonds with the inorganic ions in enamel and dentin [ADA Council on Scientific Affairs, 2003]. The new paradigm in restorative dentistry is the emergence of bioactive restorative materials that can remineralize and strengthen the remaining tooth structure through apatite like mineral deposition on surface and margins by means of fluorides and/or appropriate mineral releases, thereby preventing secondary caries; or can regenerate pulp tissue to promote/preserve the vitality in the tooth [Chen *et al*., 2013].

Temporary restorations are given in deep carious lesions, using obtundent cements made of zinc oxide-eugenol or calcium hydroxide pastes. Regenerative or reparative strategies for dentin are possible by means of indirect and direct pulp capping, as the clinical situation necessitates. In case of immature young permanent teeth with incomplete root formation, pulpotomy or removal of the diseased part of the pulp, followed by placing suitable biomaterials to sustain the remaining pulp so as to complete root formation (Apexogenesis) is attempted. In case of necrosed dental pulp, apexification or artificial root end formation is initiated using suitable biomaterials. In all the treatment strategies, there is scope of further advancements in the type of biomaterials involved.

The management of periodontal diseases is carried out using routine scaling and root planning activities in mild cases. In moderate to severe periodontitis, Guided tissue/bone regeneration (GTR/GBR) strategies using periodontal barrier membranes and alveolar bone grafts are used either alone or together, to achieve proper ligamental attachment between newly formed cementum and alveolar bone, without interferences from a gingival epithelial overgrowth [Nyman *et al*.,1991]. Here also there is scope for bioactive barrier membranes and bone grafts that can act as barrier grafts.

1.2.3 Regenerative treatment approaches in dentistry

Dental tissues retain a complex structural architecture with anisotropic mechanical properties and a heterogeneous cell distribution which makes it hard to mimic through synthetic means [Amrollahi *et al*., 2016]. Regenerating vascularized and innervated dental pulp, new alveolar bone and cementum tissues, and healthy periodontal ligament with Sharpey's fibers embedded on either side, is a highly coveted goal in Regenerative Dentistry. In case of periodontium, GTR/GBR treatment strategies depend on selective repopulation of progenitor cells to the diseased region, the space maintained by preventing unwanted epithelial cell migration using barrier membranes, to achieve natural healing and proper regeneration of the damaged tissues [Nyman et al, 1991]. The same principle was applied to treat large periradicular lesions of endodontic origin also [Pecora *et al*., 1995].

In case of the pulp-dentin complex, treatment strategies termed vital pulp therapies indicate the regenerative approaches to preserve the vitality of dental pulp and thus retain a functional, vital tooth. Depending on the severity of pulpal involvement, vital pulp therapies include indirect pulp capping, direct pulp capping, pulpotomy with apexogenesis, and pulpectomy with apexification. Endogenous regenerative approaches by inducing cell migration towards the dental pulp space using growth factors and mediators are reported as promising strategies to regenerate dental pulp [Suzuki *et al.*, 2011].

Current advancements in biomaterial development have led to the emergence of bioactive barrier membranes that can enhance alveolar bone formation; barrier graft materials which can act as alveolar bone grafts as well as physical barrier in GTR/GBR procedures; bioactive cements for pulp regeneration through pulp capping; and injectable hydrogels to attract cells to the dental pulp space towards regeneration [Rajesh *et al*., 2009].

1.2.4 The role of biomaterials in tooth regeneration

Biomaterial is defined as a synthetic or biologic material that can be introduced to the body as a biologic matrix or scaffold to replace an organ or part of an organ completely, or till the function of the tissue is restored. Hench defined a bioactive material as "the material that elicits a specific biological response at the interface of a material which results in the formation of a bond between the tissues and the material" [Hench, 2006]. A truly regenerative biomaterial should be able to regenerate live tissues like dental pulp and periodontal ligament

to restore the vitality and function of the tooth. Smart biomaterials and constructs refer to biomaterials and constructs that possess instructive/inductive or triggering/stimulating effects on cells and tissues by engineering the material's responsiveness to internal or external stimuli or have intelligently tailored properties and functions that can promote tissue repair and regeneration [Yuan *et al*., 2011].

Biomaterial based regeneration can be divided into (i) Regeneration of dentin by the formation of either reactionary or reparative dentin, (ii) Regeneration of whole or part of dental pulp to preserve the vitality of the tooth, (iii) Regeneration of part or whole of periodontium involving cementum, alveolar bone and periodontal ligament, and (iv) Remineralization of dentin and enamel mimicking the original tissue architecture [Yuan *et al*., 2011]. Biomaterials have been in use for the regeneration of dental tissues as bioactive restorative materials that promote remineralization, dental pulp capping agents, apexification and revascularization agents, GTR barrier membranes, and bioactive bone grafts. The biomaterials used in regenerative dentistry can be divided into (i) Natural biomaterials like collagen, chitosan, decellularized tissue matrices, demineralized bone etc., (ii) Synthetic polymeric materials like PLA, PLGA, etc. and (iii) Synthetic inorganic materials like calcium phosphates, hydroxyapatite, bioglass etc. [Amrollahi *et al*., 2016]. Combinatorial approaches using different types of biomaterials like natural and synthetic polymers, organic inorganic composite biomaterials, biomaterials enriched with bioactive molecules etc. can enhance the regenerative potential of biomaterials. Current advances in material science including electrospinning, 3D bioprinting, in situ hybridization of inorganic salts such as calcium phosphates to natural or synthetic hydrogels, incorporation of growth factors and mediators into biologic matrices, etc. can elevate the existing treatment strategies with predictable results. Biomaterials can act as scaffolds or matrices to provide three dimensional supports for cells and promote selective repopulation of local progenitor cells through migration and cell adhesion followed by differentiation [Bottino *et al*., 2017].

1.3 Endogenous treatment strategies for tooth regeneration

The current concept of tissue engineering strategies including exogenous cells along with suitable scaffold and mediators is difficult to apply in tooth regeneration practically, due to the cell sourcing and preservation of cell vitality in the scaffold. This issue can be

eliminated by promoting endogenous homing of resident cells like odontoblasts, fibroblasts of dental pulp and periodontium, cementoblasts, osteoblasts and stem/progenitor cells to aid regeneration, with the presence of a scaffold [Yuan *et al*., 2011]. The blood clot formed at the site of injury can act as a scaffold as well as a storehouse of growth factors, which are released to recruit circulating and local stem cells and progenitors, through complex signaling cascades to regenerate new tissue. This strategy is used in root canal revascularization procedures to restore tooth structure of immature permanent teeth. Certain biomaterials with proven hard tissue regeneration abilities are used along with the blood clots. Autologous biologics like platelet rich plasma and platelet rich fibrin (PRP and PRF) are also used for endogenous regenerative approaches [Chen *et al*., 2011]. Endogenous regenerative approaches utilize nontoxic, biocompatible and bioactive biomaterials as biologic scaffolds to provide anatomic structure of temporary matrices, biophysical support, biochemical agents and biologic cues to enhance cell recruitment/homing, cell adhesion, proliferation and differentiation. In endogenous regenerative approach, the responses of resident progenitor cells to the site of injury is modulated and induced using suitable biomolecules or biomaterials, with the body simulating an in vivo bioreactor to regenerate tissues [Suzuki *et al*., 2011].

1.3.1 Biomimetic matrices to promote cell homing for endogenous regeneration

During endogenous tissue regeneration, the target site must be modulated to facilitate coordinated cell movements, with immune modulation of the local micro environment to allow the recruitment of endogenous cells through physicochemical modification. A variety of therapeutic tools including chemical moieties, chemo attractant gradients, mechano-physical modifications, biological agents and/or biomaterials are used to facilitate/augment recruitment of resident reparative cell populations to the injured site by cell mobilization and homing [Suzuki *et al.*, 2011]. The use of biomimetic or ECM mimetic matrices is made possible with tunable smart biomaterials [Haridas *et al*., 2014].

Synthetic biomaterials lack the inherent extracellular milieu that is important as the native cells reside within a niche composed of dynamic and complex ECM, with integral micro-environmental cues to dictate cell behaviors and functions. The native matrices have significant potential to promote cell homing because they represent warehouses of various growth factors. Therefore, blood derived biologics, decellularized tissues and acellular

matrices with abundant native biologic cues can be used as biomaterial scaffolds for endogenous regeneration [Chen *et al*., 2011].

By understanding and recapitulating the hierarchical assembly, interfaces and the interactions between the base units or biological building blocks; scientists have attempted to fabricate bio inspired, biomimetic matrices, for regenerative approaches in multiple tissue and organ systems. These biomimetic materials are made possible through chemical processes like directed self-assembly, bio patterning, micro nano fabricating techniques, 3D printing, electrospinning, additive manufacturing procedures etc. [Haridas *et al*., 2014]. Biomaterials with varying stiffness, depending on their site of final use, like hydrogels, membranes, bioceramic constructs, etc. are fabricated to enhance cell homing and endogenous regeneration [Dissanayake *et al*., 2015].

1.3.2 Biomaterials for endogenous tooth regeneration

Bone, dentin and cementum are composites composed of an organic type 1 collagenous matrix with a hierarchical arrangement of hydroxyapatite crystals, which endows it with unique mechanical properties, including low stiffness and high fracture toughness to resist tensile and compressive forces. Understanding the functionality and complexity of biological systems and knowledge of basic biological principles of collagen deposition and biomineralization processes is essential to design appropriate biomaterials for the regeneration of alveolar bone, dentin and cementum.

To achieve periodontal ligament regeneration, coordinated deposition of newly formed cementum and alveolar bone with collagen fibers interspaced between these two hard tissue components, by means of healthy Sharpey's fibers, is a necessity. Collagen barrier membranes applied alone or combined with alveolar grafts, are used in GTR/GBR procedures to achieve periodontal regeneration. Current advances in biomaterial designs such as electrospinning and directed self-assembly are aimed to produce composite bioactive barrier membranes. Also "barrier grafts" are designed out of self-setting injectable cement formulations or biomimetic hydrogels, with optimum resorption properties to bring out regeneration [Rajesh *et al*., 2009]. Such matrices can be laden with growth factors, biomolecules, small bioactive peptides or nano-calcium formulations or antimicrobial agents [Bottino *et al*., 2017].

Regeneration of dental pulp is evaluated by the formation of new dentin or 'dentin-bridge' at the margin of the exposed or treated dental pulp by means of biomaterials. The differences in pulp repair and regeneration results in the formation of reparative dentin and/or reactionary dentin by the recruitment of pulp progenitor cells or odontoblasts as such. Calcium hydroxide has been the material of choice for pulp capping, for decades, and is currently challenged by the novel more biologically acceptable materials such as calcium phosphates [Bottino *et al.*, 2017]. In addition, injectable hydrogels loaded with growth factors or antimicrobial agents are being studied upon to attract cells which can differentiate to odontoblasts when in contact with dentinal tubules [Galler *et al.*, 2012]. Such biomaterials should accommodate an appropriate 3-D porous structure as a template for initial cell attachment and subsequent tissue formation with vascularization, mechanical integrity, and integration with host tissues [George *et al*., 2010]. Currently, osteoconductivity by means of growth factors, proteins, peptides, and inorganic calcium and phosphate ions that can recruit and induce precursor cells to osteogenic lineage is being attempted with various structural matrices. Since the biology of dentin and cementum is similar, osteogenic matrices can also support cemento/odontogenic differentiation.

1.4 In vitro evaluation of biomaterials for tooth regeneration

Biocompatibility is defined as the ability of a biomaterial to elucidate an appropriate and favorable host response [Polyzois, 1994]. Every biomaterial needs to be evaluated for *in vitro* cytocompatibility, as a first line of acceptance for further *in vitro* and *in vivo* biocompatibility evaluations, before being forwarded for human clinical trials [ISO 10993-5]. Although different cell lines are generally used for preliminary cytocompatibility evaluation studies, a better cell source will be the cells derived from the tissues to where its final use is intended. Isolated primary cells from the appropriate tissue sources, if well characterized, can be utilized to carry out *in vitro* cytocompatibility evaluations [Taraslia *et al*., 2018]. In addition to a more appropriate cytocompatibility evaluation, such primary cells can be used to study their specific biologic responses towards the biomaterials including lineage commitment and differentiation, specific gene and protein expression, mineralization, matrix deposition etc. The advances in cell culture techniques using 3D bioprinted constructs have further provided the opportunity to study the material-cell

interactions in conditions mimicking the host environment. Therefore, the ideal strategy of *in vitro* evaluation of dental biomaterials should include well characterized tooth-derived cells.

Guided regeneration strategies for periodontal regeneration (Guided Tissue Regeneration/ Guided Bone Regeneration or GTR/GBR) are aimed to achieve selective repopulation of the periodontal defect by the periodontal ligament fibroblasts. Alveolar bone grafts and endodontic sealers also come in contact with the periodontal ligament fibroblasts. Therefore, the periodontal ligament cells are ideal for the *in vitro* cytocompatibility and cell interaction studies of periodontal barrier membranes, alveolar bone grafts and endodontic sealers. In case of biomaterials for dental pulp regeneration, those materials that can come in contact with the dental pulp - direct pulp capping agents - can be evaluated for cytocompatibility and cell interaction studies using dental pulp cells. Apart from the routine biological evaluations, it is possible to study the osteo/odonto/cementogenic differentiation potential through gene expression profiles by PCR, mineralization potential by histological staining, enzyme activity by assays and marker expression by immunocytochemistry. The use of 3D culture systems can simulate the native environment for the host tissues.

1.5 In vivo and preclinical evaluation of dental biomaterials

Large animals, like swine, primates, dogs and minipigs are the commonly used and well-established experimental models to study periodontal defect healing. The choice is based on the similarity in the size of the teeth, masticating patterns and the structure of periodontal tissues. Dog model is commonly used and well-established and the mini pig model is considered a good alternative. Small animal models, though not very suitable considering the differences in the tissue size and structure, are adopted for the reasons of difficulties in handling and maintenance of large animals. The rabbit model is preferred in small animals, because the tooth sizes are larger than the rodent models. Periodontal and pulpal defects can be created in these models either by pathologic induction or by surgical procedures [Kantarci *et al*., 2015; Suzuki *et al*., 2011].

Rodents are commonly used animal models for *in vivo* evaluation of biomaterials owing to the ease of standardization, handling and lower cost of maintenance. The mice and

guinea pigs are very small to conduct oral procedures with precision. Rats are ideal candidates for systemic diseases, but surgical procedures are difficult due to the small size and continued tooth eruption with bone and cementum deposition. Periodontal and alveolar bone defects can be created in rats to study regeneration. Even though the incisor teeth of rats are continually growing, the molars are similar to miniature human teeth to permit creation of alveolar bone defects and pulpal defects to test biomaterials.

The *in vivo* studies are important to understand the local and systemic responses to biomaterials in a dynamic host environment. *In vivo* pre-clinical usage tests are mandatory to obtain approval from regulatory bodies for the human use of biomaterials. For tooth regeneration, periodontal disease models and periodontal or alveolar bone defect models can be designed. Pre-clinical experiments on pulp exposure for pulp capping agents and root canal preparation for endodontic biomaterials can be carried out as per standards.

1.6 Challenges in biomaterial based tooth regeneration

The biomaterials have evolved through 3 generations – (i) Inert materials with no interaction with biological tissues, (ii) Bioactive materials eliciting controlled positive tissue responses, and (iii) Materials and structures having the potential to stimulate cellular and molecular events towards regeneration, by means of specific molecules and/or design parameters. Current 21st century biomaterials including bioceramics and cements for hard tissue regeneration includes bioactivity, immunomodulation, infection control and prevention of microbial contamination and predictable cellular and molecular responses [Raman and Bashir, 2017].

Different dental tissues possess varied ability to regenerate the damaged tissues. The regenerative capacity varies between individuals and is often affected by systemic illnesses like diabetes mellitus, osteoporosis etc. Initial blood clot formation and organization also influence the healing and regeneration of tissues, especially dental pulp. The traditional biomaterials like calcium hydroxide can cause initial necrosis of adjacent cells directly in contact, and weakening of teeth whereas MTA can cause discolorations of teeth and is not bioresorbable. Regeneration of dentin with traditional approach is clinically proven with radiographic evidence of hard tissue formation.

Despite the proofs of healing of periapical region, continued root end development and positive vitality responses, it is doubtful whether the hard tissue formed is actually dentin. It is often found to be osteoid, cementum, ectopic bone, fibrous tissues or unorganized dentin-like tissues without the formation of a proper dentin-pulp complex [Dissanayake *et al*., 2015]. This unpredictability in dentin formation by conventional materials has led to various alternative biomaterials – cements, hydrogels, self-assembling polymers, natural and synthetic polymers, lysine dendrigrafts etc. However, most of these are still in proof-of-concept stage and these have not yet been proved to regenerate dentin with full predictability [Haridas *et al*., 2014]. Cell laden biomaterials scaffolds possess the ethical concerns of autologous cell source, disease transmission and preservation of cell viability.

The restoration of the three-dimensional structural and functional architecture of the periodontium still remains a challenge in biomaterial-based regeneration. The principle of periodontal regeneration is based on prevention of the epithelial cell migration and separate the proliferating epithelial cells from hampering hard tissue repair that occurs slower by a process called cell exclusion and selective repopulation. To achieve this, physical barrier membranes are placed that can stay in the defect region for 3–4 weeks, which is called the Guided Tissue Regeneration (GTR) technique. GTR Barrier membranes have been successfully demonstrated to heal tissue losses involving minimal alveolar bone defect. However, the membranes alone will not be sufficient in the case of large vertical bony defects which call for additional placement of bone grafts.

Different kinds of bone graft materials are being used, both of biological origin (e.g., allograft - DFDBA) and synthetic origin (e.g., hydroxyapatite granules), but a complete predictable regeneration of periodontal tissues is not yet demonstrated. Both have their own advantages and drawbacks. Complex three-dimensional matrices and scaffolds have been developed with excellent *in vitro* results, but they are yet to reach successful clinical translation. The different advanced biomaterial based therapeutic strategies for periodontal regeneration have still not met with any notable success, through the formation of proper fiber-mineral tissue complexes along with fiber orientation and spatial tissue compartmentalization were observed [Bottino *et al.*, 2017].

The biomaterials should ideally be capable of regenerating the diverse tissues, comparable to natural tissues both structurally and functionally.

1.7 Knowledge gaps in existing research

In the current research scenario, so many novel biomaterials and biomolecules are being proposed for regenerative applications [Haridas *et al.*, 2014; Sandhya *et a.* 2017; Nivedhitha *et al.*, 2016]. The *in vitro* biocompatibility of these biomaterials is carried out by using established cell lines or randomly decided tissue derived cells, and no specific *in vitro* test model could be found which can apply across all classes of materials developed. It would be ideal if host tissue specific cells are selected for the *in vitro* evaluation. This would give better correlation than that of an established but unrelated cell line and help taking decisions in clinical translation of these biomaterials [Cavalcanti *et al*., 2013; Dissanayake *et al*., 2015]. Currently, there is no established standard for specific cell culture models or protocols towards utilizing tooth-derived cells for the biological evaluation of biomaterials for dental regeneration.

From among the tooth-derived cells, most appropriate cells that can be used to evaluate regenerative biomaterials for the intended application like periodontal ligament regeneration and dental pulp regeneration. Establishing a standard cell culture procedure for biomaterial evaluation can be applied for the evaluation of novel biomolecules and bioactive cements that have proven or expected biological effects using tooth-derived cells.

The use of different tooth-derived cells will delineate the regenerative potential of the particular biomaterial for the specific application. It is easier to identify which biomaterial has better chances of regenerating the specific dental tissues when tooth-derived cells are used. In other words, the level of regeneration of dental pulp, periodontal ligament etc., could be better envisaged from the *in vitro* results using specific tooth-derived cells. The specific tooth-derived cells to which the material contact is maximal can be identified and used for this purpose, so that a more realistic conclusion could be made. This *in vitro* predictability of regenerative potential is important during clinical translations of the biomaterials to propose its tissue specificity in tooth regeneration. In clinical translation, all biomaterials may not have all the ideal properties for the given clinical situation. It is now possible to tune and combine different biomolecules to formulate and execute near ideal biomaterials. The ultimate use of bioactive dental materials is tooth regeneration, and this goal cannot be achieved without clinical translation.

In this situation, planning for cell culture models based on tooth-derived cells will immensely help in the development and translation of bioactive dental materials for specific regenerative applications.

1.8 Definition of problem

Biomaterial based endogenous regeneration of dental tissues is an emerging treatment strategy with wide range of applications in regeneration of dental pulp and periodontium. Different kinds of materials and scaffolds are being developed towards this purpose. *In vitro* screening of materials is very important, as it can indicate workable materials before going to expensive *in vivo* studies for the local and systemic responses in a dynamic host environment. Biomimetic molecules with the capability of enhancing the homing, adhesion and differentiation of cells can be useful tools in endogenous regenerative approaches. Certain calcium containing cements, especially phosphates, sulfates and such mineral combinations can be used to mimic the natural mineral component of dentine, cementum and bone, to initiate biomineralization. It would be useful, if the potential of biomaterials can be evaluated using the respective cells to which they come to contact with. Therefore, establishment of a cell culture system for biomaterial evaluation can be a significant step which will be of immense help in the design, development, evaluation and translation of dental biomaterials. Evaluation of novel materials like biomimetic molecules and new generation inorganic compositions could be done using such validated *in vitro* cell culture systems, which will enable to assess and compare their regenerative potential and make appropriate choice for clinical application.

Current standard *in vitro* biological evaluation of biomaterials often depends on totally unrelated cell lines for the preliminary cytocompatibility studies. Isolated and properly characterized primary cells are currently acceptable to carry out cytocompatibility of biomaterials [ISO 10993]. The biological evaluation of dental biomaterials could benefit much from identifying suitable tooth-derived cell culture systems to thoroughly evaluate clinically relevant specific host cell responses to biomaterials. In this context, identifying the relevant human dental tissues as suitable cell sources and establishing a standardized procedure for the isolation, culture and characterization of tooth-derived cells for biomaterial interaction studies can be of much utility.

The currently available regenerative treatment strategies in dentistry give variable, often unpredictable results in retaining the ideal structure and function of the lost tissues. Even the apparently promising biomaterial structures and complex scaffolds rarely go beyond *in vitro* and *in vivo* biological evaluations in their path of translation. The reason, as it could be envisaged from the literature, is the inadequacy in the evaluation which has to be set ideally against standard protocols. The bioactivity of these novel dental materials needs to be established *in vitro* precisely, before going to preclinical tests and clinical trials. The tooth-derived cells can bridge the gap here, for an extensive, cellular and molecular level responses and differentiation potential of the biomaterials.

From the preliminary assessment of the current research scenario, the lack of a standardized tooth-derived cell culture system was noted, which is a definite research scope. Once a tooth-derived primary cell culture system is established, it can be used to evaluate novel biomimetic molecules and bioactive mineral compositions to elucidate the cell responses specific to the host tissues. Depending on the responses obtained, these biomaterials can be modified to suit clinical situations and the *in vitro* results can be confirmed using suitable *in vivo* models. The establishment of a reliable *in vivo* model using small animals, preferable laboratory rats, with minimal surgical trauma possible, is also a perceived research challenge towards the preclinical evaluations needed for clinical translation of biomaterials. Based on these identified research problems, a thorough literature search was initiated to gain understanding of the current status of knowledge in the related areas.

Chapter 2

REVIEW OF LITERATURE

The major activity in Regenerative Dentistry is the evaluation and validation of regenerative biomaterials through *in vitro* cell culture techniques. It is essential to go through these *in vitro* steps during the scaffold design and optimization, to assess the safety and efficacy of the materials prior to pre-clinical animal experiments. However, there is a lack of standardized protocol for the use of tooth-derived cells for biomaterial evaluation and validation. Therefore, an attempt is made towards facing the research challenges through planned experiments towards the establishment of an *in vitro* tooth-derived cell culture system fit for evaluating bioactive and biomimetic dental materials. Such a validated cell culture system could be made a standard model to evaluate and screen candidate dental biomaterials towards their regenerative potential. Further *in vivo* experiments in a suitable small animal model will reveal their suitability for clinical application.

A thorough knowledge is needed about past and current approaches in primary cell isolation and culture, biomaterial synthesis, *in vitro* material-cell interaction procedures and *in vivo* models, to attain these goals. This chapter gives a systematic review of the tooth-derived cells, biomaterials for regeneration of periodontium and dental pulp, the *in vitro* evaluations for assessing bioactivity of dental biomaterials and *in vivo* models for biomaterial based tooth regeneration approaches. Novel experimental designs and strategies are deduced with the aid of the published literature reviewed to develop the research hypotheses and set the aims and objectives of the thesis.

2.1 Tooth as a source of endogenous progenitor cells

Stem cells are defined as the cells having the capacity of self-renewal and the ability of generating differentiated cells. They are present within the tissues, serving the role of replacing dying or damaged cells. Studies with stem cells provide an opportunity to understand the mechanisms that regulate embryonic development, cellular differentiation and

organ maintenance [Ramalho-Santos *et al.*, 2007]. The term '*stem cell*' was coined by the German biologist Ernst Haeckel in his works on Evolution (Phylogeny) and Embryology (Ontogeny). He suggested the fertilized egg be called a *stem cell*, as it gives rise to all cells of the organism and the term was used to refer the unicellular ancestor of all multicellular organisms [Haeckel *et al*., 1868]. Depending on the source *stem cells* can be divided broadly into *embryonic* and *adult* stem cells. Embryonic stem cells are totipotent and carry the risk of teratoma formations and ethical issues if used *in vivo*. The adult stem cells reside in tissue specific niches and can divide to self-renew, can differentiate to progenitor cells or terminally differentiated cells, or remain in a quiescent state till a need occurs [Silvana *et al*., 2017]. These adult stem/progenitor cells are responsible for tissue homeostasis, repair and/or regeneration, and are present in their respective niche microenvironment [Nicolescu et al., 2016].

The vital dental hard tissues of the teeth - the dentin and cementum - show evidence of remodeling in response to appropriate stimuli. This is marked by the formation of reactionary dentin by odontoblasts and formation of reparative dentin by odontoblast-like cells of dental pulp; and new cementum formation by cementoblasts of periodontal ligament. Though the stimuli are exogenous, it is the local endogenous host progenitor cells that respond and differentiate to regenerate or repair the respective tissues [Smith *et al*., 1995; Grzesik *et al*., 2002; Liu *et al*., 2019]. Earlier it was presumed that the odontoblasts were terminally differentiated, until Gronthos et al isolated and characterized progenitor cells from dental pulp and proved the existence of a postnatal dental pulp stem cell (DPSC) [Gronthos *et al*., 2000]. This pioneering work was followed by the isolation of post natal stem cells from exfoliated deciduous teeth [Miura *et al*., 2003], periodontal ligament [Seo *et al*. 2004] and dental apical papilla [Huang *et al*., 2008]. Since then, various tooth-derived stem cells are being isolated, characterized and their differentiation potential studied upon as cell sources for therapeutic and biological research applications [Saito *et al*., 2015].

The dental and periodontal structures other than enamel are derived from the cranial neural crest/ ectomesenchyme [Matalová *et al*., 2015]. The tooth-derived stem cells hold special importance due to their neural crest cell origin.

The periodontal ligament is the soft tissue connecting the hard tissues of periodontium – viz. the cementum and alveolar bone. It originates from the ectomesenchymal cells of the

dental follicle and contains a heterogeneous population of predominantly fibroblasts, osteoblasts and cementoblasts along with collagenous fibers, blood vessels and innervations [Matalová *et al*., 2015]. It is a rich source of stem cells that maintains routine homeostasis, bone remodeling and cementum formation.

The dental pulp cells consist of odontoblasts that lays down dentin. The dental pulp stem cells can differentiate into odontoblasts or odontoblast-like cells that can lay down dentin under the influence of non-collagenous proteins and transcription factors like Wnt, runx2, osteocalcin, bone sialoproteins and dentin matrix proteins. These factors are expressed in osteoblast differentiation also, as both the hard tissues consists of collagen matrix and hydroxyapatite mineral [Matalová et al., 2015].

The stem cell properties may be influenced by their position in the immediate niche. The cells may remain quiescent; undergo cell division for local homeostasis or differentiation to intermediate (transient amplifying cells) or terminally differentiating cells, depending on the position of the cells [Watt *et al*., 2000; Shi *et al*., 2003]. Thus, the periodontal ligament cells can differentiate to cementoblasts that lay down cementum towards root dentin, to osteoblasts that lay down bone towards alveolar bone end, or as fibroblasts that deposit and remodel collagen to form the periodontal ligament. Similarly, the progenitor cells in dental pulp can differentiate to odontoblast like cells that lay down reparative dentin [Oshima *et al*., 2011].

2.1.1 Isolation of tooth-derived cells

The first attempts at isolation of tooth-derived cells utilized enzymatic digestion techniques of the respective tooth tissues. Further studies have compared the effects of isolation techniques including the explant culture technique to isolate the dental pulp cells and showed that a more heterogeneous population was obtained in enzymatic digestion technique. Other than that, the properties of the cells including the presence and absence of characterization markers and differentiation potential did not differ significantly between these techniques [Hilkens *et al*., 2013]. Dental pulp cells were isolated from adult teeth, deciduous teeth, super-numerary teeth, cryopreserved teeth and even from traumatized teeth needing endodontic therapy without tooth extraction. The cells from these various niches did not affect the properties of the cells [Kawashima *et al*., 2012]. It is hypothesized that the enzymatic treatment may destroy the dental pulp cell niche, whereas the pulp tissue explant

culture method can provide high purity dental pulp cell population repeatedly in substantial quantities, and can mimic natural intrinsic mechanisms of stem cell activation similar to that occurs during trauma or injuries. The authors also recommend this explant culture system to be applied to other dental tissues like apical papilla and periodontal ligament cells [Lizier *et al*., 2012]. The same techniques were used to isolate the deciduous teeth cells exhibiting similar findings with enzymatic digestion method yielding a homogeneous population and explant culture method yielding a heterogeneous population without significant differences in other properties [Kerkis *et al.*, 2012].

Similar isolation techniques were studied and reported on periodontal ligament cells also. Isolation of PDL cells through enzymatic tissue dissociation technique consumed less time initially, compared to cell outgrowth from tissue explant culture, which needed more time for the cells to migrate out of the explant. The enzyme digestion method initially released adequate number of cells from the tissue in lesser time, but some degree of cell loss or damage may be present due to the enzyme treatment, which can be minimized in the explant culture system, where the tissue provides a native ECM environment for the cells to migrate out. Initial heterogeneity was lost in subsequent passages and the cells similar characterization, proliferation and differentiation patterns thereafter [Tran *et al*., 2014].

2.1.2 Characterization of tooth-derived cells

The isolation and characterization of cultured primary hPDL cells is reported to be based on the anatomical site of harvesting the tissue specimens. It is usually done from the middle third of the root to avoid cross contamination with gingival fibroblasts coronally, and dental pulp or apical papilla cells from the apical foramen areas. Protein and gene markers used to identify hPDL cells include cementum attachment protein, cementum protein-23, periodontal ligament associated protein (PLAP-1), Cementum Membrane Protein (CEMP), ligament specific marker Scleraxis etc. Because of this, the choice of the markers for hPDLCs may be based on the design and purpose of the study. The progenitor cell population within the hPDL cells can be characterized by the presence of the mesenchymal stem cell markers like CD 90, CD 105, CD 73, and STRO-1. Specific markers like CEMP and Scleraxis can be used to confirm the tissue of origin of periodontal ligament cells. The variation in the expression of CEMP and Scleraxis can be used to study the differentiation of periodontal ligament cells in response to osteogenic/cementogenic stimuli. This helps to determine

whether the cell response is inherent or dependent on the biomolecules or biomaterials being tested. The use of primary hPDL cell cultures at early passages helps to maintain the unique cellular heterogeneity [Marchesan *et al.*, 2011].

The Dental Pulp Cells consists of a heterogeneous and mixed population with no specific characterization marker, but they do show expression of the common MSC markers [Kawashima N, 2012]. The deciduous teeth and apical papilla cells also exhibit common mesenchymal stem cell markers like CD 90, CD 105, CD 73, STRO-1, CD 271, CD 44 etc. and they are negative for hemopoietic markers such as CD 31 and CD 45, which are consistent with the minimum criteria for classification as mesenchymal stem cells [Saito *et al*., 2015]. Although scleraxis and cementum membrane protein expression may be used to confirm the origin of periodontal ligament cells, no such particular markers are applicable for dental pulp. The site of origin itself is considered enough to identify dental pulp cells [Karamzadeh *et al*., 2012; Avinash *et al*., 2017].

2.1.3 Differentiation potential of tooth-derived cells

The isolation of tooth-derived cells by different techniques results in a heterogeneous cell population with stem cells and surrounding supporting cells that can influence the progenitor cell behavior [Ducret *et al*., 2016; About, 2018]. It is reported that the cells from the dental tissues are capable of an osteogenic differentiation upon induction [Monterubbianesi *et al*., 2019; Acil *et al*., 2016]. Since dentin and cementum resemble bone in having the type 1 collagen matrix and hydroxyapatite mineral deposition similar to bone, it is difficult to distinguish osteogenic differentiation from cemento/odontogenic differentiation [Proksch *et al*., 2019; Winning *et al*., 2019]. Therefore, it is currently acceptable to use common inducing agents like bone morphogenic proteins (BMPs), L ascorbic acid-β Glycerophosphate-Dexamethasone cocktail, or biomaterials like hydroxyapatite, calcium phosphates etc., for the osteogenic differentiation of the tooth-derived cells. Histological staining for mineralization, expression of common differentiation marker proteins and relative gene expression studies can be used to evaluate the differentiation potential of tooth-derived cells [Wu *et al*., 2012; Hosseini *et al*., 2018; Saberi *et al*., 2019]. The cell density is also found to affect the osteogenic differentiation of dental pulp cells as well as other tooth-derived cells [Noda *et al*., 2019].

There is evidence that periodontal ligament and gingival fibroblasts display osteogenic potential even as a heterogeneous population *in vitro* [Proksch *et al*., 2019]. The dental pulp cells also undergo osteogenic differentiation *in vitro* and *in vivo*, under suitable conditions, with evidence of mineralization and osteo/odontogenic gene expressions [Gronthos *et al.*, 2000; Komaki *et al*., 2012]. Biomaterials are also reported to induce osteogenic differentiation of dental pulp cells [Ajlan *et al*., 2015]. The isolation techniques did not influence the differentiation of the dental stem cells significantly. Alkaline phosphatase enzyme activity was consistently increased during the osteogenic differentiation of tooth-derived cells. The presence of mineralized nodules, collagen matrix deposition, osteogenic marker expression and gene expression are the important parameters to confirm osteo/cementogenic differentiation *in vitro* [Hilkens *et al*., 2013].

