Microspheres and their Potential in Endodontic Regeneration Application

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Microspheres have been widely utilised as versatile carriers in biomedical applications. In recent years, as a new type of injectable scaffold, microspheres have attracted increasing attention in the field of regenerative medicine owing to their various advantages including their small size, large specific surface area and mimicry of the 3D native environment. These characteristics enable them to adopt the narrow and irregular anatomy of the tooth and become an ideal scaffold for endodontic regeneration. Microspheres play an important role in carrying biologics (cells, biomolecules and drugs), which effectively regulate the fate of stem cells and control the release of growth factors and drugs. Cell-laden microspheres, which can be divided into microcarriers and microcapsules, have great application prospects in dental pulp regeneration. This paper summarises the properties and characteristics of microsphere scaffolds used in tissue engineering, placing emphasis on their advantages and applications in endodontic regeneration.

Key words: *dental pulp, endodontic regeneration, microcapsules, microcarriers, microspheres*
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Dental pulpitis and periapical periodontitis are common diseases in stomatology. The conventional treatment, root canal therapy, involves removing the infected dental pulp completely, cleaning the root canal and filling it with bioinert materials[1]; however, due to the loss of vitality, the repaired tooth is often prone to postoperative fracture and repeated infection[2]. Endodontic regeneration, based on modern tissue engineering, is expected to become an alternative strategy to restore the structure and function of dental pulp[1]. Among the three essential elements in tissue engineering, namely stem cells, scaffolds and growth factors, scaffolds serve as an artificial extracellular matrix (ECM), which is critical to support the proliferation and differentiation of stem cells[1]. A scaffold can also incorporate biomolecules or drugs and control the release profile, which can further regulate stem cells and their microenvironment.

In general, pulp regeneration scaffolds, like other scaffolds for tissue regeneration, should adhere to the following principles: good biocompatibility, biosafety, biodegradability, mechanical stability, favourable cell microenvironment and appropriate biochemical and biophysical clues. For endodontic regeneration, due to the unique dental anatomy, the selection and design of the scaffold must strive for pertinency and practicability to meet the specific requirements. Regarding the irregular and complex root canal system, the ideal scaffold

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needs to fill the irregular pulp cavity and support cells in differentiating within, whereas the pulp cavity only communicates with the periapical tissue through the apical aperture (~ 1 mm). Thus, the injectable scaffold is considered more appealing for endodontic regeneration and further clinical transformation[3]. At present, the commonly used injectable scaffolds for endodontic engineering are mainly divided into two categories: hydrogel and microspheres[3]. Hydrogel scaffolds for endodontic regeneration have been examined in many published reviews[4,5]. Polymer microspheres, however, are a new type of injectable scaffold and have shown great application prospects after being introduced in the field of pulp regeneration, which has not yet been reviewed. This review therefore aims to summarise the current advances in microsphere scaffolds in research into endodontic regeneration.

Overview of microspheres for tissue engineering

Microsphere technology for animal cell culture was first used in 1967 and has gradually matured and been used widely in many biomedical fields such as cell transplantation, drug delivery and biomolecule production[6]. The present review focuses mainly on cell-based microspheres for endodontic regeneration. Microspheres are generally spherical polymerised networks prepared from biomaterials using physical or chemical methods, with a diameter ranging from 1 to 1000 μm[7] and especially within 500 μm[8]. They include microcarriers, in which cells are directly integrated with microspheres, and microcapsules, in which cells are encapsulated within microspheres[9]. As a microcarrier used for cell culture, a larger specific area can provide more support for cells, which is conducive to adhesion and proliferation; however, due to the limitation of oxygen diffusion, microcapsules used for cell delivery are usually 100~400 μm in diameter. Large microspheres lead to internal cell apoptosis whereas small ones carry fewer cells and are even internalised by cells[10]. Recently, a variety of biomaterials has been developed to fabricate microspheres. The selected materials and their degradation products must be non-toxic and induce limited inflammatory or immune responses after transplantation in vivo. More importantly, the materials must be able to form microspheres quickly and easily, so only polymers with crosslinkable functional groups or partially modified by crosslinkable moieties are suitable choices. The selected materials should be made into microspheres from the precursor solution using the appropriate techniques. Generally, microcarriers are preformed, thus reducing any direct adverse effects on cells during preparation. Common preparation techniques include solvent evaporation (single or double emulsion), emulsion polymerisation, spray drying and phase inversion microencapsulation[11-14]; however, microcapsules are prepared directly from a suspension formed by mixing cross-linkable polymer and cells to microspheres, with cells encapsulated inside the microspheres. Usually, the preparation method needs to be mild, such as electrostatic droplets, microfluidic technique and 3D printing[15-17], to reduce damage to the cells during the preparation process.

