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Tissue Engineered Constructs for Periodontal Regeneration: Current Status and Future Perspectives

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The periodontium, consisting of gingiva, periodontal ligament, cementum, and alveolar bone, is a hierarchically organized tissue whose primary role is to provide physical and mechanical support to the teeth. Severe cases of periodontitis, an inflammatory condition initiated by an oral bacterial biofilm, can lead to significant destruction of soft and hard tissues of the periodontium and result in compromised dental function and aesthetics. Although current treatment approaches can limit the progression of the disease by controlling the inflammatory aspect, complete periodontal regeneration cannot be predictably achieved. Various tissue engineering approaches are investigated for their ability to control the critical temporo-spatial wound healing events that are essential for achieving periodontal regeneration. This paper reviews recent progress in the field of periodontal tissue engineering with an emphasis on advanced 3D multiphasic tissue engineering constructs (TECs) and provides a critical analysis of their regenerative potential and limitations. The review also elaborates on the future of periodontal tissue engineering, including scaffold customization for individual periodontal defects, TEC's functionalization strategies for imparting enhanced bioactivity, periodontal ligament fiber guidance, and the utilization of chair-side regenerative solutions that can facilitate clinical translation.

1. Introduction

The periodontium is a highly hierarchical organ consisting of intercalated soft (gingival and periodontal ligament) and hard (cementum and alveolar bone) tissues that mechanically support the teeth and play an important role in transmitting the mechanical forces experienced during mastication. Periodontitis is a chronic inflammatory disease initiated by an oral bacterial biofilm, which results in periodontal hard and soft tissues destruction and can lead to tooth loss. It affects 30–40% of the population[1] and the large impact and burden of this disease on individuals and the community is well recognized not only in terms of compromised quality of life, but also overall health and systemic well-being.[2]

1.1. The Unique Challenges Faced in Achieving Periodontal Regeneration

The ultimate objective of periodontal treatment is regeneration of the lost tissues of the periodontium, which involves the functional reattachment of the periodontal ligament onto newly formed cementum and alveolar bone. This requires a highly coordinated spatiotemporal healing response, including cementogenesis concomitant with periodontal ligament fiber reattachment to the previously contaminated root surface, as well as bone formation within the periodontal defect (**Figure 1**). In addition to the challenges posed by the complex architecture of the periodontium, healing is further complicated by the avascular nature of the tooth surface, which means that all periodontal wound healing occurs by secondary intention. Furthermore,

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healing is additionally compromised by the presence of intraoral bacteria at the nonshedding tooth surface, which can negatively affect the wound healing process following periodontal treatment.

1.2. Historical Perspective of Periodontal Regeneration Approaches

In the 1970s, the concept involving the utilization of a physical barrier for preventing epithelial downgrowth along the tooth root surface following the surgical treatment of periodontitis was in its genesis (**Figure 2**). Indeed, clinicians had previously hypothesized and conceptualized that the collapse of gingival tissues into the periodontal defects significantly impeded bone regeneration and periodontal reattachment.[3,4] Some early studies proposed the placement of a harvested free palatal graft over the periodontal defect in an attempt to hinder or at least delay the downgrowth of epithelial cells along the tooth root surface.[5] Another commonly utilized approach involved the placement of bone fillers (autologous, allogenic, or synthetic) within the periodontal defect in order to regenerate the lost bone.[6] However, none of these approaches resulted in the new attachment formation required for periodontal regeneration, and only periodontal repair was observed in the form of a long junctional epithelium. Retrospectively, it can be concluded from the large experimental and clinical data sets that the lack of compartmentalization between the periodontal defect and the surrounding soft tissue was responsible for the poor regenerative outcomes.

The issue of selective periodontal defect repopulation by tissues capable of promoting periodontal regeneration was addressed in a series of seminal papers by Nyman et al.,

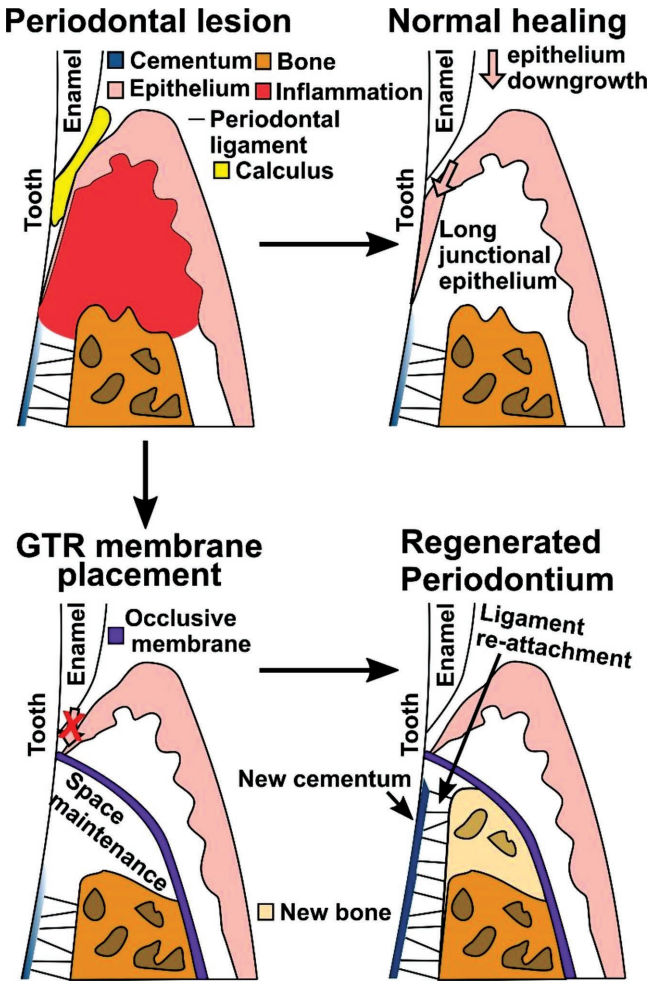

Figure 1. Schematic representation of the biological events leading to either periodontal healing or periodontal regeneration in the presence of a GTR membrane involving concomitant deposition of new cementum, bone regeneration, and periodontal reattachment for functional regeneration.

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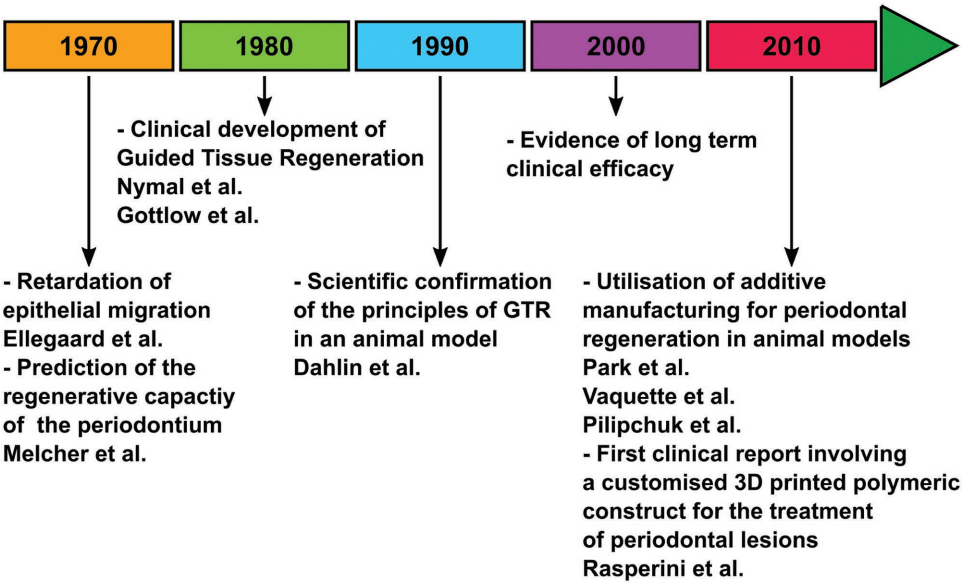

Figure 2. Historical timeline of the development of barrier/membrane and scaffold-based periodontal regeneration approaches aimed to exert temporo-spatial coordination of the periodontal wound healing process: from the initial concept involving a free palatal graft for impeding epithelial migration, via the clinical implementation of the GTR concept utilizing occlusive membranes, to the most recent clinical advancements involving additively manufactured polymeric multiphasic scaffolds for periodontal tissue engineering.

which led to the establishment of the concept of guided tissue regeneration (GTR). This elegant approach involves the use of a synthetic membrane to exclude undesirable tissues (gingival epithelium and connective tissue) from the healing periodontal wound, while selectively promoting the in-growth of the periodontal ligament and bone tissues required for achieving periodontal regeneration[7] (as depicted in Figure 1). Using GTR, Nyman et al. histologically demonstrated the formation of new functional attachment of periodontal ligament fibers into newly formed cementum on the root surface of a human mandibular incisor.[7a] The effectiveness of this clinical technique was further confirmed several years later by Gottlow et al., who successfully applied it to a larger cohort of patients.[8] Subsequently, the scientific and biological rationale of GTR have been validated over the last three decades in many publications,[9] and efficacy of the technique has been demonstrated in a plethora of clinical studies[10] and reported in systematic reviews.[11] As a result, the pioneering work with the subsequent large-scale clinical translation has profoundly shaped the future developments in periodontal regeneration. Indeed, the principles of GTR, namely wound stabilization, space maintenance, and selective cell repopulation, often neglected by so-called tissue engineers, yet remain key design considerations in any tissue engineering approaches aimed at enhancing periodontal regeneration.

1.3. Challenges in GTR and Periodontal Healing

GTR became a standard surgical treatment for periodontal regeneration; however, despite histologically verified regeneration and positive outcomes in selected clinical scenarios, there still exists a significant variability in the clinical efficacy of the technique.[11] Indeed, GTR remains unpredictable and clinical outcomes vary depending on the nature of the periodontal defect and the skills and experience of the practitioner.[9] The challenging environment of the oral cavity is at least partly responsible for the poor regeneration observed in GTR procedures; indeed, it is an "open" wound adjacent to an avascular surface (the tooth), which is subject to microbial challenge from the resident intraoral microbial biofilm.[12] In addition, the periodontium has a very specific and highly hierarchal anatomy, and as such, requires well-coordinated healing sequences in order to achieve complete regeneration via the restoration of the complex periodontal architecture. These challenges are relatively poorly addressed in GTR procedures, as autologous progenitor cells may repopulate the periodontal defect at random time intervals and locations without a specific and temporal course of events whereby new cementum, ligament, and bone are formed in sequential and overlapping phases. As a result, complete regeneration, consisting of concomitant 1) new cementum deposition, 2) periodontal ligament (PDL) attachment with insertion of Sharpey's fibers into the cementum, and 3) bone formation,[4] is rarely and unpredictably achieved.[9] Scar tissue is often formed in lieu of a functional periodontal ligament which does not resemble the structure, nor recapitulate the function, of the native tissue.

It is clear that periodontal regeneration has unique challenges from both a biological (spatiotemporal healing coordination, competition between the tissues, and nonvascular tooth surface preventing primary closure) and from a clinical point of view (microbial accumulation on nonshedding tooth surface, technically challenging surgical environment due to limited access and small operating field). Hence, a tissue engineering approach, utilizing advanced 3D scaffold architectures, combined with bioactive molecules, drugs, gene therapy, and/or cell delivery, that have the ability to guide and coordinate the

healing process, is scientifically sound and has the potential to circumvent many limitations associated with current clinical practice.[13] These tissue engineering constructs (TECs) should fulfill the requirements of GTR (space maintenance, wound stabilization, and selective cell repopulation) while enabling spatiotemporal control of the periodontal wound healing process. Such tissue engineered TECs should not only be made of a biodegradable biomaterial with proven clinical biocompatibility, yet also highly porous with an interconnected porosity for allowing the formation of a stable fibrin network within the blood clot and subsequently enabling cell infiltration and finally tissue formation with a well-developed vascular network.

