Mineralization and bone regeneration using a bioactive elastin-like recombinamer membrane


1. Introduction

Bone grafts are extensively used in bone healing therapies that require significant osteoconductive and osteoinductive enhancement. Despite a number of well-known negative consequences, autogenous cancellous bone continues to be the preferred bone graft option and a major target to replace [1]. Scaffolds based on collagen [2], hyaluronic acid [3], chitosan [4], biological composites [5], and self-assembling materials [6] have been and continue to be investigated as three-dimensional bone graft alternatives.

In an attempt to further enhance bone regeneration therapies, and taking advantage of the benefits of the periosteum, the use of periosteal grafts has emerged as an attractive strategy [7]. An ideal periosteal graft would not only provide a physical structure that facilitates osteoconduction, but also osteoinductive signals that stimulate osteogenesis and ultimately promote biomineralization [8]. Membranes made of amniotic tissue [9], chitosan-silica [10] or silk fibroin nanofibers [11] have been reported to induce osteoblastic differentiation in vitro. Also, a variety of in vitro mineralizing membranes based on chitosan/bioactive glass nanoparticles [12], platelet-rich fibrin functionalized with alkaline phosphatase (ALK) [13], polycaprolactone fibers incorporating nano-apatite particles [14] or forsterite nanopowder [15], and collagen [16] have been developed. In addition, the capacity of membranes to enhance bone regeneration in vivo has been reported. Examples include membranes based on a modified polyactide/polyglycolide acid polymer [17], collagen membranes combined with a porous titanium membrane [18], or chitosan bioelectric membranes capable of accelerating bone fracture healing by electric stimulation [19].
However, the success of periosteal grafts has been restricted by limitations associated with the lack of biocompatibility, absence of bioactivity, poor mechanical properties, or early degradation [20]. A possible alternative to overcome these limitations may be found in the use of molecularly designed materials made from peptides and proteins. This approach offers a much higher level of tuneability, spatiotemporal control, bioactivity, andstimulation of bone formation. Towards this goal, phosphorylated serine (SP(SiP) [6], MLPHHG/A heptapeptide [21], the bone sialoprotein sequence E7PRGDT [22], or the statherin-derived protein sequence DDDEEKFLRRIGRFG [23] have been used and reported to promote mineralization in vitro. Elastin-like recombinomers (ELRs), genetically engineered protein-based polymers inspired by the extracellular matrix protein elastin [24], are especially attractive due to their molecular versatility, biomimetic character, biocompatibility, good mechanical properties, and biodegradability [24]. These molecules are mainly composed of the repeating pentapeptide domain VPGXG (where X could be any amino acid apart from proline) and can be designed to contain additional bioactive sites such as RGDS or REDY [25].

In an effort to bioengineer a bioactive membrane for bone regeneration that takes advantage of the potential benefits of these biomolecular sequences, we have recently reported on a couple of strategies based on peptide and protein-based materials [26,27]. In particular, we have described the fabrication and characterization of thin robust ELR membranes comprising the bioactive epitope DDDEEKFLRRIGRFG, and their capacity to promote osteogenesis in vitro [25]. The present work provides further evidence of the potential use of these membranes for bone regeneration applications. First, we describe the in vitro mineralization and osteogenic properties of the bioactive ELR membranes tested under biomechanical conditions in both static and dynamic culture settings. Then, we report on the bone regeneration capacity of these membranes using an orthotopic critical-size rat calvarial defect model.

2. Materials and methods

2.1. ELR molecules

Four ELRs molecule were supplied by Technical Proteins NBT S.L. (Valladolid, Spain). The materials consisted of repeating pentapeptide domains of VPGIG and VPGKG to provide structural integrity including the amino acid lysine (K) to serve as a cross-linking point (K), that incorporates the peptide RGDS for mesenchymal stem cell adhesion (RGDS), the peptide DDDEEKFLRRIGRFG for nucleation of mineralization (HA) and an ELR that combined the later and the RGDS sequence (HAP-RGDS) (Table 1).

2.2. Membrane fabrication

Membranes were fabricated according to a recently reported method [25,27] (Fig. 1a–c). The ELR molecules were dissolved in anhydrous dimethylformamide (DMF) (SigmaAldrich, Taufkirchen, Germany) at room temperature, and then mixed with hexamethyldisiloxane (HD) (SigmaAldrich, Taufkirchen, Germany) (Table 1). Four ELR membranes were fabricated from the different ELR molecules, containing either one of the bioactive sequences (RGDS, HAP, and HAP-RGDS) or without any bioactivity (IK).

