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REVIEW

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Engineered scaffolds and cell-based therapy for periodontal regeneration

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ABSTRACT

Background: The main objective of regenerative periodontal therapy is to completely restore the periodontal tissues lost. This review summarizes the most recent evidence in support of scaffold- and cell-based tissue engineering, which are expected to play a relevant role in next-generation periodontal regenerative therapy.
Methods: A literature search (PubMed database) was performed to analyze more recently updated articles regarding periodontal regeneration, scaffolds and cell-based technologies.
Results: Evidence supports the importance of scaffold physical cues to promote periodontal regeneration, including scaffold multicompartmentalization and micropatterning. The in situ delivery of biological mediators and/or cell populations, both stem cells and already differentiated cells, has shown promising in vivo efficacy.
Conclusions: Porous scaffolds are pivotal for clot stabilization, wound compartmentalization, cell homing and cell nutrients delivery. Given the revolutionary introduction of rapid prototyping technique and cell-based therapies, the fabrication of custom-made scaffolds is not far from being achieved.

Keywords: Calcium phosphates, Cell encapsulation, Cell sheet technology, Chitosan, Multilayered scaffolds, Regenerative periodontal therapy

Introduction

The periodontal apparatus – namely, the periodontium – is a complex multitissue system with 4 components: root cementum, alveolar bone, periodontal ligament (PDL) and gingiva. PDL, in particular, has perpendicular and oblique fiber insertions from the cementum into the alveolar bone to transfer mechanical stresses of the tooth into the alveolus. Regenerative periodontal therapy (RPT) aims to completely restore the lost periodontal structure, anatomically and functionally, via surgical and tissue-engineered approaches which include the use of alloplastic grafts or membranes.

The specific objectives of RPT are wound compartmentalization and clot stabilization, pivotal steps to successfully achieving periodontal regeneration (1). However, major drawbacks still exist, including infection and dehiscence at the membrane or graft site, and a nonfunctional PDL attachment, with fibers not inserting into cementum and alveolar bone (2). To date, complete periodontal regeneration is not achievable in a highly reproducible and easy way, thus there is an overwhelming scientific interest in accomplishing this challenging task.

When bone tissue reconstruction is needed in RPT, clinicians may use autologous, allogeneic or xenogenic bone grafts (3) or, alternatively, synthetic biomaterials, also referred to as alloplastic materials. Autologous bone grafts represent the gold standard of care for bone regeneration, since they possess ideal inorganic and organic components, such as both hydroxyapatite (HA) and viable osteoblasts, with their osteogenic, osteoconductive and osteoinductive properties. Patient costs, however, hinder their full application, together with long intervention timing. To overcome these issues, allogeneic (from a compatible donor) or xenogeneic (from a donor of different species) bone grafts have been proposed. In particular, decellularized and deproteinized extracellular matrix has largely been used for cell seeding with a certain degree of clinical success, although it does not possess osteoinductive properties and does not completely rule out the potential risk of cross infections and immunological responses from the recipient. As an alternative, alloplastic materials, in the form of engineered scaffold with osteoconductive properties, have been synthesized. However, according to a recent systematic review, the implantation of alloplastic material alone leads to limited or no periodontal regeneration, and the best options for care of intrabony defect regeneration remain the combination of bone grafts, guided tissue regeneration (GTR) and biological factors (4).

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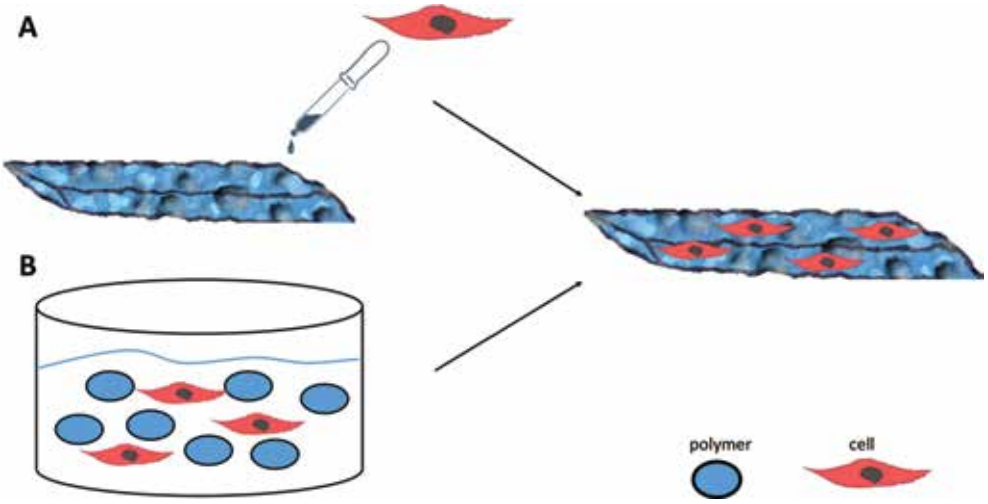

Fig. 1 - Cell delivery within bioengineered scaffolds: (**A**) cell seeding into a "prefabricated" scaffold; (**B**) cell encapsulation obtained during scaffold synthesis, where the formed hydrogel polymer matrix entangles the cells. In both cases, the final construct is composed of the scaffold itself and the target cell population (stem cell progenitors or differentiated cells).