Importantly, the degradation kinetics of the scaffold should be tailored, based on the timeframe required for the preservation of space maintenance properties until a dimensionally stable new tissue has formed.

This review reports on the recent advancements in tissue engineering strategies for periodontal regeneration, with a strong and critical focus on 3D multiphasic constructs incorporating compartmentalized designs for achieving the aforementioned spatiotemporal coordination of the periodontal wound healing events.

2. Monophasic Scaffolds

The development of scaffolds for periodontal regeneration was inspired by current clinical practice (namely GTR) whereby a biomaterial is utilized in order to maintain space[14] and promote new periodontal tissue formation within the defect. Therefore, a variety of monophasic TECs have been developed which followed the principles of GTR. Additionally, bioactivity is imparted to these TECs through the use of either inorganic fillers and/or biological adjuvants for promoting bone and/or ligament formation.

2.1. Monophasic Scaffolds for Cell Delivery

An obvious technique for increasing the regenerative potential of biomaterial constructs is the introduction of cells from various sources. This approach is well documented in the literature whereby cells are either encapsulated in various hydrogel systems or seeded into scaffolds and then transplanted into the periodontal defect. In addition to the capacity of the monophasic scaffold to maintain space, it also acts as a carrier for delivering cells within the periodontal defect. While the concept may appear to be relatively simple, its implementation has resulted in variable outcomes depending on the scaffold biomaterial and the type of cells used.

Several recently published studies have utilized rapidly resorbing materials in either preclinical[15] or clinical studies.[16] A notable clinical example combined osteogenically induced bone marrow-derived mesenchymal stem cells with a platelet-rich plasma (PRP) preparation which was infused into a poly-L-lactic acid knitted mesh (**Figure 3**A).[16] This malleable, cellularized TECs was thereafter implanted into periodontal defects of ten patients and resulted in some improvements in clinical outcomes, such as clinical attachment level, pocket depth, and linear bone growth (Figure 3A).

Conceptually, for the successful regeneration of complex organs such as the periodontium which incorporate a hard tissue component, a mature bony tissue should be formed

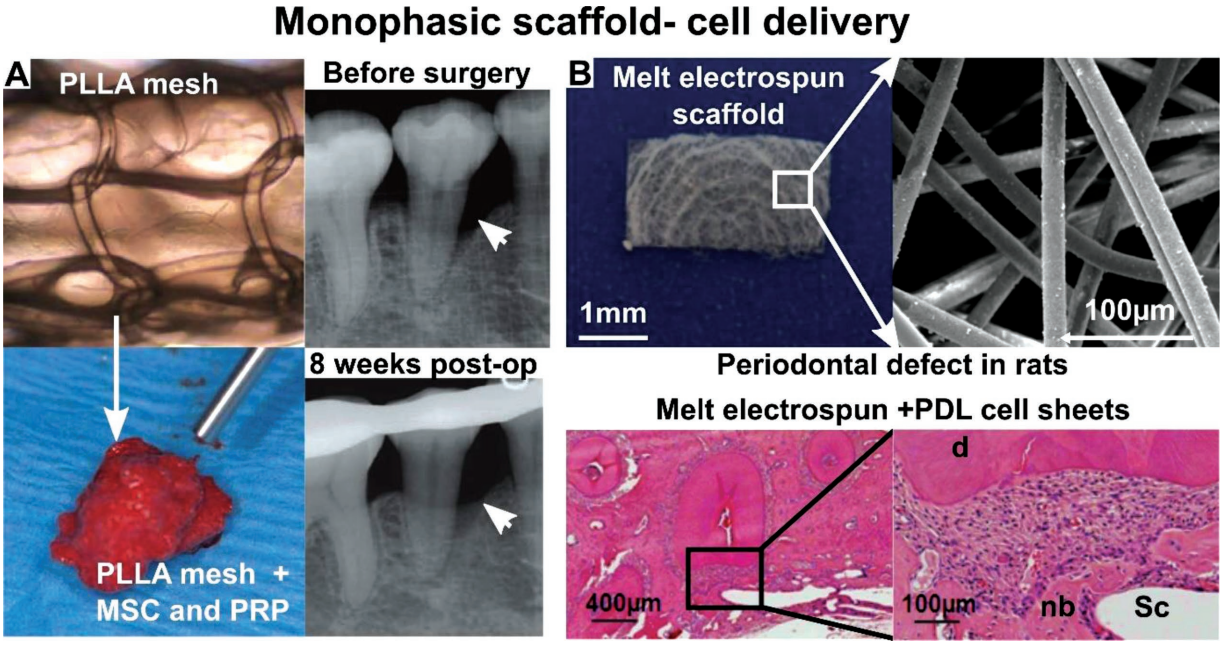

Figure 3. Monophasic scaffolds for cell delivery: A) utilization of a PLLA mesh loaded with bone marrow mesenchymal stem cells in a clinical trial involving ten patients, resulting in significant improvements of clinical parameters such as increased bone height (as indicated by the white arrows). Reproduced with permission under the terms of the CC-BY 4.0 license.[16] Copyright 2016, the Authors. Published by Hindawi Publishing Corporation. B) Melt electrospun scaffold used for the support and delivery of trilayered cell sheets in a rat periodontal defect. The macroporous structure of the electrospun scaffold enabled bone ingrowth and integration with the newly formed periodontal ligament (nb: new bone; Sc: scaffold; d: dentin). Reproduced with permission.[17] Copyright 2014, Elsevier.

before the complete degradation of the biomaterial so that long-term dimensional stability of the regenerated tissue is achieved. Therefore, a slowly degrading material is preferable. This can be achieved by the utilization of a medical grade polycaprolactone melt electrospun membrane in combination with mature cell sheets of various tissue origins, as Dan et al. recently demonstrated in a surgically created rodent periodontal defect.[17] Indeed, the calcium phosphate–coated melt electrospun scaffold, consisting of 20 µm diameter microfibers organized in a macroporous network (Figure 3B), enabled the placement of cell sheets in intimate contact with the root surface while allowing for the space maintenance necessary for bone ingrowth and periodontal ligament regeneration (Figure 3B).[17]

It should be noted that cell-based periodontal therapies have limitations associated with considerable regulatory barriers related to cell source and harvesting, culture, etc.; however, the development of advanced scaffolds made of synthetic biomaterials and loaded with drugs or growth factors has the potential to provide interesting solutions toward clinical translation without a cellular component.

2.2. Monophasic Scaffolds for Growth Factor Delivery

Synthetic polymers with extended biodegradation profiles and enhanced mechanical properties have been utilized for the delivery of growth factors. Although the incorporation of biological cues directly into the bulk of the scaffold is challenging due to material processing requirements (high temperature and strong organic solvent potentially leading to denaturation of biological components), the strategy of using microspheres as the delivery vehicle has found widespread use in tissue engineering applications, including periodontal regeneration. Such an approach was implemented for the dual and sequential delivery of platelet-derived growth factor (PDGF) and simvastatin.[18] This was achieved by developing double-wall microspheres manufactured by coaxial electrospraying of poly-(D,L-lactide-*co*-glycolide) and poly-(D,L-lactide) solutions, incorporating simvastatin (used as a differentiation factor) in the core and PDGF in the shell (**Figure 4**A). This technique allowed for better control over the release profile, allowing more sustained delivery when compared to simpler biomaterial systems such as collagen gels, whereby a rapid release generally occurs within the first hours. The microspheres displayed a sustained release of PDGF over 14 days while only half of the simvastatin was released over that time period.[19] Subsequently, the regenerative performance of the microspheres was tested in a rat periodontal defect model, and this demonstrated the beneficial influence of the dual release system on osteogenesis (Figure 4B), as well as cementogenesis and the formation of perpendicularly inserting newly formed ligament fibers[18] (Figure 4C).

Another approach utilized PLGA microspheres loaded with growth factors, which were incorporated into polycaprolactone and 3D printed using a melt extrusion printer[20] (Figure 4D–H). The embedding of the microspheres into the additively manufactured TECs drastically modified the release profile of the encapsulated drugs (BMP-2, BMP-7, and connective tissue growth factor (CTGF)), which reached only 50% release after 42 days (Figure 4F). An ex vivo model was utilized in order to investigate the impact of the growth factors onto cementum and periodontal ligament formation. This involved the culture of CD146 positive periodontal ligament

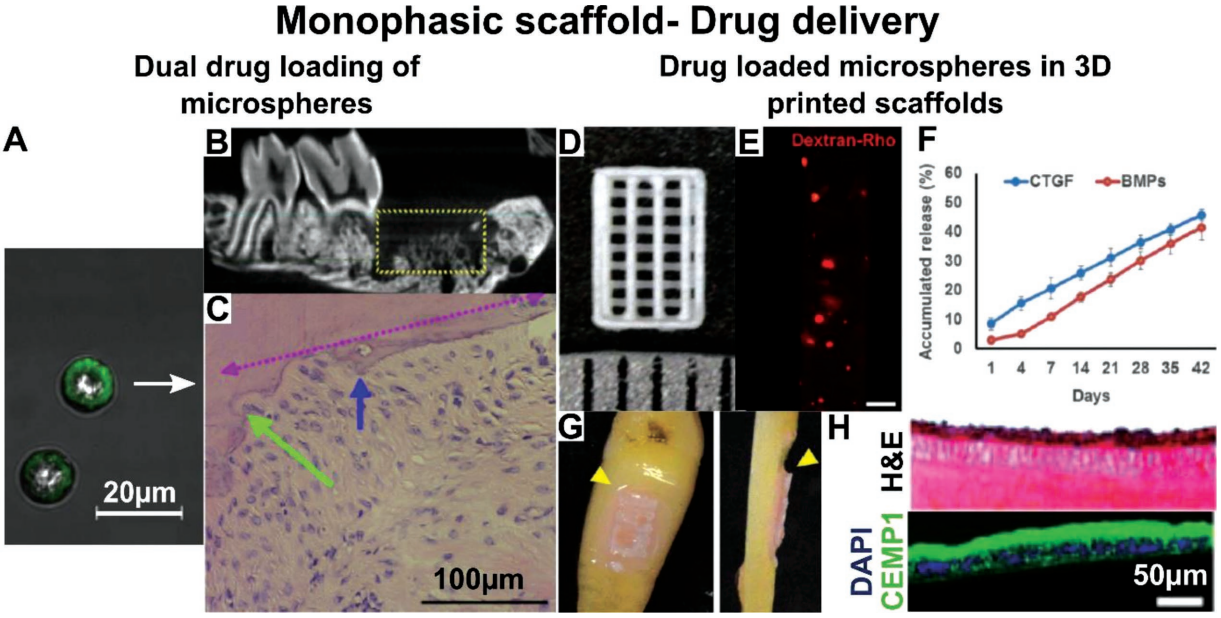

Figure 4. Monophasic synthetic scaffolds for drug delivery. A) Morphology of microspheres utilized for the dual delivery of bioactive molecules. B) Bone formation induced by the combined delivery of PDGF and simvastatin in three-wall periodontal defects in rats. C) Histology of the regenerated periodontium demonstrating evidence of cementogenesis, and formation of perpendicularly oriented periodontal ligament fibers. Reproduced with permission.[18] Copyright 2013, Elsevier. D–H) Growth factor–loaded microspheres incorporated into monophasic 3D-printed scaffold. (D) Morphology of the 3D-printed scaffold. (E) Distribution of the microspheres within the printed polymer filament. (F) Sustained release of several growth factors. (G) Ex vivo model for cementogenesis assessment. (H) Deposition of a mineralized tissue CEMP1 positive in the presence of BMP-7. Reproduced with permission.[20] Copyright 2016, Taylor & Francis.

cells over 6 weeks in the constructs which were placed in direct contact with a human dentin slice (Figure 4G). Three different growth factors were tested (CTGF, BMP-2, and BMP-7), demonstrating that although the BMPs induced mineralization at the dentin interface, only BMP-7 resulted in deposition of the cementum specific marker CEMP-1 within the mineralized layer (Figure 4H). In the context of periodontal regeneration, cementum formation is an essential requirement for new periodontal attachment formation and, as such, should occur during the early phase of the wound healing process. Therefore, a significantly delayed release of biological cues, as shown in this study, could result in cementogenesis only in the later phases of wound healing, which could compromise the insertion and attachment of the ligament fibers onto the root surface. Another potential concern is that the sustained release of biological cues that promotes hard tissue formation could result in a direct union of bone with the tooth surface without an intervening periodontal ligament, which is an undesirable clinical phenomenon known as ankylosis.