Microspheres offer several advantages for stem cells in tissue regeneration: their spherical structure provides cells with a 3D microenvironment simulating natural extracellular matrix, which is more conducive to maintaining their function and phenotype than two-dimensional monolayer culture[18]; their small size and large specific surface area allow rapid diffusion of oxygen, nutrients and metabolites[7,9]; they support cell attachment, proliferation and differentiation in vitro, which further enhance cell viability and tissue regeneration after cell transplantation to the target defect site[3,11]; and microsphere scaffolds could protect the delivered stem cells, reduce shear stress injury during injection and prevent the immune system from being attacked[19]. They have performed excellently in a wide range of tissue engineering and regeneration medicine applications, such as bone and cartilage, vascular and cardiac[9,20]. It is imperative to comprehensively consider the characteristics of the repaired tissue, the inherent bioactivity of the material, the appropriateness of the fabrication technique and the types of delivery cells to overcome the difficulties of regenerating specific tissue[21].

Application of microspheres in endodontic regeneration

Dental pulp is a kind of connective tissue that is rich in blood vessels and nerves, surrounded by hard dentine and connected with periapical tissue through a narrow apical foramen[22]. The lack of effective collateral circulation means that once the dental pulp becomes infected, the necrotic pulp cannot be reversed and must be removed completely. Recently, strategies for pulp regeneration have focused mainly on cell sheets and cell-laden hydrogels, whereas microsphere scaffolds are relatively few. Cell sheets can form an abundant extracellular matrix without the support of scaffolds but are difficult to transport into the narrow root canal system directly[23,24]. Hydrogel is also widely used as a scaffold for endodontic regeneration owing to its injectability and simulation of the extracellular matrix; however,