2.3. Fabrication and characterization of topographically patterned ELR membranes

In order to fabricate topographically patterned ELR membranes, a patterned PDMS mold was fabricated by soft lithography technique as previously reported [27]. The features consisted of channels that were 7 μm high, 10 μm wide, and separated by 10 μm wide ridges (Channels) (Fig. 1e). Membrane fabrication and pattern reproducibility were analyzed by qualitative observations using scanning electron microscopy (SEM) and profilometry.

2.4. Mechanical stimulation

A custom-made stretching device [28] was used in this study (Fig. 1d). The system applied uniform, uniaxial strain to elastic silicone dishes that had 18 cm² surface growth areas, in which the ELR membranes were immobilized. A cyclic stretching magnitude of 4% at 1 Hz frequency was applied as previously described in the Section 2.4, while the samples were incubated in 3 ml of simulated body fluid (SBF) which was replaced daily for 7 days. ELR membranes incubated in milli-Q water in static conditions were used as control. SBF was prepared following a previously reported protocol [29]. The SBF solution was prepared using 50 mM Tris(HCl)2HCl, 138.6 mM NaCl, 3 mM KCl, 1.5 mM MgCl26H2O, 2.5 mM CaCl2H2O, 1 mM KH2PO4, 0.15 mM NaHCO3, and 0.5 mM Na2SO410H2O and the second stock solution with the reagents Tris(HCl)2HCl, NaCl, KCl, KH2PO4, NaHCO3, Na2SO410H2O and the second stock solution with the reagents Tris(HCl)2HCl, NaCl, MgCl26H2O, CaCl2H2O. After adjusting the pH of the stock solutions to 7.4, they were stored at 4°C. Prior to the mineralization experiments, the stock solutions were mixed and filtered using a surfactant-free cellulose acetate filter unit (pore size 0.2 m) to eliminate impurities and used in the mineralization experiments. In order to assess the mineralization potential of the ELR membranes, in vitro mineralization in SBF was investigated using different techniques. Each sample was assayed in triplicate and the experiment was repeated twice.

2.5.1. Calcium content

ELR membranes were rinsed twice with milli-Q water after 7 days of incubation in SBF solution. Then, 100 μl of 0.5 M acetic acid was added in each membrane. The samples were maintained under agitation overnight at room temperature. The supernatant was recollected and calcium content was determined using an orthocresolphthalein complexone method [30]. The experiment was performed applying a mechanical stimulation described in the Section 2.4, while the samples were incubated in 3 ml of simulated body fluid (SBF) which was replaced daily for 7 days. ELR membranes incubated in milli-Q water in static conditions were used as control. SBF was prepared following a previously reported protocol [29]. The SBF solution was prepared using 50 mM Tris(HCl)2HCl, 138.6 mM NaCl, 3 mM KCl, 1.5 mM MgCl26H2O, 2.5 mM CaCl2H2O, 1 mM KH2PO4, 0.15 mM NaHCO3, and 0.5 mM Na2SO410H2O and the second stock solution with the reagents Tris(HCl)2HCl, NaCl, KCl, KH2PO4, NaHCO3, Na2SO410H2O and the second stock solution with the reagents Tris(HCl)2HCl, NaCl, MgCl26H2O, CaCl2H2O. After adjusting the pH of the stock solutions to 7.4, they were stored at 4°C. Prior to the mineralization experiments, the stock solutions were mixed and diluted with milli-Q water to obtain the SBF concentration of the reagents. The resulting SBF solution was filtered using a surfactant-free cellulose acetate filter unit (pore size 0.2 m) to eliminate impurities and used in the mineralization experiments. In order to assess the mineralization potential of the ELR membranes, in vitro mineralization in SBF was investigated using different techniques. Each sample was assayed in triplicate and the experiment was repeated twice.

2.5.2. Mineral characterization

The mineral on Smooth HAP membranes incubated in SBF for 7 days in static condition was characterized by time-of-flight secondary ion mass spectroscopy (TOF-SIMS) and scanning electron microscopy (SEM-EDXS). Smooth HAP membranes incubated in milli-Q water were used as control.

