Current and Future Views on Biomaterial Use in Regenerative Endodontics

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5.1 Introduction

Dental pulp plays a vital role in tooth development as it harbours progenitor/stem cells (DPSCs) that proliferate and differentiate into dentine-secreting odontoblasts (Gronthos et al. 2002). Dental trauma and the bacterial infection (caries) of dental pulp lead to inflammation, and if left untreated, pulpal necrosis and apical periodontitis will eventually develop (Albuquerque et al. 2014a; Galler 2016). While trauma is more commonly associated with an accidental injury (Andreasen and Kahler 2015), caries may depend on several variables, especially those related to poor oral hygiene and sugar intake (Selwitz et al. 2007).

Traumatic dental injuries have recently become a public health problem worldwide (Zaleckiene et al. 2014); they are more prevalent in the permanent than the primary dentition and occur in earlier stages of life, i.e. before age 20 (Glendor 2009). Concerning caries, data from the latest National Health and Nutrition Examination Survey (Dye et al. 2015) revealed ~21% of children aging 6–11 years and ~58% of adolescents aging 12–19 years have experienced dental caries in their permanent dentition. When properly managed, tooth decay is a reversible condition; however, if neglected, caries may induce inflammatory reactions at the pulp, leading to tissue necrosis and, ultimately, root canal therapy (Larsen and Fiehn 2017).

In the United States, over 15 million patients undergo root canal therapy each year (American Association of Endodontics 2016), resulting in a major socioeconomic burden. Traditional root canal treatment remains the standard of care for

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mature, fully developed teeth with pulpal necrosis, and it involves the chemomechanical debridement and sealing of the canal system with an inert rubber-like material (Huang 2011). However, immature permanent teeth present a very unique anatomy, i.e. wide open root apex and thin root dentinal walls (Albuquerque et al. 2014a; Galler 2016; Diogenes et al. 2014). Given the wide open root apex, which halts the possibility of achieving an apical seal and thin dentinal walls, performing traditional root canal therapy on necrotic immature teeth is not advisable (Albuquerque et al. 2014a; Galler 2016; Diogenes et al. 2014). Thus, apexification using either calcium hydroxide ($Ca(OH)_2$) or mineral trioxide aggregate (MTA) has been used to induce apical closure (Cvek 1972, 1973; Damle et al. 2012). Although apexification supports apical closure, it neither promotes root development nor restores the immunologic competence of pulp (Jeeruphan et al. 2012; Wang et al. 2010). Additionally, apexification eliminates any further chance for complete root development (e.g. dentinal wall thickening and apical maturation), thus increasing the chance of future root fracture (Cvek 1992; Diogenes et al. 2016).

Regeneration of the pulp-dentine complex holds the promise of extending the function of the natural dentition, particularly in cases where traumatic injuries to permanent immature teeth halt root maturation and full development (Albuquerque et al. 2014a, b; Diogenes et al. 2014, 2016). The clinically available regenerative strategy, namely, evoked bleeding (EB), employs intracanal medications, including triple (TAP, ciprofloxacin, metronidazole and minocycline) and double (DAP, minocycline-free) highly concentrated antibiotic pastes or $Ca(OH)_2$. Following proper disinfection, the intentional laceration of periapical tissue is performed to provoke bleeding allowing intracanal delivery of apical stem cells and formation of a fibrin-based scaffold which induces tissue regeneration (Albuquerque et al. 2014a; Galler 2016; Diogenes et al. 2014, 2013). It is worth noting that this form of medication was introduced into the clinics without precise information as to the therapeutic dose to be used, which would retain its antimicrobial effect while minimising its toxicity on host tissues and cells. There is a compelling level of data indicating that both intracanal medicaments and chemical irrigants can negatively affect the survival and function of dental stem cells (Albuquerque et al. 2014a; Galler 2016; Diogenes et al. 2014). Meanwhile, the blood clot-derived fibrin scaffold might not be the most ideal matrix to act as a scaffold. Thus, the purpose of this chapter is to discuss the latest discoveries on biocompatible strategies of root canal disinfection and the use of biomaterials (scaffolds), stem cells and growth factors in dental pulp tissue regeneration.

5.2 The Role of Disinfection in Regenerative Endodontics

In regenerative endodontics, both root canal disinfection and blood clot formation (i.e. fibrin-based scaffold) have been shown to play a critical role in new tissue formation and overall root maturation and development. Despite these promising results, the biological outcome of the therapy is rather unpredictable (Diogenes et al. 2013; Banchs and Trope 2004; Bose et al. 2009; Cehreli et al. 2011; Iwaya

et al. 2001; Petrino et al. 2010). Bone healing and root development do not necessarily confirm the formation of tissue that closely resembles the pulp-dentine complex within root canals. In fact, the histological examination of tissue formed inside the root canals of teeth treated with regenerative procedures reveals apposition of a cementum-like tissue, which is responsible for canal narrowing and an increase in length (Gomes-Filho et al. 2013; Martin et al. 2013). Additionally, the ingrowth of a connective tissue similar to the periodontal ligament, along with a bone-like tissue, was identified inside root canals (Diogenes et al. 2013; Lin et al. 2013; Becerra et al. 2014). Unpredictability of the histologic results could relate to many factors (Albuquerque et al. 2014a; Galler 2016; Diogenes et al. 2014, 2013). It has previously been understood that inflammation derived from TAP remnants (da Silva et al. 2010) or from the healing process itself (Wang et al. 2010) could impair the formation of new pulp tissue. More recently, the effect of residual bacteria on the outcome of pulp regeneration was confirmed in vivo (Verma et al. 2017), thus showing a strong association with inflammation. In that study, residual bacteria were found to be mostly located in the coronal part of the canal, far from the inflammation zone and regenerated tissues located at the apical region. The authors stated that, at the coronal part of the canal, bacteria have the ability to grow faster than at the apical part, where vital tissues are present and the host response is strongly available to control infection. Interestingly, a study by Vishwanat et al. (Vishwanat et al. 2017) demonstrated that residual biofilm arrested within the intracanal space may promote osteoblastic versus dentinogenic gene expression of stem cells from the apical papilla (SCAP), preventing the formation of new dentine-like tissue. For those reasons, true pulp regeneration remains a clinical challenge, especially due to the presence of residual bacteria/biofilm within the root canal system. Therefore, antimicrobial strategies that assure a strong disinfection capacity without jeopardising the environment, such as enabling stem cell differentiation, are still needed.