2.2 Principles and practice of Regenerative Dentistry

Regenerative dentistry attempts to explore and understand the underlying mechanisms of tooth development and biological processes in healing, repair and regeneration. The knowledge can be applied to harness the natural healing and regeneration potential of the dental tissues, and to devise novel clinical treatment approaches towards regeneration of damaged dental tissues [Volponi *et al*., 2018]. According to the definition, 'repair' indicates the healing of a defect or wound by the growth of tissues that may not completely restore the intricate microstructure and function of the lost tissues. Scar formation in skin wounds is a repair mechanism [Eming *et al*., 2014]. Regeneration is the natural process of replacing or restoring damaged or missing cells, tissues, organs, and even entire body parts to full function through recapitulation and reactivation of early embryonic developmental processes. This is often initiated and carried out through the activation of stem cells whose primary function is to maintain, heal and regenerate the tissue in which they are found [Gilbert *et al*., 2000].

Since tooth enamel cannot be biologically regenerated, ions like fluorides and calcium phosphates or hydroxyapatite nanocrystals are used for remineralizing enamel through fluoride incorporation in the crystal lattice of outer ~ 30 μm of enamel, thereby reducing the solubility of the enamel [Pandya and Diekwisch, 2019; Laino *et al*., 2005].

The dental pulp is the vital connective tissue component encased in a thick, porous mineralized chamber lined by dentin. Maintaining the vitality of dental pulp is cardinal to successful long-term dental restorations. In the adult pulp, the cell division and the secretory activity of odontoblasts is re-activated following shallow enamel and/or dentinal damage. The odontoblasts survival and its activation results in reactionary dentin formation that repair or regenerate dentin, thereby protecting the dental pulp [Couve *et al*., 2014]. Such reactionary dentin inducing restorative materials and luting cements can be of much use in regenerative approach. When the dental injury reaches deep into the dentin or the dental pulp, the breakdown products of dentin matrix influence pulp cell migration and the recruited progenitor cells differentiate into new odontoblast-like cells that secrete tertiary (reparative) dentin as a thin dentin bridge that protects the pulp from further bacterial infection [Smith *et al*., 2012].

The treatment strategies to regenerate the lost dental pulp tissue following pulp necrosis due to dental caries or trauma require a "de novo" regeneration of dental pulp. Currently, revascularization strategies are successfully attempted in case of infected or necrotic dental pulp with incomplete root formation to achieve complete root development in length and dentine thickness [Banchs and Trope, 2004]. Endogenous regenerative approaches with autologous biologics and smart biomaterials are preferred clinical attempts in dental pulp regeneration [Hargreaves *et al.*, 2013].

Periodontal regeneration is intricate and challenging because of the presence of four distinct compartments of the periodontium – the cementum, the alveolar bone, the periodontal ligament and the gingiva. The nature of new attachment formation depends on the type of cells repopulating the post-surgical periodontal defect area (Melcher's Hypothesis) [Melcher, 1976; Melcher *et al*., 1987]. Following this principle, a treatment strategy was attempted to regain connective tissue attachment and establish a ligamental attachment between newly formed cementum and supporting alveolar bone. A millipore filter membrane was used for covering the defect to prevent the overgrowth of gingival connective tissue from contacting the root surface [Nyman *et al*., 1982]. This treatment strategy, named "Guided Tissue Regeneration (GTR)" currently utilizes various barrier membranes to permit new attachment formation to cementum and alveolar bone. In addition, bone grafts are used as and when needed, to promote alveolar bone regeneration (Guided Bone Regeneration- GBR) [Reynolds *et al*., 2003].

2.3 Evolution of Biomaterials for dental tissue regeneration

Various materials are being used from time immemorial, to replace and restore teeth. A scientific approach to such materials enabled the identification of their safe and effective use and led to the special classification of *Dental Materials*. The modern knowledge about biological processes helped to identify biomaterials which can enhance the regeneration of damaged dental tissues. Examples are synthetic bone grafts, barrier membranes, pulp capping agents and scaffolds for tissue regeneration [Yuan *et al*., 2011].

2.3.1 Barrier membranes

Guided tissue regeneration techniques for periodontal regeneration utilize various types of natural and synthetic barrier membranes to prevent epithelial and connective tissue cells from invading the periodontal or alveolar bone defect site, and to allow periodontal cells to selectively repopulate the defect [Nyman *et al.*, 1982; Gottlow *et al*., 1986]. The successful use of barrier membranes towards maintaining and stabilizing the blood clot and excluding gingival epithelial cells from the periodontal defect site, allows guided tissue regeneration locally, which has been demonstrated since its inception in the 1980s [Dahlin *et al*., 1988; Nyman *et al*.,1991]. The first barrier membranes were non-resorbable, made of ePTFE (expanded polytetrafluoroethylene) and needed a second barrier removal surgery which is unfavorable for the patient [Zitzmann *et al*., 1997]. Collagen, with its proven bioabsorption, biocompatibility, bioactivity and wound healing properties, soon replaced the non-resorbable barrier materials, with good clinical acceptance [Wang and MacNeil, 1998; Sheikh *et al*., 2017]. Explorations are being done to overcome the disadvantages of collagen membranes like high cost, xenogenic origin and chances of immune reactions insusceptible individuals; through alternative materials and membrane design (Figure 2). The new generations of barrier membranes consist of synthetic and natural polymers. Techniques like electrospinning, 3D printing, additive manufacturing etc. are employed for fabrication [Yuan *et al*., 2011]. The earlier non-resorbable barrier membranes constitute the first-generation barrier membranes. The second generation includes resorbable collagen and other polymeric barrier membranes. The third-generation barrier membranes are bioactive and functional, incorporating antimicrobial agents, growth factors, and biomimetic and angiogenic agents. Combinatorial approaches are also seen with inorganic phosphates for alveolar bone regeneration [Sam and Pillai, 2014].

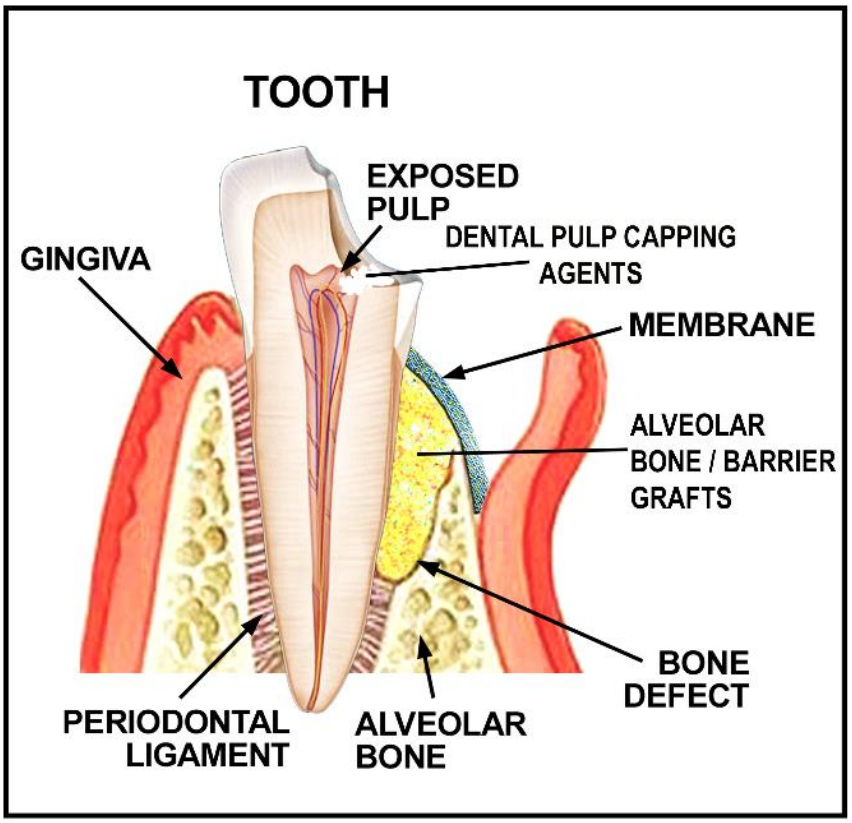


Figure 2: Potential application of dental biomaterials for the regeneration of dental pulp and periodontium.

2.3.2 Bone grafts

Bone graft materials are being used in combination with collagen membranes to improve bone regeneration in cases like furcation involvement and vertical alveolar bone defects, as well as in root coverage procedures [Bunyaratavej *et al.*, 2001]. There are four basic categories of bone grafts –

a) Autograft – This refers to bone harvested from the same patient from a different site. Autografts are harvested from non-defect areas intra-orally (e.g., maxillary tuberosity) or extra orally (e.g., iliac crest). These freshly harvested autografts are fully compatible to the host defect site as it contains the patient's own healthy bone tissue with growth factors and mediators. They provide good scaffolding with osteoinduction through the viable osteoblasts in the graft bone matrix. However, they have the drawbacks of donor site morbidity and limited availability of good bone in the case of patients who are aged or very young [Betz RR, 2002].

b) Allograft – These are bone harvested from a different host of same species - e.g., freshly harvested bone from a healthy donor or cadaveric bone. Natural bone derived grafts like Demineralized Freeze-Dried Bone Allografts (DFDBA) can be obtained and stored for later use from the cadaveric bones. However, they pose chances of disease transmission and immune reactions, along with batch-to-batch variations in bone quality [Betz RR, 2002].

c) Xenograft – They consist of bone taken from a host of different species, mostly bovine and swine. These can be anorganic, i.e., mineral component alone without cellular and organic matrix (e.g., Bio Oss), or demineralized i.e., organic matrix alone without the mineral component (DFDBA) [Jensen *et al.,* 2006]. The anorganic bone grafts are osteoconductive in the presence of host osteoblasts and precursor cells. The DFDBA contains collagen matrix and the growth factors which provide osteoinductive matrix and cues [Blaggana *et al.,* 2014].

d) Alloplast – This class includes synthetically prepared bone mineral components. Calcium phosphate ceramics in the bone mineral composition (hydroxyapatite or HA) is reported to be osteoconductive, but in highly sintered form, its bioresorption is slow. Biphasic calcium phosphate combinations with fast resorbing β Tricalcium Phosphate (β-TCP) enables synthetic bone cements with tunable resorption rate, osteo conductivity and osteoinductivity due to the release of calcium and phosphate ions [Komath M. *et al*., 2017]. Bioactive glasses (BioGlass) containing SiO_2, CaO and P_2O_5 are reported to possess osseo-integration and angiogenic properties [Hench and Jones, 2015]. The combination of glass ceramics containing bioglass, HA and βTCP has potential orthopedic and dental applications.

These four types of bone grafts also differ in their efficacy, *in vivo* resorption, strength and physicochemical properties [Betz RR, 2002]. Allografts and xenografts have the risks of disease transmission and immunologic reactions [Sheikh *et al*., 2017]. The final choice of the use of the graft material lies with the surgeon, who chooses the appropriate bone graft considering the healing outcome, feasibility, availability and affordability.

Due to the clinical limitations of natural bone derived grafts, there is an increasing demand for alloplastic (synthetic) bone graft materials (Figure 2). Bioceramics like hydroxyapatite (HA), bioactive glass and calcium phosphates in various forms are being used extensively as bone graft substitutes in humans, as they mimic the chemical composition and induce physiological and biological mineral deposits in bone [Dewi and Ana, 2018]. These minerals are biocompatible and bioresorbable and the ceramic processing impart porous

structure and mechanical properties to the mass [Lobo *et al*., 2015]. Hydroxyapatite (HA) with the chemical formula $Ca_{10}(PO4)_6(OH)_2$, is the basic mineral of human hard tissue. Various impurity ions like carbonate, fluorides, sodium, strontium, magnesium etc. get included in the HA crystal lattice during its formation. Carbonate is one of the most abundant of ion found in HA which resides in weight percentages 4-8 %, and hence the bone mineral phase in bone is often referred to as carbonated HA (or CHA) [Von Euw *et al*., 2019; Takano, 2004].

Synthetic bone grafts including bioceramics and bioglass are subjected to high temperature 'sintering' or melting process during their making for improving the mechanical properties. Porous granules and blocks of sintered hydroxyapatite are widely used as bone graft substitutes for the past 4 decades or so. Brittleness and lack of biological cues are the main drawbacks of sintered bone graft substitutes. Apart from the rigid nature, they show slow resorption *in vivo* due to densification in the sintering process [Komath M. *et al*., 2017]. Self-setting cement formulations of calcium phosphates can overcome these problems and provide mouldability and better *in vivo* resorption [Komath and Varma 2003]. Clinical success of calcium phosphate cements as alveolar bone/ barrier grafts and dental pulp capping agents are reported [Rajesh *et al*., 2009; Jose *et al*., 2013].

Physiological deposition of calcium phosphates, similar to that happens during biomineralization, can be achieved using calcium sulfates. Therefore, calcium sulfate based cements are also reported useful for bone defect filling [Pecora *et al.*, 1997]. The first documented use of calcium sulfate synthetic bone graft was reported in 1892 by Van Meekeren [Carson and Bostrom, 2007]. This knowledge expanded the utilization of different permutations and combinations of inorganic cements containing phosphates and sulfates of calcium for bone regeneration. Modern techniques like 3D powder printing and additive manufacturing procedures help to make engineered bioceramic constructs [Zhou *et al*., 2014; Qi *et al*., 2017]. The biocompatibility and osteoconductivity of calcium phosphate ceramics make them useful as a graft for periodontal infrabony defects. Cement formulations of calcium phosphates and calcium sulfates combine the mouldability, barrier property and enhanced bioresorption, which serve as excellent materials for Guided Bone Regeneration [Fairbairn *et al.*, 2018; Sandhya *et al.*, 2017].

2.3.3 Barrier grafts

The effectiveness of barrier membranes and alveolar bone grafts towards regeneration of periodontium including the formation of new bone and cementum with proper attachment apparatus and optimal gingival height is established [Sheikh *et al*., 2017]. It was noted that calcium phosphate cements can promote periodontal regeneration, and their mouldability and resorbability make the use of barrier membranes unnecessary (Figure 2). The cement formulation conforms to the bone defects, shows good osteogenic properties, optimal resorption and allows selective repopulation of cells. The properties of optimally formulated calcium phosphates enable them to be used for the 'barrier-graft' concept [Rajesh *et al*., 2009]. Calcium sulfate material, which could be designed as cement, is also explored as a bone graft and barrier [Maragos *et al*., 2002]. It was noticed *in vivo* that calcium sulfate can act as bioactive barriers to enhance bone regeneration, as early as three weeks [Pecora *et al*., 1997].

2.3.4 Dental pulp capping agents

Dental Pulp capping which includes direct and indirect pulp capping, is a regenerative approach to seal off the exposed or nearly exposed dental pulp using bioregenerative dental materials inducing neodentinogenesis [Hargreaves *et al*., 2013; Yu *et al*., 2016]. This facilitates the formation of reactionary dentin or a dentin bridge that act as a barrier towards further microbial contamination and thus protects the dental pulp to preserve the vitality of the tooth [Smith *et al*., 1995]. Pulpotomy is a procedure where a portion of the pulp, which may be infected or inflamed, is removed and the remaining portion of healthy pulp is capped with suitable biomaterials (Figure 2).

Calcium hydroxide in various formulations as powder, cement or pastes has been used as pulp capping agents with varying levels of success. However, this material is found to cause an initial tissue necrosis due to its high pH (approximately 12) and calcifications or dentin bridges with tunnel defects are formed preventing complete seal of the dentin, thereby retaining the chances of microbial infections [Njeh *et al*., 2016]. Calcium silicate cements like Mineral Trioxide Aggregate (MTA) and other new silicate formulations are gradually replacing calcium hydroxide as dental pulp capping agents [Komabayashi *et al*., 2016]. The disadvantages of MTA include potential for discoloration, prolonged setting time and presence of leachable toxic elements like bismuth oxide. Also, it is currently almost

impossible to remove MTA if a retreatment is deemed necessary [Hilton *et al*, 2013]. No currently available biomaterial satisfies all the desirable qualities of an ideal direct pulp capping material.

Calcium phosphate cements have evolved as dental pulp capping agents which possess better biocompatibility and odontogenicity with reduced alkalinity (approximate pH of 8), as pulp capping agents to promote reparative dentinogenesis [Lee *et al*., 2014; Jose *et al*. 2013]. Calcium sulfate has also been explored as a potential pulp capping agent with success [Ulusoy *et al*., 2014]. Calcium sulfate-phosphate combination cements were also explored as potential candidate biomaterials for vital pulp therapies [Chang *et al*., 2014].

2.3.5 Combinatorial approaches in dental tissue regeneration

Tissue regeneration, especially in the case of dental tissues, refers to a complex cascade of biological events facilitating the regrowth of local damaged tissues to their original structure. In Tissue Engineering, biomaterial structures are used to assist in regaining the normal tissue architecture. A single category of biomaterial may not be enough sometimes, to meet all the requirements for regeneration. A combination of two or more adjuvant biomaterials with optimal physical, chemical and mechanical properties can be developed using advanced fabrication techniques to suit their final application [Ivanovski *et al*., 2014]. Current investigations are aimed at designing biomaterials to replicate the local extra cellular environment thereby enhancing cell-cell and cell-material interactions at micro and macro levels to bring about functional tissue regeneration. The availability of different types of biomaterials and the technical advancements to functionally incorporate these biomaterials into a single easy to apply systems has led to the combinatorial approaches in bioactive dental materials development [Sharma *et al*., 2014].

The regeneration of periodontium after severe periodontitis involving alveolar bone loss, can be achieved by the use of bioactive bone grafts and barrier membranes, which is a combinatorial approach towards regeneration. Tissue-engineering approaches rely on suitable three-dimensional (3D) templates or scaffolds containing bioactive molecules to enhance cell homing and differentiation, vascularization and angiogenesis, and provide mechanical stability to support local tissue regeneration. Such an approach can trigger the series of subsequent events that include connective tissue regeneration, osteogenesis and

cementogenesis to complete the periodontal regeneration through appropriate cell responses in response to various physico-chemical and mechanical stimuli [Iviglia *et al*., 2019].

The use of calcium sulfate in combination with calcium phosphates is a typical example of material combination, as the calcium sulfate will act as a resorbable barrier with drug loading capabilities and calcium phosphate will serve the purpose of bioactive agent to promote hard tissue regeneration [Sukumar *et al*., 2011]. Bioceramic and bioglass powders can be combined with natural polymers like collagen to improve the mechanical properties and regulate the degradation kinetics. In addition, the biofunctionalization of ceramic grafts using natural proteins like collagen and hyaluronic acid or drug release molecules can add biomimetic properties to enhance regeneration [Iviglia *et al*., 2016].

Dental pulp regeneration can be achieved through bioactive cements and/or injectable hydrogels that can induce existing pulp cells, or promote endogenous cell homing and differentiation to dental pulp cells, in order to lay down healthy dentin. Apparently, dentin formation is taken as evidence for dental pulp regeneration [Raddall *et al*., 2019]. Combinatorial approaches involving various biomaterials like polycaprolactone (PCL)-magnetite nanoparticles, hyaluronic hydrogels with platelet lysates, PCL-hydroxyapatite with growth factors and collagen, growth factor loaded silk fibroin etc. were evaluated *in vitro* and *in vivo* for dental pulp regeneration [Medina-Fernandez and Celiz,2019].

2.4 Biological response to biomaterials

The biological responses to biomaterials can be evaluated *in vitro* through cytocompatibility evaluations and specific cellular responses like osteoinductivity. The biocompatibility can be confirmed using suitable *in vivo* animal models, before attempting clinical translations.

2.4.1 Cellular responses to dental biomaterials

The cellular response testing contributes to the first phase of biological evaluations of any biomaterials coming in contact with living human tissues. Therefore, understanding the basics of cell responses and their interpretation not only prove the cytocompatibility but also elucidate various clinically relevant responses, thereby helping to optimize biomaterials

for potential clinical applications. The cellular responses to various biomaterials is critical to incorporate suitable cues to influence cell homing, adhesion and differentiation. With advancements in technologies, it is now possible to modulate appropriate cellular behavior to induce regeneration of tissues.

2.4.2 Cellular responses for cytocompatibility evaluations

Any biomaterials to be used clinically, should undergo cytocompatibility evaluations as part of the preliminary biocompatibility evaluations, prior to *in vivo* evaluations or contact with human body, failing which, the clinical translation of biomaterial is jeopardized. Therefore, after the first phase of physico-chemical and biologic characterization of biomaterials, suitable biological evaluations are carefully planned and carried out, following the international guidelines [ISO 10993-1:2018]. The design of cytocompatibility assessments *in vitro* takes into consideration the possible effects of leachable substances, degradation products, presence of contaminants, contact toxicity etc. *In vitro* testing has been used successfully with different types of cell lines, and suitable culture models to screen the biomaterials [Inayat-Hussain and Siew 2008]. The effects of leachable components and degradation products of the biomaterials can be evaluated by using appropriate extracts of the biomaterial and evaluating their effects on cells using standardized procedures such as colorimetric tetrazolium dye reduction assays (MTT assay, LDH assay etc.), dye uptake or exclusion assays (Trypan blue, eosin, Congo red, erythrosine B assays), fluorimetric assays (Alamar Blue assay) etc. [Ö.S. Aslantürk, 2017]. Indirect contact tests including dentin barrier tests can be used for toxicity evaluation of leachable degradation products. Direct contact tests evaluate the contact cytotoxicity of biomaterials. Although cell lines were recommended earlier for standardization of these assays, properly characterized primary cells can also be used to understand responses of specific cells to the biomaterials [ISO 10993-1:2018].

For those biomaterials coming directly in contact with blood, tissues or cells, the following are suggested in addition to the above mentioned tests – (i) direct contact tests, (ii) cell viability, adhesion and spreading, and (iii) cell morphology analysis. Methods like immunofluorescence imaging of live/dead cells, actin cytoskeleton imaging or scanning electron microscopy analysis, could be adopted.

Cell adhesion is considered as a dynamic process that results from specific cell-cell, cell-ECM or cell-biomaterial interactions mediated through cell surface molecules and their appropriate receptor ligands. A sensing phenomenon occurs between the cell and the material when the cell comes in contact with a biomaterial. This is to explore and confirm the surface compatibility to its expected physicochemical and mechanical conditions to maintain normal cellular activities. Cell adhesion is mediated through adhesion proteins like collagen, laminin, fibronectin, etc. employing the cell membrane specific integrins, all of which can be visualized microscopically through immunofluorescence staining or SEM evaluations [Lotfi *et al*., 2013].

2.4.3 Cell morphology, adhesion and spreading onto Biomaterials

The cell cytoskeleton plays certain basic roles in maintenance of cell morphology, regulation of motility and adhesion, and the arrangement of intracellular organelles, thus preventing the cell collapse. Fluorescence imaging using a suitable fluorochrome tagged phalloidin is used to visualize actin cytoskeleton that enhances the knowledge and understanding of cell shape, cell interactions with the biomaterials, the effect of biomaterials or biomolecules on the cellular processes of adhesion, spreading, proliferation, differentiation, motility and apoptosis [McKayed and Simpson, 2013]. Scanning electron microscopy (SEM) is extensively used for high-resolution imaging of biological samples, for the ultra-structural analysis of cells to unveil valuable information about morphological characteristics of cells. This includes essential information about the cell type, structure, and pseudopodal and lamellipodial extensions indicative of cell spreading, in response to different physicochemical and biological stimuli. In addition, it can also give indications towards biomineralization [Tang *et al*., 2014; Lotfi *et al*., 2013]. The visualization of cell cytoskeletal organization, cell adhesion and spreading on the biomaterial surfaces indicates the cellular affinity to the biomaterials.

2.4.4 Responses of tooth-derived cells to biomaterials

The *in vitro* biological evaluation of biomaterials can make use of cultured primary cells isolated from tissues to which they are intended to be used clinically. For example, periodontal materials like bioactive cements and biomimetic matrices can be evaluated by the primary periodontal ligament cells, which provide clinically relevant cell responses. This

in vitro evaluation provides information on cell adhesion and spreading, and also on the potential of the cells to differentiate into osteogenic and cementogenic lineages. The differentiation of cells can be elucidated by histologic staining like Alizarin red for mineral deposits, assays for enzyme activity evaluations, immunofluorescence imaging using appropriate antibodies and staining techniques and Polymerase Chain Reactions (PCR) for qualitative and quantitative assessments [Tansriratanawong *et al*., 2018]. Similarly, the differentiation of dental pulp cells to odontogenic lineage can be used to assess the regenerative potential of dental pulp capping materials *in vitro* [Victoria-Escandell *et al*., 2017]. The specific roles of ions like calcium, phosphates, sulfates and silicates can also be evaluated *in vitro* using suitable primary dental cells [An *et al.*, 2015].

2.5 In vivo models for tooth regeneration

The ultimate motive of biomaterial based biomedical research is the translational potential of the experimental knowledge to actual clinical situations. The cytocompatibility evaluations accomplished through *in-vitro* systems needs to be confirmed through *in vivo* animal studies required for biocompatibility testing, and preclinical safety and efficacy evaluations, before proceeding to clinical translation to humans. In dental research, the selection of suitable animal species depends on the ability to simulate the clinical conditions in accordance with the site of application and the expected healing outcome with appropriate ethical approaches [Pasupuleti *et al*., 2016].

In rodent models, ovariectomy and placement of silk ligatures around the gingival margins of the teeth is one of the accepted practices to obtain periodontitis related alveolar bone defects. However, it is not possible to standardize the defect size and site by this method [Xu *et al.*, 2014]. A surgically created alveolar bone defect model in rats using a modified surgical technique, is also reported to evaluate biomaterials for alveolar bone regeneration following periodontitis, which can provide a standardized defect size and site *in vivo* [Kim *et al*., 2013]. Standardized mandibular fenestration models are also reported in rats to evaluate endogenous regenerations of cementum and alveolar bone in response to growth factors, scaffolds or both [Pellegrini *et al*., 2009].

Conventional histopathological evaluations and micro-computed tomography (µCT) can be utilized to evaluate the bone/cementum formation and periodontal regeneration [Park *et al*., 2007]. Bone healing could be elucidated from 4 weeks onwards whereas inflammatory responses can be elucidated much earlier as within 2 weeks *in vivo* [Ruan *et al*., 2018].

2.6 Background of the work

The literature review gives an insight into the challenges in the current research towards regenerative dentistry. An overall view of the isolation and characterization of tooth-derived cells derivable from the existing literature did not reveal any unified or standardized method or protocol.

The studies on cell - dental biomaterial interactions *in vitro* using tooth-derived cells could be found in literature, but all done independently and empirically made protocols. The identification and utility of the appropriate tooth derived cells in each experiment has been done to meet the objectives of the specific study. Existing literature lacks an established tooth derived cell culture model which can be used commonly for *in vitro* dental biomaterial evaluations. A uniform pattern of evaluation parameters is not available. This opens up a new avenue towards the establishment of a standardized tooth-derived cell culture system for evaluating bioactive dental materials *in vitro*.

Abundant data is available in the literature regarding novel biomolecules, modified biomaterials and 3D scaffold systems, with promising results towards dental tissue regeneration. Since the evaluation parameters are not uniform, it is difficult to compare the results and to draw a valid conclusion for the clinical translation. This research challenge can be taken up by carrying out the *in vitro* evaluations of selected biomolecules and bioactive cements using an established cell culture system following validated procedures.

Clinical use of dental materials necessitates the *in vitro* results to be supported *in vivo* in suitable animal models. Dog model is generally considered superior to test dental materials, but in practice, conducting dog experiments are cumbersome and expensive, and unaffordable for small labs. Establishment of a suitable small animal model to validate the *in vitro* results will be very useful. Current literature suggests the use of rat maxillary alveolar defect models for evaluation of barrier membranes and alveolar bone grafts. The surgical

facilities needed including custom surgical platforms, restrainers during the surgical procedures and adequate lighting systems need to be standardized. Development of such a standard surgical procedure to carry out uniformly sized defects with minimal trauma to the animals is a research challenge.

The present work was planned in this background. The review of literature provided critical insights to the different regenerative materials and the design and planning of various *in vitro* and *in vivo* experiments to plan a research study.

With sound definition of the problem and the extensive literature review, the research questions were developed and the research hypothesis for the study has been formulated with specific aims and objectives.

2.7 Research Question and Hypothesis

2.7.1 Research questions

i. Whether methods for isolating and culturing of tooth-derived cells can be developed.

ii. Whether a standardized cell culture system using tooth-derived cells can be established for *in vitro* evaluation of dental biomaterials.

iii. What is the feasibility of using the established cell culture system for *in vitro* evaluation of bioactive materials with regenerative potential?

iv. Whether it is possible to confirm the *in vitro* results using a suitable *in vivo* model using small animals.

2.7.2 Hypothesis

Based on the literature survey and research questions, the following hypothesis is made for the study -

"Biomimetic matrices and bioactive cements can be evaluated using appropriate *in vitro* model systems for their ability to regenerate damaged dental tissue structures."

2.8 Aims and Objectives

2.8.1 Aims

The aims of the study are established as:

(i) Establishment of tooth-derived cell culture systems for the evaluation of novel dental biomaterials.

(ii) *In vitro* evaluation and screening of novel bioactive dental materials through their regenerative potential using tooth-derived cell culture systems.

(iii) Design modifications and translation of selected biomaterials using suitable animal experiments.

2.8.2 Objectives

To prove the above hypotheses, and achieve the aims, the study was designed with the following objectives, with each objective is attained by a series of carefully planned experiments.

(i) Isolation and characterization of tooth-derived cells for in vitro analysis of biomaterials.

(ii) Identification and optimization of suitable biomaterials for regeneration of Dental Pulp and Periodontium, including

  (a) Biomimetic matrices based on self-assembling dendritic peptides.

  (b) Calcium based inorganic self-setting cement formulations.

(iii) Evaluation of the selected biomaterials using tooth-derived cell culture system.

(iv) Conducting the in vivo evaluation of selected biomaterials for translation towards clinical applications.

Chapter 3

MATERIALS AND METHODS

This chapter describes in detail, the methodology of the experiments designed and carried out to achieve the objectives of the study. The materials and methods are divided into four chapters, each representing the execution of specific study plans and experiments.

3.1 Establishment of in vitro Cell Culture System Using Tooth-Derived Cells

The first objective of this study is the establishment of a tooth-derived cell culture system. Cells were isolated from discarded extracted human teeth obtained from Govt. Dental College, Thiruvananthapuram, India with prior approval from the Institutional Ethics Committee (IEC) of Sree Chitra Tirunal Institute for Medical Sciences and Technology, (SCTIMST) Thiruvananthapuram, India and Government Dental College, Thiruvananthapuram, India. The protocol was also approved by the Institutional Committee on Stem Cell Research (IC-SCR) of SCTIMST. Human teeth, which are generally discarded as a clinical waste after extraction, were anonymously collected for the cell isolation procedure. The sample collection procedures and *in vitro* cell culture experiments were carried out as per the protocol approved by IEC.

A common collection and transport procedure was carried out to collect the deciduous and permanent teeth samples to the cell culture facility. Extracted teeth were collected in sterile Tissue Collection Medium (TCM) [0.1 M Phosphate Buffered Solution (PBS) containing 1000 IU/mL Penicillin, 1000 µg/mL Streptomycin and 0.25 µg/mL Amphotericin B]. The adult and deciduous teeth samples collected separately was transported to the cell culture lab immediately after collection and further procedures were performed following aseptic techniques inside a Class II biosafety cabinet (Esco, Singapore). Cell isolation procedures were initiated within two hours of tissue collection. Figure 3 illustrates the source tissues for isolation of tooth-derived cells.

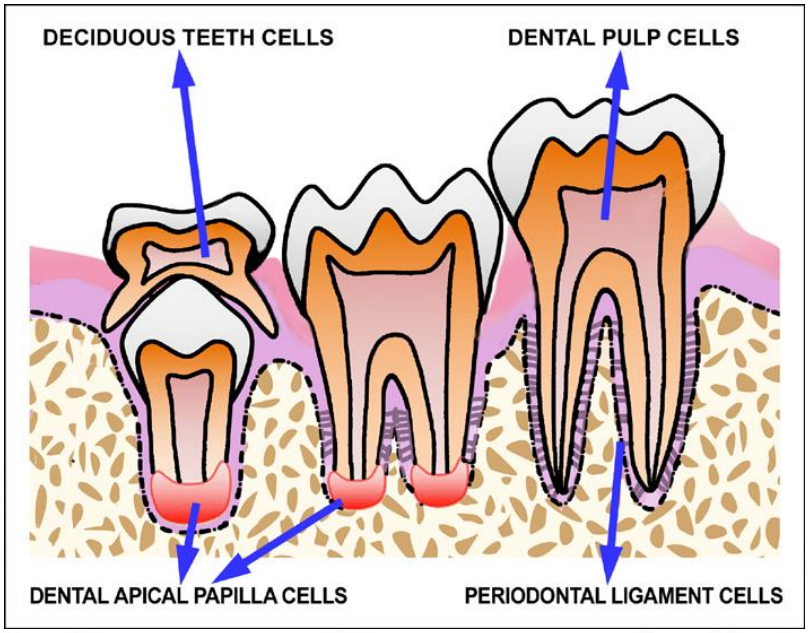


Figure 3: Illustration of source tissues for isolation of tooth-derived cells.

3.1.1 Isolation and culture of human Periodontal Ligament cells

An explant culture model and a collagenase/dispase tissue digestion model were used to isolate the human periodontal ligament cells (hPDLCs). The cell output and reliability in achieving a healthy population was considered to select one technique of cell isolation for further studies.