Membranes analyzed by TOF-SIMS were rinsed twice with milli-Q water, frozen at −80°C overnight and freeze-dried for 24 h. The TOP-SIMS (TOP-SIMS IV, Ion-TOP, Germany) operated at a pressure of 5 × 10⁻⁸ mbar. Samples were bombarded with a

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**Table 1**

<table>
<thead>
<tr>
<th>ELR Material</th>
<th>ELR sequence (bioactive sequence)</th>
<th>Bioactivity</th>
<th>ELR Con.%</th>
<th>ELR: Cross-linker ratio</th>
</tr>
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<tr>
<td>IK</td>
<td>(VPGIG) (VPGIG) (VPGIG) (VPGIG)</td>
<td>Control</td>
<td>4</td>
<td>1:0.75</td>
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<tr>
<td>RGDS</td>
<td>(VPGIG) (VPGIG) (VPGIG) (VPGIG)</td>
<td>Cell Adhesion</td>
<td>3.5</td>
<td>1:0.50</td>
</tr>
<tr>
<td>HAP</td>
<td>(VPGIG) (VPGIG) (VPGIG) DDDEEKFLRRIGRFG (VPGIG) (VPGIG)</td>
<td>Mineralization</td>
<td>5</td>
<td>1:3</td>
</tr>
<tr>
<td>HAP-RGDS</td>
<td>(VPGIG) (VPGIG) (VPGIG) (VPGIG) (VPGIG) (VPGIG) (VPGIG)</td>
<td>Mineralization and cell adhesion</td>
<td>5</td>
<td>1:3</td>
</tr>
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pulsed Bismuth liquid metal ion source (Bi\(^{116}\)), at energy of 25 keV. The gun was operated with a 20 ns pulse width, 0.3 pA pulsed ion current for a dosage lower than 5 \(\times 10^{13}\) ions/cm\(^2\) (below the threshold level of 1 \(\times 10^{14}\) ions/cm\(^2\) generally accepted for static SIMS conditions). Charge neutralization was achieved with a low energy (20 eV) electron flood gun. The secondary ions were detected with a reflector time-of-flight analyzer, a multichannel plate (MCPs), and a time-to-digital converter (TDC). Measurements were performed with a typical acquisition time of 20 s at a TDC time resolution of 200 ps and 128 \(\times\) 128 pixel image size. Secondary ion spectra were acquired from areas of 400 \(\times\) 400 \(\mu\)m\(^2\) within the sample’s surface. Mass spectral acquisition and image analysis were performed within the IonSpec software (version 4.1, ION-TOF, Germany) and Ion image software (version 3.1, ION-TOF, Germany), respectively. Each spectrum was normalized to the total intensity [31].

Membranes analyzed by SEM-EDS were frozen at \(-80^\circ\)C overnight after washing twice with milli-Q water. After 24 h membranes were freeze-dried, and finally they were coated with a 10 nm layer of goldplatinum. The mineral morphology and chemical composition were imaged and analyzed by SEM (JSM-7100F, JEOL, Germany) fitted with an energy dispersive X-ray microanalyzer (Oxford Inca Xper, Oxford instruments, UK) at 20 kV.

2.5.3. Membrane Young’s modulus

The stiffness of ELR membranes was measured by the Young’s modulus. A tensile test was performed under wet conditions in milli-Q water at 37 °C. ELR Membranes were fabricated in rectangular shape (8 \(\times\) 15 mm\(^2\)). The thickness of the membranes was measured with a micrometer having a precision of 0.001 mm. Mechanical tests were performed with a BOS®® ElectroForce® BioDynamic®TM biosensor (BOS®, Friedrichsdorf, Germany) with an orthopedic chamber, equipped with a 22 N load-cell at a speed of 1 mm/min, and forces were recorded using Win Test® software (BOS®, Friedrichsdorf, Germany). The Young’s modulus was calculated as the slope of the straight line portion of the stress-strain curve. Membranes incubated in milli-Q water were used as control.

2.6. In vitro cell differentiation

Rat MSCs obtained following a procedure described previously [38], were cultured on membranes made of 1K and HAP ELR molecules comprising or not channels topographies. Cells were incubated overnight prior the application of the mechanical stimulation (Section 2.4) for 2 days. Membranes were sterilized for 20 min under ultraviolet (UV) inside the cell culture hood, and then immobilized over the silicone dishes of the stretching device. The silicone dishes were prepared by combining Elastosil component A with Elastosil component B (Elastosil RT 601; Wacker-Chemie, Germany) at a 10:1 ratio, respectively. The elastomer was mixed, poured into the dish mould and, allowed to sit at room temperature overnight. The silicone dishes were then removed, cleaned with a 1% Liqui-nox liquid detergent (Alconox Inc., White Plains, NY) in milli-Q water solution, rinsed in milli-Q water, air-dried, and autoclaved at 121 °C for 15 min.

