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Advanced Scaffolds for Dental Pulp and Periodontal Regeneration

Marco C. Bottino, DDS, PhD[1,*], **Divya Pankajakshan, PhD**[1], and **Jacques E. Nör, DDS, PhD**[2]

[1]Indiana University School of Dentistry, Department of Biomedical and Applied Sciences, Division of Dental Biomaterials, Indianapolis, IN, 46202, USA

[2]University of Michigan School of Dentistry, Department of Cariology, Restorative Sciences and Endodontics, Ann Arbor, MI 48109, USA

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INTRODUCTION

Caries and periodontitis are major pathologies affecting teeth and their ancillary structures that, if not properly managed, may lead to tooth loss.[1,2] Recent estimates from the National Health and Nutrition Examination Survey (NHANES) show that in the United States nearly 8% of adults (age 20 to 64) and 17% of seniors (age ≥ 65) suffer from periodontitis, while caries impacts 37% of children (age 2 to 8) in their deciduous teeth and 58% of adolescents (age 12 to 19) in their permanent dentition. From these statistics, it becomes immediately clear that these two conditions remain a significant public health problem and require better strategies for prevention and management.

A challenging problem for endodontists and pediatric dentists is the clinical management of immature (open apex) permanent teeth with necrotic pulp resulting from trauma or bacterial infection.[3] Over the years, the therapy of choice has followed the principles of apexification, i.e., disinfection treatment with calcium hydroxide followed by root canal sealing with gutta-percha. However, the last decade has brought forward new prospects regarding dental pulp regeneration, thanks to evoked bleeding (EB), an approach that has been found to induce dentinal wall thickening and root end closure.[3–6] Nonetheless, despite the aforementioned clinical and histological observations, the regenerative outcome of this patient-dependent and rather unpredictable therapy remains elusive.[6–11] Several aspects, including, but not limited to the use of very cytotoxic antibiotic pastes, have been thought to account for the

Corresponding Author: Dr. Marco C. Bottino, Indiana University School of Dentistry, Department of Biomedical and Applied Sciences, Division of Dental Biomaterials, 1121 W. Michigan St. (Rm DS270), Indianapolis, IN, 46202, USA, Tel: +1-317-274-3725/ Fax: +1-317-278-7462, mbottino@iu.edu (M.C. Bottino).

unsystematic success.[4] To circumvent the characteristic toxicity associated with commonly used antibiotic pastes and sodium hypochlorite, a more biocompatible strategy has recently been developed by our laboratory. A series of studies[3,12–20] have stressed the practicality and translational prospects of three-dimensional (3D) easy-to-fit antibiotic-eluting nanofibers as a localized, intracanal drug delivery strategy that, combined with injectable scaffolds, enriched or not with stem cells and/or growth factors (GFs), may lead to an increased likelihood of achieving predictable dental pulp regeneration in humans.

Considered to be one of the most aggressive chronic inflammatory oral diseases, periodontitis affects the integrity of both soft and hard tissue, which, in severe cases of tissue destruction, can result in tooth loss.[21] Originally, the principles of guided tissue regeneration have been followed to restore the architecture and functionality of the periodontal system. In essence, an occlusive biocompatible polymer-based membrane is used as a barrier to prevent epithelial and connective tissue migration into the regenerating site. In this way, slower migrating progenitor cells, located in the remaining periodontal ligament (PDL) are able to recolonize the root area and differentiate into new periodontal tissues.[22] Based on varying levels of clinical success with this approach, the last decade has witnessed significant advancement toward the generation of membranes with therapeutic properties. The work reported in the literature has not only included antimicrobials and inorganic particles (e.g., calcium phosphates), but also biomolecules (e.g., growth factors), in the fabrication of membranes with therapeutic functions.[21,23] More recently, the combination of known materials and biomolecules with advanced technologies[24–31], particularly 3D printing, have permitted translation of the first patient-specific scaffold modified with platelet-derived growth factor (rhPDGF-BB) for treating large periodontal defects.[26]

This two-part review offers an update on progress related to advanced biomaterials for dental pulp and periodontal regeneration. To provide a better understanding of the regenerative strategies described herein, a concise, yet informative summary on dental stem cells, is presented. The first part provides a short background on the EB strategy, the significance of a biocompatible disinfection, and major highlights on the use of scaffolds, stem cells, and GFs in dental pulp regeneration. The second part highlights the newest advances regarding the development of membranes with therapeutic properties and technologies, such as additive manufacturing, to engineer patient-specific membranes/scaffolds to amplify hard and soft tissue periodontal regeneration.

DENTAL STEM CELLS

Dental Stem Cells in Deciduous and Adult Teeth

Dental stem cells are considered a population of mesenchymal stem cells (MSCs), according to the minimal criteria defined by the International Society for Stem Cell Research.[32,33] Apart from demonstrating plastic adherence and multilineage differentiation, dental stem cells display positive expression of specific surface antigen markers (e.g., CD44, CD73, CD105).[33]

In 2000, Gronthos and collaborators reported seminal work on the isolation and characterization of an MSC-like heterogeneous population of stem cells from the dental pulp

tissue of human third molars, calling them post-natal dental pulp stem cells (DPSC).[34] The notable regenerative potential of DPSCs based on their differentiation into odontoblasts, osteoblasts, adipocytes, and neurons has been demonstrated.[35–37] A few years later, a subpopulation of DPSCs, which express certain hematopoietic lineage markers, were similarly isolated from the pulp of exfoliated deciduous teeth (stem cells from human exfoliated deciduous teeth, or SHED).[38] The advantages of SHED, when compared to DPSCs, are their higher proliferation rate and enhanced differentiation potential; for example into odontoblasts and endothelial-like cells when implanted *in vivo* using a tooth slice/scaffold model.[38–40] The mechanistic pathways involved in the endothelial differentiation of SHED[41] and DPSCs[42] have been recently unveiled by our group.

Stem cells from the apical papilla (SCAP) were identified by Sonoyama and collaborators as an embryonic-like soft tissue located at the apex of growing tooth roots.[43,44] Apical papilla is speculated to be a source of “primary odontoblasts” that synthesize primary tubular dentin, as opposed to the “replacement odontoblasts” that form reparative dentin. A key benefit related to SCAPs pertains to the apical location that supports tissue survival during pulp necrosis.[45] Specifically, SCAP co-expresses STRO-1+ with a range of osteo/dentinogenic markers and neural markers.[44] From a proliferation standpoint, SCAP demonstrates a higher rate than DPSC and expresses, similarly to DPSCs, typical dentinogenic markers (e.g., DSPP) upon induction.[46]

Stem Cells in Periodontal Tissues and Dental Follicles

The periodontal ligament (PDL), a specialized connective tissue that links the radicular surface to the alveolar bone, was found to harbor a unique population of stem cells (PDLSCs).[47] These cells express not only MSC markers, but also tendon-specific markers. PDLSCs isolated from periodontal granulation tissue improved new bone formation when transplanted in calvarial defects in mice.[47] Another important stem cell niche is the dental follicle, a loose connective tissue surrounding the developing tooth, which later develops into the periodontium.[48] The dental follicle harbors dental follicle precursor cells (DFSCs). DFSCs can be maintained and expanded in cell culture, demonstrate a higher proliferation capacity when compared to DPSCs, and have been found to be precursors of periodontal tissues cells (i.e., fibroblasts in the PDL, alveolar bone cells, and cementoblasts).[48,49]

Worth noting, several other candidate dental-derived stem cells for oral/craniofacial tissue regeneration isolated from salivary glands, oral mucosa, gingiva, and periosteum, have been identified. However, their differentiation ability and *in vivo* regenerative potential remains unclear.[50] In summary, the multipotency, increased proliferation rates, and ease of accessibility makes dental stem cells an attractive source for tissue regeneration. Next, selected studies involving the use of dental stem cells and advanced scaffolds for dental pulp and periodontal regeneration are discussed.

