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REVIEW ARTICLE

Advances in scaffolds used for pulp–dentine complex tissue engineering: A narrative review

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Abstract

Background: Pulp–dentine complex regeneration via tissue engineering is a developing treatment modality that aims to replace necrotic pulps with newly formed healthy tissue inside the root canal. Designing and fabricating an appropriate scaffold is a crucial step in such a treatment.

Objectives: The present study aimed to review recent advances in the design and fabrication of scaffolds for de novo regeneration of pulp–dentine complexes via tissue engineering approaches.

Methods: A literature search was conducted using PubMed, Europe PMC, Scopus and Google Scholar databases. To highlight bioengineering techniques for de novo regeneration of pulp–dentine complexes, both in vitro and in vivo studies were included, and clinical studies were excluded.

Results: In the present review, four main classes of scaffolds used to engineer pulp–dentine complexes, including bioceramic-based scaffolds, synthetic polymer-based scaffolds, natural polymer-based scaffolds and composite scaffolds, are covered. Additionally, recent advances in the design, fabrication and application of such scaffolds are analysed along with their advantages and limitations. Finally, the importance of vascular network establishment in the success of pulp–dentine complex regeneration and strategies used to create scaffolds to address this challenge are discussed.

Discussion: In the tissue engineering platform, scaffolds provide structural support for cells to adhere and proliferate and also regulate cell differentiation and metabolism. Up to now, considerable progress has been achieved in the field of pulp–dentine complex tissue engineering, and a spectrum of scaffolds ranging from bioceramic-based to naturally derived scaffolds has been fabricated. However, in designing a suitable scaffold for engineering pulp–dentine complexes, a variety of characteristic parameters related to biological, structural, physical and chemical features should be considered.

Conclusion: The variety of biomaterials and fabrication techniques provides a great opportunity to address some of the requirements for scaffolds in regenerative endodontics. However, more studies are required to develop an ideal scaffold for use in a clinical setting.

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KEYWORDS

Pulp–dentine complex, regenerative endodontics, scaffold, signalling molecule, stem cell, tissue engineering

INTRODUCTION

Inflammation and infection of the dental pulp through carious lesions, operative procedures and dental trauma, if left untreated, may lead to pulp necrosis. In an immature tooth with a necrotic pulp, root development is disrupted, leaving it short in length, with thin walls and a wide apical foramen that makes the root susceptible to fracture (Albuquerque et al., 2014; Galler, 2016). Regenerative endodontics (RE) is a relatively new treatment modality for immature teeth with pulp necrosis through the regeneration of new healthy tissue inside the root canal. The main goal of RE is the elimination of intra-radicular infection and resolution of apical periodontitis, followed by continued root development both vertically and laterally to improve their load capacity and the fracture resistance of the root walls (Lin et al., 2021; Saoud et al., 2016). Common features of this method in clinical practice include effective disinfection of the root canal, provocation of bleeding into the canal from the apical tissues, intra-canal blood clot formation to the level of the cementoenamel junction and a double-layered coronal restoration to prevent reinfection (Banchs & Trope, 2004; Bezgin & Sönmez, 2015; Galler, 2016).

The blood clot creates a three-dimensional (3D) matrix containing various growth factors (GFs) and brings mesenchymal stem cells (MSCs) into the root canal (Chrepa et al., 2015). Although many case reports have concluded that the evoked bleeding technique leads to dentinal wall thickening, root elongation and apical closure (Diogenes et al., 2013; Thibodeau & Trope, 2007), the outcomes of this regeneration remain patient dependent and unpredictable (Almutairi et al., 2019; Petrino et al., 2010). Additionally, histological analysis of regenerated intra-canal tissues in animal and human teeth has described fibrous periodontal ligament (PDL)-like connective tissue with a hard tissue similar to cementum or bone rather than pulp–dentine complex-like tissues (Lin et al., 2014; Simon et al., 2014; Simon & Smith, 2014). To improve the outcomes of such a technique, various studies have focused on the incorporation of scaffolds developed for tissue engineering (TE) applications alongside the evoked bleeding strategy. In this regard, the incorporation of gelatine-based scaffolds coated with basic fibroblast growth factor (bFGF; Nagy et al., 2014), chitosan-based scaffolds (Palma et al., 2017) and rat tail collagen type I solution (Thibodeau et al., 2007; Wang et al., 2010) has failed to improve the histological outcomes. Whilst using crosslinked collagen sponge (Yamauchi et al., 2011) and commercially available gelatine-based scaffold (Gelfoam; Londero et al., 2015) in orthotopic dog models, a greater healing rate and more tissue regeneration were achieved; however, the characteristics of the regenerated tissues did not alter.

A promising approach to overcome the limitations of current clinical RE procedures is the interplay of three basic elements of TE, namely, scaffolds, stem cells and signalling molecules, whilst taking into account biological aspects regarding the stem cell niche and survival/trophic factors (Marí-Beffa et al., 2017). Attempts to engineer dental pulp tissue were made in the late 1990s when for the first time the possibility of *in vitro* engineering of dental pulp-like tissues using poly(glycolic acid) (PGA)-based scaffolds was described (Bohl et al., 1998; Mooney et al., 1996). Subsequently, the identification and isolation of dental pulp stem cells (DPSCs; Gronthos et al., 2000) were major developments in engineering pulp–dentine complex tissues in a way that would be clinically feasible. From a clinical perspective, dental pulp TE can be divided into two categories: (i) partial regeneration of dental pulp where the existing tissue is recoverable and can guide tissue regeneration and (ii) *de novo* regeneration of dental pulp tissue in cases with complete pulp necrosis (Huang, 2009). *De novo* regeneration of dental pulp tissue is challenging and many research groups have used the TE platform to address the problem. The TE platform relies extensively on the design and fabrication of 3D scaffolds to provide structural support for cells to adhere and proliferate, trigger extracellular events and induce cell migration, differentiation (Chan & Leong, 2008). Up to now, numerous scaffolds have been fabricated to engineer pulp–dentine complexes. In this regard, biomaterial selection and fabrication technique are key elements in developing scaffolds with optimal characteristics.

SCOPE OF THE REVIEW

This article aims to review recent advances in the design and synthesis of scaffolds for *de novo* regeneration of pulp–dentine complexes via TE approaches. The optimal biological, structural, physical and chemical characteristics that an ideal scaffold should meet for application in RE are summarized. A spectrum of scaffolds ranging from bioceramic-based to synthetic/natural polymer-based scaffolds is reviewed. In this regard, concepts related to

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biomaterial selection including chemical nature, preparation techniques and physicochemical properties are discussed, and the advantages and limitations of the designed scaffolds and how they meet the optimal characteristics are summarized. Finally, the importance of vascularization for the successful regeneration of pulp–dentine complex is discussed, and vascularization strategies used in designing scaffolds to meet the challenge of limited blood supply during pulp regeneration are reviewed.

SEARCH STRATEGY

An electronic search of PubMed, Europe PMC, Scopus and Google Scholar databases was undertaken. Appropriate keywords, including 'pulp-dentine complex tissue engineering', 'dental pulp regeneration', 'scaffolds for pulp-dentine complex regeneration' and similar phrases defined in relevant papers, were used to extract papers on *de novo* regeneration of pulp–dentine complexes via TE approaches. In addition, to locate other relevant papers, reference mining of the identified papers was undertaken.

INCLUSION AND EXCLUSION CRITERIA

Only studies on scaffolds designed for *de novo* regeneration of pulp–dentine complex via TE approaches are reported in this review. Additionally, since the main objective of this review is to highlight bioengineering techniques for pulp–dentine complex regeneration, *in vitro* studies as well as *in vivo* studies performed in animal models were included and clinical studies were excluded.

REQUIREMENTS OF SCAFFOLDS FOR PULP–DENTINE COMPLEX TISSUE ENGINEERING

The architecture of the human tooth is highly complex; it possesses several layers of hard tissues organized precisely to strengthen the tooth and protect the dental pulp from the bacteria-rich environment of the mouth (Figure 1a). An ideal scaffold for pulp–dentine complex TE should meet a number of characteristics depending on the special anatomical, structural and functional properties of the dental pulp (Figure 1b). As with scaffolds designed for the regeneration of other tissues, the basic requirements for a suitable scaffold for RE are biocompatibility and biodegradability. A suitable scaffold must be degraded as the extracellular matrix (ECM) of the cells is synthesized and replaced with a new secreted ECM. A slow degradation rate can adversely affect the quality of the newly formed tissue (Huang, Yamaza, et al., 2010), whilst degradation rates that are too rapid cause the structure to collapse before regenerated tissue can fill the entire defect. The next critical factor for pulp–dentine complex TE is cell–matrix interaction. Not only should an appropriate fabricated scaffold provide structural support for cells to adhere and proliferate but it should also be able to trigger extracellular events and induce cell migration, differentiation, etc. (Chan & Leong, 2008). Moreover, it should allow the functionality of multiple cell types, including odontoblasts, fibroblasts, vascular cells and/or neural cells, and support vascularization and biomineralization, which are primary requirements for the successful regeneration of pulp–dentine complexes (Galler, 2014; Galler, D'Souza, et al., 2011).

Scaffold injectability is also an important consideration in RE. The irregular and complex shape of root canals make it difficult to place a pre-fabricated scaffold into the canal system in a way that fills the space completely. Additionally, the architecture and structural features at the micro- and nano-scale should also be considered in designing a suitable scaffold for dental pulp regeneration. Scaffold architecture can determine the initial spreading of cells on the surface or within the scaffold, and consequently, new tissue formation. The odontogenic differentiation of mouse dental pulp cells (DPCs) on the surface of polymethyl methacrylate scaffolds with a microtubular architecture was triggered more than those on the scaffold surface with planar architecture (Haeri et al., 2017). Also, comparing poly(L-lactic acid) (PLLA)-based scaffolds with various architecture, nanofibrous and smooth surfaces revealed that nanofibrous architecture provided a better extracellular environment and significantly enhanced proliferation and odontogenic differentiation of DPSCs (Garzón et al., 2018; Wang et al., 2012). In this regard, topographies of nanofibre scaffolds could also influence the fate of cells. Apical papilla cells on an electrospun PCL-based scaffold with aligned and random nanofibres exhibited elongated and polygonal morphologies respectively (Leite et al., 2021). Another important structural feature of scaffolds is a porous structure with interconnected pores to allow effective cell ingrowth and mass transfer. The mean pore size of the scaffold should be large enough to facilitate cell migration but small enough to provide a high specific surface area for efficient cell attachment (O'Brien et al., 2005). Hence, controlling the upper and lower limits of the pore size is a critical step in designing the microstructure of scaffolds. El-Backly et al. (2008) indicated that although larger pores are important to promoting migration and proliferation of stem cells and biomineralization, small pores are essential to provide a matrix for angiogenesis and formation of a well-organized tissue.

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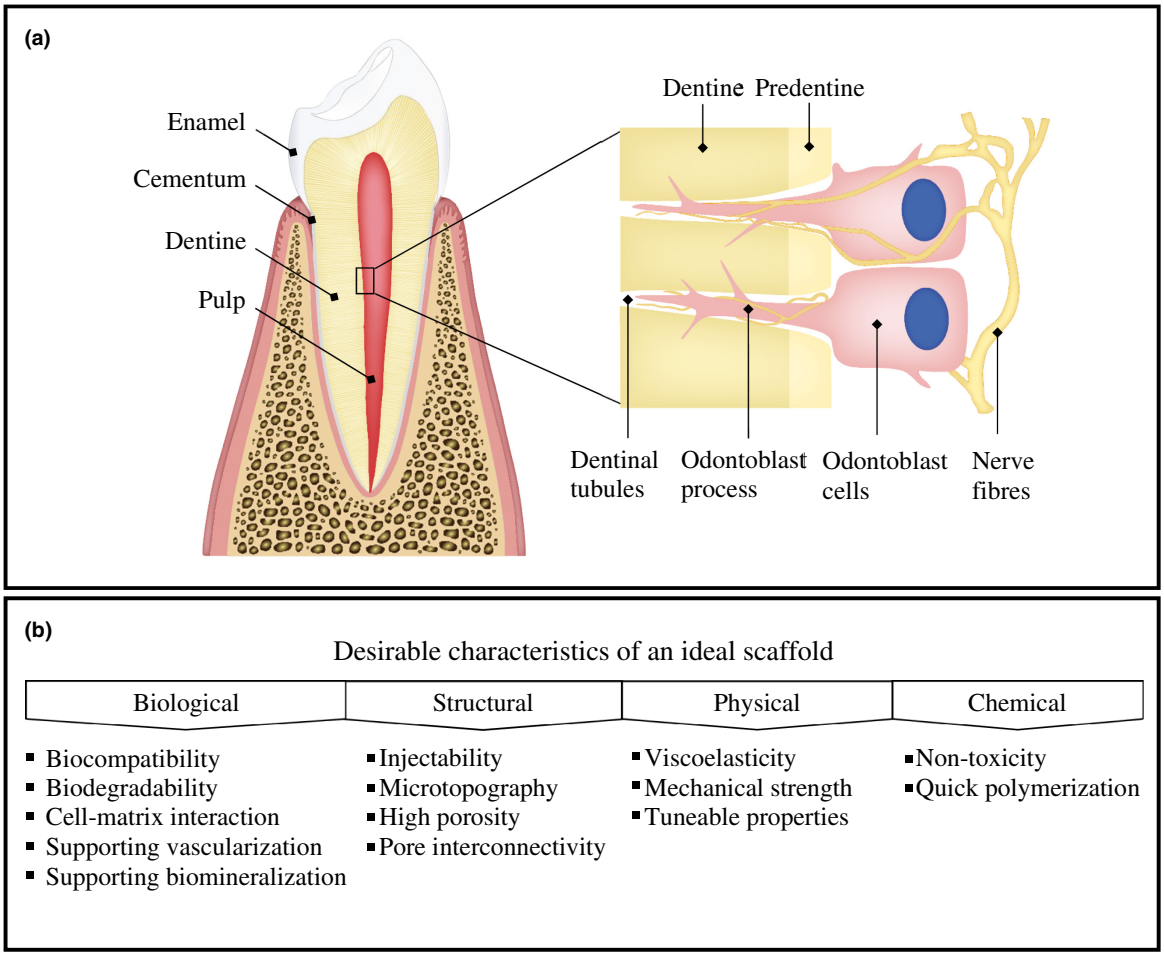

FIGURE 1 Tooth anatomy, pulp structure and desirable characteristics of a scaffold for pulp–dentine complex TE. (a) Anatomy of (i) tooth and (ii) pulp. (b) Biological, structural, physical and chemical characteristics that an ideal scaffold should exhibit.

Besides considering suitable structural features, viscoelasticity and stiffness are other vital aspects of scaffold-based pulp–dentine complex TE. The native pulp tissue has viscoelastic properties (Erisken et al., 2015; Ozcan et al., 2016). Comparing the effect of various hydrogels based on alginate and hyaluronic acid on the viability and metabolic activity of stem cells from the apical papilla (SCAP) revealed that a combination of surface and mechanical characteristics influenced the fate of such cells (Lambrichta et al., 2014). Mimicking the microenvironment of host tissues via tuning mechanical and rheological properties of scaffolds is a well-known method to direct differentiation and control adhesion behaviour and the gene expression profile of cells (Lv et al., 2015; Reilly & Engler, 2010). Gangolli et al. (2019) investigated the effect of matrix stiffness on the behaviour of DPSCs *in vitro*. They fabricated a bilayered PLGA-based scaffold through lamination of two layers with different stiffness (generating from 12% and 20% w/v PLGA solution) and cultured DPSCs on both sides. Such scaffold contained continuous channels whose diameter ranged from 5 to 10 μm on the stiffer side and 10 to 45 μm on the other side. On the stiffer side, DPSCs remained on the surface and could not penetrate the depth. But in comparison with DPSCs on the other side, they expressed significantly greater levels of odontogenic markers (BSP, DSPP, MEPE and ALP). Seeding DPSCs onto polydimethylsiloxane scaffolds with a stiffness in the range of 1.4 to 135 kPa also revealed that DPSCs on stiffer scaffolds had a greater proliferation rate and expressed higher levels of osteo-/odontogenic related markers (Liu et al., 2018). In this regard, the potential mechanism for regulation of DPSC osteo-/odontogenic differentiation through altering scaffold mechanical properties was related to the canonical WNT signalling pathway.

To reach the above-mentioned properties, biomaterial selection and fabrication techniques become two key considerations in designing scaffolds. The structural and physical characteristics of a scaffold as well as its biological performance can be tuned by altering its physicochemical properties such as polymer concentration and crosslinking methods. However, the biocompatibility of fabrication procedures should be considered. The use of various crosslinking techniques such as chemical crosslinking

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might raise concerns about cytotoxicity. Additionally, the fabrication technique directly affects polymerization time. Whilst rapid polymerization minimizes operation time and discomfort to the patient, polymerization that is too rapid could prevent injectability and complete filling of the root canal space. In the following sections, various scaffolds designed for RE ranging from bioceramic-based scaffolds to synthetic/natural polymer-based scaffolds along with fundamentals related to their design such as physicochemical properties, morphological properties and fabrication methods are discussed. In addition, the advantages and limitations of designed scaffolds are summarized.