3.1.1.1 Tissue explant culture method

The adult teeth were taken inside a biosafety cabinet (Esco Singapore) in cell culture lab was rinsed with fresh sterile Tissue Collection Medium. The periodontal ligament was identified as a thin membrane of tissues attached tightly to the tooth roots. This tissue was scrapped off from the middle third of the tooth root surface to the apical third using a No.12 Bard Parker blade. The tissue fragments were collected in a sterile petri dish and further minced to 2 × 3 mm pieces. The tissue fragments were treated with 0.25 % trypsin (Gibco) for 5 min at 37 ºC. One or two trypsin treated tissues fragments were carefully placed in each of 35 mm cell culture petri dish (Nunc, Thermofischer) containing 1 mL regular cell culture medium [α-MEM (Gibco) supplemented with 10 % Fetal Bovine Serum (FBS - Gibco), 100 IU/mL Penicillin and 100 µg/mL Streptomycin (1X Pen/Strep, Gibco)]. The petri dishes

containing the periodontal ligament tissue explants were placed inside a sterile 150 mm petri dish, maintaining ambient air circulation and incubated inside CO_2 incubator. The tissue explants were monitored regularly and the culture media was changed every third day. The explants were incubated for 7 – 10 days till cell outgrowths were noted and further cultured till a confluent cell layer was formed. The cell outgrowths from the explants were visualized under a phase contrast microscope (Nikon, TiS 100) and images were taken as and when required. Once confluent, the explants were discarded, the adherent human Periodontal Ligament Cells (hPDLCs) were harvested by trypsinization, and transferred to fresh T25 cell culture flasks (Nunc) and considered as the first subculture (passage 1). These cells (in passage 1) were cultured for 3 – 5 days or till it attained confluency. Cells at Passage 1 were further sub cultured (Passage 2, 3, 4 etc.) and used for experiments.

3.1.1.2 Tissue digestion method

In tissue digestion method, the tissue fragments collected from the middle third of the tooth was digested using a mixture of 4 % collagenase and 3 % dispase prepared in serum free αMEM. The tissue fragments were digested for 30 min inside the CO_2 incubator and then centrifuged at 2200 rpm 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 60 mm cell culture petri dishes, till a healthy population of cells was observed.

3.1.1.3 Characterization of human Periodontal Ligament cells

The hPDLCs in passage 2 were seeded onto $1cm^2$ glass coverslips (1×1 cm) at a cell density of 10^3 cells/cm^2 and cultured for 48 h. Briefly, the cells at passage 1 grown on T25 culture flasks was trypsinised and harvested to fresh culture medium. Cell concentration was estimated using a Neubauer's cell counting chamber and a cell suspension of approximately 1000 cells/100 µL was prepared. Sterile glass coverslips were placed inside a multiwell plate and 100 µL cell suspensions were carefully pipetted onto it. The cells were allowed to adhere on cover slips (approximately 1 h) and 1 mL fresh cell culture medium was added. Cells were incubated for 48 h and observed under microscope for cell adhesion and spreading. The culture medium was discarded and the cells were rinsed with sterile PBS. The cells were then fixed using 4 % Paraformaldehyde (PFA) solution and kept at room temperature for 1 h. The fixative was removed and cells were washed again with PBS. Fixed cells were treated with

0.1 % triton X100 for 1 min. The cells were again rinsed with PBS and the non-specific antibody binding was blocked by incubating with freshly prepared 10 % Bovine Serum Albumin (BSA) for 30 min.

The cells were incubated overnight at 4 $^{\circ}$C, with the mouse primary antibodies against human Vimentin, Cementum Membrane Protein (CEMP), CD 90 (Thy 1), CD 105 (Endoglin), STRO-1; and rabbit primary antibodies against human Scleraxis, Dentin Matrix Protein 1 (DMP1) and CD 73 (ecto-5'-nucleotidase). The cells were rinsed 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 rinsed with PBS and the cell nuclei was counter-stained using Hoechst 33258 (0.5 µg/ml in PBS) for 1 min. After final PBS wash, samples were observed under an inverted fluorescence microscope (Leica DMI 6000, Germany) equipped with filters suitable for green (I3), blue (A) and red (N21) emission. Confocal Laser Scanning microscopy (CLSM) (Olympus) was used to image hPDLCs expression of vimentin, CD 73, CD 105, STRO 1, and CEMP and DMP 1.

3.1.1.4 Osteogenic differentiation of human Periodontal Ligament cells

The periodontal ligament cells at passage 3 were used for the *in vitro* osteogenic differentiation of the cells [Noda *et al.*, 2019]. The confluent cells were cultured on 1 cm^2 glass coverslips at the cell density of 10^4 cells/cm^2. The cells of control group were cultured in regular cell culture medium (αMEM containing 10 % FBS and 100 IU Penicillin / streptomycin) and the cells in test group was cultured in an osteogenic induction medium (αMEM containing 10 % FBS, 100 IU Penicillin Streptomycin; 50 µg/ml L-ascorbic acid, 10 mM sodium β-glycerophosphate and 10 nM dexamethasone). The same lot of culture medium and osteogenic induction medium was used for the entire study. The osteogenic differentiation was evaluated by analyzing mineral deposits (alizarin red and von Kossa staining) and collagen synthesis (Masson's trichrome staining) at different time points like 7, 14 and 21 days. In addition to this, the expression of osteogenic markers Alkaline phosphatase, Osteonectin, Collagen 1 and DMP 1 was evaluated.

i. Alizarin red staining

The hPDLCs cultured in regular cell culture medium and osteogenic induction medium was fixed on day 7, 14 and 21 using 4 % PFA. Fixed cells were washed thrice in deionized water and then incubated with freshly prepared 4 % alizarin red solution for 30 min at room temperature. The cells were rinsed with deionized water and viewed under light bright field microscope (Nikon, TS100). The mineralization was evidenced by the presence of red nodules formed due to the chelation of calcium ions by alizarin red.

ii. Von Kossa Staining

Fixed cells were treated with 2 % silver nitrate solution and irradiated with UV light (λ – 250 nm) for 30 min. The cells were washed with deionized water and viewed under light bright field microscope (Nikon, TS100). The phosphate deposits by the differentiated cells were visualized as brownish black deposits. The silver ions of silver nitrate react with phosphates, replacing the calcium ions. Upon UV irradiation photochemical degradation of silver phosphate to metallic silver occurs, which appear as brownish black deposits when viewed under bright field microscope. This method along with alizarin red staining, gives a reliable indication of biomineralization *in vitro*.

iii. Masson's trichrome staining

The deposition of collagen by hPDLCs in osteogenic induction medium (test) and regular cell culture medium (control) was evaluated by Masson's trichrome staining. The cells fixed at 7, 14, and 21 days were washed with deionized water and treated with freshly prepared Bouin's solution for 1 h. The solution was removed and cell nucleus was counter stained using iron hematoxylin solution for 5 min. The cells were rinsed and sequentially treated with the component reagents of Masson's trichrome staining kit (Sigma, Aldrich), as per manufacturer's instructions. The stained samples were observed under a microscope (Nikon TS 100) for bluish-green color which represents collagen.

iv. Osteogenic marker expression by hPDLCs

The hPDLCs in the osteogenic experiment was fixed at day 7 with 4 % PFA for 1 h, washed thrice with PBS and permeabilized with 0.1 % triton X 100 for 1 min. The cells were treated with freshly prepared 10 % BSA for 30 min to avoid non-specific binding. The cells

were then incubated overnight with mouse primary anti body against human Osteonectin (QED Bioscience), rabbit primary antibody against human Alkaline Phosphatase, Collagen 1 and DMP1 (Abcam) at 4 °C. Cells were rinsed thrice with PBS and incubated with corresponding secondary antibodies (anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 555) for 1 h in dark. After 1 h, the cells were again washed with PBS, and the cell nuclei were counterstained with Hoechst 33258 (0.05 μg/ml), washed and imaged in inverted fluorescence microscope (Leica DMI 6000, Germany) equipped with filters suitable for blue (A), green (I3), and red (N21) emission.

3.1.2 Isolation and culture of human Dental Pulp cells

The dental pulp is the vital tissue enclosed within the hard dentinal walls of the human tooth. Extracted and discarded human permanent teeth were collected in sterile Tissue Collection Medium (TCM) consisting of phosphate buffered solution (PBS) with 1000 IU penicillin/streptomycin and 25 μg Amphotericin B. Coronal access opening was prepared in the collected teeth and dental pulps were extirpated using H files. The pulp tissues were collected in sterile TCM. The samples were transferred to biosafety cabinet (Esco, Singapore) for aseptic handling.

3.1.2.1 Tissue explant culture method

The collected pulp tissues for explant culture technique were transferred to a 35 mm cell culture petri dish inside the biosafety cabinet (Esco) and minced into 2 × 3 mm pieces. These tissue sections were treated with 0.25 % trypsin (Gibco) for 5 min at 37 ºC. Each tissue fragment was then carefully placed in 35 mm cell culture petri dishes 1 mL regular cell culture medium and cultured in the CO_2 incubator, monitoring every day and changing culture media on alternate days till cell outgrowths were observed.

3.1.2.2 Tissue digestion method

The pulp tissue samples collected in sterile TCM were transferred to 35 mm cell culture petri dish was incubated with collagenase-dispase enzyme (4 % collagenase type I and 3 % dispase, in serum free αMEM) for 30 min inside the CO_2 incubator at 37 ºC, 5 % CO_2, and >95 % relative humidity. The digested tissue fragments containing the cells, along with the medium, was centrifuged at 2200 rpm for 5 min. The supernatant was discarded,

and the cell pellet was dispersed in regular cell culture medium in 60 mm cell culture petri dishes and cultured till a healthy population of cells was observed.

3.1.2.3 Characterization of human Dental Pulp cells

The hDPCs in passage 2 was seeded onto 1 cm^2 glass coverslips at a cell density of 10^3 cells/cm^2 and cultured for 48 h as described in section 3.1.1.3. Cells were observed under microscope for cell adhesion and spreading. After 48 h, the culture medium was discarded and the adherent cells monolayer was rinsed using sterile PBS to remove residual serum. The cells were then fixed using 4 % PFA solution and kept at room temperature for 1 h. The fixative was removed and cells were washed again with PBS. Fixed cells were treated with 0.1 % triton X100 for 1 min. The cells were again rinsed with PBS and the non-specific antibody binding was blocked by incubating with freshly prepared 10 % Bovine Serum Albumin (BSA) for 30 min.

The cells were incubated overnight at 4 $^{\circ}$C, with the mouse primary antibodies against human Vimentin, CD 90, CD 105 and STRO-1, and rabbit primary antibody against CD 73 and DMP 1. The cells were rinsed 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. Again, the cells were rinsed with PBS, and the nuclei were counter stained using Hoechst 33258 (0.5 μg/ml in PBS) for 1 min. After final PBS wash, samples were observed under the inverted fluorescence microscope equipped with filters suitable for green (I3), blue (A) and red (N21) emission.

3.1.2.4 Osteogenic differentiation of human Dental Pulp cells

The cells dental pulp cells at passage 3 were used for the *in vitro* osteogenic differentiation of the cells. The confluent cells were cultured on 1 cm^2 sterile glass coverslips at a cell density of 10^4 cells/cm^2. The cells of control group were cultured in the regular and the cells of test group were cultured in the osteogenic induction medium, the composition of which is described in section 3.1.1.4. The same lot of culture medium and osteogenic induction medium was used for the entire study. The osteogenic differentiation was evaluated by analyzing mineral deposits (Alizarin red and von Kossa staining) and collagen synthesis (Masson's trichrome staining) at different time points like 7, 14 and 21 days. In addition to

this, the expression of osteogenic markers Alkaline phosphatase, Osteonectin, Collagen 1 and DMP 1.

i. Alizarin red, von Kossa and Masson's trichrome staining

The hDPCs fixed at 7, 14 and 21 days were analyzed for calcium, phosphate and collagen deposition by Alizarin red, von Kossa and Masson's Trichrome staining as described in sections 3.1.1.4-i, 3.1.1.4-ii and 3.1.1.4.iii respectively.

ii. Osteogenic marker expression by hDPCs

The hDPCs in the osteogenic experiment was fixed at day 7 with 4 % PFA for 1 h, washed thrice with PBS and permeabilized with 0.1 % triton X 100 for 1 min. The cells were treated with freshly prepared 10 % BSA for 30 min to avoid non-specific binding. The cells were then incubated overnight with mouse anti-human Osteonectin (QED Bioscience), rabbit anti-human Alkaline Phosphatase, Collagen 1 and DMP1 (Abcam) at 4 °C. Cells were rinsed thrice with PBS and incubated with corresponding secondary antibodies (anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 555) for 1 h in dark. The cells were rinsed with PBS and the cell nuclei were counterstained with Hoechst 33258 (0.05 μg/ml). After final PBS rinse, the cells were imaged in inverted fluorescence microscope equipped with filters suitable for blue (A), green (I3), and red (N21) emission.

3.1.3 Isolation and culture of human Deciduous teeth cells

An explant culture system was used to isolate the deciduous teeth cells. The deciduous teeth in Tissue Collection Medium were transported to Tissue Culture Laboratory and the follow up procedures were done aseptically. The residual tissue from the inner side of the deciduous teeth was scraped off using a No.12 Bard Parker blade and collected in 35 mm cell culture petri-dish, washed with sterile PBS, and treated with the 0.25 % trypsin (Gibco) at 37 ºC for 5 min. Each tissue fragment was then transferred to fresh 35 mm cell culture dishes containing 1 mL regular cell culture medium and cultured in the CO_2 incubator (Sanyo, Japan). The tissue explants were monitored using an inverted phase-contrast microscope (Nikon TS100, Japan) and regular medium change was given. Once confluent, the explants were discarded, the adherent human Deciduous dental cells (hDecDCs) were harvested by trypsinization, and transferred to fresh T25 cell culture flasks (Nunc) and considered as the first subculture (passage 1). These cells (in passage 1) were cultured for 3 - 5 days or till it

attained confluency. Cells at Passage 1 were further sub cultured (Passage 2, 3, 4 etc.) and used for experiments.

3.1.3.1 Characterization of human deciduous teeth cells

The deciduous teeth cells in passage 2 was seeded onto 1 cm^2 glass coverslips at a cell density of 10^3 cells/cm^2 and cultured for 48 h as described in section 3.1.1.3. The culture medium was discarded and the cells were rinsed with sterile PBS. The cells were then fixed using 4 % PFA solution and kept at room temperature for 1 h. The fixative was removed and cells were washed again with PBS. Fixed cells were treated with 0.1 % triton X100 for 1 min. The cells were again rinsed with PBS and the non-specific antibody binding was blocked by incubating with freshly prepared 10 % BSA for 30 min. The cells were then incubated overnight, at 4 ºC, with the corresponding mouse primary antibodies against human Vimentin, CD 90, CD 105, and Stro 1. The cells were rinsed thrice with PBS and incubated with the secondary antibody (anti mouse AlexaFluor 488, Abcam), for 1 h in dark, at room temperature. The cells were rinsed thrice in PBS and the cell nuclei was stained using Hoechst 33258 (0.5 µg/ml in PBS) for 1 min. After final PBS wash, the samples were viewed and imaged using inverted fluorescence microscope (Leica DMI 6000, Germany) equipped with filters suitable for blue (A) and green (I3) emission.

3.1.3.2 Osteogenic differentiation of Deciduous teeth cells

The cells deciduous teeth cells in passage 3 were used for the *in vitro* osteogenic differentiation. The confluent cells were cultured on 1 cm^2 glass coverslips at a density of 10^4 cells/cm^2. The cells of control group were cultured in regular cell culture medium and the cells in test group were cultured in an osteogenic induction medium. The differentiation potential of the cells was evaluated by alizarin red staining, von Kossa staining and Masson's trichrome staining at 7, 14 and 21 days.

i. Alizarin red, von Kossa and Masson's trichrome staining

The deciduous teeth cells fixed at 7, 14 and 21 days were analyzed for calcium, phosphate and collagen deposition by Alizarin red, von Kossa and Masson's Trichrome staining as described in sections 3.1.1.4.i, 3.1.1.4.ii and 3.1.1.4.iii respectively.

3.1.4 Isolation and culture of human Dental Apical Papilla cells

The adult teeth in TCM were transported to Tissue Culture Laboratory and the follow up procedures were done aseptically in a class II Bio Safety Cabinet (Esco Singapore). The apical papilla, at the apical end of the tooth root was identified under by visual and microscopic inspection, and carefully scrapped off from the root apex, into a sterile 35 mm cell culture petri-dish. The tissue fragments were washed repeatedly, with sterile PBS, and treated with the enzyme trypsin (0.25 % kept at 37 ºC) for 5 min. One or two trypsin treated tissues fragments were carefully placed in each of 35 mm cell culture petri dish (Nunc) containing 1 mL regular cell culture medium and cultured in CO_2 incubator (Sanyo, Japan) at 37 °C having a humidified atmosphere of 5 % CO_2. The cell outgrowths from the explants were visualized under a phase contrast microscope (Nikon, TS100).and images were taken as and when required. Once confluent, the explants were discarded, the adherent human Dental Apical Papilla Cells (hDAPCs) were harvested by trypsinization, and transferred to fresh T25 cell culture flasks (Nunc) and considered as the first subculture (passage 1). These cells (in passage 1) were cultured for 3 – 5 days or till it attained confluency. Cells at Passage 1 were further sub cultured (Passage 2, 3, 4 etc.) and used for experiments.

3.1.4.1 Osteogenic differentiation of Dental Apical Papilla cells

The apical papilla cells in passage 2 were used for the *in vitro* osteogenic differentiation of the cells. The confluent cells were cultured on 1 cm^2 glass coverslips at a cell density of 10^4 cells/cm^2.The cells in control group were cultured in regular medium and the cells in test group were cultured in an osteogenic induction medium. The differentiation potential of the cells was evaluated by histological analysis of mineralization by alizarin red staining, von Kossa staining and Masson's trichrome staining at 7, 14 and 21 days.

i. Alizarin red, von Kossa and Masson's trichrome staining

The human dental apical papilla cells fixed at 7, 14 and 21 days were analyzed for calcium, phosphate and collagen deposition by Alizarin red, von Kossa and Masson's Trichrome staining as described in sections 3.1.1.4.i , 3.1.1.4.ii, and 3.1.1.4.iii respectively.

3.1.5 Comments on the development of the *in vitro* cell culture system

The isolation, culture and characterization of the tooth-derived cells were carried out with an aim to use them for the biological evaluation of dental biomaterials. The procedures were carried out based on existing literature, modifying according to special needs as and when they arise. The characterization markers were taken in accordance with the current literature, and the osteogenic induction medium was constituted according to the most used combination of reagents in literature. Only explant culture method was used to isolate deciduous teeth cells and the apical papilla cells. The availability of deciduous teeth tissue and apical papilla tissue were limited. Both deciduous tooth cells and apical papilla cells rarely come to contact with dental biomaterials in clinical scenario. Also, most biomaterials come to direct contact with the periodontal ligament cells and dental pulp cells, in adult dentition. Therefore, for further biomaterial evaluation studies, it was decided to use only periodontal ligament cells (hPDLCs) and dental pulp cells (hDPCs).

3.2 Biomimetic Matrices for dental regeneration

The second objective of the study includes identification and optimization of suitable biomaterials having regenerative potential for dental pulp and periodontium. Biomimetic matrices based on self-assembling dendritic peptides or peptidomimetics can provide an ECM mimetic matrix that can facilitate local progenitor cell homing, proliferation and differentiation. Amino acids, short peptide sequences or similar small biomolecules can be designed to form self-assembling, ECM mimetic, smart peptide matrices [Lutolf *et al.*, 2009; Cha *et al.*, 2012]. Lysine Appended Polydiacetylene (Lys-PDA) and Guanidine Appended Polydiacetylene (G-PDA) were identified and selected as candidate biomolecules for periodontal regeneration applications. The biomolecules synthesized were characterized and evaluated as ECM mimetic matrices *in vitro* using the human periodontal ligament cells.

3.2.1 Lys-PDA (Lysine Appended Polydiacetylene)

Lys-PDA was designed and synthesized at Dept. of Chemistry, IIT Delhi and received after preliminary characterization [Haridas *et al.*,2013]. Lys-PDA was shipped in powder form and stored at room temperature until used.

3.2.1.1 Lys-PDA coating and sterilization

The Lys-PDA coating on cell culture plates (6 well Cell culture plates, Nunc) and glass coverslips (inert surfaces for osteogenic potential), was done inside a laminar flow hood to maintain a clean environment. The glass coverslips and 6 well cell culture plates were treated with 1 mL Lys –PDA solution (5 mg/mL in sterile deionized water), for 5 min. Excess solution was carefully removed, leaving a thin film of the Lys-PDA on the surfaces of glass coverslips and cell culture plates. The coated surfaces were irradiated with UV light of 254 nm for 2 h to complete residual polymerization. The coated cell culture plates and glass coverslips were air dried and sterilized using ethylene oxide gas (EtO), and kept at room temperature until further use.

3.2.1.2 Lys-PDA coating confirmation and characterization

The Lys-PDA coating on the glass coverslips was evaluated by phase contrast microscopy (Nikon), fluorescence microscopy (Leica) to visualize any autofluorescence and Scanning Electron Microscopy (SEM - FEI QUANTA 200).

3.2.2 G-PDA (Guanidine appended polydiacetylene)

Guanidine appended polydiacetylene was synthesized and preliminary characterization was done at Dept. of Chemistry, IIT Delhi. G-PDA was procured as aqua soluble powder and stored at room temperature till use.

3.2.2.1 G-PDA coating and sterilization

G-PDA was coated manually onto sterile glass coverslips and the wells of 6 well cell culture plates similar to Lys-PDA, at a concentration of 5 mg/mL solution in deionized water. The coated samples were air dried, sterilized using EtO gas and stored at room temperature until further use.

3.2.2.2 G-PDA coating confirmation and characterization

To confirm the presence of uniform coating of the G-PDA matrix, SEM images were taken after gold sputter coating. Atomic force microscopy (AFM) evaluation was also carried out using a scanner of 80 μm window (Agilent SPM 5500) to confirm the G-PDA coating on the surfaces.

3.2.3 *In vitro* biological evaluation of the biomimetic surfaces using hPDLCs

The third objective of the study includes the *in vitro* biological evaluation of the selected biomolecules using the established tooth-derived cell culture system. The cell culture system using characterized hPDLCs were used for the biological evaluation of the Lys-PDA and G-PDA coated surfaces. The hPDLCs at passage 2 was seeded onto the sterile G-PDA and Lys-PDA coated glass coverslips at 10^3 cells/cm^2. The cells were monitored daily for checking cell morphology, adhesion and proliferation and phase contrast images were taken.

3.2.3.1 Cell adhesion and viability

Cell viability on Lys-PDA and G-PDA was evaluated at 24 and 48 h by live/ dead staining. Cell adhered on Lys-PDA and G-PDA was treated with serum free αMEM containing fluorescein diacetate (FDA 5 µg/ml in) and propidium iodide (PI 0.5 µg/ml) for 5 min. The samples were observed under fluorescence microscope (Leica) using green (I3) and red (N2) filters and images were taken.

3.2.3.2 Actin Cytoskeleton staining

The cytoskeletal organization of hPDLCs, which is an important indicator of maintenance of cell morphology and active cell spreading on the matrices, was analyzed by staining actin cytoskeleton with Actin green 488 (Molecular probes, Thermo Fischer Scientific). The hPDL cell morphology on Lys-PDA and G-PDA was evaluated by actin cytoskeleton staining. The cells at 24 and 48 h of culture were rinsed with PBS and fixed using 4 % PFA for 1h. The fixed cells were permeabilized with 0.1 % Triton X100 for 3 min. The actin filaments were stained using Actin green 488 for 30 min in dark. The cells were rinsed with PBS and nuclei were counterstained with Hoechst 33258 (5 µg/ml) and visualized under fluorescence microscope using suitable green (I3) and blue (A) filters.

3.2.3.3 SEM evaluation of cell morphology

Cell morphology at 24 and 48 h of adhesion on Lys-PDA and G-PDA was also evaluated by scanning electron microscopy (SEM). The cells were fixed with 2.5 % glutaraldehyde for 1 h and rinsed using PB solution [0.2 M disodium hydrogen phosphate and sodium dihydrogen phosphate in deionized water]. Subsequently, serial dehydration was

done with ascending concentrations of ethanol (30 %, 50 %, 70 %, 90 % and 100 %) and isoamyl acetate. The samples were dried in a critical point dryer, sputter coated with gold (Hitachi) and SEM images were taken (FEI Quanta).

3.2.4 Osteogenic differentiation of human Periodontal Ligament Cells on Lys-PDA and G-PDA matrices

The hPDLCs at passage 3 were used for the osteogenic differentiation experiment. The cells were trypsinized and seeded onto Lys-PDA and G-PDA coated glass coverslips and the wells of 6 well cell culture plates at a cell density of 10^4 cells /cm^2 and cultured for 7, 14 and 21 days. Phase contrast images were taken to monitor the cells throughout the duration of the osteoinduction experiment. The details are given in Table 1.

Test	Control (Differentiation)	Control (Material)
HPDLCs cultured on Lys-PDA coated surfaces in osteogenic medium (section 3.1.1.4).	Negative Osteogenic control: hPDLCs cultured using regular cell culture medium.	HPDLCs cultured on Lys-PDA coated surfaces using regular cell culture medium.
HPDLCs cultured on G-PDA coated surfaces cultured in osteogenic induction medium.	Positive Osteogenic control: hPDLCs cultured using osteogenic medium.	HPDLCs cultured on G-PDA coated surfaces cultured in regular cell culture medium.

Table 1: Osteogenic differentiation of hPDLCs on the Lys-PDA and G-PDA matrices Distribution of test and control groups.

3.2.4.1 Alizarin Red staining and assay

The alizarin red staining was carried out as per the previously described protocol in section 3.1.1.4. The calcium deposition on day 21 was quantified by Alizarin red assay. The bound alizarin red dye was solubilized in 100% ethanol and colorimetrically measured at 505 nm using a multi-well plate spectrophotometer (Biotek USA). The optical density of the test samples was compared with the controls.

3.2.4.2 Von Kossa Staining

The hPDLCs were fixed at 7, 14, and 21 days with 4 % PFA for 1h, washed with deionized water and incubated with freshly prepared 2 % silver nitrate solution for 30 min under ultra violet light of 254 nm wavelength. After 30 min, the cells were washed with

deionized water, viewed and imaged under bright field microscope to visualize brownish black deposits denoting the presence of mineralization.

3.2.4.3 Masson's Trichrome staining for collagen deposits

The hPDLCs at 7, 14, and 21 days were rinsed with PBS and fixed using 4 % PFA for 1 h. The fixed cells were rinsed with deionized 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 rinsed with deionized water and the nuclei were stained with hematoxylin for 5 min. After washing the cells were sequentially stained for collagen deposits using Masson's trichrome solution, following manufacturer's instructions.

3.2.4.4 Osteogenic marker expression of hPDLCs on Lys-PDA and G-PDA

The expression of osteogenic markers proteins like osteonectin, alkaline phosphatase, Dentin Matrix Protein 1 (DMP1) and Cementum Membrane Protein (CEMP - specific for cementum) expressed by hPDLCs on the Lys-PDA and G-PDA matrices was carried out as per the procedure is described in section 3.1.1.4.iv. The primary antibodies used were mouse antibodies against human osteonectin and CEMP, and rabbit antibodies against human alkaline phosphatase and DMP 1.

3.3 Bioactive calcium cements for dental regeneration

The second objective of the study also includes the identification and optimization of cost-effective bioactive cements towards regeneration of dental pulp and periodontium. In-house developed medical grade calcium sulfate cement and phosphate modified calcium sulfate cement (BioCaS) was selected as candidate bioactive cements towards regeneration of periodontium and dental pulp. The cytotoxicity, cytocompatibility, and osteoinduction potential of BioCaS towards hPDL cells was investigated. Calcium-sulfate, calcium - phosphate cements and sintered hydroxyapatite were taken as control materials. BioCaS was also evaluated for the potential for dental pulp regeneration using the isolated characterized dental pulp cells *in vitro*.

3.3.1 Synthesis of calcium sulfate and phosphate based bioactive cements

Synthesis of medical grade, low dimensional calcium sulfate was carried out in-house from high purity ingredients (all purchased from M/s Merck, India). Calcium sulfate (in dihydrate form) was first synthesized from analytical grade $Ca(NO_3)_2.4H_2O$ (98 wt.% purity) and conc.H_2SO_4 (97-99 wt.% purity) through a 'drowning-out' wet precipitation technique using isopropyl alcohol (99.5 wt.% purity). The resultant precipitate was isolated through centrifugation and freeze drying, which contained uniform low-dimensional (3-5 µm) rod-shaped crystals of calcium sulfate, when observed by SEM. It was heated for 18 hours at 120ºC at the atmospheric pressure in an oven to obtain the beta phase calcium sulfate hemihydrate (CaS) [Sandhya *et al*., 2012].

To synthesize the Bioactive Calcium Sulfate (BioCaS) cement, disodium hydrogen orthophosphate dihydrate (99.5 wt. % purity) was added to calcium sulfate powder in a proportion 2 % w/w. On wetting with de-ionized distilled water (0.5 ml per gram), the CaS and BioCaS powder forms cement putty that sets in approximately 7 and 12 minutes respectively. The physico-chemical characterization of Calcium Sulfate and BioCaS cement has previously been reported [Sandhya *et al*., 2015].

The Calcium Phosphate cement (CPC)powder and the liquid component (aqueous solution containing orthophosphate ions) was prepared as reported before. The calcium phosphate cement powder contained equimolar concentrations of tetra calcium phosphate (TTCP) and dicalcium phosphate dihydrate (DCPD) particles with an average particle size of 100 µm. TTCP is synthesized using $CaHPO_4$ and $CaCO_3$ in a solid state reaction with equimolar portions of dicalcium phosphate and calcium carbonate reactant powders which were mixed and then milled to get a uniform particle size and distribution. DCPD used was analar grade which was ball milled to reduce the particle sizes to below 100µm.The wetting medium used was deionized water with Na_2HPO_4 at 0.2 M concentration as the setting accelerator. The wetting ratio was optimized as 0.8 ml/g to obtain a cement mix of moldable consistency [Fernandez *et al*., 2006.]. The cement paste was placed in suitable molds and allowed to set completely to obtain the desired dimensions of set cement discs.

For cell culture studies, the samples of CaS, BioCaS and CPC cements were prepared by filling the cement putty in pre-fabricated silicone molds with cavities of 1 cm diameter and 2 mm height and allowed to set. The discs formed were washed in distilled water and

dried in an oven at 90 ºC for 24 h. The cement discs were sterilized using EtO for the experiment. The discs were cut into 4mm diameter for cytotoxicity studies.

Sintered Hydroxyapatite was taken as a control material for the biological evaluation of the cements. Hydroxyapatite (HA) ceramic discs were made using in-house prepared high purity powder obtained through chemical precipitation. The calcined fine particles (125 μm diameter) were isometrically compressed and made into discs (Automatic hydraulic press SPEX, X-PRESS 3630). These were sintered at 1100 ºC for 2 hours to obtain HA discs of about 10 mm diameter with 2 mm thickness. The samples were cleaned using distilled water and sterilized by autoclaving.

3.3.2 Physico-chemical characterization

The crystallinity of the calcium sulfate, calcium phosphate and BioCaS was carried out after setting, to identify any phase changes during the setting reaction. The corresponding cement powder and liquid was mixed to form the set cement. The set cements were dried and crushed to powder form for X-Ray Diffraction (XRD) analysis (Bruker AXS). The XRD data obtained was compared to confirmed standards for interpretation.

Fourier Transform Infra-Red Spectroscopy (FTIR) analysis of the set cements was carried out to identify the functional groups present. The corresponding cement powder and liquid was mixed to form the set cement. The set cements were dried and crushed to powder form and mixed with potassium bromide into a pellet. The IR spectroscopy was measured in transmission mode (Nicolet 5700). The peaks corresponding to the functional groups were analyzed to evaluate the presence of the sulfate and phosphate groups in the respective cements.

The surface morphology and particle size of the set cements were evaluated by SEM imaging of the set cement discs after gold sputter coating.

3.3.3 *In Vitro* evaluation of Bioactive Cements

The third objective of the study includes the *in vitro* biological evaluation of the selected bioactive cements using the established tooth-derived cell culture system. The cytocompatibility of the CaS, BioCaS and CPC cements and sintered HA was evaluated through a set of *in vitro* experiments using periodontal ligament cells as follows:

3.3.3.1 Direct contact and MTT assay

Human periodontal ligament cells were seeded in 24 well cell culture plates at a density of 3×10^4 cells/well and cultured for 24 h, after which, the test materials – CaS, BioCaS, CPC, and sintered HA discs (4 mm diameter) were carefully placed on the cell layer and cultured for 24 h. The experiment was conducted in triplicates of test samples and control cells. The cells cultured without the test materials were taken as the negative control (cell control). The 24 h phase contrast images of hPDLCs in direct contact with the discs were taken to visualize any signs of cytotoxicity. The cell response was analyzed under a phase contrast microscope and the cytotoxicity was graded from 0 to 4 (0 - none, 1 - slight, 2 - mild, 3 - moderate and 4 - severe) based on the morphology, cell lysis, cell detachment and vacuolization of the cells around the material [ISO 10993 - 5].

The metabolic activity of cells after direct contact test was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) reduction assay [Präbst *et al*., 2017]. The test samples and culture medium were removed and the cells were incubated with 100 µl of freshly prepared 1mg/ml MTT solution for 2 to 4 h in the dark. The formazan product formed was dissolved in isopropanol and absorbance was measured (as optical density) using a microplate reader (Biotek, USA) at 570 nm. The percentage metabolic activity of the cells was calculated from the optical density (OD) values using the equation:

$$\text{Percentage cell metabolic activity} = \frac{\text{OD}_{\text{test}}}{\text{OD}_{\text{Control}}} x100$$

The data was presented as mean ± standard deviation.

3.3.3.2 Cell adhesion and viability of hPDLCs on materials

The hPDL cells were seeded onto the discs of CaS, BioCaS, CPC and HA at a density of 10^3cells/cm^2 and incubated for 24 h. The cell viability was assessed by FDA/PI live/dead staining (details in section 3.2.3.1) and observed under fluorescence microscope.

3.3.3.3 Cell adhesion and morphology of hPDLCs on materials

The hPDL cells were seeded onto the discs of CaS, BioCaS, CPC and HA at a density of 10^3 cells/cm^2 and cultured for 24 h. The morphology of the hPDLCs on materials was

evaluated by actin cytoskeletal staining and SEM as described in section 3.2.3.2 and 3.2.3.3 respectively. The cells cultured in glass coverslips was taken as the control.