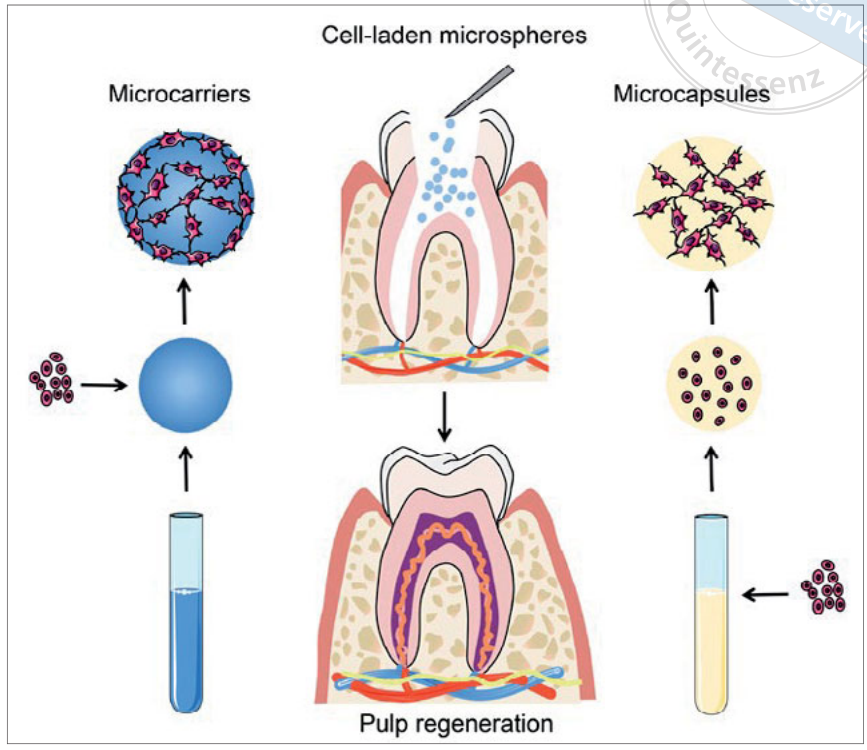

Fig 1 Schematic illustration of microspheres used for pulp regeneration. Microspheres can be classified as microcarriers or microcapsules according to their different cell-laden delivery applications. Microcarriers are usually preformed into large amounts of tiny particles, and then cells are loaded on their surfaces. In contrast, microcapsules are prepared by mixing crosslinkable polymers with cells to encapsulate cells within them.

crosslinked hydrogel bulk restricts the rapid diffusion of oxygen and nutrients. Microsphere scaffolds therefore offer unique advantages in application for endodontic regeneration[25,26]. First, their small size allows them to be injected into narrow and irregular root canals[9]; second, their small volume supports the rapid diffusion of oxygen and nutrients, which is conducive to the survival of transplanted stem cells in the early ischemic environment of the root canal system[7,9]; and third, they allow cells to adhere and proliferate in vitro for a period of time to form microtissues, which can enhance their vitality and function in this bionic 3D microenvironment[3,19,27]. A schematic illustration of cell-laden microspheres applied in endodontic regeneration is presented in Fig 1.

Most microspheres used in endodontic regeneration are loaded with cells for in vitro cell culture and in vivo cell delivery[9], and others have been encapsulated with various effective components for related research application, such as pulp capping, apexification and root canal disinfection.

Application for dentine and pulp tissue engineering

Microspheres can be classified as microcarriers and microcapsules according to their different cell-laden delivery applications[9]. Microcarriers are usually preformed into large amounts of tiny particles, then cells are loaded on their surfaces, whereas microcapsules are prepared by mixing crosslinkable polymers with cells to encapsulate cells within them.

Microcarriers

Conventional polymer microcarriers are solid spheres with a smooth surface where cells can attach and grow. Microcarriers offer cells a microsized 3D platform that is conducive to cell connection and substance exchange compared with the conventional two-dimensional culture. Combined with a bioreactor mimicking biological environments in vitro, microcarriers can accelerate cell proliferation and differentiation[28]. Bhuptani and Patravale[28] used a double emulsion solvent extraction technique to prepare interconnected porous poly(lactic-co-glycolic acid) (PLGA) microspheres with a particle size of 100 to 200 μm and a pore diameter of 10 to 30 μm. They found that porous microspheres provided a favourable microenvironment for human dental pulp stem cells (hDPSCs) to support cell adhesion, proliferation and survival[28]. Zou et al[29] also applied a double emulsion solvent extraction technique to fabricate PLGA microspheres with diameters of around 150 to 400 μm, which supported hDPSCs proliferation and odontogenic differentiation. Due to their hydrophobicity and the lack of cell adhesion moieties of synthetic material, PLGA microspheres were coated with type I collagen to improve their surface bioactivity; this sur-

