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Guidance for evaluating biomaterials' properties and biological potential for dental pulp tissue engineering and regeneration research

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ABSTRACT

Background: Dental pulp regeneration is a complex and advancing field that requires biomaterials capable of supporting the pulp's diverse functions, including immune defense, sensory perception, vascularization, and reparative dentinogenesis. Regeneration involves orchestrating the formation of soft connective tissues, neurons, blood vessels, and mineralized structures, necessitating materials with tailored biological and mechanical properties. Numerous biomaterials have entered clinical practice, while others are being developed for tissue

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Biological interactions
 Immune response
 Vascularization
 Collagen
 Synthesis
 Material properties and cellular outcomes

engineering applications. The composition and a broad range of material properties, such as surface characteristics, degradation rate, and mechanical strength, significantly influence cellular behavior and tissue outcomes. This underscores the importance of employing robust evaluation methods and ensuring precise and comprehensive reporting of findings to advance research and clinical translation.

Aims: This article aims to present the biological foundations of dental pulp tissue engineering alongside potential testing methodologies and their advantages and limitations. It provides guidance for developing research protocols to evaluate the properties of biomaterials and their influences on cell and tissue behavior, supporting progress toward effective dental pulp regeneration strategies

1. Introduction: the importance of testing the biological properties of biomaterials for dentin-pulp regeneration

Various biomaterials are used in clinical practice or developed to manage pulp disease and trauma, with applications aimed at preserving the remaining pulp for dentin-pulpal regeneration in reversible cases or promoting peri-apical lesion healing in irreversible cases [1]. The primary objectives of these materials are to eradicate biofilms and microorganisms in the root canal system, and periapical tissues and to facilitate the regeneration of pulp tissue capable of depositing new dentin. This regeneration aims to facilitate root formation, reinforce the dental structure and serve as a barrier against further bacterial invasion or promote root formation [2,3].

Research on materials for the dentin-pulpal complex varies from the synthesis of new components for cementitious or resinous materials and their combinations with existing components to advancements in technologies for the fabrication of scaffolds and matrices such as cements, hydrogels, and fibers and their fabrication through conventional or 3D printing methodologies [4–7]. These biomaterials can be broadly categorized as inert, serving only as a template to guide tissue formation, or bioactive, with the capacity to deliver cells and components to induce desired biological effects (for the purposes of this article, the term bioactive will be used to refer to the latter class of materials; for advanced discussions on the topic of "bioactivity," refer to [6]). While dental biomaterials may be applied for various purposes, their physical, chemical, and mechanical properties are routinely characterized to assess their impact on the desired clinical outcomes. Consequently, researchers frequently conduct *in vitro* evaluations to characterize these materials, providing initial insights into their biological effects and potential limitations, even though these tests may not fully predict clinical performance [4,7,8]. Further progression to *in vivo* studies is typical for biomaterial developed for pulp regeneration, where the ability of the materials to promote the formation of viable human pulp tissue with the capacity to form dentin can be indicated [2,8–11].

As the emphasis on biomaterials for pulp and dentin regeneration continues to grow and the need for a deeper understanding of the characteristics and biological properties that promote dental pulp and dentin regeneration becomes more pressing, it is crucial to scrutinize the elements that contribute to a comprehensive understanding of these materials. With the materials science community demonstrating increased interest in biomaterials and their role in dental pulp and dentin regeneration, it is now more critical than ever to examine these factors deeply. Therefore, this article aims to provide an overview of the key concepts associated with evaluating biomaterial properties and the biological outcomes related to dental pulp regeneration while also offering practical guidance on principles, methodologies, and assays that can aid in the planning, execution, and reporting appropriate testing and results in the field of dental pulp tissue engineering and regeneration.

2. An overview of dentin-pulp regenerative strategies and therapies, mechanistic processes leading to odontoblastic differentiation and dentin

The application of biomaterials to facilitate the repair and regeneration of pulp tissue plays an important role in the success or failure of

endodontic therapies [12,13]. Consequently, the standardization or, at least, the normalization of procedures using new materials for pulp management plays an important role in ensuring the different analyses are comparable for future *in vivo* applications [14]. Among pulp therapies of interest, vital pulp management and regenerative endodontic treatment (RET) procedures are currently the most relevant from a biomaterial point of view. In this context, they also need to be treated separately since the former is more closely related to pulp repair, while the latter is associated with its regeneration [15–17].

2.1. Vital pulp therapies and pulp repair

The outcomes of vital pulp therapies have been intimately related to biomaterials since their inception and the early use of calcium hydroxide for pulp capping [18]. Both direct pulp capping and pulpotomies benefit directly from the type of material placed on the pulp [12]. The literature is replete with attempts to induce the differentiation of odontoblast-like cells and the consequent production of tertiary reparative dentin, with testing of a range of materials including calcium hydroxide [12], zinc-oxide eugenol cements [19], formocresol [20], composites [21] and, more recently, hydraulic calcium silicate cements as MTA and Biodentine [22,23]. Each of these materials has been tested at different times and with a range of parameters, which has unfortunately led to controversies and often misleading recommendations [21,24]. Nonetheless, the learning curve for the experimental application of new materials on pulp therapies has culminated in more biological and sophisticated evaluations, moving away from relatively simple histological sampling in animal models [25], towards cell culture analysis with a focus on gene/protein expression [26,27] and cell/cell interactions [28].

From a translational view point, the following describes the sequence of clinical events: i. the pulp tissue is exposed to the environment due to caries, trauma, and/or cavity preparation; ii. the clinician evaluates the clinical conditions (e.g., symptoms, tooth, patient's age, macroscopic condition of the pulp, other relevant clinical factors such as hygiene and periodontal status, and patient choice); iii. potential removal or management of the pulp tissue to achieve hemostasia; iv. application of a biomaterial on the exposed pulp tissue; v. tissue repair by producing hard tissue (dentin bridge); and determination of success by evaluating the pulp status.

In this context, other properties are also desirable from the biomaterials and have their own standards for testing, namely the ability to resist mechanical stress, the antibacterial capacity, and the interaction with other common dental materials [29,30]. However, with the recent shift towards targeted biomaterials and even the customization of pulp capping materials [31], understanding the processes involved in biocompatibility and odontoblast differentiation becomes essential to predict how a material will promote or inhibit pulp repair. Moreover, there is also the need to understand and address the inflammatory component present in the pulp tissue following disease and injury. While tissue inflammation is necessary for adequate repair [32], a material must also suppress and modulate the inflammatory process to not overwhelm the pulp tissue and allow the materials to enable cellular responses.

Initially, biomaterials must be evaluated regarding their biocompatibility [30]. After biocompatibility analysis, biomaterials can be

evaluated according to the odontogenic potential, generally compared with the current 'gold standard' material. This can be analyzed by the expression of genes expressed in odontoblasts, although there are no perfect and consistent markers for this purpose [15,33]. Genes that can be utilized as markers to confirm this differentiation process include dentin sialophosphoprotein (*DSPP*), dentin matrix acidic phosphoprotein 1 (*DMP-1*) and others (Table 3). Additionally, the expression of mineralization markers such as *RUNX2*, osteopontin (*OPN*), and osteocalcin (*OCN*) can provide complementary data. Protein analyses and functional assays are conducted to observe and quantify processes related to mineralization (e.g., alkaline phosphatase activity, Alizarin red staining) [33–37]. Nevertheless, true odontoblast differentiation must be verified through *in vivo* experimentation, confirming cellular polarization and the formation of new tubular dentin [11,38,39].

2.2. Dentin-pulp regenerative procedures

Besides vital pulp therapies, where residual vital pulp tissue is necessary to induce and support repair, there is a current surge in interest in RET [40]. Many of the same principles regarding odontoblast differentiation apply here, specifically the understanding that the newly formed pulp tissue must also produce a mineralized tissue for its protection [41]. However, biomaterials now also serve a different role by providing scaffolds that can allow for new tissue formation and development [42].

In this context, additional factors must be considered, such as the biomaterial's capacity to adapt to three-dimensional environments, facilitate cellular interactions, and exhibit mechanical responsiveness to induce cell differentiation and adhesion to the dentinal walls [10,43,44]. This will demand standardization of other properties, such as the degree of shrinkage, stiffness, and pH, required from these materials [39,45]. It is important to emphasize that odontoblastic differentiation is not necessary throughout the entire tissue: other cell types, such as neural cells, vascular cells, and fibroblasts, are also essential for achieving complete pulp regeneration [41]. Thus, scaffolds serve a different purpose and have different requirements than those related to pulp capping materials, and they demand their own testing methodologies and standards.

3. Characterization and assessment methods for materials affecting the pulp and its healing and regenerative potential

Biomaterials' physical and chemical properties play important roles in triggering and modulating cellular behaviors such as adhesion, proliferation, and differentiation involved in pulp-dentin tissue regeneration. By carefully designing and tailoring biomaterials, an optimal microenvironment that supports and enhances the regenerative processes of dental pulp tissues can be created [4].

Key properties of biomaterials, such as pH, ion release, surface topography, and stiffness, can modulate cellular activities and responses. For instance, the pH level can affect enzyme activity and cellular metabolism, while ion release can provide essential cell differentiation and mineralization signals [46]. Comprehension and regulation of these properties facilitate the development of biomaterials capable of eliciting and modulating the expression of genes and proteins involved in the differentiation toward odontoblast-like cells, endothelial, and other cell types, as well as enhancing specific and essential cellular functions integral to the processes of pulp and dentin tissue regeneration (Fig. 1).