BIOCERAMIC-BASED SCAFFOLDS

Bioceramics are biocompatible inorganic nonmetallic materials that are used extensively in the field of Endodontics. Generally, bioceramics can be classified into two categories: bioinert bioceramics with stable physicochemical properties such as zirconia and alumina, which have no interaction with biological systems, and bioactive bioceramics such as calcium phosphate compounds (CPCs) and bioactive glasses, which are biodegradable and able to interact with the surrounding tissues (Raghavendra et al., 2017). Regarding RE, CPCs have been used as the basis of the scaffolds in several studies (Table 1). CPCs, specifically hydroxyapatite (HA) and tricalcium phosphate (TCP), are amongst the most widely used bioceramics in clinical settings. They dissolve gradually in the physiological environment and release Ca^{2+} and PO_4^{3-} ions that can contribute to the cellular response (Reddy et al., 2020; Swarup et al., 2014). Calcium phosphate bioceramics mimic to some extent the mineral phase of biological hard tissues. Natural dentine is a composite structure of which 50 vol% is the mineral phase in the form of calcium-deficient carbonate HA (Marshal Jr. et al., 1997). In addition, CPCs have appropriate biological affinity and activity and they support hard tissue formation making them a major group of inorganic biomaterials for application in pulp–dentine complex TE. Cultivation of DPCs onto CPCs indicated the positive effect of such compounds on osteo-/odontogenic differentiation of DPCs, which was evidenced by more mineralized nodule formation and upregulation of osteo-/odontogenic gene (DMP-1, DSPP, MEPE, ON, OPN and BSP) expression (Lee et al., 2010). Also, it has been reported that various CPCs including HA (Imura et al., 2019), TCP (Heller et al., 1975; Lee et al., 2014) and calcium β-glycerophosphate (Imai & Hayashi, 1993) can induce dentine-like hard tissue regeneration when used as pulp-capping agents. However, various types of bioactive bioceramics have been reported to exhibit a range of effects on the fate of stem cells. Zheng et al. (2011) used three different CPCs, including HA, TCP and calcium carbonate HA, combined with poly(lactic-co-glycolic acid) (PLGA) as composite scaffolds for culturing DPSCs. Although SEM analysis revealed a similar architecture between composite scaffolds, the effect of scaffold composition on DPSC proliferation and differentiation was entirely different (Figure 2a). All CPCs improved the cell affinity of PLGA-based scaffolds since they had an alkaline property and were able to reduce the acidity of PLGA degradation by-products. However, the molecular size of CPCs was a determinative parameter in the effectiveness of acidity reduction. The smallest molecule, here TCP, was the most effective to increase cell affinity. In addition, TCP was the most successful compound for guiding DPSC differentiation, and the greatest ALP activity and the most extensive *ex vivo* mineralized nodule formation were observed in the PLGA/TCP group. Transplantation of such constructs with rat tooth bud cells into the mesentery of 8-week-old rats also revealed the capability of TCP-containing scaffolds to support pulp–dentine-like tissue formation.

In RE, biphasic HA/TCP has been used commonly as the basis of scaffolds for dental pulp regeneration. Biphasic HA/TCP is commercially available in various forms, such as powders, granules, blocks and excipients. To investigate the *in vivo* developmental potential of DPSCs, Gronthos et al. (2000, 2002) used the powder of HA/TCP as the carrier for the transplantation of DPSCs into the dorsal surface of immunocompromised mice. Swine SCAP and DPSCs mixed with HA/TCP granules were also implanted subcutaneously into the back of nude mice for ectopic pulp–dentine complex formation (Zhu et al., 2018). Although such studies reported the regeneration of dentine-like tissue, which surrounded a pulp-like tissue with an interface layer of odontoblast-like cells, the low resorption rate of HA/TCP adversely affected the quality of the regenerated tissues. In all studies, regenerated pulp–dentine-like tissues were disorganized and scattered within the scaffolds (Figure 2b), and the bioceramic-based scaffolds remained undegraded even after 3 months of *in vivo* transplantation (Matsui et al., 2018; Zhu et al., 2018).

In general, the resorption rate of CPCs depends on the calcium-to-phosphorous ratio. Decreasing the Ca/P ratio leads to increasing the dissolution rate, but a Ca/P ratio of less than 1 is not recommended for biological applications (Yang et al., 2011). In the case of using biphasic HA/TCP, the dissolution rate in physiological environments can be controlled by adjusting the HA/TCP ratios, since HA and TCP have different Ca/P ratios and consequently different dissolution rates. However, to decrease the dissolution rate of CPCs significantly in a way that is commensurate with the rate of tissue regeneration, other strategies, such

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TABLE 1 Summary of the most important studies on bioceramic-based scaffolds for pulp–dentine complex TE

Biomaterials	Fabrication techniques	Main achievements	Drawbacks	References
α-TCP-based CPCs	Crystallization reaction	Promotion of odontoblastic differentiation of DPCs; Enhancement of biomineralization	Cytotoxicity to DPCs due to the ionic activities and pH changes	Lee et al. (2010)
HA nanoparticles	Wet precipitation	Bounding FGF2 to the nano-HA particles; Induction of vascular endothelial cell ingrowth into the exposed pulp; Promotion of odontoblastic differentiation and tubular dentine formation	Different orientation of new dentinal tubules from original ones	Imura et al. (2019)
PLGA/calcium phosphates	Particle leaching and phase separation	Indicating the effect of calcium phosphate composition on the fate of DPSCs; Improvement of cell affinity; Enhancement of biomineralization and ALP activity (TCP>CDHA>HA); Formation of pulp- and dentine-like tissues by tooth bud cells *in vivo*	Pre-fabricated; Limited access to tooth bud cells; Nonrelevant animal models	Zheng et al. (2011)
HA/TCP	–	Demonstrating multilineage differentiation capacity and developmental potential of DPSCs *in vivo*; Ectopic regeneration of pulp- and dentine-like tissues	Low resorption rate; Regeneration of disorganized and scattered tissues	Gronthos et al. (2000, 2002)
HA/TCP	–	Ectopic regeneration of tubular dentine-like tissue surrounding a pulp-like tissue with an interface layer of odontoblast-like cells	Low resorption rate; Regeneration of disorganized and scattered tissues	Zhu et al. (2018)
HA/TCP	–	Promotion of ectopic regeneration of pulp- and dentine-like tissues by CD146-positive DPSCs	Low resorption rate; Regeneration of disorganized and scattered tissues	Matsui et al. (2018)
HA/PLLA/PCL	Electrospinning	Homogenous embedding of HA nanoparticles into electrospun nanofibers; Facilitation of initial DPSC attachment via enhancing surface roughness; Promotion of proliferation, differentiation and biomineralization of DPSCs *in vitro*	Pre-fabricated; Osteoinductivity	Asghari et al. (2016)
HA/TCP	–	Support of biomineralization and DSPP expression of DPSCs both *in vitro* and *in vivo*	Pre-fabricated; Low degradation rate; Formation of bone-like tissue rather than dentine-like tissue	Zhang et al. (2006)
PCL/Chitosan/HA	Freeze-drying	Tuneable properties; Improvement of bioactivity, physical properties and mechanical strength; Facilitation of biomineralization	Pre-fabricated	Tondnevis et al. (2019)
HA/silk fibroin	Freeze-drying	Embedding HA in the composite scaffold; Support of DPSC viability *in vitro*	Pre-fabricated	Zhang et al. (2019)

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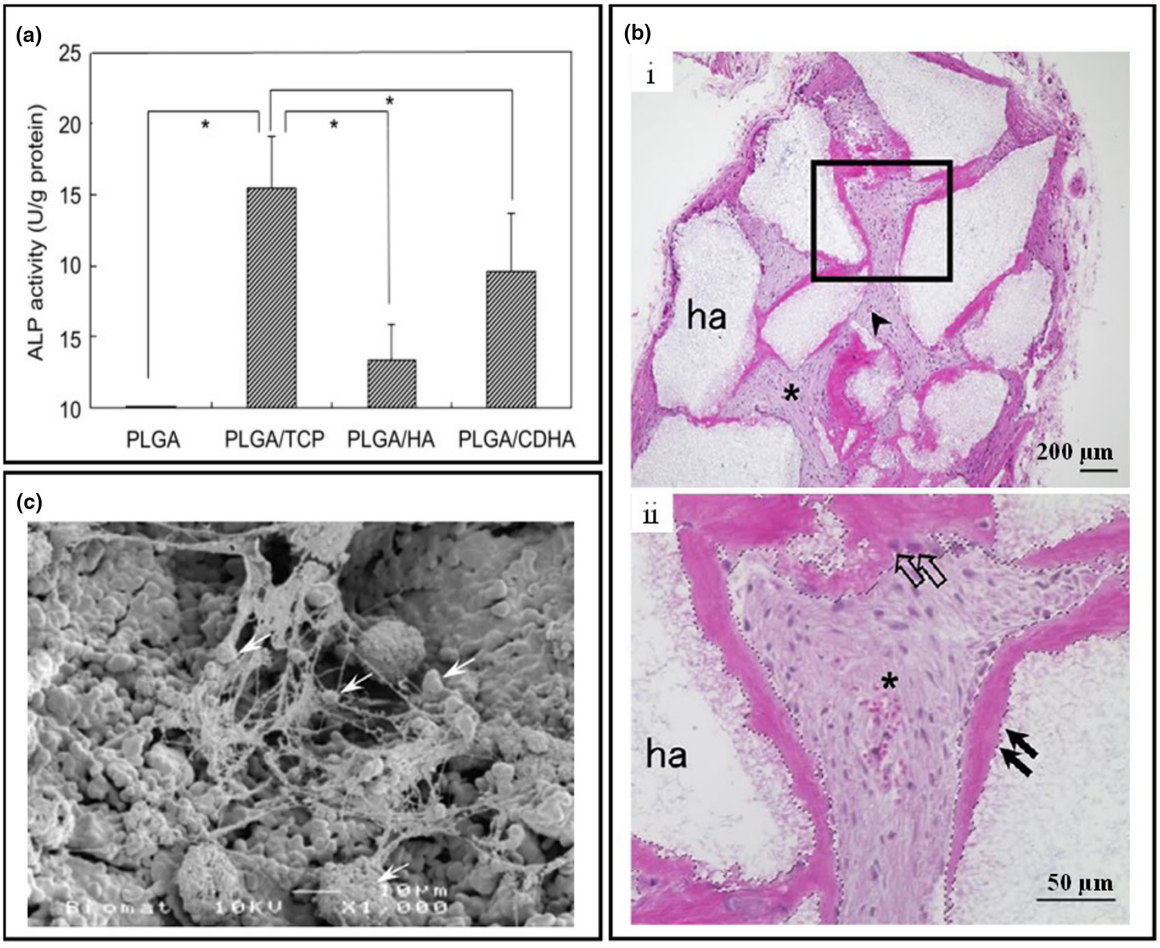

FIGURE 2 Bioceramic-based scaffolds for pulp–dentine complex TE. (a) Comparison of ALP activity in scaffolds based on different CPCs. Adopted from Zheng et al. (2011) with permission from Elsevier, copyright 2011. (b) Ectopic formation of pulp–dentine-like structures scattered within HA/TCP scaffolds. (i) Low magnification of the harvested construct. (ii) High magnification of the bordered area in (i) which shows pulp–dentine-like structures. Asterisk, arrowhead, black arrow and open arrow show connective tissue, blood vessels, dentine-like structure and odontoblast-like cells respectively. Adopted from Matsui et al. (2018), licensed under creative commons license. (c) Biomineralized nodule formation after *in vitro* culturing of HA/TCP scaffold loaded with DPSC for 4 weeks. Adapted from Zhang et al. (2006) with permission from Elsevier, copyright 2006.

as incorporating biodegradable polymers and fabricating composite scaffolds, can be considered.

Other shortcomings that hamper the use of CPC-based scaffolds in RE are their brittleness, poor mechanical strength and difficult processability to form highly porous structures. Additionally, CPCs have an osteoinductive property, which may cause the formation of bone-like tissue rather than dentine-like tissue (Asghari et al., 2016; Zhang et al., 2006). Adding HA to nanofibrous PLLA/poly(ε-caprolactone) (PCL) scaffolds directed DPSC differentiation into osteoblast-like cells (Asghari et al., 2016). Moreover, despite the *in vitro* formation of mineralized nodules in the HA/TCP ceramic discs loaded with DPSCs (Figure 2c), histological outcomes of ectopic transplantation of cell-laden discs revealed the formation of calcified tissue-entrapping cells, which resembled bone rather than dentine (Zhang et al., 2006). In this regard, engineering hybrid composite scaffolds composed of bioceramics and natural/synthetic polymers can be considered an affordable way to develop better scaffolds with enhanced odontogenic regenerative potential. The flexibility of polymers to tailor their physicochemical properties in combination with bioactivity and biological similarity of CPCs to native hard tissues can provide fundamental characteristics for scaffolds used for pulp–dentine complex TE. Tondnevis et al. (2019), by using the freeze-drying technique, fabricated a composite scaffold composed of PCL, chitosan and HA/fluoroHA. They reported that the combination of PCL and chitosan with HA/fluoroHA caused the development of a suitable scaffold with improved bioactivity, physical properties and mechanical strength. Using a similar technique, a HA/silk fibroin composite scaffold was fabricated and proposed as a potential scaffold for dental pulp repair/regeneration since it supported the viability of

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DPSCs *in vitro* (Zhang et al., 2019). However, being prefabricated could affect the outcome of using such scaffolds for the regeneration of pulp–dentine complexes in practice. Scaffold injectability is an important consideration in RE. Remnants of scattered voids inside the canal or more importantly adjacent to the dentinal wall may adversely affect stem cell differentiation and the regeneration process. In such a case, injectable CPCs may be a good alternative if their setting time can be shortened.

In summary, despite their advantages (i.e. high biocompatibility, bioactivity and supporting hard tissue regeneration) and their widespread use in Dentistry, the use of calcium phosphate bioceramics is associated with several critical drawbacks including osteoinductive properties, brittleness, difficult processability and low degradation rate. Continued progress towards engineering hybrid biomaterials composed of bioactive bioceramics and biopolymers will alleviate some of the problems to afford better scaffolds with enhanced odontogenic regenerative potential.

SYNTHETIC POLYMER-BASED SCAFFOLDS

Synthetic biomaterials are used in a substantial volume of research in regenerative medicine, including regenerative dentistry. These materials can be manufactured on a large scale with high purity and low cost. Furthermore, synthetic biomaterials offer high tuneability of degradation kinetics, mechanical strength and microstructure, and at the same time avoid the risk of pathogen transmission. The most widely explored synthetic biomaterial scaffolds used in RE are based on members of poly(α-hydroxy esters) and self-assembling peptides (SAPs) that will be discussed in detail below and which are summarized in Table 2.

Poly(α-hydroxy ester)-based scaffolds

Amongst the poly(α-hydroxy esters) members, poly(glycolic acid) (PGA), poly(lactic acid) (PLA), their copolymers poly(lactic-co-glycolic acid) (PLGA) and poly(ε-caprolactone) (PCL) have been used extensively in the fabrication of scaffolds for pulp–dentine complex TE. PGA is the simplest member of linear aliphatic polyesters and is known as a hydrophilic biocompatible polymer with a high degradation rate in aqueous solutions. Compared to PGA, PLA has an additional methyl group which makes it optically active. It also has three stereoisomers, L-lactide (the naturally occurring isomer), D-lactide and D,L-lactide. PLA is more hydrophobic and has a slower degradation rate than PGA. The copolymerization of PLG and PLA leads to the formation of a new polymer, PLGA, whose properties can be altered by using different ratios of PLG and PLA. PCL is another polyester with adjustable physicochemical properties; however, its low degradation rate compared to the mentioned polymers restricts its application in scaffold fabrication. Concerning RE, culturing odontogenic stem cells on the poly(α-hydroxy esters)-based scaffolds have been reported to have suitable biocompatibility of such biomaterials (Gotlieb et al., 2008; Louvrier et al., 2018). Appropriate attachment of stem cells from human exfoliated deciduous teeth (SHED) to a D,D-L,L-PLA-based scaffold was observed *in vitro* (Gotlieb et al., 2008). Also, seeding DPSCs onto commercially available PCL cones revealed that the PCL-based construct supported the viability and differentiation of DPSCs *in vitro* (Louvrier et al., 2018).

A common strategy to produce poly(α-hydroxy esters)-based scaffolds for RE is the solvent casting/particulate leaching technique. In this method, the polymer solution containing particles (also called progens) with a specific dimension is cast into a mould, and after evaporating the solvent and dissolving the particles, the desired porous scaffold remains. Using this technique, Cordeiro et al. (2008) prepared scaffold/tooth slice constructs for *in vivo* evaluation of SHED potential for regenerating ectopic dental pulp. They used the pulp chamber of 1-mm-thick tooth slices as the mould and cast a homogenous mixture of sodium chloride and polymeric solution of PLLA such that the scaffold filled the chamber completely (Figure 3i & ii). After polymerization, the prepared scaffold was seeded by SHED and transplanted into nude mice for *in vivo* investigations (Figure 3iii). They reported ectopic regeneration of vascularized pulp-like tissue with odontoblast-like cells lining the pre-dentine after 28 days of transplantation (Figure 3iv–vi). This approach was further used by others for engineering pulp-like tissue *in vivo* (Conde et al., 2015; Demarco et al., 2010; Sakai et al., 2010).