Cells were cultured in osteogenic differentiation medium (OMp) in tissue culture plastic (TCP) as positive control, or without dexamethasone (OM-) on ELR membranes and tissue culture plastic (TCP-) as negative control. As an additional control, ELR membranes were cultured in OM- in static conditions. OMp was prepared with 15 ml Eagle minimum essential medium (MEM, Gibco-Invitrogen, Scotland) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Taufkirchen, Germany), 50 mM ascorbic acid (Sigma, St. Louis, MO), 10 mM sodium-\(\beta\)glycerophosphate (Sigma, St. Louis, MO), 10 mM gentamycin (Sigma, St. Louis, MO), and 10\(^{-8}\) M dexamethasone (Sigma, St. Louis, MO). Rat MSCs of passage 3 were used. After 2 h of culture, all the medium was replaced with DMEM containing 10% FBS for subsequent culture. Each sample was assayed in triplicate and the experiment was repeated twice.

2.6.1. Osteoblastic differentiation and mineralization assays

In order to analysis in vitro cell differentiation DNA content, alkaline phosphatase (ALK) activity and calcium content assays were performed on day 5 and 10. The culture media was removed and cells were rinsed in phosphate-buffered saline (PBS). Then, 500 ml of filter milli-Q water was added to each sample. The cell suspension was transferred to an eppendorf, sonicated for 20 min, and the supernatant was collected and frozen at -20°C overnight. The cell suspension was thawed and frozen two times more. This cell suspension was used in the following assays.