Recent research in RPT has focused on scaffold design and physicomechanical properties, producing biomimicking devices able to successfully deliver biological mediators, related genes or cells themselves, into the local site of the intervention. Scaffolds, indeed, can be acellular, wherein cells are attracted by surrounding host tissue thanks to fibrin clot and bioactive molecules, or they can contain cells before implantation. In the latter case, stem cells or progenitor cells are incorporated usually by cell seeding within a "prefabricated" scaffold (Fig. 1A) or cell encapsulation obtained during scaffold synthesis, where the formed hydrogel polymer matrix entangles the cells (Fig. 1B). Biologically, an engineered scaffold should mimic the role of extracellular matrix of the target tissue, providing structural and physical support for cells to attach, proliferate, migrate and respond to signals. Functionally graded scaffolds, which match physicomechanical properties of tissues to be regenerated, have recently been developed with promising results, in terms of their finely tunable degradation, resistance to compression and their elasticity (5). Furthermore, to improve regenerative performance of scaffolds, their architecture can be implemented with some physical cues, such as specific surface topography and internal patterning, to influence the spreading of a certain cell population (6-8). Cells significantly respond to their substrate and are finely attuned to its differentiation, which can, finally, promote the regeneration of a specific tissue. This concept belongs to *mechanobiology,* a field that investigates how these physical changes can influence cell homing and thus tissue development, differentiation and neovascularization (9, 10).

A further achievement in RPT is scaffold customization according to the patient's anatomy, working in association with modern radiology. Morphology of alveolar bone and periodontal defects can be visualized via cone beam computed tomography (CBCT), which reproduces the tridimensional (3D) image bone anatomy using a low dose of X-rays compared with traditional CTs. The clinical and therapeutic advantages of a CBCT scan in RPT, however, are still debated – e.g., as to whether the therapeutic advantages override the disadvantages related to safety issues, including radiation exposure.

Porous scaffolds for cell hosting

Natural and synthetic compounds represent the 2 categories of biomaterials used for synthesizing porous scaffolds. Natural biomaterials are derived from natural sources (plant or animal). Bioceramics, collagen, chitosan, agarose, alginate and fibrin are some of natural biomaterials explored for RPT. Some of them, such as chitosan, may represent the best choice thanks to their antimicrobial activity against the risk of bacterial contamination when scaffolds are exposed to the oral environment.

All natural biomaterials show excellent biocompatibility (11, 12), but concerns regarding their immunogenicity hinder their application. In addition, questionable physical and mechanical stability reduces the possibility of load-bearing applications. Therefore, natural biomaterials have been reinforced by polymer cross-linking and/or by developing composites with synthetic materials, such as polyglycolic acid, poly(lactic-*co*-glycolic acid) (PLGA) and polyethylene glycol. Composite biomaterials are produced in a plethora of variations with high control of mechanical responses and degradation time. An example is the use of poly(L-lactic acid) (PLA) combined with alginate hydrogel for repairing dog spinal defects, which resulted in higher mechanical resistance and hierarchical porosity when compared with alginate alone, promoting in vivo new tissue formation (13).

Natural biomaterials

Bioceramics

HA, structurally similar to bone apatite, is the most commonly used bioceramic for periodontal engineering, but its slow resorbability and subsequent replacement by new bone may delay periodontal regeneration (14). Recently, HA has been proposed not only for bone regeneration, but also for soft tissue augmentation, and its resorption, in particular, has been fine-tuned by modulating size and crystallinity degree (15). In 1- and 2-wall periodontal defects, HA resulted in limited periodontal regeneration at 6 months, with long junctional epithelium, new cementum formation and fibrous tissue

response around HA particles (16). HA has been used in association with autografts to regenerate extensive periodontal defects: at 9 weeks, HA particles were encapsulated in fibrous tissue, and newly formed bone was found just around autograft residues (16).

Bicalcium phosphate is a precursor of HA, which, in combination with enamel matrix derivative (EMD) within periodontal defects, resulted in the formation of newly deposited cementum and inserting fibers, although HA was still encapsulated, with negligible evidence of new bone around it (16).