3.1. Characterization of chemical properties of biomaterials that affect biological outcomes

The chemical properties and characteristics of biomaterials can significantly affect the ability of cells to express genes and proteins related to odontoblastic differentiation and differentiate into

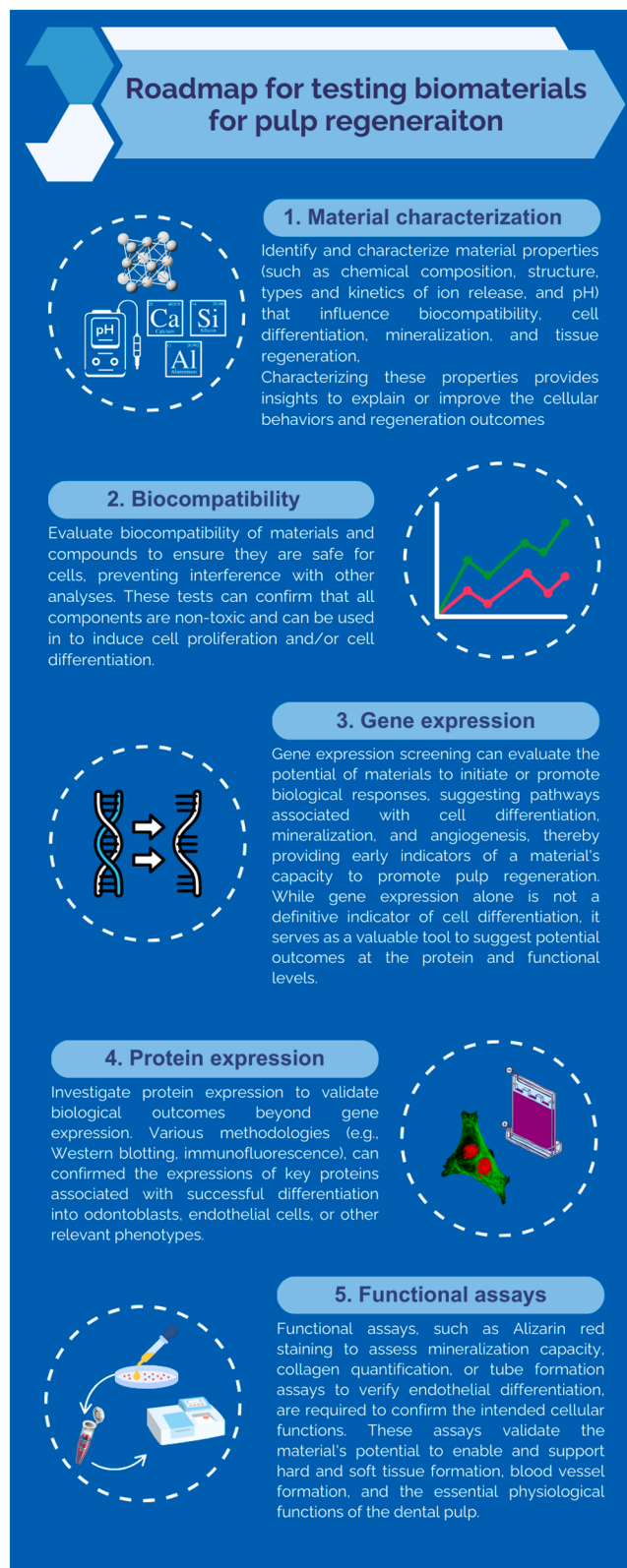


Fig. 1. Roadmap to assessing the potential of biomaterials to promote dentin-pulp complex regeneration. A comprehensive characterization of the fundamental properties of biomaterials, biocompatibility, gene and protein expressions, and the ability of biomaterials to induce functional outcomes provides a dataset that can robustly indicate the potential and limitations of biomaterials in promoting the regeneration of the dentin-pulp complex.

mineralized tissue-producing phenotypes (Table 1). Calcium silicates (so-called bioceramics) are usually designed to modulate the alkalinity of the microenvironment through the release of hydroxyl, calcium, and other ions [3]. Calcium ions released from pulp-capping materials react with carbonates in the pulp tissue to form calcium carbonate. This process influences dental pulp stem cell proliferation, promotes differentiation, and contributes to the onset and progression of mineralization [8,47–49]. It is important to emphasize that the magnitude of the effects experienced by cells differs depending on the type of ion, compound, and their concentrations. For example, relatively high concentrations of Ca^{2+} can lead to extensive cell death or a decrease in initial proliferation, which can be recovered depending on cellular adaptation mechanisms [49–51]. In addition, calcium ions stimulate fibronectin synthesis in dental pulp cells. Fibronectin is a protein that can promote the differentiation of dental pulp cells into cells that produce the mineralized tissue, which is essential for the formation of dentinal and mineralized tissue bridges [48]. Similarly, a concentration of released ions can alter the pH of the microenvironment, which can affect cellular responses, such as the activity of ALP, which is significantly higher in human dental pulp cells cultured with conditioned media at pH 7.8 compared to pH 7.2 [47]. Therefore, it is crucial to evaluate the capacity of biomaterials to release ions and other compounds over time in different environments, as this directly impacts their potential to initiate biochemical processes associated with cell proliferation, differentiation, and mineralization.

A widely utilized, cost-effective approach for assessing a material's ability to modulate environmental alkalinity and elicit cellular responses is the simple measurement of the pH of a liquid containing the

Table 1

Properties and characteristics of biomaterials and their potential impact on biological responses involved in dental pulp tissue engineering and regeneration. The comprehensive characterization of biomaterials can facilitate their production with enhanced properties and predict tissular responses when placed clinically.

Properties and characteristics	Impact
Composition	The chemical and structural makeup of the material determines its bioactivity, mechanical properties, and compatibility. Materials such as bioceramics, polymers, or composites influence scaffold mimicry of the extracellular matrix, cell adhesion, differentiation, and mineralization.
Crystalline phases	Influence mechanical strength, stability, and mineralization support. Characterizing crystalline phases (e.g., calcium hydroxide or tricalcium silicate) supports understanding how biomaterials affect cell differentiation by initiating mineralization pathways, hydroxyapatite formation, and tissue repair.
Ion release	The type, quantity, and sequence of ion release from bioactive cements critically influence molecular and cellular behavior, including signaling pathways, proliferation, differentiation, and mineralized tissue formation.
pH profile	Impacts cell viability, differentiation, and function. Some polymers may reduce environmental pH upon degradation, necessitating pH characterization to prevent false viability test results based on colorimetry.
Degradation rate and types of byproducts	Scaffold longevity and the time available for cell attachment and multiplication should ideally match the formation rate of new tissue. Degradation byproducts must also be non-toxic to support tissue viability and formation.
Rheology	Dictate material handling, injectability, and the ability to conform to irregular pulp spaces. It also impacts the ability to 3D-print hydrogels and influences how cells respond to the injection process (cell alignment and potential cell death due to shear stress).

material over an extended time period (Fig. 2). Generally, articles demonstrate a similar trend of a rapid increase in pH within the first few hours to a few days (usually less than three) when specimens are immersed in the assay liquid. This is followed by a plateau (around pH 12), where additional significant increases are typically not observed [49,52]. Despite the simplicity of this assay, maintaining a constant solution volume is also essential to ensure accurate pH measurements over extended periods, as the solutions are often stored in heated incubators during the study. It is worth noting that ISO 23496 specifies reference buffer solutions for calibrating pH equipment [53]. Ion-selective electrodes (ISEs) are sensors that measure the concentration of specific ions in a solution by converting them into electrical potential. Although ISE provides a relatively straightforward and efficient method for measuring ion release, it can present challenges in differentiating between free ions and complexes released from materials. Consequently, it may be advisable to complement the analysis with ion chromatography (IC) to ensure accurate quantification of free ions when complexes can also be formed [54,55]. This technique can quantify specific ions with high sensitivity for detecting low to moderate ion concentrations [56]. The IC technique can be time-consuming when optimizing the columns, conditions, and phases for accurate detection, especially for more complex materials. However, hydraulic cement and bioactive materials should not be too complex to be characterized by this technique [55].

Other quantitative techniques can be employed to assess the specific release of ions and compounds from materials that can impact cellular behavior (Fig. 2). For instance, inductively coupled plasma mass spectrometry (ICP-MS) is a highly sensitive technique capable of detecting trace levels of multiple ions simultaneously with high precision in a solution [57]. However, specimen preparation often involves acid digestion and can be technically demanding. In addition, the analyses require costly machinery and operator expertise, and certain materials can release substances into the solution, which may negatively impact ion detection (matrix effects). Finally, Atomic Absorption Spectroscopy (AAS) is also a promising alternative as it has high specificity [7]. Nonetheless, AAS has lower sensitivity to trace elements and typically measures one element at a time, making multi-ion analysis more time-consuming. Finally, calcium colorimetric assay kits can quantify calcium and phosphates released from bioceramic scaffolds. Such assays are very straightforward as reagents are supplied and usually require basic benchtop spectrophotometers to measure the optical densities of solutions once incubated with biomaterials for defined periods and compared with negative controls (solution/no biomaterial). Despite its simplicity, the main drawback of this strategy is that separate kits are necessary for the determination of distinct ions, and kits for the characterization of species of interest may not always be accessible.

