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Nanostructured hybrid materials for bone-tooth unit regeneration

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ABSTRACT

As a part of regenerative medicine, biomaterials are largely used in this field of nanotechnology and tissue engineering research. We have recently developed a new scaffold using electrospun nanofibers of Poly (ε-caprolactone), PCL which is able to mimic the collagen extracellular matrix of cells. The aim of this study was to engineer a biological and implantable structure leading the regeneration of the tooth-bone unit. For this aim, we have cultured mouse osteoblasts embedded in a collagen gel on the nanofibrous membrane and coupled this structure with an embryonic dental germ before implantation. To follow bone and tooth regeneration, we have performed RT-PCR, histology and immunofluorescence analysis. We showed here that this leaving implantable structure represents an accurate strategy for bone-tooth unit regeneration. We report here the first demonstration of bone-tooth unit regeneration by using a strategy based on a synthetic nanostructured membrane. This electrospun membrane is manufactured by using an FDA approved polymer, PCL and functionalized with osteoblasts before incorporation of the tooth germs at ED14 (the first lower molars) to generate bone-tooth unit *in vivo* after implantation in mice. Our technology represents an excellent platform on which other sophisticated products could be based.

Keywords: Bone; Nanostructured Material; Osteoblast; Tissue Engineering; Tooth

1. INTRODUCTION

Biomaterials play central roles in modern strategies in regenerative medicine and tissue engineering as designable biophysical and biochemical environment that direct cellular behavior and function. The fibrillar collagens are the most abundant natural polymers in the body and are found throughout the interstitial spaces with an essential function to impart structural integrity and strength to tissues. In the native tissues, the structural extracellular matrix (ECM) proteins range in diameter from 50 to 500 nm. In order to create scaffolds or ECM analogues, which are truly biomimicking at this scale, one must employ nanotechnology. Recent advances in nanotechnology have led to a variety of approaches for the development of engineered ECM analogues. To date, three processing techniques (self-assembly, phase separation, and electrospinning) have evolved to allow the fabrication of nanofibrous scaffolds. With these advances, the long-awaited and much anticipated construction of a truly "biomimicking" or "ideal" tissue engineered environment, or scaffold, for a variety of tissues is now highly feasible.

The intricate fibrillar architecture of natural ECM components has inspired several researchers to produce materials with similar structure. Upon fibers that are tens of microns in diameter, cells seem to respond as though to a 2-D substrate, acquiring an unnatural flat shape, leading to a nonphysiological, asymmetrical occupation of adhesion receptors; notwithstanding, such matrices have already shown remarkable success in tissue engineering applications, such as in the reconstruction of a dog urinary bladder [1] or as scaffolds for neural stem cells to facilitate regeneration after brain injury in a mouse stroke model [2]. Polymer processing technologies such as electrospinning [3] allow fiber formation

down to the 10 nm scale. One difficulty in nanofiber technology is placing cells within a nanofibrillar structure with pore spaces much smaller than a cellular diameter; somehow the network must be formed *in situ*, around the cells, without cellular damage.

Tooth organ engineering is an area of regenerative medicine. One methodology of this field of research is based on biomimetic. It tries to replace tooth organ by mimic epithelial-mesenchymal interaction occurring during tooth organ development [4]. Today, to build an intact and biological tooth-bone unit and regenerate a functional anchoring system (root, peridental, ligament, alveolar bone) remains a major challenge in tooth organ engineering. Indeed, a correct anchoring system is needed for the complete tooth functionality, for avoiding tooth ankylosis and to prevent bone damage after tooth loss. It is expected that bioengineering technology will be developed for the reconstruction of fully functional organs *in vitro* through the precise arrangement of several different cell species.

Our strategy is based on an active and cellularized hydrogel and nanofibers as a matrix. In this study, we have used electrospun nanofibers of Poly (ε-caprolactone) (PCL). PCL is degraded by hydrolysis of its ester linkages under physiological conditions (such as in the human body) and has therefore received a great deal of attention for use as an implantable biomaterial. In particular it is especially interesting for the preparation of long-term implantable devices, owing to its degradation, which is even slower than that of polylactide. PCL is a Food and Drug Administration (FDA) approved material that is used in the human body as (for example) a drug delivery device, suture (sold under the brand name Monocryl or generically), or adhesion barrier. We report here the first demonstration that we are able to regenerate bone-tooth unit by using a FDA approved electrospun membrane coated by mixed osteoblasts/collagen.