100 ml of the cell suspension was used to analyze the DNA concentration using the picogreen assay (Molecular Probes, The Netherlands) according to the...
manufacturer's instructions. The DNA content (μg/ml) of the samples was read in a microplate reader (BioRad 450, Bio-Rad Laboratories, Hercules, CA) at 480 nm and determined from a DNA standard curve. Second, in order to analyze ALK activity 100 μl of the cell suspension was added to 100 μl of working reagent consisting of 0.5 μM 28-amino-2-methylpropanol (Sigma, St. Louis, MO), 5 μM p-nitrophenol phosphate (Sigma, St. Louis, MO), and 5 μM magnesium chloride (12.13 M) (Sigma, St. Louis, MO). The reaction was stopped using 100 μl of sodium hydroxide (Sigma, St. Louis, MO), and the final absorbance was read at 405 nm using a microplate reader (BioRad 450, Bio-Rad Laboratories, Hercules, CA). A standard curve was generated by making serial dilutions of 4-nitrophophate, and sample measurements were extrapolated from known concentration values of the curve. ALK activity (nmol) was normalized to DNA concentration (μg/ml). Finally, information about the mineralized matrix formation on the ELR membranes was obtained by the calcium content assay using 20 μl of the cell suspension. The calcium content (μg/ml) was determined using the protocol described in Section 2.5.1, and normalized to DNA concentration (μg/ml). 2.6.2. Scanning electron microscopy characterization Cells cultured on ELR membranes were rinsed twice with filtered PBS, fixed with 2% glutaraldehyde (Sigma, St. Louis, MO) in cacodylate (Sigma, St. Louis, MO) buffer for 5 min, and rinsed twice with 0.1 M sodium cacodylate buffer for 5 min. Then, the samples were dehydrated in a series of 70%, 80%, 90%, 96%, and 100% ethanol solution. Finally, two drops of tetramethylsilane (Sigma, St. Louis, MO) were added to each sample followed by air-dried for 5–10 min. The samples were coated with a 10 nm layer of gold. 2.7. In vitro characterization The bioactivity of Smooth HAP ELR membrane scaffolds was tested in a 5 mm critical-size rat calvarial defect model [22]. The rats were treated with HAP membrane to evaluate the effect of the bioactive sequence, IK membranes to evaluate the effect of non-bioactive sequence and without treatment to evaluate the healing in the empty defect. Membranes were aseptically in UV for 20 min and hydrated in physiological saline, previously to fill the calvarial defect. A total of 21 male Sprague-Dawley rats (12-week-old, 425±47 g) were obtained from Univeris Lab Animal (France) (7 rats per group). The animals were housed singly and received food and water ad libitum. All animals’ research protocols were approved by the Ethical Committee of Animal Experimentation (CERA) of the University of Barcelona (Spain) and the Ministry of Agriculture, Livestock, Fisheries, Food, and the Environment of the Government Institution of Catalonia (Spain). 2.7.1. Surgical procedure The surgery was performed in the Laboratory Animal Applied Research Platform at ParCientific Barcelona (PCB, Spain). The animals were anaesthetized using isoflurane (SigmaAlrich, Spain) inhalation anesthesia (5% induction; 3% maintenance). All animals prior to the surgical intervention received an intraperitoneal administration was repeated 12 h after. The skin that covered the calvarial bone surface. A 5 mm-diameter trephine bur (Fine Science tools, Heidelberg, Germany) mounted on a dental hand piece (Foredom, Bethel, New York, USA) was used to drill a round, segmental defect in the right parietal bone. During the drilling, the area was continuously irrigated with sterile saline solution, and subsequently the calvarial disk was carefully removed and extracted using tweezers. The defect was rinsed with physiological saline, and the hydrated ELR membrane (7.5 mm) was implanted covering the defect. The extreme of the membrane was fixed to the bone applying a drop of cyanoacrylate. The skin was closed over in one layer. The presence of CaP was also confirmed using the protocol described in Section 2.5.1, and normalized to DNA concentration (μg/ml). 2.7.2. High-resolution micro-computed tomography analysis Immediately after rats were sacrificed, a high-resolution micro-computed tomography (microCT) analysis was performed using the SkyScan 1172 computed microtomographic system (Kontich, Belgium). The parameters of the scan were voltage source 81 kV, current source 124 mA, image pixel size 9 μm, an aluminum filter of 0.5 mm, a tomographic rotation of 180° and a sample rotation step of 0.8°. The reconstruction was carried out with NRecon software using a specific post-alignment per each sample and applying a medium intensity ring artefact correction. Microtomographic 3D analysis was performed with CTAn (v.1.10.19) software, using a global threshold of 50±255. A cylindrical volume of interest (VOI) was used to quantify the bone volume and bone mineral density corresponding with the size of the defect. 2.7.3. Histological analysis The calvarias were extracted from the skull and fixed in 4% paraformaldehyde (Panreac, Spain) at pH 7.4 for 2 days followed by bone decalcification with Sur-gipath Decalcifier II (Leica biosystems, Spain) for 4 h. Next, the calvarias were embedding in paraffin and 3 μm sections were made using a microtome. The histological sections were stained with H&E and Trichrome Goldner and observed with a microscope Zeiss AxioScope A (Carl Zeiss, Madrid, Spain). 3. Results 3.1. ELR membrane fabrication Membranes were fabricated as previously reported [25,27] from either ELR materials comprising a bioactive sequence (RGDS, HAP or HAP-RGDS) or a non-bioactive ELR material (IK) used as control (Table 1). The quality and reproducibility of the membranes exhibiting topographical channels were verified by profilometry and SEM observations. These results confirmed topographical patterns with well-defined features that closely resembled those of the PDMS molds. 3.2. In vitro membrane mineralization ELR membrane mineralization in vitro was assessed using a variety of techniques. First, the calcium (Ca) content assay revealed the highest amount of deposited Ca on Smooth HAP membranes tested in static condition (14.49 ± 4.19 mg/ml) compared to all other membranes in either static or dynamic conditions (Fig. 1e). Second, TOF-SIMS analysis confirmed the presence of calcium phosphate (CaP) mineral on these membranes. As expected, positive TOF-SIMS peaks demonstrated the presence of CH25 (blue) on all ELR membranes, which corresponds to the common amino acid sequence of all ELRs tested. However, only ELR membranes exhibited peaks for Ca2P (green) and CaOH5 (red) (Fig. 2c), which corroborate the results of the Ca content assay. Similarly, negative TOF-SIMS peaks also revealed signals for CN and CNO on all ELR membranes, which again correspond to the common amino acid sequence of all tested ELRs, while signals for PO4 and PO4 were only observed on Smooth HAP membranes (Fig. 2d). The presence of CaP was also confirmed by chemical images that indicated strong PO4 and PO4 signals only on Smooth HAP membranes (Fig. 2e). Furthermore, the total ion image revealed a homogenous distribution of the deposition of mineral within the ELR membrane surface. Finally, SEM examination (Fig. 2e) and EDS analysis (Fig. 2f) confirmed the presence of mineral on Smooth HAP membranes with a Ca/P ratio of 1.78. 3.3. Young's modulus Tensile tests were conducted to investigate the effect of mineralization on the mechanical properties of the different membranes. Smooth HAP membranes were incubated in SBF for 7 days in static condition displayed the highest Young's modulus (E) (2081 ± 315 kPa) compared to all other tested membranes (Fig. 2k), which exhibited moduli ranging between 362 ± 74 and 600 ± 249 kPa (Supplementary data Table 1).
Fig. 2. Presence of mineral was observed by SEM examination on (b) Smooth HAP membrane incubated in SBF in static conditions compared with (a) the control in mili-Q water. (c) TOF-SIMS revealed in both cases peaks of CH$_4$N$^+$ and CN$^-$ corresponding with amino acid from ELR molecules. While peaks of Ca$^{2+}$ (green), CaOH$^+$ (red), PO$_3^-$, and PO$_4^-$ correspond with the mineral observed on Smooth HAP membranes incubated in SBF compared with the control in mili-Q water. (d) EDS analysis confirmed that the mineral exhibited on Smooth HAP membranes displayed a Ca/P proportion of 1.78. (e) Additionally, the highest value of Young modulus was observed on these membranes incubated in SBF. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. (a) The highest ALK/DNA expression and (b) Ca deposition was observed on rMSCs growing on Smooth HAP membranes in non-osteogenic differentiation medium, compared to cells on any other membrane or control substrate growing in osteogenic differentiation medium. (c) SEM observations confirmed the presence of this mineral on cells cultured on Smooth HAP membranes on day 10. ALK activity (d) and Ca deposition (e) between cell growth on membranes tested in static or dynamic culture conditions were not significantly different.
3.4. In vitro cell differentiation