As shown here, previous research has focused on the delivery of biochemical cues targeted toward soft and/or hard tissue regeneration. However, another important aspect to consider in periodontal healing is to address the bacterial challenge that originates from the exposure of the wound to intraoral fluids. A recent study by our group has proposed the utilization of an antibiotic loaded membrane manufactured by electrospinning.[21] In this approach, the antimicrobial and immunomodulatory drug azithromycin was deposited over the surface of the electrospun fibers, resulting in a sustained release over several weeks. The loaded membranes were tested in a rat calvarial defect model, and it was demonstrated that the immunomodulatory properties of the azithromycin loaded membrane led to enhanced bone formation. Hence, this strategy could potentially both enhance tissue regeneration and protect from bacterial contamination.

2.3. Monophasic Scaffolds Loaded with Bioactive Natural Materials

Additive manufacturing is a well-suited method for the preparation of scaffolds with controlled internal porous architecture, as well as excellent biomechanical and space maintenance properties. Nevertheless, most synthetic polymers have poor bioactivity, and hence apart from the physical properties that they impart to the TECs (i.e., space maintenance), they do not contribute actively in the initiation of the regenerative processes. Hence, the incorporation of a biopolymer into these polymeric constructs has been proposed in order to create a composite scaffold with enhanced bioactivity. Such a strategy was explored by Puppi et al. whereby a highly porous hydroxyapatite-polycaprolactone scaffold was manufactured by coupling wet spinning and 3D printing prior to infiltrating a mixture of chitosan and poly(γ-glutamic acid) within the porous network of the PCL construct.[22] The composite scaffold was subsequently freeze-dried and this resulted in the excellent integration of the two components at the macro- and microscopic scale. In addition, the presence of chitosan within the composite scaffold imparted antimicrobial properties, which were assessed on both Gram-positive and Gram-negative bacteria, and it was demonstrated that the initial rate of bacterial growth within the first 8 h was drastically reduced. Although the scaffold was not tested in vivo, the antimicrobial effect imparted by the chitosan may enhance periodontal regeneration by preventing bacterial contamination of the construct after implantation. However, the stiffness of the 3D-printed composite could result in a low adaptability to the root surface in the clinical setting, as well as contributing to a rigidity mismatch with the overlying soft tissues that can lead to gingival perforation.

Another interesting approach involved the utilization of acemannan, a natural polysaccharide extracted from aloe vera.[23] This natural product was also manufactured by freeze-drying in order to create a sponge (**Figure 5**A), which was subsequently tested in class II furcation defects in dogs. This approach demonstrated enhanced periodontal regeneration at 30 and 60 days postimplantation with extensive new cementum deposition (up to 80% of the root surface), increased periodontal attachment and nearly full bone height recovery (Figure 5B). This study provided an interesting insight into the utilization of herbal-derived products for the regeneration of the periodontal complex. Although the exact mechanisms resulting in the positive influence of accecaman are not known, the authors reported excellent blood clot retention of the sponge, which was speculated to enhance wound stability and growth factor retention within the wound. Although the resorption of the sponge was not systematically investigated, the in vivo study revealed rapid degradation of the polysaccharide as no remnants of the material were observed 30 days postimplantation. The loss of the space-maintaining properties could compromise the healing

Figure 5. Monophasic scaffold incorporating natural material extracted from plants. A) Accecaman, a polysaccharide extracted from aloe Vera, is processed into a porous sponge. B) Regenerative outcome of the sponge in a class II furcation defect in dog, demonstrating the formation of new cementum (black arrowhead), bone formation (NB), and ligament attachment. Reproduced with permission.[23] Copyright 2014, John Wiley and Sons (PB denotes the preexisting bone).

in more challenging, less contained defects and therefore could be an impediment toward successful clinical translation.

The regeneration of the periodontium requires a synchronized wound healing response which may not be achieved by the utilization of simple monophasic scaffold architectures. Indeed, the hierarchical structure of the native tissue requires the regeneration of cementum, ligament, and alveolar bone, while monophasic scaffolds generally exert control over the formation of a single tissue component, which is usually bone. The lack of integrated and coordinated regeneration events of the different tissues can be circumvented by scaffold compartmentalization, capable of managing the reconstruction of soft and hard tissue, and most importantly, their interface.

3. Multiphasic Scaffolds

The complex architecture of the periodontium requires a highly coordinated regeneration process, whereby each individual component (cementum, ligament, and alveolar bone) regenerates according to specific, sometimes overlapping, spatiotemporal sequences. Therefore, the utilization of multiphasic constructs, recapitulating the native tissue architecture, has recently been advocated as the way forward for periodontal regeneration.[13a] A multiphasic TEC is generally defined as possessing multiple distinguishable compartments of different architectural nature (pore size, pore shape, porosity, etc.) and/or biochemical composition. Multiphasic scaffolds are particularly suited for tissue engineering of complex structures such as soft–hard tissue interfaces, and in the context of periodontal regeneration, they can be utilized to mimic the organization of the periodontium complex (alveolar bone, PDL, and cementum). As such, multiphasic scaffolds for periodontal regeneration strongly focus on functional biomimicry to address the challenges posed by the soft–hard tissue interfaces between alveolar bone–ligament and ligament–cementum. This approach is aimed at facilitating functional regeneration and integration of the various periodontal components, and more importantly of the newly formed tissues at the interfaces aforementioned. In other words, these constructs are required to allow for spatially compartmentalized bone, periodontal ligament, and cementum formation, while facilitating, via their architecture/biochemical composition at the interfaces, the integration of the ligament into both bone and cementum. In addition, multiphasic scaffolds must facilitate selective cell repopulation and wound stabilization, as well as having appropriate space maintenance properties to facilitate the formation of functional tissues that are dimensionally stable over time.

Although scaffold compartmentalization appears to be a well-suited strategy for periodontal tissue engineering, there are a very few studies that have explored this approach. These studies can be divided into those that have utilized either biphasic or triphasic TECs.

3.1. Biphasic Scaffolds

Carlos-Reis et al. developed a biphasic scaffold directly inspired from the requirements of GTR.[24] In this strategy, an osteoconductive polylactide-*co*-glycolic (PLGA)–calcium phosphate (CaP) composite foam (bone compartment) was utilized in conjunction with an occlusive PLGA/CaP membrane. The PLGA/CaP foam was fabricated by dispersing the CaP particles into a PLGA solution prior to performing solvent casting into a sugar template which was subsequently leached out in water. The incorporation of the PLGA membrane was achieved by placing the resulting foam onto a CaP/PLGA solution immediately prior to complete solvent evaporation, ensuring a strong cohesion between the various components. This biphasic scaffold was tested using a canine class II furcation defect with the foam facing the tooth root and the membrane providing cell and tissue occlusion. This resulted in histologically verified periodontal regeneration featuring newly formed ligament inserted into cementum, whereas control specimens only displayed scar tissue.

In a similar design, Requicha et al. developed a biphasic scaffold consisting of a porous fibrous PCL/starch scaffold for allowing bone ingrowth and an occlusive membrane made of the same material.[25] Here again, the occlusive membrane was designed to support periodontal ligament regeneration by preventing epithelial and gingival tissue invasion of the periodontal defect, hence selectively favoring osteoblast and periodontal fibroblast ingrowth, according to the principles of GTR. In addition, in vitro evaluation revealed a high potential for osteogenesis, an important feature for periodontal regeneration.[26]

While these approaches were inspired by current clinical practices, involving the utilization of an osteoconductive filler and a barrier membrane to allow for selective cell repopulation, recent papers have more specifically addressed bone and PDL compartmentalization, in order to exert greater influence on the soft–hard tissue interfaces.

To this end, Park et al. proposed the utilization of additive manufacturing technology in an indirect manner for the development of a biphasic scaffold consisting of bone and ligament compartments.[27] This approach, involving computer-assisted design and manufacturing (CAD/CAM), utilizing two different sacrificial materials in order to 3D print a mold carrying the negative imprint of the scaffold design. Thereafter, polymer solutions specific to each compartment (polyglycolic acid and polycaprolactone for bone and ligament compartment, respectively) were casted in these molds, and the solvent was evaporated prior to removing the sacrificial material. This resulted in the manufacturing of a porous scaffold of defined dimensions and shape with a well-controlled internal pore architecture. As the two compartments are separately manufactured, they were subsequently assembled using a thin PCL film, thus resulting in the creation of a biphasic scaffold. The authors utilized fibrin to deliver BMP-7 transfected human gingival fibroblasts and human periodontal fibroblasts into the bone and periodontal ligament compartments, respectively. The performance of the cellularized biphasic scaffold was assessed using a murine ectopic model whereby a human dentin block was placed in direct contact with the periodontal ligament compartment. This demonstrated that the presence of periodontal cells greatly facilitated the attachment of a newly formed ligament onto the dentin slice along with the deposition of cementum-like tissue 6 weeks postimplantation. In a subsequent study, the manufacturing technique was slightly modified in order to achieve a higher level

of porosity within the ligament compartment while simultaneously providing topographical tissue guidance for periodontal fiber orientation and attachment.[28] This was spontaneously achieved by casting a PCL solution in the additively manufactured mold, resulting in the formation of microchannels in the periodontal compartment, as shown in Figure 7A. The performance of the fiber-guiding scaffold was assessed using a surgically created periodontal defect in immunocompromised rats and this demonstrated that the presence of the microchannels on the surface of the periodontal compartment was effective in guiding the newly formed periodontal ligament fibers to insert into the root surface at an angle resembling that of native periodontal tissue. Both of these studies relied on the regenerative capacity of freshly seeded/delivered cells, introduced into the TECs a few hours prior to implantation, to subsequently proliferate, differentiate, and produce ECM that contributed to in vivo tissue regeneration. This was further enhanced in the bone compartment by delivering transfected cells expressing BMP-7, a growth factor that promotes osteogenesis. Although these strategies resulted in ectopic periodontal regeneration the multiple manufacturing steps and the complexity of the process may potential impair commercially viable clinical translation.