Among bioceramics, however, the most commonly explored compound is β-tricalcium phosphate (β-TCP), which is completely resorbed within 6-9 months from implantation and substituted with new bone, as demonstrated even in wide bone defects (14). During resorption, β-TCP provides calcium, magnesium and phosphate ions at the ideal ion concentration for the activation of alkaline phosphatase, a pivotal enzyme for the ossification process. β-TCP was investigated to treat 1- to 2-wall periodontal defects: after 6-18 months, it produced long junctional epithelium and, in a few cases, periodontal regeneration with weak signs of cementogenesis and osteogenesis (16). A tunnel-structured β-TCP material has recently been developed to enhance, in particular, bone deposition and it was tested in 1-wall intrabony defects in dogs. At 12 weeks, after surgery, newly formed bone, PDL and cementum-like tissue were observed; new bone and vessels were observed within tunnels (17).

To enhance its regenerative properties, β-TCP has been used in combination with growth factors. β-TCP added to platelet-derived growth factor (PDGF) showed a long junctional epithelium healing (16). Similarly, β-TCP particles were coated with growth differentiation factor-5 to treat intrabony defects, enhancing cementum, bone and PDL regeneration, but with no evidence of ankyloses or root resorption, although particles produced a foreign body reaction (16).

In a further implementation, β-TCP was combined with HA to provide the bioactive core structure with fast degradation giving biphasic calcium phosphate (BCP). BCP showed a tunable degradation rate by adjusting the ratio of HA and β-TCP phases. Clinical improvements in treating periodontal bone defects have been reported only in combination with EMD (18).

Another class of bioceramics includes bioglasses or bioactive glasses (BGCs), brittle materials composed of SiO_2, CaO and P_2O_5, not suitable for load bearing applications, but with high osteoconductive properties, very slow resorption and the interesting capacity to bond directly to soft and hard tissues (14). In the treatment of 1-, 2- or 3-wall defects, BGCs promoted healing with long junctional epithelium (16). Similar findings were reported for grade II furcation defects (19). When used in combination with EMD, instead, BGCs showed signs of periodontal regeneration with mineralized tissue around them (16).

Finally, coral-derived bioceramics, in the form of coralline HA and calcium carbonate, have been investigated as osteoconductive scaffolds, which are degradable by means of carbonic anhydrases of osteoblasts. In a dog model of 1-wall intrabony defects, they produced the same periodontal healing as the control site receiving autograft bone and well-organized PDL fibers were connected to neodeposited alveolar bone, with evidence of cementum regeneration (20). In humans, coralline HA was investigated in 1 clinical trial performed to treat 2-3 osseous wall intrabony defects, and although the type of healing was not clarified, bone formation was visible around graft particles (16).

Natural polymers

Naturally occurring degradable polysaccharides are commonly used in surgical reconstructions and in periodontal tissue engineering (11). They are derived from algae, as sodium alginate and agarose (21), from animals, as chitosan (22) or from polypeptides, as collagen and hyaluronic acid (16, 23). Recently, a biomimetic electrospun matrix in silk fibroin nanofiber was successfully proposed for oral mucosa repair, as evaluated in a rat model, revealing a promising alternative to acellular dermal matrix (24).

Collagen represents one of the most widely applied natural polymers, and in humans, it is the core protein of extracellular matrix found in several connective tissues. Collagen is thus the most abundant polypeptide in mammals and, as a biomaterial in periodontics, has largely been applied in the form of a biodegradable barrier during GTR. Collagen barriers allow periodontal regeneration (with defect resolution, new cementum and new inserting fibers), without leaving material residues 5 months after surgery; instead, no coronal bone overgrowth was reported (16). Collagen scaffold, as hydrogel, also facilitates periodontal wound healing (18), but to date, evidence of periodontal regeneration is limited (16).

Chitosan, a polysaccharide formed from D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), is a natural biomaterial able to induce antiviral activity and innate immunity in plants (25). It is a linear polysaccharide, made by treating the chitin shells of crustaceans with alkaline agents. Chitosan has recently been introduced in periodontal engineering for its biocompatibility, antibacterial and mucoadhesive properties, in the form of gel or freeze-dried scaffolds to enhance alveolar bone regeneration (26, 27). A further application is in the form of a biodegradable membrane for GTR) and for guided bone regeneration (22).

Chitosan scaffold, as hydrogel, was reported to lead to periodontal healing within intrabony defects (26). Yeo et al, in particular, found that chitosan porous membrane stimulated new deposition of bone and cementum within 1-wall intrabony defects, in beagle dogs (28).

One major concern is related to chitosan sterilization, since autoclaving showed a reduction in molecular weight, viscosity and gelification. Thus, to overcome this issue, a thermosensitive chitosan hydrogel, proposed as an injectable scaffold, was produced via autoclave treatment and β-glycerophosphate, resulting in suitable physicochemical and biocompatible properties, with evidence of periodontal regeneration in class III furcation defects in dogs (29).