Despite ectopic regeneration of pulp-like tissues within the aforementioned constructs, using 1-mm-thick tooth slices as a model for *in vivo* studies is far from the real situation. In clinical practice, the goal is to regenerate dental pulp tissue throughout the full length (in the range 11–13 mm (Kim et al., 2013)) of root canals. In addition, this fabrication technique is associated with the risk of residual solvent and/or progens. In addition, placing the polymeric solution within the pulp chamber is not practical clinically. In this regard, Huang, Yamaza, et al. (2010) constructed a pre-fabricated disk-shaped PLG-based scaffold via a gas foaming/particulate leaching process, which is free of toxic solvents, and divided it into small pieces to insert inside the canal space of root fragments (~6–7 mm long). Although ectopic transplantation of cell-laden

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TABLE 2 Summary of the most important studies on synthetic polymer-based scaffolds for pulp–dentine complex TE

Biomaterials	Fabrication techniques	Main achievements	Drawbacks	References
Poly(α-hydroxy ester)				
PCL	Jet spraying & prototyping	*In vitro* support of DPSC proliferation and differentiation	Pre-fabricated; Lack of bioactivity; Slow degradation; Acidic by-products	Louvrier et al. (2018)
PLLA	Solvent casting/particulate leaching	*In situ* fabrication of scaffolds with tuneable porosity and pore size; Development of scaffold/tooth slice constructs for *in vivo* studies; Ectopic regeneration of vascularized pulp-like tissue with odontoblast-like cells lining the pre-dentine	Possible toxicity; Slow degradation; Impracticality in clinical settings; Thinness of tooth slices; Leaving both sides of constructs open; Acidic by-products	Cordeiro et al. (2008)
PLLA	Solvent casting/particulate leaching	Formation of tubular dentine with a pre-dentine layer secreted by cells differentiated from SHED *in vivo*; Induction of SHED differentiation into vascular endothelial cells *in vivo*	Possible toxicity; Lack of bioactivity; Slow degradation; Impracticality in clinical settings; Thinness of tooth slices; Leaving both sides of constructs open; Acidic by-products	Sakai et al. (2010)
PLLA	Solvent casting/particulate leaching	Demonstrating the effect of progen types on the fate of DPSCs; Regeneration of ectopic pulp-like tissue *in vivo*	Possible toxicity; Slow degradation; Impracticality in clinical settings; Thinness of tooth slices; Leaving both sides of constructs open; Acidic by-products	Demarco et al. (2010)
PLG	Gas foaming/particulate leaching	*In vivo* regeneration of vascularized pulp-like tissue with a newly formed dentine-like tissue along the dentinal wall by DPSCs and SCAP	Pre-fabricated; Lack of bioactivity; Slow degradation; Acidic by-products; Formation of cellular dentine	Huang et al. (2010)
PCL	Electrospinning	Incorporating magnetic nanoparticles into the scaffold; Enhancing mechanical strength; Promoting proliferation, odontogenic differentiation and pro-angiogenesis of DPSCs *in vitro*	Pre-fabricated; Acidic by-products; Risk of tooth discolouration	Yun et al. (2016)
PLGA	Double-emulsion solvent evaporation	Fabrication of injectable porous microspheres supporting proliferation and stemness properties of dental pulp mesenchymal stem cell line *in vitro*	Use of cell lines; Lack of bioactivity; Acidic by-products	Bhuptani and Patravale (2016)
PLGA	Double-emulsion solvent extraction	Fabrication of injectable bioactive microspheres promoting odontogenic differentiation of DPCs	Slow degradation; Acidic by-products	Zou et al. (2017)
PLLA	Thermally induced phase separation & emulsification	Fabrication of injectable microspheres releasing BMP-2; Promotion of odontogenic differentiation *in vitro* and *in vivo*; Enhancement of angiogenesis *in vivo*; *In vivo* regeneration of ectopic osteodentine-like tissue	Exogenous GFs; Slow degradation; Acidic by-products; Regeneration of disorganized cell-embedded osteodentine-like tissue	Wang et al. (2016)

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TABLE 2 (Continued)

Biomaterials	Fabrication techniques	Main achievements	Drawbacks	References
PLLA	Thermally induced phase separation	Directing of the fate of DPSCs both *in vitro* and *in vivo* via controlling scaffold architecture; Promotion of degradation rate, angiogenesis and tissue regeneration *in vivo*; *In vivo* regeneration of vascularized pulp-like tissue ectopic and orthotopic models	Hypoxic culture prior to *in vivo* transplantation; Acidic by-products	Kuang et al. (2016)
PLGA	Diffusion-induced phase separation	Directing *in vitro* differentiation of DPSCs via mechanical cues	Pre-fabricated; Slow degradation; Acidic by-products	Gangolli et al. (2019)
Self-assembling peptides (SAPs)				
Customized SAP	Self-assembling	Designing an injectable SAP containing cell adhesion motifs and enzyme-cleavable sites; Mimicking ECM's nanoscale dimensions; Enhancement of bioactivity by incorporating GFs via electrostatic reaction	Complex design process; High cost; Low mechanical strength; Low productivity	Galler et al. (2010, 2012)
PuraMatrix	Self-assembling	Mimicking ECM's nanoscale dimensions; *In vivo* regeneration of vascularized pulp-like tissues with the ability of new dentine generation along the full length of root canals	Leaving both sides of constructs open; Low mechanical strength	Rosa et al. (2013)
PuraMatrix	Self-assembling	Intercellular crosstalk between HUVECs and DPSCs related to angiogenesis and pulp regeneration; *In vivo* regeneration of ectopic pulp-like tissue and new osteodentine/pre-dentine	Low mechanical strength; Immune incompatibility of commercially available HUVECs in clinical settings; Partial regeneration within root canals	Dissanayaka et al. (2015)

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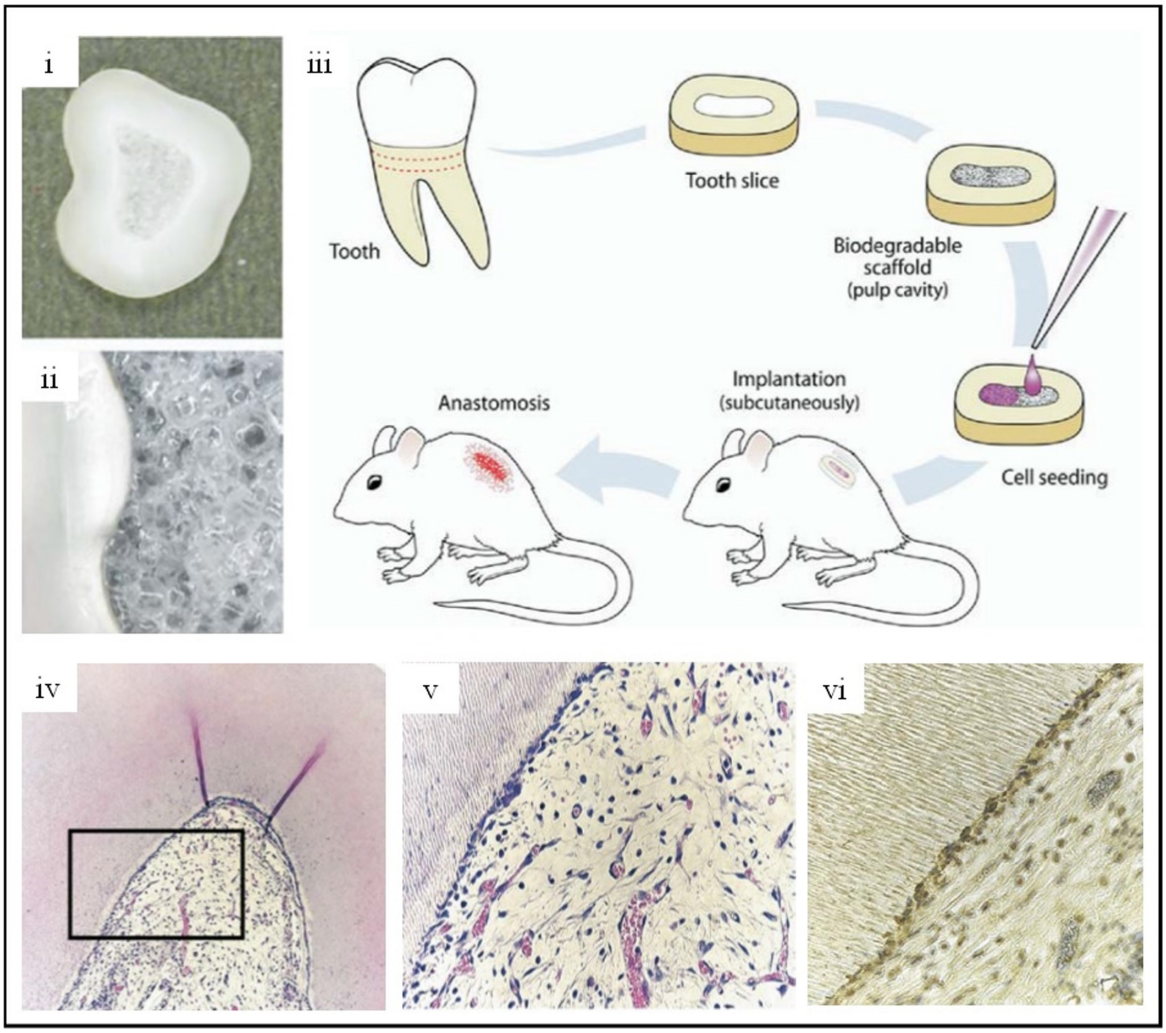

FIGURE 3 Scaffold/tooth slice model for dental pulp regeneration. (i) Low and (ii) high magnification of PLLA-based scaffold fabricated within a tooth slice. (iii) Schematic illustration for the preparation of scaffold/tooth slice constructs and *in vivo* investigation. (iv) Newly formed vascularized pulp-like tissue after 14 days of ectopic transplantation. (v) High magnification of the bordered area showed in (iv). (vi) Localization of DSP in regenerated pulp-like tissue via immunostaining. Adapted from Cordeiro et al. (2008) with permission from Elsevier, copyright 2008.

constructs led to the formation of pulp–dentine-like tissues, the low degradation rate of the PLG scaffold and remnants of unresorbed species left scattered voids within the regenerated pulp-like tissue. Moreover, the regenerated dentine-like tissue was cellular without dentinal tubules that were more similar to the tertiary dentine.

Pre-fabricated PCL-based scaffolds alone (Louvrier et al., 2018) or in combination with other materials such as fibronectin (Leite et al., 2021), fluorapatite (Guo et al., 2014), apatite (Kim et al., 2014), bioactive glass (Wang, Hu, et al., 2016) and magnetic nanoparticles (Yun et al., 2015, 2016) have also been used to enhance biocompatibility or facilitate odontogenic differentiation and subsequent mineralization of odontogenic stem cells. However, using pre-fabricated scaffolds may in practice be associated with a range of problems. In addition to incomplete filling of the root canal space, compressing cell-laden pre-fabricated scaffolds when inserted into the lumen of canals could cause failure during dental pulp regeneration as reported by Zhu et al. (2018). For such reasons, injectable poly(α-hydroxy esters)-based scaffolds have been proposed as a suitable alternative to pre-fabricated ones.

Microspheres are a common type of injectable poly(α-hydroxy esters)-based scaffolds. Bhuptani and Patravale (2016) fabricated porous PLGA-based microspheres as a potential scaffold in RE. They reported that the fabricated microscaffolds supported the proliferation and differentiation of dental pulp mesenchymal stem cell line *in vitro* and preserved the stemness properties of such cells. However, using cell lines in this study may provide different results from primary cells since such cells are manipulated genetically (Kaur & Dufour, 2012). In addition, poly(α-hydroxy esters) lack appropriate bioactivity. In this way, PLGA-based microspheres whose

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surfaces were modified with collagen type I were used for *in vitro* culturing of primary DPCs (Zou et al., 2017). In comparison with nonmodified microspheres, collagen-modified microspheres enhanced ALP activity and odontogenic gene mRNA expression of primary DPCs. A further increase in the bioactivity of poly(α-hydroxy esters)-based microspheres can be achieved by adding signalling molecules. Wang, Dang, et al. (2016) reported that treatment of SCAP-seeded PLLA nanofibrous microspheres with BMP-2 promoted odontogenic differentiation and biomineralization both *in vitro* and *in vivo*. However, direct loading of such GFs causes rapid diffusion of GFs away and rapid loss of their bioactivity. Hence, they incorporated PLGA microspheres encapsulating BMP-2 within the PLLA microspheres and observed osteodentine-like tissue deposition and abundant angiogenesis *in vivo*. Encapsulating FGF-2 and TGF-β1 within PLGA microspheres also resulted in the enhancement of DPC proliferation and promotion of STRO-1-positive progenitor pulp cell migration respectively (Mathieu et al., 2013).

Apart from GF incorporation, the microarchitecture and stiffness of microspheres can also determine their functionality. In this respect, Kuang et al. (2015, 2016) fabricated nanofibrous spongy microspheres based on star-shaped PLLA-block-poly(L-lysine) and compared DPSC behaviour in response to this architecture with that in response to nanofibrous and smooth architectures (Figure 4i). After 24 h of *in vitro* culturing, SEM images indicated enhanced attachment and spreading of DPSCs with abundant processes on the surface and inside of nanofibrous spongy microspheres compared to other microspheres (Figure 4ii). In addition, the ECM-mimicking nanofibrous architecture and the porous structure of this type of microspheres facilitated cell migration inside the construct, which led to greater *in vivo* degradation, higher angiogenesis and more tissue regeneration after 4 weeks of subcutaneous injection into nude mice (Figure 4iii).

In brief, poly(α-hydroxy esters) are promising as the basis of scaffolds for pulp–dentine complex regeneration. These synthetic compounds can be tailored to produce a broad spectrum of scaffolds with different microarchitectures from pre-fabricated to injectable. They can be also used for designing controlled release systems to deliver different compounds such as GFs or other differentiating agents. However, the most important barriers that may prevent their use in RE include their slow degradation rate, the acidity of degradation by-products and the lack of cell-binding sites. In such cases, the incorporation of natural biomaterials may help to remove these obstacles and provide a better microenvironment for stem cells to differentiate and regenerate new tissues.

Self-assembling peptides

Self-assembling peptides are a category of biological materials, which can spontaneously assemble into ordered nanostructures in response to external stimuli such as temperature, pH and electrolytes (Chen & Zou, 2019). They are composed of short sequences of amino acids (Lee et al., 2019). The characteristics of SAPs can be tuned by modulating the amino acid composition and conjugating chemical groups. Additionally, the presence of side chains in each amino acid makes peptides amenable to a wide variety of chemical modifications. This versatility provides the ability to fabricate hydrogel scaffolds engineered at the molecular level with tuneable properties for application in TE. In respect of RE, SAPs offer several advantages, indeed, their injectability, viscoelastic properties, options for conjugating peptides and the possibility of incorporating signalling molecules make SAPs promising candidates for pulp–dentine complex regeneration. The most common functional domains integrated into SAPs are bioactive motifs, including cell attachment sequences, enzyme-cleavable sites and signalling domains. Cell adhesion motifs improved cell–matrix interaction, whilst enzyme-cleavable sites make the structure susceptible to cell-mediated enzymatic degradation, which facilitates cell migration and remodelling of the matrix. Galler et al. (2010) designed β-sheet-forming multidomain peptides, a class of amphiphilic SAPs, which contained the RGD motif and the enzyme-cleavable site (MMP-2). The designed multidomain peptides had flanking blocks, which were positively charged, and assembled into a nanofibrous hydrogel by adding a negatively charged agent. They reported that the addition of an enzyme-cleavable motif led to the enhancement of cell viability, spreading and migration within the designed hydrogel. Later, they customized the designed self-assembled matrix by incorporating TGF-β1, FGF-2 and VEGF through binding to the negatively charged heparin, which also acted as the gelling stimuli, and demonstrated its potential as the scaffold in an *in vivo* ectopic model (Galler et al., 2012).

