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1 **Article type: Full Paper**

2 **Boosting mesenchymal stem cells regenerative activities on biopolymers-**
3 **calcium phosphate functionalized collagen membrane**

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30 **Abstract:**

31 The regeneration of bone-soft tissue interface, using functional membranes, remains
32 challenging and can be promoted by improving mesenchymal stem cells (MSCs) paracrine
33 function. Herein, a collagen membrane, used as guided bone regeneration membrane, was
34 functionalized by calcium phosphate, chitosan and hyaluronic acid hybrid coating by
35 simultaneous spray of interacting species process. Composed of brushite, octacalcium
36 phosphate and hydroxyapatite, the hybrid coating increased the membrane stiffness by 50%.
37 After 7 days of MSCs culture on the hybrid coated polymeric membrane, biological studies
38 were marked by a lack of osteoblastic commitment. However, MSCs showed an enhanced
39 proliferation along with the secretion of cytokines and growth factors that could block bone
40 resorption and favour endothelial cell recruitment without exacerbating polynuclear
41 neutrophils infiltration. These data shed light on the great potential of inorganic/organic
42 coated collagen membranes as an alternative bioactive factor-*like* platform to improve MSCs
43 regenerative capacity, in particular to support bone tissue vascularization and to modulate
44 inflammatory infiltrates.

45 **Keywords:** Bone regeneration; Inorganic/organic hybrid coating; Human stem cells;
46 Cytokines; Growth factors.

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55 **1. Introduction**

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57 Despite its intrinsic regeneration and self-repair capacity, bone large
58 segmental defects, usually caused by severe trauma, tumor resection,
59 infections or disorders-associated pathologies, require surgical remediation using bone
60 biomaterials. Guided Bone Regeneration (GBR), considered as one of the most attractive
61 techniques to regenerate alveolar bone, consists in the application of an occlusive membrane
62 that provides space maintenance, promoting bone regeneration and preventing soft tissue
63 infiltration. Despite accumulating evidences that GBR technique predictably leads to bone
64 regeneration, multiple drawbacks are still reported such as a lack of an adequate tissue
65 vascularization, a membrane collapse or an early exposure and bacteria colonization(1)(2). To
66 improve GBR outcome, various membrane functionalization with specific drugs including
67 antibiotics(3), anti-inflammatory(4), chemoattractive and osteogenic growth factors (*i.e.* bone
68 morphogenic protein-7)(5) have been proposed. Nevertheless, high-cost concerns and adverse
69 effects related to uncontrolled drug release tempered the initial excitement(6). Porous
70 structures with the optimal size of pores in the scaffolds, support growth of cells in bone
71 regenerative applications(7)(8). Owing to their bioactive and osteoconductive intrinsic
72 features, inorganic porous materials such as calcium phosphate (CaP), calcium carbonate,
73 bioactive glass were introduced in the main bulk of GBR membranes to favor osteoblast
74 adhesion and proliferation(9)(10)(11)(12). However, limitations due to harsh fabrication
75 conditions still exist despite recent advances in additive manufacturing such as
76 electrospinning or laser and inkjet printing(13). The fabrication of a mechanically stable and
77 microscopically homogeneous bone side functionalized membrane still
78 seems challenging.

79 Materials mimicking the structure of natural bone are thought to be promising for application
80 in bone tissue engineering(14). Macromolecules like chitosan (CHI) and hyaluronic acid (HA)

81 are largely investigated in this context. CHI forms polycation-polyanion complexes in the
82 presence of HA and its amino groups make it especially convenient for biomineralization
83 process(15). Enrichment of polysaccharidic scaffolds and/or hydrogels by mineral such as
84 calcium phosphate (CaP) or calcium carbonates was applied as material to support osteoblast
85 adhesion and proliferation(16)(17). Although they are bioactive and osteoconductive, calcium
86 carbonates, in contrast to CaP bone substitutes, present a chemical composition far from bone
87 apatite. We recently demonstrated that the biophysical features of CaP/CHI/HA coated glass
88 induce mesenchymal stem cells (MSCs) commitment into an osteoblastic lineage through
89 mechanobiological signalling(18). In the present work, we performed the same
90 functionalization on clinically relevant materials, *i.e.* polymeric collagen based GBR
91 membranes, and used them as substrate for MSCs culture. Exhaustive physicochemical and
92 mechanical insights into hybrid inorganic/organic coating are provided before testing its
93 intrinsic biological features, in comparison to inorganic-coated and bare membranes. In
94 contrast to our previous study, the hybrid inorganic/organic coated polymeric collagen based
95 membrane did not induce a cell commitment but improved MSCs proliferation along with the
96 secretion of mediators required for bone formation/vascularization. Owing to its appropriate
97 physicochemical properties, such materials can be considered as a bioactive factor-*like*
98 platform to improve MSCs regenerative capacity.