These techniques enhance basic pH measurement by identifying elements and compounds influencing the microenvironment's alkalinity. This is important as different species may be released at varying times, affecting cell responses distinctly despite minor pH value changes [49, 58]. In addition to the test selection, the purity of the water used to produce the eluents and the quality of the glassware used during elution can affect outcomes due to potential sample contamination. Therefore, it is recommended to engage the analytical chemistry team during the study's planning phase, as certain techniques may necessitate the pre-treatment of labware to prevent contamination and optimize equipment parameters to quantify the ions accurately.

Quantifying alkalinity and ion leaching from materials largely depends on test design, including specimen dimensions and leachate concentration. The size and shape of the specimen influence the elution rate, thus affecting biological outcomes such as biocompatibility or mineralization. Specimen dimensions, extraction methods, and elution time collectively determine the leached compounds from biomaterials, leading to significantly different biological outcomes [30,50]. Currently, no standardized methodology exists for preparing biomaterial extracts to induce dental stem cell differentiation. However, certain ISO

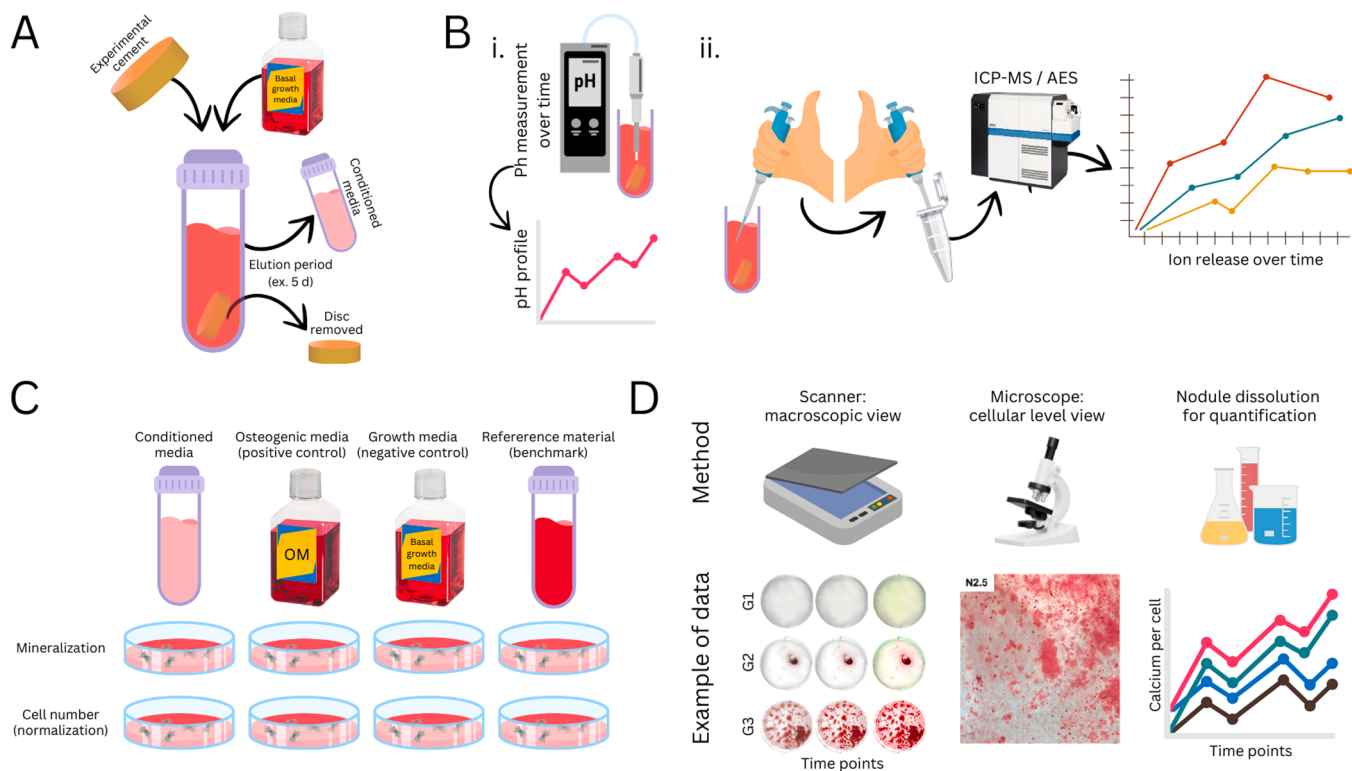


Fig. 2. The extract method, commonly used to create induction media for dental pulp stem cell differentiation, involves immersing an experimental material disc in basal growth media for a specific elution period. Subsequently, the disc is removed, and the eluent is collected to treat the cells. (B) The conditioned media can be characterized by (i) periodic measurements of pH and (ii) ion profiling using Inductively Coupled Plasma (ICP). ICP analysis necessitates periodic removal of a specific liquid volume from the sample container. Therefore, appropriate controls and volume normalization are required to avoid compromising results. (C) An example experimental design for testing the ability of an experimental material to promote mineralization includes osteogenic media as a positive control for the alizarin red staining reaction, while basal growth media functions as a negative control to verify that mineralization is induced by the conditioned media. A benchmark material, such as a bioceramic known to induce mineralization, is included to provide a reference for the magnitude of the experimental material's potential. Additional wells for cell counting ensure that differences in mineralization are not due to variations in cell density but rather reflect the material's intrinsic properties. Similar experimental designs can also be adapted for other characterizations, such as evaluating alkaline phosphatase activity or quantifying collagen production. (D) The mineral deposits can be characterized qualitatively at the macroscopic and microscopic levels. Chemical dissolution of the nodules enables quantification of the mineral content, providing an objective measure of the material's mineralization potential and reducing the risk of bias associated with qualitative analysis.

(image elements of this figure were adapted with permission from [146] and [151]).

standards outline the temperature, thickness, and sample/media ratio options used to prepare extracts for evaluating material biocompatibility [29,59]. This could also serve as the basis for preparing solutions that will be employed in differentiation studies and analytical tests. The absence of a standardized methodology allows researchers to customize experimental setups to fit their research questions. However, investigators should be cautious when comparing different studies' results, as materials may behave differently depending on the specific test designs used [2,47,50]. Therefore, conclusions drawn from comparing data across studies must consider the methodologies used.

3.2. Assessment of dental pulp-related biological properties changes triggered by dental materials

The field of research in materials for dental pulp applications aims not only to improve the physical-mechanical properties of biomaterials but also to enhance their capacity to trigger and modulate specific cell and tissue behaviors that can result in the desired healing or regenerative outcomes. In this context, biological characterizations of biomaterials often involve the assessment of biocompatibility, proliferation, cell differentiation, and functional outcomes. While these biological characterizations are not exhaustive, potentially in combination, they can point to the general ability of a material's potential not only to avoid damaging the pulp but also to positively influence tissue

regeneration. It is important to reiterate that there are no exclusive markers for "odontoblastic differentiation," as many of the genes, proteins, and functional tests associated with odontoblast-like cells are also present during (or as a result of) osteogenic differentiation [60,61]. Nonetheless, "odontogenic differentiation" will be used in this article to represent the researchers' goals to promote the differentiation of cells into odontoblast-like cells (and not osteoblasts) using dental biomaterials.

3.2.1. Cell types

Typically, the characterization of the potential biological properties of biomaterials involves the use of primary cells, such as dental pulp stem cells (DPSC) and stem cells from human exfoliated deciduous teeth (SHED), dental pulp fibroblasts, as well as immortalized murine dental pulp cells, such as MDPC-23 cells and odontoblast-lineage cells. To a lesser extent, ex vivo dental pulp tissues are also used for this purpose [62–66].

The DPSC and SHED are commonly utilized in the evaluation of the biological characteristics of dental materials due to their relative simplicity in cultivation and excellent ability to self-renew and differentiate into a variety of cell types, including neurons, odontoblast-like cells, and fibroblasts, which are relevant for dental pulp regeneration research [8,62,67,68]. Since the early 2000s, when protocols for isolating and characterizing DPSC and SHED were first published [69,

70], obtaining these cells has become a relatively widespread practice among researchers and commercial providers, making them accessible and accepted tools for assessing the biological properties of biomaterials. Despite their widespread usage, DPSC and SHED are primary cells, so they are prone to experiencing disruptions and differences in their biological activity. Consequently, these cells lose their proliferative and differentiation capabilities over time and may also undergo senescence [71,72]. Therefore, researchers often employ cells with passages lower than 8 to evaluate biomaterials. The choice between DPSC and SHED should depend on the intended application in primary or permanent teeth. Despite similarities, SHED represents a more immature cell population with greater self-renewal and proliferation capabilities [63,64]. Indeed, growth kinetics should be considered when selecting the type of cells for evaluating biomaterials for primary versus permanent teeth.

In addition, when exposed to an osteogenic differentiation medium, immortalized murine dental pulp cells (e.g., MDPC-23 cells and odontoblast-lineage cells) can undergo mineralization [73]. Immortalized cell lines continuously proliferate, offering an accessible and cost-effective option for study. However, they may differ genetically and phenotypically from the original tissue, potentially exhibiting altered cytomorphology or loss of key markers, thereby affecting their response to external stimuli [74-76]. Several immortalized cell lines have been derived from dental and odontogenic tissues of animal origin. While using these cell lines to test the biological properties of dental materials is acceptable, the results must be interpreted in light of their non-human origin, as they may differ genetically and physiologically from their human counterparts [77,78].