2. MATERIALS AND METHODS

2.1. Chemicals and Electrospinning

PCL, analytical grade, was purchased from Sigma Aldrich. PCL was dissolved in a mixture of dichloromethane/dimethylformamide (DCM/DMF 50/50 v/v) at 15% wt/v and was stirred overnight before use. Rat-tail type I collagen was purchased from Institut de Biotechnologies Jacques Boy. A homemade standard electrospinning setup was used to fabricate the PCL scaffolds. The PCL solution was poured into a 5 ml syringe and ejected through a needle with a diameter of 0.5 mm at a flow rate of 1.2 ml/h, thanks to a programmable pump (Harvard Apparatus). A high-voltage power supply (SPELLMAN, SL30P10) was used to set 15 kV at the needle. Aluminum foils (20 × 20 cm^2), connected to the ground at a distance from the needle of 17 cm, were used to collect the electrospun PCL scaffold.

2.2. SEM Observation

For morphological study, the PCL scaffolds were gold-coated (Edwards Sputter Coater) and observed with a Philips XL-30 ESEM scanning electron microscope in conventional mode (high vacuum) with a Thornley-Everhart secondary electron detector.

2.3. Cells Culture

Mice primary osteoblasts were obtain from parietal bone, cut in small pieces, washed in PBS and treated for 40 min at 37˚C with PBS containing collagenase (50 µg/ml) and fungizone (5 µg/ml). After washing in PBS, bone pieces were cultured in Dulbecco's modified Eagle's medium (D-MEM®) containing 50 U/ml penicillin, 50 µg/ml streptomycin, 5 µg/ml fungizone, 1% sodium pyruvate, 0.1% ascorbic acid and 10% FBS (Life Technologies, Paisley, UK). The cultures were incubated at 37˚C in a humidified atmosphere of 5% CO_2. After 4 days the medium was changed. The generated osteoblasts were cultured in 75 cm^2 flasks and medium was changed every 3 days.

2.4. Immunocytochemistry and Osteoblasts Mineralization Staining

Cells were cultured for 14 days on electrospun PCL membrane, fixed with paraformaldehyde (PFA) 4% during 1 h, permeabilized with 0.1% PBS-Triton X-100 for 1 h, saturated with BSA 0.1% and incubated for 20 min with Alexa Fluor 546-conjugated phalloidin (Molecular Probes) for F-actin labeling and 5 min with 200 nM DAPI (Sigma) for nuclear staining. After saturation, cells were also incubated with the primary antibodies: Osteocalcin, Osteonectin, Osteopontin and Collagen I. After rinsing with PBS, cells were incubated with secondary antibodies; anti-goat alexa fluor 488 for Osteocalcin and Osteonectin and anti-rabbit alexa fluor 488 for Collagen I and Osteopontin. For hematoxylin staining, cells were fixed with PFA and then stained with hematoxylin 10% (w/v) for 15 min, rinsed with water and observed. For red alizarin staining, cells were fixed with ethanol 70% and stained with red alizarin 1% (w/v), dried and observed.

2.5. RNA Isolation and RT-PCR Analysis

RT-PCR was performed on osteoblasts cultured for 7 and 14 days. Total RNA was isolated by affinity chromatography using the RNeasy® Minikit (Qiagen Inc., Hilden, Germany) and reverse-transcribed with oligo (dT) 12 and Superscript III (Invitrogen), according to the manufacturer protocols. In each experiment 1 µg of total

RNA was used for RT-PCR amplification was carried out with the Go Taq Hot Start kit (Promega, France) according to the manufacturer's instructions, using the specific primers (**Table 1**). PCR conditions were as followed: initial denaturation of 3 min at 94˚C followed by 35 cycles at 94˚C for 1 min, annealing for 1 min at 58˚C and elongation for 1 min at 72˚C, with a final extension of 10 min at 72˚C. PCR products were separated by electrophoresis on 1.5% agarose gels.

2.6. Molar ED14 Culture

The first lower molars were dissected from ICR mouse (Charles River Laboratories), embryos at Embryonic Day (ED) 14. All procedures were in compliance with the recommendations of the European Economic Community (86/609/CEE) on use and care of laboratory animals. Molars have been cultured for 5 days on the electrospun PCL membrane and on a semi-solid medium as previously described [5,6].