5.2.1 An Overview of Conventional Antimicrobial Therapy

Originally, the first antimicrobial agent used in regenerative endodontic protocols was the so-called triple antibiotic paste (TAP) (Hoshino et al. 1996). Its mixture is efficient against a wide range of oral pathogens that are important in polymicrobial infection naturally found in necrotic teeth. Several studies have confirmed the positive effects of TAP in disinfecting the root canal system (Banchs and Trope 2004; Albuquerque et al. 2015a), although it may not eliminate all cultivable bacteria. In the study by Windley et al. (Windley 3rd et al. 2005), TAP was able to eliminate approximately 75% of the total amount of pathogens. It can be assumed that infected teeth are more resistant to disinfection when compared to soft tissues due to the presence of dentinal tubules. Indeed, bacteria may form planktonic and biofilm cultures, limiting complete action of antimicrobial agents (Diogenes and Hargreaves 2017). Consequently, residual bacteria may stay entrapped within the depth of the dentinal tubules, surviving the disinfection protocol. Even though TAP is broadly used worldwide, some case reports have revealed that this triple mixture may stain

the tooth, provoking strong discolouration and unpleasing aesthetic issues (Albuquerque et al. 2015a; Kahler and Rossi-Fedele 2016; Porter et al. 2016). This is a consequence of the minocycline component, and in order to eliminate this staining side effect, minocycline has been replaced by nonstaining antibiotics, including but not limited to amoxicillin/clavulanic acid (Nosrat et al. 2013), clarithromycin/fosfomycin (Mandras et al. 2013) and cefaclor (Ruparel et al. 2012). Antimicrobial pastes containing only two antibiotics have been proposed, as in the case of double antibiotic paste (DAP) comprised of ciprofloxacin and metronidazole (Iwaya et al. 2001). Notwithstanding, bacteria elimination is usually greater when TAP is used, compared to DAP (Latham et al. 2016).

In spite of their antimicrobial effects or discolouration potential, antibiotic pastes used in regenerative endodontics are known to pose a critical risk to regeneration, since the high concentration of antibiotics is potentially toxic to DPSCs and SCAP (Galler et al. 2015; Althumairy et al. 2014; Martin et al. 2014). Additionally, it has been demonstrated that TAP may inhibit the release of growth factors from dentine (Galler et al. 2015), thus potentially affecting the regenerative process. One attempt has been made to reduce the final cytotoxicity of antibiotic pastes, i.e. reducing the concentration of medicaments. According to the study by Latham et al. (Latham et al. 2016), which compared the antimicrobial and cytotoxic potential of TAP and DAP at different antibiotic concentrations (e.g. 0.1, 1 and 10 mg/mL), the less concentrated pastes, though being non-toxic to stem cells, were not potent enough to eliminate bacteria, thus allowing a greater number of viable bacteria in the dentinal tubules when compared to the more concentrated formulations. On the other hand, pastes containing 10 mg/mL of antibiotics were effective in reducing the level of viable bacteria, but they were also associated with greater cytotoxicity. In a similar study by Ruparel et al. (Ruparel et al. 2012), the authors tested the effects of TAP and DAP at concentrations of 0.01, 0.1, 1, 10 and 100 mg/mL; they found that concentrations below 1 mg/mL had no detectable effect on stem cell survival (nearly 100% cell survival), whereas concentrations of 1, 10 and 100 mg/mL resulted in approximately 58%, 8% and 1.3% of stem cell survival, respectively. Thus, an impasse exists between the maximum disinfection potential and minimal cytotoxic effects of conventional TAP and DAP pastes. To that end, alternative antimicrobial strategies have been proposed, which will be the focus of the next section.

5.2.2 Nanofibrous Intracanal Drug Delivery Systems

In recent years, electrospinning or electrostatic spinning, a textile technology, has been employed to fabricate antibiotic-containing polymer-based nanofibers for drug delivery applications in dentistry (Albuquerque et al. 2014a; Bottino et al. 2012, 2013). The theory behind the use of antibiotic-containing nanofibers as a three-dimensional (3D) tubular drug delivery construct (Albuquerque et al. 2014a; Porter et al. 2016; Bottino et al. 2015) that can be placed inside the root canal system of necrotic teeth (Fig. 5.1) is based on the fact that the addition of low antibiotic