3.3.4 Osteogenic differentiation potential of the bioactive cements on hPDLCs

The hPDL cells in passage 3 to 5 were used for the osteogenic differentiation experiments. The cells were seeded at a density of 10^4 cells/cm^2 to 12 well plates and incubated for 24 h. The cement discs and sintered HA discs were carefully placed over the cells and culture medium was added. The cells of control group were cultured in regular cell culture medium (αMEM containing 10 % FBS and 100 IU Penicillin/streptomycin) and the cells in test group was cultured in an osteogenic induction medium (αMEM containing 10 % FBS, 100 IU Penicillin Streptomycin; 50 µg/ml L-ascorbic acid, 10 mM sodium β-glycerophosphate and 10 nM dexamethasone). Osteogenic property of the bioactive cements was evaluated at 7, 14 and 21 days.

3.3.4.1 Alizarin red staining and assay

The calcium deposits by the hPDL cells differentiated to osteogenic lineage was analyzed by alizarin red staining. The discs were carefully removed from the cell monolayer at 7, 14 and 21 days and culture medium was removed. The cells were fixed using 4 % PFA and stained with alizarin red as described in section 3.1.1.4.i to visualize the calcium deposits. The mineralization was quantified by estimating the calcium deposition on 21st day through alizarin red assay as described in 3.2.4.1. The values obtained for osteogenic test system were compared with positive and negative controls.

3.3.4.2 Alkaline phosphatase enzyme activity

The secreted alkaline phosphatase enzyme was taken as an indicator for osteogenic differentiation of the hPDLCs. On day 7, 100 µl of culture medium from triplicate culture were transferred to 96 well cell plates. 50 µl of para nitro phenyl phosphate (pNPP) solution (Sigma Aldrich) was added to each well. The alkaline phosphatase in the culture medium will cleave the phosphate group of the pNPP to form yellow colored paranitro- phenol, which can be assessed colourimetrically in a microplate reader at 405nm. The OD valued obtained were plotted as a direct correlation to the expression of alkaline phosphatase enzyme activity.

3.3.4.3 Osteogenic marker expression of hPDLCs on bioactive cements

The expression of the osteogenic markers by the hPDLCs on CaS, CPC and BioCaS cements were carried out by ICC/IF staining. The samples were prepared by coating sterile cover glass with the cements and sterilised using EtO. The cells were seeded onto the cement samples and cultured in regular cell culture medium for 7 days. The cells were fixed with 4 % PFA for 1 h and subjected to the ICC/IF staining for Alkaline Phosphatase, Osteonectin, DMP 1 and Collagen Type I (section 3.1.1.4.iv).

3.3.5 Biological evaluation of the bioactive cements using human Dental Pulp cells

The cytocompatibility and biological responses of bioactive cements were carried out using the hPDL cells. To identify the potential use of the CaS, CPC and BioCaS as dental pulp capping agents, the cell responses of isolated and characterized human dental pulp cells (hDPCs) was explored *in vitro* using hDPCs. Since hard tissue regeneration is possible with bioactive calcium cements, an attempt can be made to evaluate the dental pulp cell response to these bioactive cements, which will provide valuable information towards the use of these bioactive cements as cost effective dental pulp capping agents.

3.3.5.1 Cell adhesion and morphology

The CaS, CPC and BioCaS cements were coated onto sterile glass coverslips, dried and sterilised using EtO. The hDPCs in passage 3 were seeded onto the sterile, cement coated glass coverslips at a cell density of 10^3 cells/ cm^2 and cultured for 24 h. The cells seeded on uncoated glass coverslips was taken as control. The cells were fixed in 4 % PFA for 1 h, washed thrice with PBS, permeabilized with triton X100, washed again, and incubated with Phalloidin Cytopainter 555 (Abcam) for 1 h, in darkness. After incubation, the nuclei were counterstained using Hoechst 33258 (5 µg/ml) for 1 min. the cells were then washed with PBS and viewed under fluorescence microscope using suitable filters.

3.3.5.2 Osteogenic potential of bioactive cements on human dental pulp cells

The osteogenic differentiation of hDPCs in the presence of the cements was evaluated using ICC/IF staining of the hDPCs cultured on EtO sterilized, CaS, CPC and BioCaS cement coated glass coverslips (1 cm^2 area), at day 7. The cells in passage 3 were seeded onto the coated glass cover slips a cell density of 10^4 cells/ cm^2. The cells, at 7 days were fixed with

4 % PFA for 1h and rinsed thrice with PBS. They were then permeabilized with triton X100, rinsed again with PBS, treated with 10 % BSA for 30 min to block non specific antibodies, and incubated overnight at 4 ºC with the corresponding primary antibodies – Alkaline Phosphatase enzyme, Osteonectin and DMP 1. They were rinsed with PBS and incubated with the corresponding secondary antibodies (Anti mouse AlexaFluor 488 and Anti rabbit AlexaFluor 545) for 1h in dark. The cells were rinsed with PBS, the nuclei were stained using Hoechst 33258 (5 µg/ml), rinsed again and viewed under Fluorescence microscope using suitable filters.

The above experiments were designed to evaluate the response of human periodontal ligament cells towards bioactive calcium cements that could be used as bone graft materials in alveolar bone defects associated with periodontitis, and/or as endodontic sealers and medicaments or perforation repair materials as these biomaterials can come to contact to periodontal cells in clinical scenario. Also, the periodontal ligament can differentiate to osteogenic and a cementogenic lineage in accordance with the clinical situation which confirms the utility of periodontal ligament cells in cytocompatibility and bioactivity evaluations of the cements. Each procedure carried out in the above experiments were repeated thrice to confirm the reproducibility of the results.

Since calcium based cements are reported to promote dentin bridge formation, when in contact with the vital dental pulp, it was decided to evaluate the response of dental pulp cells to the in house synthesized CaS, CPC and BioCaS cements. A short experiment was designed to evaluate cell adhesion and morphology, and osteogenic marker expression by hDPCs on the cements.

3.4 Translational approaches of the biomaterials for clinical applications

In order to translate the biomimetic and bioactive materials for clinical applications, animal studies were conducted in rat models.

The dendritic peptide matrices needed suitable modifications to adapt them to a clinically acceptable format before the *in vivo* evaluations. Therefore, the self-assembling dendritic peptide molecules were used to biofunctionalize an inert, electrospun

polycaprolactone mat, intended to be applied for GTR procedures as prototype barrier membranes.

The bioactive cements were used as powder – liquid based settable and moldable cement systems, and applied *in vivo* as alveolar bone graft/ barrier graft materials for the treatment of bone defects associated with severe periodontitis.

3.4.1 Application of Lys-PDA and G-PDA as barrier membrane

In this section, Lys-PDA and G-PDA were used to biofunctionalize electrospun PCL mats to obtain a barrier membrane. The FDA approved polymer polycaprolactone (Mol.Wt. 80000) was used as the barrier membrane for biofunctionalization.

3.4.1.1 Preparation and Biofunctionalization of electrospun PCL membrane

Solution of PCL (Average mol. wt: 80000, Sigma) was prepared in solvent system containing tetrahydrofuran (THF; Spectrochem) and dimethyl sulphoxide (DMSO; Spectrochem) in 9:1 ratio. A quantity of 10 ml with the PCL ratio 10 % w/v, was stirred in magnetic stirrer for a minimum duration of 2 h for complete dissolution. Electrospinning was carried out in a customized electrospinning unit (Holmarc Opto-Mechatronics Pvt Ltd., India), at a potential difference of 15 KV and flow rate of 1.5 ml/h, with a metal collector at a distance of 12 cm. The electrospun PCL mat was washed thoroughly and repeatedly in sterile deionized water to remove the traces of solvents [Sundaran *et al*., 2017].

i. Lys-PDA/PCL

The PCL electrospun mats were cut into 1 cm^2 strips and immersed in 1 mg/ml solutions of Lys-PDA overnight. The mats were taken from the solution, placed on 6-well cell culture plates and irradiated with UV (~250 nm) for 2 h to complete residual polymerization. The mats were then air dried overnight inside a laminar flow hood. A color change from white (PCL) to red (Lys-PDA/PCL) was noted upon coating. The hydrophilicity of the mats was evaluated by water contact angle measurement (Contact Angle System OCA, Data Physics) with untreated PCL mat as control. The presence of Lys-PDA coating on the PCL membranes was confirmed by SEM evaluation and FTIR analysis of the presence of amine functional groups on the mats. The mats were sterilized using EtO at 37 ºC and stored at room temperature until used for cell culture experiments.

ii. G-PDA/PCL

The PCL mats were cut into 1 cm^2 strips and immersed in 1 mg/ml solutions of G-PDA, overnight. The residual solution was discarded; the mats were subjected to UV irradiation of 254 nm wavelength for 2 h to bring about residual polymerization and allowed to dry overnight. The hydrophilicity of the mats was evaluated by water contact angle measurement, with untreated PCL mat as control. The coating confirmation was done by SEM imaging and FTIR spectroscopy. The mats were sterilized using ethylene oxide gas at 37 ºC.

3.4.2 *In Vitro* Biological evaluation of Lys-PDA/PCL, G-PDA/PCL mats

The *in vitro* biological evaluation of the Lys-PDA and G-PDA coated electrospun PCL mats were carried out using MTT assay, cell adhesion and viability, actin cytoskeletal staining and SEM.

3.4.2.1 Direct contact test and MTT Assay

The cytotoxicity and cell activity in the presence of materials was assessed by MTT assay as described before. The hPDL cells were seeded at a density of 3×10^4 cells/well of 24 well cell culture plates for 24 h. The test materials – Lys-PDA, Lys-PDA/PCL and PCL discs (4mm diameter) were carefully placed on the cell monolayer and cultured again for 24 h. Triplicate samples of each material was used for the study. The cells cultured without the test samples were taken as non-toxic cell control. The cells incubated with 0.13 % phenol was taken as toxic control. After 24 h, the culture medium was replaced with 100 µl of 1mg/ml MTT solution and incubated for 2 h in the dark. The formazan product was solubilized in isopropanol and the absorbance of color developed was measured as optical density values using a microplate reader (BioTek USA) at 570 nm. The percentage metabolic activity of the cells was calculated from the optical density values using the equation:

$$\text{Percentage cell metabolic activity} = \frac{\text{OD}_{\text{test}}}{\text{OD}_{\text{Control}}} x100$$

The results were plotted as mean ± standard deviation.

3.4.2.2 Cell adhesion and viability

The viability of hPDLCs on the mats was evaluated using the FDA/PI live dead imaging. The hPDL cells were seeded onto the Lys-PDA/PCL, G-PDA/PCL and PCL mats at a density of 10^3 cells/cm^2 and evaluated for viability by FDA/PI staining and imaging as described in section 3.2.3.1, and observed under fluorescence microscope.

3.4.2.3 Cell morphology and spreading

The cell morphology of hPDLCs on the mats was evaluated by Actin cytoskeletal staining and SEM.

(i) Actin cytoskeleton staining: For actin cytoskeletal staining, the cells cultured as described above for 24 h, rinsed with PBS and fixed using 4 % PFA for 1 h. The fixed cells were rinsed thrice with PBS, permeabilized using Triton X-100 for 3 min and incubated with ActinGreen 488 (Thermofischer Scientific) for 30 min in the dark. The cells were rinsed with PBS and nuclei were counterstained with Hoechst 33258 (5 µg/ml). After a final PBS wash, the cells were viewed and imaged using fluorescence microscope.

(ii) SEM evaluation: The cells were seeded onto PCL (control), Lys-PDA/PCL and G-PDA/PCL mats at a density of 10^3 cells/cm^2 and cultured for 24 h. The cells were rinsed and fixed in 2.5 % gluteraldehyde for 1 h. Serial dehydration of fixed samples was done in ascending concentrations of ethanol and isoamyl acetate. The samples were dried in a critical point dryer and gold sputter coated, viewed and SEM images were taken.

3.4.3 *In Vivo* evaluation of Lys-PDA/PCL mats

In order to do clinical translation, preclinical usage tests using suitable 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 extracellular matrix.

3.4.3.1 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. Animals weighing above 350 g 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 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.

3.4.3.2 Defect creation and material placement

Bilateral maxillary alveolar bone defects were created buccal to the first molars, using surgical micro motor hand piece and tungsten carbide (WC) burs of 0.8 mm diameter at a speed of 2300 rpm, under ambient cooling using cold saline irrigation. Briefly, the selected animals were weighed and appropriate anesthetic dosage was calculated as per the accepted guidelines – Ketamine (75 mg/kg body weight) and xylazine (10 mg/kg body weight)**.** The anesthetic solution appropriate for the weight of each animal were taken in the same syringe and injected subcutaneously on either thigh. Once anaesthetized, the animals were placed on their backs securely on a special surgical platform and the jaws were kept open using retractive elastic bands. The mucosa adjacent to the mesio-buccal 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, and PCL or Lys-PDA/PCL mats were placed carefully over the defects such that they were in contact with the tooth at the gingival level, covering the defects. The bone defects without the mats were considered as the sham control.

Group	SHAM	PCL	Lys-PDA/PCL
No of defects (*N*)	6	6	6
Duration *(Months)*	3	3	3

Table 2: Distribution of defects and duration of implantation for the in vivo evaluation of Lys-PDA/PCL mats as barrier membranes in rat maxillary periodontal defect model.

3.4.3.3 Tissue harvesting and histopathology evaluation

At the end of the experiment period of 3 months, the animals were euthanized in a CO_2 chamber. The entire craniofacial area containing the maxillary alveolar bone was harvested and fixed in 10 % neutral buffered formalin by immersing the tissue specimen completely in the fixing solution for 10 days. After adequate fixation, gross dissection of the

maxillary alveolar bone was carried out and the specimens were embedded in clear PMMA (Poly Methyl Methacrylate). The PMMA embedded tissue specimens were sectioned using a hard tissue microtome (Struers Accutome 100), with average width of the sections being 100 µm. The specimens were viewed under microscope and the selected sections were stained using Van Gieson (for bone) and Stevenel's blue (for osteoblasts cells). The stained cells were viewed and images were taken.

3.4.4 *In vivo* evaluation of Bioactive cements as barrier grafts for periodontal regeneration.

In order to do clinical translation, preclinical usage tests using suitable animal models need to be carried out. CaS, CPC and BioCaS cements were evaluated as alveolar bone grafts/barrier grafts using a rat maxillary alveolar bone defect model.

3.4.4.1 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. Animals weighing above 350 g were selected for the studies. Total 3 defect sites were dedicated to each test group and sham control. Since bilateral defects were created, a total of six animals were used for the study. Each defect site was taken as a single unit and the time duration was fixed at 1 month / 4 weeks.

3.4.4.2 Defect creation and material placement

Bilateral maxillary alveolar bone defects were created buccal to the first molars, using surgical micro motor handpiece and tungsten carbide burs of 0.8 mm diameter at a speed of 2300 rpm, under ambient cooling using cold saline irrigation. Briefly, the selected animals were weighed and anaesthetized using Ketamine (75 mg/kg body weight) and xylazine (10 mg/kg body weight), taken in the same syringe and administered subcutaneously on either thigh. 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 mucosa adjacent to the mesio buccal 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. The respective cements were mixed using a sterile glass slab and spatula.

Once a putty like consistency was obtained (approximately 4-5 min), the defects were packed with the corresponding cement to conformally fill the defects, so that they were in close contact with the tooth root and alveolar bone, till the gingival attachment level. The bone defects without the cements were considered as the sham control.

Group	SHAM	BioCaS	CPC	CaS
No. of defects (*N*)	3	3	3	3
Duration *(Month)*	1	1	1	1

Table 3. Distribution of defects and duration of implantation for *in Vivo* evaluation of BioCaS, CaS and CPC cements as alveolar bone graft substitutes in rat maxillary alveolar bone defect model.

3.4.4.3 Tissue harvesting and histopathology evaluation

At the end 1 month, the animals were euthanized using CO_2 inhalation, and the entire craniofacial area containing the maxillary alveolar bone was harvested and fixed in 10 % neutral buffered formalin by completely immersing the tissue specimen for 48 h. After fixation, gross dissection of the maxillary alveolar bone was carried out and the specimens were embedded in clear Poly Methyl Methacrylate (PMMA). The PMMA embedded tissue specimens were sectioned using a hard tissue microtome (Struers Accutome 100) to get PMMA embedded sections of 100 µm thickness.

The specimens were viewed under microscope and the selected sections were stained using Van Gieson (for bone) and Stevenel's blue for osteoblasts cells. The stained cells were viewed and images were taken.

The above experiments were carried out to evaluate the translational potential of the biomimetic matrices for periodontal applications and the bioactive cements as potential alveolar bone grafts / periodontal barrier grafts.

The biomimetic dendritic peptide molecules were coated onto electrospun PCL mat with an intention to biofunctionalize the inert synthetic PCL mat to a biomimetic barrier membrane for periodontal GTR treatment strategies. Even though the cytocompatibility

biologic responses can be elucidated in *vitro* using periodontal ligament cell, the barrier function can be confirmed only *in vivo*. Therefore, a carefully planned rat periodontal defect model was developed and executed for the same. Sufficient sample numbers were ensured to enable the reproducibility of results.

Similarly, the *in vitro* osteogenic potential of the bioactive cements needs to be confirmed *in vivo* for the clinical translation of the cements as alveolar bone graft substitutes or as barrier grafts in periodontal regeneration. Therefore, a rat maxillary alveolar bone defect model was developed and executed to confirm the *in vivo* osteoinductivity of the cements.

Chapter 4

RESULTS

This chapter describes in detail, the results of the experiments designed and carried out to achieve the objectives of the study. The results correspond to the sections of the materials and methods section and are divided into four chapters, each representing the results of specific study plans and experiments.

4.1 Establishment of in vitro Cell Culture System Using Tooth-derived Cells

The establishment of an *in vitro* tooth-derived cell culture system was achieved through repeated experiments for standardization of each procedure. The dental tissue sample collection method helped to transfer the tissues from the collection point to the biosafety cabinet in tissue culture lab without chances of contamination. The results of each experiment are detailed in the following sections.

Through explant culture technique a healthy population of human Periodontal Ligament cells (hPDLCs), human Dental Pulp Cells (hDPCs) human exfoliated deciduous Dental cells (hDecDCs), and human Dental Apical Papilla cells (hDAPCs) were obtained. The Collagenase / dispase digestion method yielded a good population of hPDLCs and hDPCs, but the total cell yield was more in explant culture. The cell isolation procedures were carried out at least thrice under identical conditions to confirm the repeatability of the isolation methods to arrive at consistent results.

4.1.1 Isolation and culture of human Periodontal Ligament cells

An explant culture model and a collagenase/dispase tissue digestion model were used to isolate the human periodontal ligament cells (hPDLCs).

4.1.1.1 Tissue explant method

The cell outgrowths from the periodontal ligament explants were observed between 7 and 21 days. The cells were spindle in shape with fibroblast morphology. The cells were plastic adherent and attained confluency within 48 to 72 h (Figure 4).

4.1.1.2 Tissue digestion method

The cells isolated by digestion of periodontal tissue adhered on culture flasks within 24 h and a healthy culture was obtained. The cells expressed fibroblast morphology and were plastic adherent when observed under phase contrast microscope (Figure 4). However, the cells were very slow growing and took long duration to multiply in repeated experiments (n=3). Hence enzyme digestion method was not considered for further cell isolation and experiments (Figure 4).

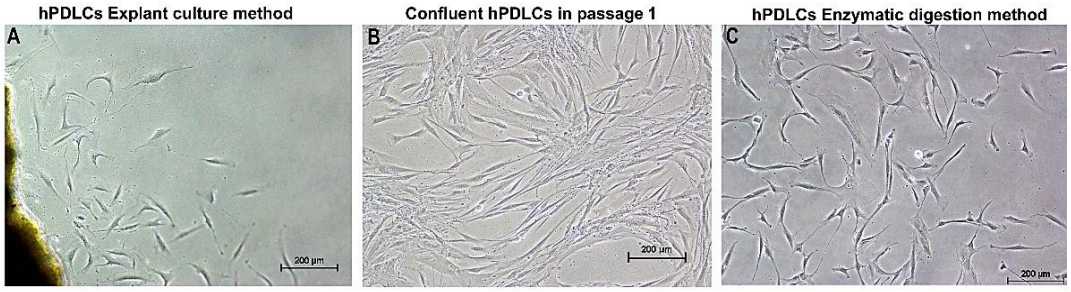


Figure 4: Isolation of hPDLCs by explant culture method and enzymatic digestion method. **A:** Cell outgrowths from the periodontal tissue explants at day 8**. B**: Confluent cells in passage 1 from tissue explant culture method. **C:** hPDLCs from tissue digestion method at day 4.

4.1.1.3 Characterization of human Periodontal Ligament cells

The hPDLCs showed positive expression of the type III intermediate filament protein Vimentin and the Mesenchymal Stem Cell (MSC) markers CD 73, CD 90, CD 105 and STRO-1. In addition, the hPDLCs showed positive expression for Dentin Matrix Protein (DMP1), the ligament specific protein Scleraxis, and cementum membrane protein (CEMP) confirming their tissue of origin (Figure 5).

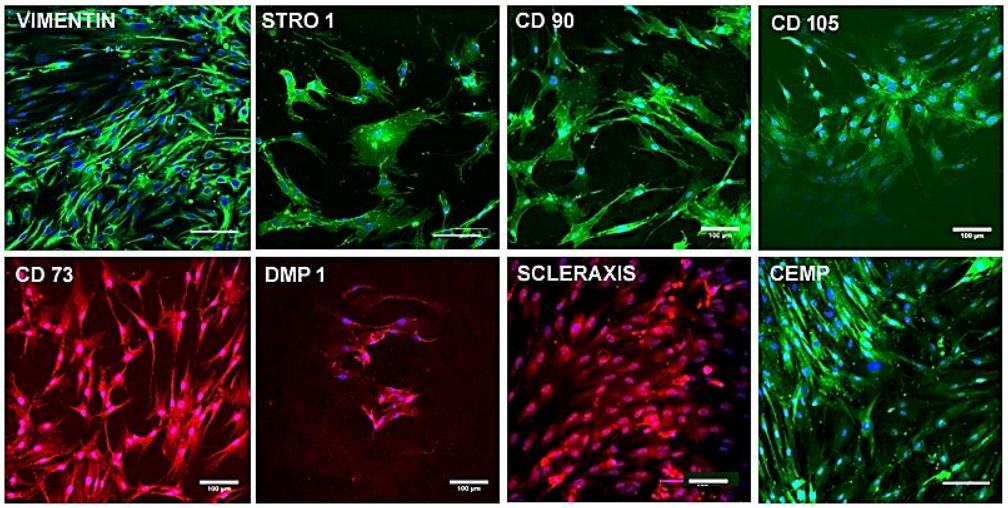


Figure 5: Characterization of human Periodontal ligament cells. Scale bar **100µm**

4.1.1.4 Osteogenic differentiation of human Periodontal Ligament cells

The hPDLCs showed evidences of mineralization and collagen deposition upon induction of differentiation using the osteogenic medium. This was confirmed by histological staining for mineral deposits and collagen formation, as well as positive expression of osteogenic marker proteins upon ICC/IF staining with the corresponding antibodies.

i. Alizarin red staining

The alizarin red staining showed significant calcium deposits by hPDLCs cultured in osteogenic induction medium at 7, 14 and 21 days. The calcium deposits got stained as red nodules. A progressive increase in the number and size of the stained mineral deposits were noted at 14 and 21 days, with minimal mineral deposition at 7 days (Figure 6).

ii. Von Kossa Staining

The presence of phosphate deposits by the cells were confirmed by the presence of brownish black deposits of metallic silver, upon von Kossa staining. Thick black deposits were evident at 14 and 21 days culture in the osteogenic induction medium than at day 7 indicating a progressive increase in phosphate deposition (Figure 6).

iii. Masson's trichrome staining

The Masson's trichrome staining for collagen deposits showed positive for collagen matrix deposition, which is essential in mineralization. The bluish green appearance of collagen deposits was evident at 14 and 21 days culture of hPDLCs in osteogenic induction medium. (Figure 6).

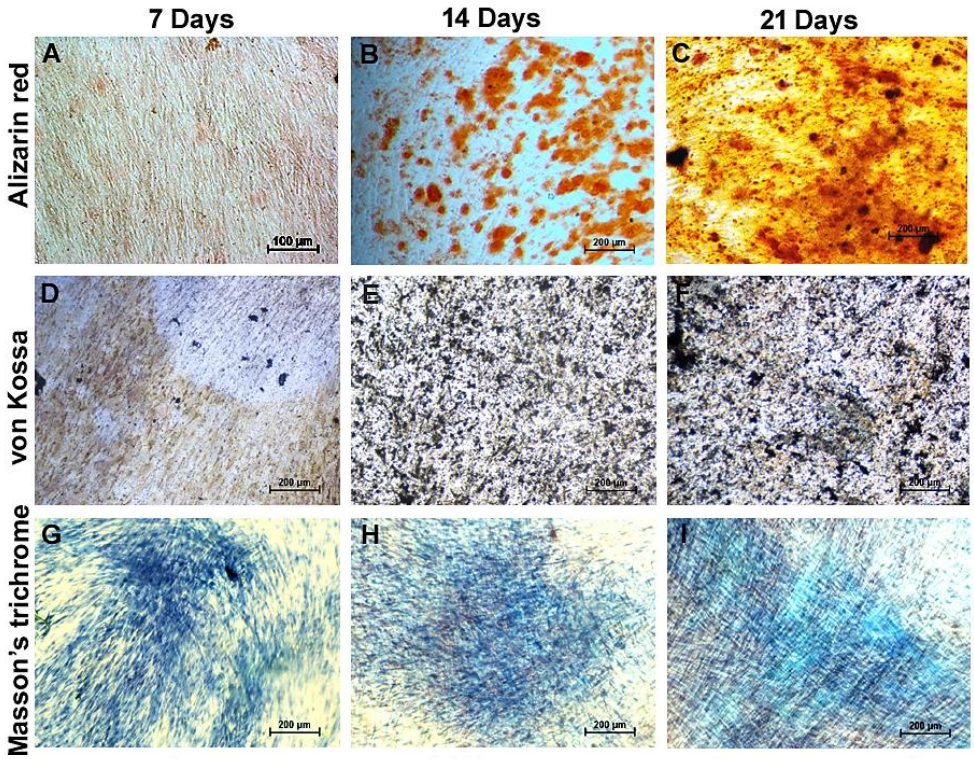


Figure 6: Histological staining of mineral deposits and collagen deposits by hPDLCs. Scale bar - **200µm**

iv. Osteogenic marker expression by hPDLCs

The hPDLCs exhibited the presence of the osteogenic markers osteonectin, alkaline phosphatase, collagen 1 and DMP 1 at day 7, indicating the osteogenic differentiation potential of the cells. Since the same markers are reported to be expressed during cementogenic differentiation also, it can also be assumed that the hPDL cells could differentiate to osteogenic and cementogenic lineage, when cultured in an osteogenic induction medium [Ozer *et al*., 2013; Yucheng *et al*.,2008]. (Figure 7).

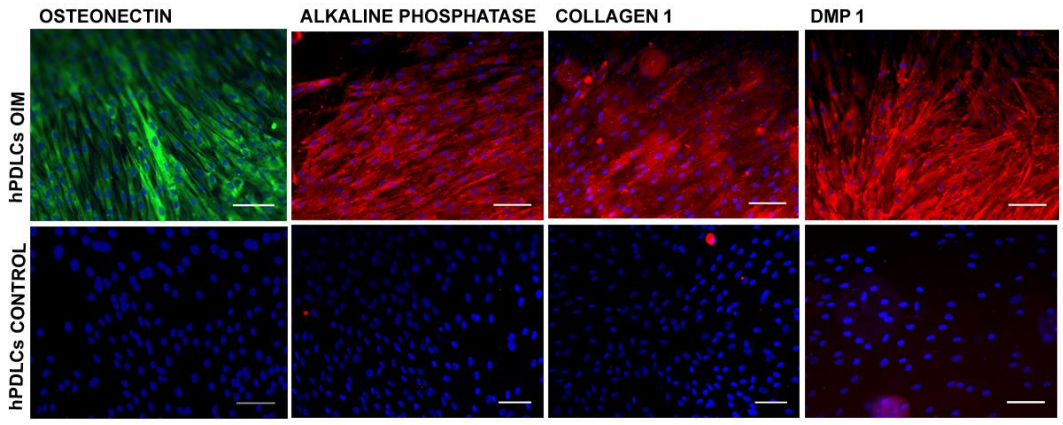


Figure 7: Osteogenic marker expression of hPDLCs in osteogenic induction medium (Top) and regular cell culture medium (Bottom). Scale bar - **100µm**

4.1.2 Isolation and culture of human dental pulp cells (hDPCs)

The results of the isolation and culture of hDPCs, its characterization and differentiation potential carried out as per the methodology described in the section 3.1.2 is presented in the following sections.

4.1.2.1 Tissue explant method

The dental pulp cells started growing out of the tissue explant from 7th day onwards. The cells were spindle in shape and adhered well on tissue culture plastic. The culture attained confluency within 3-4 days after a stable cell outgrowth was noticed. (Figure 8).

4.1.2.2 Tissue digestion method

The tissue digestion method yielded an adherent hDPC cell population in 24 h. The cells exhibited fibroblast morphology and were observed to be plastic adherent in phase contrast microscopy and attained confluency in 7 days. (Figure 8).

4.1.2.3 Characterization of Dental pulp cells

The hDPCs showed positive expression of the fibroblast marker protein Vimentin and the MSC markers CD 73, CD 90 and Stro 1. In addition, the hDPCs showed positive Dentin Matrix Protein (DMP1) expression. (Figure 9).

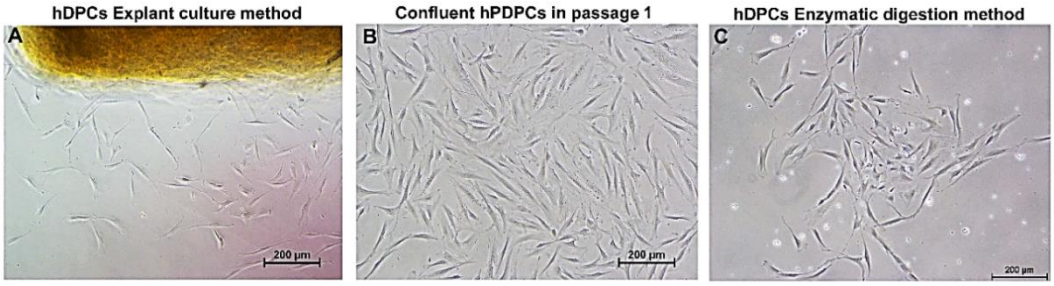


Figure 8: Isolation of human dental pulp cells (hDPCs) by explant culture and enzymatic digestion methods. A: hDPCs cell outgrowths from the dental pulp tissue explants at day 10. B: DPCs from explant culture technique in passage 1. hDPCs from enzymatic digestion technique at day 4.

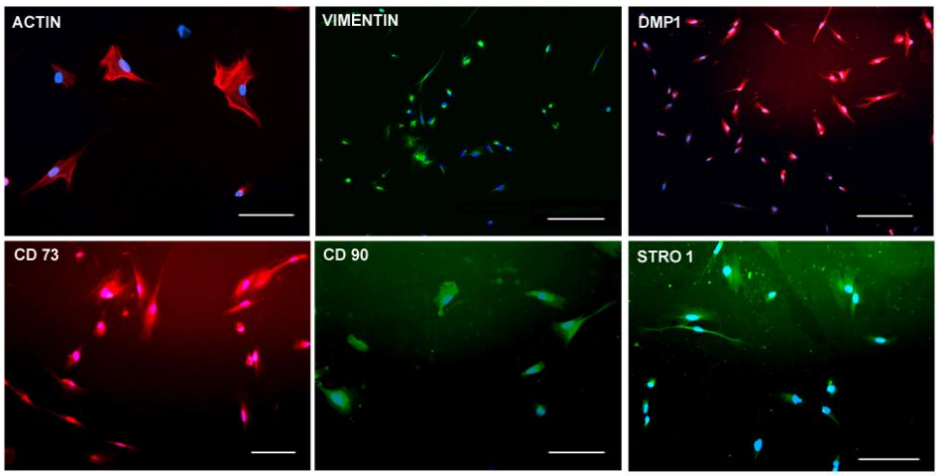


Figure 9: Characterization of Dental pulp cells. Scale bar - **100µm**; Vimentin, DMP 1 - Scale bar - **200µm**

4.1.2.4 Osteogenic differentiation of Dental pulp cells

The osteogenic differentiation potential of hDPCs was confirmed by analyzing the presence of mineralization on histological staining and presence of collagen deposits.

i. Alizarin red staining, Von Kossa staining and Masson's Trichrome staining

The alizarin red staining showed significant calcium deposits by the hDPCs cultured in osteogenic induction medium with progressive increase in the mineral deposition at 7, 14 and 21 days. The presence of phosphate deposits by the cells were confirmed by the presence of brownish black deposits of metallic silver, upon von Kossa staining. However, the typical bluish green collagen deposits were not observed in Masson's trichrome staining. (Figure 10).