Given the observed mineralizing nature of the Smooth HAP membranes, osteoblastic differentiation of rMSCs was investigated in both static and dynamic conditions by measuring ALK and Ca deposition. On day 5 and 10, the highest ALK/DNA expression was observed on cells growing on Smooth HAP membranes in non-osteogenic differentiation medium (0.23 ± 0.13), which was statistically similar to that expressed by cells growing on tissue culture plastic in osteogenic differentiation medium (TCP) (0.28 ± 0.02) (Fig. 3a). Furthermore, cells on these membranes growing in non-osteogenic differentiation medium also exhibited the highest Ca deposition on both day 5 and 10 compared to cells on any other membrane or control substrate growing in osteogenic differentiation medium (Fig. 3b). SEM observations confirmed the presence of mineralized extracellular matrix formation on cells cultured on Smooth HAP membranes on day 10 (Fig. 3c). Finally, there were no significant differences in ALK activity and Ca deposition between cells growing on membranes tested in static or dynamic culture conditions (Fig. 3d,e).

3.5. In vivo characterization

Given the observed in vitro mineralizing nature and enhancement of osteoblastic differentiation of Smooth HAP membranes, experiments were conducted to analyze their bone regeneration capacity in vivo using an orthotopic critical-size rat calvarial defect model (Fig. 4a,b).

3.5.1. Localization of ELR membranes within the defect site

In order to confirm that the ELR membranes were stable and positioned within the defect site, three animals were implanted with ELR membranes and sacrificed on day 7. In all three animals, the membranes were observed to be positioned within the defect site in the same location as they were placed during implantation (Fig. 4c).

3.5.2. Quantification of bone formation by microCT

The microCT analysis demonstrated that the animals implanted with the HAP membranes presented the highest mean volume of ossified tissue within the defect (12.6 ± 2.4 mm³) (Fig. 4d, f) compared to animals receiving the non-bioactive IK membranes (9.2 ± 2.6 mm³) (Fig. 4g) and those left untreated (9.0 ± 1.4 mm³) (Fig. 4h). While some regeneration was observed on all tested groups along the rim area of the defect, only animals implanted with the HAP membrane exhibited ossified tissue towards the center of the defect tending to breach the critical-size gap. These results were confirmed by histological analysis (Section 3.4.3). MicroCT data was also used to quantify bone mineral density within the defect. In this case, there was greater variation between...

