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FIBRIN-BASED SCAFFOLDS FOR DENTAL PULP REGENERATION: FROM BIOLOGY TO NANOTHERAPEUTICS

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Abstract

Tissue engineering-based endodontic therapies, designed to regenerate the dental pulp (DP) in the devitalised endodontic space, have been proposed to improve tooth longevity compared to conventional root-filling therapies. Their aim is to restore tooth vitality and major DP functions necessary to maintain tooth health such as immunosurveillance, sensitivity and healing/repair/regenerative capacities. Several formulations based on the use of fibrin, the main component of the blood clot matrix, recently gave valuable results in the regeneration of the human DP. This review describes recent fibrin-based scaffolds designed for that purpose. After having presented the various strategies for DP regeneration, the main fibrin-based scaffolds reported so far for clinical use in endodontics were reviewed. Particular emphasis was given to hydrogel devices that may be improved by incorporation of bioactive molecules that stimulate vascularisation and tissue neoformation or provide antibacterial properties. Data indicate that fibrin-based scaffolds constitute a highly favourable environment for mesenchymal stem cells, which is maintained upon functionalisation. Additional knowledge is needed to understand how fibrin and functionalising agents affect adhesion, survival, proliferation, migration and differentiation of cells incorporated in the scaffold or which will colonise it from neighbouring host tissues. This knowledge is needed to adapt the hydrogel formulation for various clinical conditions.

Keywords: Dental pulp, regeneration, fibrin, platelet-rich plasma (PRP), platelet-rich fibrin (PRF), tissue engineering, scaffold, hydrogel, mesenchymal stem cells (MSCs), nanotherapeutics.

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List of Abbreviations

3D	three-dimensional
BMP	bone morphogenetic proteins
CLIN	clindamycin
DP	dental pulp
ECM	extracellular matrix
EMA	European Medicines Agency
FDA	U.S. Food and Drug Administration
FGF	fibroblast growth factor
IL	interleukin
LPS	lipopolysaccharide
MSCs	mesenchymal stem cells
NGF	nerve growth factor
NPs	nanoparticles
PDGF	platelet-derived growth factor
PEG	polyethyleneglycol
PLA	poly (D,L) lactic acid
PLGA	poly(lactic-co-glycolic acid)
PPP	platelet-poor plasma fraction
PRF	platelet-rich fibrin
PRP	platelet-rich plasma
SCF	stem cell factor
SEM	scanning electron microscope
SDF1α	stromal cell-derived factor 1α
VEGF	vascular endothelial growth factor

Introduction

The DP is the loose connective tissue located in the endodontic space present in the centre of the tooth. It is responsible for tooth vitality, pain sensation, immune defence and repair/regeneration upon injury. In caries disease, degradation and invasion of enamel and dentine by oral bacteria trigger

inflammatory/immune mechanisms in the DP that are notably characterised by a large increase of the intrapulpal interstitial pressure. Blood-vessel compression eventually leads to blood-flow arrest, DP necrosis and endodontic space invasion by bacteria (Farges *et al*., 2015a; Farges *et al*., 2015b). Since, in the absence of blood flow, the infected DP is inaccessible to the host immune defence, the intervention of a dental practitioner is required, whose goal is to remove the necrotic tissue, disinfect and then fill the endodontic space with a tight biomaterial to prevent future microorganism contamination. However, several studies have shown that tooth longevity is much shorter due to the absence of DP immune and sensory systems and repair/regenerative abilities, which makes the tooth more susceptible to re-infection and/or fracture (Mao *et al*., 2012; Ng *et al*., 2010). For this reason, tissue engineering-based therapeutic strategies, based on the use of scaffolds possibly containing stem cells, have been proposed to regenerate a fully functional DP in the endodontic space (Hargreaves *et al*., 2014; Nygaard-Ostby and Hjortdal, 1971). However, none of these scaffolds was yet found to possess the ideal properties for DP regeneration, and designing innovative formulations is clearly required (Albuquerque *et al*., 2014; Ducret *et al*., 2017; Galler *et al*., 2018).

Recently, fibrin-based scaffolds – in particular in the form of hydrogels – were demonstrated to be highly suitable for DP regeneration since they clearly support DP-like tissue neoformation both *in vitro* and *in vivo* (Bekhouche *et al*., 2020; Ducret *et al*., 2019; Galler, 2016a; Galler *et al*., 2018; Ruangsawasdi *et al*., 2014; Ruangsawasdi *et al*., 2017). The purpose of this review was to describe recent developments and future challenges of fibrin-based hydrogel formulations designed for DP regeneration. The first part exposes the current strategies considered for DP regeneration, the second one describes the main fibrin-based hydrogels that have been experimentally tested. The third part reports several promising strategies of functionalisation recently implemented to improve fibrin-based hydrogel properties, with the ultimate goal is to use these hydrogels in future endodontic nanotherapeutics.

DP regeneration strategies

Revascularisation

Revascularisation was the first described strategy aimed at regenerating the DP in the devitalised endodontic space (Östby, 1961). It consists of inducing a bleeding from the periapical periodontal ligament, using an endodontic file, to form a blood clot in the endodontic space (Banchs and Trope, 2004) (Fig. 1). The endodontic space thus becomes full of an autologous natural fibrin scaffold, containing PDGFs, that promote vascularisation and blood clot replacement with a neotissue formed from infiltrating periodontal MSCs. Bleeding also brings numerous molecular (complement components, immunoglobulins, chemotaxins and antibacterial peptides) and cellular (polynuclear leukocytes and macrophages) actors of the innate and adaptive immunity that protect the blood clot from infections (Saoud *et al*., 2016). Revascularisation presents the large benefit of not inducing a foreign-body immune response, since all players of the regeneration process originate from the patient (Dianat *et al*., 2017; Jadhav *et al*., 2012). Other advantages of this strategy are simplicity, rapidity, low cost, and clinical efficacy (Raddall *et al*., 2019). Revascularisation has been indicated, for several years by both the American and European Associations of Endodontists, for the treatment of immature permanent teeth (Galler *et*