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105 2. Experimental

106 **2.1. Materials:** Calcium nitrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$), diammonium hydrogen phosphate
107 ($(\text{NH}_4)_2\text{HPO}_4$), sodium dihydrogen phosphate hydrate (NaH_2PO_4), Tris(hydroxymethyl)
108 aminomethane (Tris), calcium chloride hydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), sodium chloride and chitosan
109 (75–85% deacetylated, low molecular weight) from Sigma and hyaluronic acid (200 kDa)
110 from Lifecore Biomedical were used without further purification. Salt solutions were prepared
111 in ultrapure water (Millipore[®]). In the case of hybrid (*i.e.* CaP/CHI/HA) coating build-up,
112 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.32 M) and chitosan 0.3 mg/mL were dissolved in NaCl (0.15 M)/HCl (2 mM)
113 buffer at pH 4 whereas NaH_2PO_4 (0.19 M) and hyaluronic acid 0.3 mg/mL were prepared in
114 NaCl (0.15 M) buffer at pH 10. In the case of inorganic (*i.e.* CaP) coating build-up, a calcium
115 solution of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (0.32 M) and a phosphate solution of $(\text{NH}_4)_2\text{HPO}_4$ (0.2 M) were
116 prepared in Tris buffer (10 mM Tris, pH 4 and pH 10 respectively). Polymeric collagen based
117 membranes (Bio-Gide[®]) were provided from Geistlich Pharma[®] and biocompatible glue (R41
118 wood vinylic) was provided from BOSTIK[®].

119 **2.2. Coating build-up:** An automated spraying device was used to functionalize Bio-Gide[®]
120 membranes with both hybrid and inorganic coatings. This device is constituted of four
121 identical Airbrushes VL (Paasche[®], USA) nozzles. Each nozzle is pressurized by in-house
122 compressed air line under a pressure of 1 bar and connected to solenoid valves. The spraying
123 of the different solutions, following a chosen deposition sequence, is obtained by a succession
124 of closings and openings of the valves controlled by homemade software. Three nozzles allow
125 spraying of the calcium (with/without chitosan), the phosphate (with/without hyaluronic acid)
126 and of the rinsing solutions. The fourth nozzle, free of solution, is used for the drying step.
127 The Bio-Gide[®] membrane is mounted vertically on a mobile holder, allowing the drainage of
128 solution excess. The holder was rotated at 150 rpm to obtain a homogenous deposition of the
129 coating. Both calcium (with/without chitosan) and phosphate (with/without hyaluronic acid)

130 corresponding solutions were sprayed simultaneously, on the membrane, for 2 sec followed
131 by a rinsing step of 2 sec with ultrapure water and a drying step of 2 sec under compressed
132 air. These steps were repeated 50 times and polymer concentrations were adjusted after
133 calculation of flows during spraying in order to keep a charge ratio hyaluronic acid/chitosan
134 constant and equal to 0.7 to optimize the complex formation. Biocompatible glue was used in
135 order to stick the polymeric collagen based membranes on an appropriate surface for
136 biological experiments.

137 **2.3. Substrate characterization:**

138 *2.3.1. Scanning electron microscopy with a field emission gun (FEG-SEM).* FEG-SEM
139 investigations were performed with a FEG-SEM (JEOL JSM-7900F, France), on coated
140 polymeric collagen based membranes sputtered with thin gold–palladium film (JEOL ion
141 sputter JFC 1100). Samples were previously dehydrated in graded ethanol solutions from 50
142 to 100% and desiccated in hexamethyldisilazane (HMDS, Sigma) for 10 min, then air-dried at
143 room temperature. Images were acquired from secondary electrons at primary beam energy at
144 5 kV.