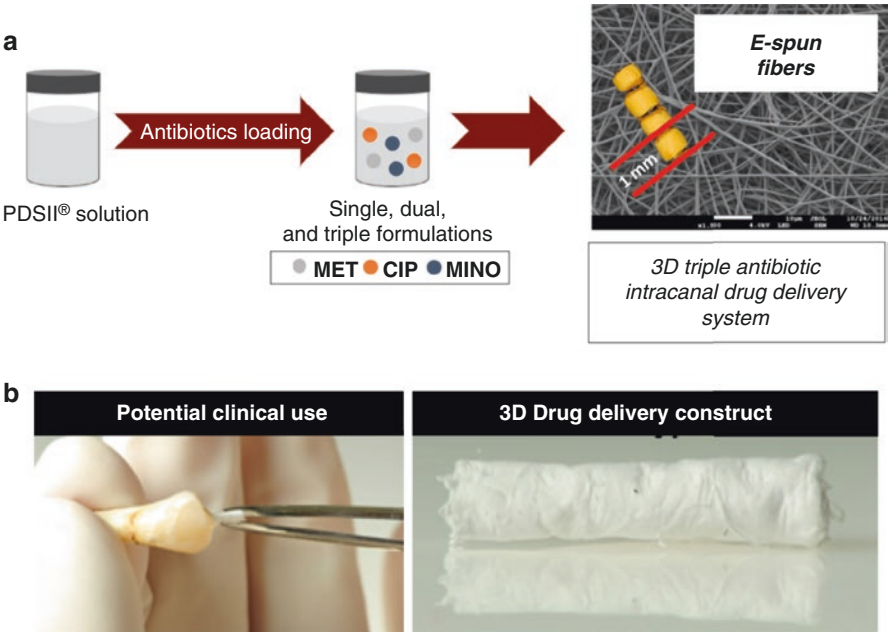


Fig. 5.1 (**a**) Synthesis of triple antibiotic-eluting nanofibers. Polymer solubilisation in hexafluoro-2-propanol. Single, dual or triple antibiotic incorporation (MET, CIP and MINO) into the solution before electrospinning. Representative scanning electron micrograph (SEM) of triple antibiotic-containing fibres and 3D constructs (in yellow, superimposed on the SEM image). Image obtained from reference (Palasuk et al. 2014), with permission. (**b**) Potential clinical use of the recently developed 3D patient-specific drug delivery construct. Image obtained from reference (Bottino et al. 2017), with permission

concentrations and the slow drug release provided by these nanofibrous constructs will eradicate infection and thus create a bacteria-free environment favourable to tissue regeneration (Albuquerque et al. 2014a, 2015a, b, 2016; Porter et al. 2016; Bottino et al. 2013; Palasuk et al. 2014; Kamocki et al. 2015a, b).

In electrospinning, a polymer solution/melt containing the desired concentration of antibiotics is prepared in order to produce nanofibers (Albuquerque et al. 2014a; Bottino et al. 2012, 2013). The chosen polymer solution can be incorporated with one or a combination of antibiotics, making it possible to fabricate fibres with a narrow or wide spectrum of action (e.g. ciprofloxacin [CIP], metronidazole [MET] and minocycline [MINO], among others) that have been shown to inhibit the growth of endodontic pathogens (Bottino et al. 2013; Palasuk et al. 2014; Kamocki et al. 2015a, b). In a recent study, CIP-containing polymer nanofibers were tested against *Enterococcus faecalis* (*Ef*) biofilms developed on human root fragments (Albuquerque et al. 2015b). An *Ef* suspension was inoculated on dentine specimens for 5 days to enable biofilm formation which were then exposed (direct contact) to CIP-containing (5 and 25 wt.%CIP) nanofibers. A thick biofilm mass was observed using scanning electron microscopy (SEM) along the whole root segment, with a

remarkable concentration of bacteria on the middle third, likely due to intrinsic substrate characteristics (e.g. uniform distribution of dentinal tubules and a similar tubule diameter) (Wang et al. 2012). Antimicrobial assays involving the use of colony-forming units (CFU) and SEM methodologies found that this young *Ef* biofilm was susceptible to 25 wt.%CIP nanofibers, demonstrating maximum bacterial biofilm elimination (Albuquerque et al. 2015b).

In an attempt to improve the antimicrobial effects of these unique nanofibers and based on several studies using TAP as the standard of care in regenerative endodontics, our group was the first to develop triple (MET, CIP and MINO) antibiotic-eluting nanofibers (Albuquerque et al. 2015a). The chosen bacteria, *Actinomyces naeslundii*, consisted of uncommon bacterial species used for in vitro studies in endodontics; however, it has been recently associated with root canal infections, particularly in cases of undeveloped traumatised teeth (Nagata et al. 2014). *A. naeslundii* was cultured on dentine specimens for 7 days to allow for biofilm formation on the surface and inside dentinal tubules. Infected specimens exposed to triple antibiotic-eluting nanofibers (i.e., TAP scaffold) revealed significant bacterial death based on confocal laser scanning microscopy (CLSM) data when using the Live/Dead Cell assay (Fig. 5.2) (Albuquerque et al. 2015a).

Noteworthy, the aforementioned studies focused on facultative anaerobic bacteria; however, root canal colonisation, mainly in primary infections, is often composed of strict anaerobic species (Gomes et al. 2004). Therefore, recent research (Albuquerque et al. 2016) used *Porphyromonas gingivalis* to induce a 7-day biofilm on human dentine through the careful limitation of environmental conditions. The established *P. gingivalis* biofilm was also susceptible to triple antibiotic-eluting nanofibers (Albuquerque et al. 2016).

Further research related to the fabrication of antibiotic-eluting fibres has focused on improving the staining limitation discussed previously. One study in particular (Porter et al. 2016) tested the effects of different TAP pastes and triple antibiotic-eluting nanofibers formulated with either minocycline or doxycycline on dentine colour changes. The authors observed that nanofibers containing minocycline or doxycycline produced similar colour changes when compared to their respective TAP systems, although the doxycycline-treated groups presented less discolouration than those treated using conventional minocycline. A different study by Karczewski et al. (Karczewski et al. 2018) intended to replace minocycline with clindamycin, i.e. another potent antibiotic with a wide spectrum of action, testing the antimicrobial properties, cell compatibility and dentine discoloration. The authors demonstrated that these modified antibiotic-eluting nanofibers may present remarkable antimicrobial effects, a cell-friendly behaviour (biocompatibility) and a stain-free property (Fig. 5.3), since no discoloration was seen. Not less important, clindamycin has demonstrated in vitro proangiogenic activity (Radomska-Lesniewska et al. 2010), which may be considered a critical step to the recreation of the dentine-pulp complex, since angiogenesis is essential for oxygen and nutrient transport to regenerated cells (Saghiri et al. 2015). Conversely, MINO was revealed to negatively affect angiogenesis by decreasing vascular endothelial growth factor secretion and suppressing neovasculogenesis of endothelial cells (Li et al. 2014). In