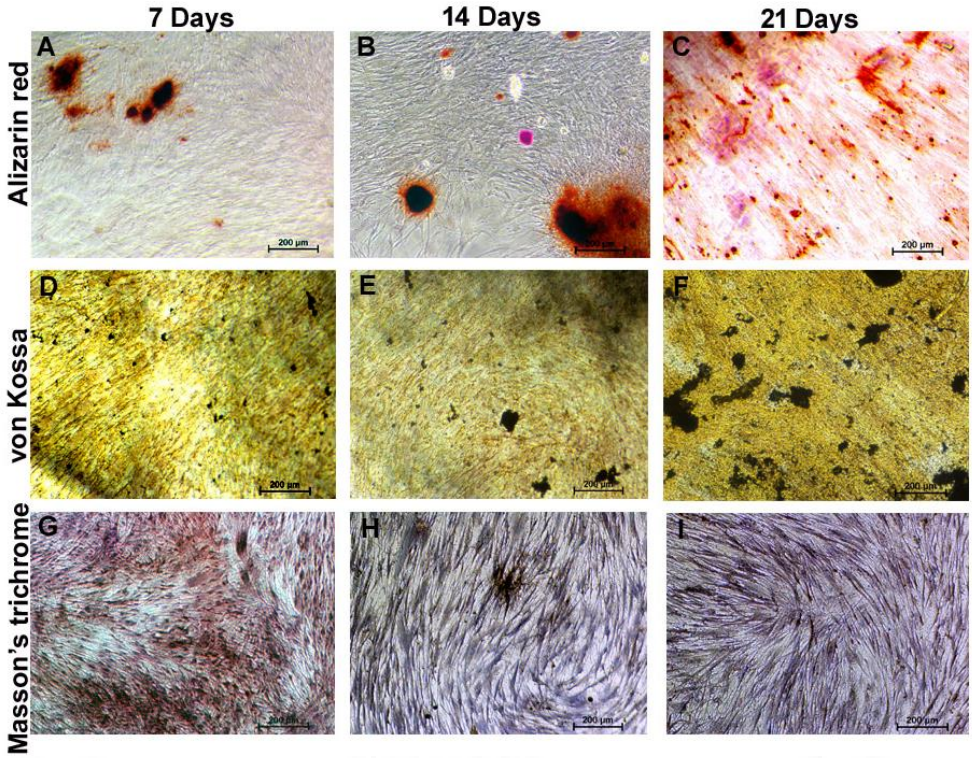


Figure 10 Histological staining of hDPCs in osteogenic induction medium at 7, 14 and 21 days. Scale bar - **200µm**

ii. Osteogenic marker expression by hDPCs

The hDPCs exhibited the presence of the osteogenic markers osteonectin, alkaline phosphatase, collagen 1 and DMP 1 at day 7, indicating the osteogenic differentiation potential of the cells (Figure 11). The presence of type I collagen, which was not evident in Masson's trichrome staining was also noted in ICC/IF staining. Since the same markers are reported to be expressed during odontogenic differentiation, it can be assumed that the hDPCs could differentiate to osteo/odontogenic lineages under suitable induction conditions. (Figure 11).

4.1.3 Isolation and culture of human exfoliated deciduous Dental cells (hDecDCs)

The explant culture method yielded a healthy population of deciduous dental cells (hDecDCs) (Figure 12). The cell outgrowths from the tissue samples were noted from 7th day onwards. The hDecDCs exhibited fibroblast morphology and was plastic adherent.

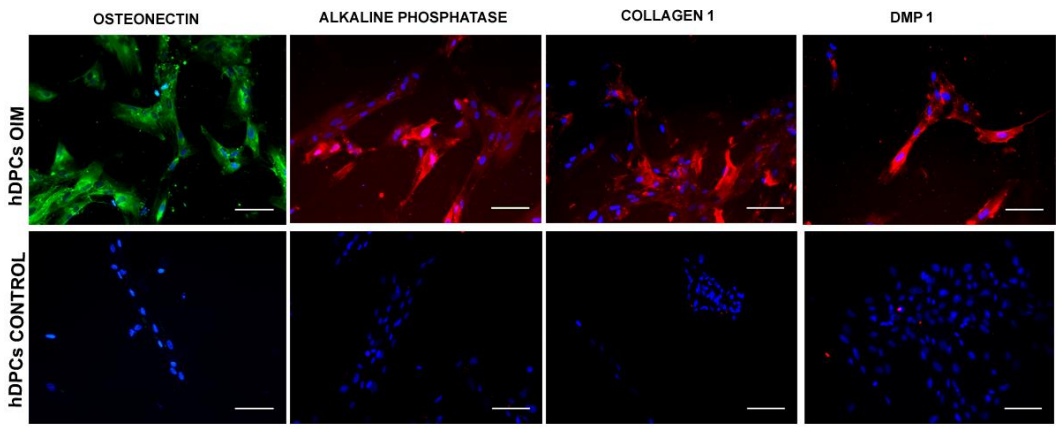


Figure 11. Osteogenic marker expression by dental pulp cells. Scale bar - **100µm**

4.1.3.1 Characterization of Deciduous Dental Cells (hDecDCs)

The hDecDCs showed positive expression of Vimentin and the MSC markers – CD 90, CD 105and Stro 1, confirming a progenitor cell population within the hDecDCs (Figure 13).

4.1.3.2 Osteogenic differentiation of Deciduous teeth cells

The osteogenic differentiation of hDecDCs was confirmed based on the presence of mineral deposits.

i. Alizarin red staining, Von Kossa staining and Masson's trichrome staining

The alizarin red staining of cells cultured in osteogenic induction medium showed very prominent calcium deposits as progressively increasing red nodules on the cell monolayer at 7, 14 and 21 days. The presence of phosphate deposits by the cells were confirmed by the presence of thick black deposits of metallic silver, upon von Kossa staining. However, typical bluish green staining for collagen deposits was not evident in Masson's trichrome staining. (Figure 14).

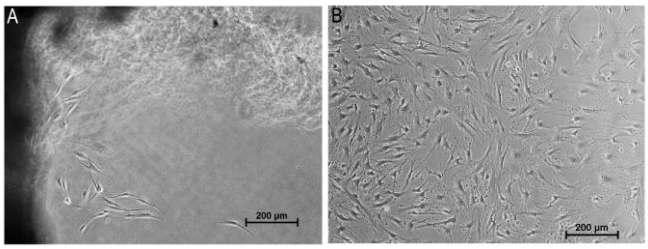


Figure 12: Isolation of human deciduous dental cells (hDec.DCs) by explant culture method. A) hDec.DCs outgrowths from the explant; B) hDec.DCs in passage 1.

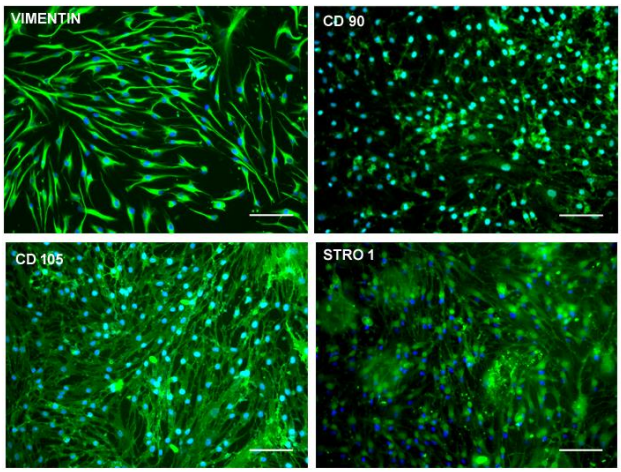


Figure 13: Characterization of Deciduous teeth cells. Scale bar - **200µm**

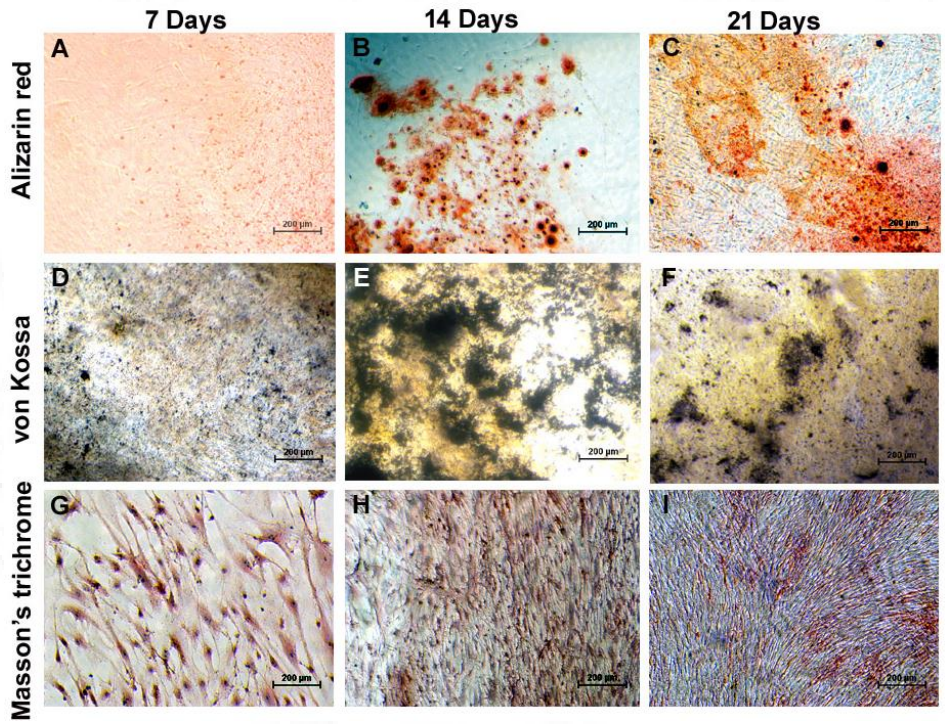


Figure 14: Histological staining of osteogenic differentiation of human deciduous teeth cells. Scale bar - **200µm**

4.1.4 Isolation and culture of human Dental Apical papilla cells

The explant culture method yielded a good population of hDAPCs. The cell outgrowths from the tissue samples were observedbetween11 and 14 days. The subculture and passaging, the cells exhibited the fibroblast morphology. 70 – 80 % confluency was observed in 2-3 days. (Figure 15).

4.1.4.1 Osteogenic differentiation of Apical papilla cells

The hDAPCs showed evidence of mineralization upon histological staining for the presence of collagen and mineral deposits.

i. Alizarin red, Von Kossa and Masson's trichrome staining

The alizarin red staining showed significant, progressive calcium deposits on the osteogenic induction medium at 7, 14 and 21 days. The presence of phosphate deposits by the cells were confirmed by the presence of brownish black deposits of metallic silver, upon von Kossa staining. The Masson's trichrome staining for collagen deposits showed bluish green collagen deposits at 21 days. (Figure 16).

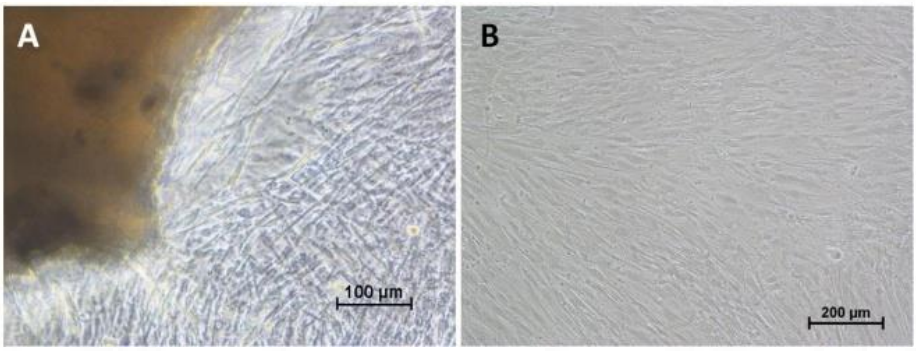


Figure 15: Isolation of dental apical papilla cells (hDAPCs). A) hDAPCs cell outgrowth from explant culture. B) Confluent hDAPCs in passage 1.

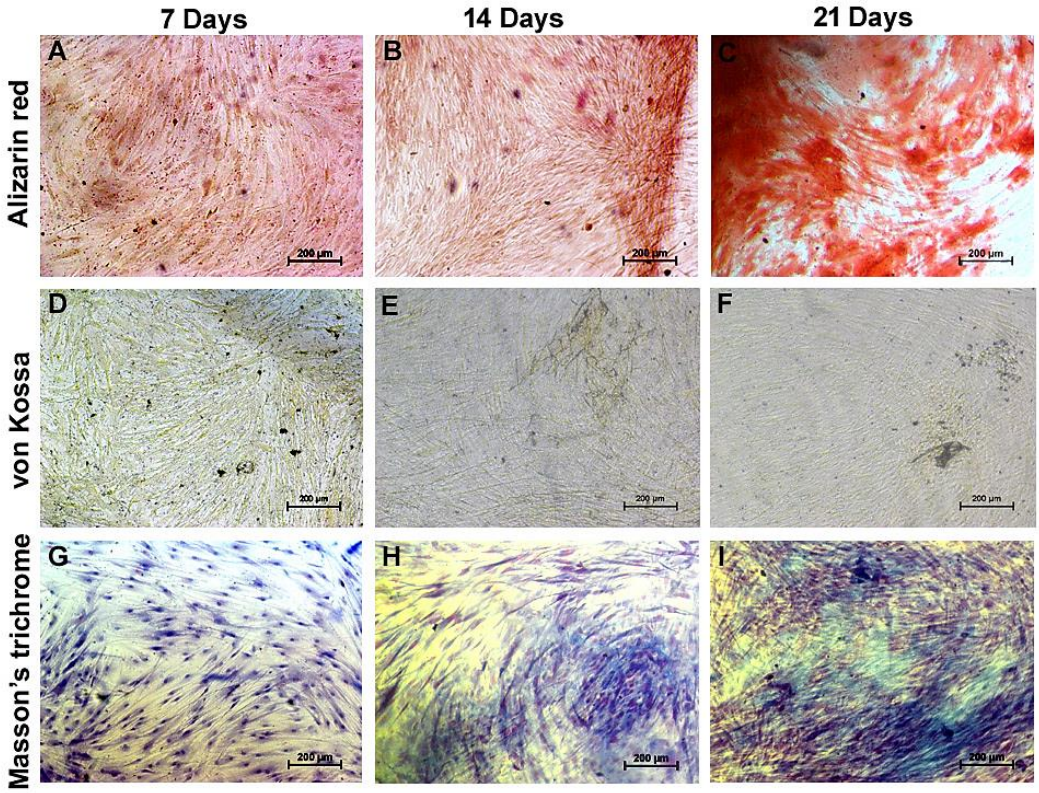


Figure 16: Histological staining of osteogenic differentiation of human dental apical papilla cells. Scale bar - **200µm**

The first objective of this study is the establishment of a tooth-derived cell culture system. The results from the section 4.1 confirms methodology developed to attain the first objective was successful in the isolation, culture, and characterization of tooth-derived cells – hPDLCs, hDPCs, hDec.DCs and hDAPCs. The results also confirm the differentiation potential of tooth-derived cells towards an osteogenic, odontogenic and cementogenic lineages, through a single induction medium. From these experiments, the availability, culture conditions and characterization indicate the use of periodontal ligament cells and the dental pulp cells as better suited for dental biomaterial evaluations. Therefore, the hPDLCs and the hDPCs were selected for further biomaterial – cell interaction studies.

4.2 Biomimetic Matrices for dental regeneration

The amino acid Lysine and the small biomolecule Guanidine which constitutes the terminal group of amino acid arginine were selected as functional peptide and peptide mimetic components and were successfully appended to a self-assembling UV polymerizable polydiacetylene molecule. These molecules were procured from dept. of Chemistry, IIT Delhi, as aqua soluble powder and were further termed as Lysine – polydiacetylene (Lys-PDA) and Guanidine – polydiacetylene (G-PDA) respectively. The results of the cytocompatibility and differentiation potential of hPDLCs on Lys-PDA and G- PDA are presented as follows.

4.2.1 Lys-PDA (Lysine Appended Polydiacetylene)

Lys-PDA was designed and synthesized at dept. of Chemistry, IIT Delhi and received after preliminary characterization. Lys –PDA was shipped in powder form and stored at room temperature until used.

4.2.1.1 Lys-PDA coating and sterilization

Upon dissolving in sterile deionized water, it was possible to drop cast Lys-PDA polymer onto sterile glass coverslips and 6 well tissue culture plates (Nunc) as a thin surface layer. UV irradiation at 254 nm was carried out for 1 h to complete the residual polymerization of Lys-PDA. The Lys-PDA coated glass coverslips and multiwell cell culture plates were successfully sterilised using ethylene oxide at 37 ºC and stored at room temperature till further use.

4.2.1.2 Lys-PDA coating confirmation and characterization

The presence of Lys-PDA coating was confirmed by phase contrast microscopy, SEM and fluorescence microscopy, as the Lys-PDA matrix exhibited autofluorescence. The dendritic peptide matrix of Lys-PDA was visible as a layer of branched network identifiable in phase contrast microscopy, Fluorescence and Scanning Electron Microscopy. The presence of Lys-PDA on glass coverslips was confirmed by FTIR which showed the characteristic peaks of amine bonds and the diacetylene bonds. The characteristic peaks of aliphatic primary amine groups were observed as double peaks between 3250 – 3400 cm^{-1} and at 1669 cm^{-1}. The C-H peaks were observed at 2975 cm^{-1}, between 720 – 880 cm^{-1}, and

between 1350 to 1450 cm^{-1}. The C-N peaks of lysine were observed between 1100 and 1270 cm^{-1}. The C=C peaks of polydiacetylene was observed between 750 to950 cm^{-1} with peak at 949 cm^{-1} indicating the polymerization of diacetylene. (Figure 17).

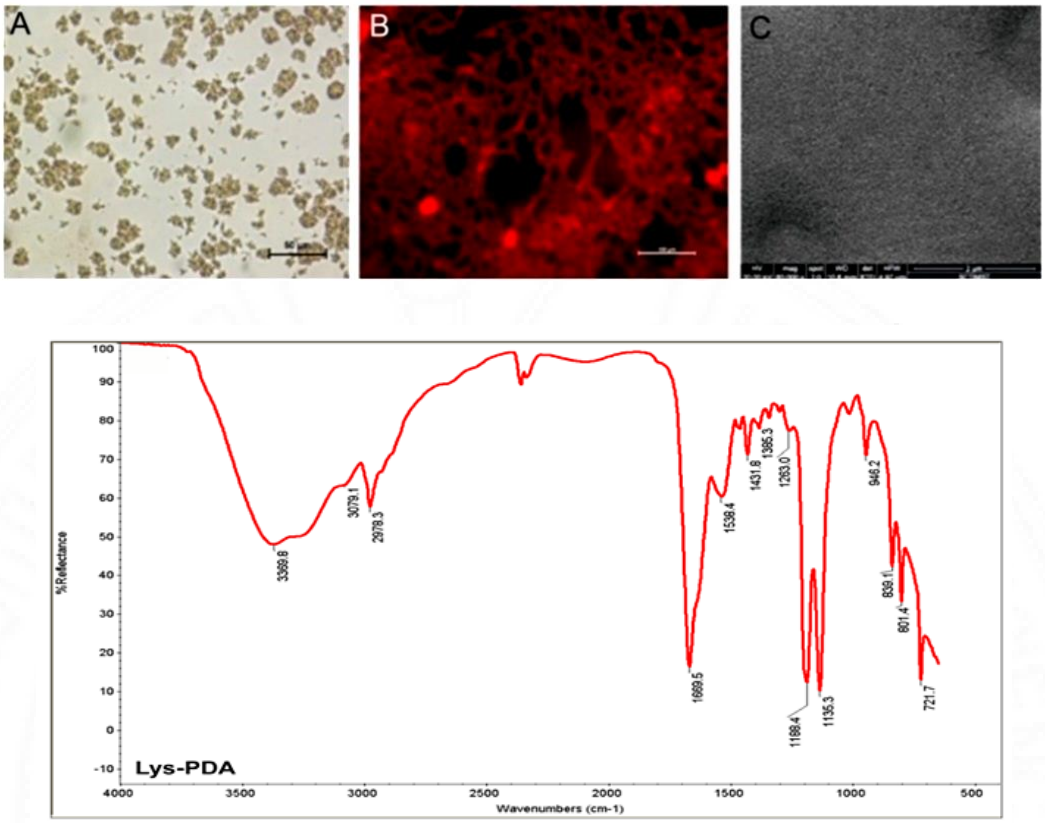


Figure 17: Characterization of Lys-PDA. **A**,**B,C**) Phase contract, fluorescence microscopic and SEM images of Lys-PDA coating on cover glass respectively. Scale bar **A, B - 100 µm.** SEM Scale bar :**4 µm.** Below: FTIR spectroscopy

4.2.2 G-PDA (Guanidine appended polydiacetylene)

G-PDA was designed and synthesized at dept. of Chemistry, IIT Delhi and received after preliminary characterization. G–PDA was shipped in powder form and stored at room temperature until used.

4.2.2.1 G-PDA coating and sterilization

G-PDA polymer was dissolved in water and drop casted onto sterile glass coverslips and 6 well tissue culture plates as a thin matrix. The G-PDA coated glass coverslips and

multiwell cell culture plates were sterilised using ethylene oxide at 37 ºC and stored at room temperature till further use.

4.2.2.2 G-PDA coating confirmation and characterization

The presence of G-PDA coating on the glass coverslips and multiwell cell culture plates were confirmed by SEM and AFM. The presence of G-PDA on glass coverslips was confirmed by FTIR which showed the characteristic peaks of amine bonds in G-PDA and the polydiacetylene bonds. The characteristic peaks of the primary amine groups in guanidine were observed between 3200 – 3600 cm^{-1} with peaks at 3211 and 3355 cm^{-1} and at 1669 cm^{-1}. The C-H peaks were observed at 2927 cm^{-1}, triple peaks between 720 – 840 cm^{-1} and at 1434 cm^{-1}. The C-N peaks of primary amines in guanidine were observed between 1100 and 1270 cm^{-1} with peaks at 1138, 1191 and 1259 cm^{-1}. (Figure 18).

4.2.3 *In vitro* biological evaluation of the biomimetic surfaces using hPDLCs

The isolated characterized hPDLCs were successfully used for the biological evaluation of the Lys-PDA and G-PDA coated surfaces. The hPDLCs at second passage were seeded onto the sterile G-PDA and Lys-PDA coated cover glass at 10^3 cells / cm^2 exhibited normal cell morphology, adhesion and proliferation.

4.2.3.1 Cell adhesion and viability

The cells on Lys-PDA and G-PDA coated surfaces showed typical fibroblast morphology in phase contrast imaging. (Figure 19– A-D). The cell adhesion and viability test using FDA/PI live/dead images revealed that the majority of cells to be viable (green fluorescence) with only a few dead cells (red fluorescence) in both the G-PDA and Lys-PDA matrices (Figure 19: E-H). There was no evidence of cell death or morphology changes, indicating the biocompatibility of the matrices. (Figure 19).

4.2.3.2 Actin Cytoskeleton staining

The cells on Lys-PDA and G-PDA coated surfaces showed typical fibroblast morphology in actin cytoskeleton staining. The actin filaments were seen to be well organized and the normal cytoskeletal architecture was maintained, confirming the cell adhesion of hPDLCs on Lys-PDA and G-PDA coatings. (Figure 19: I -L)

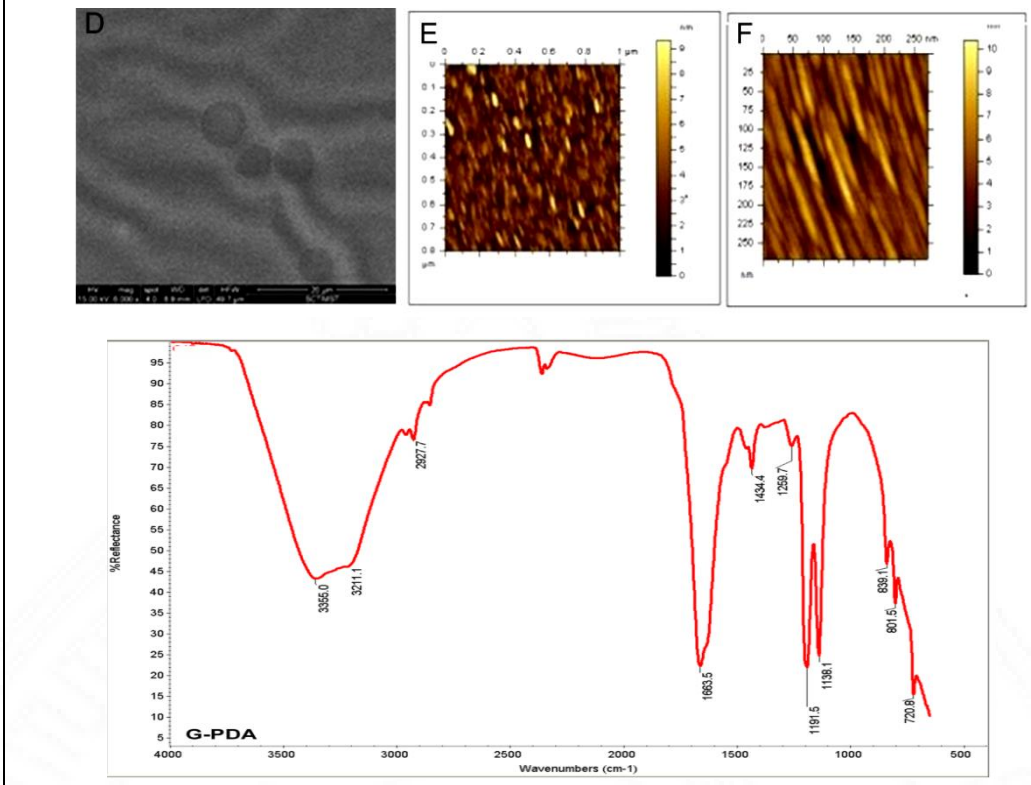


Figure 18: Characterization of G-PDA. **D,E,F**) SEM, AFM evaluation of G-PDA coating on cover glass. SEM Scale bar: **4 µm.** Below: FTIR spectrum of G-PDA.

4.2.3.3 SEM evaluation of cell morphology

The SEM images showed the lamellipodial and filopodial extensions which are manifestations of cell adhesion to the Lys-PDA and G-PDA surfaces with active cells spreading. This was in accordance with the actin cytoskeletal staining to confirm cell morphology and spreading. (Figure 19: M-P)

4.2.4 Osteogenic differentiation of human Periodontal Ligament Cells on Lys-PDA and G-PDA matrices

The osteogenic differentiation of hPDLCs on biomimetic Lys-PDA and G-PDA matrix showed positive osteogenic properties such as mineral deposition and osteogenic marker proteins. The cells on Lys-PDA and G-PDA matrices cultured in osteogenic induction medium exhibited mineral deposition and positive expression of osteogenic marker proteins, compared to the negative and material controls.

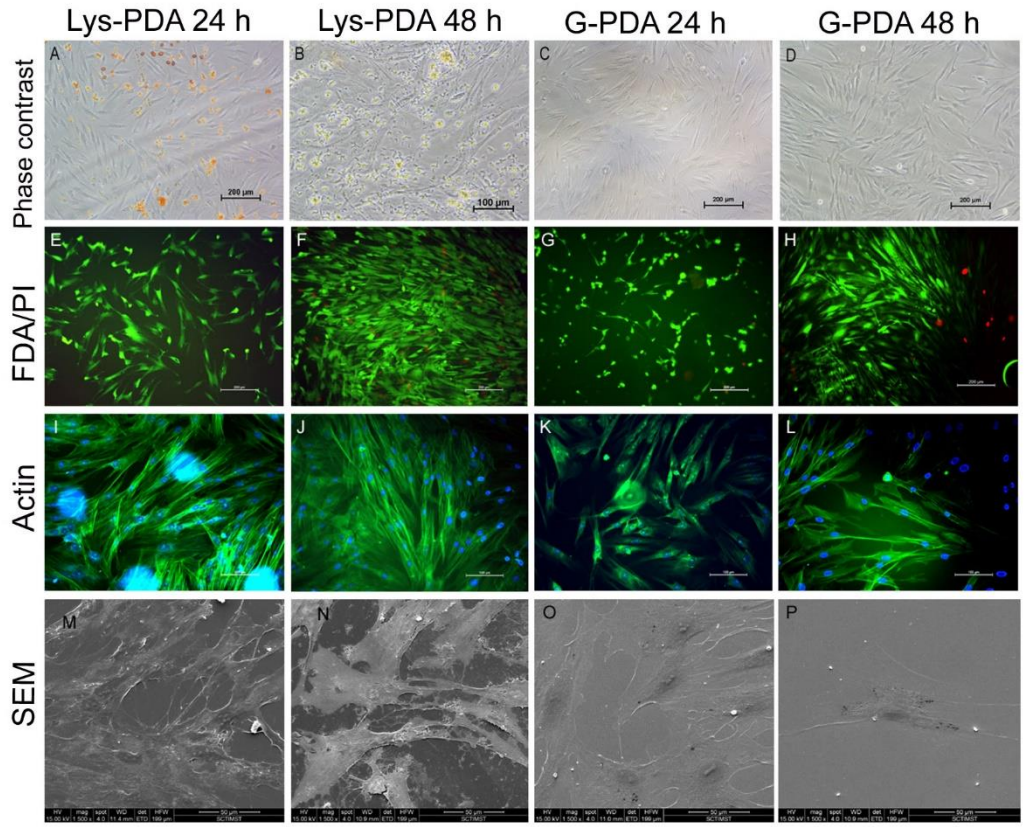
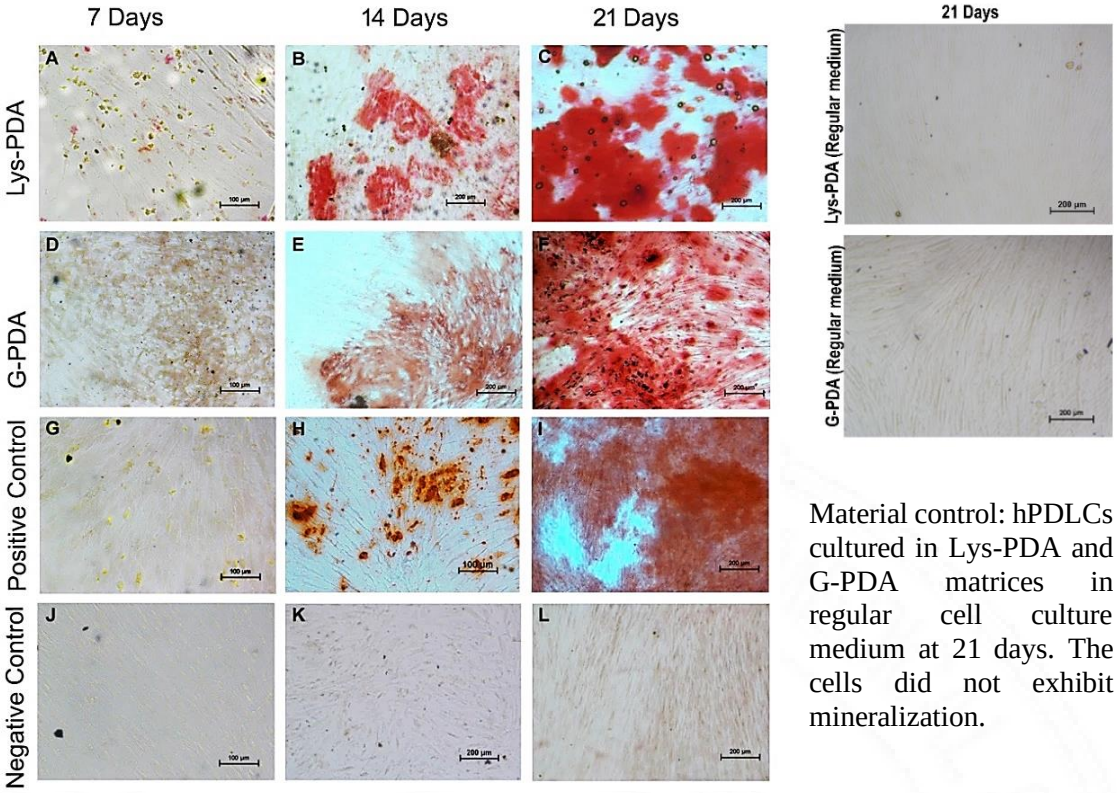


Figure 19: Periodontal ligament cell adhesion, viability and morphology on Lys-PDA and G-PDA. Scale bar – A-H: **200µm**; I-L: **100µm**; SEM : **50 µm**.

4.2.4.1 Alizarin Red staining and assay:

The hPDLCs cultured on the Lys-PDA and G-PDA surfaces using osteogenic differentiation medium showed evidence of mineralization as early as 7 days than the cells cultured in uncoated glass coverslips in osteogenic induction medium. This shows that the cells retained their differentiation potential in the presence of Lys-PDA and G-PDA coatings. The alizarin red assay values quantitatively confirmed the presence of mineral deposits with statistically significant mineral deposits in the induced cells. The hPDLCs on Lys-PDA coating showed significant mineralization when compared to the positive control. (Figure 20, Figure 21).



Material control: hPDLCs cultured in Lys-PDA and G-PDA matrices in regular cell culture medium at 21 days. The cells did not exhibit mineralization.

Figure 20: Alizarin red staining of hPDLCs cultured with osteogenic medium on Lys-PDA and G-PDA. Scale bar - **200µm**.

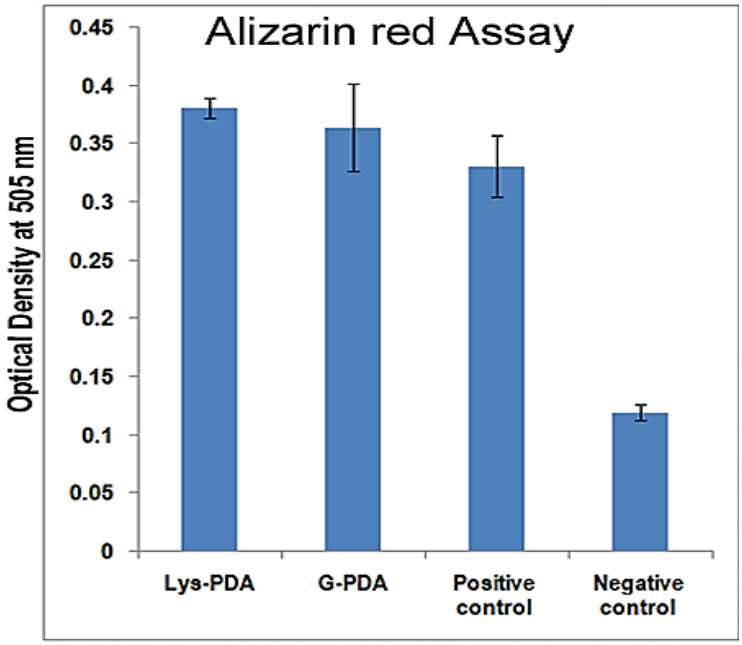


Figure 21:Alizarin red assay of hPDLCs on Lys-PDA and G-PDA at 21 days

4.2.4.2 Von Kossa Staining:

Upon Von Kossa staining with 2% silver nitrate, the hPDLCs in the osteogenic differentiation experiment showed phosphate deposition, showing the mineralization potential of the cells in the matrices, as compared to the cells grown in regular medium (Negative control). An early mineralization on the Lys-PDA matrix was also present. (Figure 22).