Dental pulp cells (DPC) represent a population of cells that can be obtained directly through *in vitro* culture of the dental pulp from freshly extracted teeth. DPC has been utilized for biocompatibility testing and characterization of the differentiation potential of biomaterials for several decades due to the relative ease of their acquisition. Indeed, cells can be obtained from teeth stored for up to 5 hours before the pulp is removed and cultured without prejudice to cell proliferation potential [79]. Despite the simplicity of the isolation method (digestion with collagenase and dispase [80]), the final DPC pool receives limited consideration for cell types capable of enduring *in vitro* culture, particularly compared to other defined populations such as DPSCs and SHED, which undergo stem cell marker characterization [70]. Alternatively, dental pulp fibroblasts (DPF) can be isolated from the dental pulp and cultured *in vitro*. DPFs play an important role in regulating immunity and inflammation within the pulp.

Furthermore, some specialized contractile fibroblasts can be a source of newly differentiated odontoblast-like cells that can synthesize reparative dentin [81]. Similar to DPSCs, both DPC and DPF can undergo osteogenic differentiation under appropriate stimuli [80,82]. The latter experience variable cell death when cultured in the presence of glass ionomer cement and CaOH₂ material [83].

As reported, different types of cells can be utilized in studies involving biomaterials for pulp application. While there is no need to establish strict determinants for which cell types are most appropriate for specific tests, researchers must consider that all types possess inherent advantages and limitations. However, the interpretation of data and comparison between studies should be undertaken in light of the inherent properties of each cell type.

3.2.2. Assessment of biomaterials biocompatibility and potential to promote cell proliferation and migration

Materials designed to interact with dental pulp cells must exhibit high biocompatibility to support the cells' survival, function, and overall viability. This promotes healing and integration with surrounding tissues without triggering adverse effects such as toxicity, inflammation, mutagenicity, immune responses, or even cell death. Thus, the initial evaluation of any biomaterial intended for use with pulp tissue should focus on its biocompatibility. The concept of biocompatibility has

undergone significant evolution, transitioning from a simplistic understanding established in basic toxicology knowledge to a more complex and comprehensive definition. Initially, biocompatibility indicated the "inertness" of a material or its ability to exist within a host without eliciting a harmful response [84]. Early assessments focused on whether a material would trigger fibrous connective tissue formation without causing inflammation, assuming that an inert material would be non-irritant, non-toxic, non-immunogenic, non-thrombogenic, and non-carcinogenic [85]. These materials, which caused no evident harm to the tissue, were considered biocompatible and thus suitable for medical and dental applications. However, the understanding of materials' biocompatibility has expanded to embrace more dynamic interactions between materials and the tissue environment [86,87]. The International Organization for Standardization (ISO) defined biocompatibility as the "ability of a medical device or material to perform with an appropriate host response in a specific application" [88]. This shift acknowledges that a material's success is not merely about it being inertness but how it interacts with the surrounding tissues, cells, and immune system to support healing and regeneration without exerting adverse effects.

The ISO and other such standards offer guidelines for specimen preparation, experimental design, and other aspects to evaluate the biocompatibility of materials that facilitate data comparisons across studies [29,59,89]. The methods highlighted include agar diffusion, filter diffusion, direct contact or extract tests, the dentin barrier cytotoxicity test, and the tooth slice model. Their primary purpose is to evaluate the potential cytotoxic effects of materials by observing their impact on cultured cells and tissues. Typically, the first line of screening involves two-dimensional (2D) cell cultures using established cell lines from sources such as the American Type Culture Collection (ATCC). These tests often require extracting chemical compounds from materials, the leachates, and either indirectly culturing cells in dilutions of such extracts or placing the materials in direct contact with cells seeded in well plates. Key measurement endpoints include assessment of cell viability and metabolic activity, often using metabolic markers such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and succinate dehydrogenase. Materials that produce no measurable leachates or do not interfere with cell metabolic activity are typically classified as biocompatible. These 2D *in vitro* tests are selected for their high throughput, reproducibility, and cost-effectiveness, making them a popular choice for initial material screening [88,90,91]. Recently, a guidance paper on biocompatibility testing has been published to support researchers in the planning, executing, and reporting of tests and results [30].

A common method to evaluate the biocompatibility of materials for endodontic treatment or regeneration involves seeding human cells on specimen surfaces (such as cements and scaffolds) and imaging their behaviour using scanning electron microscopy (SEM) or confocal laser scanning microscope (CLSM). Despite its popularity, this technique has limitations. Adherent cells, such as DPSC or SHED, typically need to attach to a substrate, forming focal adhesion spots and a flattened morphology to support survival, signaling, growth, and differentiation. When cells undergo apoptosis or other forms of death, they lose their spread morphology, round up, and detach from the substrate. This introduces confounding factors in biocompatibility assessments, as adhesion-based assays or imaging may overestimate biocompatibility by excluding non-viable, detached cells. Therefore, these imaging techniques should complement quantifiable methods (e.g., metabolic assays), and conclusions about material biocompatibility should not rely solely on imaging. Notwithstanding the inherent limitations, imaging techniques are helpful for the visualization of cellular morphology and the elucidation of interactions between materials and cells [61,92,93].

Besides morphological analyses, biomaterials used in pulp studies are often characterized by their ability to allow (or even induce) cells to multiply and migrate. A material that promotes cell proliferation stimulates the division and growth of cells within the dental pulp and,

therefore, likely enables tissue regeneration and repair. Therefore, this assessment is important as the success of vital pulp treatment and regenerative strategies rely on the multiplication and mobility of viable cells to repair damaged or diseased tissues continuously [1,94].

Wound healing assays provide valuable data for evaluating the potential of biomaterials to influence cell migration. These assays can be performed using various methodologies, such as transwell cell migration assays, invasion assays (commonly used in cancer research), and spreading and scratch assays [95]. The latter is commonly used to assess endodontic materials due to its simplicity in setup, requiring only a microscope with basic features and a camera.

In this assay, cells are cultured until they reach a high confluence rate of 90 % or higher. Subsequently, a linear area devoid of cells is created by scraping the culture with a pipette tip. The cells are then typically cultured with a pre-exposed culture medium, and the "scratch" is imaged at regular intervals (hours or days) to identify when it is fully repopulated by cells. Notably, researchers may encounter a challenge in creating a uniform scratch by hand. Due to the limited surface area for analysis and the lengthy analysis period, pipette handling can cause variations in the 'scratch,' resulting in data inconsistencies. Culture inserts with defined areas facilitate controlled experimental conditions by allowing cells to be seeded and grown to confluence within a confined space. Upon removal of the inserts, a consistent 'scratch' area is created for subsequent analysis. While using commercially available inserts ensures standardized assay conditions and enhances the reliability of the results, it substantially increases the overall cost of the assay. Therefore, researchers must equate both the cost of the assay and the reliability, deriving insights into the biological properties of materials via scratch assay. Once full closure is achieved, the obtained images are processed using imaging software (e.g., ImageJ) to measure the distance between the cell barriers at predetermined time-points or to calculate the total area uncovered by cells (relative wound area closure) over time [58,96]. When analyzed against control conditions (e.g., cells cultured solely in basal growth media), the data can provide valuable insights into the biomaterial's ability to promote or inhibit cell migration and wound healing. The test outcomes are highly dependent on the specimen preparation conditions and the elution parameters used for extract preparation [30,59]. Therefore, it is not uncommon for studies to report different potentials or even conflicting outcomes regarding the healing properties of specific materials [58,97]. Thus, similar to quantifying leachable species from biomaterials, researchers must consider the test design parameters utilized in different studies when comparing published data and consequently drawing conclusions.

Despite the widespread use of 2D cell cultures for biocompatibility and proliferative tests, this geometrical arrangement has limitations when considering the structural complexity of the 3D pulp tissue. For instance, 2D cultures are highly sensitive to uncured monomers and other chemicals, possibly overestimating a material's cytotoxicity [98, 99]. This sensitivity stems from the artificial, planar cultivation environment, which lacks the complexity and protective factors present under *in vivo* conditions. Thus, 2D tests may not accurately reflect material interactions within the complex environment of the dental pulp [87].

To address these limitations, cytotoxicity tests incorporating a dentin barrier have been developed to emulate *in vivo* conditions more accurately [93, 100–103]. To that end, a dentin slice is placed between the material and the cultured cells, creating a more physiologically relevant model. Dentin barrier tests can be performed with cells cultured in a 2D layer on top of the dentin or by encapsulating the cells in a hydrogel to mimic the more natural 3D environment [104]. Similarly, dentin slices can be utilized to simulate the indirect exposure that cells may experience to biomaterials or their byproducts [105] without resorting to entirely indirect methods, such as using extracts to treat cell populations [29,59]. The presence of dentin and an extracellular matrix provides biophysical cues that enable cells to function under conditions that more closely resemble those present *in vivo*. This offers a more accurate

assessment of a material's biocompatibility (for more detailed information, refer to [87, 91, 106, 107]).