The implantation of a well-developed and in vitro matured ECM has also been explored in combination with a biphasic scaffold.[29,30] To this end, additive manufacturing was utilized in order to fabricate a biphasic scaffold. The bone compartment consisted of a 3D-printed PCL scaffold, produced via fused deposition modeling (FDM), which was subsequently combined with a periodontal ligament compartment, consisting of a solution electrospun membrane for supporting and delivering mature PDL cell sheets (**Figure 6**B). In this study,[29] the various compartments were separately manufactured and subsequently assembled by partially melting the first layer of the FDM scaffold and then press-fitting the solution electrospun membrane. The resulting heat transfer between the 3D-printed scaffold and the fibrous membrane enabled the partial melting of the electrospun fibers which fused to the struts of the bone compartment, thus creating a strong adhesion while maintaining a porous interface. This biphasic scaffold enabled the culture of osteoblasts in the bone compartment while cell sheets, separately grown, were attached to the periodontal compartment. The in vivo assessment of this cellularized TECs was investigated using an ectopic periodontal regeneration model, whereby a dentin slice was positioned in contact with the cell sheet and implanted subcutaneously in immunocompromised rats. Histological assessment demonstrated that the presence of the cell sheets was essential for cementum deposition on the dentin surface. However, the partially cell occlusive nature of the periodontal compartment made of an electrospun membrane may be considered as a limitation, potentially impeding the integration of the newly formed periodontal ligament into the new bone. Hence, in a subsequent study,[30] the periodontal compartment consisted of a melt electrospun membrane with a macroscaled pore size, which was not cell occlusive (Figure 6C). Implantation of the modified biphasic TECs seeded with osteoblasts and periodontal fibroblast cell sheets using the same ectopic periodontal regeneration model confirmed the central role of the cell sheet for PDL fiber attachment. Importantly, the increased pore size of the periodontal compartment enabled ligament-like tissue insertion into the newly formed bone (Figure 6C). In addition, the specific organization of the melt electrospun scaffold (periodontal compartment) composed of concentrically arranged rings provided a degree of tissue guidance. This resulted in oblique periodontal fibers attachment into the dentin block similarly to that observed in native periodontal tissue, although this feature was only sporadically observed over the dentin surface, and therefore was not actuated in a controlled manner (Figure 6C).

While the aforementioned studies utilized state of the art additive manufacturing technologies for the fabrication of the biphasic TECs, autologous blood products combined with a calcium phosphate cement have also been proposed.[31] This approach consisted of creating a genipin cross-linked platelet lysate membrane supported by a calcium phosphate cement combined with hyaluronic acid microspheres loaded with platelet lysate. This TEC was implanted in a three-wall bone defect in rats with the hemoderivative membrane in direct contact with the root surface. The presence of the platelet membrane appeared to have a beneficial impact over periodontal regeneration; however, the rapid degradation of the calcium phosphate cement compromised the global stability of the TEC and overall resulted in poor periodontal regeneration. An important advantage to this approach is that the biphasic constructs were prepared using technologies which can be implemented by the periodontologist at the chairside using autologous materials. As a result, this approach has limited regulatory barriers from an FDA approval point of view and hence could be rapidly translated to the clinic if proven successful.

In the context of periodontal regeneration, biphasic scaffolds are generally designed to facilitate regeneration of the alveolar bone and periodontal ligament, however the deposition of a new cementum layer at the tooth interface is not actively addressed by these concepts. They rely either on the implantation of in vitro differentiated cells or on the capacity of endogenous cells to promote the apposition of new cementum on the tooth surface. Therefore, recent studies have started addressing the issue of actively promoting cementogenesis by incorporating a third layer, which is the cementum compartment, within the periodontal TECs.

3.2. Triphasic Scaffolds

Although the concept of triphasic scaffolds has been extensively studied for orthopedic applications, such as tendon, ligament or osteochondral tissue engineering, it remains rarely utilized for periodontal regeneration. However, the field is evolving rapidly and recent studies have reported on the development of triphasic scaffolds, created using either conventional scaffold fabrication technologies or additive manufacturing. Triphasic scaffolds only differ from their biphasic counterparts by the addition of an extra layer, usually targeted to cementum regeneration. While in the biphasic design, native cells or cells seeded in the periodontal compartment achieve cementum regeneration post-implantation, the triphasic concept involves the incorporation of a specific cementum compartment, consisting of a biological cue/bioactive biomaterial, with the goal of actively triggering cementogenesis within this part of the TEC.

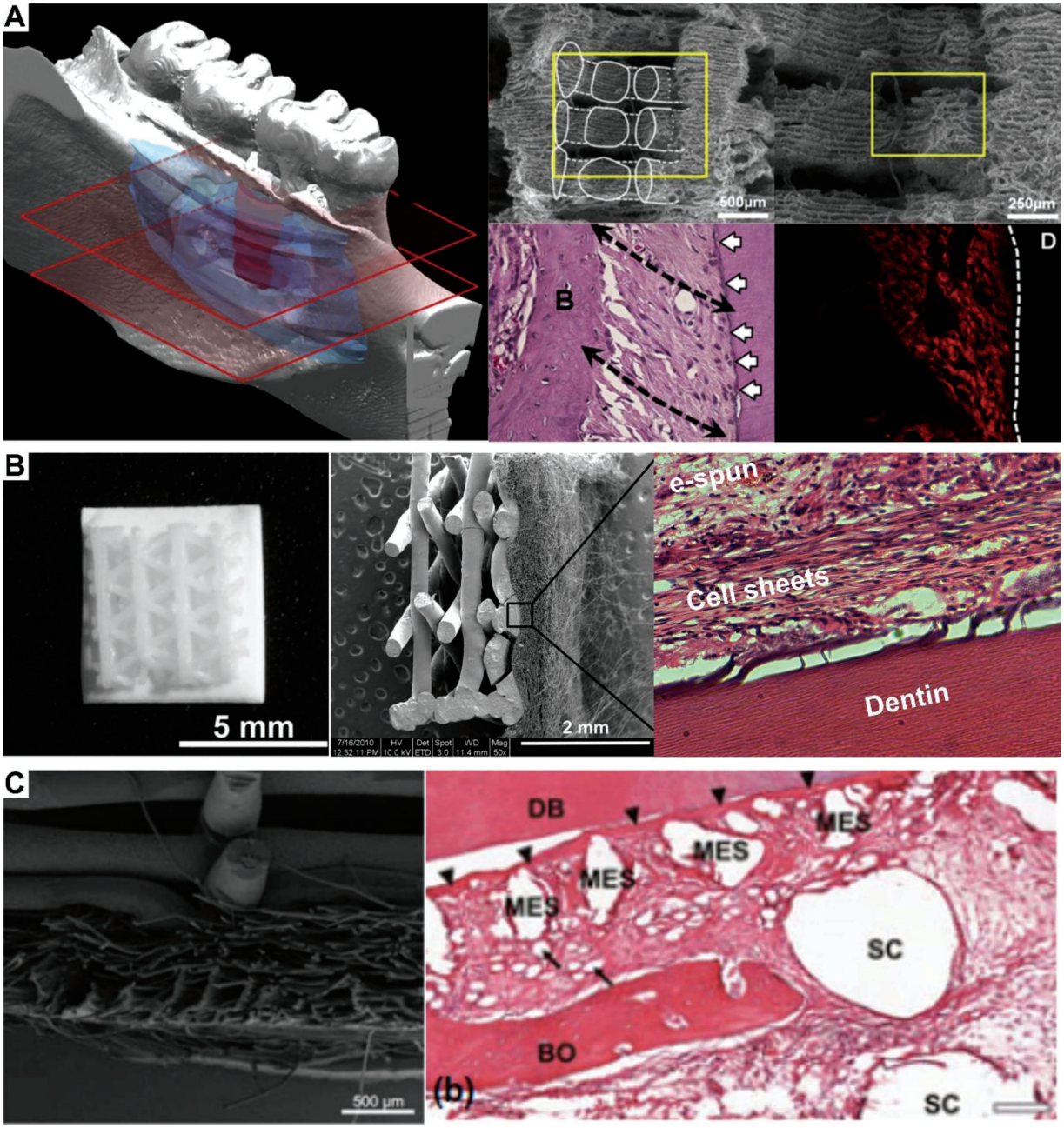

Figure 6. Additively manufactured biphasic scaffold. A) Bicompartment scaffold manufactured using a sacrificial mold, the specific features on the mold surface resulted in the creation of microchannels for fiber guiding, as demonstrated in vivo in a rodent periodontal defect. Reproduced with permission.[28] Copyright 2012, Elsevier. B) 3D-printed biphasic scaffold fabricated via fused deposition modeling and solution electrospinning combined with three layers of cell sheets which promoted tissue attachment on a dentin slice. Reproduced with permission.[29] Copyright 2012, Elsevier. C) Similar design utilizing a melt electrospun scaffold with a larger pore size that enhanced the permeability and cross-talk between the bone and ligament compartments. The particular architecture of the melt electrospun membrane, made of concentrically organized rings, resulted in an ordered periodontal fiber orientation. Reproduced with permission.[30] Copyright 2014, John Wiley and Sons.

Following this concept, a hydrogel triphasic TEC was manufactured utilizing a mixture of chitin, poly(lactic-*co*-glycolic acid), nanobioactive glass ceramic and various biological cues specific to the different compartments.[32] In this approach, each compartment was separately fabricated from a solution blend of chitin and nanobioglass particles, added in both the cementum and bone compartments in order to stimulate ECM mineralization. In addition, specific growth factors were subsequently incorporated within the individual compartments by adding a defined amount of cementum protein 1 (rhCEMP1), fibroblast growth factor 2 (FGF-2), and platelet derived growth factor in the cementum, periodontal ligament and bone compartments, respectively (**Figure 7**). Although the specific details of the compartment assembly were not reported, this approach

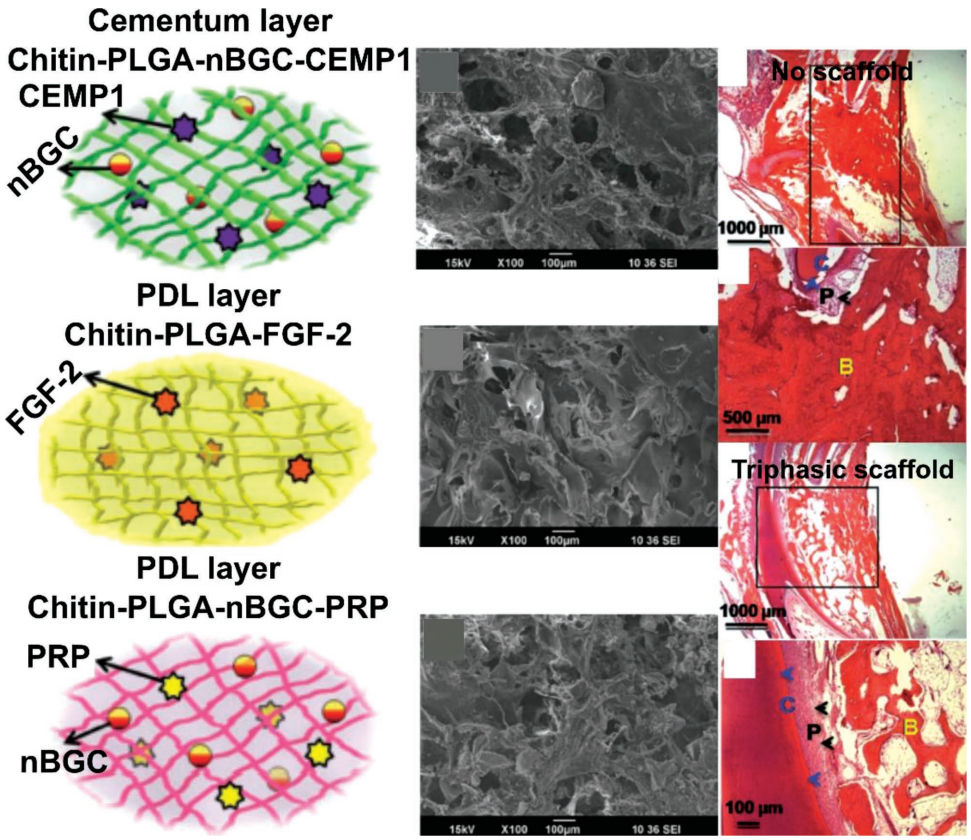

Figure 7. Triphasic scaffold utilizing both natural and synthetic materials for the delivery of biological cues in the various compartments. The three layers were manufactured separately, and subsequently assembled and tested using an incisor periodontal defect model in the rabbit. Each layer displayed a randomly organized porosity and allowed the release of specific biomolecules. In vivo testing demonstrated cementum, ligament, and new bone formation; however, the continuously erupting nature of the incisors does not necessarily enable a distinction between regeneration induced by the triphasic scaffold and natural tissue remodeling. Reproduced with permission.[32] Copyright 2017, Wiley-VCH. nBGC: nanobioactive glass ceramic; PLGA: poly(lactic-*co*-glycolic acid); FGF-2: fibroblast growth factor-2; PRP: platelet-rich plasma; C: cementum; P: periodontal ligament; B: alveolar bone.