face modification enhanced cell adhesion, proliferation and odontogenic differentiation[29]. Garzón et al[30] utilised self-assembled technology and the solvent evaporation technique to prepare poly(L-lactic acid)-block-poly(L-lysine) (PLLA) into two kinds of microspheres with different surfaces, fibrous and smooth. Both offered good biocompatibility, biosafety and odontogenic potential and could form bioactive injectable aggregates that promoted dentine regeneration in both in vitro and in vivo models; however, fibrous microspheres presented advantages in supporting cell adhesion and spreading, expressing collagen type I and promoting microsphere degradation, which may be related to the biomimetic structure of collagen fibres[30]. Kuang et al[12] used self-assembly and thermally induced phase separation techniques to prepare PLLA nanofibrous spongy microspheres with a diameter of approximately 30 to 60 μm, biomimetic nanofibrous architecture (160 nm) and an interconnected porous structure (10 to 20 μm) where hDPSCs could adhere to the outer surface and inner pores. In comparison to nanofibrous microspheres and smooth microspheres, nanofibrous spongy microspheres further promoted cell adhesion, proliferation and differentiation, and their degradation was also faster, showing great potential for dentine–pulp complex regeneration[12]. Then, they further induced cell differentiation into blood vessels by 3D hypoxic-primed culture of the cell-laden microspheres and found that expression of vascular endothelial growth factor (VEGF) of hDPSCs could be promoted and pulp-like tissue with rich vasculature could be regenerated in situ after injection into the pulp cavity of rat teeth[12]. Wang et al[31] used thermally-induced phase separation and emulsification methods to fabricate PLLA nanofibrous microspheres carrying human stem cells from the apical papilla (SCAP), and PLGA microspheres with controlled release of bone morphogenetic protein-2 (BMP-2). The addition of BMP-2 enhanced the odontoblast differentiation of human SCAP whether it was cultured in a monolayer or seeded on nanofibrous microspheres in a spinner flask[31]. This demonstrated that sustained release of BMP-2 from PLGA microspheres enhanced the odontogenic differentiation of SCAP to regenerate dental tissue[31]. Li et al[27] designed a hierarchical nanofibrous microsphere in which heparin, binding with vascular endothelial growth factor (VEGF), was conjugated onto a gelatine nanosphere, then fixed in injectable PLLA nanofibrous microspheres. The microspheres controlled the release of growth factors for over 4 weeks to promote the migration of human umbilical vein endothelial cells and simulate the structure of the extracellular matrix to support the proliferation of DPSCs[27]. The potential of these microspheres in dental pulp regeneration has been verified after being applied to full-length root canals with one end sealed in vivo[27]. Manaspon et al[14] used a combination of the water-in-oil emulsion technique and EDC/NHS crosslinking to fabricate fibrinogen and thrombin-crosslinked fibrinogen microspheres, with a mean diameter of 213.9 ± 35.9 μm and 199.9 ± 41.9 μm, respectively. These microspheres did not alter the cell viability of the hDPSCs, but thrombin-crosslinked fibrinogen microspheres had an advantage over the fibrinogen microspheres in promoting cell attachment and spreading[14]; however, their biological effects in promoting odontoblastic/osteogenic differentiation and their potential for pulp regeneration in vivo need to be investigated further.

Most of the abovementioned microcarriers are made from synthetic materials, except fibrinogen microspheres that are natural materials. Natural materials offer good biocompatibility and degradability[4], but their applications are restricted by limited sources, batch variability and potential pathogen transmission[32]. In contrast, synthetic materials possess good plasticity and reduce the risk of carrying pathogens with natural materials. However, most synthetic materials are restricted by limited biological cues and difficulty of degradation, and PLLA and PLGA are no exception. Although they offer good biocompatibility and biodegradability, they still need to improve cell adhesion with surface modification due to the lack of biological cues. In addition, their degradation problem is more obvious due to the closed root canal and insufficient blood supply[30]. It is therefore necessary to adjust the degradation through appropriate material composition and structure design. For example, nanofibrous spongy microspheres degrade faster than nanofibrous microspheres despite having been prepared from PLLA. Synthetic materials often have adjustable physical and chemical properties, but their degradation products need to be evaluated for cytotoxicity.

Microcapsules

In contrast with prefabricated microcarriers where cells are seeded after preparation, microcapsules can wrap cells together during preparation, providing the latter with a 3D environment that resembles natural tissues[20]. Hydrogel is a kind of crosslinked hydrophilic polymer network that has mechanical properties similar to many soft tissues. Due to its rheological property, degradability and simulation of the natural extracellular matrix, it is widely used as a substrate for cell culture, a scaffold for tissue engineering and a carrier for the delivery of biologics[25]. Although injectable hydrogel can be injected

into the defect location in a minimally invasive manner, cell interactions are limited in the process of hydrogel formation. Moreover, microspheres with larger specific surface areas provide more cell anchoring sites than bulk hydrogels. Thus, instead of the conventional form of hydrogel crosslinking into clusters, hydrogel microspheres combine the advantages of hydrogel matrix and microspheres[7] and are easy to transport to small-sized defect locations.