3.2.3. Assessment of biomaterial's potential to promote cell differentiation

Biomaterials are frequently utilized in vital pulp therapies or tissue engineering strategies aimed at inducing and promoting cell differentiation toward specific phenotypes, such as the conversion of DPSCs into cells forming connective tissue, odontoblasts for the secretion of mineralized tissue, or endothelial cells to promote vascularization [11, 72, 108–110]. These effects are commonly defined as the capacity of materials to exhibit "bioactivity." Although the definition of bioactivity may vary depending on criteria and applications, it is not within the scope of this article to promote or resolve the discussion regarding its definition [111]. Therefore, the term bioactivity will be used in accordance with the current literature on biomaterials for pulp applications, which is often used to describe the ability of a material to promote mammalian cell differentiation or enhance its functions.

Studies evaluating the capacity of biomaterials to promote cellular functions typically comprise two stages: cell differentiation induction and the assessment of biological outcomes elicited by the induction. The induction phase frequently involves the treatment of cells with a differentiation-inducing culture media, extracts prepared to utilize biomaterials, or direct exposure to biomaterials. The assessment phase encompasses various types of analyses that, when interpreted collectively, can confirm successful cellular differentiation and functions. These analyses commonly include a preliminary investigation via quantification of gene expression due to the technique's popularity, simplicity, and cost-effectiveness in elucidating the effects of biomaterials on cells and the potential mechanisms involved. Despite the utility of gene expression, this analysis does not comprehensively characterize the cell differentiation process. Therefore, additional relevant analyses are conducted, including *in vitro* (protein expression and functional assays) and *in vivo* analyses to confirm differentiation and to assess the cell's ability to perform the intended functions in response to stimuli provided by biomaterials [3,11,61,66,72,112].

A significant portion of research on pulp materials involves exposing cells to biomaterials to induce expression. Notably, many articles claiming odontoblastic differentiation *in vitro* do so by assessing genes and proteins expressed by odontoblasts *in situ* and the ability of differentiated cells to secrete a mineralized matrix. Whether these assessments can confirm a "true" odontoblastic differentiation is debatable, as many of the markers used and cellular activities are also common to osteoblasts. The unique hallmark of odontoblastic differentiation is the production of tubular dentin, which is better observed *in vivo* [11,45,72, 112]. However, the literature broadly accepts that the expression of relevant genes and proteins (Table 1) and characteristics like increased ALP expression and mineralized matrix secretion collectively indicate odontoblastic differentiation [10,11,27,46,54,70,72,98,108,113–121]. Thus, this article discusses "odontoblastic differentiation" based on widely accepted evidence from the current literature.

3.2.4. Frequently employed protocols for the analysis of odontogenic and angiogenic differentiation

Numerous biomaterials have been developed to induce or promote dental pulp tissue regeneration through odontogenic and angiogenic differentiation processes. However, many biomaterials are designed to trigger or enhance only specific aspects of these processes and do not possess the capability to induce such intricate processes independently. Therefore, differentiation induction media initiate or sustain the differentiation process.

There are no universally standardized media for inducing odontogenic differentiation. One possibility is to use dentin-derived growth factors to induce odontoblastic differentiation [113]. This can be achieved by extracting dentin matrix proteins from human teeth and lyophilizing them before supplementing them in the culture medium of DPSCs. Several researchers adapt the commonly utilized osteogenic

media (OM) by adding growth factors and compounds that influence odontogenic processes. Despite its popularity, the OM also exhibits variations in the literature; however, the formulation describing the supplementation of basal growth media with 100 nM dexamethasone, 10 mM β -glycerophosphate, and 50 μ g/ml L-ascorbic acid-2-phosphate appears to be widely accepted by the scientific community [122].

To promote odontogenic differentiation, numerous growth factors and compounds have been incorporated into OM (Table 1) to create what is often referred to as "odontogenic induction media" (the quotation marks are intentional, as there is no consensus in the literature regarding the specific components, biological processes, or functions this media should promote). Consequently, it is advisable to evaluate the biological effects of varying concentrations of compounds added to OM, both with and without the biomaterials of interest. This approach ensures that any significant biological effects arising from interactions with the biomaterial are not masked by the potentially stronger chemical stimuli provided by the induction media.

Specific markers for odontogenic differentiation have not yet been identified, as the markers expressed by odontoblasts are also present in other cell types, such as odontoblast-like cells and osteoblasts. Consequently, the term "odontogenic induction media" should be used cautiously. Unlike neurogenic or adipogenic media, which reliably induce differentiation into neurons and adipocytes due to the exclusive markers of these phenotypes, odontogenic induction media merely induces cells to express markers associated with odontoblasts, which may also be present in other cell types. Therefore, careful consideration is advised when applying this term, as it does not necessarily reflect the *in vitro* differentiation of stem cells into odontoblasts.

One of the most straightforward protocols for the induction of angiogenic differentiation of DPSCs involves exposing them to the EGM2-MV medium (Lonza) supplemented with 50 ng/ml rhVEGF [11]. Under these conditions, DPSCs differentiate into endothelial cells within 5–7 days, as evidenced by the expression of endothelial markers such as VEGFR2, VE-Cadherin, and CD31 [11,39,132,133].

3.2.5. Gene expression

Demonstrating a material's ability to induce the expression of odontoblast-related genes is an important indicator of its bioactive potential to promote odontogenic differentiation. Unfortunately, odontoblasts lack distinctive markers exclusive to their phenotype, as is the case with other cell lineages. To overcome this limitation, it is common practice in dental research to characterize the expression of a panel of genes (usually four or more) that are commonly expressed by functional odontoblasts, pulp cells, stem cells undergoing osteo/odontoblastic differentiation under stimulated conditions *in vitro* or by functional cells capable of secreting dentine *in vivo* [11,58, 61,92,122,124,134]. Therefore, it is the praxis to state that a material "can induce odontogenic/blastic differentiation" if it can increase the expression of the selected panel of genes. Table 2 presents a non-exhaustive list of genes frequently utilized in combination to define the potential of biomaterials to induce odontogenic/blastic differentiation.

Table 2

Examples of growth factors, compounds, and concentrations used to supplement media for odontogenic differentiation.

Growth factors and compounds	Concentrations	Ref.
Biomaterials (e.g., cements)	Conditions defined by researchers	[8,27,46,50, 123,124]
BMP-2	Dentin derived; 100 ng/ml	[113,125]
BMP-4	25, 50 and 100 ng/ml	[72]
BMP-7	50 ng/ml	[126]
Fibroblast growth factor 2 (FGF2)	2.5, 5.0, 10, 20 and 40 ng/ml	[127,128]
Monopotassium phosphate	1.8 mM	[129,130]
Transforming growth factor beta 1 (TGF- β 1)	2.5, 5.0, 10, 20 and 40 ng/ml	[128,131]

The expression of genes induced by biomaterials does not confirm terminal differentiation; proteomic and functional analyses are also necessary. If researchers opt not to conduct tests beyond genetic characterization, conclusions should be based solely on gene expression data without implying terminal phenotypic changes. Thus, any claims regarding gene expression should be along the lines of a *possibility* for a material to induce differentiation or to *suggest* potential cell mechanisms and pathways that could be triggered by biomaterials over time. Consequently, it should not be asserted that odontoblastic/genic differentiation has occurred based solely on gene expression results. (Table 3)

3.2.6. Protein and functional analyses

In stem cell differentiation, both gene expression and protein synthesis play crucial regulatory roles. Genes encode instructions for protein synthesis, and evaluating their expressions provides insights into the differentiation potential of biomaterials and plausible pathways that drive cell fate [4,61,124]. However, evaluating gene expression alone is insufficient to assert differentiation, as mRNA levels do not always correlate directly with protein expression. Even if a gene is expressed at a high level, the actual functional impact on stem cell differentiation is determined by the protein it encodes since proteins execute the molecular and cellular functions, serving as enzymes, structural components, signaling molecules, and regulators of various biological processes [11, 68,113, 127]. In addition, proteins (e.g., collagen, integrin, cadherins, and collagens) mediate spatial and temporal interactions between stem cells and the microenvironment, production of extracellular matrix components, and signaling molecules [110,141–143]. Consequently, characterizing and quantifying protein levels and activities are essential for confirming differentiation into specific cell types and functions [11, 72,120,144].

It is important to highlight that while protein analyses are ideal for assessing hallmarks of cell differentiation and function, they are more costly and require significantly more time and expertise compared to gene expression quantification via qPCR. Various qualitative, semi-quantitative, and quantitative methodologies for assessing protein expression in cells that have undergone differentiation induced by biomaterials, as well as the most common methods used in pulp tissue engineering and regeneration research, are shown in Table 4. Similar to gene expression, no single method or protein is universally indicated to assert odontoblastic or angiogenic differentiation. Nonetheless, studies often combine one or more methods highlighted in Table 4 with other assays that indicate functional readouts, such as the characterization of calcified nodule formation by Alizarin red staining (ARS) or the tube formation assay to confirm endothelial differentiation [11,39,119,145, 146].

Given that each manufacturer employs distinct protocols and recommendations and each protein exhibits specific absorbance peaks, adherence to the manufacturer's instructions is essential. From a quantification perspective, all kits contain a stock solution of the target protein for preparing standard curves. Consequently, quantification should be based on comparing the collected samples' absorbances with the standard curves.

3.2.6.1. Collagen quantification. Collagen is essential for forming the pulp tissue's extracellular matrix (ECM). Its presence and organization provide structural support for cells and influence cell behavior, migration, and differentiation, all of which are important for dental pulp regeneration [147].