Chitosan and collagen have been used in combination to form sponge-like scaffolds, used to treat 1-wall intrabony defects in dogs (30). Histology found that scaffolds enhanced the formation of bone and cementum (30).

Less frequently, alginate (a natural polysaccharide) and gelatin (a natural polypeptide) have been investigated, mainly as GTR or guided bone regeneration barriers rather than as scaffolds for periodontal regeneration. In vivo evidence of their efficacy in this field remains limited (31, 32).

Some interesting results can be found using gelatin combined with platelet-rich plasma (PRP) and blood clots, revealing successful results in bone regeneration and probing depth (PD) reduction (33, 34). Gelatin was used to obtain a "periodontal inspired" scaffold, synthesized via the freeze-casting technique (35). Despite having low resistance to compression, gelatin possesses important biological properties; among others, gelatin contains tripeptide Arg-Gly-Asp (RGD) motifs which mediate cell attachment (35). Composite PCL/gelatin nanofibrous scaffolds were electrospun to obtain a directionally oriented nanofiber membrane able to drive PDL stem cells (PDLSCs) stem cells under mechanical-stress conditions (36). In rat premaxilla periodontal defect models, these scaffolds were successfully integrated into the defect, enhancing bone formation (36).

Synthetic biopolymers

Synthetic biomaterials compose the first and second generations of membranes for GTR. The first-generation membranes are nonresorbable and include expanded polytetrafluoroethylene and dense polytetrafluoroethylene. Periodontal defect resolution with new cementum and new fiber attachment has been observed in several clinical reports, although without evidence of bone regrowth (16).

The second-generation membranes are composed by resorbable biomaterials and are currently the most widely used in RPT, as the risk of exposure is low, and there is no need for second surgery to remove the membrane. They are made of polyesters, such as polycaprolactone (PCL), poly(glycolic acid) (PGA) and PLA, alone or in combination (11). Due to hydrolysis, the compounds deriving from their degradation, although potentially toxic, are slowly released in negligible amounts, and resorption ranges from 4 to 8 months, with about 1% of residual particles (32). Synthetic resorbable PLA membranes for GTR showed some clinical success in terms of PD, clinical attachment level (CAL) loss in intrabony and class II furcation defects, with similar results for open flap debridement (32).

A further study reported no additional benefits of a PLA GTR barrier, combined with autogenous bone grafting, in regenerating severe intrabony defects, when compared with bone graft alone (37). A clinical trial compared the use of PGA/PLA membrane, with or without resorbable HA, with a connective tissue graft, for treating mandibular class II furcation defects (38). GTR therapy, with or without resorbable HA, showed clinical and radiographic improvements compared with flap debridement alone; in particular, GTR + HA promoted the healing of furcation lesions and the deposition of bone (38).

Furthermore, the same degradable synthetic polymers displayed biomechanical properties suitable for synthesizing scaffolds useful for periodontal tissue engineering. They have recently been investigated to deliver growth factors as well as to produce multilayer scaffolds by means of 3D printing techniques.

Mechanical cues to implement periodontal scaffolds: multicompartment approach to scaffold synthesis and 3D manufacturing

Scaffold multicompartmentalization and micropatterning

Compartmentalization allows controlling of the spatiotemporal biological processes, which result in effective regeneration of the periodontal apparatus, preventing tooth ankylosis, and enhancing deposition of bone and of PDL fibers directed in a functional manner. Indeed, RPT aims at a synchronized reestablishment of both soft and hard tissues. Thus a successful approach putatively includes spatial organization achieved by multicompartmental (or multilayer or multiphasic) 3D scaffolds. Here, the architecture and the chemical composition of each compartment match the organization and the cellular, mechanical and biochemical composition of those tissues to be regenerated (27, 39). Mechanobiology and the contact guidance concepts support the rationale underlying this approach, since cells can be guided by mechanical stress during their migration and differentiation. Nonetheless, a macroporous architecture with channels promoting transport phenomena during the delivery of nutrients and in the removal of waste products to and from the interior of cell-populated 3D scaffolds, may promote cell growth (40).

Gingiva regeneration has rarely been investigated through a multilayer approach. Lotfi et al developed a bilayer scaffold able to achieve gingival augmentation in dogs lacking keratinized gingiva (41). It consisted of a first dense layer made of a mixture of chitosan and polyvinyl alcohol, able to increase the scaffold strength and to suture the scaffold to its adjacent tissue, and of a second layer with a sponge-like structure made of chitosan (41).

Most researchers have attempted especially to restore the physiological bone-PDL architecture by promoting ligament integration with surrounding tissues. Microchanneled scaffolds for PDL regeneration, in particular, have been investigated, following the concept of contact guidance, where the cells adherent to the substrate follow its morphology (Fig. 2).