ADVANCED BIOMATERIALS FOR DENTAL PULP REGENERATION

Pulpal Disease

Root canal therapy involving chemo-mechanical debridement and sealing of the canal system with an inert rubber-like material remains the standard of care for necrotic mature teeth with closed apices.[51] However, immature permanent teeth display a wide-open root apex and thin root dentinal walls, making it virtually impossible to obtain an apical seal using the customary method.[3–5] Therefore, new clinical therapies (e.g., EB) for dental pulp regeneration have been explored, particularly because apexification supports only apical closure and does not promote root maturation[52,53], thus increasing the chance of root fracture upon secondary trauma.[54,55]

Evoked Bleeding — The First Step Towards Dental Pulp Regeneration

In the early 1990s, tissue engineering emerged as a field tasked to provide a clinically translatable platform for tissue/organ regeneration.[56] Three major elements form the basis for tissue engineering, namely, stem cells, bioactive signaling molecules, and scaffolds. Scaffolds, in turn can have unique structural, chemical, mechanical, and biophysical properties. These have been explored individually and in tandem to ensure controllable tissue regeneration.

In recent years, the development of new clinical therapies for dental pulp regeneration, such as the EB method, have offered promise for improving treatment outcomes. In EB, succeeding proper root canal disinfection, the laceration of periapical tissue is deliberately performed to evoke bleeding and form a fibrin-based scaffold to interact with endogenous stem cells and growth factors. The EB method has preferably employed a triple (ciprofloxacin/CIP, metronidazole/MET, and minocycline/MINO) or double (minocycline-free) antimicrobial component constituted of a very concentrated antibiotic paste to accomplish disinfection. However, the specific therapeutic dose of the antibiotic mixtures that promotes maximum antimicrobial action, while reducing toxicity to the host tissues and residing cells, is not currently known. Regardless of the promising results achieved by EB when treating immature permanent teeth with necrotic pulp[57], only one case report demonstrates pulp-like tissue formation.[58] Indeed, most histological findings have acknowledged the invagination of periapical tissue containing bone-like hard tissue and cementum-like tissue that has led to further root canal walls' thickening.[59,60] Although the EB strategy has been proposed to treat immature teeth, a recent study also demonstrated the influx of undifferentiated MSCs from the apical region into the pulpal space of mature teeth with apical lesions.[61]

Antibiotic-eluting Polymer Nanofibers for Intracanal Drug Delivery

A significant amount of data has indicated that antibiotic pastes and chemical irrigants can affect the survival ability and function of dental stem cells.[3–5] In light of this, a biocompatible nanofiber-based intracanal drug delivery system has been proposed as a means to create a bacteria-free environment favorable to tissue regeneration.[3,12–20] In brief, a polymer solution loaded with the chosen antibiotic(s) at the desired concentration needs to be prepared.[3,12, 21] Following that, by adjusting electrospinning parameters (e.g., flow rate,

field strength, etc.), antibiotic-eluting nanofibers are obtained. The clinical use of these therapeutic nanofibers processed as a three-dimensional (3D) tubular-shaped construct[3,17, 62] that can be easily fitted into the root canal system of necrotic teeth (Fig. 1) holds great clinical potential, as it will guarantee the release of antibiotics onto the dentinal walls where microbial biofilm has been found to be present.[3,12,14–16,18–20,62] As an example, triple (MET, CIP, and MINO) antibiotic-eluting nanofibers were developed and tested for antimicrobial efficacy against a dual species bacterial biofilm.[20] Infected dentin exposed to the triple-antibiotic-eluting nanofibers revealed significant bacterial death based on confocal laser scanning microscopy data (Fig. 1). Numerous studies[3,12–16,18–20] have been published and provided critical information to test the clinical efficacy of 3D tubular-shaped antibiotic-eluting nanofibers using animal models of periapical disease (Fig. 1).

Advanced Scaffolds and Regenerative Strategies

Besides a more cell-friendly disinfection strategy, a number of developments in tissue engineering and regenerative medicine, primarily related to the synthesis scaffolds, have provided the foundational knowledge for reliable and predictable regeneration of the pulp-dentin complex. According to the American Society for Testing Materials (ASTM—F2150), a scaffold is defined as “the support, delivery vehicle, or matrix for facilitating the migration, binding, or transport of cells or bioactive molecules used to replace, repair, or regenerate tissues.” It should precisely replicate the features of the native extracellular matrix (ECM) at the nanoscale to regulate cell function and encourage and regulate specific events at the cellular and tissue levels.[63–65] Moreover, scaffolds should be synthesized from biocompatible and biodegradable material(s) to avoid immune responses. A myriad of polymers, both synthetic (e.g., poly[lactic] acid, PLA) and natural (e.g. collagen), have been used in gas foaming, as well as salt leaching techniques, to obtain macroporous scaffolds. Meanwhile, nanofibrous scaffolds have been processed via electrospinning, self-assembly, and phase-separation.[63,66,67]

In electrospinning, polymer nanofibers are obtained by the creation and elongation of an electrified jet.[68] Various polymer solutions can be used and modified through mixing with other chemical reagents, polymers, nanoparticles, GFs, and cells to generate unique nanofibers.[68] Meanwhile, molecular self-assembly has been used to fabricate nanofibrous scaffolds through spontaneous molecular arrangement via non-covalent interactions.[63] This technique allows recapitulation of collagen’s supramolecule formation and enhances cell adhesion.[63] Moreover, these unique nanofibers present major clinical advantages as they are assembled in solution and result in gels that are biocompatible and can be used for stem cell transplantation.[66,67,69–71] However, this technique has limitations in terms of controlling pore size/shape within the scaffold and in producing sufficient mechanical properties.[63,70] Accordingly, an alternative method, commonly referred to as thermally-induced phase separation, has been incorporated in the fabrication of macro/micro pore networks within 3D nanofibrous scaffolds.[63] Taken together, recent advances in the field of biomaterials have allowed researchers to obtain scaffolds that can be easily injected in the desired site to aid in stem cell transplantation or to serve as delivery vehicles for bioactive factors. Some of the latest developments include the testing of innovative scaffolds/stem cells constructs in

conjunction with therapeutic agents, and these are presented next as evidence of the translational potential of tissue engineering in regenerative endodontics.

A well-known approach, the tooth slice/scaffold model, which uses immunodeficient mice and tooth fragments/segments[40], has provided key insight into the use of injectable scaffolds and stem cells toward dental pulp regeneration (Fig. 2). Puramatrix™, a self-assembling peptide hydrogel[71] mixed with SHED, generated a pulp-like tissue with odontoblasts capable of producing new tubular dentin. Moreover, the engineered pulp (Fig. 2) showed similar cellularity and vascularization when compared to human pulps.[67]

Over the past decade, multidomain peptides (MDP) consisting of short sequences of amino acids that self-assemble to form fibers in aqueous solution, have been the focus of Professors D'Souza and Hartgerink's groups.[66,72] MDPs displaying the cell adhesion motif arginine-glycine-aspartic acid (RGD), matrix metalloproteinase (MMP)-cleavable site, and heparin-binding domains, allowed growth factors conjugation and assisted in its slow release. Upon injection into dentin cylinders and subsequent implantation in immunocompromised mice for 5 weeks, the scaffold was entirely degraded and replaced by collagenous ECM. Vascularized soft connective tissue resembling dental pulp could be visualized and the cells at the cell-dentin interface appeared in intimate association with the dentin wall.[66]

A well-known hydrogel (i.e., Gelatin methacrylate, GelMA) was recently investigated for the first time for dental pulp regeneration.[73] GelMA is composed of denatured collagen and retains RGD adhesive domains and MMP-sensitive sites to enhance cell binding and matrix degradation. Furthermore, it is suitable for cell encapsulation and easily tunable by varying the concentrations of GelMA and photoinitiators. Professor Yelick's group demonstrated the formation of patent blood vessels filled with host blood cells following subcutaneous implantation of *in vitro* cultured human umbilical vein endothelial cells (HUVEC)/DPSC/GelMA injected into the tooth.[73]