2.7. Implant Preparation and *in Vivo* Implantation

For the Collagen preparation, 3 ml of Rat Tail Type I Collagen (Institut de Biotechnologies Jacques Boy) were mixed with 5.5 ml of medium containing 10% FBS, 0.5 ml of a 0.1 M NaOH and 1 ml of osteoblasts suspension at 2×10^5 cells/ml. 0.1 ml of osteoblasts suspension/ collagen preparation were deposited on the top of the electrospun PCL membrane. After adding the ED14 tooth germs, the construct was incubated at 37˚C for 30 min before implantation. For *in vitro* analysis, the osteoblasts were cultured for 7 and 14 days on electrospun PCL membrane. For *in vivo* analysis, the samples were implanted between skin and muscles behind the ears in mice (8 weeks old ICR) for 2 weeks. For histology, samples were fixed in Bouin-Hollande, embedded in paraffin and 5 µm serial sections were stained with Mallory. Implanted samples were demineralized in 15% EDTA before embedding in paraffin. For immunofluorescence, implants were embedded in Tissue-Tek and frozen at −20˚C. Sections (10 µm) were stained with anti-CD31 (BD Pharmingen) for the detection of vascular endothelial cells, and with Osteopontin for the detection of bone. After washing with PBS, sections were incubated with secondary anti-rabbit antibodies conjugated to Alexa 594 and anti-rat antibodies conjugated to Alexa 488 (Molecular Probes, Invitrogen).

3. RESULTS AND DISCUSSION

3.1. *In Vitro* Bone Induction Analysis

For bone induction analysis, we have currently analyzed the biocompatibility of our electrospun PCL membrane after incubation of primary mice osteoblasts by SEM analysis (**Figure 1**). Moreover, our results indicated clearly that after 4 hours, the adhesion of cells growing on the surface of the membrane become comparable to the positive control including plastic support (data not shown).

We have also studied the behavior of mice osteoblasts growing on nanofibrous PCL membrane by histology (**Figures 2(A)-(C)**) and immunofluorescence (**Figures 2(D)-(H)**). After 14 days *in vitro*, these cells expressed some bone specific proteins: collagen I, Osteonectin, Osteocalcin and Osteopontin (**Figures 2(E)-(H)**). Furthermore we showed a nice mineralization by red alzarin in these cells after 14 days *in vitro* (**Figure 2 (C)**) and normal actin distribution (**Figure 2 (D)**). Gene expression of these molecules was also analyzed by RT-PCR (**Figure 3**) in 7 and 14 days cultured osteoblasts on the electrospun PCL membrane. Our results demonstrated clearly that the nanofibrous PCL membrane could be considered as a suitable scaffold for bone tissue engineering.

3.2. *In Vivo* Analysis of the Engineering Tooth-Bone Unit

Recently, there has been an increasing interest and awareness of the importance of the sub-tooth bone for its role in the pathogenic processes. It's necessary to carefully consider this structure in the treatment of tooth damage, in the evaluation of the results over time and in the determination of the patient prognosis. In fact, the conditions of teeth and its supporting bone are tightly coupled and should be viewed as a connected bone-tooth unit.

For the *in vivo* tooth-bone unit regeneration, we ana-

Table 1. Forward and reverse primers used for RT-PCR.

Primers	Forward	Reverse
GAPDH	CCATGGAGAAGGCCGGGG	CAAAGTTGTCATGGATGACC
Collagen1	AATGGTGAGACGTGGAAACCCGAG	CGACTCCTACATCTTCTGAGTTTGG
Osteopontin	GACCATGAGATTGACAGTGATTTG	TGATGTTCCAGGCTGGCTTTG
Osteocalcin	GACAAAGCCTTCATGTCCAAGC	AAAGCCGAGCTGCCAGAGTTTG
Osteonectin	TGAGAATGAGAAGCGCCTGGA	AAGGGGGTAATGGGAGGGGTG

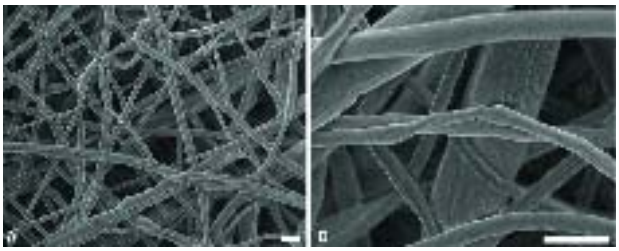

Figure 1. SEM observation of the nanofibrous PCL scaffolds (A, B). Bar = 1 µm.