145 *2.3.2. Transmission Electron Microscopy (TEM).* TEM investigations were performed with a
146 JEOL ARM 200F – Cold FEG TEM/STEM (point resolution 0.19 nm in TEM mode and
147 0.078 nm in STEM mode) fitted with a GIF Quatum ER. High-Resolution TEM (HR-TEM)
148 pictures were performed with a JEOL ARM 200F – Cold FEG (point resolution 0.19 nm)
149 fitted with a GIF Quatum ER. Samples were previously dehydrated in graded ethanol
150 solutions from 50 to 100% for 10 min and embedded in epoxy resin (48.2% epon 812, 34%
151 anhydride nadic methyl, 16.4% anhydride [2-dodecenyl] succinic, and 1.5% 2,4,6-tris
152 dimethy- laminoethyl] phenol) for 72 h at 60°C. Ultra-thin cross sections (60 nm in thickness)
153 were performed using an automatic ultra-microtome (Ultracut-UCT Ultramicrotome, Leica) at
154 room temperature.

155 2.3.3. *X-ray photoelectron spectroscopy (XPS)*. XPS spectra were obtained from XPS
156 ESCALAB 250 spectrometer (Thermo Scientific) equipped with a
157 monochromatized micro focused Monochromatic Al K α source X-ray
158 source (powered at 15 kV). The pass energy was set at 20 eV. The sample is
159 placed on the standard sample holder and at an angle parallel to the
160 input lens axis. The flood gun was used for charge compensation (1 μ A, 14 eV, Ar gas).
161 The C-(C, H) component of the C 1s peak of carbon has been fixed to 285 eV
162 to set the binding energy scale. Data treatment was performed with the
163 Advantage Software program. Some spectra were decomposed with the least
164 squares fitting routine provided by the software with a
165 Gaussian/Lorentzian (85/15, 70/30) product function and after subtraction
166 of a non-linear baseline. Atomic ratios were calculated using peak
167 areas normalized on the basis of acquisition parameters and sensitivity
168 factors provided by the manufacturer. For each condition, two coated polymeric
169 membranes were analysed on two different points.

170 2.3.4. *Infrared (IR) microspectroscopy*. IR spectra, in reflectance mode, were recorded
171 between 4000 and 800 cm^{-1} on a Bruker Vertex 70v spectrometer equipped with a Hyperion
172 2000 microscope and a ($\times 15$) objective controlled by OPUS 7.5 software. A KBr beam
173 splitter and a MCT detector were used. The resolution of the single beam spectra was 4 cm^{-1} .
174 Masks of 300 $\mu\text{m} \times 220 \mu\text{m}$ were used to record 18 spectra and obtain maps on a (1380 \times 915
175 μm) of membrane analysed surface. The number of bidirectional double-sided interferogram
176 scans was 64, which corresponds to a 40 sec accumulation. All interferograms were Fourier
177 processed using the Mertz phase correction mode and a Blackman-Harris three-term
178 apodization function. Measurements were performed at $21 \pm 1^\circ\text{C}$ in an air-conditioned room.
179 Water vapor subtraction was performed when necessary, and baseline was corrected at 3800,
180 2500, 1900 and 900 cm^{-1} before further analysis of the spectra.

181 2.3.5. *Dynamic Mechanical Analysis (DMA)*. Dynamic mechanical properties of
182 functionalized polymeric collagen membranes were determined using a Netzsch DMA 242C.
183 Measurements of the complex modulus were performed in tension mode at three different
184 frequencies (1.5 and 10 Hz) in the temperature range of around $37 \pm 1^\circ\text{C}$. The complex
185 modulus is composed of the storage modulus E' (real part) and the loss modulus E''
186 (imaginary part). E' represents the stiffness of a viscoelastic material and is proportional to
187 the energy stored during a loading cycle (20 μm amplitude). It is roughly equal to the elastic
188 Young modulus. E'' represents the energy loss from the viscous part of the polymer. The loss
189 factor $\tan \delta$ is then defined as the ratio of the loss modulus to storage modulus.