In pulp regeneration, the assessment of collagen production, organization, and density helps evaluate the effectiveness of regenerative strategies. Since pulp tissue naturally contains collagen types I and III, quantifying collagen helps determine if the newly formed tissue resembles native pulp tissue and whether it will provide an adequate environment for dental pulp function, including nerve and vascular

Table 3

Examples of genes commonly used to evaluate odontoblastic and angiogenic differentiation.

Gene/protein	Abbreviation	Role	Ref.
Collagen type I	COL-1	Expressed by osteoblasts, odontoblasts, fibroblasts, and other cells involved in forming connective tissue.	[4,114,120,135]
Dentin matrix protein 1	DMP-1	Non-collagenous protein that plays a critical role in the mineralization of dentin and bone.	[10,11,27,46,72,98,113-119]
Dentin sialophosphoprotein	DSPP	he DSPP gene encodes a precursor protein cleaved into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP). These proteins are essential for the mineralization of dentin.	[10, 11, 27, 54, 70, 98, 108, 113, 116, 117, 119-121]
Matrix extracellular phosphoglycoprotein	MEPE	Involved in mineralization and phosphate metabolism.	[8,10,59,72,113]
Runt-related transcription factor 2	RUNX2	Transcription factor critical for osteoblast differentiation, expressed during the early stages of odontoblast differentiation.	[72,77,93,114,117,120,135,136]
Osteopontin / Osteocalcin / Bone sialoprotein	OP or OPN / OC or OCN / BSP	Involved in the regulation of mineralization and calcium ion homeostasis. Expressed by odontoblasts, and is considered a marker of mature osteoblasts.	[27,51,74,114,135]
Bone morphogenetic proteins	BMP-2 / BMP-4 / BMP-7	Various roles during the formation, development of bone and dentin, and odontoblastic differentiation.	[113,135,136]
Msh homeobox 1 and 2	MSX1 and MSX2	Transcription factors that play crucial roles in tooth development and differentiation of dental pulp cells.	[72,92,136]
Alkaline phosphatase	ALP	Key role in mineralization by providing the phosphate for the formation of hydroxyapatite. Expressed by odontoblasts and osteoblasts.	[93,108,116,117,135]
Vascular endothelial growth factor receptor	VEGFR1 / VEGFR2	Modulate the angiogenic potential and endothelial differentiation.	[11,45,110,119,133,137,138]
Vascular endothelial cadherin	VE-cadherin	Functional role in keeping the integrity and permeability of endothelial cell junctions, vascular stability, and the formation of blood	[89,110,137]

Table 3 (continued)

Gene/protein	Abbreviation	Role	Ref.
Cluster of differentiation 31	CD31	vessels. Expressed by endothelial cells.	[37,110,117,133,138-140]
Coagulation factor VIII	Factor VIII	Endothelial cell marker that plays a role in angiogenesis.	[110,138]
von Willebrand factor	vWF	Blood coagulation protein that plays a crucial role in the clotting cascade, expressed by endothelial cells. Glycoprotein that mediates the adhesion of platelets (blood clotting). Expressed by endothelial cells.	[71,109]

support [148]. Methods for collagen quantification often include biochemical assays, such as the Sircol assay or hydroxyproline assay immunohistochemistry and immunofluorescence, or quantitative Polymerase Chain Reaction (qPCR), which is not a direct quantification of collagen but measures the gene expression levels of collagen type I and III which are precursors to collagen protein production.

3.2.6.2. ALP quantification and Mineralization. The Alizarin Red S (ARS) method involves the staining of mineral deposits produced by cells through the interaction of ARS solution with calcium to form red complexes [149]. Although the test is not specific to dentinal structures, it is widely utilized in pulp regeneration literature to indicate that cells have assumed the capacity to secrete mineralized structures, which is one of the components of dentin. The protocol fundamentals and the qualitative and quantitative analyses have been thoroughly documented and established in the literature for cell cultures and, in general, are performed using cells treated with culture medium obtained using the extract method from the biomaterial [150]. Careful consideration is necessary when evaluating mineralization in cells cultured on material surfaces, as many materials can precipitate mineralized structures or adsorb the staining, potentially confounding analyses. Consequently, including appropriate controls, such as staining materials subjected to identical treatment conditions in the absence of cells, becomes essential to exclude nonspecific staining. Another critical control is cells treated with osteogenic media (which is known to induce mineralization in DPSC and SHED, for example) to ensure that the reagents and reaction are functioning effectively.

Following the staining protocol, imaging stained mineralized deposits can acquire qualitative data from ARS with phase contrast microscopy or a scanner; the latter often provides an edge for opaque substrates where staining is not visible through the microscope (Fig. 2). Quantification involves dissolving mineralized nodules after staining with acetic acid and cetylpyridinium chloride (CPC) methods, then measuring absorbance at 405 nm for acetic acid extraction and at 562 nm for CPC using a microplate reader [114].

Alkaline phosphatase (ALP) quantification is a common biomarker assay used in studies of biomaterials assessments as it plays an important role in odontoblastic differentiation and dentin formation, acting as an early marker for odontoblast-like cell phenotypes. Its upregulation during differentiation is vital for mineral deposition. ALP hydrolyzes phosphate esters, producing inorganic phosphate ions, which then combine with calcium to form hydroxyapatite crystals, a key component of dentin produced by odontoblast-like cells. Thus, increased ALP activity is directly linked to dentinogenesis, supporting matrix maturation and mineralization and indicating a biomaterial's regenerative potential. ALP quantification measures the enzyme's activity associated with early osteogenic and odontogenic differentiation [151]. An elevated ALP

Table 4

Techniques commonly used to characterize and quantify protein expression in dental pulp tissue engineering and regeneration.

Technique/Analysis	Aim	Advantages	Disadvantages
Western Blotting	To detect specific proteins in a sample by binding antibodies to the protein of interest and separating it via gel electrophoresis.	The method exhibits high specificity for the target protein. It is capable of providing semi-quantitative data on protein expression levels. Additionally, it detects post-translational modifications (e. g., phosphorylation, glycosylation).	It is time-consuming and labor-intensive. It has low throughput and can detect only a limited number of proteins per analysis. It requires substantial quantities of protein samples. It is semi-quantitative, and precise concentrations are challenging to determine.
Confocal Microscopy	Fluorescence microscopy for high-resolution imaging of protein localization in cells and tissues.	High-resolution imaging enables the visualization of protein localization in three dimensions. This technique facilitates the observation of protein-protein interactions and subcellular distribution. Furthermore, it allows for co-localization studies of multiple proteins.	This method necessitates specialized equipment and expertise. It is semi-quantitative at best. The process requires the optimization of fluorescent labeling.
Enzyme-Linked Immunosorbent Assay (ELISA)	A highly sensitive immunoassay that utilizes antibodies to quantitatively determine the concentration of specific proteins in a liquid sample (e.g., cell culture supernatants or tissue extracts).	Exhibits high sensitivity and specificity, being able to quantify the amount of proteins at the nanogram scale. Quantitative: provides precise concentration values of the protein. High-throughput: capable of analyzing multiple samples simultaneously.	This method necessitates well-validated antibodies. It is limited to detecting one or a few proteins per assay.
Immunohistochemistry (IHC)	A methodology for visualizing protein expression in tissue sections utilizing antibodies that specifically bind to target proteins, frequently employed in conjunction with a colorimetric or fluorescent	This technique provides spatial information regarding protein localization within tissues. It can be utilized to assess protein expression in situ. Furthermore, it is valuable for analyzing structural and cellular changes in tissue.	The method is semi-quantitative and subject to interpretation. Its efficacy is contingent upon the quality of antibodies utilized. The procedure necessitates extensive tissue processing and optimization of antibody

Table 4 (continued)

Technique/Analysis	Aim	Advantages	Disadvantages
		detection system.	concentration, along with other protocol steps.
Flow Cytometry	A methodology for analyzing and quantifying protein expression on the cellular surface or intracellularly utilizing fluorescently-labeled antibodies.	High-throughput for analyzing thousands of cells within a brief time frame. It is quantitative analysis with single-cell resolution, capable of assessing multiple proteins simultaneously utilizing different fluorophores.	It depends on the availability and compatibility of fluorophores and antibodies. There is a potential for non-specific binding, which may result in background noise.

activity frequently indicates a material's potential to initiate or stimulate cellular differentiation toward mineralized tissue formation, such as odontoblasts [93]. Colorimetric and fluorometric assays are the most commonly used methods for ALP quantification, as they are relatively straightforward and cost-effective [152].

It is essential to highlight that mineralization characterization, whether qualitative or quantitative, also depends on the number of cells in each well. Normalizing absorbance by cell count or alternative metrics (metabolic activity or DNA quantification) is an effective method to reliably assess the mineralization-inducing potential of biomaterials after treatment [4]. This approach can be implemented by culturing cells in separate plates under identical treatment conditions. At the end of the experiment, part of the wells are used for mineralization analysis, and the control wells are used for cell quantification. However, this normalization is frequently neglected, with researchers assuming uniform confluence across wells after prolonged culture periods for mineralization induction. Nonetheless, normalization is a standard practice in other characterization processes, offering important insights into the potential of biomaterials to induce mineralization.

3.2.6.3. Angiogenic tube formation assay. Human umbilical vein endothelial cells (HUVECs) are commonly used to study tube formation, an essential process in angiogenesis. The tube formation assay assesses the ability of endothelial cells to form capillary-like structures, providing critical insights into the angiogenic potential of various factors. There are multiple variations used to evaluate HUVEC tube formation under different conditions and with specific endpoints, enabling a thorough analysis of angiogenic mechanisms [153].