A widely used SAP hydrogel in TE applications, which is also available commercially, is PuraMatrix (also called RADA16-I). It is a 16 amino acid peptide that is synthesized by linking hydrophilic and hydrophobic amino acids in a repeating sequence. This peptide is an ionic self-complementary that forms stable β-sheet structures, which further self-assemble into nanofibrous constructs when introduced into physiologic concentrations of salts (Cavalcanti et al., 2013; Hauser & Zhang, 2010; Zhang et al., 2005). The high water content of PuraMatrix and its nanofibres with a diameter of ~10 nm, which is on the scale of those of natural ECM, desirably mimic *in vivo* conditions (Zhang et al., 2005). Using PuraMatrix hydrogel

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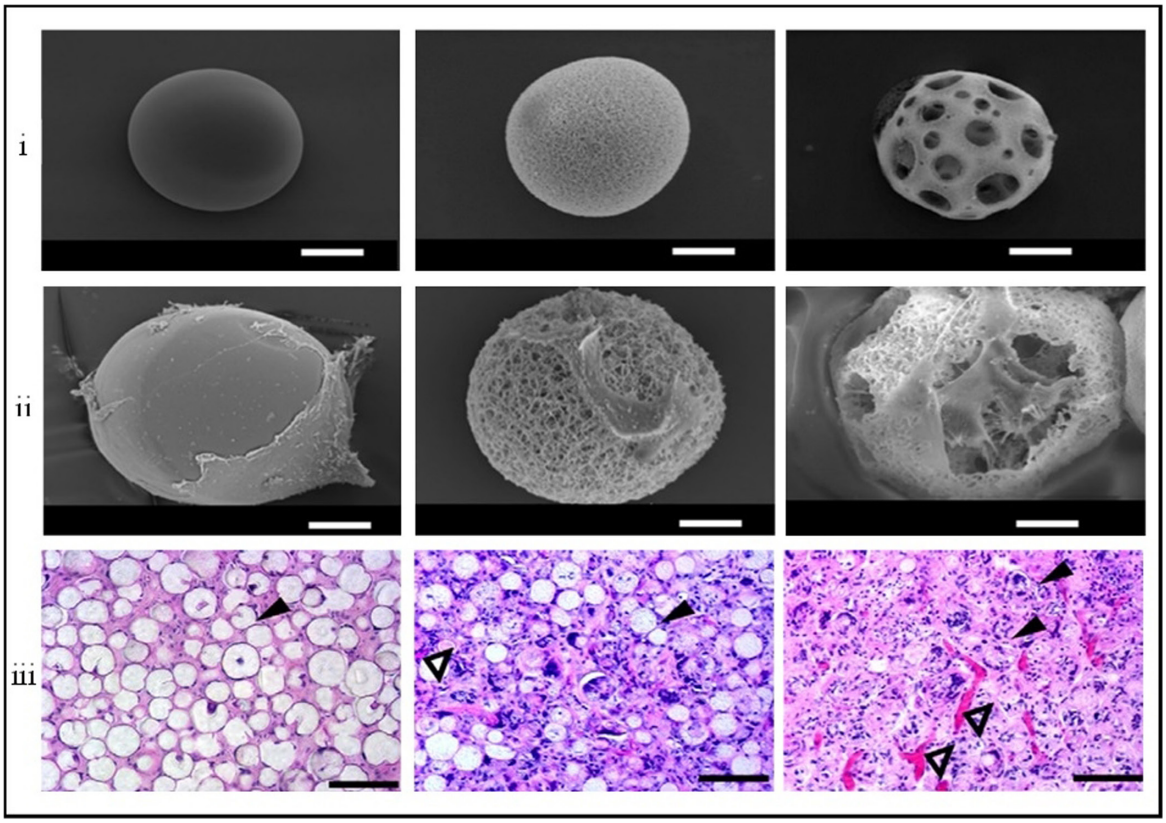

FIGURE 4 Effect of scaffold microarchitecture on the fate of cells. (i) SEM images of microspheres with different microarchitectures, including smooth (left), nanofibrous (middle) and nanofibrous spongy microspheres (right). Scale bar = 10 μm. (ii) SEM images of DPSCs seeded on each type of microspheres for 24 h. Scale bar = 10 μm. (iii) H&E staining of the harvested samples after 4 weeks of subcutaneous injection in nude mice. Solid and hollow arrows showed microspheres and blood vessels respectively. Scale bar = 50 μm. Adapted from Kuang et al. (2016) with permission from Elsevier, copyright 2016.

had promising results in cardiac (Davis et al., 2005), bone (Misawa et al., 2006) and neural (Thonhoff et al., 2009) tissue regeneration. Furthermore, it has been reported that PuraMatrix supported odontogenic differentiation of dental stem cells both *in vitro* (Cavalcanti et al., 2013) and *in vivo* (Dissanayaka et al., 2015; Rosa et al., 2013). To investigate its potential as the scaffold in RE, Rosa et al. (2013) used PuraMatrix mixed with SHED within the roots of human premolars for ectopic regeneration of dental pulp in the dorsum of nude mice. Histological analysis on Day 35 revealed the suitability of this construct for the formation of vascularized pulp-like tissues with the ability of new dentine generation along the full length of root canals. However, in this study, both ends of the canals were left open, which is far removed from the clinical situation where access to the blood supply is restricted to the apical foramen at the root tips. Dissanayaka et al. (2015) sealed the coronal ends of tooth root segments, which better mimic clinical conditions, and reported that using the combination of PuraMatrix with DPSCs and human umbilical vein endothelial cells (HUVECs) could lead to the formation of pulp-like tissue and new osteodentine/pre-dentine along with a layer of odontoblast-like cells. However, PuraMatrix without cells was not intrinsically able to recruit resident stem cells for the regeneration process. Moreover, the definition of a critical apical foramen size was necessary for successful dental pulp regeneration. Unlike roots with enlarged apical foramen, regenerated tissues in roots with normal apical root openings could not extend to the coronal ends.

Despite such promising results, using SAPs is associated with several drawbacks and limitations. For example, SAP-based hydrogels have poor mechanical strength and their internal pore shape cannot be controlled. One way of tuning the mechanical and biological performance of SAP hydrogels is to alter the SAP concentration. Typically, increasing the SAP concentration improved the mechanical strength of the matrix, but may limit cell viability and proliferation within the 3D construct. Encapsulation of DPCs and human umbilical cord MSCs within RADA16-I peptide hydrogel with different concentrations from 0.125% to 1% indicated that hydrogels with high concentrations (≥0.5%) decreased cell proliferation (Huang et al., 2020).

Briefly, the major advantage of SAPs over conventional nonbiological materials is their ECM-like microenvironment. The nanofibrous structure of SAP hydrogels

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properly mimics the nanostructure of natural ECM (Galler et al., 2010). Additionally, by altering peptide sequences, hydrogels with various functional properties can be produced. However, one of the major problems with peptides is the complexity of their design. Careful selection of amino acids in SAP chains is necessary to reach desirable physicochemical and viscoelastic properties of assembled hydrogels (Galler et al., 2010). Also, low mechanical strength and low productivity are other leading problems in the use of SAPs, which limits their application as the scaffold in regenerative medicine (Chang et al., 2017).

NATURAL POLYMER-BASED SCAFFOLDS

Naturally derived biomaterials are a major group of biomaterials used as the basis of scaffolds in RE. Derivation from natural resources makes these materials recognizable biologically and provides better cell–matrix interaction. They are usually biocompatible and biodegradable with minimal risk of tissue rejection. The most widely used natural-based biomaterials for application in pulp–dentine complex TE are proteins, polysaccharides, acellular ECMs and platelet concentrates that will be discussed in detail below and are summarized in Table 3.

Protein-based scaffolds

Using protein-based biomaterials is common in regenerative dentistry. Some such biomaterials are also used in various forms in dental practice. Resorbable collagen membrane is an example of these materials, which are widely used by endodontists, periodontists and oral surgeons for multiple purposes. So far, with the aim of pulp–dentine complex TE, various types of scaffolds have been fabricated based on natural proteins. In particular, researchers have focused on three kinds of natural protein to develop scaffolds for application in RE, namely, collagen, gelatine and fibrin, which will be summarized in the following sections.

Collagen- and gelatine-based scaffolds

Collagen is the most abundant protein in mammalians and the major structural building block of connective tissues. It is also the major organic compound of the dentine ECM (Goldberg et al., 2011) and plays a key role in dentine biomineralization (He et al., 2019). Up to now, numerous collagen-based scaffolds have been developed for pulp–dentine complex regeneration. Coyac et al. (2013) used type I collagen to fabricate a dense hydrogel with a fibrillar density similar to the native ECM of osteoid and pre-dentine through plastic compression of SHED-seeded collagen gels. They reported that such a scaffold not only promoted osteo-/odontogenic differentiation of SHED under osteogenic conditions but also supported mineral deposition that occurred suitably along the collagen fibrils. Due to its cell adhesion motifs, collagen is highly biocompatible and supports cell interactions. Comparison between collagen and other biomaterials such as poly(α-hydroxy esters; Leong et al., 2016; Sumita et al., 2006), chitosan (Kim et al., 2009) and gelatine (Kim et al., 2009) indicated the suitability of collagen in terms of biocompatibility and bioactivity for pulp–dentine complex TE. *In vitro* coincubation of DPSCs with PLGA-based scaffolds caused degeneration and apoptosis of such cells, especially in the vicinity of the scaffold (Leong et al., 2016). Whilst cells in contact with the collagen scaffold had high proliferation rates that were greater than those cultured in the growth medium alone. Culturing DPCs in well plates coated with collagen, gelatine and chitosan also indicated that odontogenic biomarkers, i.e. DSPP and DMP-1, were mostly expressed in cells cultured on collagen, where the most ECM mineralization was also observed through alizarin red staining (Kim et al., 2009).

As a naturally derived protein, collagen can be extracted from different sources and various tissues through decellularization and purification processes, and be used in various forms. Physiological collagen hydrogel and collagen sponges are two kinds of collagen-based scaffolds that have been widely used for cellular ingrowth and tissue regeneration. For instance, Pan et al. (2016) used a sheet of collagen sponge loaded with stem cell factor (SCF), a homing agent, for *in vivo* investigation of progenitor cell recruitment and pulp–dentine complex regeneration in an ectopic mouse model. In general, the matrix stiffness, microstructure and consequently, the biological performance of such scaffolds can be adjusted by altering the collagen concentration in precursor solutions (Pankajakshan et al., 2020). Typically, a greater concentration of prepolymer results in the fabrication of stiffer scaffolds. Using this information, Pankajakshan et al. (2020) prepared two matrices with a stiffness of 235 and 800 Pa using oligomer collagen concentrations of 1.37 and 2.88 mg/ml respectively. Encapsulation of DPSCs within the resulting hydrogels revealed that the hydrogel with lower stiffness induced endothelial differentiation of DPSCs, whilst the one with greater stiffness induced odontogenic differentiation. It was proposed that developing this system in a spatially oriented fashion, in which the stiffer matrix placed adjacent to the dentinal wall and the other placed in the centre of the root canal (Figure 5a), may lead to the regeneration of pulp and dentine tissues in their proper

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TABLE 3 Summary of the most important studies on natural polymer-based scaffolds for pulp–dentine complex TE

Biomaterials	Fabrication techniques	Main achievements	Drawbacks	References
Protein-based scaffolds				
Collagen	Physiological gelation & plastic compression	Fabrication of a dense hydrogel with a fibrillar density similar to the native ECM of osteoid and pre-dentine; Promotion of osteo-/odontogenic differentiation of SHED *in vitro*; Biomineralization along the collagen fibrils	Pre-fabricated; Loading extra force on encapsulated cells; Risk of pathogen transmission	Coyac et al. (2013)
Collagen	–	Induction of cell homing, angiogenesis and tissue remodelling *in vivo*	Using exogenous factors; Pre-fabricated	Pan et al. (2016)
Collagen	Physiological gelation	Providing spatial control over DPSC differentiation via controlling matrix stiffness	Difficulty of use in practice; Contraction of cell-seeded collagen-based hydrogels; Using exogenous GFs; Low mechanical strength; Risk of pathogen transmission	Pankajakshan et al. (2020)
Collagen	Chemical crosslinking	Addressing collagen shrinkage problem; Enhancement of mechanical strength and surface stiffness; Promotion of attachment, proliferation and odontogenic differentiation of DPCs *in vitro*	Possible cytotoxicity of crosslinking agents; Gelation time >5 min; Risk of pathogen transmission	Kwon et al. (2015), Kwon, Lee, et al. (2017), Kwon, Kim, et al. (2017)
Collagen	–	Orthotopic regeneration of pulp-like tissue and deposition of secondary dentine in the whole length of root canals	Pre-fabricated; Using exogenous factors; Risk of pathogen transmission	Iohara et al. (2016)
Collagen	Physiological gelation	Incorporation of exosome-like vesicles; Promotion of migration, proliferation and odontogenic differentiation of dental papilla cells *in vitro*; Regeneration of vascularized pulp-like tissue accompanied with innervation; Deposition of pre-dentine-like tissue with polarizing odontoblast-like cells	Difficulties in isolation and characterization of exosomes; Low mechanical strength; Risk of pathogen transmission	Zhang et al. (2020)
Gelatine	Photo-crosslinking	*In vivo* regeneration of ectopic pulp-like tissue	Using UV light; *In vitro* culturing prior to ectopic transplantation	Khayat et al. (2017)
Gelatine	Electrostatic microdroplet & photo-crosslinking	Control over scaffold architecture; Promotion of attachment, viability and proliferation of DPSCs *in vitro*; Improvement of degradability; Promotion of tissue regeneration *in vivo*	Limited cellular density of microspheres	Yang et al. (2021)
Gelatine	EDC/NHS-mediated crosslinking	Promotion of cell–matrix interaction and odontogenic differentiation of DPSCs by tunning matrix stiffness; Spatial control over pulp–dentine complex regeneration *in vivo*	Pre-fabricated; Difficulty of use in practice; *In vitro* culturing prior to ectopic transplantation	Qu et al. (2015)

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TABLE 3 (Continued)

Biomaterials	Fabrication techniques	Main achievements	Drawbacks	References
Fibrin	Enzymatic gelation	Indicating the superiority of fibrin over various synthetic biomaterials in terms of *in vivo* tissue regeneration and vascularization	Leaving both sides of constructs open; Rapid degradation; Low mechanical strength; Less control over its physicochemical properties	Galler et al. (2018)
Fibrin	Enzymatic gelation	*In vivo* regeneration of ectopic pulp-like tissue via the cell homing approach	Rapid degradation; Low mechanical strength; Less control over its physicochemical properties	Ruangsawasdi et al. (2014, 2017)
Fibrin	3D printing & enzymatic gelation	Regulation of odontogenic differentiation and biomineralization of DPSCs via mechanical cues	Pre-fabricated	Han et al. (2019)
Fibrinogen/ PEG-diacrylate	Photo-crosslinking	Control over fibrin degradation rate; Directing *in vitro* differentiation of DPSC via controlling hydrogel stiffness	Using UV light	Lu et al. (2015)
Polysaccharide-based scaffolds				
Hyaluronic acid	Chemical crosslinking	Establishment of mini-swine as a large animal model for orthotopic pulp regeneration studies; *In vivo* regeneration of pulp- and dentine-like tissues in full-length root canals in an orthotopic model; Formation of dentine bridge under the sealant	Slow degradation rate; Lack of bioactivity; Difficult manipulation & tooth sectioning; Short crown portion; Leakage of MTA	Zhu et al. (2018)
Hyaluronic acid/ cellulose	Hydrazone crosslinking chemistry	Enhancement of mechanical strength; Promotion of chemotactic and pro-angiogenic features of scaffolds via incorporating PL	Cytotoxic effect of cellulose nanocrystal	Silva et al. (2018)
Hyaluronic acid	Photo-crosslinking	Promotion of proliferation rate, ALP activity and calcium deposition of DPCs *in vitro* via incorporating PL	Using UV light	Almeida et al. (2018)
Chitosan/fibrin	Enzymatic gelation	Antibacterial activity against *Enterococcus faecalis* whilst preserving cytocompatibility	Aggregation of chitosan in the fibrin network; Gelation time >5 min	Ducret et al. (2019)
Alginate/nano-silicate laponite	Electrostatic microdroplet	Sustained release of VEGF for up to 28 days; Promotion of odontogenic differentiation of DPSCs *in vitro* and ectopic regeneration of vascularized pulp-like tissue *in vivo*	Using exogenous GFs; Slow degradation	Zhang et al. (2020)
Natural extracellular matrix-based scaffolds				
Pulp ECM	Decellularizing	Regeneration of ectopic pulp-like tissue and biomineralization *in vivo*	Using xenograft tissues; Pre-fabricated; Thinness of root segments; Nontuneable properties.	Hu et al. (2017)

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TABLE 3 (Continued)

Biomaterials	Fabrication techniques	Main achievements	Drawbacks	References
Pulp ECM	Decellularizing & freeze-drying	Regeneration of pulp-like tissue via recruitment of resident SCs in an orthotopic dog model	Using xenograft tissues; Pre-fabricated; Nontuneable properties	Alqahtani et al. (2018)
Pulp ECM	Decellularizing	Proposing a decellularizing method reducing tissue damages; Cytocompatibility of the decellularized tissue to DPSCs	Pre-fabricated; Limited access to allogenic tissues; Nontuneable properties	Matoug-Elwerfelli et al. (2018)
DPSC-derived ECM	Cell culturing & decellularizing	Enhancement of DPSC proliferation *in vitro*; Regeneration of ectopic pulp-like tissue with a well-organized ECM *in vivo*	Pre-fabricated; Difficulties in scaffold preparation; Nontuneable properties	Zhang et al. (2017)
Cell-derived ECM/ collagen/chitosan	Cell culturing & decellularizing	Promotion of proliferation and odontogenic differentiation of DPSCs *in vitro* and regeneration of vascularized pulp-like tissues *in vivo*	Pre-fabricated; Difficulties in scaffold preparation; Nontuneable properties	Huang et al. (2018)
Platelet concentrate-based scaffolds				
PRP	Initiation of the clotting cascade	*In vivo* regeneration of neotissue in an orthotopic dog model	Risk of immunological rejection and pathogen transmission; Nontuneable properties; Rapid degradation; Formation of PDL- and cementum-like tissues	Zhu et al. (2012, 2013, 2014)
PRF	Intrinsic activation of clotting cascades	Demonstrating the superiority of PRF over PRP in terms of promoting proliferation, migration and odontogenic differentiation of DPCs *in vitro*	Rapid degradation; Nontuneable properties	Chai et al. (2019)
PRF	Intrinsic activation of clotting cascades	Enhancing proliferation and odontogenic differentiation of DPSCs *in vitro*; Ectopic and orthotopic regeneration of pulp- and dentine-like tissues	Pre-fabricated; Nontuneable properties; Difficulty of use in practice	Chen, Zhao, et al. (2015)
CGF	Intrinsic activation of clotting cascades	Regulation of DPSC proliferation, migration and osteo-/odontogenic differentiation *in vitro*. *In vivo* biomineralization and ingrowth of soft connective tissue in an orthotopic dog model via cell homing approach	Rapid degradation; Nontuneable properties; Pre-fabricated.	Xu et al. (2019)