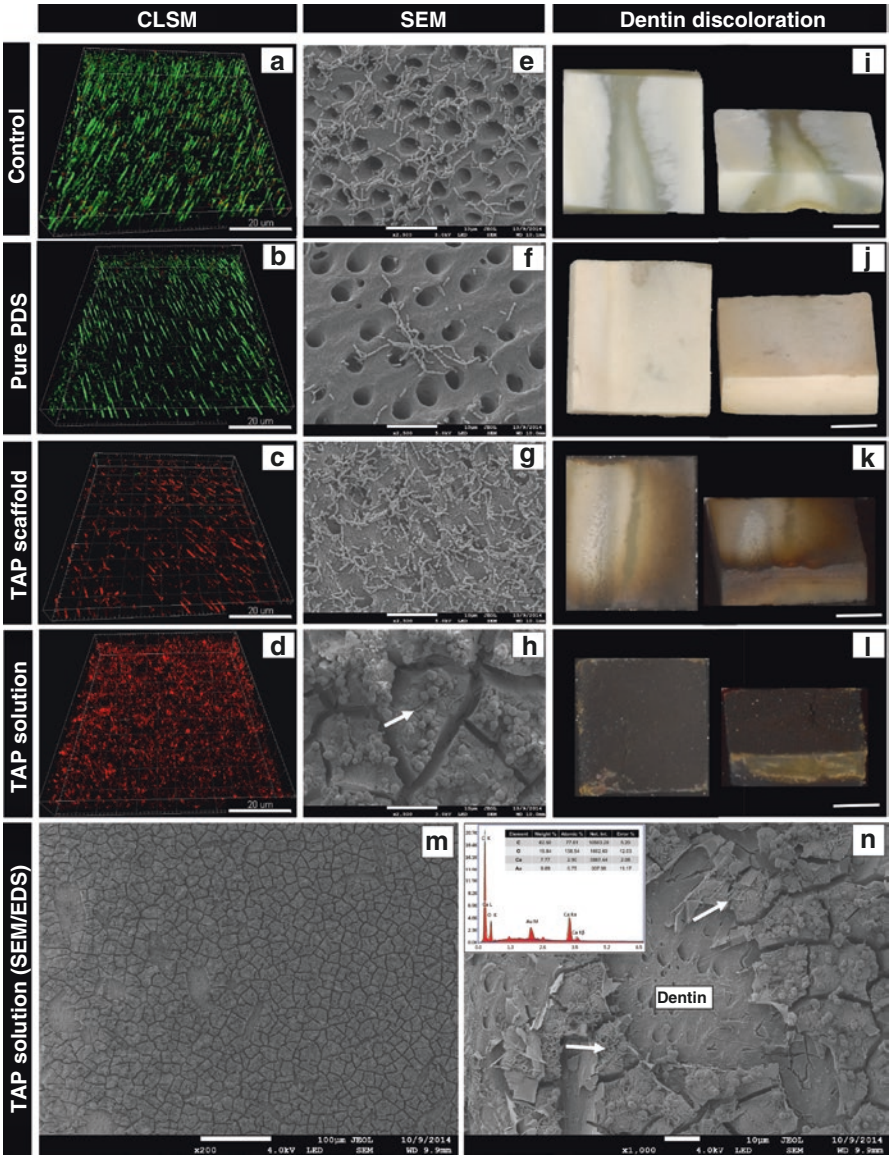


Fig. 5.2 CLSM images were collected in sequential illumination mode by using 488 and 552 nm laser lines. Fluorescent emission was collected in two HyD spectral detectors with filter range set up to 500–550 and 590–655 nm for green (SYTO9) and red dye (PI), respectively. CLSM macrophotographs of 7-day *A. naeslundii* biofilm (negative control) growth inside dentinal tubules (**a**), infected dentine treated with pure PDS (**b**), TAP scaffold (**c**) and TAP solution (**d**) for 3 days. SEM images of *A. naeslundii* biofilm on the dentine surface (negative control) (**e**) treated by pure PDS (**f**), TAP scaffold (**g**) and TAP solution (**h**). Dentine discolouration images of negative control (**i**), pure PDS (**j**), TAP scaffold (**k**) and TAP solution (**l**) groups. Representative SEM images (original magnification, ×200 and ×1000) of TAP solution-treated dentine showing calcium-enriched (Ca) insoluble agglomerates attached to the dentine surface (**m**) and covering dentinal tubules (**n**) as demonstrated by energy-dispersive X-ray spectroscopy (EDS) analyses (inset EDS image **n**); *A. naeslundii* can be seen on the surface of this insoluble complex (white arrows) (**n** and **h**). Image obtained from reference (Albuquerque et al. 2015a), with permission

Fig. 5.3 Representative macrophotographs showing human dentine colour stability/change after 1, 7, 14 and 21 days of exposure to control (PBS), antibiotic-free (PDS), CLIN and CLIN-m nanofibers and triple antibiotic paste (TAP). Image obtained from reference (Karczewski et al. 2018), with permission

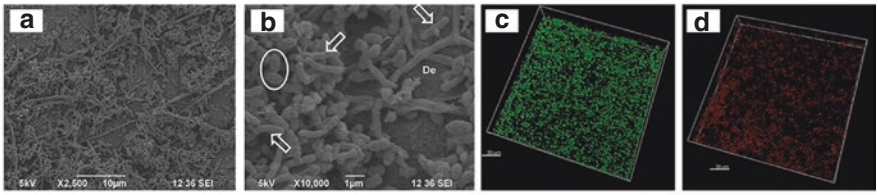
light of this, replacing MINO by clindamycin into TAP formulations would contribute to the use of a more bioactive antimicrobial agent and perhaps to improved cell survival and angiogenesis.

Collectively, our studies have provided abundant background information to not only test the antimicrobial efficacy of these nanofibers on multispecies biofilms in vitro but to also explore their clinical efficacy using preclinical animal models of periapical disease (Fig. 5.4).