created a stratified scaffold recapitulating both the architecture and the biochemical composition of the native periodontium. Further evaluation of the trilayered scaffold was performed using a surgically created defect around rabbit incisors. Despite the obvious limitations of the in vivo model, due to the use of continuously erupting incisors (a feature shared with many other rodents), the authors demonstrated some bone regeneration, and new cementum tissue was deposited upon the incorporation of CEMP-1 in the cementum compartment of the triphasic scaffold (Figure 7). Interestingly, the success of this approach relied solely on the capacity of the host cells to facilitate tissue regeneration (instead of repair) which was induced by incorporating both bioceramic particles and growth factors into the various compartments. This cell-free approach has the advantage of potentially being more cost-effective (compared to cellularized TECs), as well as having fewer barriers for FDA approval and CE-marking, which are both important considerations for clinical translation.

More recently, Varoni et al. reported the utilization of a trilayered chitosan scaffold fabricated by combining freeze drying (gingival and bone compartments) and electrochemical deposition (periodontal compartment).[33] This resulted in the manufacturing of a scaffold featuring aligned microchannels of 450 µm width within the periodontal compartment. The regenerative potential of the trilayered scaffold was assessed using hPDL in a murine periodontal ectopic regeneration model. Histological assessment demonstrated significant mineralization in the bone compartment, CEMP-1 positive mineralized nodules at the dentin interface and the presence of soft tissue intercalation between these mineralized tissues, hence resembling the anatomical structure of the periodontium. Despite these promising findings, the soft tissue intercalation was mainly composed of collagen fibers running parallel to the dentin surface, with no functional attachment observed.[33]

Another example utilizing a triphasic scaffold for periodontal tissue engineering involved a continuous additive manufacturing process in order to incorporate both an architectural and biochemical gradient.[34] This scaffold comprised of three distinct yet fully integrated phases, recapitulating the trilayered morphology of the native tissue: cementum, periodontal ligament layer and alveolar bone layers. Each layer displayed a specific architecture with a pore size of 100, 600, and 300 µm for the cementum, periodontal and bone compartments respectively, hence creating a hierarchical structure. Further to this architecturally stratified design, a biochemical compositional compartmentalization was achieved by incorporating

polyglycolic microspheres loaded with tissue specific growth factor, amelogenin (cementum compartment), connective tissue growth factor (periodontal ligament compartment) and BMP-2 (bone compartment) for controlled spatiotemporal delivery of biological cues hence favoring progenitor cell homing and recruitment. Even though the manufacturing of the scaffold was numerically controlled (as in every CAD/CAM methodology), the incorporation of the growth factors was performed manually by pipetting the microspheres in each specific compartment, hence potentially inducing batch to batch variations. The performance of these TECs seeded with dental pulp stem/progenitor cells was tested in vivo using an ectopic rodent model (albeit without the presence of a dentin block). Discontinuous cementum-like tissue formation was observed, while some level of mineralization was noted in the bone compartment. A soft connective tissue was intercalated between these two mineralized tissues and displayed some attachment and alignment toward the cementum-like layer. Despite these promising ectopic regenerative outcomes, the design of the scaffold has limitations that may hinder clinical translation, such as poor adaptability to "real-world" periodontal defects, as well as scaffold excessive stiffness which could induce soft tissue perforation and subsequent exfoliation. Furthermore, as mentioned previously, the manual loading of the growth factors can induce large variation in the actual quantity incorporated within each compartment of the triphasic TEC. This later issue was circumvented by the authors of a recent study by directly loading the PLGA microspheres into the polymer melt utilized for 3D printing, albeit for a different application—temporomandibular joint reconstruction. Addressing potential limitations, mostly related to thermal degradation of the biological cues once exposed to the elevated temperatures required for extruding the polymer melt, the authors reported a protective effect from the PLGA, which reduced the heat transfer and hence prevented significant degradation of the biochemical cues.[35]

The utilization of a triphasic scaffold for periodontal regeneration is relatively recent and remains vastly unexplored as the clinical implementation of this approach may be challenging. Indeed, regenerative events should occur in a highly coordinated, sequential, and sometimes overlapping manner, whereby cementogenesis onto the dentin of the tooth and the formation of inserting new periodontal ligament fibers into this cementum occur concurrently in order to achieve the fully integrated, functional new periodontal attachment that mimics native tissue. While the incorporation of a cementum layer into the implanted TEC may appear to be conceptually sound, a major foreseeable challenge is the ability to fix and integrate a cementum layer onto the underlying dentin surface of the tooth root. In addition, the design of the triphasic scaffold does not necessarily take into consideration the anatomical dimensions of the cementum tissue, which is less than a hundred micrometers, whereas most cementum compartments reported in the literature are of thicknesses approaching the millimeter scale. This discrepancy could potentially result in the formation of tissue that is not anatomically relevant and hence could jeopardize or impede periodontal regeneration and hence clinical translation.

This section has described the major advancements in the field of multiphasic construct manufacturing for periodontal regeneration, and has highlighted the advantages as well as the limitations of compartmentalized multiphasic scaffolds. While these approaches are scientifically sound, it is conceivable that the coordinated healing events necessary for successful periodontal regeneration may also be achieved without the utilization of a biomaterial scaffold, through the implantation of cells embedded in their own mature extracellular matrix.

4. Cell Sheets and Scaffold-Free Constructs

The literature overwhelmingly suggests that periodontal ligament fibroblasts are the best performing cell type for facilitating periodontal regeneration.[17,36] The positive outcome toward tissue regeneration is intimately linked to ECM production during the in vitro cell differentiation phase prior to transplantation. This can be explained by two mechanisms which are likely to work in a synergistic manner: biomechanical stabilization and biological cues. Indeed, the production of ECM which results in the formation of a cell sheet enables the stabilization of the cells once placed in the periodontal defect when compared to cells which are freely injected/delivered into the recipient site. These cells contribute directly to new tissue formation as previous described[17] but also act in an indirect manner via the release of biological cues.[37] Indeed, biological molecules, specific to the native PDL cell niche, which are secreted during the in vitro maturation of the cell sheet, are likely to significantly influence the regenerative process.

This concept was explored by Okano and co-workers in a series of papers whereby the efficacy of three-layered cell sheets was confirmed in various animal models and implantation sites (**Figure 8**A).[36,38] It was shown that PDL cell sheets could attach to ectopically implanted dentin slices, and promote sporadic formation of cementum-like tissue at the dentin interface.[38b] Further evidence of the beneficial effect of implanting cells embedded in a mature ECM network was attained by using a surgically created periodontal defect model in athymic rats.[38c,d] This demonstrated enhanced attachment of the regenerated periodontal ligament as early as 1 week postimplantation of the cell sheets.[38d] Observations at 4 weeks postsurgery confirmed the maturation of the periodontal ligament, as oblique collagen fibers were inserted into the newly formed cementum whereas only randomly organized repair tissue was observed at the contralateral side (controls without a cell sheet). In a different study, the cell sheets were implanted into periodontal dehiscence defects in the mandibular first premolars of canines, thus more closely resembling a clinical situation.[38a] In this approach, a hyaluronic acid membrane was utilized in order to support the trilayered cell sheets and to deliver the cellularized material onto the root surface. The authors reported that full periodontal regeneration, defined as the formation of alveolar bone, ligament and cementum, occurred in three of the five animals that received cell sheets. Functional PDL attachment was also attained in these three animals, with fibers inserting perpendicularly into newly formed cementum. In contrast, the defects without cell sheets displayed poor periodontal attachment resulting in delamination of the tissue upon histological sectioning. Although the cell sheet significantly enhanced the

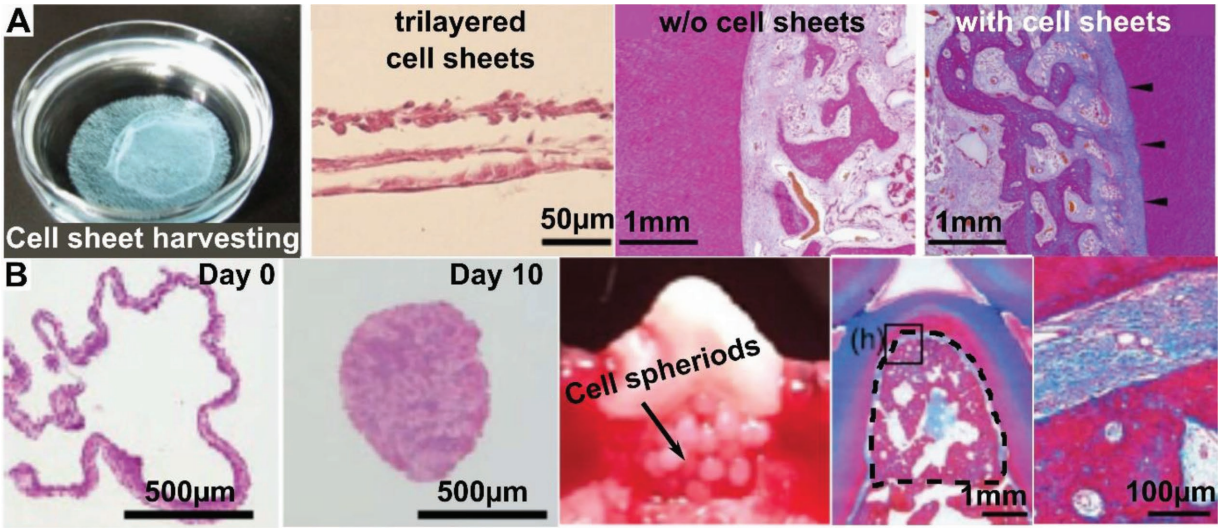

Figure 8. Scaffold-free periodontal regeneration. A) Utilization of trilayered cell sheets harvested from thermoresponsive culture dishes stacked together and placed in a three-wall periodontal defect in a canine. This approach demonstrated superior regeneration in the specimens where the cell sheets were implanted. Reproduced with permission.[38e] Copyright 2009, Elsevier. B) Utilization of cell spheroids created by the spontaneous contraction of cell sheets during in vitro osteogenic differentiation and implanted in a class III furcation defect resulting in full bone regeneration. Reproduced with permission.[39] Copyright 2009, SAGE Publications.

regenerative outcomes, two of the five animals displayed partial or poor regeneration, and this was attributed to a lack of biomechanical stability of the cell sheets on the root surface. The cell sheets may have been displaced during suturing or subsequent mastication, thus resulting in impaired regeneration. In a subsequent study also using a canine model, a three-wall defect was created using trilayered cell sheets that were placed into this confined defect using a thin biodegradable polyglycolic acid (PLGA) membrane, with the remainder of the defect filled with an inorganic filler.[38e] In this instance, good biomechanical stability of the cell sheet was achieved due to the confined nature of the three-wall defects, which resulted in full periodontal regeneration in all of the four animals. The cell sheets out-performed the control group in all parameters used to assess periodontal regeneration, including higher level of bone fill and new cementum deposition (Figure 8A). This study demonstrated the favorable performance of the in vitro engineered cell sheets containing an extensive extracellular network along with differentiated living cells.