Biodegradable polymer microspheres have been used as cell carriers for the regeneration and repair of irregularly-shaped tissue defects due to their injectability, controllable biodegradability and the capacity for drug incorporation and release.[74] In this way, nanostructured, self-assembling microspheres were employed (Fig. 3A–B) to deliver DPSCs into the pulpal space.[75,76] The authors reported on the synthesis of a novel, star-shaped block copolymer, poly(L-lactic acid)-block-poly-(L-lysine), capable of self-assembling into nanofibrous microspheres (NF-SMS). The NF-SMS microspheres supported DPSC proliferation and demonstrated DSPP expression *in vitro*.[75,76] Interestingly, DPSCs in NF-SMS microspheres, when exposed to hypoxic conditions, demonstrated increased VEGF expression. Following 4-weeks' implantation in an *in situ* pulp regeneration model, the cells in the hypoxia-primed group demonstrated columnar odontoblastic cell arrangement at the dentin–pulp interface, similar to that of native teeth. Hypoxia-primed hDPSCs/NF-SMS effectively regenerated pulp-like tissue with higher vascularity compared to the normoxia conditions (Fig. 3C).[75]

Nanostructured microspheres have also been investigated for GFs' delivery. A recent study elegantly described a strategy to allow dual drug delivery.[77] A microsphere platform was

used to concurrently release fluocinolone acetonide (FA) to suppress inflammation and bone morphogenetic protein 2 (BMP-2) to enhance odontogenic differentiation of DPSCs. A constant linear release of FA, and a rapid BMP-2 release was observed in *in vitro* systems that reduced inflammation on DPSCs and enhanced differentiation.[77]

Cell-Free Approaches for Dental Pulp Regeneration

The identification of biomolecules, including but not limited to GFs and matrix molecules sequestered within dentin and dental pulp, affords a unique opportunity to make these signaling cues available in the regenerative process after a biocompatible disinfection approach. It has been suggested that the release of these biomolecules by certain irrigants and medicaments can potentially circumvent the use of non-human exogenous biomolecules and avoid their short half-life.[78] Meanwhile, the utilization of exogenous bioactive molecules that can be adsorbed, tethered, or encapsulated into scaffolds to attract stem/progenitor cells adjacent to the root apices of endodontically-treated teeth has demonstrated great clinical prospects. Professor Mao's group reported[79] on the regeneration of dental-pulp-like tissue based solely on the intracanal delivery of fibroblast growth factor (FGF2) and/or vascular endothelial growth factor (VEGF) without stem cell transplantation. A re-cellularized and re-vascularized connective tissue integrated with the native dentinal wall in root canals was observed following *in vivo* implantation of endodontically-treated human teeth in mouse dorsum for 3 weeks. In addition, combined delivery of a cocktail of GFs (FGF2, VEGF, and PDGF) with a basal set of nerve growth factor (NGF) and bone morphogenetic protein 7 (BMP-7), led to the formation of tissues with patent vessels and new dentin regeneration.[79]

Clinical Translation

Over the past 5 years, unprecedented preclinical (animal model) demonstration[80–82] of pulp regeneration by $CD31^-$ side population (SP) cells and $CD105^+$ cells has suggested that clinically effective human pulp regeneration is closer than it has ever been. Remarkably, this specific subfraction of DPSCs revealed higher angiogenic and neurogenic potential than bone marrow or adipose-derived MSCs.[80–82] Evidence for complete pulp regeneration with adequate vasculature and innervation (Fig. 4A–C) into the pulpectomized root canals of dogs after autologous transplantation of $CD31^-$ (SP) cells or $CD105^+$ cells associated with stromal cell-derived factor-1 (SDF-1) and a collagen scaffold has been shown.[80–82] Moreover, new dentin formation along the dentinal wall was observed (Fig. 4D).

To expedite the clinical translation of the aforementioned approach in humans, it is key to obtain clinical-grade stem cells based on good-manufacturing-practice (GMP) conditions. A safe technique that isolates DPSC subsets has recently been devised by employing an optimized granulocyte-colony stimulating factor (G-CSF)-induced mobilization.[83] The mobilized DPSCs (MDPSCs) demonstrated stem cell properties, including high proliferation rate, migratory activity, and expression of multiple trophic factors.[83] Preclinical efficacy and safety tests were performed in dogs using clinical-grade G-CSF and collagen with MDPSCs, which resulted in complete pulp regeneration (Fig. 4E) with coronal dentin formation in the pulpectomized root canal and reduced number of inflammatory cells, decrease in cell death and the major increase in neurite outgrowth.[84] These preclinical results of efficacy and

safety of stem cell transplantation, has led to the initiation of a clinical trial with the consent of the Japanese Ministry of Health, Labor and Welfare.[85]

ADVANCED BIOMATERIALS FOR PERIODONTAL REGENERATION

Periodontal Disease

Periodontitis, a chronic inflammatory disease, occurs when bacteria-stimulated inflammation or infection of the gingival tissue progressively destroys the periodontium.[21] Tissue integrity is compromised by the loss of soft tissue attachment to the root surface, which results in periodontal pocket formation and subsequent loss of the alveolar bone, ultimately resulting in tooth loss.[21] According to Eke and colleagues, the prevalence of varying degrees of periodontitis in the U.S. adult population has been estimated to be nearly 47%.[86]

Traditional Membranes for Periodontal Regeneration

Periodontal regeneration is attributed to a complete recovery of both architecture and function, manifested as alveolar bone regeneration and new connective attachment through collagen fibers functionally oriented on the newly regenerated cementum.[21,87,88] As stated earlier, the use of synthetic or tissue-derived (collagen) membranes as barriers for guided tissue/bone regeneration procedures with or without calcium phosphate bone graft materials has been the treatment of choice.[21] According to their degradation behavior, membrane materials can be grouped into two classes—non-resorbable and resorbable.[21] Ideally, these membranes need to display biocompatibility to allow host integration without eliciting inflammatory responses, and a proper degradation profile that not only matches that of the new tissue formation, but more importantly allows sufficient maturation of the tissue before the membrane starts to degrade[21,25]. Also, these membranes need to possess sufficient initial strength to allow for clinical handling and placement.[21]

One of the major drawbacks of non-resorbable membranes (polytetrafluoroethylene) is the necessity for a secondary surgery for removal.[21] Resorbable membranes were developed to eliminate the pain and discomfort, as well as the financial burden associated with a second surgery.[89–92] The majority of resorbable synthetic membranes are based on polyesters and/or their copolymers.[91–101] Collagen membranes derived from the extracellular matrix (ECM) of human skin and other sources have become important alternatives to their synthetic counterparts, due to their excellent cell affinity and biocompatibility.[89,102,103] Regrettably, type-I collagen has many limitations, such as low strength and fast degradation, that support the need for an improved material.[21]

Membranes with Therapeutic Properties—Numerous attempts, with varying degrees of clinical success, have been made to develop a membrane with the right combination of mechanical, degradation, and biological characteristics required for guided tissue regeneration.[21,23] While progress has been made, these requirements are only being approached in recent published work. Advances related to membranes' biomodification to endow needed functionalities (e.g., antimicrobial, anti-inflammatory, cell differentiation capacities) and technologies (e.g., additive manufacturing) to engineer patient-specific

membranes and constructs to amplify both hard and soft tissue periodontal regeneration are presented below.