5.2.3 Alternative Antimicrobial Strategies

Other strategies, in lieu of using antibiotic-eluting nanofibers, have also demonstrated important root canal disinfection. For example, the 2010 study by Sousa et al. (Sousa et al. 2010) prepared novel amoxicillin-loaded microspheres constituted of poly(D-L-lactide-co-glycolide) and zein (i.e. a class of prolamine protein), showing effective antimicrobial activity against *Ef*. The authors also demonstrated that, by varying the content of zein, the release of amoxicillin could be modulated to a level where it could achieve a more effective intracanal dressing, thus improving the disinfection potential of the microspheres. Another strategy showing

❑ **Antimicrobial activity**



❑ **Clinical translation**

Fig. 5.4 Antimicrobial activity of triple antibiotic-eluting nanofibers against a 7-day dual-species (*Actinomyces naeslundii* and *Enterococcus faecalis*) biofilm formed on dentine specimens. (**a**) Lower-magnification SEM image showing a homogeneous distribution of the two bacterial cells. (**b**) Higher-magnification SEM image revealing the rod-shaped *A. naeslundii* (arrows) and cocci-shaped *E. faecalis* (circle) bacterial cells over the dentine (De) surface. Confocal laser scanning micrographs of (**c**) 7-day dual-species biofilm growth on dentine (live bacteria = green) and (**d**) confocal image showing the elimination of most of the bacteria (dead bacteria = red) by the formulated triple antibiotic-eluting nanofibers. Scale bars = 30 μm. Clinical translation: placement of 3D tubular triple antibiotic-eluting construct into the root canal of a periapical lesion dog model, to act as a localised intracanal drug delivery system. Image obtained from reference (Bottino et al. 2017), with permission

effective antibiofilm properties against *Ef* is the use of photo-triggered drug delivery system made of nano-graphene oxide and indocyanine green (i.e. a cyanine dye generally used in medical diagnoses with known antimicrobial properties upon photoactivation), as shown in the study by Akbari et al. (Akbari et al. 2017). Besides being effective against *Ef* biofilm, indocyanine green-loaded nano-graphene oxide was prepared using much lower concentrations of active indocyanine green, compared to how this molecule is conventionally used, thus presenting less cytotoxic behaviour and less potential for causing tooth staining.

A recent strategy considered effective against oral biofilms relates to the use of silver nanoparticles in the form of core-shell compounds applied as an irrigant solution during the disinfection step (Ertem et al. 2017). Silver is already known for its broad-spectrum antibacterial activities, although silver nanoparticles may possess a tendency toward particle aggregation under ambient conditions, leading to a significant reduction in their antimicrobial effects. To overcome this limitation, the authors of that study proposed the following method to increase nanoparticles' stabilisation: using a porous silica shell to encapsulate the silver nanoparticles. As a result, for the first time, this proof-of-concept study demonstrated the long-term antimicrobial potential of the nanoparticle-based approach for endodontic infection treatment.

Core-shell silver nanoparticles were also effective in preventing biofilm regrowth when combined with suitable cleaning compounds and less cytotoxic than classical antimicrobial agents.

The foregoing strategies have proven to be effective in the control of intracanal infection, and perhaps they may be the focus of regenerative studies for the proper disinfection of immature permanent teeth with pulp necrosis. Taken together, future insights in this field need to be designed in an attempt to maximise the antimicrobial effects while minimising damage to stem cell survival and differentiation ability. Next, selected studies involving the use of dental stem cells and advanced scaffolds for dental pulp regeneration are discussed.

5.3 Regenerative Strategies for Dental Pulp Regeneration

Besides a more cell-friendly disinfection strategy, a number of developments in tissue engineering, primarily related to the synthesis of scaffolds, have provided the foundational knowledge for reliable and predictable regeneration of the pulp-dentine complex. According to the American Society for Testing Materials (ASTM—F2150), a scaffold is defined as "the support, delivery vehicle, or matrix for facilitating the migration, binding, or transport of cells or bioactive molecules used to replace, repair, or regenerate tissues". It should precisely replicate the features of the native extracellular matrix (ECM) at the nanoscale to regulate cell function and encourage and regulate specific events at the cellular and tissue levels (Bunyaratavej and Wang 2001; Owens and Yukna 2001). Moreover, scaffolds should be synthesised from biocompatible and biodegradable material(s) to avoid immune responses. A myriad of polymers, both synthetic, e.g. poly[lactic] acid (PLA), and natural (e.g. collagen), have been used in gas foaming, as well as salt leaching techniques, to obtain macroporous scaffolds. Meanwhile, nanofibrous scaffolds have been processed via electrospinning, self-assembly and phase separation (Albuquerque et al. 2014a).

In electrospinning, polymer nanofibers are obtained by the creation and elongation of an electrified jet (Albuquerque et al. 2014a). Various polymer solutions can be used and modified through mixing with other chemical reagents, polymers, nanoparticles, growth factors (GFs) and cells to generate unique nanofibers (Albuquerque et al. 2014a). Meanwhile, molecular self-assembly has been used to fabricate nanofibrous scaffolds through spontaneous molecular arrangement via non-covalent interactions (Albuquerque et al. 2014a). This technique allows recapitulation of collagen's supramolecule formation and enhances cell adhesion (Albuquerque et al. 2014a). Moreover, these unique nanofibers present major clinical advantages as they are assembled in solution and result in gels that are biocompatible and can be used for stem cell transplantation (Albuquerque et al. 2014a). However, this technique has limitations in terms of controlling pore size/shape within the scaffold and in producing sufficient mechanical properties (Albuquerque et al. 2014a). Accordingly, an alternative method, commonly referred to as thermally induced phase separation, has been incorporated in the fabrication of macro-/

micropore networks within 3D nanofibrous scaffolds (Albuquerque et al. 2014a). Taken together, recent advances in the field of biomaterials have allowed researchers to obtain scaffolds that can be easily injected in the desired site to aid in stem cell transplantation or to serve as delivery vehicles for bioactive factors. Some of the latest developments include the testing of innovative scaffolds/stem cell constructs in conjunction with therapeutic agents, and these are presented next as evidence of the translational potential of tissue engineering in regenerative endodontics. Of note, the next section has been divided into cell-free and cell transplantation approaches. In brief, the former approach comprises a cell-free strategy in which no exogenous cells are used/transplanted into the root canal system to propagate cell proliferation, whereas the latter approach induces regenerative outcomes by transplanting stem cells into the desired site.