In the literature there are proposals to use several different types of micropatterned PCL structures. Park and colleagues developed a 3D printed PCL scaffold with a microchanneled structure that guided PDL fiber orientation (42-44). In this work, the fiber-guiding PCL compartment for PDL was computer-designed using 3D printing, although the same group lately proposed using a freeze-casting method to control pore architecture, mimicking the topographies of alveolar bone and PDL fibers (45). The same fiber-guiding PCL scaffold was further enriched with a compartment for delivery of recombinant human PDGF, and then used for filling the human periodontal osseous defect, without acute signs of chronic inflammation or dehiscence (46). This remained covered until the 12-month follow-up visit, showing a 3-mm gain of CAL and partial root coverage; after 13 months, however, the scaffold became exposed, and ultimately it had to be removed altogether (46).

Vaquette et al proposed a bilayer construct supporting cell sheets: It incorporated bone and PDL compartments made of PCL containing β-TCP (47). They demonstrated, in a

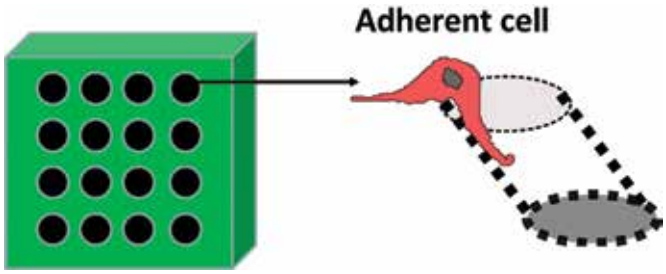

Fig. 2 - Contact guidance concept: cells adhere to the substrate and follow its morphology during migration. In the presence of hierarchical micropatterned scaffolds, cells orientate within microchannels which drive their movement.

rat periodontal ectopic model, the successful, simultaneous and spatially controlled in situ localization of those cells required for regenerating bone, PDL and cementum (47).

Recently, a bilayer scaffold composed of PCL electrospun membrane (to mimic PDL) and a chitosan-based construct (to mimic bone tissue) was developed, reporting in vitro differentiation of fibroblast and osteoblasts, respectively (48). A similar biphasic scaffold, used in combination with cell sheets, was developed by adding calcium phosphate to the bone compartment and by substituting the electrospun membrane for PDL with a thin melt electrospun scaffold with larger pores to enhance cellular interaction and neo-vascularization (49). In vivo, the revised construct produced functional PDL orientation (49).

Along the same lines, Lee et al investigated multicompartment PCL/HA scaffolds fabricated using 3D printing: it possessed a 100-µm microchannel compartment to regenerate the interface between cementum and dentin, a 600-µm microchannel compartment was designed for PDL, while a 300-µm microchannel compartment was intended for bone regeneration (50). The scaffold was used to support the adhesion of stem cells in combination with the delivery of 3 proteins (connective tissue growth factor, amelogenin and bone morphogenetic protein-2 [BMP-2]). In vivo results showed formation of oriented PDL fibers, which were inserted into bone, and dentin/cementum-like tissues (50).

The bone–PDL ligament interface has represented a further challenging aspect of periodontal regeneration. PLGA-based scaffolds were proposed for its regeneration. A semirigid PLGA/CaP bilayered construct had externally a membrane acting as barrier and helping to avoid collapse, while inside, a micropatterned compartment retained the blood clot. This was successfully tested in dog class II furcation defects (51). More recently, a bilayered system for supporting the adhesion of PDLSCs was made in the form of a platelet lysate/PLGA-based construct (52). In a rat 3-wall intrabony defect, the scaffold favored periodontal regeneration, independently from the seeding of PDLSCs, with significant connective tissue attachment formation and bone deposition (52).

Trilayered scaffolds intended to support the simultaneous regeneration of cementum, alveolar bone and PDL have been developed, although in most cases only animal evidence has been provided. A porous hydrogel was made with 3 compartments and enriched with growth factors as follows: (i) chitin-PLGA/nanobioactive glass ceramic (nBGC), loaded with cementum protein 1 (CEMP1), for the cementum regeneration; (ii) chitin-PLGA, loaded with fibroblast growth factor 2, for the PDL regeneration and (iii) chitin-PLGA/nBGC, loaded with PRP-derived growth factors, for the alveolar bone regeneration (53). After 1 and 3 months from implantation in periodontal defects in rabbits, the trilayered scaffold demonstrated complete periodontal healing and bone neodeposition, as evaluated with micro-CT and histology (53).

Furthermore, Varoni et al developed a micropatterned chitosan-based trilayer scaffold (27). It exploited different molecular weights of chitosan (low vs. medium) to obtain a bilayer structure for gingiva and alveolar bone healing. This was then assembled with a further layer intended for PDL, obtained by electrochemical deposition of the same polymer and showing an highly oriented micropatterning (27). The latter, having 450-µm-large pores, had been tested previously to provide evidence of enhanced neovascularization within the scaffold (10).