Antimicrobial

Infection is the foremost reason for clinical failure of periodontal regeneration. Therefore, it is extremely important to control and/or eradicate bacterial contamination of the periodontal defect.[104,105] A wide range of antimicrobials, including but not limited to tetracycline hydrochloride, metronidazole, and amoxicillin, have been incorporated into polymer membranes.[106–108] Furtos and colleagues reported on the synthesis of nanocomposite polycaprolactone-based membranes modified with amoxicillin and nano-hydroxyapatite to provide antimicrobial and osteoconductive properties, respectively.[109] Based on the well-known side effects, such as bacterial strain resistance, associated with the overuse of antibiotics, alternative agents, such as zinc oxide (ZnO) nanoparticles, have been proposed by our group.[110] Successful synthesis of PCL-based nanofibrous membranes using ZnO has been recently reported (Figure 5).[110] The antimicrobial action of cytocompatible ZnO-modified membranes was tested against *Porphyromonas gingivalis* (*Pg*) and *Fusobacterium nucleatum* (*Fn*). All membranes containing different concentrations of ZnO demonstrated significant antimicrobial action against the periodontopathogens tested.[110]

Calcium Phosphates and Bioactive Glass

A naturally occurring mineral form of calcium phosphate, hydroxyapatite (HAp), constitutes up to 70% of the dry weight of bone.[111] Scaffold processing techniques, such as co-electrospinning of HAp nanoparticles[112–118], have been used to produce composite membranes with improved strength and bioactivity.[119,120] Interestingly, a recent study combined electrospinning with melt-plotting to generate a hierarchical PCL/β-TCP scaffold embedded with collagen nanofibers.[121] Scanning electron micrographs of the constructs revealed uniform distribution of β-TCP particles in PCL struts and well-layered collagen nanofibers between composite struts. The combination of collagen nanofibers and β-TCP was found to provide synergistic effects related to cell activity.[121]

Over the past decade, Bioglass, another material with demonstrated properties related to bone formation, osteogenic differentiation, and activation of gene expression, has been used to modify periodontal membranes. For example, El-Fiqi and colleagues synthesized via electrospinning PCL-gelatin nanofibrous membranes modified with mesoporous bioglass (mBG) nanoparticles to provide the long-term delivery of dexamethasone.[122] PDLSCs showed increased proliferation and differentiation on these membranes. The mBG/PCL-gelatin membranes revealed excellent strength, elasticity, and hydrophilicity, when compared to their mBG-free counterparts. Dexamethasone was released in a linear fashion up to 28 days after a rapid initial burst (~30%) within the first 24 hours.[122]

Growth Factors

The local delivery of growth factors (e.g., BMP-2) has demonstrated enhanced periodontal healing and regeneration by modulating the cellular activity and providing stimuli to cells to differentiate and synthesize the ECM to develop new tissues.[21] The potent stimulatory effects of platelet-derived growth factor (PDGF-BB), a commercially available molecule for

periodontal regeneration, as a chemoattractant and mitogen, along with its ability to promote angiogenesis, were reported by Phipps and colleagues.[123] PDGF-BB was physically adsorbed to blended (PCL-Collagen I) nanofibers embedded with HAp nanoparticles. A sustained release of PDGF-BB was seen for 8 weeks in addition to enhanced mesenchymal stem cells (MSCs) chemotaxis.[123]

A recent strategy to promote bone regeneration relates to endogenous stem/progenitor cell recruitment/homing to the injury site by increasing local concentrations of cytokines and chemokines at the injured site. Stromal cell derived factor-1α (SDF-1α) is key in MSCs homing and localization within the bone marrow. Ji and colleagues reported on the synthesis of SDF-1α modified polymer membranes. The membranes were able to amplify chemotactic migration of MSCs. In vivo, after eight weeks, SDF-1α-loaded membranes led to a 6-fold increase in bone formation compared to SDF-1α-free counterparts.[124]

Multilayered Membranes and Multiphasic Patient-Specific Scaffolds—It has become evident that a multiphasic periodontal membrane/scaffold utilizing a tissue-specific structure with compositional and structural variation to recapitulate the structural organization or cellular and biochemical composition of native tissues is critical for periodontal regeneration. With this in mind, our group reported on the fabrication of a multilayered, tissue-specific biodegradable membrane with therapeutic properties (Fig. 6A–B).[125] The innovative membrane was designed and processed via sequential electrostatic spinning to present a core layer (CL) and two functional surface layers (SLs) that interface with hard and soft tissues. CL was engineered by spinning a poly(DL-lactide-co-ε-caprolactone) (PLCL) layer surrounded by two composite layers consisting of a gelatin/polymer blend. Hydroxyapatite nanoparticles were incorporated to enhance bone formation on the SL facing the bone defect and metronidazole (MET) was added to inhibit bacterial colonization on the SL facing the epithelial tissue (Fig. 6B). Worth noting, no delamination of the layered structure was observed upon mechanical loading, thus potentially guaranteeing adequate surgical handling and physiologic loading *in vivo*.[125] Taken together, the findings of this study demonstrated that sequential electrospinning can be used to fabricate tissue-specific multilayered membranes with desired physico-chemical, mechanical, and biological cues that could ultimately lead to enhanced periodontal regeneration.[125]

More recently, advances in tissue engineering and scaffold synthesis have permitted the development of mechanically-competent, tissue-specific, and multiphasic 3D scaffolds for periodontal regeneration, all of which have been addressed in exceptional review articles.[24–31,126] Ivanovski and colleagues[25] provided a comprehensive perspective regarding the association between scaffolds with cells and/or GFs to engineer 3D structures capable of influencing a spatiotemporal wound-healing cascade to encourage predictable regeneration. Notably, the ability to form highly complex 3D multiphasic scaffolds with tissue compartmentalization properties to encourage: (i) bone and periodontal attachment formation and integration, (ii) promotion of cementum formation onto the root surface, and (iii) the establishment of suitably oriented PDL fibers that attach to regenerated bone and cementum, have significantly advanced the field of periodontal tissue engineering. The design and fabrication of multiphasic scaffolds need to mimic the anatomy of the defect,

which, in turn, will permit cell delivery and neovascularization, while providing space for new tissue formation. Moreover, these constructs must also follow the general principles of the scaffolds' design, namely: (1) biocompatibility and degradability with a tunable degradation rate to complement cell and tissue growth and proper maturation; (2) a highly porous 3D framework with surface properties that enhance cell attachment, migration, proliferation and differentiation; and (3) an open interconnected structure that allows for the flow transport of nutrients and metabolic waste.[24,25]

Remarkably, Giannobile's group recently reported on the clinical findings of 3D-printed, bioresorbable (polycaprolactone, PCL), patient-specific scaffold and signaling growth factor to treat a large periodontal osseous defect due to generalized aggressive periodontitis (Fig. 6C–D).[26] Specifically, selective laser sintering was used to print a hydroxyapatite-containing PCL-based scaffold according to the anatomical configuration of the defect, as revealed by the patient's cone beam computed tomography (CBCT). The design consisted of perforations for fixation, an internal port for delivery of recombinant human PDGF-BB, and pegs oriented perpendicular to the root for PDL formation.[27] The adaptation ratio based on the methodology for PDL fiber guidance was defined based on micro–CT information. Prior to implantation, the scaffold was immersed in rhPDGF-BB, filled with autologous blood, and stabilized over the defect with resorbable pins. No clinical signs of chronic inflammation or rejection associated with the presence of the scaffold was seen during the first year. *In vitro* studies demonstrated a burst release of rhPDGF-BB within 180 minutes. The scaffold remained covered for 12 months, revealing a 3-mm gain of clinical attachment and partial root coverage. Unfortunately, scaffold exposure was noticed after one year (13 months) and, though palliative strategies were performed to save the treatment, the implanted material was removed (~ 76% of the molecular weight) for analyses. Although the success of this clinical study was modest, given that complete regeneration of the periodontium was not observed, it provided key information to drive the field forward, particularly regarding the aspects associated with scaffold design and fabrication.