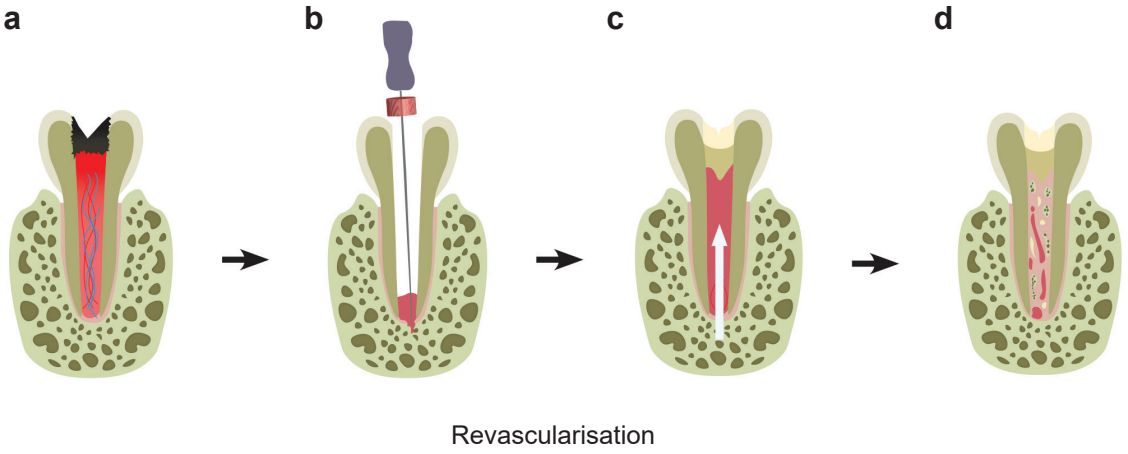

Fig. 1. **Strategy of DP revascularisation**. (**a**) Deep caries lesion (black) and DP with irreversible pulpitis (red). (**b**) Upon DP removal, the endodontic space is roughly cleaned, then an apical bleeding is triggered from the periapical periodontal ligament with an endodontic file, in order (**c**) to form a blood clot which completely fills the endodontic space. (**d**) The blood clot is replaced with a neotissue formed by incoming periodontal MSCs. The neotissue is a fibrous connective tissue more or less calcified, sometimes cementum-like, rather than true DP.

al., 2016b; Schmalz and Smith, 2014). However, its major limitation is that the neo-formed tissue in the endodontic space is either fibrous connective tissue or cellular cementum, rather than a regular DP tissue (Albuquerque *et al.*, 2014; Becerra *et al.*, 2014; Nygaard-Ostby and Hjortdal, 1971). This is a real disadvantage since, in the absence of an odontoblast layer at the pulp-dentine interface, dentine cannot be deposited on the endodontic wall to increase the robustness of the tooth and physically protect the new DP from external irritants. The absence of odontoblasts with long cell processes in dentine tubules also prevents the early sensing of external injuries (Farges *et al.*, 2015a; Farges *et al.*, 2015b). Finally, a risk has been reported of tooth-colour change following the formation of the blood clot with this technique (Becerra *et al.*, 2014; Nosrat *et al.*, 2012).

Cell-free and cell-based regeneration

More recently, following the development of multiple kinds of natural and synthetic scaffolds for tissue engineering (Moussa and Aparicio, 2019) and the discovery of various populations of MSCs in the DP and the periapical area (Ducret *et al.*, 2015a; Gronthos *et al.*, 2000; Kerkis *et al.*, 2006; Miura *et al.*, 2003; Sonoyama *et al.*, 2008), two additional strategies, described as cell-based and cell-free, have been proposed for regenerating the DP.

The cell-based regeneration strategy, based on the tissue engineering original triad (scaffold, stem cells and growth factors) (Langer and Vacanti, 1993), consists of associating MSCs and a hydrogel-type biomaterial, possibly functionalised by bioactive molecules, to recreate a new DP tissue (Nakashima *et al.*, 2019) (Fig. 2). This strategy recently gave promising results both in animal models and humans when DP-derived MSCs were used (Nakashima *et al.*, 2017; Nakashima *et al.*, 2019; Ruangsawasdi *et al.*, 2017). Clinical trials reported the re-establishment of tooth sensitivity and the increase of root length, confirming the relevance of this strategy (Iohara *et al.*, 2018; Nakashima *et al.*, 2019; Xuan *et al.*, 2018). However, the standardisation of the protocols for human MSC isolation, characterisation, *ex vivo* amplification, storage and implantation in the host, in accordance with good manufacturing practices, is still a big challenge. This is before considering the cell-based regeneration approach (Ducret *et al.*, 2015a; Ducret *et al.*, 2017) for cell-based medicinal products by European authorities and the FDA (Ducret *et al.*, 2015b).

The cell-free regeneration strategy is characterised by the implantation of an uncellularised scaffold (Galler *et al.*, 2014; Widbiller *et al.*, 2018) into the endodontic space, functionalised with bioactive molecules such as growth factors that are released progressively. Passive diffusion through the root foramen will allow these factors to attract, into the hydrogel, host periodontal MSCs that will differentiate into DP cells (Conde *et al.*, 2015; Langer