Our team took the lead in introducing hydrogel microspheres into endodontic regeneration. We prepared arginine-glycine-aspartic acid (RGD)-alginate-based hydrogel microcapsules using the electrostatic microdroplet method. These microspheres encapsulate and deliver hDPSCs, load and slowly release VEGF and form more blood vessels and new tissues in in vivo experiments[26]. This multifunctional hydrogel microsphere loaded with cells and biomolecules is an instructive scaffold strategy in vascularised endodontic regeneration. Unfortunately, the alginate material did not support cell spreading even after RGD modification, and some of the material remained undegraded after transplantation in vivo for 1 month. We therefore prepared hydrogel microspheres using photocrosslinked gelatine methacryloyl (GelMA), which offers excellent bioactivity and biodegradability, instead of alginate to improve its performance in cell adhesion and degradation. GelMA microspheres support hDPSC spreading, proliferation and ECM protein secretion and finally form a microtissue. In addition, hDPSC-laden GelMA microspheres can withstand cryopreservation, which supports their future clinical transformation. hDPSC-laden GelMA microspheres showed better degradability and new pulp–dental tissue generation than GelMA hydrogel bulk in vivo, possessing great potential for endodontic regeneration and transformation applications[33].

Hydrogel microcapsules prepared using the electrostatic microdroplet method have normal particle size distribution; other fabrication methods, such as microfluidic and 3D bioprinting technology, could control the particle size more accurately and form monodispersed phase microspheres. Taking the complex structure and function of dental pulp into account, cell-laden microcapsules need to be improved further to achieve functional pulp tissue regeneration. For instance, microspheres carrying oxygen-releasing components could improve the oxygen supply to maintain the survival of cells in the early ischemic environment[34], and the delivery of bioactive factors could endow microspheres with the function of angiogenesis and nerve promotion[26,27]. A summary of microspheres applied in endodontic regeneration can be found in Supplemental Table 1 (provided on request).

Other related applications for endodontic regeneration

Microspheres laden with protein, inorganics and antibiotics are effective methods to deliver these biomolecules to the target location. They can not only prevent the biomolecules from being eliminated directly by the immune response, but also prolong the retention of biomolecules and avoid the toxic impacts and side effects of burst release on the surrounding cells.

Incorporate biomolecules in pulp capping

A variety of microspheres loaded with biomolecules have been used for pulp capping, including natural materials such as fibrinogen and hydroxyapatite, and synthetic materials such as poly(ethylene)glycol and PLGA. These microcarriers are used to bind biomolecules that facilitate odontoblastic/osteogenic differentiation, such as calcium hydroxide (CH), dexamethasone, prostaglandin E and immobilised Jagged1. Due to their small size and sustained release of biomolecules, these microspheres promote odontoblastic/osteogenic differentiation of hDPSCs[14,35-37]. In addition to promoting dentine bridge formation, microspheres for pulp capping can also carry other effective bioactive factors that promote blood vessel formation, control inflammation and perform other functions[38-41].

Release biomolecules for apexification

Sustained release of biomolecules could be achieved through electrostatic adsorption, physical restriction, material degradation and other methods to better promote tissue regeneration. CH is also widely used as an intracanal medicament for apexification. Cerda-Cristerna et al[42] prepared CH-PLGA microspheres using oil-in-water and oil-in-oil/oil-in-water emulsion solvent evaporation techniques with a diameter of approximately 18.63 ± 7.23 μm and 15.25 ± 7.37 μm. Both can release Ca^{2+} in a sustained manner for up to 30 days; this is better than CH paste which releases it completely in 6 days, thus continuously inducing mineral tissue formation and apical closure[42]. Strom et al[43] prepared CH microspheres surrounded with an alginate shell using an emulsion method. These core–shell structure microspheres, ranging from 75 to 150 μm, can release Ca^{2+} and OH^- in a sustained manner in the root canal for up to 6 months, which should reduce the number of visits required for apexification[43].