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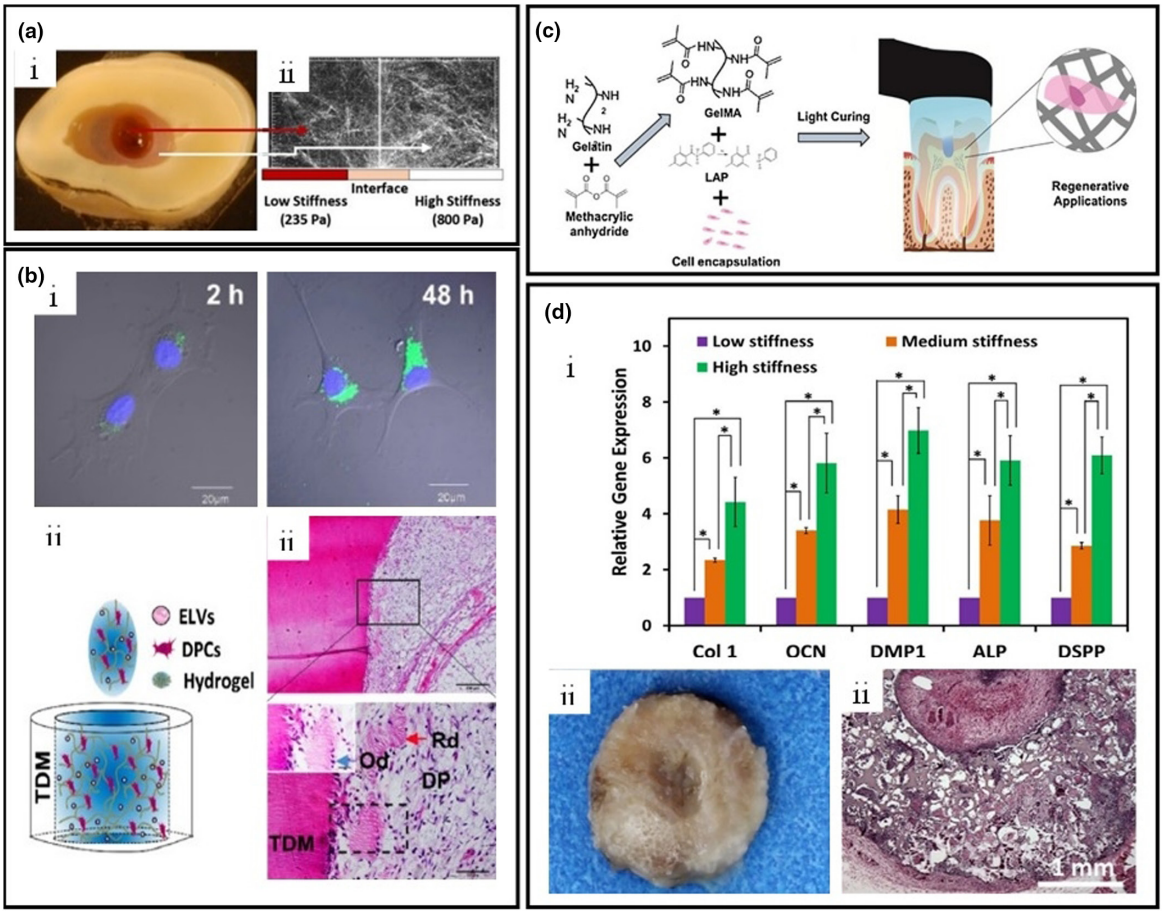

FIGURE 5 Collagen- and gelatine-based scaffolds for pulp–dentine complex TE. (a) Schematic illustration of (i) concentrated injection of the softer collagen hydrogel in the centre and the stiffer one in the peripheral area adjacent to the dentinal wall (ii) interface adaption between two hydrogels. Adapted from Pankajakshan et al. (2020) with permission from American Chemical Society, copyright 2020. (b) Role of exosome-like vesicles combined with collagen hydrogel in dental pulp regeneration. (i) Endocytosis of labelled vesicles (green) by dental papilla cell after 2 (left) and 48 h (right) of *in vitro* culturing. Cell nuclei were stained with DAPI (blue). (ii) Schematic illustration of sample preparation for *in vivo* implantation. (iii) H&E staining showed ectopic regeneration of pulp–dentine complex (TDM: treated dentine matrix; Rd: regenerated dentine-like tissue; Od: odontoblast-like cell; DP: dental pulp-like tissue). Adapted from Zhang, Yang, et al. (2020), licensed under creative commons license. (c) Schematic illustration of GelMA application in RE. Adapted from Monteiro et al. (2018) with permission from Elsevier, copyright 2018. (d) Pulp–dentine complex regeneration by modulating the stiffness of gelatine scaffold. (i) Relative odontogenic gene expression after DPSC culturing on the gelatine-based scaffolds with different stiffness. (ii) Regenerated dentine–pulp complex after 4 weeks of *in vivo* implantation of pre-cultured samples in nude mice. (iii) H&E staining of the regenerated pulp–dentine complex. Adapted from Qu et al. (2015) with permission from Elsevier, copyright 2015.

anatomical positions. However, the clinical application of such a system can be challenging in some circumstances. Additionally, the contraction of self-assembly collagen hydrogels during cell cultures (Zhu et al., 2001) is a major challenge for their application in TE. Collagen gels with greater cellular density and lower polymer concentration contract more (Zhu et al., 2001). Contraction of scaffolds after insertion into defects, such as a root canal, may disrupt the proper regeneration process due to the formation of voids. However, the shrinkage of collagen-based scaffolds has been addressed by the use of chemical crosslinking techniques. Free amines of collagen, mostly on lysine moieties, can be chemically modified with various crosslinking agents to produce materials that undergo sol–gel transition under various conditions. In addition to inhibiting collagen shrinkage, the chemical crosslinking of collagen-based scaffolds enhances the mechanical strength of such matrices. So far, different crosslinkers, such as cinnamaldehyde (Kwon, Lee, et al., 2017), genipin (Kwon et al., 2015), epicatechin (Lim, Lim, et al., 2016) and epigallocatechin gallate (Kwon, Kim, et al., 2017), have been used to fabricate chemically crosslinked collagen hydrogels for application in pulp–dentine complex TE. In all cases, it was observed that such techniques positively affected attachment, proliferation and odontogenic differentiation of DPCs through enhancement of mechanical strength and surface stiffness. However, the cytotoxicity of crosslinking agents or crosslinking procedures,

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the setting time of scaffolds as well as tooth discoloration are amongst the issues that should be considered when designing chemically crosslinked scaffolds. For instance, genipin reaction with amino acids or proteins such as collagen leads to the production of dark-blue pigments, which may induce tooth discoloration (Kwon et al., 2015).

Despite high biocompatibility and supporting proliferation and differentiation of odontogenic stem cells, it has been reported that collagen scaffolds alone did not cause any neotissue organization *in vivo* (Iohara et al., 2016; Prescott et al., 2008). In this regard, the bioactivity of collagen-based scaffolds can be enhanced through the incorporation of signalling molecules. Prescott et al. (2008) filled the pulp chamber of 2.5-mm-thick dentine slices with a commercially available collagen matrix, Collagraft Bone Graft Matrix Strip, with or without DMP-1 and DPSCs, and studied the effect of DMP-1 on *in vivo* regeneration of dental pulp. In canals filled with collagen scaffold alone or with DPSCs, only degradation of scaffolds, some red blood cells and a few nucleated cells were observed. Instead, the addition of DMP-1 to the construct guided the differentiation of DPSCs and led to the formation of blood vessels and a new matrix by endothelial and fibroblast cells, which indicated the progression of tissue regeneration. Inserting a DPSC-laden atelocollagen scaffold combined with granulocyte colony-stimulating factor into the root canal space of pulpectomized dog teeth with apical closure also clearly indicated the role of signalling molecules in the success of dental pulp regeneration throughout the entire length of root canals (Iohara et al., 2016). Recently, Zhang, Yang, et al. (2020) used the capability of exosomes, extracellular vesicles that contain a variety of GFs, cytokines, chemokines and RNAs (Joo et al., 2020), to bind to matrix proteins, such as collagen, to increase the bioactivity of the collagen scaffold. In recent years, MSC-derived exosomes have received much attention as bioactive agents to induce odontogenic differentiation in RE. Binding exosomes to collagen could lead to the sustained release of vesicles from the gel and their endocytosis by cells. They incorporated the physiological collagen hydrogel with exosome-like vesicles derived from Hertwig's epithelial root sheath cells to mimic epithelial–mesenchymal interactions during tooth development. They observed that under *in vitro* culturing in a medium supplemented with such exosome-like vesicles, the vesicles were endocytosed by dental papilla cells (Figure 5bi) and promoted cell migration, proliferation and odontogenic differentiation. Hence, cell-laden collagen gel containing attached exosome-like vesicles was used in an *in vivo* tooth root slice model (Figure 5bii), which resulted in the formation of vascularized pulp-like tissue accompanied by innervation and deposition of pre-dentin–like tissue with polarizing odontoblast-like cells adjacent to it (Figure 5biii). Nonetheless, it is worth mentioning that using exosomes remains challenging, mainly related to difficulties in their isolation and characterization (Li et al., 2019).

Gelatine, which is obtained by partial hydrolysis of collagen, is another promising biomaterial for RE purposes since it is very similar to collagen molecularly and functionally. Using a cell-laden gelatine sponge, combined with a blood clot in canine immature permanent teeth, clearly demonstrated its suitability for pulp–dentine complex regeneration, since the radiographic and histologic analysis of treated teeth revealed root development and apical foramen closure through the formation of pulp-like tissue in the lumen of root canals and new dentine deposition along the dentinal wall (Wang et al., 2013). Compared to collagen, gelatine is a water-soluble biopolymer whose denatured structure eliminates the risk of immunogenicity and pathogen transmission (Chang et al., 2017). However, its sol–gel transition with a lower critical solution temperature of approximately 37°C (Chang et al., 2017) makes its chemical modification inevitable to yield stable hydrogels at physiological conditions. In this regard, functionalizing of gelatine through methacrylation reaction has gained a great deal of attention in recent years. Gelatine methacrylate (GelMA) is a very popular photo-crosslinkable biomaterial that can be transited into a 3D hydrogel network through the free radical polymerization of its methacryloyl substituents upon light exposure and with the aid of a photo-initiator such as Eosin Y, lithium acylphosphinate or Irgacure 2959 (Yue et al., 2015). Such a crosslinking technique for RE can benefit from the availability of curing lights in dental clinics. Cytocompatibility of the GelMA hydrogel and its potential application in pulp–dentine complex TE was established by the encapsulation of odontoblast-like cells within it (Figure 5c) and obtaining a high percentage of cell viability (Monteiro et al., 2018). GelMA hydrogels with tuneable physicochemical properties could be fabricated by altering parameters such as degree of methacrylation, prepolymer concentration and crosslinking conditions (e.g. photo-initiator concentration, light intensity and light exposure time). Khayat et al. (2017) used 5 wt.% GelMA-encapsulated DPSCs and HUVECs for ectopic regeneration of dental pulp in nude rats. Root segments with 6 mm length and enlarged pulpal space lumen (2–3 mm orifice width) were used in this research. Upon UV light exposure for 20 s, cell-laden GelMA was photo-polymerized inside root segments, and samples were cultured in osteogenic media for 13 days. *In vitro* data revealed the formation of cellularized pulp-like tissue, which was followed by an increase in cellularity, neovascularization and matrix deposition after *in vivo* implantation. In addition to

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tuneable properties, GelMA is also versatile in terms of processability and can be fabricated with controlled architectures (Yue et al., 2015). Yang et al. (2021) used the advantages of microsphere hydrogels over bulk hydrogels (such as rapid transfer of oxygen and nutrients) and fabricated DPSC-laden GelMA microspheres by using the electrostatic microdroplet method. *In vitro* investigations indicated that such microspheres supported DPSC attachment, viability and proliferation. Furthermore, in comparison with bulk GelMA with similar cellular density, microspheres had better degradability and caused the formation of neotissue with a greater area rate and more vascularization *in vivo*. However, the hollow area of microspheres limited the cellular density of such a construct, which can prevent the extension of neotissue formation throughout the length of root canals (Yang et al., 2021).

Another popular crosslinking method in fabricating gelatine-based scaffolds is EDC/NHS-mediated crosslinking (carbodiimide chemistry), which leads to the carboxyl-to-amine linkage. Using this method, Qu et al. (2015) fabricated nanofibrous gelatine scaffolds with different stiffness via controlling crosslinking time and evaluated the behaviour of DPSCs in response to the mechanical cue. *In vitro* cell seeding onto the scaffolds with compressive modulus from 0.89 ± 0.43 to 18.23 ± 0.54 kPa revealed that greater stiffness improved cell spreading, cell–cell interaction and cell–matrix interaction and facilitated odontogenic differentiation (Figure 5di). Hence, a double-layered scaffold composed of a low stiffness matrix with a diameter of 2 mm in the centre and a ring-like high stiffness matrix with an outer diameter of 5 mm around it was fabricated for guided pulp–dentine complex regeneration. *In vitro* cultivation of cell-laden scaffolds in the odontogenic medium for 4 weeks revealed that the central area had higher cellularity, but mineralization only occurred in the high stiffness area. This result was further confirmed through observation of a well-organized pulp–dentine complex regenerated after *in vivo* implantation of pre-cultured samples in nude mice (Figure 5dii & iii).

In summary, the experimental evidence suggests that collagen holds promise as a suitable basis for designing scaffolds for pulp–dentine complex TE due to its high biocompatibility and its role in dentine biomineralization. Additionally, this naturally derived biomaterial can be engineered for the controlled release of signalling molecules to guide dental pulp regeneration properly. However, batch-to-batch product variation and possible immunological problems when derived from animal sources are the major obstacles that limit the application of collagen-based scaffolds in a clinical setting. The last issue can be addressed by using gelatine, which is more processible and whose denatured structure reduces the risk of immunological problems.

Fibrin and fibrinogen-based scaffolds

Fibrin is an elastic fibrous protein involved in the coagulation cascade. It is formed through the action of the serine protease thrombin on the soluble fibrinogen (Brown & Barker, 2014). Ease of polymerization and great cell–matrix interactions make fibrin a suitable biomaterial in regenerative medicine. Fibrin also possesses an inherent mechanism to bind several GFs, such as several members of the TGF-β family, PDFG and VEGF, and to release them in a controlled manner (Aksel et al., 2018; Martino et al., 2013; Mosesson, 2005). In RE, fibrin is a superior scaffold material for the induction of pulp-like tissue formation. Galler et al. (2018) investigated the suitability of different natural and synthetic biomaterials as the basis of scaffolding for dental pulp TE. They used fibrin, collagen, two types of SAPs as well as three forms of modified poly(ethylene glycol) (PEG). They reported that DPSC viability was significantly greater within natural-based scaffolds, especially within fibrin gel, than synthetic-based scaffolds. Similar results were obtained from *in vivo* experiments, where pulp-like tissue with the extension of cellular processes into dentinal tubules was regenerated in most cases of the fibrin group, whilst just a loose connective tissue was formed in most of the constructs containing functionalized PEG. Such observations demonstrate that fibrin may be the most suitable scaffold biomaterial to induce odontogenic differentiation and pulp-like tissue regeneration via cell-based strategies. *In vivo* studies on fibrin-based scaffolds have also confirmed their ability to support dental pulp TE via cell-free approaches (Ruangsawasdi et al., 2014, 2017; Widbiller et al., 2018). Ruangsawasdi et al. (2014) evaluated the suitability of fibrin gel as the scaffold for regeneration of the dental pulp using a cell homing approach. They filled the canal space of 9 mm root segments produced from extracted immature premolars with fibrin gel or left them empty and implanted the samples on top of the calvarial bone of rats. After 12 weeks, histological analysis revealed that the fibrin gel had triggered tissue ingrowth and directed the differentiation of migrated resident stem cells towards the formation of pulp-like tissue with a morphology similar to native tissue. Later, they facilitated this process by incorporating SCF into the fibrin gel (Ruangsawasdi et al., 2017). Under such conditions, an ectopic vascularized pulp-like tissue was regenerated in the middle third of the root canal, which was more mature compared to the newly formed tissue in the group without SCF.

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In general, fibrin gel is formed by mixing purified fibrinogen with a thrombin solution. In this way, fibrin clots with a range of crosslinking densities and thus different network architectures and cellular responses can be developed by using various concentrations of thrombin and fibrinogen (Zhao et al., 2008). Aksel et al. (2018) investigated the effect of four different concentrations of fibrinogen (12.5, 25, 50 and 100 mg/ml) on the proliferation of DPSCs at a constant thrombin concentration. They realized that fibrin gel with a lower concentration of fibrinogen facilitated cell elongation and promoted the proliferation rate of DPSCs. Using fibrin-based bioinks with two different concentrations of fibrinogen (5 and 20 mg/ml) to print pulp–dentine complexes with 3D patient-specific shapes also demonstrated that the fibrinogen concentration regulated odontogenic differentiation of DPSCs (Han et al., 2019). In the presence of differentiation media, localized mineralization only occurred in the dentine region, where a greater concentration of fibrinogen was present.