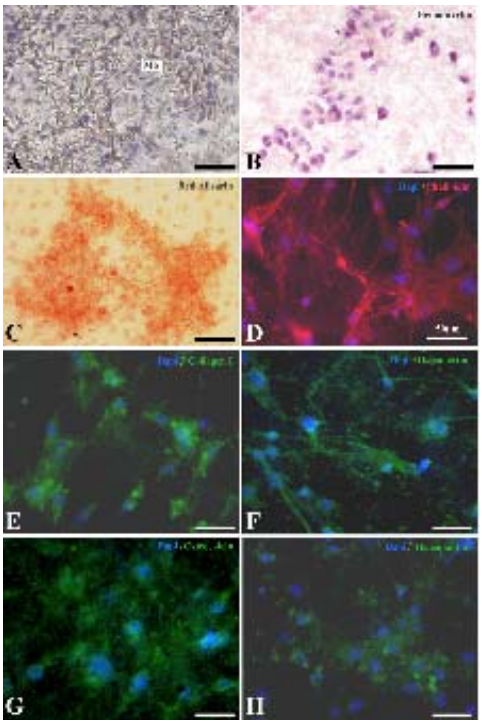

Figure 2. Behaviour of mice osteoblasts growing on PCL nanofibrous membrane during 14 days. Hematoxylin staining for histology ((A), (B)), red alizarin staining for mineralization (C), alexa fluor 546-conjugated phalloidin for F-actin labeling (D) and immunofluorescence ((E)-(H)). Collagen I (E), Osteonectin (F), Osteocalcin (G), Osteopontin (H). Bar = 50 µm.

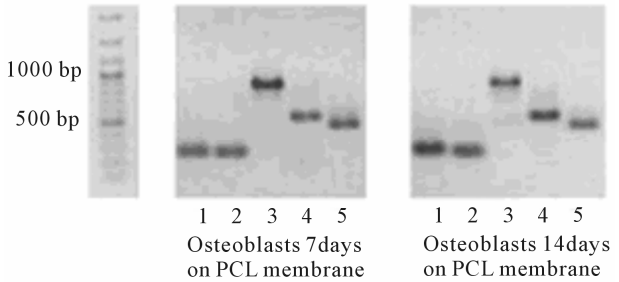

Figure 3. RT-PCR performed on mice osteoblasts cultured for 7 and 14 days on PCL membrane. The genes specifically observed were: Collagen I (1), Osteopontin (2), Osteocalcin (3), Osteonectin (4) and GADPH (5) for a control.

lyzed the subcutaneous implantation of the ED14 first lower molars cultured during 5 days on the nanofibrous PCL membrane (**Figure 4 (A)**). We observed a correct development of the crown, including functional odontoblasts and ameloblasts secreting dentin and enamel respectively. Furthermore, the gradients of odontoblast differentiation were maintained in the root portion.

We then test the possibility to regenerate bone-tooth unit by using a hybrid nanostructured and living material. After *in vivo* implantation of the tooth germs at ED14, cultured 5 days on the membrane without adding osteoblasts (**Figure 4 (A)**), no bone induction was detected. Interestingly, by adding mixed osteoblasts/collagen as a coating of the membrane (nanostructured living membrane) and after incubation of the tooth germs at ED14 and implantation, we have shown bone induction (**Figure 4 (B)**). In these conditions, the developed tooth exhibited correct epithelial histogenesis (**Figure 4 (B)**) and allowed the functional differentiation of odontoblasts (**Figure 4 (D)**) and ameloblasts (**Figures 4 (E)-(H)**). Induced bone was observed around the tooth (**Figures 4 (B)**, **(F)-(H)**) and especially near the root (**Figures 4 (G)** and **(H)**). Cementoblasts are in contact with the root dentin and begin to secrete cement (**Figure 4 (H)**). At this stage of development, orientation of the future fibers of the peridental ligament can be yet observed (**Figure 4 (H)**). For more characterization of the induced bone-tooth unit growing on this nanofibers membrane, the blood vessels in the dental pulp and in the peripheral tissue were stained with an anti-CD31 antibody and the bone was visualized with anti-Osteopontin antibody (**Figure 4 (I)**). We then controlled that collagen had no effect on teeth development after implantation under skin (**Figure 4 (C)**). We attempt to implant ED14 molar with mixed murine osteoblasts/collagen without PCL membrane, with no results. Based on these results, we have reported here the first demonstration of a unique nanostructured material for bone-tooth unit regeneration *in vivo*. Thus, PCL nanofibrous membrane coated by mixed osteoblasts/collagen could represent a promising strategy of nanostructured living membrane for *in vivo* bone-tooth unit regeneration.