SUMMARY

The unprecedented histological findings reported by Nygaard-Ostby[127,128], who demonstrated that periapical tissue laceration may lead to vascularized tissue formation within the root canal system and root maturation, have laid the groundwork for dental pulp tissue engineering. Over the past decade, in spite of significant advancement and amendments of the evoked bleeding technique, thanks to accumulating evidence regarding key aspects deemed to negatively affect clinical outcome (e.g., cytotoxic antibiotic pastes and sodium hypochlorite irrigation), only one report has shown pulp-like tissue formation. As a result, numerous research groups have been working intensively on tissue-engineering-based strategies for regenerative endodontics. A variety of scaffolds, associated or not with stem cells and GFs, have been explored. Based on current knowledge, a key aspect for clinical success refers to the development of a biocompatible disinfection approach. Our group has focused on the design and synthesis of 3D patient-specific cytocompatible antibiotic-containing nanofibers for intracanal drug delivery. *In vivo* preclinical (animal) studies are currently being conducted to validate these results. Nonetheless, the development of a regenerative strategy using advanced scaffolds, loaded or not with stem cells and/or

growth factors to stimulate pulp and dentin regeneration after attaining a bacteria-free niche, is warranted to establish novel therapeutics to treat teeth with necrotic pulp.

Regarding periodontal tissue engineering, regenerative strategies with membranes associated or not with grafting materials, have been used with distinct levels of clinical success. With the aging population, it is crucial to find a tissue-engineering/regenerative medicine approach that allows for the fabrication of scaffolds that can ultimately guide reliable and predictable regeneration of multiple periodontal tissues. Current evidence, including the results of the first 3D-printed patient-specific scaffold, suggests that both biologically-modified and multilayered tissue-specific scaffolds should be used. Although that case report was considered unsuccessful in the long-term, several issues were raised and will certainly help move the field forward. For example, it is well-known that vascularization of the scaffold/cell construct is an essential step in tissue healing, as this process provides the nutrients and oxygen needed for bone cells to survive, while facilitating removal of cell waste products. Therefore, a more open and interconnected porous structure might amplify bone regeneration and vascularization. Lastly, the compartmentalized delivery of biologics to the PDL-forming region of the scaffold, along with osteogenic molecules (e.g., bone morphogenetic proteins) to the bone region, may further facilitate tissue growth and remodeling.

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KEY POINTS

- No current therapy exists that promotes root canal disinfection and regeneration of the pulp-dentin complex in cases of pulp necrosis.
- Antibiotic pastes used to eradicate canal infection have been demonstrated to negatively impact stem cell survival.
- Three-dimensional easy-to-fit antibiotic-eluting nanofibers, combined with injectable scaffolds, enriched or not with stem cells and/or growth factors (GFs), may lead to an increased likelihood of achieving predictable dental pulp regeneration in humans.
- Periodontitis is an aggressive disease that impairs the integrity of tooth-supporting structures and may lead to tooth loss.
- The latest advances related to membranes' biomodification to endow needed functionalities (antimicrobial capacity) and technologies (additive manufacturing) to engineer patient-specific membranes/constructs to amplify both hard and soft tissue periodontal regeneration are presented.

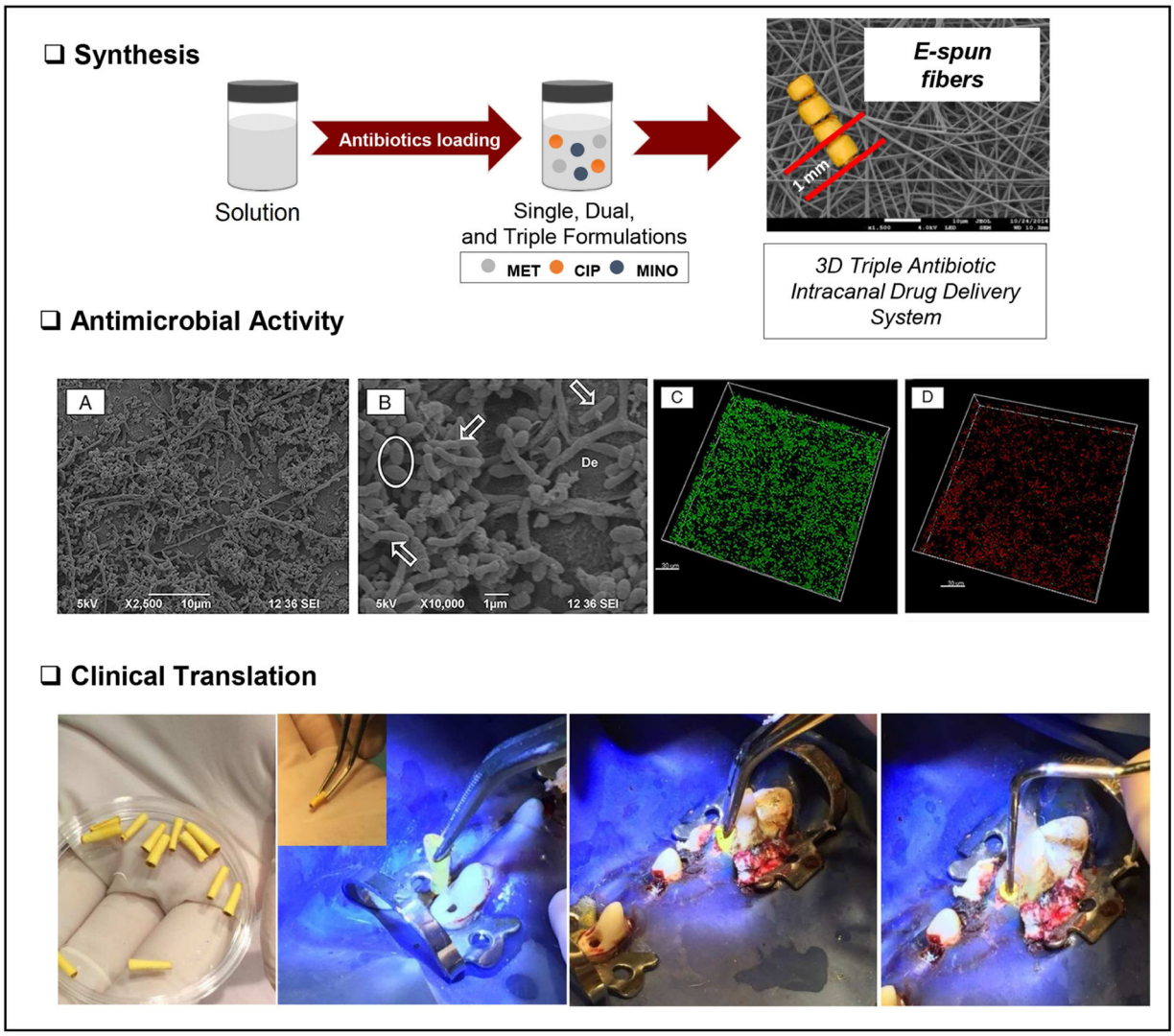

Fig. 1.

Synthesis of triple antibiotic-eluting nanofibers — Polymer solubilization in hexafluoro-2-propanol. Single, dual, or triple antibiotic incorporation (Metronidazole, Ciprofloxacin, and Minocycline) into the solution prior to electrospinning. Representative scanning electron micrograph (SEM) of triple antibiotic-containing nanofibers and 3D constructs (in yellow, superimposed to the SEM image). Antimicrobial activity of triple antibiotic nanofibers against a 7-day dual species (*A. naeslundii* and *E. faecalis*) biofilm formed on dentin specimens. **(A)** Lower-magnification SEM image showing a homogeneous distribution of the 2 bacterial cells. **(B)** Higher-magnification SEM image revealing the rod-shaped *A. naeslundii* and cocci-shaped *E. faecalis* bacterial cells over the dentin (De) surface. Confocal laser scanning micrographs of **(C)** 7-day dual-species biofilm growth inside dentinal tubules (live bacteria = green) and **(D)** triple antibiotic-containing nanofibers. Confocal images demonstrate the elimination of most of the bacteria (dead bacteria = red) by the formulated triple antibiotic nanofibers. Scale bars = 30 µm. Clinical translation — Placement of 3D

tubular-shaped triple antibiotic-eluting construct into the root canal of a periapical lesion dog model, to act as a localized intracanal drug delivery system.