Although fibrin is considered a superior biomaterial for regeneration applications, its rapid degradation may limit the regeneration process to a shorter time (Park & Woo, 2018). One way of deaccelerating fibrin degradation is to combine it with other biomaterials such as bioceramics (Chatzistavrou et al., 2016) or synthetic biopolymers (Galler, Cavender, et al., 2011; Lu et al., 2015) that can also improve its mechanical strength. For example, Galler, D'Souza, et al. (2011) fabricated a hybrid material through PEGylation of fibrinogen. The PEGylated fibrin gel as the scaffold for cell-based regeneration of dental tissues had appropriate biocompatibility to bone marrow stromal stem cells and various odontogenic stem cells, including DPSCs, SHED and PDLSCs *in vitro*, and supported vascularized soft connective tissue generation by SHED *in vivo*. Lu et al. (2015) modified fibrinogen with PEG-diacrylate and fabricated a photo-crosslinked PEG-fibrinogen hydrogel with tuneable properties. In this way, the properties of the hydrogel, such as its stiffness, network density and swelling ratio, could be adjusted by altering the concentration of PEG-diacrylate, which acted as the crosslinker. *In vitro* application of PEG-fibrinogen hydrogels with different stiffness clearly demonstrated the effect of the stiffness of the hydrogel on the morphology and differentiation capacity of DPSCs. Within stiffer hydrogels, DPSCs remained round with a great percentage of cell aggregation and expressed higher levels of odontogenic markers, including DSPP, DMP-1 and OCN. However, within softer hydrogels, they had a spindle shape with higher Col I gene expression without any biomineralization.

Currently, fibrin-based scaffolds, especially platelet-rich fibrin (PRF), have been considered by many clinicians for use in regenerative dentistry to promote hard and soft tissue healing and regeneration (Hartshorne & Gluckman, 2016) due to their clinical benefits including excellent cytocompatibility and complete re-adsorption. However, in addition to weak mechanical strength and rapid degradation, using fibrin as the scaffold biomaterial is associated with several drawbacks, especially if it is derived from animal sources. Immunological risks due to pathogen transmission and possible transmission of infectious diseases are the two most important problems when using fibrin derived from animal sources (Noori et al., 2017). To avoid such problems, autologous fibrin derived from the same patient is a safe alternative to animal-derived fibrin. However, this process can be costly and time consuming, and the quality of the product will be patient dependent (Noori et al., 2017).

Polysaccharide-based scaffolds

Polysaccharides are another class of naturally derived polymers, which are formed through the binding of monosaccharides, as their structural building block, via glycosidic linkages (Bačáková et al., 2014). Found in living organisms, their biodegradability and tuneable physicochemical properties make polysaccharides promising scaffold materials in regenerative medicine. The backbone of polysaccharides contains a variety of functional groups, which provides the opportunity for chemical modification and fabrication of structures with adjustable properties by using various crosslinking mechanisms. From a structural perspective, polysaccharides are classified into two categories, linear polysaccharides and branched polysaccharides (Bačáková et al., 2014). Hyaluronic acid, chitosan and alginate are the well-known linear polysaccharides that have often been used as scaffold materials in pulp–dentine complex TE, and the following sections are dedicated to them.

Hyaluronic acid-based scaffolds

Hyaluronic acid (hyaluronan) is a glycosaminoglycan (GAG) consisting of repeating disaccharide units of β-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine that is found ubiquitously in connective tissues (Chang et al., 2017). In addition to its excellent biocompatibility and viscoelastic properties, previous studies suggest that hyaluronic acid contributes to the development of the dental pulp and dentine matrix (Felszeghy et al., 2000). Such properties make hyaluronic acid an appealing material for the fabrication of scaffolds in RE applications. *In vivo* comparison of a spongy scaffold based on hyaluronic acid with one based on collagen indicated that the

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hyaluronic acid-based scaffold better met the criteria of dental pulp TE via pulp capping (Inuyama et al., 2010). Insertion of hyaluronic acid sponges in dentine defects above the amputated rat pulp triggered the formation of pulp tissue with more cellularity and less inflammatory responses compared to collagen sponges. Also, *in vitro* studies reported that hyaluronic acid supported odontogenic differentiation of MSCs, including DPSCs and SCAP, and provided a suitable environment for the mineralization activity of such stem cells (Chen et al., 2016; Chrepa et al., 2017; Ferroni et al., 2015). For instance, *in vitro* treatment of DPCs with various concentrations of high-molecular-weight hyaluronic acid led to the enhancement of ALP activity of such cells at the early phase and deposition of more mineral nodules in a dose-dependent manner. In this process, the stimulatory effect of hyaluronic acid on mineralization was mediated by receptor CD44, which is a cell surface glycoprotein participating in different cellular functions (e.g. cell migration, adhesion and differentiation; Chen et al., 2016).

A simple way to prepare hyaluronic acid-based scaffolds is to use ionic crosslinking methods. Due to the presence of negative charges on its polymeric chain, hyaluronic acid can form polyelectrolyte complexes in combination with positive charge compounds such as chitosan (Kim et al., 2003). In this method, depending on the selection of polymer concentration in the precursor solution, the mechanical properties of the final structure can be adjusted. However, compared to chemically crosslinked hydrogels, ionically crosslinked hydrogels lack appropriate chemical and mechanical stability in physiological conditions (Chang et al., 2017). Accordingly, chemical crosslinking methods can be used to promote structural stability and tailor the properties of scaffolds. With this aim, each of the three functional groups of hyaluronic acid, namely, the carboxyl, hydroxyl and acetamido groups, can be chemically modified and used as sites for covalent bonding. 1,4-Butanediol diglycidyl ether, which reacts with the hydroxyl group of hyaluronic acid under strong base conditions, is a common crosslinking agent used for preparing commercially available hyaluronic acid hydrogels, such as Restylane® and Juvéderm™ (Allemann & Baumann, 2008; Xue et al., 2020). Zhu et al. (2018) used Juvéderm™ encapsulating swine DPSCs in an orthotopic model in mini swine to examine its performance during the pulp regeneration process. Four months after the injection of cell-laden gels into pulpectomized teeth with enlarged canals, regeneration of pulp-like tissue along the full length of root canals, formation of dentine bridges under the sealant as well as deposition of new dentine-like tissue along the dentinal wall were observed. However, using a collagen-based scaffold instead of hyaluronic acid led to the formation of a thicker dentine bridge and regeneration of more homogenous tissue, possibly because of the slower degradation rate of hyaluronic acid hydrogel compared to collagen (Zhu et al., 2018). Silva et al. (2018) improved the chemotactic and pro-angiogenic features of a hyaluronic acid-based scaffold by incorporating platelet lysate (PL) into the structure. They used hydrazone crosslinking chemistry between aldehyde and hydrazide derivatives of hyaluronic acid to fabricate an *in situ* forming viscoelastic hydrogel whose mechanical strength was enhanced through the addition of cellulose nanocrystals. Although the incorporation of cellulose nanocrystals decreased the viability and metabolic activity of encapsulated DPCs *in vitro*, the presence of PL improved the adhesion and proliferation of DPCs and 3D sprouting of DPC pellets with or without HUVEC. A combination of PL with photo-crosslinked hyaluronic acid hydrogels was also proposed as a potential bioactive scaffold for pulp–dentine TE since it enhanced proliferation rate, ALP activity and calcium deposition of DPCs (Almeida et al., 2018). It is worth mentioning that, PL as an endogenous source for growth and differentiation factors is considered a suitable alternative to exogenous factors, since using exogenous factors is associated with several fundamental challenges, such as high cost, immunogenicity and tumorigenesis (Galler, 2014; Lo et al., 2012).

Chitosan-based scaffolds

Chitosan is a cationic polysaccharide composed of randomly distributed D-glucosamine and N-acetyl-d-glucosamine. Owing to its abundance, great cytocompatibility and biodegradability to nontoxic products, chitosan has been used extensively in regenerative medicine. Moreover, chitosan has intrinsic antimicrobial properties that make it a prominent candidate for application in RE. The most prevalent proposed mechanism for the bactericidal effect of chitosan is leakage of intracellular materials as a result of interaction between the positively charged chitosan and negatively charged bacterial membranes (Atay, 2020). Ducret et al. (2019) designed injectable chitosan–fibrin hydrogel for application in dental pulp TE and reported its suitable antibacterial effect against *Enterococcus faecalis* whilst preserving its cytocompatibility with dental pulp mesenchymal stem/stromal cells. However, the poor solubility of chitosan in neutral aqueous solutions and organic solvents restricts its application in TE. To address such a limitation, researchers have developed chitosan-based scaffolds using physical and chemical crosslinking methods.

When working with a charged biomaterial, one of the most interesting crosslinking methods is ionic crosslinking, which involves polymer chains by electrostatic

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interactions. As chitosan is a positively charged biopolymer, it forms complexes with negatively charged biomaterials such as carboxymethyl cellulose (Chen & Fan, 2007), β-glycerophosphate (Wu et al., 2019), alginate (El Ashiry et al., 2018) and hyaluronic acid (Kim et al., 2003). Using such strategies, it is possible to take advantage of the desirable properties of each component and build structures with tuneable properties. For example, the addition of carboxymethyl cellulose to chitosan to form an ionically crosslinked scaffold not only improved the cytocompatibility of the construct but also upregulated the expression of ON and DSPP by DPSCs *in vitro* (Chen & Fan, 2007). Although ionic crosslinking avoids harsh conditions such as toxic organic solvents or crosslinking agents, chemically crosslinked scaffolds are more stable than ionically crosslinked scaffolds since the involvement of the polymer chain in such structures is performed via covalent bonding. The free amino and hydroxyl groups on the backbone of chitosan are amenable to a wide variety of chemical modifications such as carboxymethylation (Osmond et al., 2019) and amination (Afshar & Ghaee, 2016), which makes it a versatile biomaterial in developing chemically crosslinked scaffolds. However, chitosan matrices alone do not have cell attachment properties, which suggests the necessity of developing functional materials to improve cell–matrix interactions.

One way of improving the cellular behaviour of a construct is to modify its surface through the immobilization of small peptides or proteins containing biologically active sites. In this regard, Sana et al. (2017) immobilized the surface of a chitosan-based scaffold with either RDG or fibronectin to control and direct the response of DPSCs seeded on the scaffold surface. They had observed that DPSCs attached on the surface of the chitosan scaffold as spheroids and did not proliferate. Binding RGD to the surface of the chitosan increased the attachment of DPSCs but did not inhibit spheroid formation. In contrast, binding fibronectin resulted in the attachment and spreading of DPSC on the scaffold surface in a star-like shape. However, in order to direct odontogenic differentiation of such cells, appropriate signalling molecules were necessary. Biologically active ECM proteins can also be used in combination with chitosan to form composite scaffolds. For example, a gene-activated collagen–chitosan scaffold was fabricated to direct odontogenic differentiation of DPSCs (Yang et al., 2012). After incorporating plasmid vectors encoding BMP-7 gene into such a scaffold and cell seeding, upregulated expression of odontogenic markers (i.e. DSPP and DMP-1) was reported both *in vitro* and *in vivo*.

Altogether, the experimental evidence suggests that chitosan can be considered as a suitable scaffold for pulp–dentine complex TE due to its excellent biocompatibility and antibacterial activity. Additionally, it has been reported that the presence of chitosan in composite scaffolds significantly increased the initial attachment and proliferation of DPSCs onto the composite scaffold by enhancing surface tension and hydrophilicity of the construct (Tondnevis et al., 2019). However, some challenges need to be overcome including its high crystallinity, no cell attachment activity and the necessity of chemical crosslinking of chitosan for irreversible gelation that may be toxic or adversely affect its intrinsic properties.

Alginate-based scaffolds

Alginate is an anionic linear polysaccharide, which is composed of two repeating units, (1,4)-linked β-D-mannuronate and α-L-guluronate residues (Lee & Mooney, 2012). Its biocompatibility, ease of gelation and ability to tailor its properties make it a versatile biomaterial in TE applications. Alginate-based scaffolds can be prepared using various crosslinking methods, however, a widely used method is ionic crosslinking through the addition of a divalent cation such as Ca^{2+} (Lee & Mooney, 2012). Dobie et al. (2002) used this method to apply a TGF-β1-containing alginate hydrogel to the cut surface of the pulp in bisected tooth slices and observed *de novo* dentinogenesis along the cut pulpal surface. However, poor cell adhesion is the main drawback of alginate for applications in RE (Chang et al., 2017). To deal with this problem, the addition of cell adhesion motifs such as RGD peptides to the alginate structure that can lead to the improvement of cell–matrix interactions and cellular responses has received significant attention. For example, Bhoj et al. (2015) fabricated an RGD-bearing alginate hydrogel in the shape of gutta-percha, which also encapsulated VEGF, FGF-2, DPSCs and HUVECs and reported its sufficient biocompatibility within the first 7 days of *in vitro* culturing. However, on day 14, cells formed clusters and their proliferation declined. Using RGD-bearing alginate, Zhang, Xie, et al. (2020) developed a hybrid hydrogel microsphere through the electrostatic microdroplet method and investigated its application in RE. They combined RGD-bearing alginate with nanosilicate laponite and fabricated microspheres for the coencapsulation of DPSCs and VEGF. Laponite is a synthetic clay with charged nanostructure that makes it able to interact with many types of chemical entities, including small drugs and GFs (Tomás et al., 2018). The addition of laponite to such microspheres not only caused a sustained release of VEGF for up to 28 days but also improved mechanical properties and promoted the expression of odontogenic-related genes *in vitro*. Also, implanting the designed microspheres with human root segments (~4 mm in length)

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in nude mice led to the formation of pulp-like tissue with rich microvessels. However, the slow degradation of the microspheres caused voids in the neotissue.

The available studies indicate the feasibility of using alginate-based scaffolds for *de novo* regeneration of dental pulp tissues. However, the number of reports using alginate as the basis of scaffold for this application is limited, and more studies are needed to explore the potential of such a biomaterial in RE.

Natural ECM-based scaffolds

The ECM is the noncellular component of tissues, which structurally supports cellular constituents, and plays a vital role in regulating cell behaviour by providing biochemical and biomechanical cues (Frantz et al., 2010; Sackett et al., 2018). Water, polysaccharide GAGs and proteins such as collagen, laminin and fibronectin are the main components of the ECM. In regenerative medicine, the ECM is widely used as a natural source for the derivation of biological scaffolds in the form of decellularized matrices or hydrogels to better mimic tissue-specific microenvironments and guide new tissue regeneration. Scaffolds derived from dental pulp ECM can be an optimal candidate for pulp–dentine complex regeneration since it imitates the complexity of the native tissue through the preservation of tissue-specific signalling molecules and ECM's proteins, GAGs, vascular, lymphatic and nervous networks (Bakhtiar et al., 2020). Decellularizing swine dental pulp indicated that matrix proteins such as COL 1, DSP, DMP-1 and vWF were preserved after decellularization (Alqahtani et al., 2018). Moreover, it was reported that decellularized swine dental pulp ECM was able to induce the differentiation of dental follicle stem cells towards odontogenic phenotypes and regeneration of functional pulp-like tissue *in vivo* (Chen, Chen, et al., 2015). In this way, Hu et al. (2017) cut and fitted acellular swine dental pulp tissue in 1-mm-thick tooth root segments and recellularized it through seeding DPSCs. Histological analysis of samples after 2 months of subcutaneous transplantation into nude mice demonstrated regeneration of pulp-like tissue in the root segments with a layer of odontoblast-like cells, which strongly expressed DSPP near the dentinal wall. Moreover, a layer of mineralized tissue was deposited at a rate that was greater than that for natural pulp tissue. Inserting the ECM-derived scaffold from swine dental pulp combined with evoked bleeding in an orthotopic model also led to the recruitment of resident cells and induction of differentiation to regenerate pulp-like tissue expressing CD31 and DSP (Alqahtani et al., 2018). However, using xenograft tissue matrices is associated with the risk of immunogenic responses. Hence, Song et al. (2017) successfully decellularized human dental pulp within tooth slices extracted from healthy third molars. They reported that the decellularized tissue supported the proliferation and differentiation of SCAP *in vitro*.

Apart from acellular matrices, hydrogels derived from decellularized ECM can also be used in RE applications. It has been reported that digested decellularized swine pulp ECM as a supplement in the culture medium enhanced the viability of DPCs and promoted their migration (Alqahtani et al., 2018). Paduano et al. (2016) fabricated a hydrogel scaffold through demineralization and decellularization of ECM derived from cancellous bovine bone to promote odontogenic differentiation of DPSCs. Decellularized bovine pulp was also digested enzymatically and used to fabricate highly porous scaffolds through gelation of its neutralized solution at 37°C and freeze-drying (Bakhtiar et al., 2020). However, it has been reported that the spongy scaffolds obtained were not suitable for pulp regeneration since they are soluble in culture medium and easily degraded. In such a case, crosslinking the construct chemically could be helpful.

Despite these hopeful outcomes, using decellularized ECM presents challenges in practice. An important issue that should be considered in the decellularizing process is the selection of a suitable decellularization protocol. An appropriate method should lead to the greatest removal of cellular components whilst the native structure of the matrix is preserved. The suitability of three different protocols for decellularizing human dental pulp was evaluated by Song et al. (2017). They found that the efficiency of ECM preservation and cellular removal depended on the protocol used. The most effective method for the removal of cellular DNA was one using three cycles of 1% sodium dodecyl sulfate (SDS) and one cycle of 1% Triton X-100 agents (Song et al., 2017). However, previous studies have shown that using high concentrations of SDS and Triton X-100 causes tissue damage (Bondar et al., 1986) and removal of GAGs (Gilbert et al., 2006) respectively. Hence, an established protocol using one cycle of SDS with a lower concentration (0.03%) was used to investigate the feasibility of human pulp decellularizing (Matoug-Elwerfelli et al., 2018). In this method, DNA removal was achieved with an efficiency of approximately 98%, and the pulpal histoarchitecture of decellularized tissue was preserved. Also, the acellular dental pulp was cytocompatible for mouse fibroblasts L929 cell lines and DPSCs. Using the same protocol, decellularization of rat dental pulp tissue was performed and similar results were obtained (Matoug-Elwerfelli et al., 2020).