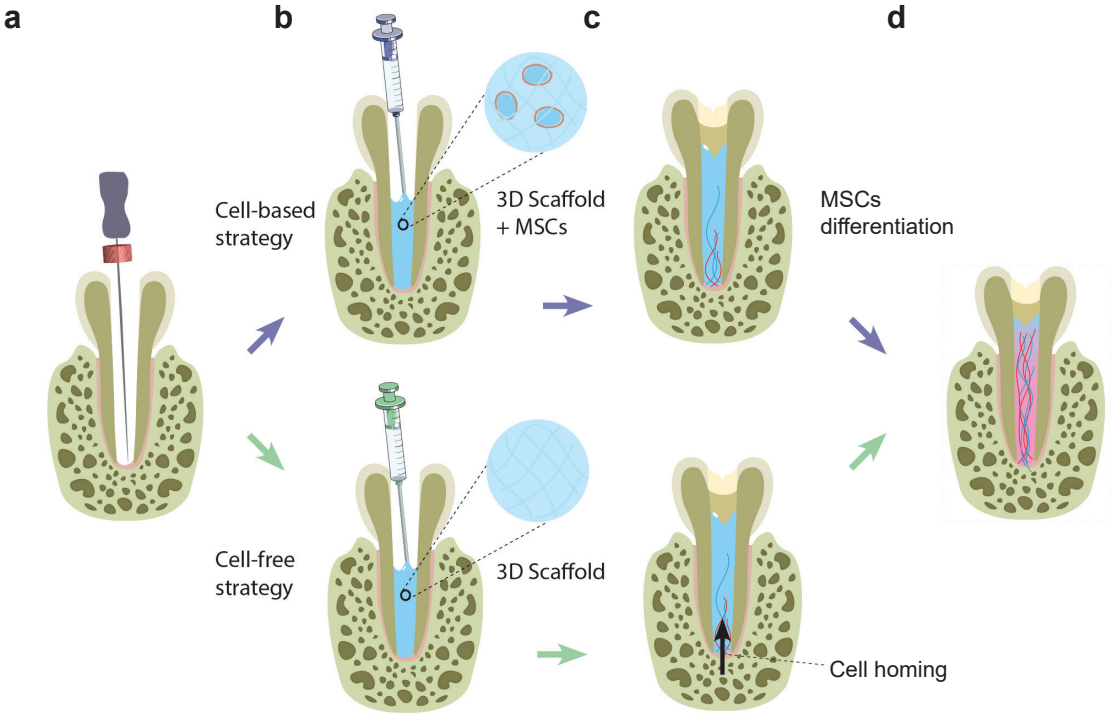

Fig. 2. Cell-free and cell-based DP regeneration. Upon DP removal, the endodontic space is cleaned, disinfected and shaped (**a**). (**c**) A cell-free or cellularised scaffold formulation is then injected (**b**) to get a 3D environment able to promote cell survival and vascularisation (**c**). Progressive replacement of the scaffold leads to the formation of a vascularised and innervated neotissue expected to be a DP (**d**).

and Vacanti, 1993) (Fig. 2). This strategy appears to be an easier way than the cell-based approach since it does not involve isolation and manipulation of autologous or allogenic MSCs *in vitro* before implantation. Therefore, it is considered a relevant concept that may offer rapid clinical translation to human in the future (Huang and Garcia-Godoy, 2014; Yang *et al*., 2015). However, since the tissue formed in the endodontic space rarely possess the characteristics of the DP tissue, it is not clear whether periodontal MSCs can differentiate into DP cells (Huang *et al*., 2009).

Challenges for scaffold-based DP regeneration

Regenerative strategies are facing several challenges. One major difficulty is to obtain odontoblasts to produce dentine (Ducret *et al*., 2017). Moreover, most if not all of the scaffolds designed for DP regeneration lack antibacterial activity. This may constitute a major obstacle to clinical use, since animal model studies have demonstrated that microorganisms remaining in the endodontic space hinder DP regeneration (Redl *et al*., 1983; Verma *et al*., 2017). As stated above, other important factors to consider for future clinical use are the cost of the technique, the biological properties of the scaffold and its ease of handling and storage (Ducret *et al*., 2015a; Ducret *et al*., 2017), making the scaffold a central player for the success of DP regeneration.

Biologically, an ideal scaffold must create a cell-friendly environment able to promote adhesion, survival and differentiation of MSCs that will progressively replace it by an ECM characteristic of the DP (Ducret *et al*., 2017). Clinical requirements include low viscosity, allowing implantation into the small and anatomically complex endodontic space. Numerous kinds of scaffolds have been tested so far, including natural and synthetic polymers/co-polymers, hydroxyapatite/tricalcium phosphate powders, self-assembling peptide systems, PRP derivatives and decellularised tissue matrices (Conde *et al*., 2015; Galler, 2016a; Huang and Garcia-Godoy, 2014; Jazayeri *et al*., 2020; Nakashima *et al*., 2019; Palma *et al*., 2017; Proksch and Galler, 2018; Rosamma and Kavyashree, 2017). However, most of these scaffolds were found to regenerate a tissue lacking structural and functional properties of DP, which highlights the need to deeply investigate the initial cell-scaffold cross-talks regulating cell behaviour before considering clinical applications (Mangione *et al*., 2017; Moussa and Aparicio, 2019). In this context, fibrin-derived biomaterials, used for decades in tissue engineering-based therapeutic strategies, appear to be promising tools for DP regeneration (Galler *et al*., 2018).

A major advantage of fibrin-based scaffolds comes from the regulatory aspect, because plasma-rich fibrin, fibrin sealants and fibrin glues have been approved by the FDA and the EMA for use in humans. This will undoubtedly facilitate the clinical transfer of fibrin-based formulations designed for DP regeneration (Ducret *et al*., 2015b; Park and Woo, 2018). The recent development of nanobiotechnologies will, hopefully, help to provide fibrin with specific properties, including antibacterial ones, suitable for DP regeneration (Bekhouche *et al*., 2020; Willyard, 2018).

From fibrin to fibrin-based scaffolds

Fibrin and blood clot formation

Fibrin is a plasma protein involved in the later stage of haemostasis and synthesised in the liver in the form of soluble fibrinogen (Woodson *et al*., 2013). Briefly, when bleeding occurs, blood-stream exposure of the connective tissue surrounding blood vessel leads to the recruitment of platelets and the formation of the primary plug (Periayah *et al*., 2017; Swieringa *et al*., 2018). The so-called coagulation cascade is then triggered by the proteolytic activation of serine proteases, expressed by platelets and the endothelium or present in the plasma (Farges *et al*., 2015b). Notably, the circulating thrombin cleaves the fibrinogen molecules into insoluble fibrin monomers which then self-associate to form a fibrin polymer (Fig. 3) (Periayah *et al*., 2017). The latter is stabilised by the transglutaminase factor XIII which catalyses the formation of amide bonds between fibrin side chains, leading to a final cross-linked blood clot (Jobe *et al*., 2005; Periayah *et al*., 2017). During the wound healing process, the blood clot is progressively replaced with a neoformed tissue similar to the tissue of origin (Collen and Lijnen, 1991). Importantly, this process is mediated by cell activation through binding to fibrin. Cells of the blood clot interact with fibrin through a cell surface integrin-driven mechanism mediated by Arg-Gly-Asp (RGD) adhesive sequences (Mosesson, 2005). Fibrin binds to integrin $\alpha_{IIb}\beta_3$ (present on platelets) (Höök *et al*., 2017), integrin $\alpha_M\beta_2$/Mac-1 (neutrophils, monocytes, macrophages and mast cells) (Gailit *et al*., 1997) and integrin $\alpha_V\beta_3$ (fibroblasts, endothelial cells) (Flick *et al*., 2004; Gawaz *et al*., 1997) (Fig. 3). Cell binding leads to the activation of specific intracellular signalling pathways, which drive blood-clot formation and ECM remodelling, suggesting that cell adhesion to the fibrin matrix is a key step in the triggering of tissue regeneration. The interaction of DP-MSCs with fibrin and its cellular consequences remain totally unknown and should be investigated to clearly understand how DP neotissue formation is initiated, in order to be able to adapt the formulation of fibrin scaffolds to several pathological contexts.