In the standard tube formation assay, HUVECs are seeded on a thin layer of basement membrane matrix, such as Matrigel, which supports cell adhesion, alignment, and tube formation. Over several hours, HUVECs align and connect to form network-like tubular structures, mimicking in vivo capillary formation. This process is quantified by measuring structural parameters, including the number of nodes (junctions), number of tubes, tube length (average and total), thickness (average), and closed loops or meshes [37]. These metrics effectively compare the angiogenic potential of pro- and anti-angiogenic conditions. Three three-dimensional (3D) matrix systems are used to study long-term tube formation. HUVECs are embedded within a 3D scaffold, such as collagen or fibrin gels, that simulates the natural extracellular matrix. This 3D setup allows the formation of sustained tubular structures, lumen development, and branching over extended periods. These complex networks provide a more physiologically relevant model, offering detailed insights into the structural and functional aspects of angiogenic formation and maturation [132]. Spheroid or bead-based sprouting assays model the three-dimensional nature of vascular

growth. HUVECs sprout radially from multicellular spheroids in this setup, mimicking capillary formation and branching. Together, these assays offer a comprehensive suite of tools for characterizing angiogenesis, helping advance research into angiogenic processes and therapies relating to biomaterials development [154,155].

3.3. *In vivo models and assessments*

The *in vivo* analysis of biomaterials for dental pulp regeneration is essential for evaluating biocompatibility and determining whether biological regeneration or repair has occurred. The selected experimental model for *in vivo* analysis enables researchers to observe whether repair is attributable to ectopically formed tissues or if site-specific regeneration has been achieved [156]. Histological examination is essential for determining the precise nature of newly generated tissues. Consequently, pre-clinical study models are indispensable for developing novel biomaterials and procedures for pulp regeneration, as conducting such examinations on human teeth presents significant challenges. While some clinical studies on human teeth proposing regenerative procedures have been conducted, histological analysis was only feasible on teeth subsequently extracted for unrelated reasons [157,158]. Consequently, animal study models constitute an essential step in the development of novel biomaterials for dental pulp regeneration.

Animal models for studying dental pulp regeneration can be categorized into three distinct groups: i) ectopic, wherein scaffolds and cells are transplanted into ectopic tissues of immunocompromised animals; ii) semi-orthotopic, wherein scaffolds and cells are adapted to a tooth framework to simulate clinical conditions and subsequently implanted into ectopic tissues of immunocompromised animals; and iii) orthotopic, whereby *in situ* simulation of clinical procedures is conducted in the dentition of animal [39,45,72,148,156,157].

Ectopic implantation of scaffolds into subcutaneous tissue can be used to observe the biocompatibility of novel compositions in a physiological environment, allowing the study of interactions with connective tissues and the immune system. This model is often employed in cell-transplantation strategies for dental pulp regeneration, where scaffolds are pre-seeded with cells before being implanted into the animal [159,160]. One of the strategies employed is to pre-condition the cells in the scaffold construct with osteogenic media or other types of induction media for 7–14 days to enable cell attachment and acclimatization to the scaffold and induce some degree of differentiation *in vitro* before implantation in subcutaneous tissue [145, 160–163].

The semi-orthotopic model for dental pulp regeneration leverages biomaterial, implanted cells, and dentinal walls interaction to promote the growth of pulp tissue with fibroblasts, stem cells, and vascular structures with odontoblasts that can secrete tubular dentin, a pulp-dentin complex similar to natural tissues [11,72,148]. In this model, cells are seeded into scaffold constructs, placed within the pulpal space of human tooth slices, dentin cylinders, or roots, and subsequently implanted into subcutaneous pockets of small animals, providing a more clinically relevant microenvironment created by the dentinal tissue and serving as a valuable platform for mechanistic and translational studies [45,72,148 156,157]. For tooth slices, the pulp chamber, typically measuring 1 mm in thickness, can be obtained from the crown of human molars, while dentin cylinders are prepared from the roots of extracted human molars or premolars, measuring 4–7 mm in height and 1–2.5 mm in inner diameter. In addition, full-length roots of human premolars or molars can be utilized, wherein the coronal portion is removed, resulting in a root fragment with a length comparable to that of natural teeth [10, 45,113,119,164–166]. Using roots instead of discs may pose challenges for the experimental design, execution, and histological analyses [45, 110, 119, 157]. While this model offers an advantage in terms of anatomical relevance, it also presents challenges in achieving adequate vascularization for the various regions of the regenerated tissue, as the blood supply is provided by the anastomosis between the host animal's vasculature and the vessels that develop over time within the

regenerated pulp tissue [11,110,165]. To facilitate the vascularization process, proof-of-concept studies opt for leaving both the coronal and apical ends open to allow the vascularization process from native tissues. While leaving both ends of the dentin cylinder or root open facilitates the influx of blood supply from both directions, having only the apex opened or sealing the coronal opening with a bioactive material more accurately represents clinical situations. Indeed, unilateral vessel sprouting into the tooth root presents a significant challenge [45,110, 119,157, 64,167].

Immunodeficient animals are frequently utilized to mitigate undesired immunogenic responses in cell-transplantation procedures involving human cells. A commonly used technique involves implanting tooth slices or roots into subcutaneous pockets in the relatively loose dorsal skin of rodents. This tissue allows the prepared tooth fragments to be easily accommodated with minimal animal distress. The pockets are created using blunt surgical instruments and expanded contralaterally to harbor the fragments. The accessibility and flexibility of the dorsal tissue facilitate efficient closure of the incision, rendering this method both effective and practical for researchers. Following a predetermined period, contingent upon the study design, the dentin slices are retrieved post-euthanasia for histological and other characterizations [11,45,72, 108,156,162].

The traditional histological characterization utilizes hematoxylin and eosin (H&E), which can identify morphology, alignment, polarization, the uniformity of the tissue, the presence of blood vessels, the various cell types found within the regenerated pulp, and the presence of an odontoblastic layer lining the dentin surface [11,45,72,110]. To assess new dentin deposition, the mice can be periodically injected with tetracycline, which binds to mineralizing dentin and appears as fluorescent lines under a confocal microscope. The distance between these lines can be measured to determine the deposition rate of new dentin in relation to the time between the injections [11]. Immunohistochemistry analysis provides an additional characterization method frequently utilized to verify that the cells in the generated pulp exhibit markers confirming their phenotypic transition. Specifically, DSP and DMP-1 markers are commonly employed to confirm the presence of differentiated odontoblast-like cells lining the dentin, analogous to the odontoblasts found in native tissues. Vascularization is essential for neo-tissue genesis, and in clinical contexts, the expansion of a functional vascular system from the apical entry is crucial for successful dental pulp tissue engineering. Consequently, markers such as Factor VIII and vWF serve to validate the presence of viable endothelial cells and blood vessels [10,45,119,160]. In addition, it is important to confirm the origins of the new tissue formed within the root fragment to demonstrate that this process is initiated and mediated by the human-seeded cells and not solely by invading murine cells. Immunolabeling for the human origin of the cells with markers specific for human cells or pre-marking transplanted cells with fluorescent probes, such as green fluorescent protein (GFP), has been utilized to confirm that the cells in regenerated tissues were of human origin [10,45,165, 166].

Other quantitative/qualitative analyses should be performed, such as the organization of regenerated tissue, blood vessel formation and spread throughout regenerated tissue, presence of nerve fibers, cell density, and volume of newly formed tissue. Although there are no universally adopted protocols to guide the execution of certain tissue quality quantification methods, valuable insights can be obtained by characterizing the tissue according to predetermined criteria or by enumerating odontoblast-like cells lining the dentin or blood vessels per unit area. In this context, the natural human dental pulp, whether implanted or not into the animals alongside the experimental specimens, serves as a reference for comparison [45, 110, 119, 143, 165].

In addition to cell transplantation using scaffolds, cell-homing techniques can be employed to explore the potential of regenerative strategies, such as in immature teeth, which prove particularly valuable due to their open apices and stem cell-rich apical papillae [148]. This

approach can leverage both residual pulp within the canal, induced bleeding, and the apical papillae as sources of precursor cells to promote regeneration [168]. Due to the experimental requirements, it is not uncommon that this type of study is carried out in larger animal models, such as immature minipig premolars, as a model for testing cell-free biomaterials in pulp regeneration through cell-homing approaches. In this procedure, the pulp is extracted, and the root canal is prepared, followed by induced bleeding before the insertion of the biomaterial. Periapical radiographs are used to monitor the process, and after 12 weeks, the teeth are collected following animal euthanasia. Organized pulp-like tissue has been successfully regenerated only when induced bleeding was combined with the biomaterial application [169]. These experimental models demonstrate significant potential for evaluating cell-homing strategies for use in pulp regeneration.

Studies in pulp regeneration may also utilize large animal models, which have increased-sized teeth that provide improved access, visibility, and space to perform procedures that mimic clinical scenarios and offer significant advantages for the placement and evaluation of biomaterials. In contrast, small animals such as rodents present several challenges due to their limited size and tissue accessibility, which impede their utility in research and involve preparation, irrigation, and intracanal medication. Nevertheless, they remain promising for studies on direct pulp capping. Consequently, larger animal models, including ferrets, dogs, and minipigs, may be more suitable for studies requiring *in situ* usage of biomaterials. These models have been successfully employed in pulp regeneration studies, emphasizing their significance in bridging the gap between preclinical experiments and clinical applications [43,168,170,171].