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Fig. 4. (a) An orthotopic critical-size rat calvarial defect model was used to analyze the bone regeneration capacity of (b) Smooth HAP membranes. (c) Membranes were observed to be positioned within the defect site in the same location as they were placed during implantation on day 7. The microCT analysis demonstrated that animals implanted with the (d) HAP membranes presented the highest mean volume of ossified tissue (f) within the defect compared to animals receiving the (g) non-bioactive IK membranes and (h) those left untreated. (e) MicroCT analysis of bone mineral density within the defect revealed no significant differences between the tested groups.
samples and no significant differences were observed between the three tested groups (Fig. 4e).

3.5.3. Quantification of bone formation by histological analysis

Histological sections stained with H&E indicated greater ossification on animals treated with the HAP membranes both around the rim and in the middle of the defect (Fig. 5a). Within these regions, Trichrome Goldner staining (Fig. 5b) demonstrated the presence of both immature (osteoid) and mature bone as well as a layer of active osteoblasts (Fig. 5c) embedded within an osteoid matrix located on the growth front along the developing bone around the rim of the defect (Fig. 5d). In contrast, animals that were implanted with the IK membranes and animals that were not treated with any membrane only exhibited bone formation around the edge of the defect. In addition, in accordance with the MicroCT results, semi-quantitative analysis of histological sections also verified the presence of more regeneration in animals implanted with the HAP membranes compared to the other groups (Fig. 5e).

4. Discussion

Many proteins found in bone have the capacity to modulate or inhibit mineralization in vivo [32]. The possibility to fabricate materials using proteins as both structural and functional building-blocks may provide an effective tool to improve bone regeneration with high efficiency and control. In previous work, we developed a fabrication process to reproducibly create robust and tunable ELR membranes that exhibit specific physical and biomolecular signals [27] and the capacity to promote osteoblastic differentiation in vitro [25]. The main objective of the present study was to determine the in vitro mineralization and in vivo bone regeneration potential of bioactive ELR membranes in an animal model. In particular, the focus was to assess the bioactivity of ELR membranes containing the amino acid sequence DDDEEKR-HAP. This segment corresponds to SN15, an analog of the SN15 fragment of statherin in which aspartate (D) residues substitute the original phosphoserines and whose bioactivity is equivalent to the SN15 fragment of statherin [33]. Statherin is a protein found in saliva, a supersaturated metastable solution compound of ions, proteins, and water, which plays a role in the nucleation and growth of hydroxyapatite in the oral environment [34]. The N-terminal of the SN15 segment of statherin is formed by a sequence of negatively charged residues such as aspartic acid (D), glutamic acid (E) and phosphorylated-serine (S(P)) [33]. This region is responsible for binding calcium ions present in the oral environment and stabilizing the growth and critical size of the CaP cluster required for precipitation and transformation into a crystalline phase of hydroxyapatite [35]. Upon a pH decrease next to the enamel due to the presence of metabolic products secreted by bacteria, statherin releases the CaP ions on the surface of the tooth and enables their use to promote remineralization [36]. Our hypothesis was that the use of ELRs exhibiting this SNA15 peptide sequence, which is known to modulate mineralization in enamel as described above, may be used to enhance mineralization in bone. In order to further enhance membrane bioactivity, we also designed...
and used ELRs with an additional RGDS sequence (HAP-RGDS) in order to promote integrin-mediated cell adhesion.

Mineralization experiments, with and without cells, were conducted in simulated body fluid (SBF) [29] in both static and dynamic experimental conditions since some studies have reported an increase of cell-mediated mineralization under applied strain [30]. In this work, Smooth HAP membranes tested under static conditions reproducibly and significantly increased their mineralization compared to all other tested membranes as revealed by the calcium content assay (Fig. 1e), SEM observations (Fig. 2a, b), TOF- SIMS analysis (Fig. 2c, d), and EDS measurements (Fig. 2i, j). These HAP membranes also exhibited a significant increase in Young's modulus compared to all other tested membranes (Fig. 2k and Supplementary data Table S1), an expected result given the presence of the observed mineral layer. These results are consistent with studies demonstrating the affinity of the SN A15 fragment to adsorb to hydroxyapatite surfaces by chelation with surface calcium ions in vitro [37]. This affinity depends on the number and close vicinity of the negatively charged residues in the N-terminal domain, as demonstrated by studies using the individual SN A15 sequence [33] and within our HAP ELR molecule on titanium surfaces [38]. Therefore, it is possible that the mineralization observed on our HAP membranes was produced by the high density of negative charges present on the N-terminal segment of the SN A15 fragment of our HAP-containing ELRs. This hypothesis would explain the lower mineralization observed on HAP-RGDS membranes, since these membranes contain a lower density of the SN A15 sequence compared to HAP membranes [25].