Regulation of fibrin network formation

Polymerisation of fibrinogen molecules, during blood-clot formation, leads to the formation of a fibrillary network important for haemostasis and subsequent wound healing (Janmey *et al*., 2009). Major parameters governing this formation are the local concentrations of fibrinogen, calcium and

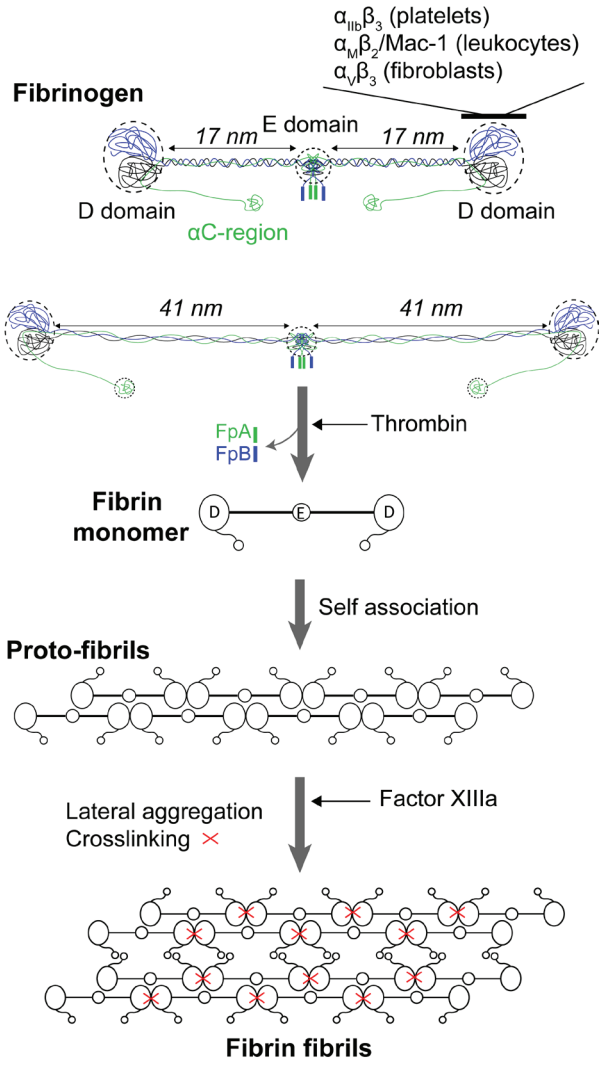

Fig. 3. Fibrin polymer formation from fibrinogen molecules. Plasma circulating fibrinogen is a dimeric molecule composed of α (green), β (blue) and γ (grey) chains forming D and E domains separated by a coiled-coil region implicated in fibrin elongation. The D domain is implicated in integrin-mediated binding of fibrin(ogen) onto fibroblasts, leukocytes and platelets. Cleavage of the N-terminal fibrinopeptides A (FpA) and B (FpB) by thrombin promotes the interaction between D and E domains which induces the lateral association of fibrin monomers as double stranded.

thrombin. *In vitro*, the structural and functional properties of the fibrin network can be modulated by varying these concentrations (Litvinov and Weisel, 2017; Man *et al.*, 2011). The modulation of fibrin scaffold properties, through adjusting the fibrinogen concentration, is probably the best example to illustrate how fibrin hydrogels could be fine-tuned. Highly-concentrated fibrinogen solutions (up to 60 mg/mL) have been used as sealants to achieve haemostasis, by application to bleeding wounds. Conversely, low-fibrinogen concentrations, of around 5-20 mg/mL, have been largely used to design scaffolds, sometimes in combination with other biomolecules, which promote the regeneration of various tissues including the DP (Ducret *et al.*, 2017; Litvinov and Weisel, 2017). Interestingly, increasing the mechanical strength of the fibrin hydrogel by increasing fibrinogen concentration (9.5 to 141 mg/mL) reduced dorsal root ganglia neurite outgrowth in a fibrinolysis-dependent manner (Man *et al.*, 2011). This study demonstrated that a 10 mg/mL fibrinogen concentration confers to the scaffold physical and mechanical properties that enable the support of nerve-guidance conduits, an outstanding property toward a restoration of DP functionality. Fibrin hydrogels are sometimes stabilised by factor XIIIa, which increases crosslinking of fibrinogen molecules. However, factor XIIIa's use strongly slows down hydrogel lysis. The addition of 10 U/mL of factor XIIIa thus decreases the lysis rate by approximately 45 % (Francis and Marder, 1988; Wolberg *et al.*, 2012). A fine control of the lysis rate is crucial to allow proper tissue replacement in the context of DP regeneration. The degradation of the fibrin network should be synchronised with the development of a vascular system. A low degradation rate could potentially affect nutrient supply to stem cells and induce cell death, while fast degradation could create an empty environment that may constitute an ideal niche for bacterial expansion.

Fibrin-based scaffolds for DP regeneration

Fibrin-based scaffolds have been studied for decades in the field of tissue engineering (Noori *et al.*, 2017; Park and Woo, 2018; Sakiyama *et al.*, 1999; Schense *et al*, 2000). Their advantages include excellent cytocompatibility, physiological degradation kinetics, non-toxicity of degradation products, and replacement with an ECM produced by incorporated or infiltrating stem cells within a few days (Roura *et al.*, 2017). Fibrin scaffolds possess excellent properties for tissue regeneration, as shown by preclinical and clinical studies (Borie *et al.*, 2015; Kang *et al.*, 2011; Miron *et al.*, 2017). In the field of DP regeneration, autologous fibrin-rich platelet concentrates and fibrin hydrogels manufactured *in vitro* have been used with valuable results in animal models and humans (Bezgin *et al.*, 2015; Chen *et al.*, 2015; Ruangsawasdi *et al.*, 2014; Ruangsawasdi *et al.*, 2016). Beyond the intrinsic properties of fibrin, both offer the great advantage of being easily injectable into the mm-sized endodontic space using a fine needle (Bekhouche *et al.*, 2020; Ducret *et al.*, 2019).