5.3.1 Cell-Free Approaches

Cell-homing approaches to engineer dental pulp are based on the recruitment of resident stem cells by endogenous, dentine-derived growth factors, which induce cell migration into a custom-made scaffold, as well as proliferation and differentiation of the cells (Galler and Widbiller 2017). The utilisation of exogenous bioactive molecules that can be adsorbed, tethered or encapsulated into scaffolds to attract stem/progenitor cells adjacent to the root apices of endodontically treated teeth has demonstrated great clinical prospects. A recent report (Kim et al. 2010) highlighted the regeneration of dental-pulp-like tissue based solely on the intracanal delivery of fibroblast growth factor (FGF2) and/or vascular endothelial growth factor (VEGF) without stem cell transplantation. A recellularised and revascularised connective tissue integrated with the native dentinal wall in root canals was observed following in vivo implantation of endodontically treated human teeth in mouse dorsum for 3 weeks. In addition, combined delivery of a cocktail of GFs (FGF2, VEGF and platelet-derived growth factor [PDGF]) with a basal set of nerve growth factor (NGF) and bone morphogenetic protein 7 (BMP-7) led to the formation of tissues with patent vessels and new dentine regeneration (Kim et al. 2010). Several recent studies have begun to demonstrate that the release of specific biomolecules (e.g. TGF-β1, FGF-2, BMP-2, PDGF and VEGF) by certain irrigants and medicaments can favour the activity and proliferation of host stem cells, thereby incorporating the concept of a cell-homing mechanism in which the medium becomes a more welcoming environment for cell sustainability.

The identification of biomolecules, including but not limited to GFs and matrix molecules sequestered within dentine and dental pulp, affords a unique opportunity to make these signalling cues available in the regenerative process after a biocompatible disinfection approach. It has been proposed that the liberation of these biomolecules by certain irrigants and medicaments can potentially circumvent the use of non-human exogenous biomolecules (Widbiller et al. 2018). For a conditioning agent, it is extremely desirable to present a demineralising effect on dentine inorganic content, which would favour the release of any GFs or matrix proteins that are

naturally archived within the dentine substrate. For instance, EDTA (ethylenediaminetetraacetic acid) is mostly used as an irrigant in endodontic therapy, and according to a recent study by Galler et al. (Galler et al. 2016), EDTA was capable of inducing substantial release of GFs when kept in direct contact with dentine for 10 min; conversely, irrigation using sodium hypochlorite was not able to induce the release of any bioactive molecule from dentine. Notably, conditioning of dentine surfaces with EDTA has shown to result in chemotactic effects on dental pulp cells, promoting cell adhesion and differentiation into pulp-like cells (Galler et al. 2016). A representative illustration of clinically viable cell-homing approach is depicted in Fig. 5.5.

5.3.2 Cell Transplantation Strategies

Cell-based/transplantation approaches use exogenous scaffolds and/or stem cells as the starting point to produce regenerated tissue after transplantation into the desired site (Albuquerque et al. 2014a). The basis for the cell transplantation approach is not new in dentistry; it was first presented by Mooney et al. in 1996 (Mooney et al. 1996). Subsequently, DPSCs have been transplanted and combined into inorganic compounds to form dentine-like structures in mice (Gronthos et al. 2000) or to increase dentine-pulp regeneration within the root canals of mini dogs when combined with platelet-rich plasma (Zhu et al. 2013). Other stem cell lineages (e.g. stem cells obtained from human exfoliated deciduous teeth [SHEDs] or SCAPs) were also effective in the regeneration of dentine-pulp tissues (Cordeiro et al. 2008; Huang et al. 2013, 2010). Nonetheless, the latest developments in this field include testing innovative scaffolds/stem cell constructs in conjunction with therapeutic agents, and these are presented next as evidence of the translational potential of tissue engineering in regenerative endodontics.

Fig. 5.5 Schematic illustrating a clinically viable protocol of a cell-homing approach to engineer dental pulp in immature permanent teeth with pulp necrosis (**a**), and the sequence of events/stages associated to the cell-homing approach that may occur into the root canal system under regeneration (**b**). In (**a**), disinfection of the root canal system is paramount to allow bacteria elimination, followed by dentine conditioning using EDTA solution for up to 10 min, which contributes for endogenous growth factor (GF) release; next, rinsing with saline solution is performed under ultrasonic activation, allowing the collection of GFs. The GF-containing solution will be then mixed with the liquid components of a scaffold material, forming a GF-laden scaffold/hydrogel, which will be injected into the root canal system, with subsequent polymerisation (e.g. usually photopolymerisation due to the presence of photoinitiators composing the scaffold material). Restoration with bioactive materials is advisable in order to maintain the pulpal space sealed. Lastly, follow-ups including clinical and radiographic examination must be performed until complete regeneration of the dentine-pulp complex. In (**b**), the presence of GFs into the custom-made GF-laden scaffold induces chemotaxis (i.e. stem cell migration from the root apex), being the first stage in the cell-homing approach. After chemotaxis, the cells begin to proliferate within the GF-laden scaffold, increasing in number (second stage). In the sequence, cells attach to the inner root-dentine surface (third stage), allowing cell differentiation into pulp-like cells (fourth stage)

a
Immature permanent tooth with pulp necrosis
Disinfection of the intracanal system using TAP or TAP-containing nanofibers
Release of endogenous growth factors (GFs) from the dentin via EDTA conditioning
Saline irrigation, followed by ultrasonic activation and collection of the GFs-containing solution
GFs-containing solution
Liquid component of a scaffold material
Preparation of the GF-laden scaffold
Injection of the GF-laden scaffold into the root canal, and subsequent photopolymerization
Restoration with bioactive materials for proper sealing, and follow-ups
b
First stage: chemotaxis
GFs induce the migration of stem cells from the apex
Second stage: cell proliferation
Stem cells increase in number within the custom-made scaffold
Third stage: cell attachment
Stem cells start attaching to the inner root-dentin surface
Fourth stage: cell differentiation
Stem cells start differentiating into pulp-like cells

Nanostructured, self-assembling microspheres have also been used to deliver DPSCs into the pulpal space, as demonstrated elsewhere (Kuang et al. 2015, 2016). Indeed, a novel, star-shaped block copolymer constituted of poly(L-lactic acid)-block-poly-(L-lysine) and capable of self-assembling into nanofibrous spongy microspheres (NF-SMS) was synthesised, supporting DPSC proliferation and DSPP expression in vitro. Nanostructured microspheres have also been investigated for GF delivery (Niu et al. 2016). In that study, a microsphere platform was used to concurrently release fluocinolone acetonide (FA) to suppress inflammation, as well as BMP-2 to enhance odontogenic differentiation of DPSCs. A constant linear release of FA and a rapid BMP-2 release were observed in in vitro systems that reduced inflammation on DPSCs and enhanced differentiation.