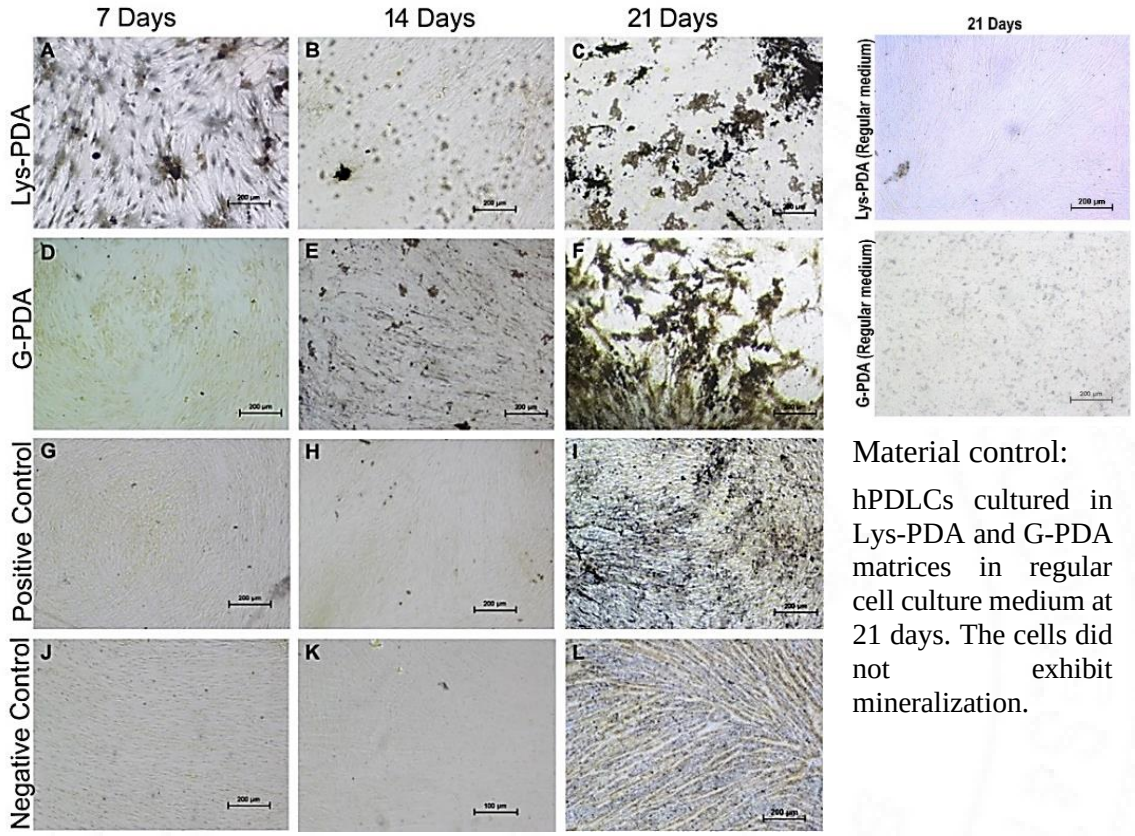


Figure 22: Von Kossa staining of hPDLCs for phosphate deposits cultured on Lys-PDA and G-PDA matrices. Scale bar - **200µm**

4.2.4.3 Masson's Trichrome Staining for Collagen Deposits

The Masson's Trichrome staining for collagen deposits showed that the hPDLCs in the osteogenic differentiation medium showed collagen deposition, which, along with the mineralization is essential for the osteogenic differentiation of the cells in the matrices, as compared to the cells grown in regular medium (Negative control). (Figure 23).

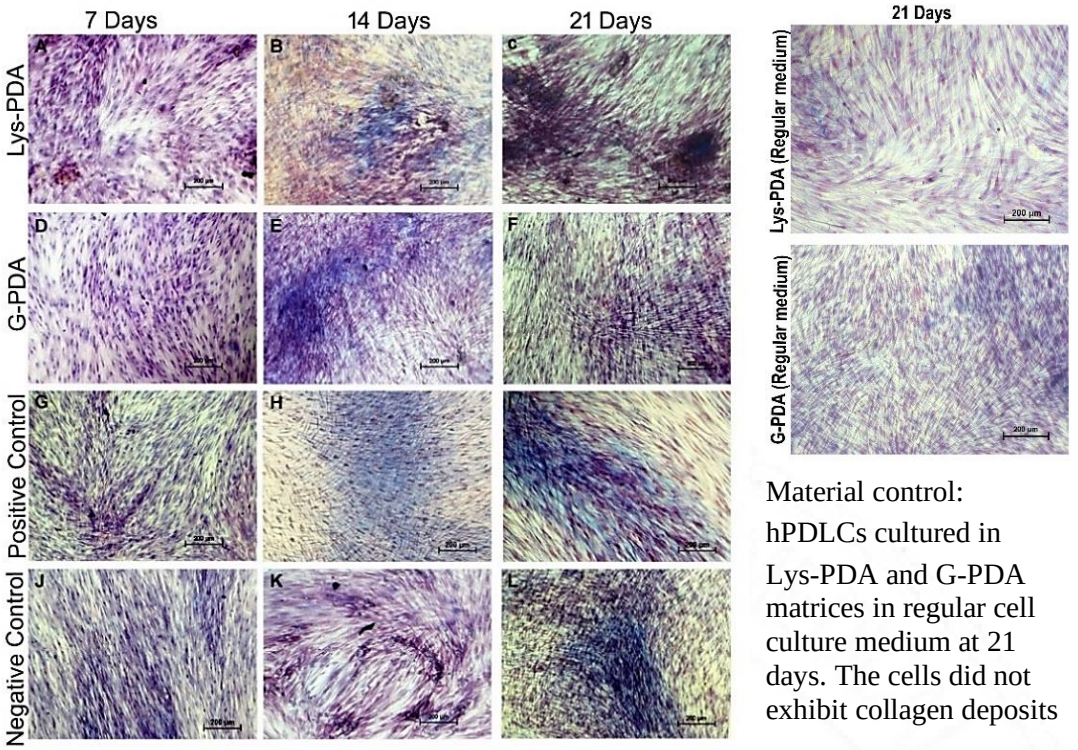


Figure 23: Masson's Trichrome staining of hPDLCs for collagen deposits, cultured with osteogenic medium on Lys-PDA and G-PDA matrices. Scale bar - **200µm**

4.2.4.4 Osteogenic marker expression of hPDLCs on Lys-PDA and G-PDA

ICC/IF staining of the hPDLCs on the Lys-PDA and G-PDA matrices and cover glass controls showed the positive expression of the osteogenic marker protein Osteonectin, Alkaline phosphatase and Dentin Matrix Protein 1 (DMP1). The expression of Cementum Membrane Protein (CEMP), marker specific for the deposition of cementum by the periodontal ligament cells, was also positive. (Figure 24).

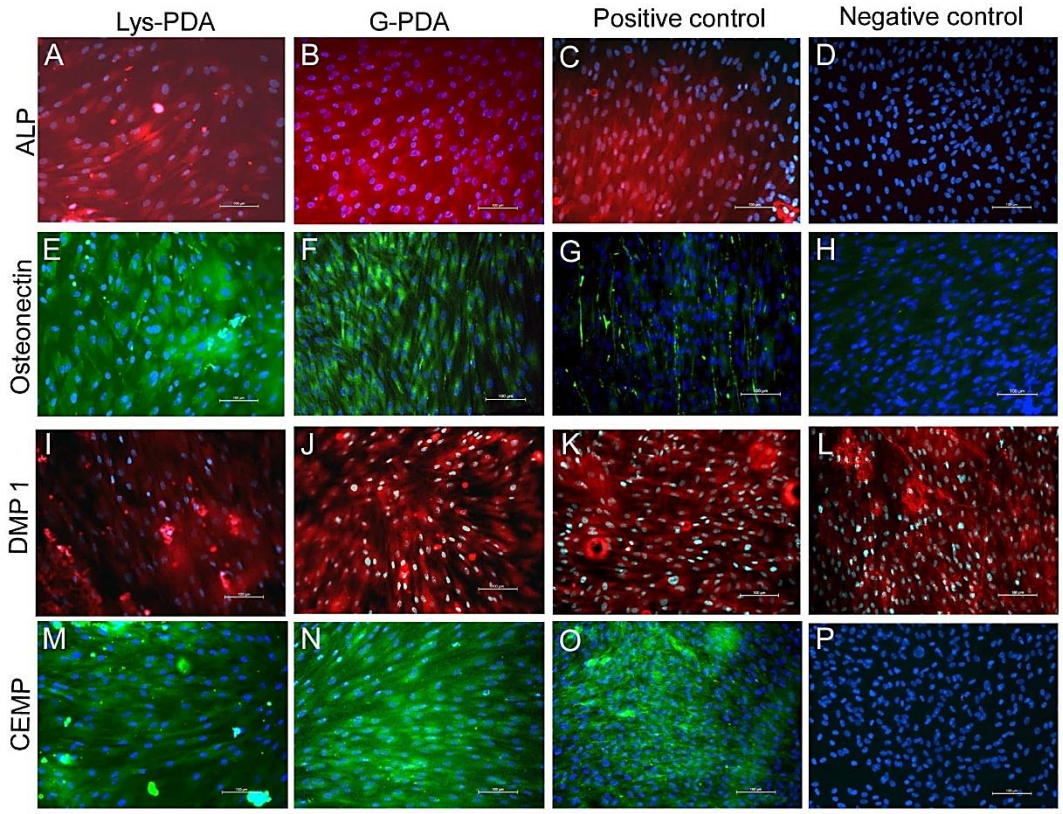


Figure 24**:** Osteogenic marker expression of hPDLCs on Lys-PDA and G-PDA. Scale bar - **100µm**

4.3 Bioactive calcium cements for dental regeneration

In house synthesized and characterized medical grade Calcium Sulfate Cement (CaS) powder, Bioactive Calcium Sulfate cement (BioCaS) and Calcium Phosphate Cement (CPC) were evaluated *in vitro* as prototype resorbable alveolar bone graft substitutes in comparison to sintered Hydroxyapatite discs for the regeneration of periodontium, using isolated characterized human Periodontal ligament cells (hPDLCs). The response of primary human periodontal ligament (hPDL) cells to these bioactive cements was investigated through a series of *in vitro* experiments using hPDLCs, to evaluate the cytocompatibility and osteoinduction potential.

4.3.1 Synthesis of calcium sulfate and phosphate based bioactive cements

The standardized synthesis techniques yielded sufficient quantities of the respective cement powder. For CaS and BioCaS cement, sterile distilled water was used as the liquid

component. For calcium phosphate cement, an aqueous solution containing phosphate ions was used as the liquid medium. HA powder compaction and subsequent sintering yielded solid sintered discs.

Upon mixing the cement powder and liquid components, a setting time of 5-8 minutes was noted for calcium sulfate cement and 10-12 min for BioCaS cement. A sufficient working time of 4-10 min was available before the initial setting of the cements. Calcium phosphate cement exhibited an increased setting time of 15-20 min. All the cements exhibited moldable putty like consistency before the final setting, which allowed sufficient time for filling the silicone molds used for the cement disc fabrication. Once set, the cements did not disintegrate when kept in PBS, thus permitting their use for cell culture. The discs were dried in a hot air oven with temperature set below 100 ºC to prevent phase conversion and were successfully sterilized using ethylene oxide at 37 ºC. The cements could not be autoclaved as it could cause phase changes within the material lattice. Sintered HA was sterilised in an autoclave as it could withstand higher temperatures. After EtO sterilization, the samples could be stored at room temperature, till further use.

4.3.2 Physico-chemical characterization

The scanning electron micrographs (SEM) of the cement discs gave an indication of the size distribution and orientation of the cement crystals, as well as the surface roughness and porosity. (Figure 25). The microstructure, i.e., the size and distribution of the particulates of the cements influence their *in vivo* performance related to resorption and bioactivity. Smaller particle sizes and larger porosities (gaps in between the particles) ensure better bioactivity and resorption.

FTIR spectroscopy is done as an additional test for phase analysis which can identify the functional groups included in the crystal which will not directly identifiable with XRD. The FTIR of CaS and BioCaS cements showed peaks corresponding to the sulfate ions, at 601 and 669 cm^{-1}. The broad band around 1,120 cm^{-1} corresponds to the anti-symmetric bend of S–O. The peaks of stretching and bending of OH group are present at 3,560, 3,492 and 3,406 cm^{-1}. The doublets at 1621 and 1686 cm^{-1} correspond to OH deformation vibration [Sandhya *et al*., 2012]. The CPC cement exhibited characteristic vibration modes of PO_4 at 568 and 600 cm^{-1}. The band at 1000-1100 cm^{-1} is characteristic to the P-O bond present in

hydroxyapatite. The broad band at 3400-3600 cm^{-1} corresponds to the O-H group. (Figure 26).

The phase identification of cements is done using X-Ray Powder Diffraction (XRD) technique (Bruker D8 Advance). The analysis was done to identify the phase changes during the setting of the cements. The scanning was done in the 2θ range of 20-80^{o} to include all possible peaks. The XRD spectrum of the 3 cements (BioCaS, CPC and CaS) after setting is given in Figure 27. X Ray Diffraction spectra of the set mass of CaS and BioCaS, best matched with the standard spectra of calcium sulfate dihydrate corresponding to ICDD data card 21-0816. A low amount of phosphate content was incorporated in BioCaS cement, and hence the corresponding peaks showing phosphate substitution are not prominent in the spectrum. The XRD data of the set mass of CPC corresponded to hydroxyapatite, with the ICDD data card 09-0432. (Figure 27).

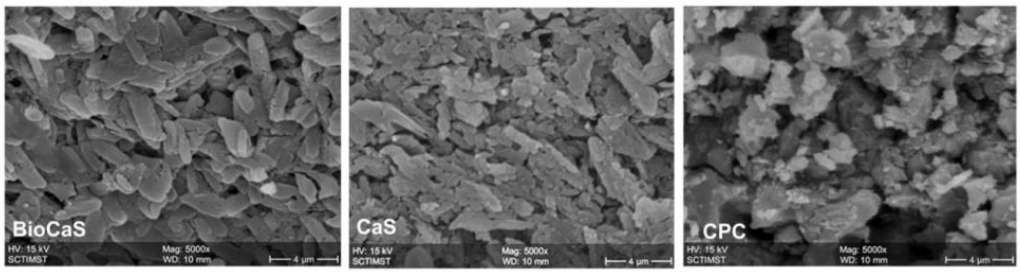


Figure 25: Characterization of BioCaS, CaS and CPC Cements (SEM)

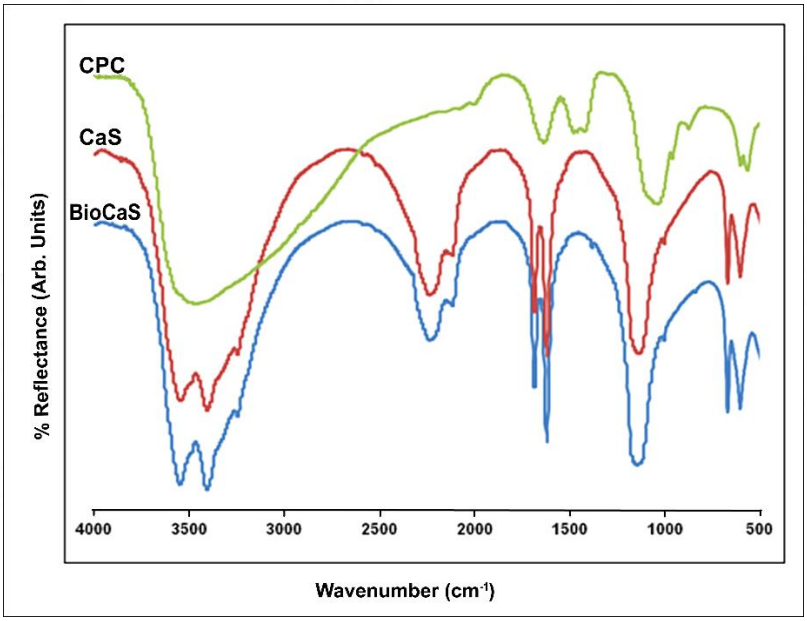


Figure 26: Characterization of BioCaS, CaS and CPC Cements (FTIR)

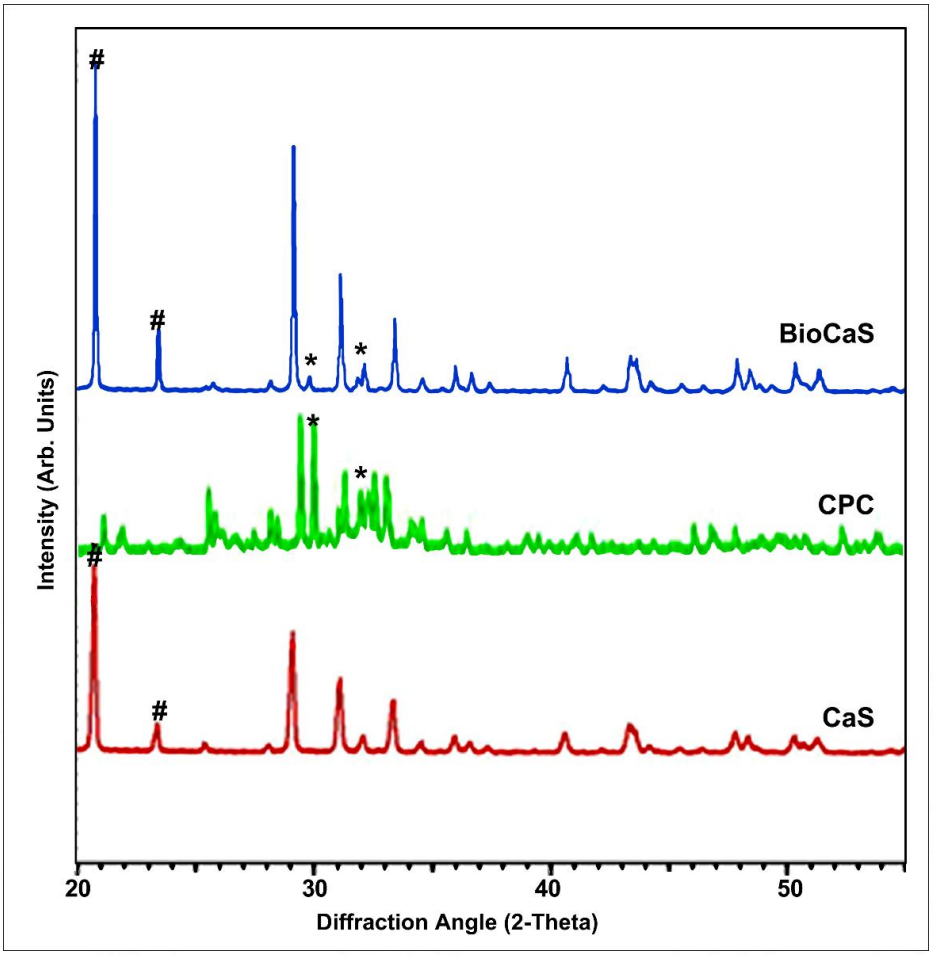


Figure 27: Characterization of BioCaS, CaS and CPC Cements (XRD)

4.3.3 *In vitro* biological evaluation of Bioactive cements

The biological evaluation of the bioactive cements was carried out using tooth-derived cells. The results of *in vitro* cytocompatibility evaluation of the CaS, BioCaS and CPC cement and sintered HA using periodontal ligament cells as follows:

4.3.3.1 Direct contact and MTT assay

The phase contrast images of hPDLCs cultured in direct contact with the bioactive cement discs shows no evidence of cell death (Cytotoxicity grade 0). The hPDL cell metabolic activity assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay at 24 h showed a greater than 80 % metabolic activity of the hPDL cells in the presence of the test materials – CaS, BioCaS, CPC and sintered HA discs (4 mm diameter), confirming the cytocompatibility of the materials. (Figure 28).

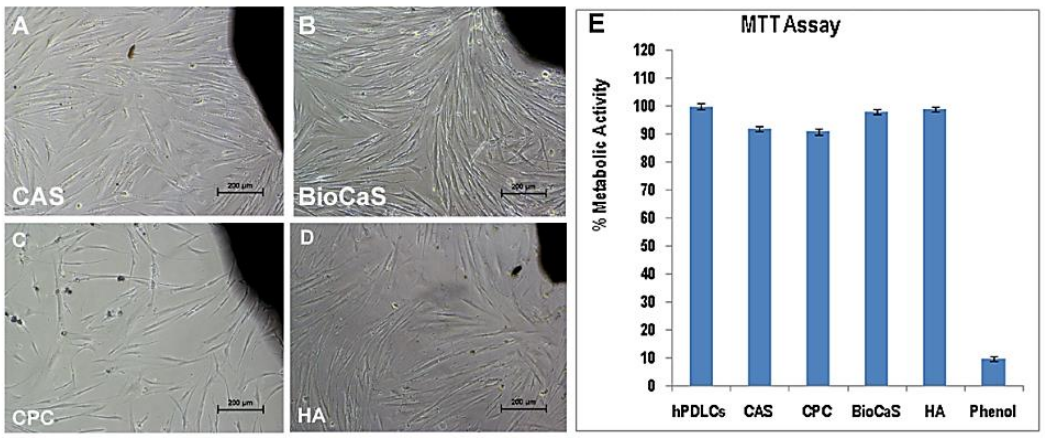


Figure 28: Cytotoxicity and cytocompatibility evaluation of Bioactive cements using hPDLCs. **A,B,C,D**) hPDLCs direct contact test with CaS, BioCaS, CPC and sintered HA discs for 24 h. **E**) MTT assay showing the percentage metabolic activity of hPDLCs in the presence of CaS, BioCaS, CPC and HA discs at 24 h. Scale bar - **200µm**

4.3.3.2 Cell adhesion and viability of hPDLCs on materials

The hPDL cells seeded onto the CaS, BioCaS, CPC, and sintered HA discs showed an abundance of green fluorescing live cells when compared with the red nuclei stained dead cells in FDA/PI live/dead evaluation, when observed by fluorescence microscopy under suitable filters. (Figure 29).

4.3.3.3 Cell adhesion and morphology of hPDLCs on materials

The hPDLCs exhibited good cytoskeletal organization upon actin cytoskeletal staining, indicating the cells retained their morphology and exhibited spreading on the biomaterial surfaces, indicating the cytocompatibility of the biomaterials. The hPDLCs seeded onto the CaS, BioCaS, CPC, and sintered HA discs, when viewed and imaged in SEM, confirmed the morphology of the cells, and evidence of cell spreading by means of filopodial and lamellipodial extensions were seen. (Figure 29).

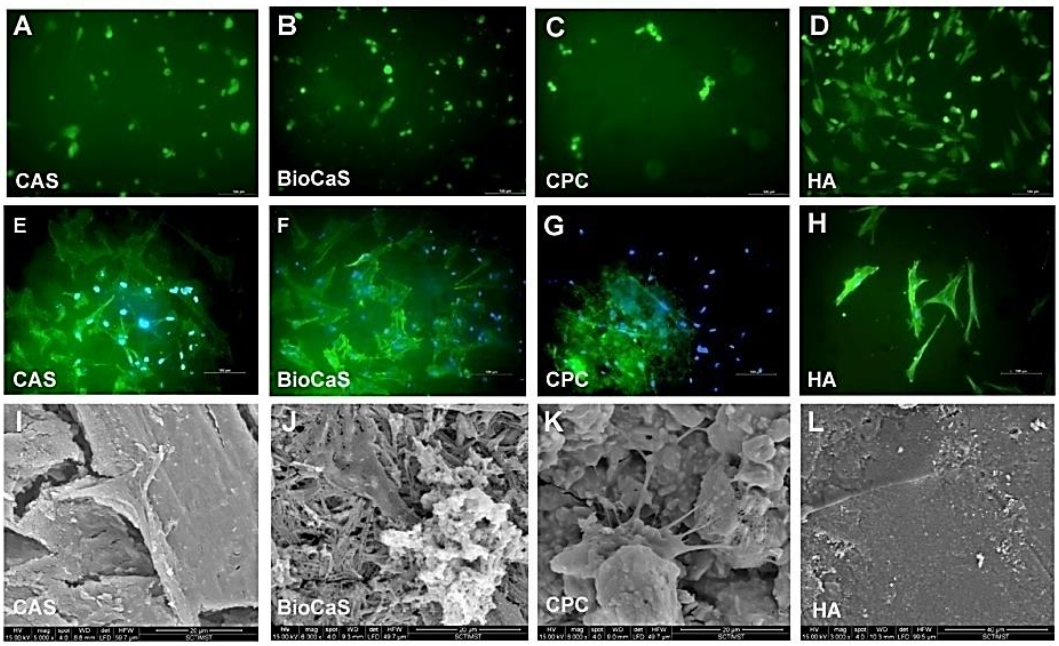


Figure 29: Periodontal ligament cell viability and morphology on BioCaS, CaS, CPC and HA. **A,B,C,D**) FDA/PI Live/Dead images of hPDLCs on CaS, BioCaS, CPC and HA discs. **E,F,G,H)** Actin cytoskeleton staining of hPDLCs on CaS, BioCaS, CPC and HA discs. **I,J,K,L**) SEM imaged of hPDLCs on CaS, BioCaS, CPC and HA disc (Scale bar **20µm). A-H)** - Scale bar **100µm.**

4.3.4 Osteogenic differentiation potential of the bioactive cements on hPDLCs

The osteogenic potential of the bioactive cements was studied using the primary human periodontal ligament cells. The cells showed evidence of mineralization and expressed osteogenic markers in the presence of the cement, even without an induction medium, confirming the osteoinduction properties of the cements. The detailed results are as follows:

4.3.4.1 Alizarin red staining and assay

The hPDLCs cultured with CaS, BioCaS, CPC and sintered HA discs in regular culture medium, without osteogenic supplements showed evidence of calcium deposits at 7, 14 and 21 days, showing the mineralization potential of the cells in the presence of the biomaterials, as compared to the cells grown in regular medium (Negative control). (Figure 30) The alizarin red assay values at 21 days quantitatively confirmed the presence of mineral deposits. (Figure 31).

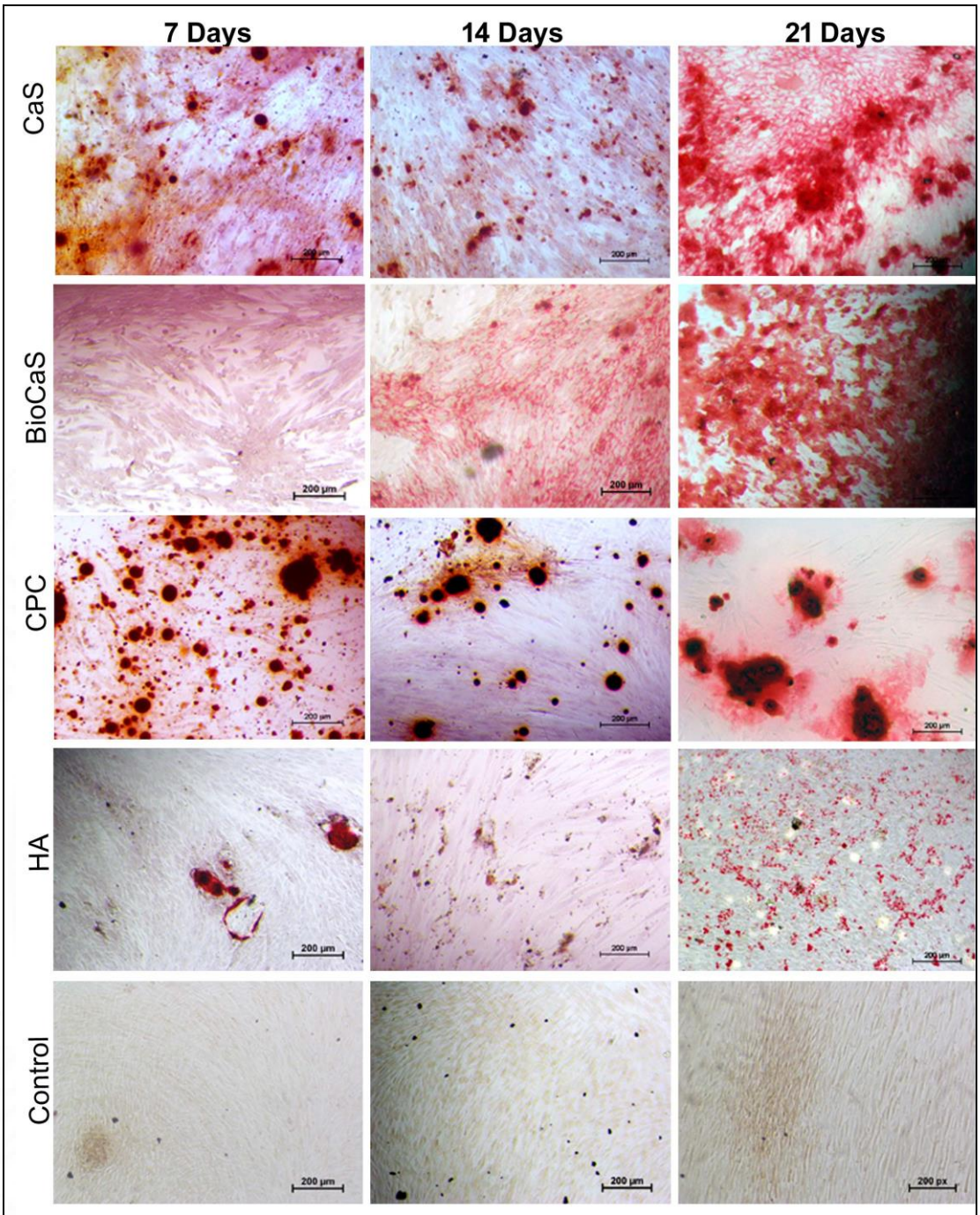


Figure 30: Alizarin red staining of calcium ion deposits, by hPDLCs in the presence of CaS, CPC and BioCaS cements, sintered HA discs and Control at 7, 14 and 21 days. Scale bar - **200µm**

4.3.4.2 Alkaline phosphatase enzyme activity

The secreted alkaline phosphatase enzyme by the hPDLCs in 7 days was also noted to be elevated in the presence of the bioactive cements. The optical density values were plotted as direct correlation of the enzyme expression. (Figure 31).

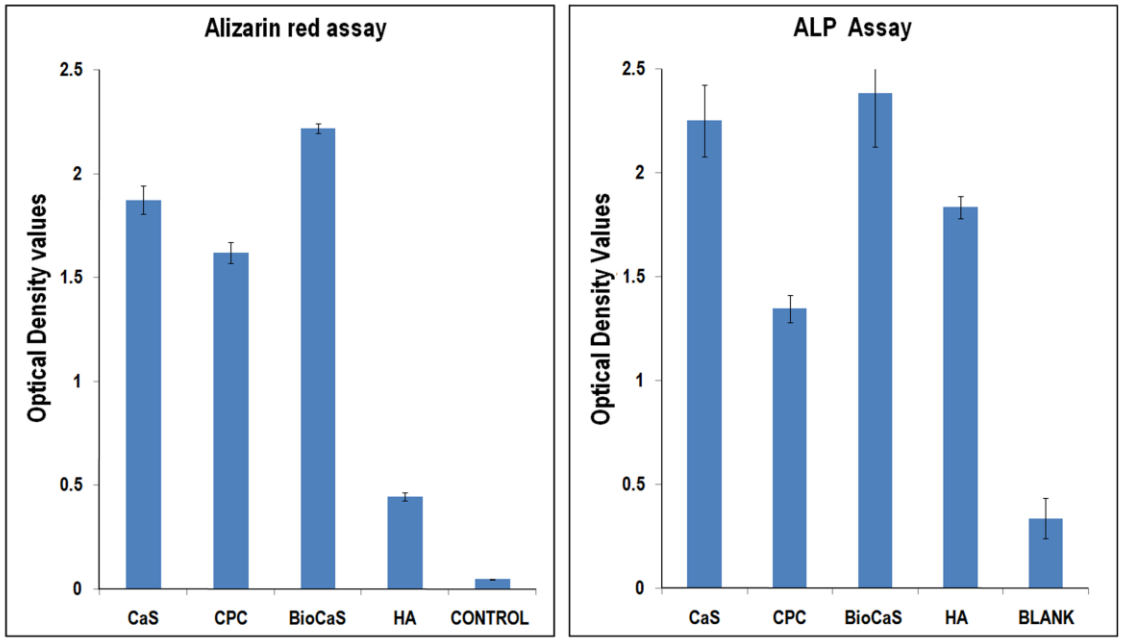


Figure 31**: A)** Alizarin red assay denoting the mineralization potential of hPDLCs in the presence of the bioactive cements at 21 days. **B)** Alkaline phosphatase assay at 7 days. Same alphabets indicate no significant difference between the respective groups. $P < 0.05$.

4.3.4.3 Osteogenic marker expression of hPDLCs on Bioactive cements

The ICC/IF evaluation of hPDL cells in the presence of the CaS, CPC and BioCaS cements showed positive expression of alkaline phosphatase enzyme secreted during the osteogenic differentiation. The expression of osteogenic marker protein osteonectin also indicate the differentiation of hPDL cells into an osteogenic lineage. This was confirmed by the positive expression of the collagen I matrix, the deposition of which is needed for mineralization. The positive expression of the matricellular dentin matrix protein (DMP1) is also indicative of osteogenic/cementogenic differentiation of hPDLCs. (Figure 32).