In the last years, more intentions are carrying on functionalization of different scaffold [3,7-18]. Multi layers film is one of the multiple technologies used to incorporate bioactives molecules [8-10]. Leading to a nanoarchitecture, these new generation of delivery molecules scaffold can provide the control of cell differentiation, inflammation [11,12] by the diffusion of growth factors, nucleic acids [13,15] in a controlled period of time and in a restricted localization. For example, the layer-by-layer technology can embedded active molecules into the multilayered films. Recently, we have designed an *in vitro* culture system based on an active film functionalized by

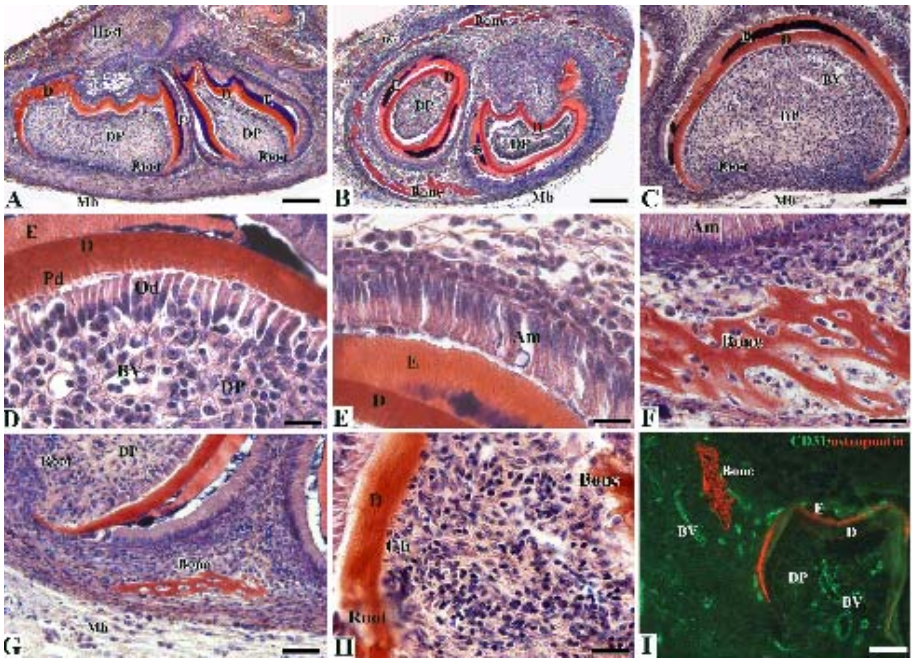

Figure 4. Bone-tooth unit regeneration *in vivo*. *In vivo* implantation for 2 weeks of ED14 first lower molars cultured 5 days on electrospun PCL membrane (A), on mixed murine osteoblasts/collagen coated electrospun PCL membrane ((B), (D)-(I)) or on PCL membrane in the presence of collagen (C). The explants were stained with Mallory ((A)-(H)). The blood vessels in the dental pulp and in the peridental tissue were stained with the anti-CD31 antibody (green) and the regenerated bone with the anti-Osteopontin antibody (red) (I). Am: ameloblasts, BV: blood vessel, Cb: cementoblasts, D: dentin, DP: dental pulp, E: enamel, Mb: membrane, Od: odontoblasts, Pd: predentin. Bars = 100 µm ((A), (B)), 50 µm ((C), (I)), 25 µm (G) and 12.5 µm ((D)-(F), (H)).

BMP-4 and/or Noggin. Our results demonstrated clearly the possibility to control *in situ* apoptosis during tooth development mediated by both BMP-4 and Noggin incorporated into our active films [19]. We have also shown that BMP-2 functionalized PCL membrane was able to increase bone tissue regeneration after implantation [20]. To increase the regeneration of this complex bone-tooth unit, it could be relevant to combine these two strategies (membrane functionalization and tooth regeneration). Particular attention has to be done to understand the interaction between the new bone and the tooth root. To regenerate of real functional bone-tooth unit, the periodontal ligament formation must be also achieved.

4. CONCLUSION

With this strategy it should be possible to fabricate a combination cell-therapy implant capable of robust and durable tooth regeneration in large bone defects, when adding cells from patients become needed, to generate bone-tooth unit. We believe that our results make a significant contribution to the area of regenerative medicine and more precisely to bone and tooth related biomaterials. The concepts discovered here are applicable to a broad class of tissues and may serve to design sophisticated implants.

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