Scaffold 3D manufacturing

In recent decades, 3D printing has received a great deal of attention for the manufacture of customized scaffolds with fine-tuned architecture mimicking periodontal multitissue complexes and accurately adapting to the shape of patient defects. Three-dimensional printing overcomes the high heterogeneity of scaffold porosity and architecture related to conventional methods of synthesis, such as particle leaching, freeze-drying and solution casting (54), and enables personalized medicine intended to produce a scaffold that fits the individual periodontal defect.

The revolutionary introduction of computer-aided design (CAD) and computer-assisted manufacturing (CAM) have allowed the development of solid freeform fabrication techniques, referred to as rapid prototyping (RP). RP makes it possible to obtain, in a highly reproducible manner, complex scaffolds composed of both natural and synthetic biomaterials, with precise external and internal 3D architecture. Solid freeform fabrication includes additive manufacturing technologies, producing scaffolds layer by layer via 3D printing. Among others, these include laser-assisted printing (such as stereolithography and selective laser sintering), inkjet printing and extrusion printing (fused deposition modeling) (55). When the process involves not only scaffolds (usually hydrogels), but also the contextual deposition of living cells, the technique is called bioprinting, and it allows cell encapsulation and cell-based therapies with a high degree of control of the position of cells within the scaffold. Furthermore, it mimics the tissue interface and the surrounding microenvironment.

In a clinical scenario, CAD models for 3D printed scaffolds will be based on images taken from radiographs of a patient-specific bone defect, particularly using CBCT in periodontal regeneration. Recent studies have demonstrated that CBCT can be applied in periodontology to determine the size and shape of the periodontal defect and then fabricate a scaffold, which is tailored to the specific patient. In periodontics, CBCT has been proposed for both diagnosis and treatment planning, as well as evaluation of treatment outcomes. Several in vitro and in vivo studies have shown that CBCT is a much

more sensitive diagnostic tool than intraoral radiography for the detection of periodontal defects (56-59). A minimum voxel size of 0.150 mm^3 seems to be required for the detection of periodontal defects (57). A recent systematic review (60) looked at CBCT utility in periodontology (from diagnostics to patient management) and found it provides the most accurate assessment of vertical bony defects; however, the recommendation is to use it on a case-by-case basis. Authors of that review discourage routine use because of the high irradiation risk and not always favorable cost-benefit balance. According to the review, furcation-involved second maxillary molars stand to benefit the most from CBCT to evaluate the degree of furcation involvement, as intraoral radiographs often fall short in this area (60).

CBCT has even been applied to assess bone level changes following RPT. It showed the potential to replace surgical reentry as the technique of choice for assessing therapy outcomes (61). Regarding geometric accuracy, a systematic review (62) has reported mean measurement errors ranging from 0.19 ± 0.11 mm to 1.27 ± 1.43 mm, with no consistency among studies regarding whether the deviation is due to overestimation or underestimation of the defect. A difference in measurement error depending on the position of the defect has also been reported, with vestibular sites having a greater measurement error (62). The authors of that review were unable to perform a meta-analysis due to incomplete information, and they warned that given the high heterogeneity between studies, the results cannot currently be used as the basis for any recommendation of the use of CBCT for assessment of periodontal defect geometry when high precision is needed (62).

The field of CBCT imaging is changing quickly, and while this imaging modality is a good diagnostic tool for the detection of periodontal defects, higher radiation doses and measurement errors can still pose a challenge for its widespread use. More up-to-date research is needed, as studies regarding accuracy performed using earlier generation devices may not apply to the current technology.

Custom-made scaffolds for periodontal regeneration, however, are possibly not far from being produced. As described in the previous paragraphs, 3D RP was recently applied to synthesize multicompartmentalized PCL-based scaffolds having a PDL fiber-guiding side and custom made on periodontal defects (47, 49, 50, 63), although this was unsuccessful in clinical application (46, 50). Authors suggest that 3D printing's low resolution and the very slow degradation rate of PCL, as well as low cell affinity and low osteoconductivity, may explain the adverse outcomes. Current RP techniques, indeed, are not always able to control precisely the overall geometrical design and porosity – depending on the machine's resolution and material repertoire. The combination of RP with other fabrication methods, such as electrospinning, may allow more and more efficient constructions. In addition, despite the addition of bioceramics in the construct modulating synthetic polymers' biodegradation, the amount of HA, in these reports, might not have been sufficient to accelerate the PCL degradation profile (35). Furthermore, the biological variations between species (rodents and humans) may have further hampered the straightforward transfer of findings from preclinical to clinical trials (35).