Other critical issues with the decellularized ECM that are mostly faced in their clinical translation are the limited access to autologous and allogenic pulp tissues

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and the risk of immunogenicity of xenograft tissues. As a solution, Zhang et al. (2017) prepared DPSC-derived ECM as the cell niche through the 2D cultivation of DPSCs in the presence of L-ascorbic acid and subsequent decellularization. In this work, recellularizing the derived ECM and using the construct for *in vitro* and *in vivo* investigations led to the enhancement of DPSC proliferation whilst preserving their properties of stemness and the formation of pulp-like tissue with well-organized ECM respectively. Ravindran, Huang, and George (2014) and Ravindran, Zhang, et al. (2014) embedded a collagen/chitosan scaffold with the ECM of DPSCs by culturing such cells within a collagen/chitosan hydrogel for 2 weeks in the presence of differentiation medium and then decellularizing the obtained construct. They observed that the fabricated ECM-embedded scaffold contained pulp-specific proteins such as DSP, DPP and DMP-1 and supported biomineralization and odontogenic differentiation of DPSCs and PDLSCs *in vitro* and *in vivo*. Using a similar technique, Huang et al. (2018) fabricated a dual ECM scaffold consisting of a pulp-specific ECM and an endothelial ECM to better mimic the ECM of native tissues. They cultured HUVECs within the lyophilized ECM-embedded scaffold for another 1 week, and the decellularizing process was performed again. In this way, proteins needed for odontogenic differentiation such as DMP-1, DPP, DSP and BMP-2, as well as those needed for vascularization and angiogenesis such as vWF, VEGF and bFGF, were present in the scaffold that cause promotion of proliferation and odontogenic differentiation of DPSCs *in vitro* and vascularization *in vivo*. However, difficulties in the fabrication of such scaffolds and their pre-fabricated nature limit their application in clinical settings. Moreover, compared to decellularized pulp tissues, microstructures such as blood vessels and lymph tubes were not found in such constructs (Zhang et al., 2017).

Platelet concentrate-based scaffolds

Platelet concentrates are a class of autologous biomaterials consisting of fibrin matrix and cocktails of key cytokines and GFs that are widely used in dentistry and especially in RE. Platelet-rich plasma (PRP), PRF and concentrated growth factor (CGF) are the three generations of platelet concentrates that can be extracted from whole blood after processing, mostly through centrifugation (Zumarán et al., 2018). Preparing from the blood of patients, ease of preparation, fibrous microarchitecture, appropriate elasticity and low cost make platelet concentrates as promising 3D biomaterials in RE. Moreover, the presence of a mixture of cytokines and GFs, such as TGF-β1, VEGF, PDGF and FGF, in platelet granules (Qiao et al., 2017) makes platelet concentrates appropriate candidates for guiding odontogenic differentiation. *In vitro* culturing of DPCs, treated under inflammatory conditions, in a medium supplemented with PRF extract resulted in the inhibition of inflammation and enhancement of odontogenic differentiation through the upregulation of DSPP and DMP-1 gene (Kim et al., 2017). PRP also promoted *in vitro* proliferation and odontoblastic differentiation of DPCs (Chai et al., 2019). So far, several researchers have used such naturally derived biomaterials alone or together with the blood clot to improve the clinical outcomes of pulp–dentine complex regeneration processes. For a comprehensive review on the clinical application of platelet concentrates in RE, readers are referred to reviews (Bakhtiar et al., 2017; Gaviño Orduña et al., 2017; Hartshorne & Gluckman, 2016; Lolato et al., 2016; Panda et al., 2020).

PRP is the first generation of platelet concentrates that is extensively applied to promote healing or regeneration in dental and oral therapeutic applications as a supplement or scaffold (Albanese et al., 2013). PRP gel scaffold can be prepared through the addition of calcium chloride and/or thrombin to the extracted PRP that leads to the initiation of clotting cascade and formation of fibrin matrix (Ehrenfest et al., 2009). To evaluate the capacity of PRP to regenerate pulp-like tissue, Zhu et al. (2012) designed an experiment in an orthotopic model, in which PRP with/without autologous DPSCs was transplanted into mature permanent premolars of canines with enlarged apical foramina and compared the results with those obtained from the evoked bleeding technique. Radiological and histological analyses indicated that replacing a blood clot with DPSC-laden PRP did not cause any improvement in tissue regeneration. In the blood clot group, the new vital tissue formed along the full length of all root canals, whereas in the PRP with/without DPSCs groups, some canals remained without new tissues. Moreover, instead of pulp–dentine complexes, PDL-like tissue with cellular bony islands within it and acellular cementum-like tissue along the dentinal wall were formed (Zhu et al., 2012, 2014). Using PRP in immature dog teeth with induced apical periodontitis (Zhu et al., 2013) and in ferret teeth with necrotic pulps and periapical lesions (Torabinejad et al., 2015) also led to the formation of new tissues whose histological characteristics were different from those of pulp- and dentine-like tissues. In addition to the lack of particular advantage in pulp–dentine complex regeneration, using PRP is associated with the potential risk of immunological rejection and pathogen transmission due to the activation by anticoagulant and thrombin (Jin et al., 2018). Hence, other generations of platelet concentrates, i.e. PRF and CGF, have been investigated widely.

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PRF is a modified generation of platelet concentrates whose clotting process and fibrin matrix formation are activated intrinsically and need no chemical manipulation. Moreover, it had a more sensible effect on the proliferation, migration and odontogenic differentiation of DPCs than PRP (Chai et al., 2019). Hence, the 3D gel of PRF with great elasticity and flexibility is a suitable alternative to PRP in RE. *In vitro* studies demonstrated that PRF membrane had a stimulatory effect on the proliferation and odontoblastic differentiation of seeded DPSCs (Huang, Yang, et al., 2010). Moreover, combining cell-sheet fragments of canine DPSCs with different concentrations of PRF granules led to the enhancement of cell proliferation and upregulation of osteo-/odontogenic gene expression in a dose- and time-dependent manner (Chen, Zhao, et al., 2015). Also, such a construct was able to regenerate pulp-like tissue with a compact structure and dentine-like tissue after ectopic and orthotopic transplantation. Similar to PRF, the 3D-architecture gel of CGF is formed via intrinsic activation of clotting cascades. In comparison with PRF, however, CGF has greater stability and stiffer texture due to its consistent centrifuge conditions and contained more GFs (Hong et al., 2018; Jin et al., 2018). Notwithstanding, in the field of RE, only a handful of studies have evaluated the performance of CGF as an autologous bioactive scaffold. For instance, Jin et al. (2018) used small pieces of CGF membranes, fabricated via mechanical compression of its gel, to evaluate its cytocompatibility as the scaffold to DPSCs. SEM images revealed that DPSCs attached well to the surface of CGF membranes and extended their processes to confirm the great biocompatibility of autologous CGF scaffolds. Also, Xu et al. (2019) transplanted CGF in an orthotopic dog model that led to the apical closure, deposition of pre-dentine–like tissue along the dentinal wall and ingrowth of soft connective tissue stained positively for VEGF and Nestin inside canals.

Despite the excellent bioactivity, practicality and clinical benefits of platelet concentrate-based scaffolds, their rapid degradation and nontuneable properties hamper their application in the pulp–dentine complex regeneration via TE-based approaches. In addition, using these materials is associated with a variety of uncertainties. The autologous approach presents inconsistency of product properties. Hence, the obtained results may be unreproducible, and the success of clinical outcomes in the regeneration of vital pulp tissue is still controversial (Kim et al., 2018; Riaz & Shah, 2020).

MULTICOMPONENT COMPOSITE SCAFFOLDS

One of the most promising approaches to produce scaffolds, which properly mimic the complexity of native tissues, is fabricating multifunctional composite scaffolds. A composite scaffold is defined as a scaffold fabricated based on two or more biomaterials that behave together to provide a suitable substrate with more favourable properties and mechanical integrity for cell growth and differentiation. Up to now, several composite scaffolds have been used for engineering pulp–dentine complexes (Table 4). In such a structure, various biomaterials are integrated with each other, whilst preserving their desired properties. More complex structure, ability to enhance cellular interaction and tuneable physicochemical properties of a composite scaffold improved their performance compared to a single-phase scaffold. Optimizing the cellular response of a commercially available injectable hydrogel, HyStem-C™ – composed of polyethylene glycol diacrylate, thiolated hyaluronan and thiolated gelatine –, via adjusting the component ratios clarified the role of each component in achieving high DPSC proliferation and spreading (Jones et al., 2016). Gelatine promoted cell viability and proliferation rate, whilst PEG concentration determined the injectability of the construct. The addition of fibronectin into the construct also resulted in more cell spreading in a dose-dependent manner. However, prolonged gelation time limits the use of this hydrogel as an *in situ* forming hydrogel for dental pulp regeneration.

Amongst the possible combinations, synthetic/natural polymer–bioceramic and polymer–polymer composites are the most commonly used combinations in manufacturing composite scaffolds for pulp–dentine complex TE. In particular, natural polymer–bioceramic composite scaffolds have gained considerable attention since they can mimic the natural ECMs of pulp and dentine tissues, which are mainly composed of collagen and mineral phase respectively. Providing suitable cell–matrix interaction and playing role in biomineralization, collagen can be considered the organic phase of the construct, whilst bioceramics function as the inorganic reinforcing phase to induce odontogenic differentiation and biomineralization (Lim, Nam, et al., 2016; Yang et al., 2010). In such constructs, bioceramics can also improve mechanical strength and alter physicochemical properties. A composite scaffold based on collagen has been developed by mixing nanofibrous mesh of bioactive glass with a collagen solution followed by EDC/NHS crosslinking (Bae et al., 2012). Results from cellular studies highlighted that the presence of bioactive glass enhanced the proliferation of DPCs and promoted mineralized nodule deposition and odontogenic-related gene expression. Using other members of bioceramics, such as calcium aluminate (Bordini et al., 2020; Soares et al., 2016, 2017) and HA (Tondnevis et al., 2019), as the organic phase in composite scaffolds also led to the enhancement of osteo-/odontogenic capability of the construct.

Chitosan is another biomaterial that has been widely used as the organic phase in composite scaffolds for RE.

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TABLE 4 Summary of the most important studies on multicomponent composite scaffolds for pulp–dentine complex TE

Biomaterials	Fabrication techniques	Main achievements	Drawbacks	References
PEG diacrylate/ hyaluronic acid/ gelatine	Chemical crosslinking	Promotion of scaffold injectability; Promotion of DPSC proliferation and spreading via altering scaffold composition	Gelation time >5 min	Jones et al. (2016)
Collagen/bioactive glass	EDC/NHS crosslinking	Enhancement of DPC proliferation; Promotion of mineralized nodule deposition and odontogenic-related gene expression	Pre-fabricated	Bae et al. (2012)
Chitosan/collagen/ calcium aluminate	Freeze-drying	Enhancement of proliferation and osteo-/ odontogenic differentiation of DPCs *in vitro*	Pre-fabricated	Soares et al. (2016, 2017)
Chitosan/calcium aluminate	Freeze-drying	Enhancement of osteo-/odontogenic differentiation of DPCs	Pre-fabricated	Bordini et al. (2020)
PCL/chitosan/HA	Freeze-drying & chemical crosslinking	Enhancement of initial attachment and proliferation of DPSCs via increasing surface tension and hydrophilicity	Pre-fabricated	Tondnevis et al. (2019)
Polydioxanone/halloysite nanotubes	Electrospinning	Enhancement of the proliferation of human-derived pulp fibroblast cells by tunning the mechanical strength of the scaffold	Pre-fabricated; Lack of bioactivity	Bottino et al. (2015)
Alginate/gelatine	3D printing	Fabrication of geometrically clear-cut 3D constructs with 100% connectivity; Promotion of growth and adhesion of DPSCs	Pre-fabricated	Yu et al. (2019)
Alginate/dentine matrix	3 D printing & ionic crosslinking	Improvement of SCAP viability and odontogenic differentiation *in vitro*	Pre-fabricated	Athirasala et al. (2018)
Collagen/agarose	Hand-held bioprinting	*In situ* bioprinting; Formation of a vascular network after 14 days of *in vitro* culturing	Difficulty of use in practice; Using bovine teeth with larger root canals	Campos et al. (2020)

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For example, Shanhnavazi et al. (2017) took advantage of the chemical similarity of HA to dental tissues to make a chitosan/nano-HA composite scaffold for dental TE. However, in addition to composition, the fabrication technique is also an influential factor in determining the properties of a composite structure. In this regard, they fabricated the composite scaffold via two different techniques, freeze-drying and electrospinning. They reported that the freeze-drying technique allowed a greater amount of HA to be used, which improved the mechanical strength and physical properties of the scaffold, whilst electrospinning led to the formation of a more flexible scaffold with lower density, aligned fibres and greater surface area that better simulated natural ECM.

Considering the nontoxicity of halloysite nanotubes and their ability to release bioactive agents in a controllable manner (Kamble et al., 2012), it would be an interesting strategy to use them as the inorganic phase in composite scaffolds for the regeneration of pulp–dentine complex. Halloysite nanotubes have a high surface area and their lumens can protect bioactive agents from harsh processing conditions (Kamble et al., 2012). By altering the amount of halloysite nanotubes in composite scaffolds, a range of mechanical strengths and cellular responses can be obtained. Bottino et al. (2015) fabricated an electrospun nanocomposite scaffold based on polydioxanone and halloysite nanotubes as a potential bioactive scaffold for RE. In such a construct, halloysite nanotubes increased the proliferation of human-derived pulp fibroblast cells by reducing the mechanical strength of the scaffold.

A further interesting approach in designing composite scaffolds for application in RE, which has gained popularity in recent years, is alginate-based scaffolds fabricated by 3D bioprinting. Fabricating geometrically clear-cut 3D constructs, controlling cell distributions, high-resolution cell deposition and scalability are the main advantages of 3D bioprinting (Mandrycky et al., 2016). To better reveal the difference between 3D bioprinting and conventional techniques, Yu et al. (2019) compared the suitability of solidified alginate/gelatine scaffold and 3D-printed alginate/gelatine scaffold for DPSCs to survive. They found that 100% connectivity and 3D structure of printed composite scaffold made a better microenvironment for DPSC to attach and grow. High connectivity provides suitable permeability for the transfer of nutrients and oxygen in the scaffold and wastes out of it, and 3D structure causes appropriate cell distribution within the scaffold. The authors also conducted an *in vitro* study to investigate the effect of scaffold extracts on the proliferation and differentiation of SCAP and observed that the degradation products of the 3D-printed scaffold were more effective than those of a solidified composite scaffold. Athirasala et al. (2018) designed a bioactive composite hydrogel by blending sodium alginate with the soluble and insoluble fractions of the dentine matrix as a bioink and using $CaCl_2$ as the crosslinking agent. They observed that printed hydrogel improved the viability of encapsulated SCAP. However, the pre-fabricated nature of bioprinted structures restricts their application in RE. To deal with this problem, Campos et al. (2020) used an *in situ* hand-held bioprinting technique to fabricate a scaffold inside tooth roots. They highlighted the controlled drop deposition, high precision through the ejection of nanolitre drops, minimum loss of bioink and elimination of the risk of air bubble entrapment as the main advantages of such a technique. Hand-held bioprinting DPC/HUVEC-laden scaffolds based on collagen type I and agarose inside mechanically enlarged bovine tooth roots showed preservation of the original shape of the construct and formation of a vascular network after 14 days of *in vitro* culturing. However, it should be noted that the researchers created a channel within the printed structure using a metal wire for better penetration of nutrients from the culture medium into the scaffold, the applicability of which should be evaluated in clinical settings.

VASCULATURE STRATEGIES IN DESIGNING SCAFFOLDS FOR PULP–DENTINE COMPLEX TISSUE ENGINEERING

The ultimate goal in RE is to engineer functional dental pulp tissue within the cleaned root canal system of a tooth. In this regard, the regenerated pulp tissue should be vascularized and innervated with cellularity and architecture similar to the native tissue and be able to produce new dentine via a new layer of odontoblast cells lining the dentinal wall. To this aim, the establishment of a vascular network is a primary requirement. Pulpal vasculature delivers oxygen and nutrients to the tissue, whilst removing waste products, and plays a major role during the secretory activity of odontoblasts and dentine formation (Yoshida & Ohshima, 1996). However, entrapment of dental pulp tissue within teeth restricts access to the blood supply to the small apical foramen at the root apex. Therefore, pulp regeneration without a rapid establishment of vascular networks will lead to apoptosis of cells at remote distances and limit the volume of the regenerated tissue. To address this limitation, the development of strategies to promote neovascularization is necessary. In recent years, with the advancement of knowledge about the angiogenesis process, several strategies have been proposed amongst which incorporation of angiogenic factors within engineered constructs for prolonged release and

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fabrication of pre-vascularized matrices have been widely used in designing scaffolds (Dissanayaka & Zhang, 2017).