Adapted from Pankajakshan D, Albuquerque MT, Evans JD, et al. Triple antibiotic polymer nanofibers for intracanal drug delivery: effects on dual species biofilm and cell function. J Endod 2016;42(10):1492; with permission.

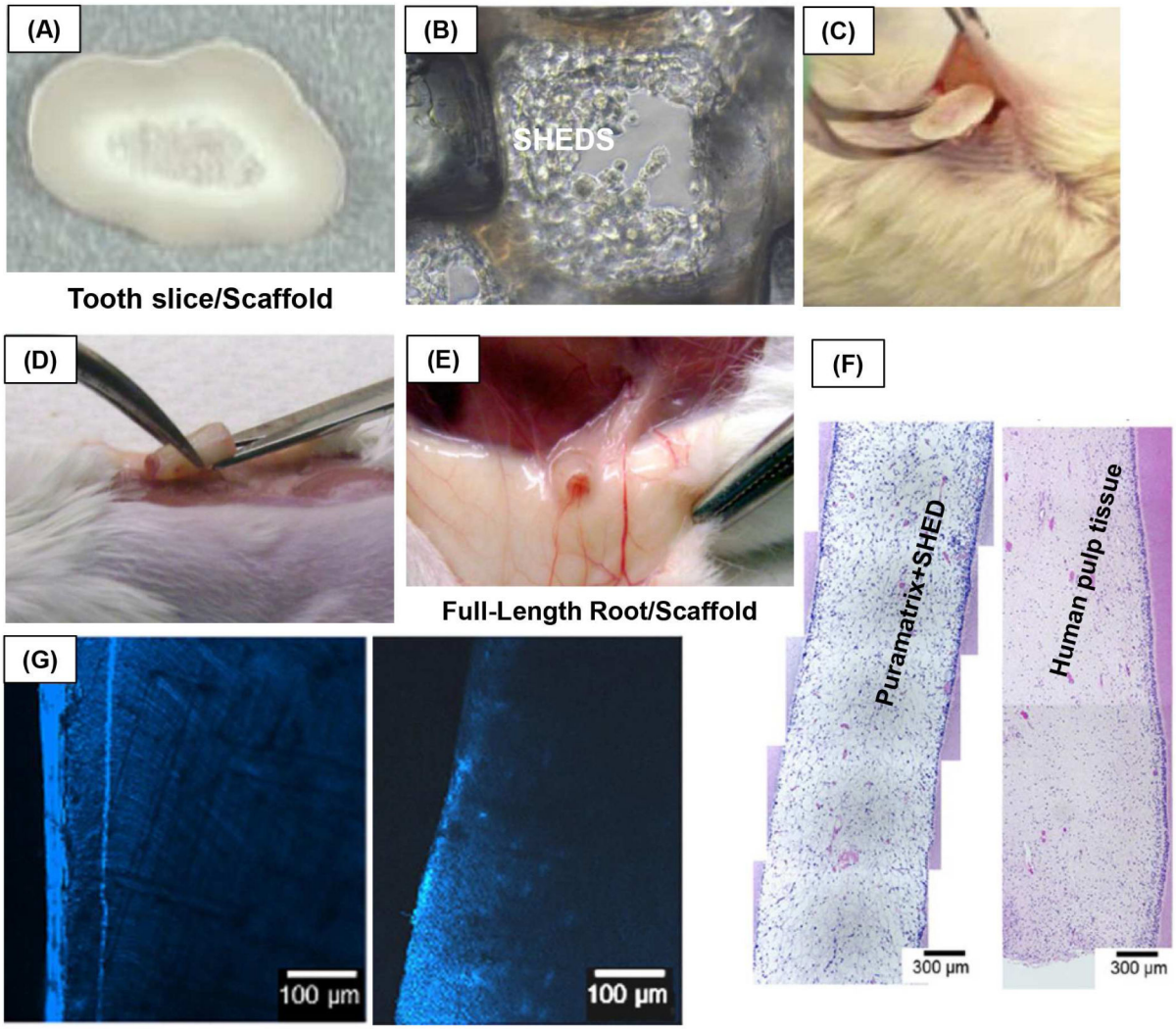

Fig. 2.

Summarized schematic of the tooth slice and full-length root/scaffold models. **(A)** Tooth slice provided from the cervical third of a human third molar with a highly porous PLLA scaffold placed within the pulp chamber. **(B)** SHED proliferation into the tooth slice/scaffold. **(C)** Insertion of a tooth slice and scaffold containing SHED into the subcutaneous space of the dorsum of an immunodeficient mouse. **(D)** Subcutaneous transplant of a human full-length root injected with hydrogel-based nanofibrous scaffolds containing SHEDs. **(E)** Photomicrographs of the engineered pulp-like tissue and human pulp tissue (control) in the root canal. **(F)** Layer of dentin formation after pulp tissue induction in PuraMatrix+SHEDs. **(G)** Dentin slice with no SHEDs.

From Albuquerque MT, Valera MC, Nakashima M, et al. Tissue-engineering-based strategies for regenerative endodontics. J Dent Res 2014;93(12):1227; with permission.