190 **2.4. Biocompatibility studies:**

191 2.4.1. *Wharton's jelly mesenchymal stem cells (MSCs)*. Human umbilical cord harvesting was
192 approved ethically and methodologically by our local Research Institution and was conducted
193 with informed patients (written consent) in accordance with the usual ethical legal regulations
194 (Article R 1243-57). All procedures were done in accordance with our authorization and
195 registration number DC-2014-2262 given by the National "Cellule de Bioéthique". MSCs
196 were enzymatically isolated from fresh human umbilical cords obtained after full-term births.
197 MSCs were amplified at density of 3×10^3 cell/cm² in α -MEM supplemented with 10%
198 decomplemented FBS, 1% Penicillin/Streptomycin/Amphotericin B and 1% Glutamax[®] (v/v,
199 Gibco) and maintained in a humidified atmosphere of 5% CO₂ at 37°C with a medium change
200 every two days. MSCs were seeded in 24 well plates at 24,000 cells/cm² on UV-
201 decontaminated (20 min) hybrid-coated membranes glued on glass coverslips. UV-
202 decontaminated inorganic-coated and bare polymeric membranes glued on glass coverslips
203 were used as control.

204 2.4.2. *Mitochondrial activity*. After 2, 4 and 7 days of culture on hybrid-coated polymeric
205 membranes and on controls, WST-1 cell proliferation assay (Roche Diagnostics) was

206 performed on MSCs in accordance with the manufacturer protocol. Absorbance was measured
207 at 440 nm using a FLUOstar Omega microplate reader (BMG Labtech) against a background
208 control as blank. A wavelength of 750 nm was used as the correction wavelength.

209 *2.4.3. DNA quantification.* After 7 days of culture on hybrid-coated polymeric membranes
210 and on controls, DNA was extracted using MasterPure™ DNA Purification Kit (Epicentre®
211 Biotechnologies) in accordance with the manufacturer protocol. Extracted DNA was assessed
212 by measuring the absorbance at 260 and 280 nm (Nanodrop®, Thermo Scientific) with
213 260/280 nm absorbance ratio for all measured samples comprised between 1.8 and 2.

214 *2.4.4. Cytoskeleton staining.* After 7 days of culture on hybrid-coated polymeric membranes
215 and on controls, MSCs were fixed with 4% (w/v) paraformaldehyde (Sigma-Aldrich) at 37 °C
216 for 10 min and permeabilized with 0.5% (v/v) Triton ×100 for 5 min. Alexa® Fluor-488
217 conjugated-Phalloidin® (1/100 dilution in 0.1% Triton ×100) was used to stain F-actin for 45
218 min at room temperature. Nuclei were counter-stained with 4,6-diamidino-2-phenylindole
219 (DAPI, 100 ng/mL, 1/10 000 dilution) for 5 min. Stained cells were mounted and imaged by
220 confocal laser scanning microscopy (CLSM, Zeiss LSM 710 NLO, 20× objective, Numerical
221 Aperture 1.4, Germany).

222 *2.4.5. Cytokines and growth factors release by ELISA.* After 7 days of MSCs culture on
223 hybrid-coated polymeric membranes and on controls, supernatants were collected and stored
224 at -20°C, and samples were subsequently assessed in duplicate for each specific
225 cytokine/growth factor. Secreted levels of osteoprotegerin (OPG), receptor activator of
226 nuclear factor kappa-B ligand (RANKL), vascular endothelial growth factor (VEGF), bone
227 morphogenic protein-2 (BMP-2) and interleukin-6 (IL-6) were assessed using human DuoSet®
228 osteoprotegerin/TNFRSF11B, TRANCE/RANKL/TNFSF11, VEGF, BMP-2 and IL-6 (R&D
229 systems) respectively, according to the manufacturer's instructions. Secreted levels of
230 interleukin-8 (IL-8) and basic fibroblast growth factor (b-FGF) were assessed by using human

231 ELISA MAXTM Deluxe Set IL-8 and FGF-basic (BioLegend[®]) respectively, according to the
232 manufacturer's instructions. Optical densities were measured at 450 nm using a microplate
233 reader (FLUOstar Omega microplate reader, BMG Labtech). Released amount of cytokines
234 was calculated from the corresponding standard curves and normalized to total DNA content
235 (measured using the DNA assay setup, Nanodrop, Thermo Scientific).

236 *2.4.6. Neutrophils migration in Boyden chamber chemotaxis assay.* Conditioned media from
237 MSCs seeded on hybrid-coated polymeric membranes and on controls for 7 days were
238 deposited on the lower compartment, whereas 5×10^4 neutrophils, collected as previously
239 described(19), were seeded on a polycarbonate membrane (5 μ m pores, Nucleopore Track-
240 etch membrane, Whatman, Maidstone, UK) in the upper compartment. After 45 min of
241 incubation at 37°C in 5% CO₂, non-migrating neutrophils were removed from the top of the
242 membrane and migrated cells at the bottom were stained with May Grünwald Giemsa
243 (RAL555 kit) and imaged (Axiovert 200M microscope, Zeiss, Oberkochen, Germany,
244 Objective \times 40). IL-8 solution (200 ng/mL) and MSCs culture medium were used as controls.