PRP and PRF

PRP and PRF are platelet concentrates collected *in vivo* from the patient's own blood by centrifugation. PRP is collected at high G force (above 300 ×*g*) and is composed of low-density fibrin and cells, whereas PRF is collected at low G force (below 300 ×*g*) and is composed of high-density fibrin rich in cells and growth factors (Mohan *et al.*, 2019). Both PRP and PRF have been proposed for various therapeutic applications including DP regeneration, owing to the presence of large amounts of pro-angiogenic

growth factors and ease of handling (Bezgin *et al*., 2015; Chow *et al*., 1983; Miron *et al*., 2017; Trevino *et al*., 2011). Practically, the patient's blood is collected, mixed with anticoagulants and centrifuged. The PRP is then separated from the PPP to increase platelet concentration. On demand coagulation into the endodontic space is realised by the addition of autologous thrombin extracted from the PRF. Thrombin can be supplemented with calcium chloride and/or PPP to modulate the mechanical properties of the fibrin matrix. PRP is considered a valuable strategy for DP regeneration, mainly because it limits handling steps, and therefore the risk of pathogen transmission, and is cost effective (Raddall *et al*., 2019). Interestingly, PRF combination with cell-sheet fragments of DP-MSCs allowed the regeneration of a vascularised DP-like tissue and the deposition of dentine after post-orthotopic transplantation in pulpectomised canals of canine premolars (Chen *et al*., 2015). However, the ability of this DP-like tissue to harbour immune surveillance and nerve sensitivity is not known.

Fibrin-based hydrogels

In the last decade, fibrin purified from human plasma has been used to design hydrogels able to promote DP regeneration in a cell-free approach (Galler *et al*., 2011; Galler *et al*., 2018; Ruangsawasdi *et al*., 2016). In a pioneer work, fibrin hydrogels were injected in the endodontic space of extracted human immature premolars implanted in rats. Results showed improved cell homing and cell-dentine interaction compared to empty root canals (Ruangsawasdi *et al*., 2014). It was recently shown that a 10 mg/mL fibrin scaffold forms a well-vascularised tissue *in vivo*, in a model of human root dentine cylinders implanted in the back of nude mice (Fig. 4). These results were in agreement with those obtained using PRF (Chen *et al*., 2015).

Numerous studies have attempted to improve the structural and functional properties of the fibrin scaffolds by incorporating polymeric elements. These elements include PEG (Dikovsky *et al*., 2006), polyurethane, polyethylene oxide, polycaprolactone, heparin, PLA, PLGA, alginate, chitosan, hyaluronic acid and collagen (Brown and Barker, 2014; Litvinov and Weisel, 2017). Recently, Han *et al*. (2019) reported the development of a fibrin-based bio-ink incorporating gelatine, hyaluronic acid and glycerol for 3D co-printing. Current research also aims at providing the hydrogel with pro-regenerative, anti-inflammatory and anti-bacterial properties to overcome the challenges of DP regeneration described above.

Strategies to improve fibrin-based scaffold properties

Pro-regenerative properties

DP regeneration requires the concerted action of growth factors and other bioactive molecules that must be provided to the cells responsible for tissue neoformation in the endodontic space. In particular, the scaffold injected within the endodontic space must guide the spatio-temporal cell recruitment and differentiation into the various DP cell lineages needed to recreate a functional DP (Gong *et al*., 2016).

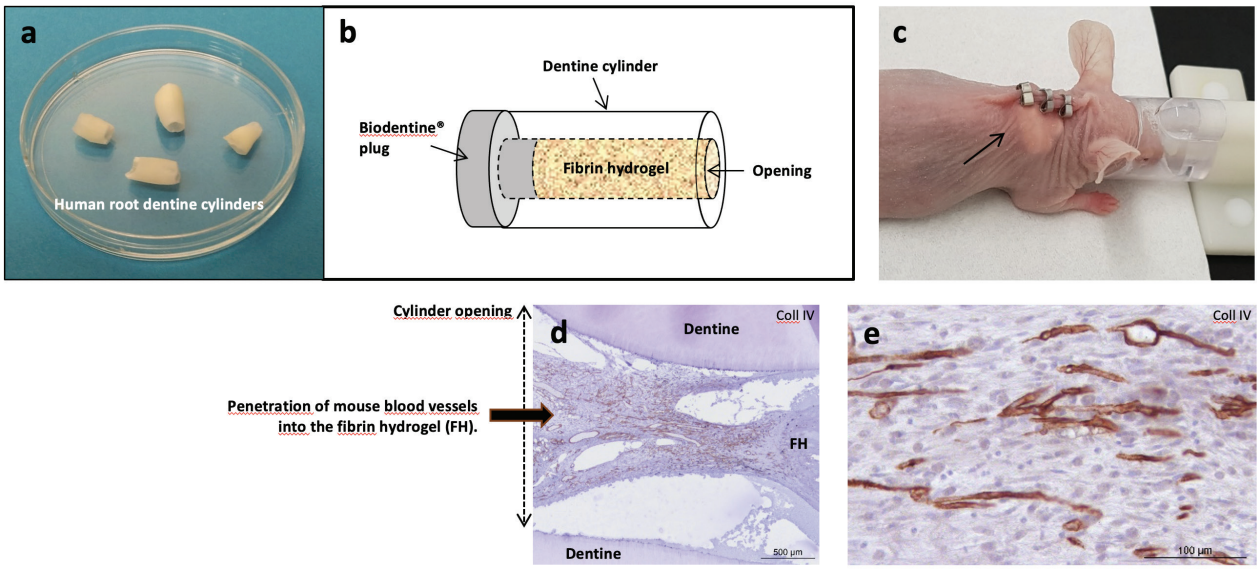

Fig. 4. Vascularisation of a cellularised fibrin hydrogel upon implantation *in vivo* for 7 weeks. (**a**) Human root dentine cylinders. (**b**) Schematic representation of a cylinder containing the cellularised fibrin hydrogel. (**c**) Subcutaneous implantation of a dentine cylinder filled with a DP-MSC-cellularised fibrin hydrogel in the back of a nude mouse. (**d**) Low magnification of a well-vascularised regenerated tissue into the cylinder. Blood vessels are identified by type IV collagen immunostaining. FH: fibrin hydrogel remnant. (**e**) High magnification of type IV collagen staining.