Despite the strong mineralization on Smooth HAP membranes in static conditions, HAP membranes with Channel topographies and Smooth HAP membranes in dynamic conditions exhibited lower CaP deposition. While the reason for this decrease is not yet clear, it is possible that the 8% uniaxial strain used in this study may have assisted the release of the mineral from the membranes due to mechanical deformation. An alternative explanation might be related with the decrease of mineralization as a result of a decrease in wettability [39] due to the presence of the channel micro-topographies. Further studies will have to be performed in order to confirm this hypothesis.

Remarkably, in both static and dynamic conditions, rMSCs growing on HAP membranes in non-osteogenic differentiation medium exhibited similar levels of ALK/DNA expression and higher Ca deposition compared to cells growing in osteogenic differentiation medium (TCP+). These results are in accordance with our previous finding that the amino acid sequence of the SN A15 fragment significantly upregulates the early osteoblastic marker osterix in vitro even in the absence of osteogenic differentiation medium [25]. Previous studies have reported that the application of cyclic uniaxial strain on MSCs increased the production of matrix mineralization [40]. However, in our study mineralization of the membrane in the presence of cells was similar for both static and dynamic culture conditions. It is possible that the mineralizing effect expected to arise from the applied strain was masked by the strong mineralizing effect of the SN A15 fragment present in HAP membranes.

Due to the strong osteoblastic differentiation and mineralization observed in the Smooth HAP membranes in vitro, they were subsequently implanted and assessed in a critical-size rat calvarial model. At day 7, membrane localization were assessed and confirmed to be within the defect in the same place as they were positioned at the time of implantation. This stability results from the distinctive strength and ease of manipulation exhibited by our ELR membranes, which contrast the inherent weakness of other peptide and protein-based scaffolds. With respect to membrane bioactivity, animals implanted with HAP membranes had the highest mean volume of...
ossified tissue exhibiting an osteoid matrix with active osteoblasts within the defect and in cases even bridging across the critical-size defect (Figs. 4, 5c and 5d). Bone mineral density was also measured by microCT although the results exhibited a high variability and did not demonstrate significant differences. This high variability may have resulted because the values of bone mineral density obtained for all samples were lower than the selected range of the accuracy of the equipment. Nonetheless, altogether the results are consistent with events normally observed in natural intramembranous ossification [41] and in accordance with studies that have demonstrated bone regeneration using other peptide-based materials with mineralization-promoting signals [6]. Both our in vitro data and in vivo results suggest that the presence of the SN15 sequence of HAP ELRs may promote early biomineralization and potentially lead to cellular signaling that stimulates progenitor cells and enhances the growth of osteoblasts in vivo.

The main design feature of the present study was the generation of ELR building-blocks that offer both mechanical stability through the elastin-like sequences and molecular signaling leading to mineral formation and osteogenesis through the SN15 segment. ELR-based materials have been found non-cytotoxic and bioactive [42] when used in osteochondral [43] and vascular [44] defects. To our knowledge, this is the first study that demonstrates enhanced bone modulation mineralization in enamel for bone regeneration. A poor found in saliva and proteins found in bone, all of which regulate the ancestor [45]. This relation may help explain why the SN15 fragment promoting bone formation as observed in this study. The use of non-collagenous proteins known to regulate the remarkable mineral formation found in enamel in order to design materials to improve mineralization and bone regeneration is an exciting possibility.

5. Conclusions

We report on the enhanced mineralization, osteogenensis and in vivo bone regeneration properties of molecularly designed ELR membranes. Smooth membranes, containing an analog of the SN15 fragments of statherin (DDEEEKFLRRIGRFG), exhibited the highest quantity of calcium phosphate (Ca/P in 1.78) deposition with and without cells compared to all other tested membranes. Furthermore, these membranes displayed the highest production of alkaline phosphatase (ALK) on day 10 even in the presence of non-osteogenic media. This strong bioactivity was further demonstrated in vivo as animals implanted with these membranes exhibited the highest bone volume within the defect. This study validates the ability of generating ELR molecules that can serve as structural and functional building blocks to create robust bio-materials capable of orchestrating biological responses. Thin robust membranes made completely from molecularly designed ELRs that are capable to promote osteogenesis and enhance mineralization could serve as effective periostral grafts capable of enhancing bone regeneration.

References


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