245 *2.4.7. Endothelial cells migration through transwell cell migration assay.* Conditioned media
246 from MSCs seeded on hybrid-coated polymeric membranes and on controls for 7 days were
247 deposited in a 24 well plastic culture plate, whereas 2×10^3 of human umbilical cord vein
248 endothelial cells (HUVECs), isolated as previously described(20), were seeded on the top of a
249 cell culture insert membrane (Millicell[®] Hanging Cell Culture Inserts). After 48h of
250 incubation at 37°C in 5% CO₂, non-migrating HUVECs were removed from the top of the
251 membrane and migrated cells at the bottom were fixed with methanol then stained with
252 crystal violet. Migrated cells were imaged with an EVOS[®] digital microscope. HUVECs
253 culture medium containing or not fetal bovine serum and MSCs culture medium were used as
254 controls.

255 **2.5. Statistical analysis:** All statistical analysis were performed using GraphPad Prism 5

256 software. Biological experiments were performed with six independent umbilical cords in
257 duplicate. Box plots represent median, 1st and 3rd quartiles, and 1st and 9th deciles, statistical
258 analysis were performed using Mann & Whitney test. For each test, a value of $p < 0.05$ was
259 accepted as statistically significant p (rejection level of the null-hypothesis of equal means).

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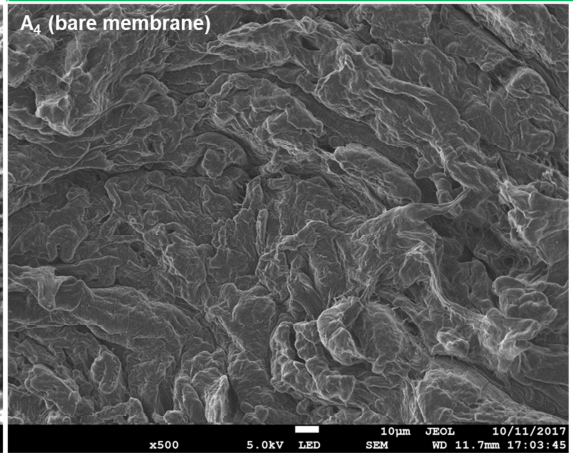
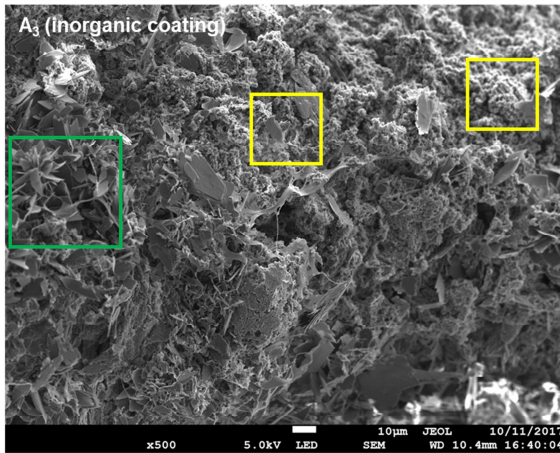
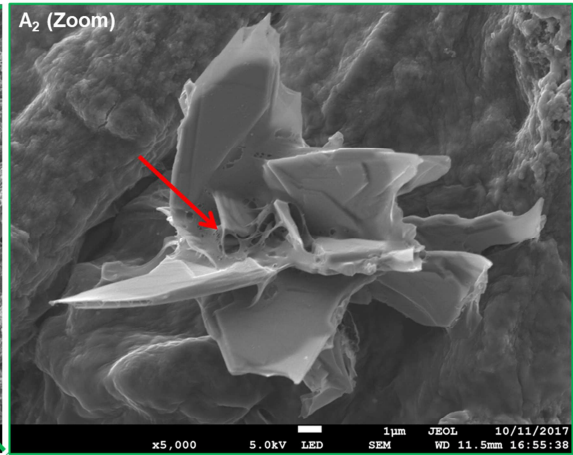
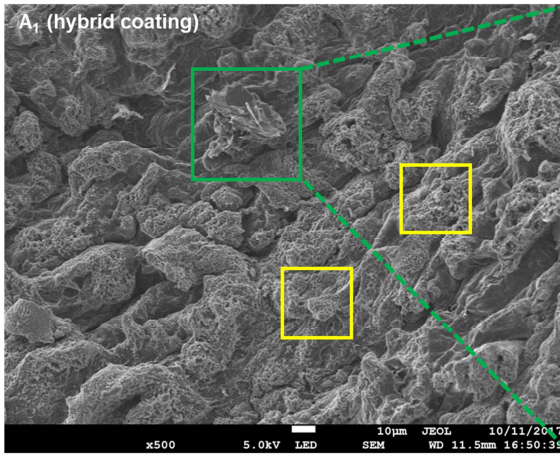
262 **3. Results and discussion**