Gelatin methacryloyl-based (GelMA) hydrogel was recently investigated for dental pulp regeneration (Khayat et al. 2017). GelMA is composed of denatured collagen and retains RGD (i.e. arginine-glycine-aspartic acid) adhesive domains and presents sensitivity to MMPs (i.e. metalloproteinases), thus enhancing cell binding and matrix degradation. In that study (Khayat et al. 2017), human DPSCs and human umbilical vein endothelial cells (HUVECs) were encapsulated in 5% GelMA and used to fabricate experimental constructs that were injected into human tooth root segments; acellular GelMA constructs and empty root segments were also prepared to serve as control groups. All constructs/root segments were cultured in osteogenic media for 13 days, followed by subcutaneous implantation in mouse dorsum for 4 or 8 weeks. The authors observed that the cellular GelMA construct allowed formation of highly cellularised and vascularised hDPSC/HUVEC-derived pulp-like tissue in the root segments and facilitated cell attachment to the tooth root inner dentine surface, formation of cellular extensions into the dentine tubules as well as elaboration of reparative dentine matrix. Overall, the GelMA hydrogel was also considered suitable for cell encapsulation and is easily tunable by varying the concentrations of GelMA and photoinitiators and, more recently, by using light-visible irradiation (Monteiro et al. 2018), which would pertain to a more clinically relevant setting (Fig. 5.6), since dental curing lights operating in the visible range are more frequent in the dental practice and may produce less deleterious effects on DNA and cellular function (Kappes et al. 2006). Figure 5.6 illustrates the potential clinical translation of the proposed strategy.

A particularly interesting study by Athirasala et al. (Athirasala et al. 2017) tested the effects of a tunable cell-laden GelMA hydrogel on the fabrication of an engineered pre-vascularised dental pulp-like tissue construct using a root canal model. In that study (Athirasala et al. 2017), the authors used root fragments of human premolars to obtain two root halves, which were properly sterilised using UV light and standardised immersion protocol. The two halves were then reattached and secured by wrapping them with laboratory film (Parafilm M); and EDTA conditioning was performed in order to expose the bioactive molecules sequestered within the dentine. The next step was to fabricate the microchannels of the engineered pulp-dentine complex; to that end, sacrificial agarose was used to synthesise 500 μm-thick fibres via a 3D printing-inspired method recently proposed (Bertassoni

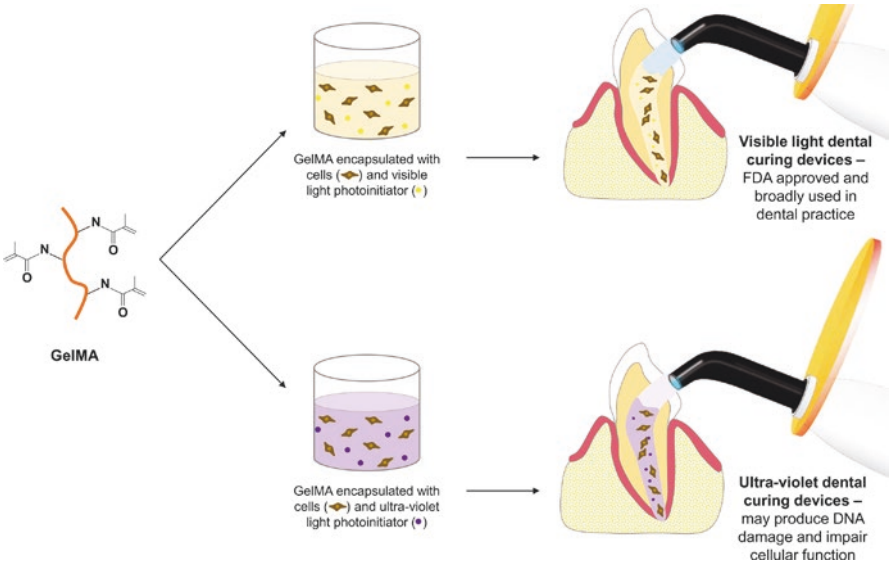


Fig. 5.6 Schematic showing a novel strategy to engineer pre-vascularised, cell-laden hydrogel pulp-like tissue constructs in full-length root canals in vitro by sequential GelMA polymerisation using visible light. First, GelMA macromer is synthesised by mixing gelatin with methacrylic anhydride, followed by lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) photoinitiator incorporation and consequent cell encapsulation, which is performed by suspending cells in the GelMA hydrogel. The resulting cell-laden hydrogel construct is then placed into the intracanal space, followed by photopolymerisation using a dental curing light unit. Image adapted with permission from reference (Monteiro et al. 2018)

et al. 2014a), followed by manually positioning pre-solidified sacrificial fibres inside the tooth, which was fulfilled with GelMA hydrogel. After photopolymerisation of the GelMA hydrogel, the final step of this root canal model consisted of complete aspiration/removal of the agarose fibres using a light vacuum and glass pipette (Bertassoni et al. 2014b). With fabrication of the tunable cell-laden GelMA hydrogel, diverse cells were seeded into the fabricated microchannels, and the tissue construct was cultured in standardised culture medium. In this study by Athirasala et al. (Athirasala et al. 2017), the authors demonstrated a novel and effective strategy to engineer pre-vascularised pulp-like tissue constructs using tunable cell-laden GelMA hydrogels (Fig. 5.7), showing optimal spreading and proliferation of cells near the dentine walls and important formation of endothelial monolayers with active angiogenic sprouts in fabricated microchannels after 7 days in culture, as shown in Fig. 5.8.