More recently, a modified concept using cell spheroids formed from cell sheets has been proposed in order to further enhance the regenerative potential of this approach. The strategy behind the utilization of cell sheets relies, as described previously, on the implantation of an intact and extensive ECM network. Guo et al. developed a technique enabling the production of cell pellets from trilayered cell sheets based on their spontaneous contraction, which resulted in the creation of highly viable cell pellets with increased ECM content when compared to noncontracted trilayered sheets. Although the mechanism behind the higher ECM production in the cell pellet remains unclear, there was a 20- to 30-fold increase in the collagen content. The regenerative performance of the cell sheets and cell pellets was assessed in a periodontal defect model created on the root of the rat maxillary first molar. This demonstrated that the increased content in ECM from the cell pellet resulted in higher mineralization within the defect and a higher degree of ligament alignment. The concept of implanting cell pellets was further investigated by Takewaki et al. using iliac crest mesenchymal stem cell spheroids differentiated in collagenic and/or osteogenic culture media prior to transplantation in a preclinical dog model[39] (Figure 8B). This strategy also relied on the extensive production of ECM for enabling mechanical manipulation of the cell sheet in order to create the cell pellet. As expected with cell spheroids, nutrient and oxygen diffusion was limited in the central portions, although significant cell apoptosis was only noted after 10 days of in vitro culture (Figure 8B). The spheroids, differentiated for 5 days in vitro, were subsequently implanted in a class III furcation defect in dog premolars and this resulted in significant regeneration of cementum, periodontal ligament and alveolar bone 12 weeks post-implantation. Apart from the inherent challenges related to the in vitro expansion of cells prior to implantation, which are of concern from both cost and regulatory perspectives, the handling and stabilization of the cell sheets or cell pellets in these scaffold-free approaches may be another major technical limitation. Further, poor biomechanical fixation that hinders cell sheet/pellet attachment on the root surface can be detrimental to subsequent regenerative outcomes, as previously discussed.

5. Future Outlook for Periodontal Regeneration

This review has provided an insight into the current scaffold designs and fabrication technologies with a focus on periodontal attachment and orientation. However, most of these recent developments are still in their early stages of translation to the clinic, and widespread clinical use still remains a challenge. In addition, clinical translation can be further impeded by the complexity of the scaffold design, which is required for

a coordinated spatiotemporal regenerative response. The current trend in tissue engineering for periodontal regeneration is to mimic the architectural and compositional features of the periodontium, therefore resulting in complex structures whereby the TEC manufacturing reproducibility may be challenging. Ultimately, the aim should be to design TECs that achieve positive clinical outcomes with as straightforward and cost-effective solutions as possible, but at the same time taking into account the complexities that are inherent to periodontal regeneration. Clinical translation could be accelerated by combining such TECs with concurrent advances in surgical techniques and other chairside approaches that are being continually developed to enhance regenerative outcomes.

The next section explores the future of periodontal regeneration and addresses the advantages and issues associated with current strategies, and proposes innovative modifications for enhanced clinical outcomes.

5.1. Patient Specific Scaffolds

Scaffold modeling using computer-aided design software has allowed for an increase in their architectural complexity. Specifically, the use of computed tomography (CT) to extract the specific dimensions and topography of a given defect site holds promise for the potential development of patient-specific scaffolds that are based on patient-specific defect parameters.

A recent seminal single case report by Rasperini et al.[40] used cone beam computed tomography (CBCT) scans of a 53 year old male's peri-osseous defect to design a customized polycaprolactone scaffold fabricated via selective laser sintering. The scaffold consisted of an internal region with pegs to support PDL regeneration and the delivery of rhPDGF-BB, and an external region that would resorb over time with subsequent replacement by alveolar bone (**Figure 9**). In this first application of a 3D-printed scaffold specifically designed for the treatment of a periodontal defect, the PCL scaffold remained in situ for a period of 12 months without signs of dehiscence or chronic inflammation. However, the primary limitation was the use of a rather rigid construct made of a slow-degrading polymer (PCL) that possibly created a mechanical mismatch with the surrounding soft gingival tissue over time. This unfortunately resulted in the construct becoming exposed to the oral environment 13 months postimplantation, whereby it became contaminated by intraoral microbes and was lost.

The ongoing technological evolution of 3D printing now enables the utilization of a greater variety of materials with tailorable mechanical and degradative properties (such as PLGA) while maintaining high printing resolution[41] necessary for manufacturing of customized scaffolds. Therefore, it will enable the fabrication of "softer" scaffolds with space maintenance and degradative properties more suited to periodontal regeneration.

In addition to the customization of 3D constructs for periodontal regeneration, another important aspect to consider is the utilization of medical grade material. For regulatory requirements, the use of traceable, high purity, and certified medical grade material is essential in order to ultimately enable clinical translation and commercialization of these medical devices.

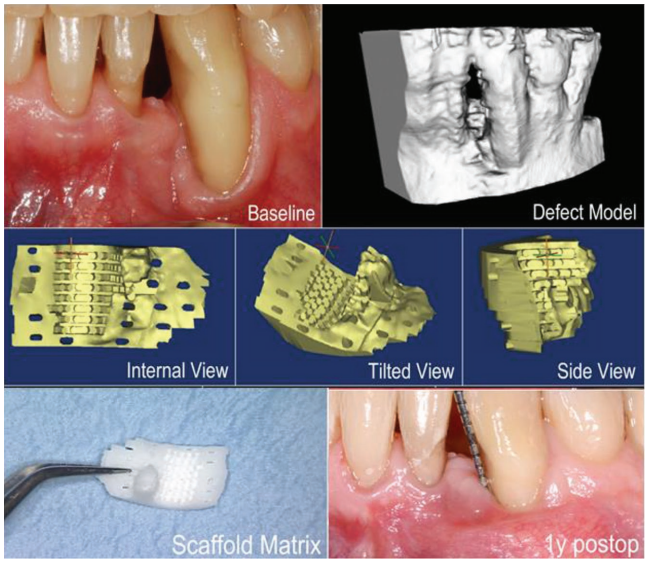

Figure 9. Customized scaffold designed for a human peri-osseous defect based on a model derived from cone beam computed tomography scans. The polycaprolactone scaffold consisted of an internal region with pegs as a dedicated space for periodontal ligament regeneration, with the external region supporting the tooth root during alveolar bone regeneration. The scaffold remained in place for a period of 1 year without signs of chronic inflammation or dehiscence. Reproduced with permission.[40] Copyright 2015, SAGE Publications.

5.2. Fiber Guidance

The specificity of scaffold architecture contributes to its ability to act as a biomaterial template for the guidance of oriented fiber formation that is critical to the regeneration of tissues that require alignment for proper function—such as the PDL. Fiber guiding is generally achieved by either topographically guiding the fiber orientation or by mechanically stimulating the cells prior to implantation in order to increase functional attachment of the periodontal ligament. The first approach utilized grooves of various widths for inducing a spontaneous alignment or a specific topographical organization as initially demonstrated by Park et al.[27,28] The fabrication of the fiber-guiding biphasic scaffold involved the casting of a polymer solution into a sacrificial mold, resulting in a surface topography in the periodontal compartment which promoted the creation of aligned micro channels. A similar feature was also observed using melt electrospinning for the fabrication of a biphasic scaffold (Figure 6C), although minimal control over the physical arrangements of the channels (channel spacing, spatial distribution, etc.) was possible with both of these techniques. Indeed, these fiber guidance features were imparted by the inherent nature of the fabrication techniques, without being specifically introduced for this purpose within the scaffold design.

As the development of scaffolds for periodontal tissue engineering has matured, different technologies have been developed for the introduction of guiding channels within the scaffold in a more systematic and controlled manner.[42] This was implemented by utilizing a directional cooling system which impacted on the heat transfer gradient and crystal growth direction of a gelatin solution resulting in the creation

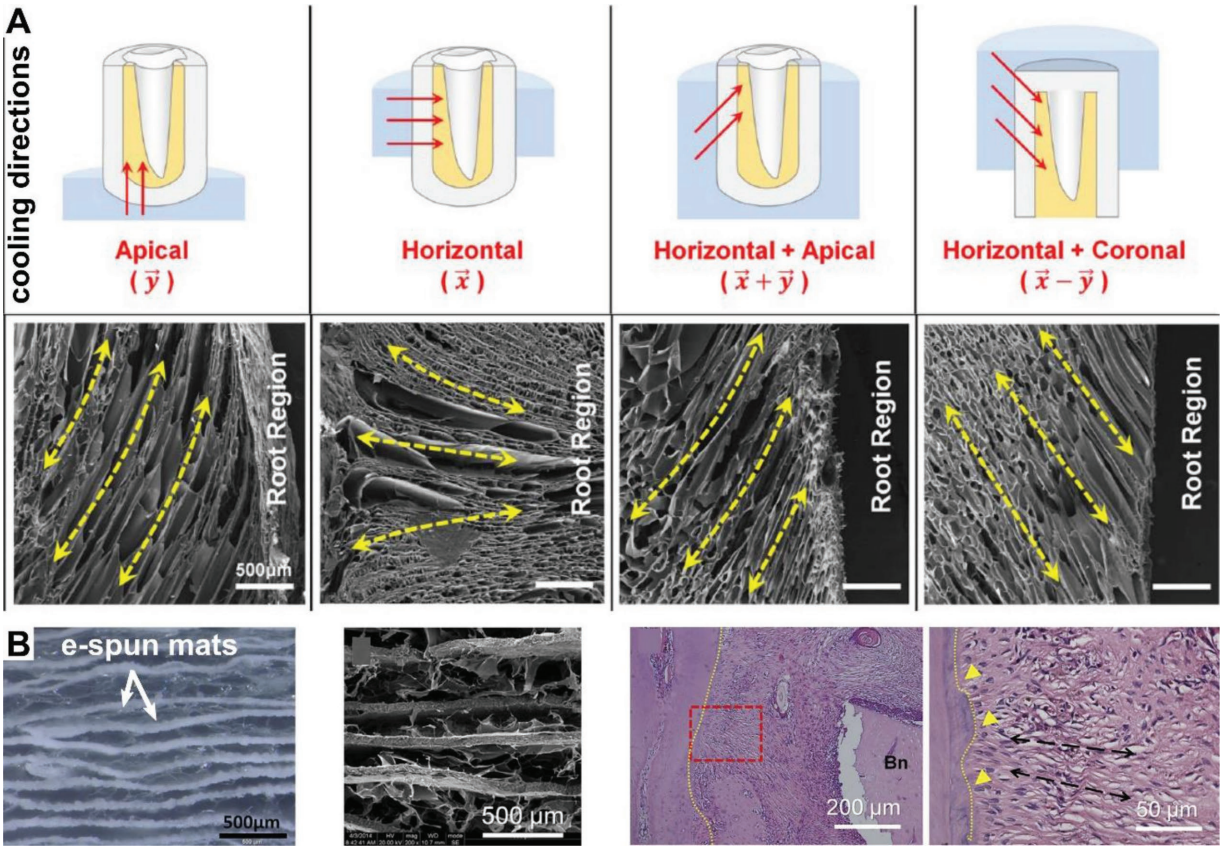

Figure 10. Fiber guiding scaffold for periodontal tissue engineering. A) Gelatin scaffold featuring orientated microchannels manufactured via a controlled bidirectional cooling of the gelatin solution. Reproduced with permission.[42b] Copyright 2014, SAGE Publications. B) Combination of thin electrospun mats glued together using a chitosan hydrogel leading to the creation of channels of various widths which demonstrated significant impact on orientating periodontal ligament fibers in a rodent model. Reproduced with permission.[42a] Copyright 2015, Elsevier.

of microchannels of various orientation within a gelatin sponge,[42b] as shown in **Figure 10**A. Although, the microchanneled scaffold was not extensively tested in vitro or in vivo, its internal architecture strikingly resembled the native organization of the collagen fibers within the periodontal ligament. Most recently, Park et al.[43] detailed a new method for the creation of angulated (parallel, oblique, and perpendicular) microgroove patterns using additive manufacturing, with in vitro confirmation of predictable ligament cell alignment along the 3D-printed grooves. The resulting fiber-guiding regions can improve the ability of next generation scaffolds to promote a more accurate recapitulation of the PDL's anatomical complexity.