Biological cues to implementing periodontal scaffolds: bioactive molecule delivery

Several bioactive molecules as biological mediators have been proposed to promote periodontal regeneration, and scaffolds represent a useful tool to support their local delivery.

In a recent randomized clinical trial (phase IIa, involving 10 patients), recombinant human growth differentiation factor-5 delivered in a β-TCP carrier resulted in greater PD reduction and CAL gain, with histological evidence of periodontal regeneration without root resorption (18).

A further multicenter randomized controlled study evaluated the effectiveness and safety of 3 different concentrations of recombinant human fibroblast growth factor 2 (rh-FGF-2; at 0.1%-0.3% and 0.4%) loaded in a β-TCP scaffold for treating vertical infrabony periodontal defects in adult patients (64). Considering as outcomes a gain in CAL of 1.5 mm and a bone growth of 2.5 mm, the 0.3% and 0.4% rh-FGF-2/β-TCP concentrations showed significant improvements over controls as well as over 0.1% rh-FGF-2/β-TCP, with a 71% 6-month clinical success rate, with 75% and 71% bone fill for 0.3% and 0.4% rh-FGF-2/β-TCP, respectively (64).

The same growth factor, FGF-2, was loaded in collagen sponges, cross-linked using an ascorbate–copper ion system, to favor regeneration in furcation defects in beagle dogs (54). At 4 weeks after surgery, alveolar bone deposition and periodontal attachment formation, with cementum-like and PDL-like tissues, were observed (54).

A similar collagen membrane was used for local delivery of stromal cell–derived factor-1 (SDF-1); it specifically recruited progenitor host stem cells via C-X-C motif receptor 4 (CXCR4) (65). It was tested in mandibular wounds of Wistar rats and resulted in the successful local recruitment of host-derived mesenchymal stem cells (MSCs) and hematopoietic stem cells, inducing early bone osteoclastogenesis and early scaffold degradation (65).

Finally, some studies have described scaffolds including more than 1 bioactive molecule. A scaffold composed of mesoporous bioglass (MBG) together with silk fibrin scaffold was loaded with BMP-7 and/or PDGF-B (66). It showed partial regeneration of the PDL, mainly improving new bone formation; the 2 factors appeared to act synergistically (66). A PCL-based electrospun multiphasic scaffold, enriched with type I collagen, was used to deliver nanoparticles made of poly(ethylene glycol)–stabilized amorphous calcium phosphate, further enriched with recombinant human CEMP1 (67). This composite scaffold showed, in a critical size defect using a rat model, cementum-like tissue formation, but little bone formation (67).

Cell-based therapy in RPT

Cell encapsulation is defined as the entrapment of viable cells within a membrane or a homogenous hydrogel, produced by cross-linking of polymers, which form a "protective capsule" for the cells. Cell encapsulation has the advantages of defending cells from the surrounding milieu, including the recipient's immune system and tissue mechanical stress, and in case of stem cells, of promoting in situ cell differentiation (68). To date, the most commonly

used methods for cell encapsulation are electrostatic spray and microfluidic channel or nozzle, while the major applications are in bone and cartilage, heart, liver and lung tissue engineering (68).

In RPT, cell encapsulation ideally aims at arranging cells within scaffolds mimicking in vivo localization of gingival fibroblasts, alveolar bone osteoblasts, periodontal ligament fibroblasts and cementoblasts. From this perspective, cell encapsulation can be combined with bioprinting, which allows cell localization into a desired geometry (68).

Among cell types under investigation, stem cells have received growing attention in recent decades. Stem cells are defined as totipotent, pluripotent, multipotent or unipotent depending on their differentiation capacity, and embryonic, postnatal or reprogrammed, depending on their derivation. Adult stem cells, including those from adipose tissue, can indeed renew and differentiate, representing a key source of progenitors that have been studied for their potential ability to differentiate into specific cells for periodontal regeneration (69, 70).

Different stem cell populations have been isolated from adult human teeth and periodontal apparatus. Dental MSCs, also called dental pulp stem cells (DPSCs), typically derive from third molars' dental pulp, but dental MSCs have also been isolated from the PDL (i.e., PDLSCs). In vivo, they display a noticeable ability to give rise to cementum-like and PDL-like tissues (71). Recently, a certain regenerative effect was associated also with inflammatory dental pulp tissues' stem cells (DPSCs-IPs). At 9 months after surgery, Li et al showed in humans their ability to regenerate new bone to promote the healing of periodontal lesions (72).

A further source of MSCs is bone marrow-derived mesenchymal stem cells (BMSCs) (70), showing similar properties to PDL-derived cells, as reviewed in a recent meta-analysis (71). Stem cell–based approaches have been reported to show favorable effects in periodontal tissue engineering, promoting new cementum, PDL and alveolar bone formation in periodontal defects, leading to support for the concept of a stem cell–based therapy in periodontal regenerative medicine (71).