Several growth factors have been added to the fibrin scaffold to promote tissue regeneration, including pro-angiogenic factors such as VEGF, PDGF and FGF-2, factors inducing odontoblast differentiation such as BMP-2 and -4, nerve growth promoting factors such as the NGF and factors promoting MSC recruitment and growth such as SDF1α and SCF (Abou Neel *et al.*, 2014; Ducret *et al.*, 2017; Kowalczewski and Saul, 2018; Ruangsawasdi *et al.*, 2017; Trevino *et al.*, 2011; Xia *et al.*, 2018). Dentine matrix molecules could also be incorporated in the fibrin scaffold before polymerisation to increase chemotaxis and pulp-like tissue formation in the endodontic space (Widbiller *et al.*, 2018). An alternative to the addition of exogenous molecules to promote DP regeneration is MSC preconditioning. DP-MSC pretreatment with FGF-2 thus enhanced angiogenesis within subcutaneously implanted hydrogels, mimicking DP regeneration, by increasing VEGF and hepatocyte growth factor release (Gorin *et al.*, 2016). DP-MSC hypoxic preconditioning could also enhance angiogenesis by inducing VEGF expression (Aranha *et al.*, 2010). Recently, exosomes isolated from human third molar DP cells, used as a delivery system for proteins, lipids, RNA and DNA, were proposed as another option to stimulate DP regeneration in a cell-free approach, since they stimulated the migration and proliferation of human bone marrow-derived MSCs *in vitro* in a fibrin hydrogel (Ivica *et al.*, 2020).

Anti-inflammatory properties

Another limitation of the current biomaterials used for DP regeneration is the inflammatory response that can occur after implantation. Indeed, inflammation often causes tissue damage at the material/host tissue interface (Colombo *et al.*, 2014; Farges *et al.*, 2015b; Willyard, 2018). "Good" and "bad" inflammations have been defined according to macrophage polarisation in a M2 or a M1 profile, respectively. While pro-inflammatory M1 macrophages cause tissue damage, anti-inflammatory M2 macrophages promote tissue repair (Mills, 2012; Vannella *et al.*, 2017). The formation of an inflammatory plug with abundant M1 macrophages at the root apical tip was reported to impede cell homing into the endodontic space and to induce resorption of the apical dentine (Zaky *et al.*, 2020). It is therefore important to control the M1/M2 balance to get adequate DP regeneration (Zaky *et al.*, 2020). Recently, early inflammatory/immune response and M1/M2 macrophage polarisation were investigated upon fibrin and fibrin-chitosan hydrogel implantation in pulpotomised rat incisors (Renard *et al.*, 2020). This study showed similar accumulation of neutrophil granulocytes at the hydrogel/residual DP interface with an enrichment of M2 but not M1 macrophages, suggesting that fibrin could promote M2-driven tissue regeneration (Renard *et al.*, 2020). This work is in agreement with a previous study suggesting that fibrin hydrogels, by promoting M2 polarisation *in cellulo*, could have a favourable effect on DP regeneration (Hsieh *et al.*, 2017). PRF extracts were reported to attenuate DP-MSC expression of the pro-inflammatory cytokines IL-1β, IL-6, and IL-8 upon treatment with LPS, which is also in favour of a possible role of fibrin in preventing inflammation (Kim *et al.*, 2017). The use of MSCs was also proposed to reduce inflammation during DP regeneration. Indeed, MSCs possess immunomodulatory properties through their inhibition of the T-cell response and their ability to modulate the M1/M2 ratio in favour of the M2 phenotype (Marei and El Backly, 2018). Altogether, data from the literature suggest that fibrin is a natural, biological biomaterial that limits inflammation and promote tissue regeneration.

Antibacterial properties

Recent studies have demonstrated that eradicating bacteria from the endodontic space with classical disinfectants (sodium hypochlorite or chlorhexidine) is very difficult because of the complex anatomy of the endodontic space, deep penetration of bacteria into dentine tubules, and bacterial organisation into biofilms (Vishwanat *et al.*, 2017). Several studies performed in animal models have demonstrated that residual bacteria hinder DP regeneration (Redl *et al.*, 1983), and the absence of antibacterial properties of the fibrin molecule or its degradation products may constitute an obstacle to the clinical use of hydrogel formulations made of fibrin alone. This obstacle could be overcome by incorporating drugs or molecules with antibacterial properties at the time of scaffold manufacture. Studies have been done in this context by incorporating metronidazole and/or ciprofloxacin into nanofibrous scaffolds (Kamocki *et al.*, 2015; Tagelsir *et al.*, 2016). If both drugs were shown to inhibit the growth of the endodontic bacteria *Enterococcus fæcalis*, *Porphyromonas spp.* and *Fusobacterium nucleatum*, they were not able to eradicate a 3-week-old biofilm (Kamocki *et al.*, 2015; Tagelsir *et al.*, 2016). Antibacterial activity against an *E. faecalis* biofilm was observed when ciprofloxacin was associated with fibrin (Chotitumnavee *et al.*, 2019). Recently, chitosan, an antibacterial glycosaminoglycan derived from shrimp shells, was associated with fibrin to provide a fibrin hydrogel with antibacterial properties (Ducret *et al.*, 2019). Results showed that the fibrin-chitosan hydrogel strongly inhibited *E. faecalis* growth *in vitro* without affecting DP-MSC viability, morphology, proliferation rate and type I/III collagen production capacity (Ducret *et al.*, 2019) (Fig. 5). Further studies are needed to deeply investigate the efficiency of such antibacterial scaffolds and notably on endodontic bacteria housed in the shelter of dentine tubules.