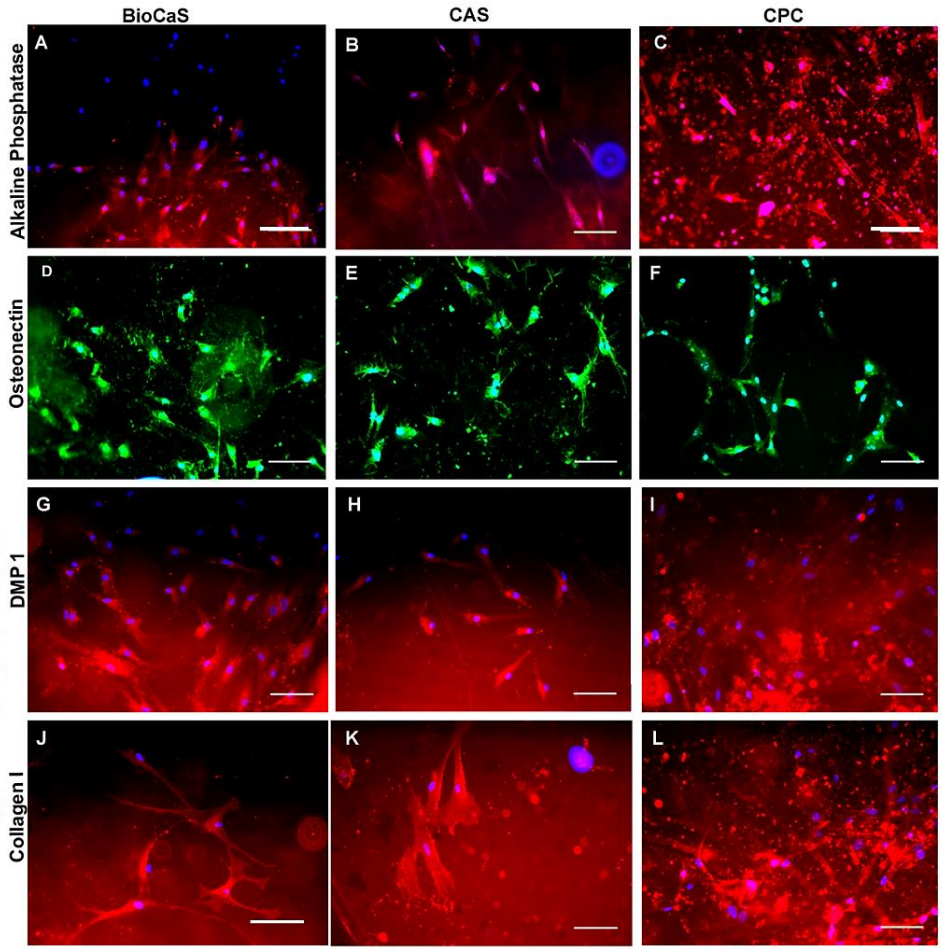


Figure 32: Osteogenic marker expression of hPDLCs on Bioactive cements.
Scale bar :**100µm**

4.3.5 Biological evaluation of the bioactive cements using human Dental Pulp cells

The Bioactive cements CaS, BioCaS and CPC was found to be biocompatible and osteoinductive to the periodontal ligament cells as evidenced by the results presented above. The experiments with dental pulp cells were carried out to evaluate the suitability of using the cements as potential dental pulp capping agents. The results are presented as follows.

4.3.5.1 Cell adhesion and morphology

The hDPCs exhibited good cytoskeletal organization on the cements at 24 h, upon actin cytoskeletal staining. This confirms the cell adhesion and morphology is preserved on the cements with reference to the control (hDPCs in cover glass). (Figure 33).

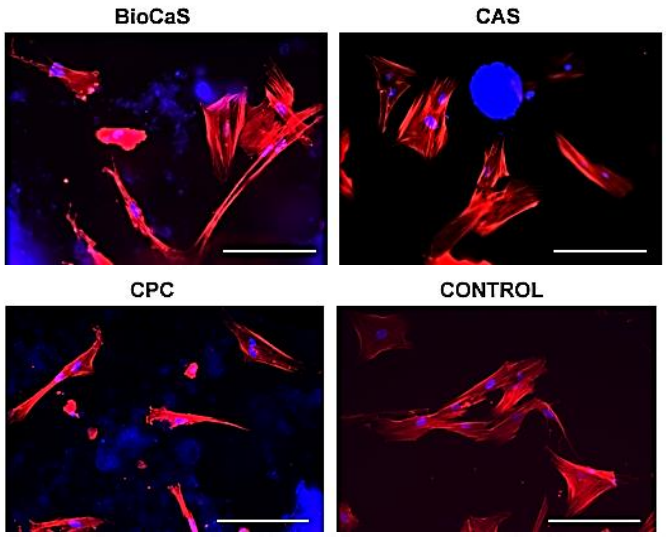


Figure 33: Cell adhesion and spreading of human dental pulp cells on bioactive cements. Scale bar: **100µm**

4.3.5.2 Osteogenic marker expression of hDPCs on Bioactive cements

The hDPCs showed positive expression of the osteogenic markers alkaline phosphatase, osteonectin and DMP 1 at day 7, when cultured on the cements. (Figure 34).

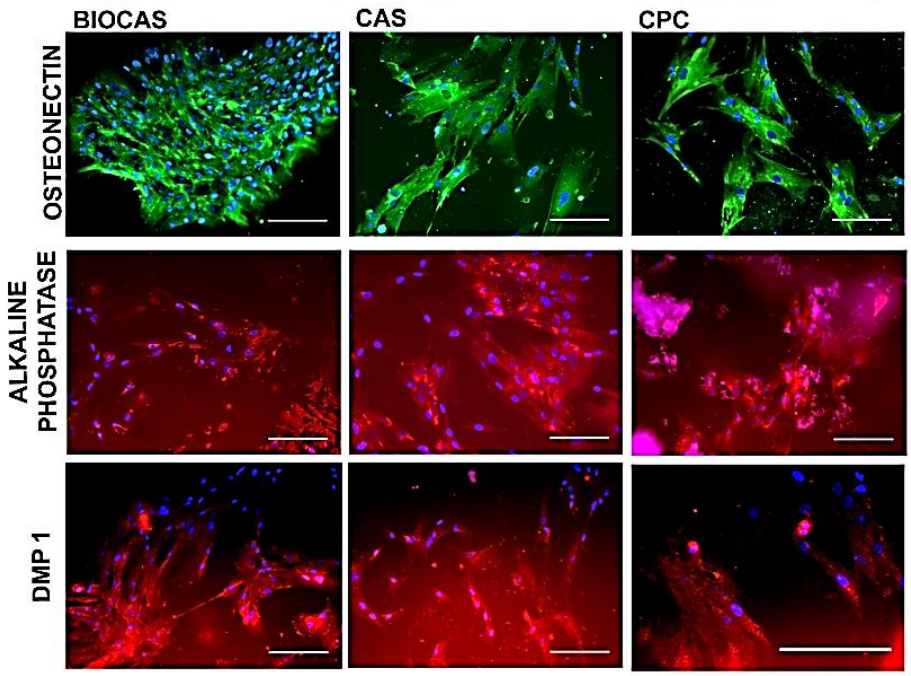


Figure 34**:** Osteogenic marker expression of hDPCs on Bioactive cements at 7 days of culture. Scale bar -**100µm**

4.4 Translational approaches of the biomaterials for clinical applications

The *in vitro* material – cell interaction studies showed that the dendritic peptides and the bioactive cements were compatible with the human periodontal ligament cells. Therefore, *in vivo* experiments were planned to confirm the translational potential of the dendritic peptides and bioactive cements. The dendritic peptides were successfully coated onto electrospun PCL mats to simulate biofunctionalized PCL barrier membranes. Necessary *in vitro* and *in vivo* experiments were carried out, the results of which are enumerated in this section. The bioactive cements were used as powder – liquid based settable and moldable cement systems applied *in vivo* as alveolar bone graft/ barrier graft materials in a rat maxillary alveolar bone defect model, the results of which, are also enumerated here.

4.4.1 Application of Lys-PDA and G-PDA as barrier membrane

The synthesis of electrospun PCL mats was successfully carried out using previously standardized procedures. The mats thus obtained could be cleaned, dried and coated with the corresponding dendritic peptide solution for biofunctionalization. The results obtained are discussed in the following sections.

4.4.1.1 Preparation and Biofunctionalization of electrospun PCL membrane

The 10 % w/v Solution of PCL (Average mol. wt: 80000, Sigma) in solvent system with THF and DMSO in 9:1 ratio was completely dissolved in 2 h when stirred in magnetic stirrer. The electrospinning parameters - potential difference of 15 KV, flow rate of 1.5 ml/h and metal collector distance of 12 cm, in the customized electrospinning unit (Holmarc Opto-Mechatronics Pvt Ltd., India) yielded the sufficient quantity of electrospun PCL mat for the experiments.

i. Lys-PDA/PCL

The electrospinning of polycaprolactone (PCL) yielded uniform nanofibrous mat to which, the Lys-PDA was coated. The coating technique yielded a uniformly coated PCL mat and the residual polymerization was completed using the UV irradiation, which was evident by a color change to red. Once dried completely, it was possible to pack Lys-PDA/PCL in sterilization pouches, sterilize by ETO and store at room temperature till further use. The water contact angle measurements showed increased hydrophilicity of the Lys-PDA/PCL

mats. The SEM showed the presence of Lys-PDA coating on the PCL mats and the nanofibrous architecture of the electrospun PCL mats. This was substantiated with FTIR spectroscopy in which characteristic amine peaks of lysine between 3300 - 3500 cm^{-1}, and the characteristic peaks of C=O ester bonds of PCL at 1722 cm^{-1}. (Figure 35).

ii. G-PDA/PCL

The electrospun PCL was successfully coated with G-PDA was coated, the residual polymerization was completed using the UV irradiation, dried, packed in sterilization pouches, sterilized by ETO and stored at room temperature till further use. The water contact angle measurements showed increased hydrophilicity of the G-PDA/PCL mats. The SEM showed the presence of G-PDA coating on the PCL mats and the nanofibrous architecture of the electrospun PCL mats. This was substantiated with FTIR spectroscopy in which characteristic amine peaks of Guanidine between 3300 – 3500 cm^{-1}, and the characteristic peaks of C=O ester bonds of PCL. (Figure 35**).**

4.4.2 *In Vitro* Biological evaluation of Lys-PDA/PCL, G-PDA/PCL mats

The *in vitro* biological evaluation of the Lys-PDA and G-PDA coated electrospun PCL mats were carried out using MTT assay, cell adhesion and viability, actin cytoskeletal staining and SEM. The results obtained are as follows.

4.4.2.1 Direct contact test and MTT Assay

The hPDL cell metabolic activity assessed by direct contact MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay at 24 h showed a greater than 80 % metabolic activity of the hPDL cells on PCL, G-PDA/PCL and Lys-PDA/PCL discs confirming the cytocompatibility of the materials. The cells exhibited no signs of cell death or toxicity as evidenced by detachment or rounding off of cells. (Figure 36).

4.4.2.2 Cell adhesion and viability

The hPDL cells seeded onto the Lys-PDA and G-PDA coated PCL mats (Lys-PDA/PCL) showed an abundance of green fluorescing live cells when compared with the red nuclei stained dead cells, in FDA/PI live/dead evaluation, when observed by fluorescence microscopy under suitable filters. (Figure 36).

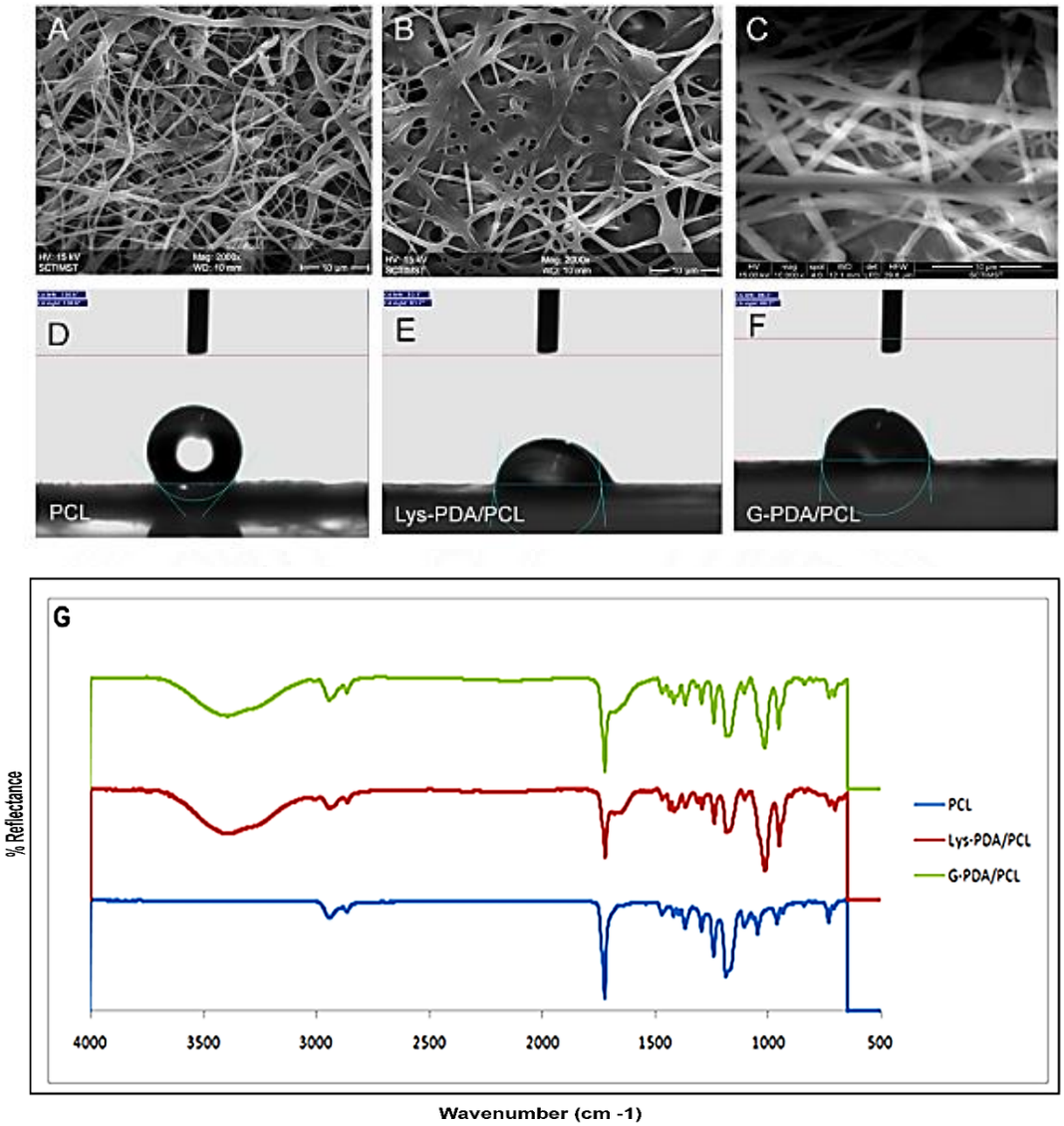


Figure 35: Characterization of PCL, Lys-PDA/PCL and G-PDA/PCL mats. **A,B,C)** The SEM images of electrospun PCL, Lys-PDA/PCL and G-PDA/PCL mats respectively. **D,E,F)** The representative water contact angle measurements of PCL, Lys-PDA/PCL and G-PDA/PCL mats respectively. **G)** The FTIR spectroscopy of PCL, Lys-PDA/PCL and G-PDA/PCL mats.

4.4.2.3 Cell morphology and spreading

Actin cytoskeleton staining: The hPDLCs exhibited good cytoskeletal organization upon actin cytoskeletal staining, indicating the cells retained their morphology and exhibited good adhesion and spreading on the PCL, Lys-PDA/PCL and G-PDA/PCL mats, indicating the cytocompatibility of the biomaterials. (Figure 36).

SEM evaluation: The hPDLCs seeded onto PCL (control), Lys-PDA/PCL and G-PDA/PCL mats, when viewed and imaged in SEM, confirmed the morphology of the cells. The SEM images also showed evidence of cell spreading by means of filopodial and lamellipodial extensions. The close adaptation of the cells to the mats was visualized by SEM. (Figure 36).

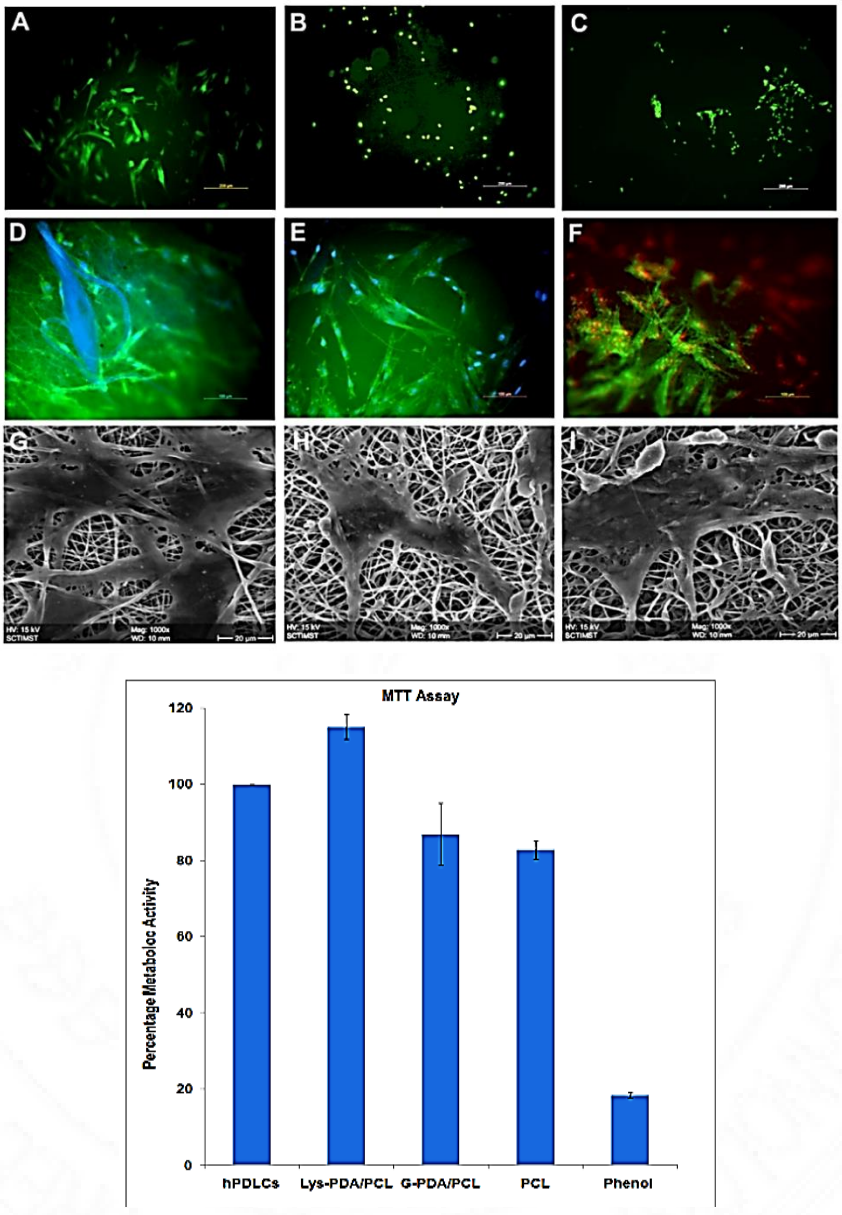


Figure 36: Cell adhesion, viability and morphology of hPDLCs on PCL, Lys-PDA/PCL and G-PDA/PCL mats. **A,B,C**) FDA/PI live/dead imaging of hPDLCs on Lys-PDA/PCL. G-PDA/PCL And PCL mats respectively. **D,E,F**) Actin cytoskeletal staining of hPDLCs on Lys-PDA/PCL. G-PDA/PCL and PCL mats respectively. **G,H,I**) SEM images of hPDLCs on Lys-PDA/PCL. G-PDA/PCL and PCL mats respectively. **J**) MTT assay of hPDLCs with PCL, Lys-PDA/PCL, and G-PDA/PCL mats. A-F: Scale bar - **100µm**. SEM Scale bar - **20µm**

4.4.3 *In Vivo* evaluation of Lys-PDA/PCL mats

In vivo preclinical usage tests using a suitable animal model – a rat maxillary periodontal defect model – was designed and 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 extracellular matrix.

4.4.3.1 Defect creation and material placement

The animals were anaesthetized for the whole duration of the surgical procedures. The supine position of the animals in the customized surgical platform allowed easy access to the rat oral cavity (Figure 37– A). The retractive elastic bands attached to the platform allowed the convenience of keeping the mouth open, without restricting the vision and access (Figure 37– B, C). The periodontal defects could be created using a slows peed micro motor hand piece with copious saline irrigation to reduce heat and subsequent necrosis (Figure 37 - D). The PCL and Lys-PDA/PCL mats were placed as barrier membranes so as to cover the defects till the cemento-enamel junction (Figure 37- E, F). The post-surgical recovery period of the animals was uneventful. Diet was restricted to soft food for two days, after which normal feeding habits was restored.

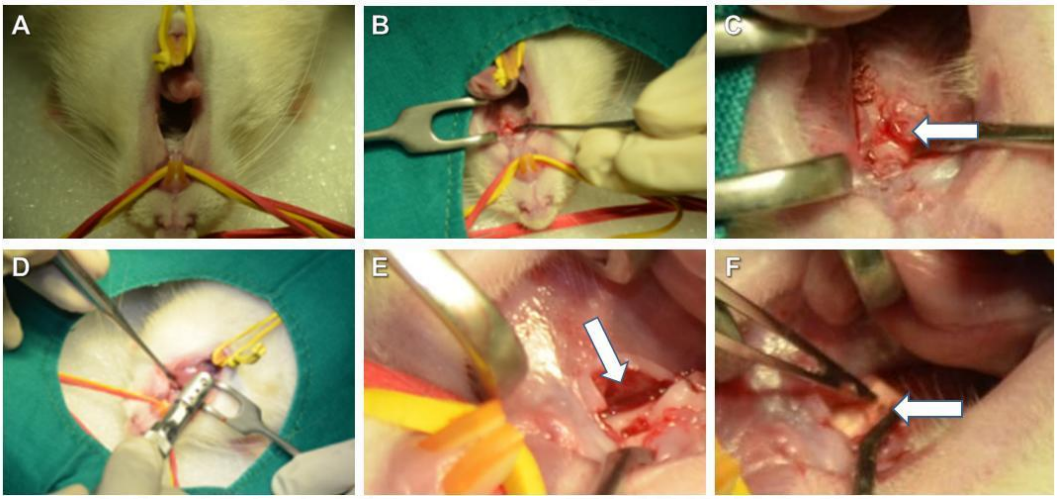


Figure 37: The periodontal defect creation and placement of Lys-PDA/PCL barrier membrane in rat maxillary first molar. A,B) Animal placed on custom surgical platform with restrains. C,D,E) Flap reflection and defect creation. F) Lys-PDA/PCL membrane placement.

4.4.3.2 Histopathology evaluation

The histopathology evaluation of shows dense epithelial overgrowth extending beyond the cemento enamel junction in the SHAM control (without barrier membranes). This epithelial overgrowth was arrested and limited to the cemento enamel junction in the barrier membrane groups - PCL (material control) and Lys-PDA/PCL (test material) - at 3 months. (Figure 38).

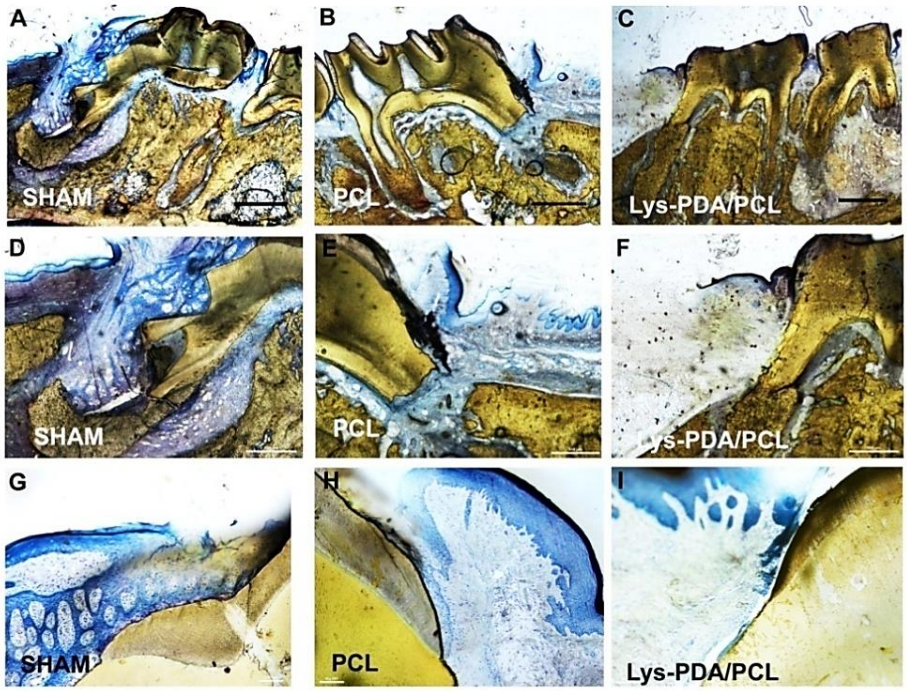


Figure 38: Histopathology evaluation of Lys-PDA/PCL mats in rat periodontal defect model. **A,D,G)** Histopathology evaluation showing the epithelial overgrowth extending beyond the cemento enamel junction in SHAM control. Histopathology evaluation showing the epithelial overgrowth prevented at the cemento enamel junction in PCL group (**B,E,H**) and Lys-PDA/PCL group **(C,F,I).** Magnification – A,B,C) **1X**; D,E,F) **4X**; G,H,I) **10X**

4.4.4 *In vivo* evaluation of Bioactive cements as barrier grafts for periodontal regeneration.

In vivo preclinical usage tests using a suitable animal model – a rat maxillary alveolar bone defect model – was designed and carried out with calcium sulfate (CaS), Calcium Phosphate (CPC) and bioactive Calcium Sulfate (BioCaS) as the alveolar bone graft substitutes.

4.4.4.1 Histopathology evaluation

The histopathology evaluation of CaS, CPC and BioCaS cements at 1 month duration shows that there is evidence of new bone formation in the presence of all three cements, when compared to the SHAM control. The presence of remnants of CPC and BioCaS cement particles was noted in the respective groups, with new bone formation evident at the cement interface. Evidence of cement resorption was also noted, which was found to be at par with the new bone formation. In CaS cement group, trabecular new bone formation was evident, with no traces of the cement particles, suggesting complete resorption of the cement. (Figure 39).

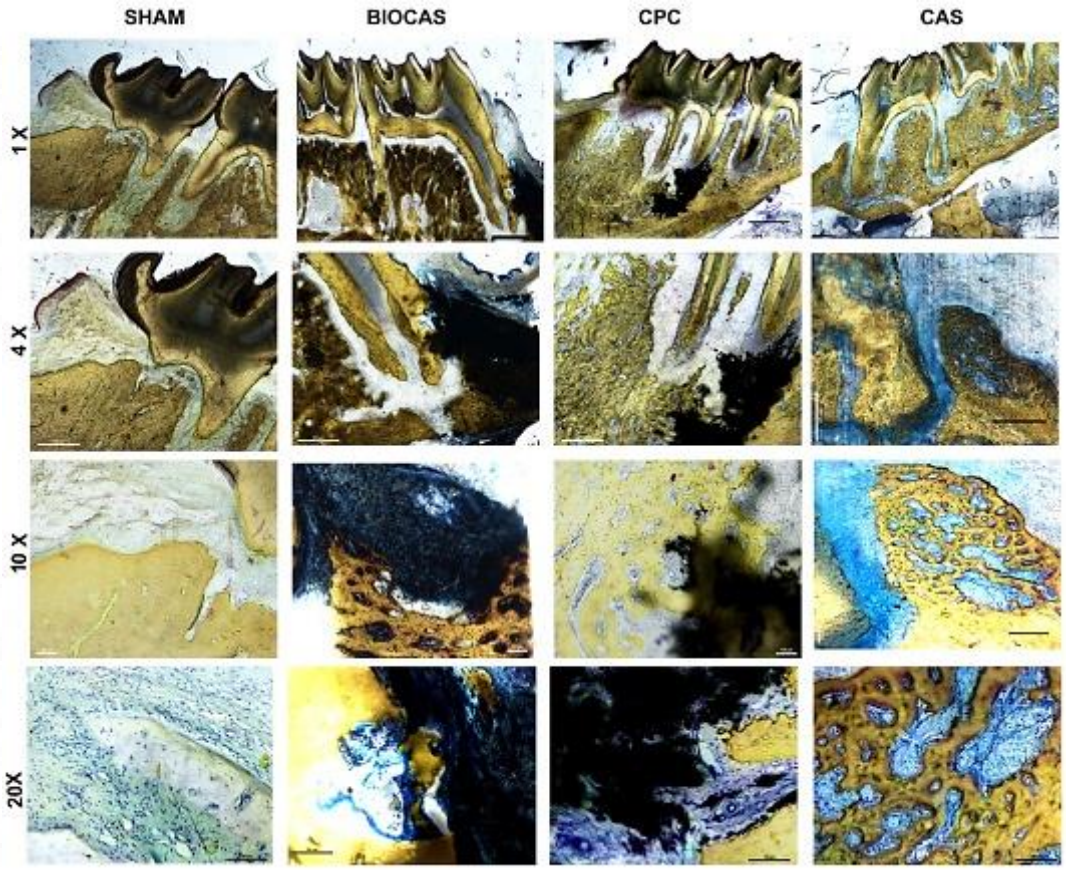


Figure 39: Histopathology evaluation of bioactive cements in rat alveolar bone defect model. New bone formation in the presence of CPC, CaS and BioCaS cements at 1 month duration. The presence of cement particles is evident in CPC and BioCaS groups, whereas it is absent in CaS group. Evidence of resorption of cements is seen in higher magnification.

Chapter 5

DISCUSSION

The major results of this study are presented with illustrations in Chapter 4. In Chapter 5, the results are interpreted and the outcomes of the results are discussed in correlation with current published literature. Three aspects are covered in this work- (i) Establishment of a tooth-derived cell culture system for *in vitro* evaluation of dental biomaterials. (ii) Application of tooth-derived cell culture system for biological evaluation of biomaterials for tooth regeneration and (iii) Translational approaches of biomimetic matrices and bioactive cements towards regeneration of periodontium. These are discussed in 3 parts, as represented in sections 5.1, 5.2 and 5.3 respectively.

The first part discusses the techniques used to isolate, culture and characterize primary dental progenitor cells to establish a primary cell culture system for dental biomaterial evaluations. The reasons for selecting periodontal ligament cells and dental pulp cells for further biomaterial evaluations is also stated with adequate literature support. (Section **5.1**).

In the second part, the application of the established tooth-derived cell culture system for biological evaluation of biomaterials including self-assembling dendritic peptide molecules for periodontal regeneration, (subsection **5.2.1**), evaluation of calcium phosphate and sulfate based bioactive cements for alveolar bone regeneration (subsection **5.2.2**), and evaluation of calcium phosphate and sulfate based bioactive cements as pulp capping agents (subsection**5.2.3**) are discussed.

The third part discusses the translational aspects of biomimetic molecules and bioactive cements through (i) *In vitro* evaluation of electrospun PCL mats as barrier membranes biofunctionalized with dendritic peptide for periodontal regeneration (subsection **5.3.1**) (ii) *In vivo* evaluation of the biofunctionalized barrier membranes towards regeneration of periodontium in small animal model (subsection **5.3.2**) and (iii) *In vivo* evaluation of bioactive cement systems towards regeneration of periodontium in small animal model (subsection **5.3.3**).

A brief description about the limitations of the study is given at the end of the chapter (section **5.4**). Future perspectives towards clinical translation approaches of the biomaterials evaluated are given at the end of this chapter (section **5.5**). The identification of limitations of the study and planning of future experiments are important to take the current research work towards clinical translation and thus used for the betterment of the society.

5.1 Establishment of a tooth-derived cell culture system for in vitro evaluation of dental materials

A cell culture system utilizing tooth-derived cells is advantageous in specific *in vitro* biological evaluation of bioactive dental materials. Accordingly, the first step is to establish standardized and easily reproducible techniques to isolate culture and characterize the relevant tooth-derived cells. In order to achieve this, the dental pulp, periodontal ligament, dental apical papilla and remnant tissues from exfoliated deciduous teeth were identified as source tissues for tooth-derived cell isolation [Avinash *et al*., 2015]. The isolation of dental pulp and periodontal ligament cells were carried out as per previously reported techniques – the enzymatic digestion technique using collagenase and dispase to enzymatically degrade the extracellular matrix and release the cells; and the explant culture technique where cells are allowed to migrate out of the cultured explant tissues. The enzyme digestion approach provided cells relatively quickly, whereas, the explant culture technique gave a comparatively larger, heterogeneous population of cells per tissue sample, the difference being attributed to the maintenance of host micro environment [Hilkens *et al*., 2013]. Subsequently, tissue explant culture model was used to isolate cells from the respective tissues.

In order to understand the phenotype and genotype of the isolated cells, the cells needed to be characterized by means of the presence of certain characteristic marker proteins [Saito *et al*., 2015; Avinash *et al*., 2017]. Characterization of the cells is also important for their further use in biomaterial evaluation [Li *et al*., 2015]. In this study, all the isolated dental cells showed a fibroblastic morphology and were plastic adherent. The periodontal ligament cells showed positive marker expression for the common MSC markers CD 90, CD 73, CD 105 and STRO-1, confirming the presence of a progenitor cell population [Saito *et al*,

2015]. Similar pattern of marker expression was found in dental pulp cells also. The deciduous teeth also expressed CD 105, CD 90 and STRO 1. The fibroblast marker Vimentin was expressed by the dental pulp, periodontal ligament and the deciduous teeth cells [Açil *et al*., 2016; Cheng *et al*.,2016]. Dentin Matrix Protein 1 expression was present in both dental pulp cells and periodontal ligament cells, which is in accordance to the literature [Choi *et al*., 2014]. The periodontal ligament cells also expressed cementum membrane protein and scleraxis, confirming the tissue of origin [Xiong *et al*, 2016; Komaki *et al.*, 2012]. Such specific markers are not recognized for dental pulp cells or deciduous teeth cells, which is supported by the literature [Kawashima *et al*.,2012].

The tooth-derived cells, by virtue of their regeneration potential, is capable of differentiating to odontogenic, cementogenic and/or osteogenic lineages. It was understood from the literature that an osteogenic induction medium can induce the differentiation of the dental pulp cells, deciduous teeth cells and apical papilla cells to osteo/odontogenic lineages and periodontal ligament cells to cemento/osteogenic differentiation [Saberi *et al.*, 2019; Acil *et al*., 2016; Proksch *et al.*, 2019]. This can be identified by histological staining procedures for mineralization including alizarin red staining (alizarin red chelates calcium deposits present, which appear as red nodules), von Kossa staining (uses 2 % silver nitrate that detects phosphate deposition) and Masson's trichrome staining (for collagen deposits) [Lee *et al*., 2014]. In this study, all the tooth-derived cells – from dental pulp, periodontal ligament, apical papilla and deciduous teeth - showed evidence of osteogenic differentiation on histological evaluation of mineralization by von Kossa and alizarin red staining, whereas collagen deposits was evident with periodontal ligament cells.