A nano-HA/collagen/PLA construct was used in combination with PDLSCs and implanted subcutaneously into dogs, revealing enhanced osteogenic capacity (73). Always in combination with PDLSCs, a new synthetic polymer, called poly(isosorbide succinate-co-L-lactide) (Pis-PLLA), was compared with simple PLLA, after collagen, HA and BMP-7 loading (74). Tested in fenestration defects in rat jaws, PLLA/collagen/HA showed showing better osteoconductivity, while Pis-PLLA/collagen/HA better osteoinductivity (74). In addition, PDLSCs, seeded on β-TCP scaffolds, were also transfected with human osteoprotegerin (hOPG) to reduce osteoclastogenesis, and the complex was tested in rabbits to regenerate bone lesions: earlier mineralization and enhanced bone formation within the scaffold were reported after 12 weeks from implantation (75).

Using the above-described multiphasic approach, a 3D-printed PCL/HA construct resulted in periodontal regeneration. It was seeded with alveolar bone stem cells (ABSCs), PDLSCs and DPSCs, and loaded with biological mediators (amelogenin, connective tissue growth factor and BMP-2). Upon in vivo implantation, DPSC-seeded multiphase scaffolds resulted in highly oriented PDL-like collagen fibers, inserted into bone and dentin and cementum–like tissues (50).

BMSCs, seeded onto PLGA/PLC electrospun scaffolds, have been also investigated, appearing to be successful in periodontal regeneration in a rat model (76). In particular, the preimplantation chondrogenic differentiation strategy allowed researchers to obtain optimal periodontal regeneration (alveolar bone and PDL) (76).

Besides scaffolds, in recent decades, cell-based therapy (11, 69, 70, 77), using either stem cells or already differentiated cells, has followed 2 innovative approaches for cellular local delivery: cell sheet technology and cell transfer.

Cell sheet technology

Poly(N-isopropylacrylamide) (PIPAAm) is a thermoresponsive synthetic polymer, which allows cell adhesion or detachment from the surface, according to temperature of the environment (78). At 37°C, a PIPAAm surface is slightly hydrophobic, enabling cell adhesion and proliferation; under 32°C, the PIPAAm surface becomes hydrophilic, where cells, forming a sheet, can easily detach. This process allows harvesting of the cell sheets, avoiding proteolytic enzymes (such as trypsin), thus preserving the structure of cell membrane proteins which mediate adhesion, mainly laminin 5 and E-cadherin. Cell sheet technology, with PDLSCs and human umbilical vein endothelial cells (HUVECs), was successfully tested for periodontal regeneration in an ectopic periodontal model in nude mice (79). A coculture of PDLSCs and jaw BMSCs was further investigated (80).

Cell sheet technology has also been verified as a part of a biphasic β-TCP/PCL construct for the regeneration of both periodontal and bone tissues in rats. The PCL construct should provide, during the healing, mechanical support to cells (47). Fused deposition modeling was applied to produce β-TCP/PCL compartment for bone, which was further integrated with a PCL membrane, synthesized by electrospinning, intended for hosting PDL cell sheets. The authors demonstrated the successful regeneration of cementum, alveolar bone and PDL (47). Since no functional PDL fiber orientation was detectable, the same group synthesized a further implementation of the scaffold, adding a functional micropatterning to the PDL compartment with concentric rings (49). In this case, the scaffold resulted in enhanced bone formation with evidence of oriented PDL fibers and neovascularization (49).

Similarly, Dan et al described bone- and PDL-derived cell sheets, which when placed on a electrospun PCL scaffold enriched with calcium phosphates (CaP-PCL), favored in particular periodontal attachment and alveolar bone formation (81).

Cell transfer technology

Cell transfer technology is a new method of cell manipulation, which enables the in situ delivery of cells using scaffolds, composed of an amniotic membrane in overlapping bilayers. This showed a high capacity to provide growth factors and elements for osteogenic differentiation (82). A plethora of adherent "cell population blends" have been used with this intention, including PDLSCs and osteoblasts, and Akazawa

et al emphasized that their structure was not altered by material deformations, but significantly improved bone deposition (83).

Concluding remarks

RPT includes novel highly biomimetic approaches related to significant improvements in scaffold synthesis and performance. From the perspective of complete periodontal regeneration, scaffolds have been implemented with physical and biological cues to drive contextual multitissue regeneration, including scaffold compartmentalization, surface micropatterning and delivery of stem cells and biological mediators. In addition, the RP technique, with or without cell encapsulation, has enabled the fabrication of custom-made scaffolds fitting the periodontal defects, although only preclinical evidence is available to date, and the radiation risk associated with CBCT hampers its applicability.

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