Nanotherapeutics

The recent development of nanobiotechnologies (Harilal *et al.*, 2019; Sinjari *et al.*, 2019; Zhao *et al.*, 2020) offers great hope to deliver locally, in a spatially and temporarily controlled manner, antibacterial drugs in

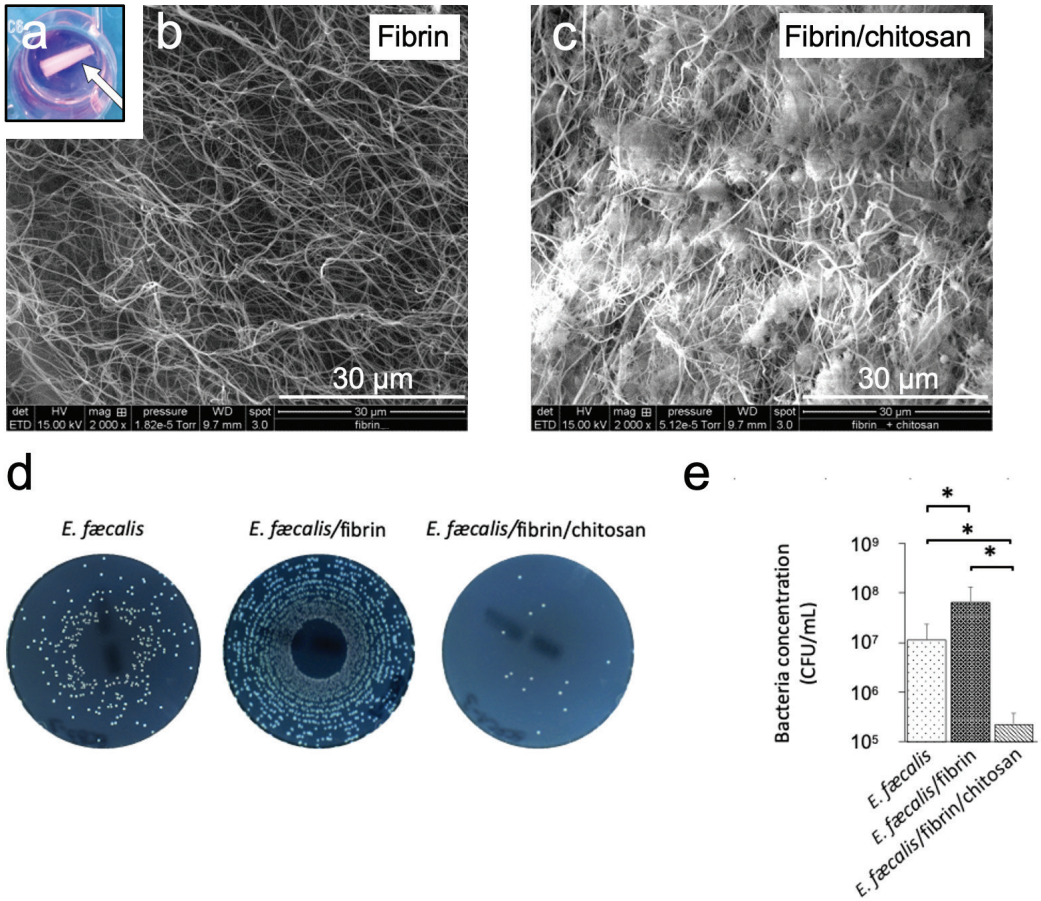

Fig. 5. Antibacterial properties of fibrin-chitosan hydrogels (Ducret *et al.*, 2019). (**a**) Photograph showing an example of fibrin hydrogel (opaque white) cast in a cylindrical-conical transparent plastic mould (white arrow) and covered with cell culture medium. SEM images showing the fibrin hydrogel network (**b**) and chitosan aggregates entrapped within the fibrin network (**c**). (**d**) Representative photographs of *E. faecalis* CFUs upon bacterial contact with fibrin or fibrin-chitosan hydrogels (far left: control, *i.e.* bacteria alone). (**e**) Bacterial concentrations determined by CFU counting. Reproduced from Ducret *et al.*, 2019, with permission from Elsevier.

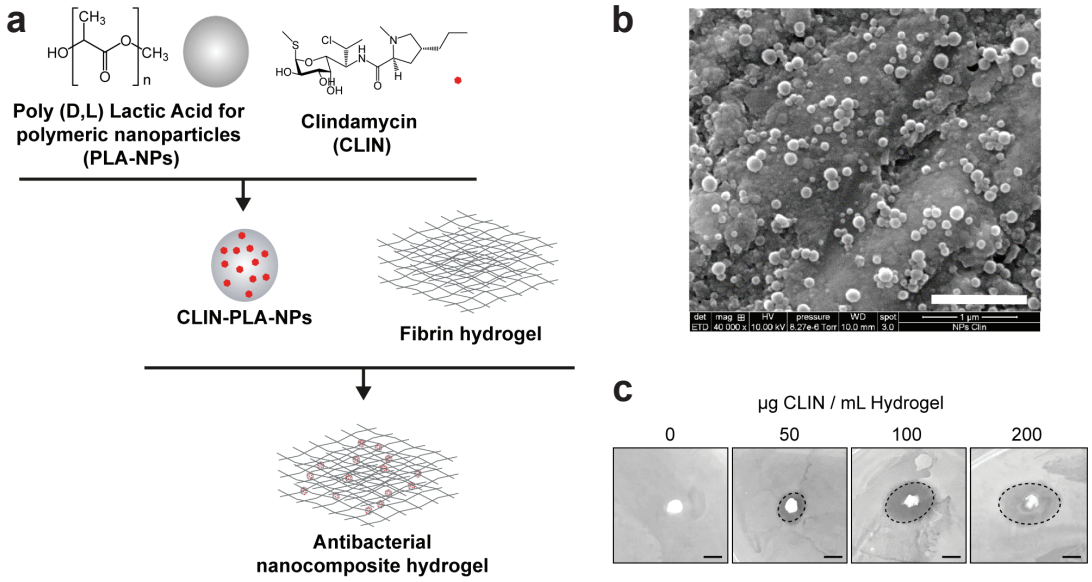

Fig. 6. Nanocomposite fibrin-based hydrogel containing CLIN-loaded PLA-NPs. (**a**) Schematic representation of the 2-step synthesis of the nanocomposite hydrogel. (**b**) Representative SEM image of CLIN-loaded PLA-NPs. Scale bar: 1 µm (bottom). (**c**) Representative pictures of agar diffusion assays in the presence of the fibrin-alone hydrogel (far left) or nanocomposite fibrin hydrogels containing PLA-NPs loaded with CLIN to get final concentrations of 50, 100 or 200 µg/mL CLIN. Scale bar is 10 mm. Reproduced from Bekhouche *et al.*, 2020, with permission from the Royal Society of Chemistry.