Mineralization alone cannot be considered as a confirmatory test for osteogenic differentiation. ICC/IF staining of specific osteogenic markers can give confirmatory evidence of osteogenic differentiation of the cells [Ajlan *et al*, 2015]. Specific dentin or odontogenic marker expression is possible in dental pulp cells *in vitro*, upon induction [Ching *et al*, 2017]. Osteogenic gene expression also adds up to confirm the osteogenic differentiation potential of the cells. The periodontal ligament cells are also reported to express the osteogenic markers such as alkaline phosphatase, osteonectin and collagen type 1, similar to the dental pulp cells [Li *et al*., 2019]. This study revealed that the dental pulp cells as well as periodontal ligament cells express the osteogenic markers alkaline phosphatase, osteonectin, dentin matrix protein and collagen type 1, upon osteogenic

induction for 7 days, confirming the osteogenic potential of the dental pulp and periodontal ligament cells. This is important because the cells could be used for evaluation of the bioactivity of biomaterials, only if they possess osteogenic differentiation potential.

As the first part of this study, a reproducible technique for the isolation of the tooth-derived cells from dental pulp, periodontal ligament, apical papilla and deciduous teeth was carried out successfully. The cells showed fibroblast morphology, expressed the characterization markers and could undergo osteogenic differentiation *in vitro*. The purpose of isolating and characterizing the cells was to use them for *in vitro* biological evaluation of possible dental biomaterials towards regeneration of periodontium and dental pulp. Towards this purpose, the cells that would come directly in contact with the biomaterials should be given preference.

The deciduous teeth are easy to obtain, but the deciduous teeth cells will be lost during the physiological exfoliation process. Moreover, using deciduous teeth cells may not have much significance, as most of the biomaterial based regenerative approaches are meant for permanent dentition. Similarly, the apical papilla cells are only present for a short period, approximately 2 years, after the eruption of tooth to the oral cavity. Any trauma, infections or medicaments can easily damage the apical papilla cells. Therefore, in most cases, regenerative biomaterials like intra-canal medicaments or endodontic sealers need not come in direct contact with the apical papilla cells [Fernandez *et al*., 2015]. Therefore, the deciduous teeth cells and the apical papilla cells were excluded from further biomaterial interaction studies.

The periodontal ligament cells were selected to study the biological effect of bioactive molecules, prototype barrier membranes and bioactive cements with the potential to regenerate cementum and alveolar bone, and also those can be used as endodontic medicaments or sealers. The dental pulp cells would be used to evaluate the possible dental pulp capping agents, which are expected to come in direct contact with the dental pulp. Endodontic medicaments and sealers are better evaluated with periodontal ligament cells as they come to contact mainly with periodontal ligament cell of the periradicular tissues (Table 4).

Cell name	Cell source	Potential uses
Deciduous teeth cells – **hDecDCs**	Exfoliated deciduous teeth	*In vitro* screening of biomaterials, stem cell banking, therapeutic applications
Apical papilla cells – **hDAPCs**	Dental apical papilla tissues	*In vitro* screening of biomaterials, stem cell based therapeutic applications
Dental pulp cells – **hDPCs**	Adult dental pulp tissue	*In vitro* evaluation of biomaterials for direct pulp capping, pulpotomy and apexogenesis, in cases where a remnant vital pulp tissue is expected.
Periodontal ligament cells – **hPDLCs**	Adult periodontal ligament tissue.	*In vitro* evaluation of biomaterials for periodontal regeneration, barrier membranes, alveolar bone grafts, endodontic sealers and medicaments, revascularization agents etc.

Table 4: Tooth-derived cells, their source, nomenclature and uses.

5.2 Application of tooth-derived cell culture system for biological evaluation of biomaterials for tooth regeneration

A true bioactive dental material should ideally lead to natural tissue regeneration. Compared to the repair of other tissues in human body, dental tissue regeneration is easier to observe and follow up, through radiographic and clinical examination [Bayne *et al*.,2005]. There are so many novel regenerative biomaterials developed with potential dental applications in the past decades, but patients and clinicians did not get benefit of these scientific advances, possibly due to the missing links in knowledge co-ordination and translational approaches [Grimshaw *et al*., 2012; Afrashtehfar *et al*., 2017]. Basic research involving dental materials needs a complex interdisciplinary approach incorporating physical sciences, biology and medicine [Fajardo-Ortiz *et al*., 2017]. The dental biomaterials need to be optimized for the final application through elaborate biocompatibility and efficacy tests which involve funds, manpower and infrastructure. A reliable biological screening system at the optimization stage will greatly benefit the prompt and appropriate biological evaluation of the biomaterials and their modifications. In this study, a validated cell culture model using tooth-derived cells for biomaterial screening purposes is envisaged.

As a preliminary demonstration, the cell culture model developed based on hPDL cells in this study was used for the *in vitro* biological evaluation of a couple of bioactive molecules towards periodontal regeneration. In addition, bioactive cements for the regeneration of periodontium and dental pulp were evaluated.

In the material development part of the work, ECM mimetic molecules were designed which can promote the *in vitro* differentiation potential of periodontal ligament cells. After confirming the osteogenic potential of the biomolecules, a prototype barrier membrane was made of electrospun PCL, biofunctionalized with the biomolecules and tested using the periodontal ligament cells. The periodontal ligament cells and dental pulp cells were also used to evaluate the *in vitro* osteogenic potential of bioactive bone cements.

5.2.1 Application of human periodontal ligament cells for *In vitro* evaluation of self-assembling dendritic peptide matrices for periodontal regeneration

In order to promote periodontal regeneration, it has been suggested to mobilize the endogenous progenitor cells from the host tissues and allow them to repopulate the defect area without hindrances from other the native or migrating epithelial cells. However, there are practical limitations to achieve this. Conventional tissue engineering strategies retains the concerns regarding use of exogenous cells and high expenses in purifying growth factors [Langer *et al*., 2016]. Since periodontal ligament harbours a good population of progenitor cells, they could be attracted towards the site of injury using autologous biologics like platelet-rich plasma and fibrin in combination with barrier membranes. This will provide an ECM mimetic scaffold with inherent biologic cues to promote regeneration, but this procedure is sensitive to the technique and expensive [Chen *et al*., 2010]. Artificial ECM mimetic instructive matrices can simulate the natural progenitor cell niche and facilitate local progenitor cell homing, proliferation, and differentiation [Yin *et al*., 2017]. Short peptide sequences, amino acids or small peptide molecules can be designed to form self-assembling smart peptide matrices that resemble the heterogeneous fibrous protein network of ECM thereby guiding the endogenous progenitor cells towards regeneration. ECM provides endogenous cells with mechanical support and biological cues to differentiate into organized tissues or tissue components. Synthetic structures incorporating cell adhering peptides sequences such as Arginine-Glycine-Aspartate (R-G-D) peptides have been used to fabricate ECM mimetic tissue engineering scaffolds. Such structures can enhance cell adhesion

through the guanidine groups of the peptide arginine by electrostatic interactions with glycosaminoglycans (GAGs), the negatively charged linear polysaccharides of the cell membrane [Tonna *et al*., 2014; Balakrishnan *et al*., 2012]. The basic amino acid lysine, with its two NH_2 groups, is also reported to provide attachment sites for GAGs on the cell surfaces and ECM to enhance cell signaling, cell-cell interactions and cell-matrix interactions towards regeneration [Haridas *et al*., 2014; Blaum *et al*., 2010]. Appending these functional biomolecules to a self-assembling polydiacetylene matrix can mimic the ECM to promote cell adhesion and differentiation [Kang *et al*., 2012; Tavafoghi *et al*., 2016].

Lysine is a basic amino acid with two NH_2 groups, and is a main component of collagen, the ECM component of periodontal ligament. Guanidine is a small functional unit of the amino acid Arginine, which is the main component of the cell adhesive R-G-D peptide sequence. Lysine contains two positively charged terminal amino groups (NH_3^+) whereas Guanidine contains three amino groups. These molecules can provide attachment sites for the cell membrane bound, negatively charged linear polysaccharides. [Sapra *et al*., 2019]. Diacetylene is a class of chemical compounds having conjugated C≡C triple bonds in their chemical structure that can undergo topochemical polymerization upon UV-irradiation resulting in the formation of self-assembling polymeric architectures. Appending lysine and guanidine to the polydiacetylene framework through controlled chemical reactions can impart ECM like properties that may facilitate interaction with GAG receptors of cell membranes to enhance cell adhesion, differentiation and deposition of phosphate ions to promote biomineralization [Haridas *et al*., 2013].

In this study, two biomimetic molecules were identified – the amino acid lysine which is a basic amino acid with two NH_2 groups and a main component of collagen; and the molecule guanidine with three NH_2 groups that forms the active component of arginine, the main component of the cell adhesive R-G-D peptide sequence. The hypothesis behind this study was to create a lysine or guanidine appended self- assembling polydiacetylene coating on glass coverslips for *in vitro* biological evaluations including cell adhesion and differentiation, using the isolated characterized human periodontal ligament cells. These prototype biomolecules with lysine and guanidine appended to an UV polymerizable diacetylene matrix through a series of chemical reactions, and is reported to support adhesion and differentiation of dental pulp cells *in vitro* [Haridas *et al*, 2014].

In this study, the dendritic peptides could be successfully coated onto cell culture plates and glass coverslips through UV polymerization, and the coating could be characterized by SEM, AFM and FTIR. The hPDL cells survived well on both Lys-PDA and G-PDA matrices and gave qualitative evidence of cell viability, adhesion and spreading as seen in the phase contrast imaging, live dead imaging, cytoskeletal staining and scanning electron micrographs (SEM). The results of this study confirm the cytocompatibility of the biomolecules. Osteogenic potential of the hPDL cells in the presence of the biomolecules through previously optimized osteogenic induction medium containing phosphate ions was used throughout the osteogenic differentiation experiments. An early mineralization was noted in the Lys-PDA and G-PDA matrices, than in the positive control in both alizarin red staining and the Von Kossa staining. Among the two molecules, Lys-PDA showed an early mineralization, indicating a better osteogenic differentiation of the same. The collagen deposits by means of Masson's trichrome staining did not differ much between the positive control and the test groups

Since osteogenic differentiation cannot be confirmed by mineralization alone, the ICC/IF expression of the osteogenic markers alkaline phosphatase, osteonectin, DMP1 and the cementum specific marker CEMP 1 was also evaluated. There was positive expression of alkaline phosphatase enzyme, osteonectin, DMP 1 and CEMP confirming the osteogenic and/or cementogenic differentiation of the periodontal ligament cells, on both Lys-PDA and G-PDA matrices.

The isolated characterized hPDLCs proved to be a useful tool to evaluate the biomolecules towards periodontal regeneration as the expression of cementum specific markers is not possible with cell lines or any other primary cells. Both the biomolecules used in this study were found to be non-cytotoxic and promote cell adhesion and differentiation of human periodontal ligament cells. This study advocates the use of these biomolecules in a clinically translatable form like biofunctionalized barrier membranes for further applications.

5.2.2 Application of human periodontal ligament cells for *in vitro* evaluation of calcium phosphate and sulfate based bioactive cements for alveolar bone regeneration

In GTR/GBR treatment approaches, alveolar bone grafts are used in addition to barrier membranes to enhance the regeneration of tooth supporting alveolar bone that have got damaged as a sequel to persistent periodontitis [Sheikh *et al*., 2017]. Synthetic hydroxyapatite (bone mineral) in sintered ceramic form has been proven as a useful graft material for periodontal applications. Despite being a good osteoconductive material, its use is limited due to the longer resorption time. Also, the high temperature-sintering process reduces the number of phosphate and calcium ions being leached out, which affects the osteogenic efficiency [De Carvalho *et al*., 2019]. The second generation of synthetic bone grafts is constituted by bioactive glasses and glass-ceramics which are made through fusing calcium and phosphate materials along with silica. They show higher bioactivity due to the presence of silicate chains in its structure and resorb faster *in vivo*. The bioactive glasses have amorphous glassy structure and are brittle, whereas the glass-ceramic composites are machinable to regular shapes. The third-generation materials in synthetic bone graft family consists of self-setting inorganic cements which are aqueous based, and do not require initiators. They are provided as powder-liquid combination, the mixing of which in prescribed ratio, gives bone mineral phase of calcium phosphate. These cements are moldable and due to their similarity to bone mineral upon setting, they show osteoconductivity and bioresorption *in vivo*. For these reasons, they became the current biomaterials of choice as bone grafts in alveolar defect management [Komath *et al*., 2000; Komath and Varma, 2003].

Current research in regenerative dentistry reiterates the possibility to induce the periodontal ligament cells to differentiate to an osteogenic lineage by the use of suitable inorganic salts [Bizelli *et al*.,2018]. About 30 % of the cells of periodontal ligament possess multipotency and can differentiate to osteogenic lineage, which is important in regeneration of periodontium following infections and injuries [Nagatomo *et al*., 2006]. This differentiation potential of the periodontal ligament cells can be utilized *in vitro*, for evaluating the osteoinductivity of sulfate and phosphate based self-setting aqueous cements as alveolar bone graft materials.

An in-house developed self-setting apatitic calcium phosphate bone cement (CPC) system is one of the test materials in the present work. It is composed of a powder mix of tetra calcium phosphate and dicalcium phosphate dihydrate, with sterile solution of Na_2HPO_4 as the liquid component that yield a workable putty material which can be used as a bone graft substitute [Komath *et al*., 2000]. CPC is a fully injectable system before setting, with sufficient setting time and mechanical properties [Komath and Varma, 2003] and its safety and efficacy assessment was carried out in small animals *in vivo*, confirming the biocompatibility [Fernandez *et al*., 2006]. The cement was also evaluated for its efficiency in alveolar bone regeneration in human clinical trial [Rajesh *et al*., 2009]. Due to its contouring capability and resorbability, CPC was found to serve the purpose of both a barrier as well as an alveolar bone graft. In other words, CPC is capable of acting as a "Barrier Graft", eliminating the use of an additional barrier membrane [Rajesh *et al*., 2009]. The role of calcium phosphate cement as a pulp capping agent is also reported through a controlled clinical trial [Jose *et al*., 2013]. Different types of calcium phosphate cements could be found in literature, especially in self-setting injectable form, which have been used in periodontal alveolar bone defects. Such cement formulations can adapt well to the defect areas and the set cement also reinforce the tooth, till complete regeneration is achieved.

Calcium sulfate cements are water based self-setting cements used as filler materials adjunct to the graft that can enhance their action [Pecora *et al*, 1997]. The basic material is medical grade calcium sulfate hemihydrate which reacts with water to undergo dissolution and recrystallization as calcium sulfate dihydrates, during which, the mix set into the shape. Different modifications of the basic formulation could be found. It was also reported that calcium sulfates by itself show osteogenic properties and has a faster bioresorption compared to calcium phosphates [Kumar *et al.*, 2013]. A novel cost-effective method to synthesize uniform low dimensional crystals of medical grade calcium sulfate was developed in-house, to make calcium sulfate-based cement systems, named BioCaS, for bone graft applications [Sandhya *et al*., 2012]. The injectability and bioactivity of the formulation was improved by the addition of hydrogen orthophosphate (HPO_4^-) ions, thereby incorporating phosphate ions into the cement system [Sony *et al*., 2015]. The preclinical evaluation of BioCaS as an injectable bone filler in long bone defects was carried out and it was found that the material resorbed in the same pace as the new bone formation, which is known as *osteotransductivity* [Sandhya *et al*., 2017]. It will further help in faster healing and gain in strength. The

physicochemical and biological properties of BioCaS advocate its use as a fast resorbing "barrier graft" for alveolar bone regeneration.

It was observed from the previous studies that calcium phosphate cements can be used for alveolar bone regeneration [Rajesh *et al*., 2009]. The various *in vitro* properties of the novel bioactive calcium sulfate cement, namely BioCaS, and the positive results in healing of bone defects in animals indicate that it can have useful application as an alveolar bone graft substitute [Sony *et al*., 2015; Sandhya *et al.*, 2017]. Therefore, in this study, human periodontal ligament cells are used to evaluate the cytocompatibility and the osteogenic potential of the bioactive calcium sulfate cement BioCaS and compare the results with sintered hydroxyapatite (HA), bare calcium sulfate (CaS) and calcium phosphate cements (CPC). The results can be correlated with clinical outcomes and be used towards further development of the bioactive cements towards clinical translation. The hPDLCs were used to study the preliminary cytocompatibility of the bioactive cements. The cements samples (BioCaS, CaS and CPC) were prepared as discs, and after complete setting, they were dried and EtO sterilized. The control sample was sintered hydroxyapatite (HA) discs of same dimensions sterilized by autoclaving. The cells exhibited good viability, percentage metabolic activity, morphology and spreading on the cement discs. The hPDLCs could differentiate to an osteogenic lineage, even without any induction media, in the presence of the bioactive cements. This was confirmed by the presence of mineralization through Alizarin red and Von Kossa staining. The elevated alkaline phosphatase enzyme expression is confirmatory in cellular differentiation to osteogenic lineage. In the control group with sintered HA, much less mineralization was noted, despite an elevated alkaline phosphatase expression, which may be due to the slow release of calcium and phosphate ions, as they are sintered. The ICC/IF staining of periodontal ligament cells on the respective cement coated glass coverslips showed positive expression of alkaline phosphatase enzyme, osteonectin, collagen 1 and DMP 1, which confirmed the differentiation potential of hPDLCs in the presence of the cements.

The isolated characterized human periodontal ligament cells deemed to be ideal candidates for the *in vitro* evaluation of biomaterials that can regenerate the periodontal ligament and alveolar bone. A healthy periodontal ligament and the supporting alveolar bone are inevitable to maintain the tooth anchored well to the supporting alveolar bone through Sharpey's fibers inserted on either side, connected to the cementum and alveolar bone. In

this study, it was found that the calcium phosphate cements (CPC) did promote osteogenic differentiation of periodontal ligament cells. The bioactive calcium sulfate cement (BioCaS) showed *in vitro* osteoinductivity similar to CPC, warranting the effectiveness of the new formulation. Interestingly, it was also noted that the bare calcium sulfate cement (CaS) also could induce differentiation of periodontal ligament cells, in accordance with certain previous reports [Pecora *et al.*, 1997].

This knowledge paves way to the development of bioactive cements with different rates of bioresorption, so that the clinician can select the most appropriate bioactive cement for the patient's needs. Calcium phosphate cements may be used for larger alveolar bone defects which need to retain the biomaterial for longer periods. BioCaS can be used for intermediate bone defects where the cement needs to be in place for at least 6 weeks. BioCaS possess the property of *osteotransductivity*, i.e., bone formation at par with the material resorption [Sandhya *et al.*, 2017], indicated by the healing without a clear-cut boundary between the graft and host bone. Calcium sulfate cement (CaS) can be used as bioactive barrier in small bone defects, with resorption in 4-6 weeks. The attraction of using calcium sulfate cements is the possibility of incorporating antibacterial agents without losing the potency, so as to control underlying microbial infections.

The biological evaluation of the bioactive cements composed of calcium phosphates and calcium sulfates yielded promising results towards cytocompatibility and osteogenic differentiation of hPDLCs *in vitro*, advocating the use of the cements as alveolar bone grafts. Since any medicaments or sealers placed inside the root canal space as endodontic sealers or as medicaments or bioactive agents for revascularization of root canal space comes to contact with the periradicular periodontal ligament cells, it can be assumed that these bioactive cements can also be used as endodontic sealers [Jung *et al.*, 2018].

5.2.3 Application of human Dental Pulp cells for *in vitro* evaluation of Calcium Phosphate and Sulfate based bioactive cements as pulp capping agents.

Calcium phosphate cements have been used as dental pulp capping agents and the capacity to induce dentin bridge formation over the remaining healthy pulp was identified [Lee *et al.*, 2014]. The in-house synthesized calcium sulfate cements are also reported to promote consistent dentin bridge formation with reduced inflammation compared to the control material [Jose *et al.*, 2013]. Recent studies have reported variable success rates using

bare calcium sulfate cements and calcium sulfate-phosphate cements, in achieving dentin bridge formation during direct pulp capping [Chang *et al*.,2014; Ulusoy *et al*., 2014]. After evaluating the periodontal ligament cell response to the bioactive calcium sulfate (BioCaS), calcium phosphate (CPC) and calcium sulfate (CaS) cements, it was assumed that the dental pulp cell response to the cements can provide information towards their use as dental pulp capping agents. Hence, it was decided to use isolated dental pulp cells for the biological evaluation of the cements, including the osteoinduction potential of the cements on the hDPCs.

The hDPCs exhibited good cytoskeletal architecture on the respective cements. Since the cytocompatibility evaluations of the cements were carried out previously using hPDLCs, it was decided to proceed with the ICC/IF expression of osteogenic markers by the hDPCs on the cement surfaces. The hDPCs showed positive expression of alkaline phosphatase, osteonectin and DMP1, when seeded onto the cements, confirming their differentiation potential in the presence of the cements. This data paves way for the potential use of these cements as dental pulp capping agents.

5.3 Translational approaches of biomimetic matrices and bioactive cements towards regeneration of periodontium

In order to decrease the gap between scientific knowledge and application, translation of the technical know-how to a product or a novel treatment strategy is imperative. Here, the knowledge translation happens with the synthesis of novel biomaterials for dental regeneration and exchange among researchers and clinicians for the ethical application of acquired knowledge to capture the benefits [Afrashtehfar and Assery, 2017]. In our study, the knowledge about periodontal ligament cell response to the biomimetic matrices can be translated clinically, to biofunctionalize inert prospective GTR barrier membranes. Therefore, the Lys-PDA and G-PDA molecules were applied to biofunctionalize electrospun PCL membranes and evaluated *in vitro* to confirm the cytocompatibility. Among these, Lys-PDA/PCL mats, due to the presence of lysine were used for *in vivo* evaluations to confirm the utility of the barrier membranes in a preclinical animal model.

The bioactive cements can be used as a moldable synthetic graft material that can be carried to the periodontal defects using suitable dental instruments. The bioactive cements were also evaluated *in vivo* using a rat maxillary alveolar bone defect model to elucidate the regeneration of alveolar bone and periodontal tissues histologically.

5.3.1 *In vitro* evaluation of Dendritic peptide molecules biofunctionalized electrospun PCL barrier membranes for periodontal regeneration

The Lys-PDA and G-PDA matrices proved to have good cytocompatibility and supported the differentiation potential of the periodontal ligament cells *in vitro*. Since the biomolecules can be applied as a thin coating on cover glasses and polymerized to form a stable matrix, it was decided to use them to biofunctionalize electrospun PCL membranes, in an attempt to translate the biomolecules to a clinically acceptable form. These biofunctionalized electrospun PCL membranes could be used as barrier membranes in Guided Tissue regeneration (GTR)approaches for periodontal regeneration.

Polycaprolactone (PCL) is an FDA approved biodegradable polyester with proven biocompatibility, good mechanical properties, and chemical and thermal stability. Electrospinning is a versatile method of fabricating non-woven, porous nanofibers using electrostatic forces. The nano-fibrous architecture of the electrospun mats itself can mimic the ECM structurally [Prasad *et al*., 2015]. Electrospun PCL can be composited with bioactive particles, can incorporate drugs and therapeutics, or can be surface modified to have better cell/tissue interactions [Sundaran *et al*., 2017]. The inherent hydrophobicity of PCL could be reduced by chemical modifications. Biofunctionalization is an accepted technique to incorporate biomimetic or bioactive molecules to the electrospun membranes [Gao *et al*, 2015, Madathil *et al*., 2017]. PCL was selected to fabricate electrospun membranes because it is an approved polymer for implantation purpose and standardized electrospinning parameters are available. [Sundaran et al., 2017]. Lys-PDA and G-PDA were used to biofunctionalize PCL membranes, as these biomolecules induced favorable cell response with hPDLCs.

Uniform nanofibrous membranes were obtained by electrospinning PCL and the structure of the fibers were evaluated using SEM. The presence of Lys-PDA and G-PDA coating was confirmed by FTIR and SEM. The sessile drop water contact angle measurements showed a reduction in hydrophobicity of the PCL membranes, which can

enhance early cell adhesion. The materials were deemed cytocompatible with MTT assay, and further biological evaluations were carried out using hPDLCs. In the present study, it was possible to obtain a biomimetic coating onto the electrospun PCL membranes, and it was noted that the hPDLCs adhered well, onto the Lys-PDA coated membranes. The number of viable cells was more on the coated membranes than the bare PCL membranes, possibly due to the increased hydrophilicity of the coated membranes. Visually, more cells were present on the Lys-PDA/PCL membranes than on the G-PDA/PCL or PCL membranes.

In order to function successfully for GTR applications, the membranes should physically prevent the migration and down growth of gingival epithelial cells. The purpose of biofunctionalizing the inert barrier membrane is to enhance the repopulation of the periodontal and alveolar bone defect sites with periodontal ligament fibroblasts, cementoblasts and alveolar bone osteoblasts cells. This will enhance new cementum and alveolar bone formation, with dense periodontal attachment between them, which may not be possible, if interrupted by the migrating gingival epithelium [Omar *et al.*, 2019]. Complex *in vitro* cell culture systems involving epithelial and fibroblast co culture systems are needed to confirm the barrier effect of the membranes. *In vivo* experiments are needed to further confirm the barrier effect of the membranes. From the results, it was evident that lysine offered a better chance of cell adhesion and biomineralization, possibly because of its interaction with phosphate ions, and being a principal component of the bone ECM collagen. Hence, further *in vivo* studies were carried out using Lys-PDA/PCL membranes.

5.3.2 *In vivo* evaluation of Dendritic peptide biofunctionalized electrospun PCL barrier membranes for periodontal regeneration

A rat maxillary periodontal defect model was created with minimal surgical trauma to the animals using a custom made surgical platform with restraints to keep the mouth open. The periodontal defect created along the mesio-buccal margin of the rat maxillary first molar could be debrided with suitable curettes. The samples (PCL and Lys-PDA/PCL membranes) were gently placed along the gingival margin as GTR barrier membrane, to prevent the epithelial overgrowth along the periodontal defect margins. The results of the *in vivo* study confirmed the barrier function of the membranes, preventing epithelial migration beyond the cemento-enamel junction.

5.3.3 *In vivo* evaluation of bioactive cement systems towards regeneration of periodontium

The results of the *in vivo* study using BioCaS, CPC and CaS cements showed evidence of new bone formation at the cement interface. Remnants of cement material were evident in CPC and BioCaS group, confirming the material resorption is not complete at 1 month duration. However, remnant cement particles were not observed in CaS group, which is suggestive of faster resorption of calcium sulfate cement with trabecular new bone formation. This result is in accordance with the previous studies confirming the *osteotransductivity* of BioCaS cements and early resorption of CaS cements.

5.4 Limitations of the study

The current study achieved the set objectives proposed through carefully planned experiments. However, certain limitations were encountered during the course of the study. The cell isolation procedures were carried out using healthy donor tissues, which may not completely replicate the disease conditions of dental pulp and periodontal ligament. Approaches to simulate any disease conditions through inflammatory mediators or hypoxia were not carried out in this study. The majority of cell culture studies is carried out using periodontal ligament cells, and only selective significant experiments were carried out using dental pulp cells. Although the barrier function of the biofunctionalized electrospun PCL mats was evident from the *in vivo* experiments, the *in vitro* osteogenic potential was not reproducible in the current experiment set up. The use of a large animal model and incorporation of more bioactive molecules may be needed to confirm the bioactivity of the molecules. The cements exhibited *in vivo* new bone formation as early as 1 month. Much more information may be achieved through the use of a large animal model in a longer duration to reiterate the osteoinductivity of the bioactive cements.

Chapter 6

SUMMARY AND CONCLUSIONS

The purpose of this study is to establish an appropriate tooth-derived cell culture system for the evaluation of dental biomaterials. The study envisaged the identification of the regenerative potential of some candidate biomimetic molecules and bioactive cements using the proposed cell culture system. The ability of these dental biomaterials to regenerate lost tissues was confirmed using a suitable *in vivo* model. This well-planned sequence of *in vitro* and *in vivo* testing can bridge the knowledge gaps and reduce the delays in the clinical translation of promising biomaterials in regenerative dentistry.

The aims of the study were set as (i) the establishment of a suitable cell culture system for the evaluation of dental biomaterial evaluation, (ii) to analyze the suitability of the cell culture system for cytocompatibility evaluations and study specific cellular responses to dental biomaterials, and (iii) to confirm the *in vitro* results using suitable *in vivo* models. During the course of the study, a uniform technique was proposed towards the isolation and characterization of tooth-derived cells. The isolated, characterized cells were successfully used for the evaluation of custom designed biomimetic molecules and bioactive cements, with promising results. The *in vivo* experiments confirmed the proposed function of the biomaterials as barrier membranes and alveolar bone grafts.

6.1 Summary

The first part of this study was aimed at establishing tooth-derived cell culture system. A reproducible technique for the isolation of the tooth-derived cells from dental pulp, periodontal ligament, apical papilla and deciduous teeth was developed successfully. The cells consisted of a heterogeneous population with fibroblasts being the prominent cells. The characterization of the cells was carried out using a cocktail of known mesenchymal stem cell markers, fibroblast markers and tissue specific markers. The differentiation potential of the cells was successfully evaluated *in vitro* using histological staining techniques and

osteogenic marker expression. The tooth-derived cells were isolated and characterized so as to use them for *in vitro* biological evaluation of the dental biomaterials. The work in this part enabled the selection of appropriate cells that comes in direct contact to the dental materials – the periodontal ligament cells and the dental pulp cells – as ideal cells for the evaluation of dental biomaterials.

The second objective of the study was to identify candidate biomaterials with regenerative potential and to optimize them for dental applications. Biomimetic dendritic peptides with lysine and guanidine as the respective functional units were selected for evaluation of periodontal regenerative abilities. New in-house developed calcium phosphate and sulfate based bioactive cements were selected based on their potential ability to regenerate bone tissues. The materials were characterized and optimized for *in vitro* evaluation using periodontal ligament cells and dental pulp cells.

The third objective of this study was the utilization of the established tooth-derived cell culture system for the evaluation of the selected dental biomaterials. The biomimetic matrices were evaluated using periodontal ligament cells for cytocompatibility as well as specific cellular responses. The periodontal ligament cells were used for the cytocompatibility evaluation of the bioactive cements. The specific cellular responses indicating the osteoinductivity of the cements were obtained using both periodontal ligament cells and dental pulp cells. The periodontal ligament cells (hPDLCs) proved to be a useful tool to evaluate the preliminary cytocompatibility of the biomolecules and bioactive cements. The specific cell responses including osteoinductivity of the bioactive cements could be elucidated using hPDL cells and dental pulp cells (hDPCs). Both the biomolecules and the bioactive cements used in this study were found to be non-cytotoxic and to promote cell adhesion and differentiation of human periodontal ligament cells. Both the hPDLCs and hDPCs exhibited good cytoskeletal architecture on the respective cements with evidence of osteogenic differentiation *in vitro*, under the influence of the material. This indicates the potential use of the bioactive cements as alveolar bone grafts, endodontic sealers and medicaments, and as dental pulp capping agents.

The final objective of this study was to correlate the *in vitro* results *in vivo* using suitable small animal models. The biomolecules were successfully used to biofunctionalize inert electrospun PCL mats as periodontal barrier membranes. The coated mats could be

characterized using SEM and FTIR and the cytocompatibility was established using hPDL cells. The results of the *in vivo* study confirmed the barrier function of the membranes, preventing epithelial migration beyond the cemento-enamel junction.

The *in vitro* osteogenic potential of the bioactive cements was confirmed *in vivo* using a rat maxillary alveolar bone defect model. The results showed evidence of new bone formation and cement resorption at the cement interface of the bioactive cements.

6.2 Conclusions

Depending on the results and the inferences from the study, it can be concluded that

1. Tooth-derived cells can be isolated and cultured from various dental tissues.
2. The cells can be characterized and utilized for the *in vitro* evaluation of potential dental biomaterials.
3. Biomimetic molecules mimicking extra cellular matrix can be designed and optimized for dental regeneration applications.
4. Bioactive bone cements can be modified suitably for the regeneration of periodontium and dental pulp.
5. Appropriate combinatorial approaches like the biofunctionalization of inert materials, for example, poly caprolactone membrane, can be utilized to promote dental regeneration.
6. Suitable surgical defect models can be created in small animals for *in vivo* evaluation of regenerative dental biomaterials.

6.3 Future perspectives

The future perspectives of the study are targeted towards clinical translations. The first step in clinical translation of a biomaterial is the validation and standardization of the cell culture system for dental biomaterial evaluation, in accordance to acceptable standards. A detailed protocol for the use of tooth-derived cells for biomaterial evaluation is to be developed, so as to serve as standard.

The biomimetic molecules studied in this work need to be modified to enhance its activity *in vivo* for clinical translation. Further studies are needed on how to incorporate adequate quantities of the biomimetic molecules to promote alveolar bone regeneration *in vivo.* The potential role of the dendritic peptide molecules in dental pulp regeneration also needs to be studied.

The bioactive cements showed promising potential for clinical translation as synthetic alveolar bone graft substitutes. The future perspective is to explore the possibility of initiating a clinical trial of the bioactive cements towards alveolar bone regeneration. The ultimate outcome of this study can be applied to develop products based on bioactive cements for alveolar bone grafting or as barrier materials.

The potential of bioactive cements towards dental pulp regeneration needs to be confirmed using suitable *in vivo* models before clinical translations can be initiated.

Chapter 7

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APPENDIX

1. Phosphate buffered Saline – 1 L

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
H_2O	1 L (Deionised)

Sterilised by autoclaving

2. Tissue collection medium

Sterile PBS	9 ml
Pen/Strep (Gibco)	1 ml (10000 IU Penicillin, 10000µg Streptomycin
Amphotericin B	25µl

3. Regular cell culture medium (100 ml)

αMEM (Gibco)	86 ml
$NaHCO_3$ (7.5 %)	3 ml
Pen/Strep (Gibco)	1 ml
FBS (Gibco)	10 ml

4. Osteogenic induction medium

αMEM (Gibco)	86 ml
$NaHCO_3$ (7.5%)	3 ml
Pen/Strep (Gibco)	1 ml
FBS (Gibco)	10 ml
Na β glycerophosphate	10 mM
L Ascorbic acid	50 µg/ml
Dexamethasone	10 nM

5. Alizarin red solution 4 %

Alizarin red (Sigma)	400 mg
Deionized water	10 ml
P^H	Adjusted to 4.2

6. Von Kossa staining

Silver Nitrate (Sigma)	200 mg
Deionized water	10 ml

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