Vascularization within TE constructs is established mainly through capillary sprouting from pre-existing blood vessels (Kaully et al., 2009). Many angiogenic factors are involved in this process, the most well-known of which is VEGF. VEGF not only promotes the migration of endothelial cells for tube network formation and vascular development (Lamalice et al., 2007) but also induces stem cell differentiation into endothelial cells (Jazayeri et al., 2008; Sakai et al., 2010). *In vitro* culturing of SHED-laden PLLA scaffold in a medium supplemented with VEGF demonstrated the expression of VEGF receptors and capillary sprouts, which confirmed the angiogenic capability of SHED under the induction of VEGF (Sakai et al., 2010). In addition to endothelial differentiation under the induction of VEGF, SCAP and DPSCs secrete VEGF themselves during their *in vitro* culturing (Hilkens et al., 2017), which could inhibit endothelial cell apoptosis and facilitate vascular network formation (de Cara et al., 2019; Dissanayaka et al., 2015). However, in comparison with cell-free scaffolds, *in vivo* transplantation of DPSC/SCAP-laden scaffolds did not improve vascularization rates, which may be due to the odontogenic differentiation of SCAP and DPSCs instead of their endothelial differentiation since pulp–dentine-like tissue formation was observed in the cell-laden group (Hilkens et al., 2017). Several experiments also revealed that the incorporation of scaffolds with dental stem cells alone, without any angiogenic factor, led to the engineering of dental pulp tissue only in a part of root canals (Li et al., 2016; Zhu et al., 2019). Accordingly, the embodiment of VEGF within scaffolds for prolonged release was proposed as a strategy for engineering tissue vascularization. In this regard, Yadlapati et al. (2017) fabricated a delivery system based on polydioxanone fibres that carried VEGF at a linear concentration of 12.2 ng/cm for sustained release of this factor for up to 25 days. In general, using controlled release systems for the delivery of GFs prevents them from rapidly penetrating outside the injection site and losing their bioactivity *in vivo*. Ectopic transplantation of such construct within human tooth roots revealed its ability to promote new blood vessel formation *in vivo* (Figure 6a). In another study, Li et al. (2016) designed a hierarchical nanofibrous microsphere system based on PLLA with the capability of VEGF-controlled release and used it for *in vivo* regeneration of dental pulp. In the fabricated system, sustained release of VEGF was obtained through the interaction of VEGF with heparin, which was conjugated to gelatine nanospheres, and immobilization of such nanospheres in the nanofibres of PLLA-based microspheres (Figure 6b). Using the DPSC-seeded microspheres in an *in vivo* model revealed that VEGF-loading microspheres facilitated pulp regeneration, and caused the formation of pulp-like tissue from the apex to the coronal third of the canal (~9 mm). However, in the case of using cell-seeded microspheres without VEGF, pulp-like tissue was formed only in some regions of the apical third area.

In addition to the entire VEGF protein, it has been reported that small peptides that reproduce VEGF-binding region (VEGF17-25 helix region) are also able to modulate VEGF–receptors interaction and promote angiogenesis (D'Andrea et al., 2005; Santulli et al., 2009). In this regard, a VEGF-mimicking peptide termed QK, which induces endothelial cell proliferation and modulates propensity towards angiogenesis, has been synthesized (D'Andrea et al., 2005; Santulli et al., 2009). Using the QK domain, Kumar et al. (2015) designed a β-sheet-forming multidomain peptide, which was augmented by an enzyme-cleavable site and a pro-angiogenic sequence. They proposed that such a multidomain peptide could preserve angiogenic functionality for a longer period. *In vivo* investigations revealed that the resulting SAP hydrogel supported the rapid establishment of a mature vascular network (Kumar et al., 2015). Moreover, the acellular hydrogel was able to regenerate vascularized pulp-like tissue with neural filaments and odontoblast-like cells in the canal space of canine teeth (Siddiqui et al., 2021).

Apart from angiogenic GFs, hypoxia conditions can stimulate angiogenesis through the activation of transcriptional factors (e.g. hypoxia-inducible factor-1) and upregulation of VEGF expression (Maxwell & Ratcliffe, 2002). It has been reported that culturing DPSC-seeded microspheres under 3D hypoxic conditions was associated with the promotion of VEGF mRNA expression of such cells (Li et al., 2016). In addition, the effect of hypoxic culture before the implantation was further observed via regeneration of pulp-like tissue with higher vascularity in ectopic and orthotopic models, as compared with the control group with the normoxic culturing condition (Li et al., 2016). Hence, in several studies, hypoxia mimetic agents were used as angiogenic factors to promote the formation of blood vessels. Dimethyloxalylglycine (DMOG) is one such agent, which was used to evaluate the effect of hypoxia condition on DPC differentiation towards the odontoblastic and endothelial phenotypes (Yoo et al., 2018). In this regard, DMOG-embedded PCL fibres were fabricated through electrospinning, and after cell seeding, used in an *in vivo* ectopic model in nude mice. Experimental data revealed that the incorporation of DMOG not only promoted odontoblastic differentiation of DPCs through elevating mRNA expression of DSPP and BSP but also enhanced angiogenesis and neurogenesis from the surrounding tissues, as evidenced by elevated expression of mouse VEGF, mouse platelet cell adhesion molecule-1 and mouse neurofilament light-chain polypeptide. Liu et al. (2021) fabricated

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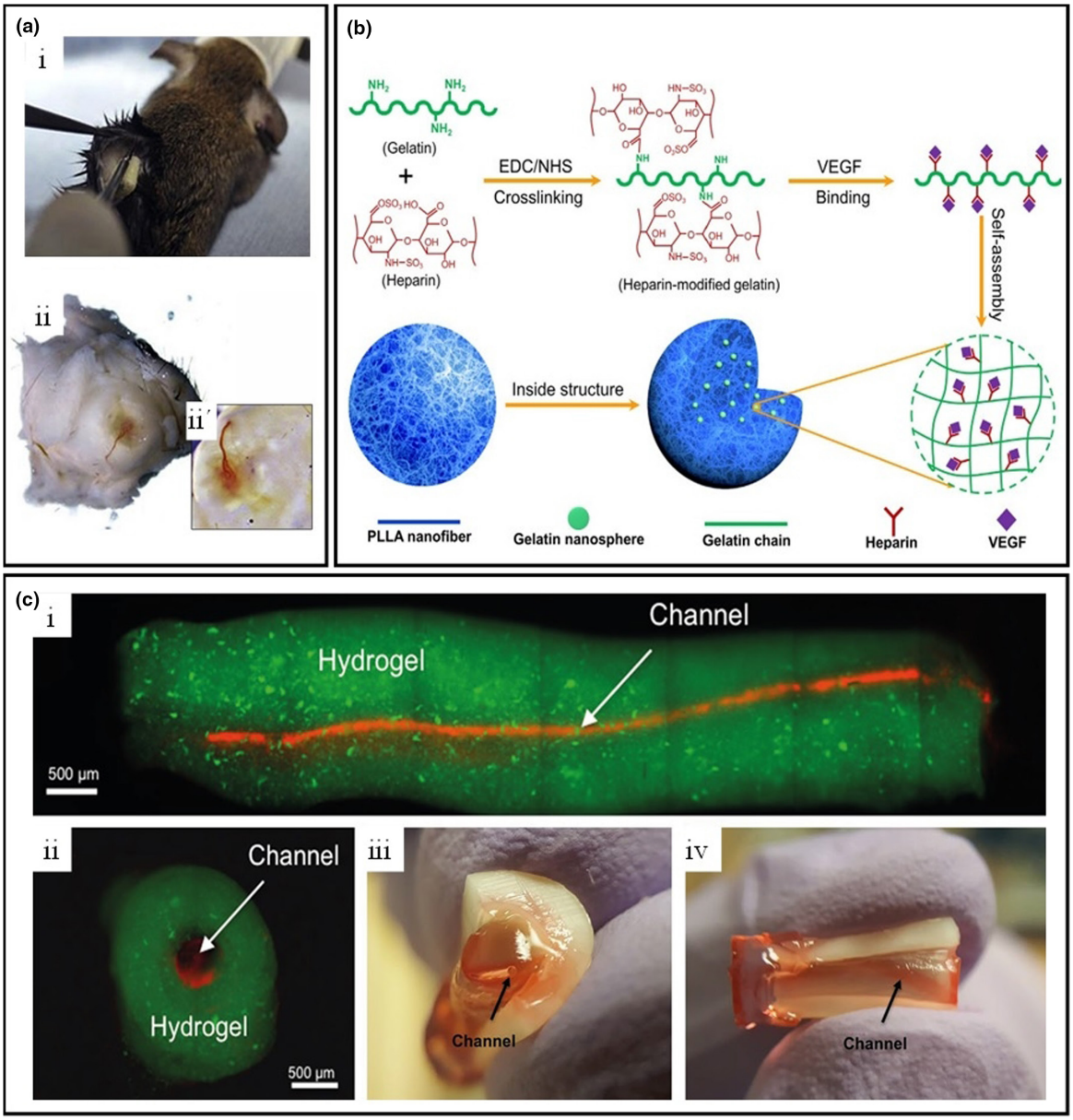

FIGURE 6 Techniques for the establishment of a vascular network. (a) Using VEGF-loaded fibres. (i) *In vivo* implantation of VEGF-loaded fibres within root fragments in the dorsum of nude mice. (ii & ii′) Microscopic illustration of blood vessel migration inside the canal space after 45 days of implantation. Adapted from Yadlapati et al. (2017) with permission from Elsevier, copyright 2017. (b) Schematic illustration of designing PLLA-based nanofibrous microspheres with the ability of VEGF-controlled release. Adapted from Li et al. (2016) with permission from Elsevier, copyright 2016. (c) Fabricating a pre-vascularized construct based on GelMA. (i) Longitudinal and (ii) cross-sectional views of the fabricated microchannel (red) within the GelMA hydrogel (green) in the entire length of the tooth root. Photographs from (iii) front and (iv) longitudinal view of the pre-vascularized GelMA hydrogel. Adapted from Athirasala et al. (2017), licensed under creative commons license.

a hypoxia-stimulating nanocomposite as a potential biomaterial in RE through the incorporation of cobalt ion (Co^{2+}) into multiwalled carbon nanotubes using the metal-organic framework. In the designed system, sustained release of Co^{2+} induced hypoxic mimic condition and promoted angiogenic capability of SCAP *in vitro*.

Owing to the problems of using exogenous GFs in clinics and also the necessity of vascularization in RE, in recent years, preparing pre-vascularized constructs has received more attention. In this strategy, microfabrication techniques are utilized to engineer vasculature or create a channel within the target construct for nutrient flow or endothelial cell attachment (Kaully et al., 2009). For example, Athirasala et al. (2017) fabricated an engineered pre-vascularized construct based on cell-laden stiff GelMA hydrogel (Figure 6c). To this aim, a sacrificial agarose fibre with a diameter of 500 μm was manually positioned in the centre of root canals, and the GelMA solution containing odontoblast-like

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TABLE 5 Overview of biomaterials used as the scaffold basis for pulp–dentine complex TE

Biomaterials	Advantages	Limitations
BIOCERAMICS		
Calcium phosphates		
Hydroxyapatite (HA) Tricalcium phosphate (TCP) Biphasic calcium phosphate (HA/TCP)	Naturally found as a component of the mineral phase of dentine; High biocompatibility; Nonimmunogenicity; Binding to bony tissues	Low degradation rate; Injection difficulty; Brittle; Osteoinductive properties
Bioactive glasses	Tailorable bioresorption rate; Binding to soft and hard tissues	Low degradation rate; Brittle
SYNTHETIC POLYMERS		
Polyesters		
Poly(ε-caprolactone) (PCL) Poly(lactic acid) (PLA) Poly(glycolic acid) (PGA) Poly(lactic-co-glycolic acid) (PLGA)	Manufacturing on a large scale; Low cost; Low foreign body reaction risk; Tuneable physicochemical properties; Well-controlled microstructure; High mechanical strength	Acidic by-products; Low degradation rate; Lack of biological cues for cell adhesion
Polyether		
Poly(ethylene glycol) (PEG)	Manufacturing on a large scale; Low cost; Great biocompatibility; Nonimmunogenic; Synthetic versatility; Excellent hydrophilicity; Tuneable physicochemical properties; Controllable structure; High mechanical strength	Nonbiodegradability; Lack of biological cues for cell adhesion
Self-assembling peptides (SAPs)	Biomimetic nanofibrous architectures; Injectability; Viscoelastic properties; Tuneable functional properties by altering peptide sequences	Design complexity; Low productivity; Low mechanical strength
NATURAL POLYMERS		
Proteins		
Collagen	The main organic component of dentine; A key element in dentine biomineralization; Possessing cell adhesion motifs and enzymatic cleavage sites; Formation of 3D hydrogel through self-assembly or crosslinking	Potential pathogen transmission; Bath-to-batch variance; Low mechanical strength; Potential toxicity of crosslinking techniques
Gelatine	Processible; Availability in a range of molecular weights with different properties; Possessing cell adhesion motifs and enzymatic cleavage sites; Relatively low cost	Low elasticity; High solubility in aqueous solution; Necessity of chemical crosslinking to form a stable gel at physiological conditions; Potential toxicity of crosslinking techniques
Fibrin	Autologous biomaterial; Great biocompatibility; Ease of polymerization at physiological conditions; Excellent cell–matrix interaction; Ability to act as a reservoir for GFs	Hard processing; Rapid degradation; Low mechanical strength; Less control over its physicochemical properties
Polysaccharides		
Hyaluronic acid	Naturally found in the body; Water solubility; Viscoelastic properties; Shear-thinning gel; Ability to form various covalent bonding via three functional groups; Electrostatic interaction with positively charged elements	Necessity of chemical crosslinking to prevent rapid elimination in the body; Potential toxicity of crosslinking techniques; High viscosity; Possible immunogenicity
Alginate	Nonimmunogenicity; Water solubility; Electrostatic interaction with positively charged elements; Ease of gelation; Relatively low cost	Nondegradable in mammals; Poor cell adhesion; Necessity of chemical modification to increase degradation (gamma irradiation or partial oxidation)

(Continues)

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TABLE 5 (Continued)

Biomaterials	Advantages	Limitations
Chitosan	Low toxicity; Antimicrobial activity; Possessing enzymatic cleavage sites; Electrostatic interaction with negatively charged elements; Relatively low cost	Poor solubility in neutral aqueous solutions; High crystallinity; No cell adhesion motifs; Necessity of chemical crosslinking for irreversible gelation; Potential toxicity or adverse effects on its intrinsic properties due to the chemical modification
Extracellular matrix (ECM)	Native ECM-like composition; Mimicking tissue-specific microstructure	Batch-to-batch variance; Difficulty with decellularizing and sterilizing; Limited availability of donor tissues; Rapid degradation
Platelet concentrates	Ease of preparation; Autologous biomaterial; Enriched with various GFs and cytokines; Low cost; Nontoxicity; fibrous microarchitecture; appropriate elasticity	Patient-dependent properties; Rapid degradation; Limited control over physicochemical properties

cells filled the remnant of the root canal space and photo-crosslinked. After aspirating the agarose fibre, endothelial colony-forming cells were seeded into the created micro-channel, and the prepared samples were cultured *in vitro*. Seven days later, densely cellularized structures were formed within the engineered construct. In the newly formed tissue, odontoblast-like cells spread more adjacent to the dentinal wall and endothelial colony-forming cells formed a monolayer on the surface of engineered microchannels and spread angiogenic sprouts. Another strategy to form pre-vascularized structures is scaffold-free approaches in which coculturing of odontogenic stem cells with endothelial cells leads to the formation of pre-vascularized microtissues. For example, Dissanayaka et al. (2014) cocultured DPSCs and HUVECs and observed that the fabricated scaffold-free structure regenerated a pulp-like tissue with a denser ECM and more cellularity and vasculature *in vivo*, as compared to the mono-culture of DPSCs.

Considerable progress has been achieved in the field of vasculature engineering, and several strategies have been developed for the facilitation of angiogenesis. Using the proposed strategies to establish the vascular network more rapidly in studies related to dental pulp TE has also led to promising results. However, there are still considerable challenges and complications for the clinical uses of these strategies that need to be addressed.

CONCLUSION

The current clinical techniques for pulp regeneration, i.e. the revascularization/revitalization technique, are promising in terms of periapical healing and root maturation. However, there are several limitations to this procedure. As a consequence, substantial efforts have been devoted to RE via TE-based strategies in an attempt to seek novel protocols that could replace conventional clinical procedures. Up to now, a spectrum of scaffolds ranging from bioceramic-based to naturally derived scaffolds, which meet some of the required design criteria for pulp–dentine complex TE, has been fabricated. Bioceramics were amongst the first biomaterials used for pulp–dentine complex TE. However, due to their osteoinductivity, they have been substantially improved or replaced with synthetic/natural biopolymers. In designing scaffolds based on such biomaterials, a variety of characteristic parameters related to biological, structural, physical and chemical features have been considered. Owing to the distinct properties of basis biomaterials (Table 5) and fabrication techniques, each scaffold shows promise for addressing some of the requirements for scaffolds in RE. However, more studies are required to develop an ideal scaffold that completely fulfils the criteria for pulp–dentine complex TE in a clinical setting. Additionally, to improve the clinical outcomes, reproduction of the cellular niche of the stem cells and the presence of trophic factors should be considered.

AUTHOR CONTRIBUTIONS

All the authors have made relevant contributions to the manuscript. All the authors have read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest with this manuscript.

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ETHICS STATEMENT

The study did not require ethics approval.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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