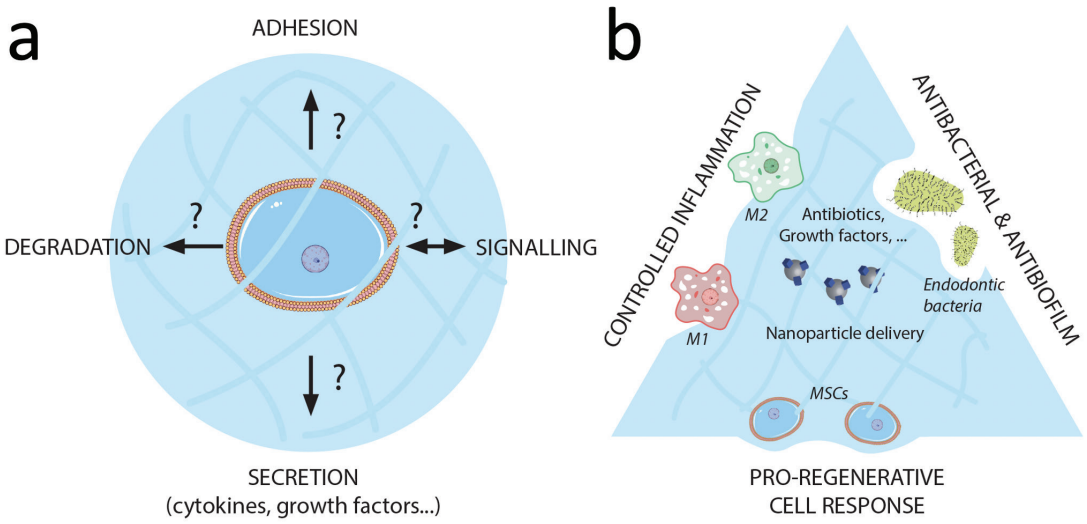

Fig. 7. Cellular (a) and functional (b) challenges in DP regeneration.

small-sized tissues such as the endodontic space and in difficult to areas reach such as dentine tubules. In particular, NPs are effective antibiotic carriers since they protect the drug in its native structure, conserve its therapeutic properties, can deliver large amounts of drug at the site of infection and limit side effects, when compared to other drug carrier systems and free antibiotic diffusion (Wang *et al.*, 2017). Interestingly, scaffolds incorporating antibacterial NPs such as CLIN-loaded hyperbranched NPs were developed for potential antibacterial applications (Wei *et al.*, 2020). Among the various types of NPs, PLA-NPs received considerable attention over recent decades because of their highly biocompatible and biodegradable nature and their low levels of immunogenicity and toxicity (Tyler *et al.*, 2016). A nanocomposite fibrin-based hydrogel containing CLIN-loaded PLA-NPs was recently designed for the purpose of safely bringing the antibiotic into contact with endodontic bacteria lining the dentine wall or residing within dentine tubules (Bekhouche *et al.*, 2020). Results demonstrated that the incorporation of CLIN-loaded NPs into a fibrin hydrogel gave it antibacterial and antibiofilm properties against *Enteroccocus fæcalis* without affecting DP-MSC viability and function (Bekhouche *et al.*, 2020) (Fig. 6). Nanotherapeutic strategies thus appear valuable for DP regeneration.

Conclusions

Together, these data indicate that fibrin is a valuable biopolymer for scaffold-based strategies of DP regeneration. Its properties could be tuned to adapt the hydrogel for various clinical conditions. A major challenge will be to elucidate the mechanisms by which DP-MSCs regulate hydrogel degradation and replacement, in order to obtain a well-differentiated DP with structural and functional properties similar to the tissue of origin (Fig. 7). Addition of bioactive molecules to the fibrin scaffold will help to provide a favourable environment for cell differentiation, promoting hydrogel replacement by a DP-like matrix, controlled inflammatory/immune response, and an aseptic environment. The controlled delivery of these active agents/molecules will benefit from the development of nanobiotechnologies and bring nanotherapeutics into the armamentarium of dental practitioners who aim at regenerating the human DP.

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Discussion with Reviewer

Franz Weber: The first clinical trials of pulp regeneration have been performed using a collagen-derived scaffold which appears coherent since the pulp tissue is made of collagen. Collagen hydrogels can also be combined with various molecules to improve antibacterial and other properties presented here in the context of the fibrin-derived scaffolds. So, what makes the authors believe the fibrin-derived scaffolds would be better than the collagen ones?

Authors: We agree with the reviewer's comment, collagen is the first material proposed for the regeneration of DP in clinical trials, based on its structural homology with DP tissue. However, like all other materials, none of the collagen-based scaffolds were found to possess the ideal properties as a biomaterial for DP regeneration. Indeed, the collagen origin, composition, methods of extraction and purification were reported to induce many different cellular responses, including inflammatory cellular reaction that is still poorly characterised (Barbeck *et al*., 2015; Herrera-Vizcaíno *et al*., 2020; Stratton *et al*., 2016; Udeabor *et al*., 2020, additional references). In addition, collagen scaffolds lack mechanical strength, which requires their enhancement by physical or chemical methods, or by the development of multi-layered scaffolds (Dong and Lv, 2016; Stratton *et al*., 2016, additional references). These points could be a major obstacle to a wide acceptance of this technique among the community of practitioners.

Inversely, fibrin is a natural scaffold biologically designed to induce tissue repair/regeneration and to promote cell response toward this purpose. Fibrin degradation products promote angiogenesis, induce a controlled inflammation, and its mechanical properties could be easily finely tuned without the need of extra-chemical agents. There is good

evidence that it is a promising biomaterial to guide DP regeneration, either alone or in combination with other materials, growth factors or nanomedicinal products.

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