Overall, both pulp regeneration strategies discussed show promising applications in the field. Nevertheless, it is important to consider that the cell-homing approach involves the use of resident stem cells (i.e. derived from the patient) and non-complex mechanisms of pulp regeneration, thus representing a cost-effective method of inducing tissue regeneration, whereas the cell transplantation approach may involve

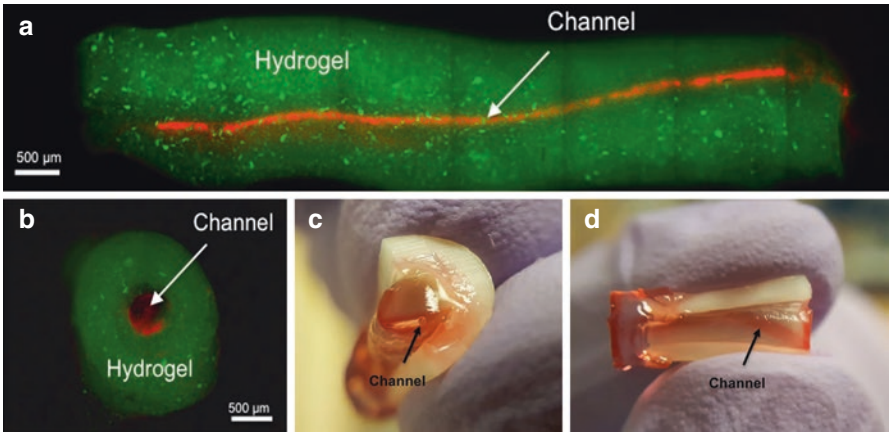


Fig. 5.7 Representative images of pre-vascularised pulp-like tissue construct, showing longitudinal (**a**) and cross-sectional (**b**) views of the GelMA hydrogels loaded with green fluorescent microparticles and the fabricated microchannel loaded with red fluorescent microparticle solution. Images of GelMA hydrogels from occlusal (**c**) and longitudinal (**d**) perspectives. Images obtained from reference (Athirasala et al. 2017), with permission

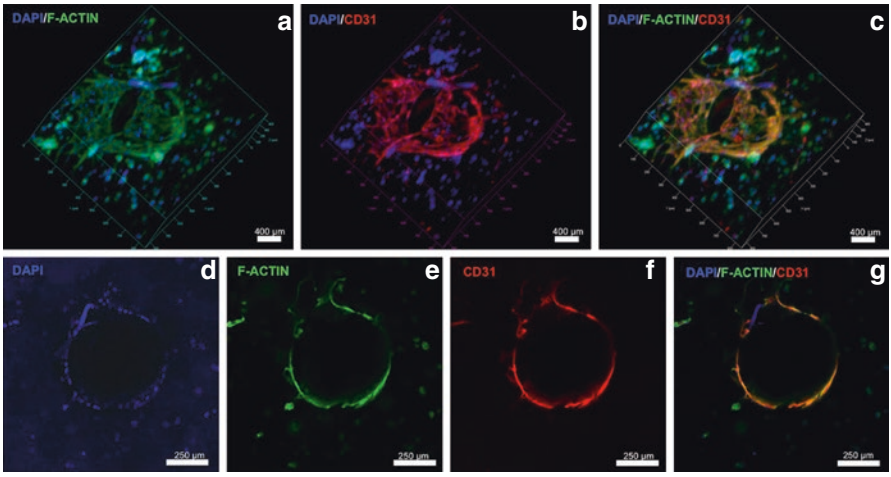


Fig. 5.8 Confocal images demonstrating the presence of endothelialised microchannels in the GelMA hydrogel, cultured in a full-length dental pulp-like tissue construct. Images (**a**–**c**) represent the 3D rendering, and images (**d**–**g**) indicate the cross-sectional slices of confocal images, showing endothelial colony-forming cell monolayer formation and angiogenic sprouts in the engineered constructs on day 7 (cells were stained with DAPI [blue], actin [green] and CD31 [red]). Images obtained from reference (Athirasala et al. 2017), with permission

a combination of exogenous stem cells and scaffolds/hydrogels/microspheres, which are usually expensive and complex/time-consuming to fabricate. This may limit clinical application of the cell transplantation approach compared with the cell-homing approach.

5.4 Conclusions and Future Outlook

Over the past decade, in spite of significant advancement and amendments of the EB technique, accumulating evidence regarding the key aspects deemed to negatively affect clinical outcome (e.g. the cytotoxic behaviour of antibiotic pastes and sodium hypochlorite irrigation), only one report has shown true pulp-like tissue formation. As a result, numerous research groups have been working intensively on tissue engineering-based strategies for regenerative endodontics. Noteworthy, preclinical (animal model) demonstration (Iohara et al. 2011; Ishizaka et al. 2012; Nakashima and Iohara 2011) of pulp regeneration by DPSCs has suggested that clinically effective human pulp regeneration (Nakashima et al. 2017) is now closer to application than it has ever been.

Concerning the major outcomes achieved over the past few years, we can list the following: (1) DPSCs revealed higher angiogenic and neurogenic potential compared with bone marrow-derived or adipose-derived mesenchymal stem cells; (2) complete pulp regeneration has been shown upon autologous transplantation of DPSC cells into the pulpectomised root canals of dogs; and (3) complete pulp regeneration with coronal dentine formation in the pulpectomised root canal of dogs was observed, showing reduced number of inflammatory cells, decreased cell death and major increase in neurite outgrowth. Other preclinical results also exist, and they generally confirm the efficacy and safety of stem cell transplantation mechanisms, aiding the initiation of a clinical trial with the consent of the Japanese Ministry of Health, Labour and Welfare (Nakashima et al. 2017). Besides the positive outcomes observed in several reports, and based on current knowledge, a key aspect for clinical success refers to the development of a biocompatible disinfection approach. Our group has focused on the design and synthesis of 3D patient-specific cytocompatible antibiotic-eluting nanofibers for intracanal drug delivery. In vivo preclinical (animal) studies are currently being conducted to validate these results. Next, the development of a regenerative strategy using advanced scaffolds, loaded or not with stem cells and/or growth factors to stimulate pulp and dentine regeneration after attaining a bacteria-free niche, is warranted to establish novel therapeutics to treat teeth with necrotic pulp.

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