Fiber guidance can also be obtained by combining several fabrication techniques such as electrospinning and freeze-drying. For example, thin PCL–PEG copolymer electrospun membranes consisting of aligned fibers were stacked on top of each other, infiltrated with a chitosan glue, and subsequently crosslinked and freeze-dried. This created a specific architecture of alternating electrospun mats and chitosan foam separated by a few hundred micrometers, hence forming a topography similar to microchannels (Figure 10B). Although this specific topographical feature was obtained in a manual manner and can hence be prone to batch-to-batch and operator variation, the scaffold was shown to be effective at guiding periodontal orientation and attachment onto newly formed cementum in a rat periodontal regeneration model (despite the absence of any PCL–PEG material in the histology sections provided in the paper).

A more systematic manner of providing guidance was recently reported by combining 3D printing and soft lithography for the fabrication of a scaffold with mesoscale and microscale topographical cues.[42c] In this approach, soft lithography was utilized for the creation of a micropatterned thin PCL film featuring pillars with microgrooves of various widths (15 or 60 μm) and depths (10 and 30 μm) (**Figure 11**). The micro patterned membrane was assembled with a scaffold fabricated via selective laser sintering (SLS) and the fiber guidance potential of the TEC was investigated in a murine subcutaneous model using a dentin slice. This demonstrated that the most prominent impact upon cell and tissue alignment was provided by the depth of the groove and that this effect, although detectable at an earlier time point, was more pronounced 6 week postimplantation. The authors further reported an increase in the thickness of orientated collagen fibers reaching up to 50 μm with the deeper grooves, exceeding the actual dimension of the microscale features (Figure 11). Therefore, these results clearly showed that the topographical cues were effective not only within the grooves but also in their direct vicinity. While this approach represents a breakthrough in the field, several limitations are inherent to the TEC fabrication method and are related to the discontinuous topographical guidance provided by the pillars. The microgrooved film was

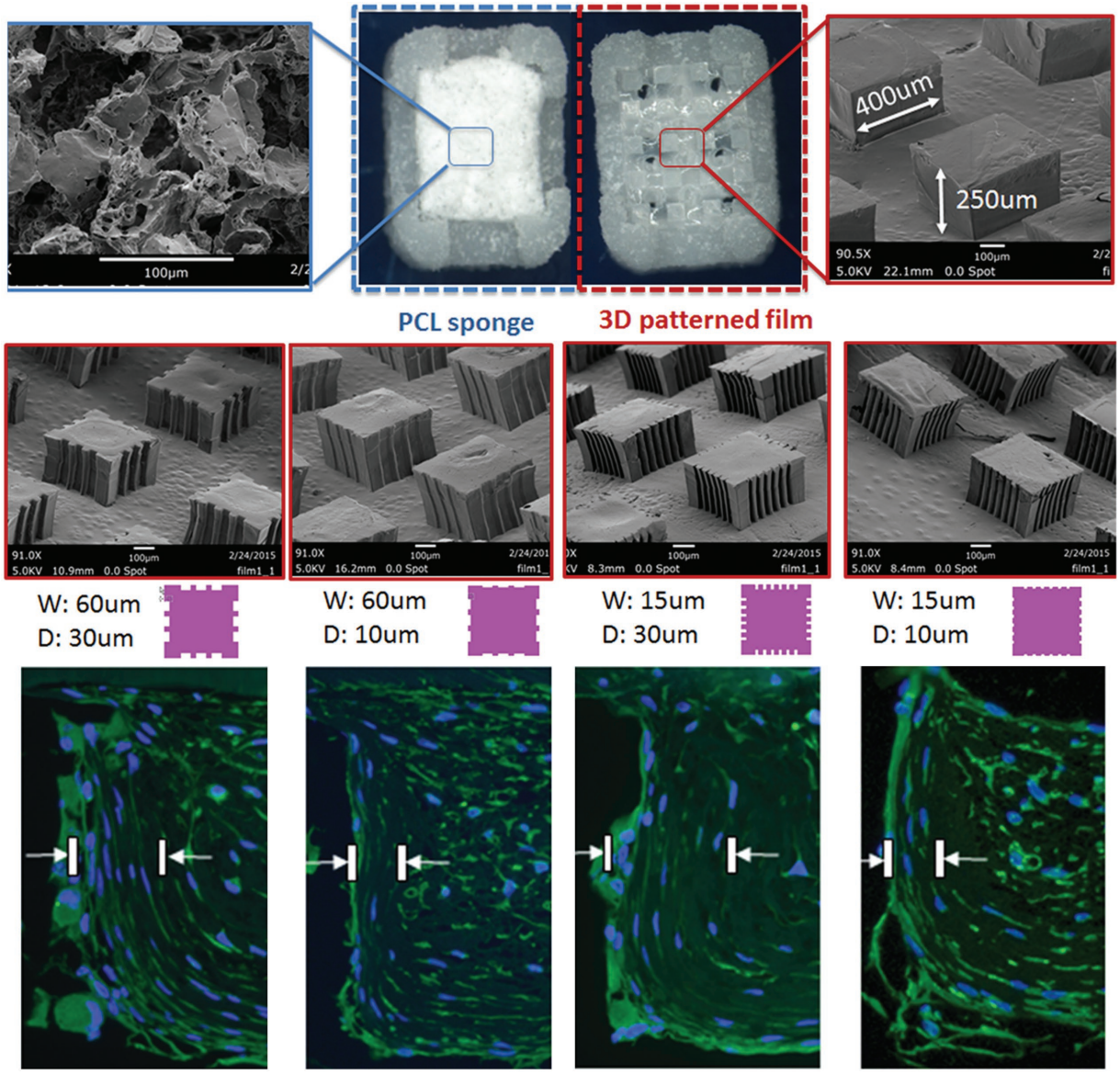

Figure 11. Advanced design for the creation of a fiber guiding biphasic construct for periodontal regeneration. A patterned membrane featuring microgrooved pillars was utilized to induce a systematic in vivo alignment of the newly formed periodontal ligament. Reproduced with permission.[42c] Copyright 2016, Wiley-VCH.

composed of 400 μm pillars evenly spaced every 400 μm, hence creating an interface consisting of alternating voids which enabled tissue infiltration, and solid PCL pillars which impeded tissue infiltration. This configuration reduces the level of tissue adhesion to the dentin block as 25% of the micro patterned surface consisted of the PCL pillars. Although, the microgrooves were proven to be very efficient in inducing tissue alignment, this effect was restricted to the direct vicinity of the grooves and hence only a fraction of the regenerated tissue was actually affected. Notwithstanding these limitations, this study represents a significant step toward promoting the functional regeneration of the periodontal ligament and provides a foundation for future work aimed at developing fully integrated, compartmentalized and fiber guiding periodontal constructs.

As discussed here, the literature overwhelmingly suggests that physical and topographical cues are the way forward for enhancing periodontal fiber orientation; however, a recent report has demonstrated the efficacy of combining this approach with an in vitro mechanical preconditioning of the cells for promoting the orientation of the periodontal fibers.[44] In this approach, PDL cells seeded onto aligned electrospun fibers were subjected to biaxial stretching of around 6% strain at a frequency of 1 Hz for up to 10 days and resulted in upregulated expression of periodontal markers such as periostin, tenascin and TGF-β. This effect was also translated in vivo and demonstrated that mechanically induced cell differentiation was able to enhance periodontal attachment and bone apposition.

The maturation of cells in a bioreactor or using a differentiation cocktail in order to guide their commitment toward differentiation can impart a significant increase in both reproducibility and costs. Other methods have proposed to directly influence the cell differentiation in vivo via the functionalization of the scaffold.

5.3. Scaffold Functionalization

5.3.1. Gene Therapy

As discussed earlier, periodontal wound healing can be enhanced through the use of recombinant growth factors (GFs) and platelet-rich plasma which contain concentrated suspensions of autologous platelets that secrete bioactive GFs at the wound site. Some commonly used bioactive agents in the clinic are derived from the following GF families: platelet derived growth factors, bone morphogenetic proteins (BMPs) within the β-transforming growth factor (β-TGF) superfamily and fibroblast growth factors (FGFs), among others. These biologics regulate periodontal and mesenchymal stem cell migration, differentiation, proliferation, chemotaxis, and cell-specific extracellular matrix (ECM) production. The efficacy of these agents varies depending on delivery system, dosages, and release kinetics. Pharmacologic dosing is often required to mitigate the transient biological activity of GFs at local delivery sites due to short half-lives and proteolytic degradation, although use of these supra-physiological doses can evoke local and systemic toxicity.[45] Localized GF delivery is therefore necessary to decrease total dosage needed without compromising function, and 3D TECs have been utilized as a delivery strategy with various degrees of success, as described earlier in this review.

Gene therapy provides a promising strategy to achieve greater bioavailability of growth factors within periodontal defects, circumventing the limitations of the short growth factor half-lives and the resultant necessity for the local delivery of potentially toxic supra-physiological doses. The gene therapy approach involves the transduction (using viral elements) or transfection (using nonviral elements) of a target cell population to express GF-encoding genes over the time-frame necessary to achieve the desired therapeutic effects.