The selection of larger animal models is more complex as it necessitates more intricate requirements, including specialized housing facilities, trained personnel, and equipment for anesthesia, as well as a larger workforce, including researchers, veterinary surgeons, and supporting staff, to conduct the studies. Furthermore, the various animal models present distinct anatomical and physiological conditions that may or may not align well with humans. For instance, immature ferret canine teeth exhibit characteristics analogous to those of immature human teeth, such as open apices and narrow dentin walls. In addition, minipig teeth offer valuable insights due to their root anatomy and apical foramen closely resembling those of human teeth, as well as their capacity to simulate both single- and multi-rooted configurations. Additionally, canine incisors and premolars exhibit similarities in terms of dentin-pulp complex biology. However, owing to the presence of an apical delta and the absence of an apical foramen, it is necessary to create an artificial apical opening to more accurately mimic human teeth conditions [168,171]. Two strategies can be employed to address this anatomical difference: i. extracting the tooth, sectioning the apical portion by 1 mm, enlarging the apical foramen, filling the root canal *ex vivo*, and subsequently re-implanting the tooth into the alveolar bone; or ii. shaping the apical cemento-enamel to 0.5–0.6 mm without extracting the tooth [172,173].

Autologous and allogenic cell transplantation techniques have been explored in ferret, canine, and minipig models for pulp regeneration. For autologous transplantation, dental pulp stem cells are initially harvested, expanded, and characterized *in vitro*. These cells are then transplanted, either alone or in combination with scaffolds, depending on the experimental design. The characteristics of these cells are very similar to those of human dental pulp stem cells [163,171,173,174]. Allogenic transplantation using human dental pulp cells in canine and minipig teeth has demonstrated successful dental pulp regeneration without eliciting immunogenic reactions. This process has led to the development of pulp-like tissue throughout the entire root length, including an odontoblast-like layer and pre-dentin beneath the dentin, as well as a well-organized and vascularized pulp core [175].

Cell-homing techniques can regenerate the pulp-dentin complex by recruiting endogenous stem cells into the root canal system, thereby overcoming many challenges of the cell-based approach and enhancing

clinical applicability. This strategy leverages both residual pulp within the canal and the apical papillae as sources of precursor cells and can be particularly promising when aiming at regeneration in immature teeth due to their open apices and stem cell-rich apical papillae [148,168]. This technique involves inducing hemorrhage in the periapical tissues to promote the formation of a blood clot, with or without introducing biomaterials and molecules into the root canal and periapical tissues to induce the formation of pulp-like and mineralized tissues [169]. Despite the high clinical relevance, *in vivo* cell homing protocols are labor-intensive and require a high level of expertise to ensure the success of the research procedure and the sealing of the cavity to prevent bacterial infection.

Despite the development of various biomaterials and strategies for pulp regeneration, it is essential to recognize the valuable insights provided by animal models, which can guide biomaterial research advancements and inform clinical practices and guidelines. However, animal testing adds significant complexity and cost to research due to ethical considerations, expenses associated with acquiring and housing animals, and labor-intensive data collection and analysis procedures. These processes often rely on histological sections and basic and advanced radiographic imaging, which require meticulous preparation and processing. Therefore, it is important to prioritize and exhaust alternative methods (*in vitro* and *ex vivo*) that can provide the essential information without requiring animal experimentation. When animal research is necessary, projects should be designed following the 3Rs principles (Replacement, Reduction, Refinement) to uphold ethical standards and ensure animal welfare. Additionally, collaborating with a veterinarian to plan the studies and adhering to the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments, available at <https://arriveguidelines.org/>) is key to designing effective experiments and ensuring rigor, transparency, and reproducibility in animal research.

4. Future directions in biomaterial-host interactions for dental pulp regeneration research

The rapid and promising development of cell-based and cell-free strategies and technologies represents an exciting period for in the development of dental biomaterials with enhanced properties to address different challenges involved in the dental pulp regeneration research cycle.

The initial biological phase in the research cycle for developing biomaterials involves the characterization of biocompatibility, as materials deemed unsafe cannot be used in clinical applications. Several techniques and protocols are employed to evaluate biocompatibility characteristics, and these have been found suitable for guiding development and certification for clinical use (see [30] for a detailed discussion regarding the assessment of biocompatibility of dental materials). In these protocols, biomaterials are tested using simplified methods and single cell lines, in the absence of the complex interactions and modulated responses of diverse cell types. Consequently, this approach does not replicate the sophistication of the pulp-dentin complex and the dynamic interactions between cells, tissues, and materials that occur during pulp regeneration *in vivo*. Consequently, refined methodologies for assessing biocompatibility, incorporating additional factors pertinent to pulp biology, such as the interaction between cells and the immune system (e.g., fibroblasts, macrophages, and T cells), phenotypic cell polarization, and the spatial and temporal distribution of cell types during regeneration, will be crucial in elucidating the biocompatible, and potentially bioactive, properties of novel biomaterials [144,176]. Another critical consideration is the role of microorganisms in the degradation of biomaterials, which can significantly influence the formation of byproducts or compounds that may affect biocompatibility or the long-term success of the dental pulp regeneration approach [177,178].

Another growing area of research focuses on the spatial distribution

of cells and how this affects their exposure and response to stimuli from biomaterials. Advancements in three-dimensional culture systems and spheroids for dental pulp studies provide more physiologically relevant environments, improving the simulation of the *in vivo* conditions, enhancing cellular behaviors and responses, and providing a more realistic model for studying cell-cell and cell-matrix interactions, compared to traditional two-dimensional systems by better mimicking the natural architecture and microenvironment of tissues [167,179]. Further development in 3D cultures and devices utilizing patient-derived cells and real-time assessment of cellular responses will facilitate more relevant characterization of materials' biocompatibility and bioactive potential.

In addition to the spatial distribution of the cells, microfluidic devices emerge as a promising platform to evaluate the interactions between biomaterials, cells, blood vessels, and microorganisms through the control of fluid flow, mechanical forces, and nutrient gradients [105, 180,181]. Microfluidics devices enhance precision in cell-based studies by enabling real-time analysis of cellular responses to precise concentrations of drugs or growth factors. This control supports the development of biomaterials tailored to specific functions, such as tissue regeneration. The ability to test multiple conditions simultaneously accelerates biomaterial and drug screening, making research more efficient and cost-effective. Additionally, patient-specific models using individual-derived cells enable customized treatments and contribute to advancements in personalized medicine. By bridging the gap between traditional *in vitro* and *in vivo* studies, microfluidics offer a versatile platform that enhances predictive accuracy, reduces reliance on animal models, and advances targeted therapies. Incorporating dynamic fluid flow in biomaterials testing provides distinct advantages over the static conditions of traditional cell culture techniques, offering a closer representation of the clinical conditions, as it only partially simulates the intricate complexities of the *in vivo* environment. Nevertheless, this approach represents an emerging technology in dental pulp regeneration research with significant potential to facilitate the development of biocompatible and bioactive materials [91,105,180]. This technology will further benefit from research to evaluate biomaterials tested in platforms combining different cell lines and the incorporation of sensors to evaluate real-time changes in the microenvironment and cell differentiation,

While significant advancements in pulp regeneration have been achieved by studying biomaterial interactions with stem cells derived from dental pulp [3,4, 25,58,93,141,148], their interactions with the immune system remain largely underexplored. Research shows that the immune system plays a critical role in the initial response to implanted materials, significantly influencing the success of regeneration [142, 182–185]. Understanding a material's capacity to affect the host immune response is key for developing biomaterials that enhance tissue formation and integration with dental structures through odontoblastic differentiation, dentin production, and anastomosis of pulp blood vessels with periapical vasculature and innervation. These processes depend significantly on the immune system's reaction to implanted cells and materials, which can either facilitate or impede regenerative processes [72,140,176,178,186].

5. Conclusion

The advancement of biomaterials has significantly impacted dental pulp tissue engineering, whether through the development of bioactive materials to stimulate host cells to produce mineralized structures and scaffolds for cell transplantation. During the development stage, the biological properties of the materials must be rigorously assessed to ensure they promote the intended biological responses related to pulp and dentin regeneration. These assessments often include a range of *in vitro* tests to evaluate biocompatibility, analyze gene and protein expression to determine the ability of the biomaterial to support and promote cell differentiation and conduct functional assays to validate

the intended biological effects. *In vivo* tests are typically conducted after proof-of-principle data confirming desired outcomes *in vitro*. Each stage provides specific and complementary information and, together, generates a comprehensive framework for understanding the potentials, limitations, and performance of biomaterials and guides their transition toward clinical application. Therefore, the interpretation of results must be made within the scope of the data obtained and the scope of the stage, ensuring that conclusions are well-supported and guide future research. Looking ahead, the ongoing development of innovative biomaterials will facilitate further advancements in pulp regeneration through novel or enhanced treatment modalities for patients. These advancements hold significant potential for preserving and enhancing the viability of injured yet salvageable pulp and enabling wholistic pulp and dentin tissue regeneration to support root development, restore sensory functions, and enable tooth functionality for the patient.

Declaration of Competing Interest

This research received no funding and there is no conflict of interest.

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