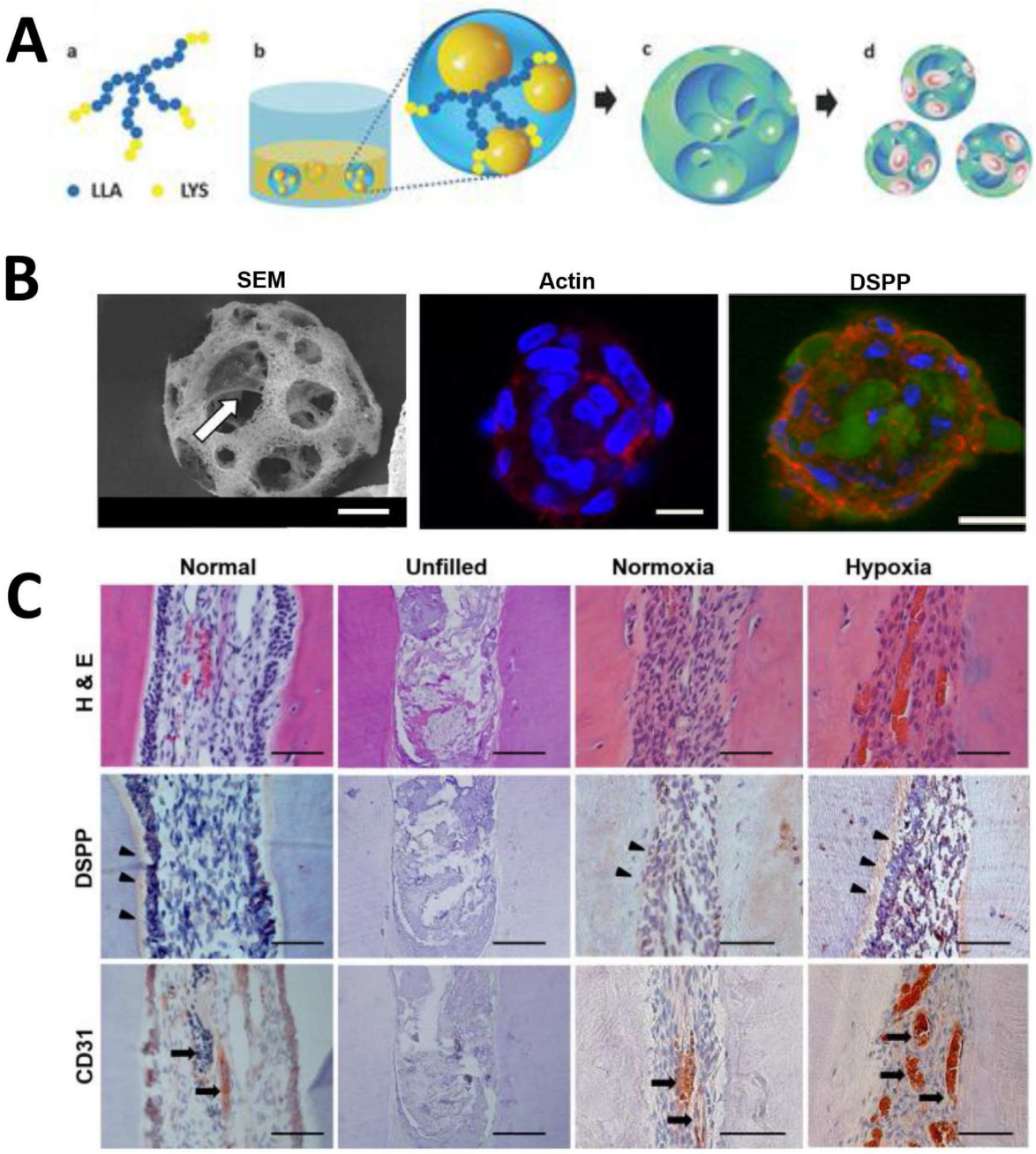

Fig. 3.

(A) Schematic illustration of the fabrication of NF-SMS for stem cell delivery through injection. (a) SS-PLLA-b-PLYS. (b) Emulsions self-assembled from SS-PLLA-b-PLYS, with one polymer solution droplet containing multiple glycerol domains. (c) NF-SMS were obtained after phase separation and freeze-drying. (d) The porous structure of NF-SMS allows efficient cell loading and delivery through injection. **(B)** Interactions of human dental pulp stem cells (hDPSCs) with the microspheres. SEM image of hDPSCs seeded on a nanofibrous spongy microsphere (NF-SMS) for 24 hr showing the attachment of cells on

both the surface and interior of the spheres, with abundant cellular processes. LSCM image of DPSCs seeded on NF-SMS for 24 hr, showing cells attached on the surface and inner pores of NF-SMS. DSPP immunofluorescence staining of hDPSCs on NF-SMS after odontogenic induction for 4 wk. Blue: nuclei; green: DSPP; red: F-actin. **(C)** Pulp tissue regeneration enhanced by hypoxia-primed hDPSCs/NF-SMS in maxillary first molar of immunodeficient rats. From left to right, the first column is the normal pulp, the second column is the unfilled pulp canal group, the third column is the normoxia group, and the last column is the hypoxia group. H&E staining showed that no pulp-like tissue was formed in the unfilled group while neo pulp-like tissue formed in the normoxia and hypoxia groups. DSPP IHC staining was positive in the hypoxia group and the normal pulp group at the dentin–pulp interface (black triangles pointing to the dentin–pulp interface). CD31 staining showed more blood vessels in the hypoxia group than in the normoxia group (marked with black arrows).

Figure 3A and 3B: *Adapted from* Kuang R, Zhang Z, Jin X, et al. Nanofibrous Spongy Microspheres Enhance Odontogenic Differentiation of Human Dental Pulp Stem Cells. Adv Healthc Mater 2015;4(13):1993–2000; with permission.

Figure 3C: *From* Kuang R, Zhang Z, Jin X, et al. Nanofibrous spongy microspheres for the delivery of hypoxia-primed human dental pulp stem cells to regenerate vascularized dental pulp. Acta Biomater 2016;33:232; with permission.

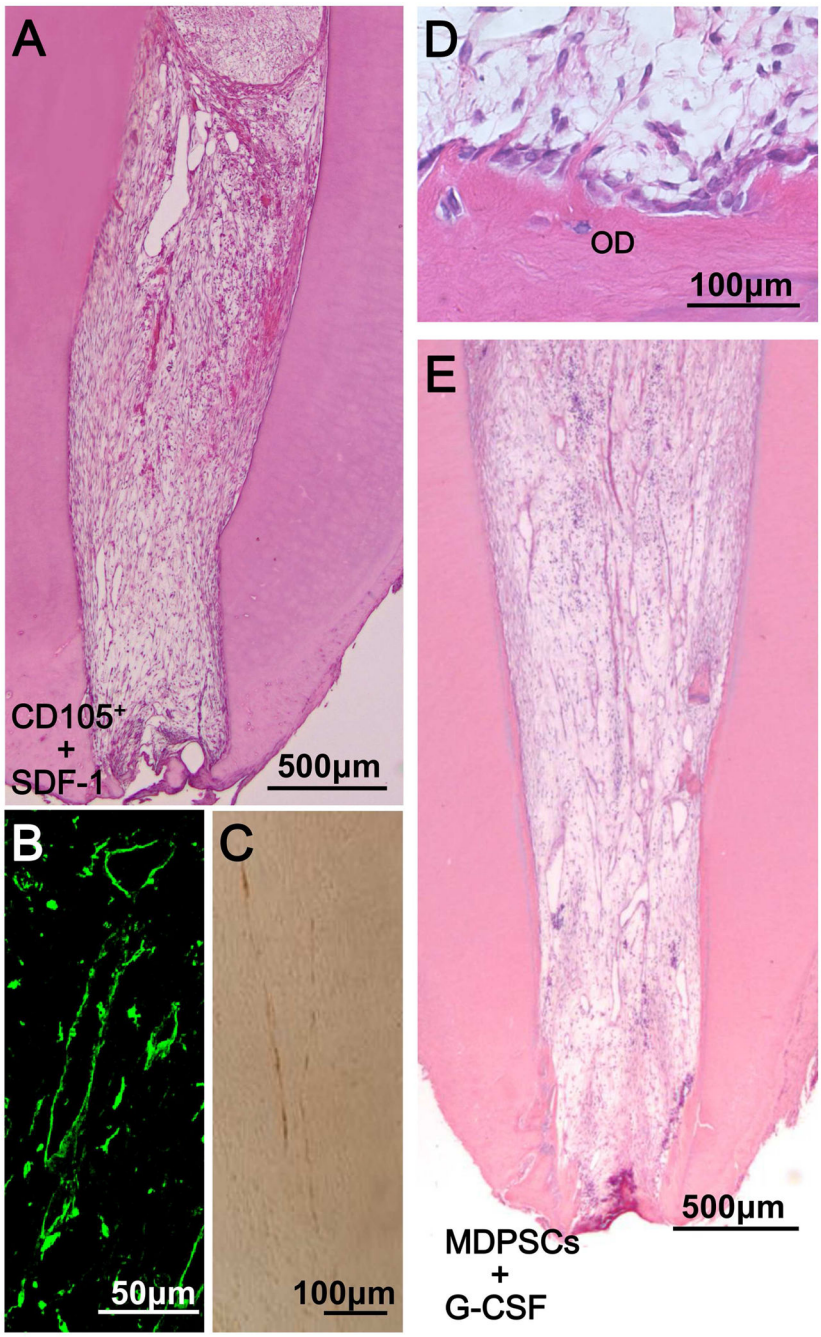

Fig. 4.

Clinical evidence of dentin-pulp complex regeneration. **(A–E)** Complete regeneration of pulp tissue after autologous transplantation of CD105+ cells with SDF-1 in the pulpectomized root canal in dogs. **(B)** Immunostaining with BS-1 lectin. **(C)** Immunostaining with PGP 9.5. **(D)** Odontoblastic cell lining to newly formed osteodentin/tubular dentin (OD), along with the dentinal wall. **(E)** Complete regeneration of pulp tissue after autologous transplantation of mobilized dental pulp stem cells (MDPSCs) with G-CSF in the pulpectomized root canal in dogs.