Deliver drugs for sterilisation

Using microspheres to carry antibiotics or anti-inflammatory drugs can support the sustained release of drugs in root canals, which is conducive to creating a microenvironment required for pulp regeneration in the infected root canals. Dornelles et al[44] and Cuppini et al[45] introduced amoxicillin-loaded microspheres into endodontic sealer, which did not affect the physicochemical properties and biocompatibility and possessed antibacterial activity against endodontic bacteria. Appropriate use of antibiotics in endodontic regeneration requires not only disinfection of root canals, but also reduction of adverse effects on stem cells[2]. Parhizkar et al[46] developed PLGA-coated ceramic microparticles as a drug delivery system for endodontic application in which new triple antibiotics were loaded in the PLGA coating. These drug-loaded microspheres demonstrate bioactivity and biocompatibility and release antibiotics for up to 3 weeks against root canal microorganisms[46].

Summary and prospects

Endodontic regeneration involves not only regeneration of structure, but also restoration of function. Dental pulp is a kind of special connective tissue composed of various cells with special functions such as dentinogenesis, nutrition supply and sensory function. The cell-laden microspheres used in pulp tissue engineering can form pulp-like tissue to a certain extent, with blood vessels and dentine formation, and even achieve full-length regeneration in the root canal; however, their functional restoration remains to be verified further. On the one hand, the new tissue needs to integrate with the host tissue, particularly the early reconstruction of the vascular network to maintain sufficient oxygen and nutrition. This is key to maintaining the long-term survival of stem cells after transplantation, especially in the ischemic microenvironment of the root canal. On the other hand, the sensory function of the new tissue can be restored and the vital pulp can play a defensive and protective role; this needs to be verified through preclinical animal experiments and clinical trials. The microspheres are endowed with the microenvironment induced by angiogenesis and neurogenesis, which is conducive to the realisation of multifunctional microspheres to restore the complex tissue structure and function of dental pulp.

Although the application of microspheres for endodontic regeneration has achieved preliminary results, there are still some limitations based on current research. For example, the degradation of the material should match the rate of tissue regeneration; otherwise, the material residues will occupy part of the space and impede complete regeneration of the pulp. This problem can be avoided by choosing materials with better biodegradability or by changing the concentration, composition and structure of microspheres. In addition, due to the limited volume of the root canal system, the cell density within the microsphere must be considered to provide sufficient stem cells for endodontic regeneration. Microspheres can be cultured in vitro for a period of time before implantation to achieve cell proliferation, and they are not only for cell delivery, but also serve as bioinduced scaffolds for microtissue formation; however, the relationship between culture time and microtissue formation remains to be explored further. Moreover, in the process of promoting clinical transformation, large-scale, repeatable, low-cost and efficient manufacturing technology and safe and effective storage technology still need to be explored.

For endodontic regeneration, the microsphere scaffold should support cell adhesion and proliferation in vitro, protect cells from stress during injection and provide a good microenvironment for cell differentiation in vivo[47]. Through the design of the microsphere scaffold, the biocompatibility and material degradation rate can be adjusted to maximise the function of stem cells and achieve endodontic regeneration. In addition to carrying cells, microspheres could encapsulate other biomolecules such as growth factors and drugs to further extend the functions of microspheres, introducing selective cues that support the desired cellular responses and improving the local microenvironment. In conclusion, microspheres are versatile and practical carriers that display great potential in endodontic regeneration for clinical applications.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Drs Ting YANG and Li XIE discussed and determined the content and structure of the manuscript; Dr Ting YANG drafted the manuscript; Drs Ting YANG and Rui Tao ZHANG were in charge of the figure, Drs Li XIE and Rui Tao ZHANG revised the manuscript; Dr Wei Dong TIAN was responsible for approval before submitting this article.

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