Viral vectors used for gene therapy have included adenoviruses (Ad), lentiviruses, retroviruses, adeno-associated viruses (AAVs), and baculoviruses (**Figure 12**). All have their own advantages and disadvantages, with viruses that have higher transduction rates also contributing to higher risk for host immunogenicity. For example, adenoviruses have high transduction rates, with the ability to transfect a large variety of different cell types; however, these is also potential for higher host

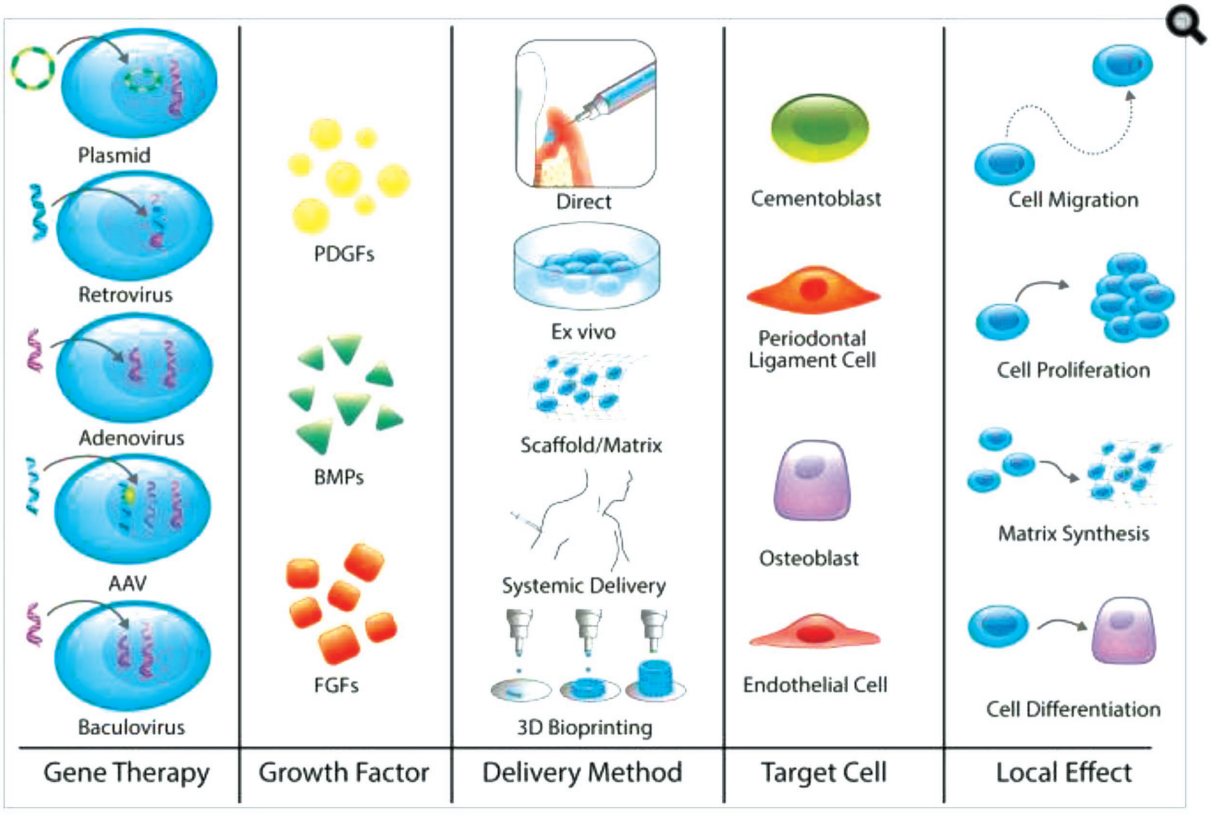

Figure 12. A variety of viral (i.e., adenovirus, retrovirus, adeno-associated virus) and nonviral (i.e., plasmid) vectors have been used for the delivery of various growth factors (i.e., platelet-derived growth factor, bone morphogenetic protein, and fibroblast growth factor) to elicit a local effect (i.e., cell differentiation, proliferation, etc.) that can promote periodontal regeneration. Cells can be transduced (using viral vector) or transfected (using a nonviral vector) and the cells and/or growth factors can be delivered using a variety of methods, including via a scaffolding matrix that can be implanted at the defect site. Reproduced with permission.[46] Copyright 2016, SAGE Publications.

immunogenicity compared to other types of vectors. AAVs have the added advantage of being able to transduce both dividing and nondividing cells with low levels of immunogenicity, but with lower rates of transduction compared to adenoviruses. Another concern is that of insertional mutagenesis, which occurs when viral DNA integrates within the genome of a host organism; this is a risk with the use of lentiviruses, as well as retroviruses, whose transduction is also limited to dividing cells, although there is low risk of immunogenicity.[46] Safety concerns regarding risk of virus dispersion, cytotoxicity, and lack of spatiotemporal control of gene expression limit the predictability of viral vector use for gene therapy and in particular hamper its translation into clinically viable solutions, thereby requiring the investigation of novel methods of gene delivery. Currently, a safer alternative is the use of nonviral vectors such as plasmids, which have lower host immunogenicity, as well as reduced production costs compared to viral vectors. The main drawback of nonviral vectors, however, is their reduced efficiency of gene delivery (i.e., low transfection efficiency), resulting in more transient expression.[47]

Various methods of viral and nonviral vector delivery have been employed to elicit a local regenerative response through induced growth factor expression (Figure 12). Multiple studies support the potential use of gene therapy for periodontal tissue regeneration. Sustained and localized PDGF-B gene expression using direct delivery in periodontal lesions was observed for up to 21–35 days after cell transduction,[48] and has been shown to stimulate alveolar bone and cementum regeneration,[49] whereas ex vivo BMP-7 gene transfer using dermal fibroblast transduction resulted in predictable bridging of periodontal bone defects.[50]

Scaffolds can also serve as delivery vehicles for transduced cells. Biphasic calcium phosphate block scaffolds have been used for the delivery of human periodontal ligament stem cells transduced with recombinant Ad-BMP2; mesoporous BioGlass/silk scaffolds for delivery of AdBMP7 and/or AdPDGF-B, whose combination was shown to promote wound healing synergistically; and chitosan thermosensitive hydrogel scaffolds for delivery of BMP2 plasmid DNA-loaded nanoparticles into rat calvarial defects, resulting in enhanced bone formation.[51]

Overall, tissue engineering approaches using scaffolds alone or in combination with growth factor, cell and/or gene delivery have the potential to address existing challenges in managing periodontal tissue loss and increase clinical options for their controllable regeneration. Given the limitations of current periodontal regenerative therapies, a scaffold-based delivery vehicle that can be used for the regeneration of the alveolar bone–PDL–cementum complex in conjunction with localized, controlled PDGF-BB and BMP-7 delivery using gene therapy is relevant to making further progress in creating the next generation of tissue engineered scaffolds for PDL regeneration.

Decellularized Extracellular Matrix: Another method of functionalizing synthetic scaffolds utilizes the concept of decellularization. Tissue decellularization is a recognized and well established method for the manufacturing of biological scaffolds which properties can affect a great variety or cellular function from migration, proliferation and ultimately differentiation,[52] and has been shown to affect the polarization of macrophages.[53] Decellularized matrices are widely used in a plethora of applications ranging from soft tissue healing to breast and tendon regeneration, and in dentistry in the form of membranes for guided bone and tissue regeneration.[54]

The preservation of the ECM components along with the biochemical cues present in the ECM network is central to the success of the regenerative outcome. As such, mild chemicals are preferably utilized in order to achieve decellularization with minimal impact on the ECM. The utilization of this technique has recently been proposed for periodontal regeneration.[55] This strategy consisted in developing a mature ECM via the in vitro culture of periodontal cells under differentiation media prior to harvesting using a melt electrospun membrane (similar to that shown in Figure 3B) and subsequent decellularization using ammonium hydroxide and DNAse treatment. This mild chemical treatment enabled the maintenance of the collagen network AND the preservation of resident growth factors, which supported the repopulation of the TEC with allogenic cells. The decellularized TEC was further assessed in a surgically created rat periodontal defect and demonstrated some encouraging in vivo outcomes.[56] This methodology could circumvent the issues associated with patient specific cell implantation and provide clinicians with an off-the-shelf product.

5.3.2. Scaffold with Immunomodulatory Properties

The designing of scaffolds with immunomodulatory capacity has been the focus of extensive research. This can be achieved by the modification of the surface of the biomaterials as stem cells and inflammatory cells are known to be affected by the roughness, the size and shape of topographical cues. Indeed, smoother PLGA spheres have been demonstrated to reduce the level of inflammation[57] and elongated PLGA particles increased the proliferation of T-cells when compared to PLGA spheres.[58] Although the surface modification on microspheres or 2D films is efficient at better controlling the activation of inflammatory cells, its implementation in 3D scaffolds remains elusive due the technical hurdles in translating the fabrication methods from 2D to highly porous scaffolds. Another interesting strategy involved the addition of biological cues within the TECs. As TNFα is a major proinflammatory molecule, strategies have sought to inactivate its in vivo action by incorporating antibodies targeted toward this cytokine in biomaterials.[59] Direct impact on the inflammatory process was also achieved by the incorporation of pro-healing cytokines[60] and via the utilization of pro-resolution molecules such as resolving D1.[61] In the latter case of resolving D1, a significant impediment is the poor stability of the molecule, which could decrease its efficacy during clinical use.

5.4. Cellular Therapy and Clinical Trials

Cell sheet tissue engineering is one of the most advanced tissue engineering methodologies for periodontal regeneration and several clinical trials have been conducted in recent years.[62] The pioneering group in the field reported the initiation of a clinical trial in 2011, with 10 patients recruited by 2014.[63]

While the outcomes of this particular clinical trial have not yet been reported, it is interesting to elaborate on the regulatory considerations for such a trial. Iwata et al. described some of the many requirements for the fabrication of the clinical grade cell sheets which are based on good clinical practice (GCP) and good manufacturing practice (GMP).[64] To achieve this, a GMP compliant cell processing unit was built, the cells were cultured using GMP-grade reagents or certified chemicals and under xeno-free condition (using uniquely autologous serum) resulting in tremendously high production costs.[65] Therefore, one can question the economic viability of this technique especially in light of a recently reported clinical trial which demonstrated that the cell sheet, although safe to use, had little to no impact on the regenerative outcomes.[62] Hence, the requirement for the expansion and transplantation of living cells with this approach imparts a significant economic burden which may prevent successful commercial translation to the clinic. The high economic cost of the treatment may also be difficult to justify in the context of a noncritical, nonlife threatening medical condition.

Other approaches, still utilizing autologous biological components, could nevertheless be implemented for circumventing the regulatory issues and mitigating the high costs associated with cell expansion in a GMP cell processing facility.

5.5. Autologous Derived Biochemical Cues

Autologous blood products, such as platelet-rich plasma, platelet-poor plasma (PPP) and platelet-rich fibrin (PRF), are commonly used for several applications in dentistry and regenerative medicine, and they have been applied with some mitigated success for periodontal regeneration.[31] The clinical application and effectiveness of these approaches has been reviewed extensively elsewhere.[66] A further development in autologous preparations for regenerative medicine has seen the emergence of extracellular vesicles (EVs) as a potent means for influencing the initial inflammatory response and the subsequent healing events in tissue engineering and regenerative medicine applications.[67] EVs are a category of endocytic produced vesicles containing biological information in the form of siRNA, protein, growth factors, and hence have the capacity to facilitate paracrine communication between distant cells[68] These effectors, isolated from platelet lysate or secreted from cells in vitro, have been shown to significantly impact cell differentiation in vitro and in vivo.[69] These recent findings also suggested that exosomes are capable of binding to ECM such as type I collagen and fibronectin and therefore can be easily combined with biomaterials for effective in vivo local delivery, as recently demonstrated.[70] This could be achieved by either decorating a synthetic multiphasic scaffold with exosomes or by encapsulating them into a hydrogel prior to implantation.

6. Conclusion

Due to their ability to mimic the complex hierarchical structure of the periodontium, 3D tissue engineered constructs offer promising opportunities for promoting periodontal regeneration. This review has outlined the significant advances that have been achieved over the past couple of decades, culminating in the development of functionalized multiphasic scaffolds that show promising results in preclinical trials. Building on preclinical research conducted over the past 10 years, 3D tissue engineered constructs for periodontal regeneration are beginning to be translated to clinical applications. While early "proof-of-principle" reports of application in human subjects are encouraging, there is clearly a need for further optimization and refinement of current strategies before they can be applied to routine clinical care. Initially, it is likely that customized multiphasic 3D TECs, functionalized with either autologous preparations that could be obtained chairside or regulator approved commercially available bioactive agents, will provide the most feasible pathway for clinical translation toward predictable periodontal regeneration.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

additive manufacturing, periodontal regeneration, scaffolds, tissue engineering

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