From Albuquerque MT, Valera MC, Nakashima M, et al. Tissue-engineering-based strategies for regenerative endodontics. J Dent Res 2014;93(12):1228; with permission.

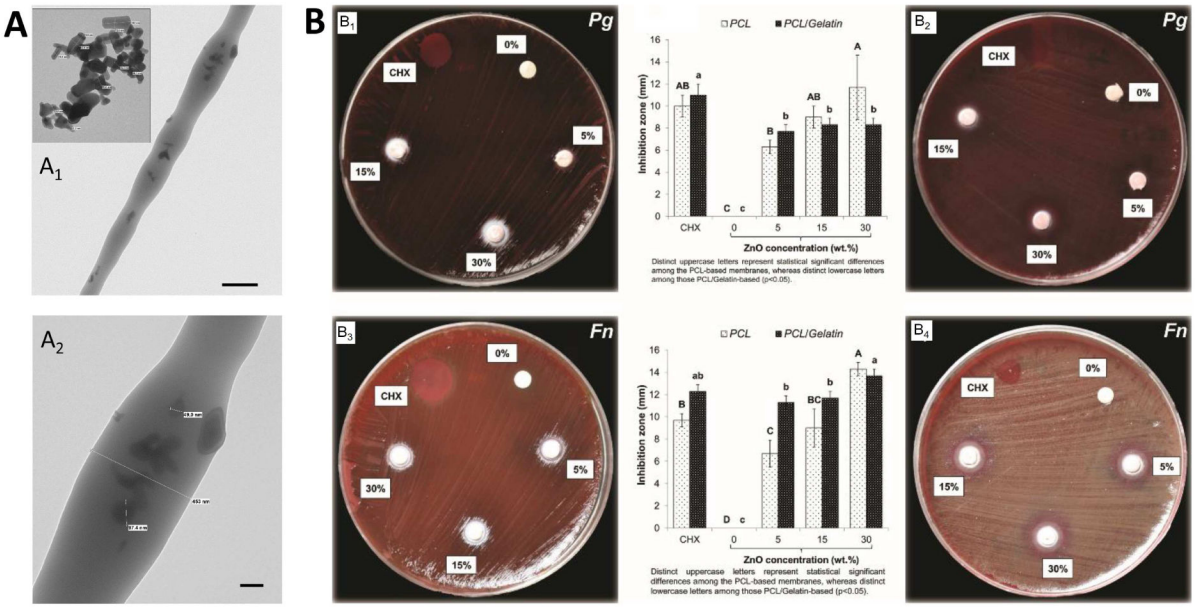

Fig. 5.

Antimicrobial properties of ZnO–PCL nanofibers. **(A_1-Inset)** Transmission electron micrographs (TEM) showing the overall morphology and size distribution of the ZnO nanoparticles. (A_1–A_2) TEM images showing ZnO nanoparticles within the neat PCL fibers at different magnifications. **(B)** Macrophotographs of representative agar plates and data of the antibacterial activity obtained with the positive control (chlorhexidine, CHX) and the PCL- and PCL/GEL-based membranes containing different concentrations of ZnO. Images (B_1) and (B_2) show the results against *P. gingivalis* and against *F. nucleatum* (B_3 and B_4). *Adapted from* Münchow EA, Albuquerque MTP, Zero B, et al. Development and characterization of novel ZnO-loaded electrospun membranes for periodontal regeneration. Dent Mater 2015;31(9):1038–1051; with permission.

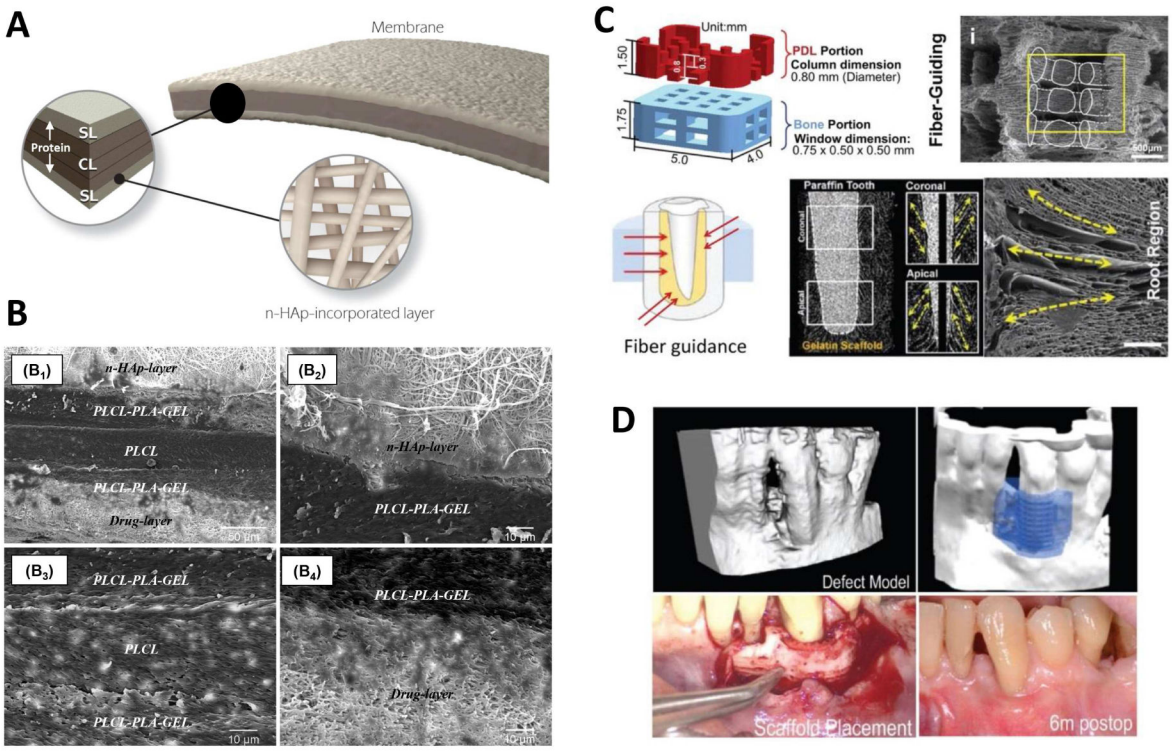

Fig. 6.

Multilayered/Multiphasic scaffolds for periodontal regeneration. **(A)** Schematic illustration of the multilayered periodontal membrane processed via multilayered electrospinning showing the details of the core-layer (CL) and the functional surface-layers (SL). **(B)** Cross-section SEM micrographs of the multilayered membrane. (B_1) General view of the FGM, (B_2) interface n-HAp-containing layer/PLCL: PLA: GEL, (B_3) core-layer structure (CL), and (B_4) interface MET-loaded layer/PLCL: PLA: GEL. (**C**) Design of a customized scaffold using 3D printing. Scaffold design consists of a periodontal ligament (PDL) portion and a bone portion. This was further modified to improve the fiber-guiding potential as well as the direction of the PDL to mimic the topography of the different kinds of fibers in the PDL (first row). Digitalized cross-sectional view of a 3D-reconstructed image. Longitudinal cross-section image showed pore morphologies at coronal and apical portions. Scanning electron microscopy image showing longitudinal pores produced by a freeze-casting method. (**D**) 3D printing using polycaprolactone was made to fit the periosseous defect based on the patient's cone beam computed tomography scan.

Figure 6A and 6B: *Adapted from* Bottino MC, Thomas V, Janowski GM. A novel spatially designed and functionally graded electrospun membrane for periodontal regeneration. Acta Biomater 2011;7(1):216–224; with permission.

Figure 6C and 6D: *From* Larsson L, Decker AM, Nibali L, et al. Regenerative Medicine for Periodontal and Peri-implant Diseases. J Dent Res 2016;95(3):262; with permission.