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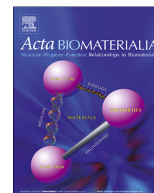

Full length article

Promoting bioengineered tooth innervation using nanostructured and hybrid scaffolds

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ABSTRACT

The innervation of teeth mediated by axons originating from the trigeminal ganglia is essential for their function and protection. Immunosuppressive therapy using Cyclosporine A (CsA) was found to accelerate the innervation of transplanted tissues and particularly that of bioengineered teeth. To avoid the CsA side effects, we report in this study the preparation of CsA loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles, their embedding on polycaprolactone (PCL)-based scaffolds and their possible use as templates for the innervation of bioengineered teeth. This PCL scaffold, approved by the FDA and capable of mimicking the extracellular matrix, was obtained by electrospinning and decorated with CsA-loaded PLGA nanoparticles to allow a local sustained action of this immunosuppressive drug. Dental re-associations were co-implanted with a trigeminal ganglion on functionalized scaffolds containing PLGA and PLGA/cyclosporine in adult ICR mice during 2 weeks. Histological analyses showed that the designed scaffolds did not alter the teeth development after in vivo implantation. The study of the innervation of the dental re-associations by indirect immunofluorescence and transmission electron microscopy (TEM), showed that 88.4% of the regenerated teeth were innervated when using the CsA-loaded PLGA scaffold. The development of active implants thus allows their potential use in the context of dental engineering.

Statement of Significance

Tooth innervation is essential for their function and protection and this can be promoted in vivo using polymeric scaffolds functionalized with immunosuppressive drug-loaded nanoparticles. Immunosuppressive therapy using biodegradable nanoparticles loaded with Cyclosporine A was found to accelerate the innervation of bioengineered teeth after two weeks of implantation.
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1. Introduction

Dental caries, periodontal diseases and oropharyngeal cancers are the most prevalent oral diseases. Dental caries and tooth loss are important oral health indicators for adults and are key measures for monitoring progress of the disease [1]. The National Health and Nutrition Examination Survey, 2011–2012, revealed that in adults aged 20–64, 91% had dental caries and 27% had untreated tooth decay. Only 48% of adults aged 20–64 had a full set of permanent teeth (excluding third molars) and nearly 19% of adults aged 65 and over were edentulous [1]. Current dentures and implants used to replace missing teeth do not remodel and show a reduced integration with the host. Therefore, there is a need for new biomaterials to promote regeneration.

Regenerative nanomedicine is a rapidly expanding domain which has as objective the development of compatible biomaterials accepted by the body able to interact with cells/tissues present in the site of implantation. Such biomaterials can be combined with nanoparticles allowing a controlled or sustained release of active molecules. On the other hand, tissue engineering aims at replacing or repairing damaged tissues.

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Keller et al. [2] showed that cultured re-associations between dissociated mesenchymal cells and intact epithelium from Embryonic Day (ED) 14 mouse molars gave well formed teeth after implantation under the skin of adult ICR mice. The vascularization of the dental pulp occurred while the innervation was never observed [2]. Siemionow et al. [3] showed that, immunosuppressive therapy with tacrolimus, a calcineurin inhibitor, accelerated nerve regeneration in the case of face transplantation. Cyclosporine A (CsA), another calcineurin inhibitor, widely used in organ transplantation [4] has also direct effect on nerve growth [5,6]. When cultured re-associations were co-implanted with trigeminal ganglia in CsA-treated ICR mice, the innervation of the dental mesenchyme occurred after one week of implantation. After two weeks, the axons coming from the trigeminal ganglia reached the odontoblasts. These results demonstrated that the innervation of the dental pulp can be obtained in immunosuppressive conditions [7]. However, the oral availability of CsA is slow and highly variable owing to its biopharmaceutical properties. The use of this molecule is controversial because it can induce different forms of kidney dysfunction, cancers and lymphomas [8,9]. Different approaches have been investigated to reduce its nephrotoxicity by developing CsA-loaded PLGA nanoparticles as delivery vehicles because of their excellent biocompatibility and sustained release [10].

On the other hand, the development of compatible biomaterials is also an essential step in the regeneration of a functional tooth as we previously showed by using a FDA approved nanofibrous polycaprolactone (PCL) scaffold functionalized with nerve growth factor (NGF) for a local release of this neurotrophic factor [11]. Indeed, when the scaffold was functionalized with nanoparticles containing NGF the innervation occurred in the dental pulp indicating the capability of the NGF nanoreservoirs to direct axon formation from the trigeminal ganglion into the bioengineered tooth [11]. So, following this strategy it should be possible to fabricate a combination cell-therapy implant capable of regeneration of a vascularized and innervated tooth as tooth replacement during regenerative therapy.

The aim of this study was to combine a local sustained effect of CsA with the use of a PCL-based scaffold, using reduced doses of the immunosuppressant molecule and so avoiding the unwanted side effects attributed to the ingestion or burst release of this drug. For this purpose, we functionalized PCL scaffolds with CsA-loaded nanoparticles and studied the innervation in the bioengineered teeth pulp by immunofluorescence and transmission electron microscopy (TEM).

2. Materials and methods

2.1. Materials

Poly (D, L-lactic acid/glycolic acid) 50/50 polymer (PLGA; MW 24–38 KDa), under the commercial name Resomer® RG 503 was purchased from Evonik Industries AG (Darmstadt, Germany). Polycaprolactone (PCL; MW 80 KDa) analytical grade, Cyclosporine A, Dexamethasone (used as HPLC internal standard), Pluronic® F-68 surfactant, ethyl acetate (Class 3 solvent according to the pharmacopeia), acetonitrile and methanol (HPLC grade), were all purchased from Sigma Aldrich (St. Louis (MO), USA) and used as received. All references to water imply the use of MilliQ water previously filtered through a 0.2 μm cellulose nitrate membrane.

2.2. Synthesis of Cyclosporine A (CsA) loaded PLGA nanoparticles

Cyclosporine loaded PLGA (PLGA/CsA) nanoparticles were prepared in a continuous microfluidic reactor using a PEEK-made interdigital micromixer (SIMM-V2, Slit Interdigital Micro Mixer, IMM, Mainz, Germany) by carrying out an oil-in-water (O/W) emulsification process followed by a solvent evaporation procedure. Briefly, 1% (w/v) of PLGA (50:50) polymer, 0.1% (w/v) of CsA and 2% (w/v) of Pluronic F68 (used as surfactant) were dissolved in 30 mL of ethyl acetate (used as organic solvent). This resulting organic phase was then mixed and emulsified with MilliQ water, interfaced using syringe pumps (Harvard Apparatus), with flow rates of 16 and 32 mL min^{-1}, respectively. Both solutions were fed through a 1/16″ PTFE tubing and then interfaced inside the PEEK-based interdigital micromixer. The micromixer was placed in an ice bath to control the reaction temperature. After the formation of a stable emulsion, the organic solvent was evaporated under continuous stirring (600 rpm) in an open flask during 3 h (Fig. 1).

2.3. Characterization techniques

Scanning electron microscopy (SEM, Inspect F50, FEI, Eindhoven, The Netherlands) was employed to determine the shape of the synthesized PLGA NPs. The freshly prepared nanoparticles were mixed during 1.5 h with the same volume of phosphotungstic acid solution (7.5% w/v) used as contrast agent. The dispersion was then centrifuged and washed three times with Milli Q water and later re-suspended. A drop of the resulting nanoparticle suspension was placed on a glass slide, air dried and coated with platinum under vacuum before SEM observation. Nanoparticle size, size distribution and zeta potential (pH 7.2) were determined by dynamic light scattering (Zeta Plus, Brookhaven Instruments Corporation, NY) after appropriate dilution with Milli Q water. At least five replicate measurements were recorded in each case. CsA content in PLGA nanoparticles was determined directly by dissolving the drug loaded nanoparticles in a solvent composed of acetonitrile and dexamethasone as HPLC internal standard. Then, methanol was added and the mixture was placed in a sonifier bath for 15 min to promote PLGA precipitation. The resulting dispersion was centrifuged at 12000 rpm for 20 min to remove the polymeric residue and the supernatant was filter using 0.22 μm PTFE syringe filters and placed in a vial for HPLC analysis. Experiments were performed in triplicate. CsA content in the samples was then determined by HPLC (Waters Instrument 2690 Alliance, USA). A Kinetex C18 column with a 2.6 μm particle size filler and column dimensions of 50 mm × 4.6 mm was used. The mobile phase consisted in a 80:20 (v/v) mixture of acetonitrile:water including a phosphoric acid concentration of 200 ppm. The detector wavelength, injection volume, flow rate, and column temperature were 210 nm, 5 μL, 0.5 mL min^{-1} and 70 °C, respectively. The HPLC method was validated with respect to linearity, repeatability and the limit of quantification and limit of detection. Drug encapsulation efficiency (EE) and drug loading (DL) were calculated using the following equations:

$$EE\ (\%) = \frac{\text{Amount of drug loaded}}{\text{Total amount of drug used}} x100 \quad (1)$$

$$DL\ (\%) = \frac{\text{Amount of drug loaded}}{\text{Amount of PLGA}} x100 \quad (2)$$

In vitro drug release studies were carried out using a mini dialysis cassette composed of a 20,000 molecular weight cut-off cellulose dialysis membrane (Slide-A-Lyzer, Fischer). The CsA-PLGA nanoparticles in suspension (2 ml with a concentration of 9 mg/ml) were loaded into the dialysis cassette which was immersed in a conical tube with 44 mL of PBS containing 1% (w/v) of tween 80. The tubes were placed in an incubator thermostatted at 37 °C under oscillation at 100 rpm. At predetermined intervals, samples of the CsA-PLGA were collected and washed by centrifugation. Finally, the entrapment efficiency protocol described above was

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