263 In the context of bone-soft tissue interface regeneration, we herein aimed to functionalize
264 polymeric collagen based membrane (Bio-Gide[®]), a dental medical device, with calcium
265 phosphate, chitosan and hyaluronic acid coating. The integration of both organic and
266 inorganic compounds is proposed to improve properties or implement functionalities of
267 material(21). Bio-Gide[®] membrane was functionalized by an inorganic/organic coating, using
268 a straightforward method, the simultaneous spray of interacting species. This technique allows
269 the asymmetric functionalization of the membrane, *i.e.* only the bone-facing side. In order to
270 investigate the advantage of supplementing calcium phosphate with chitosan and hyaluronic
271 acid biopolymers, inorganic (calcium phosphate) coated and bare collagen polymeric
272 membranes were used as controls. We used the term “hybrid coating” to designate the
273 calcium phosphate, chitosan and hyaluronic acid coating; and the term “inorganic coating” for
274 the calcium phosphate coating.

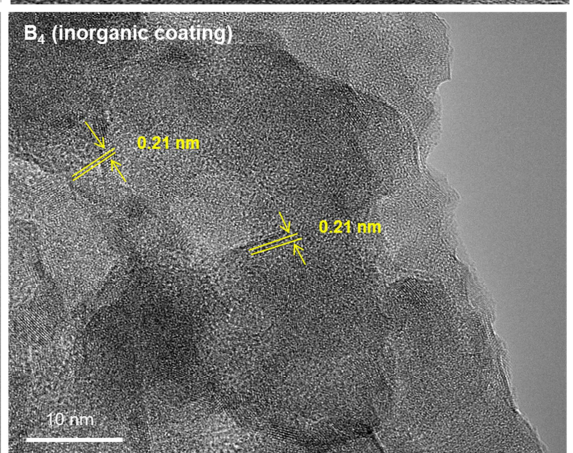
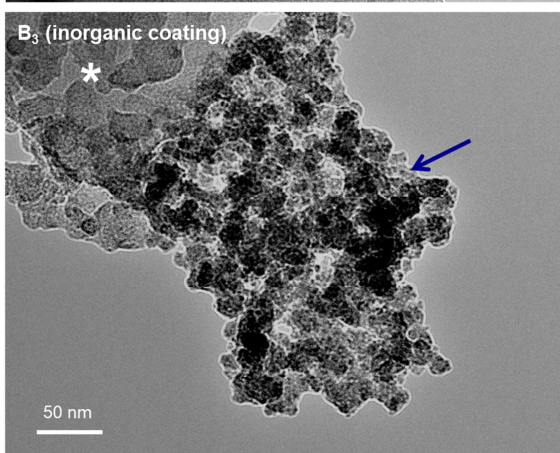
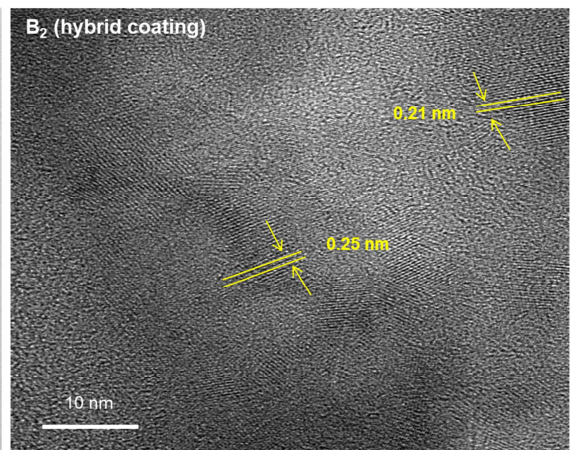
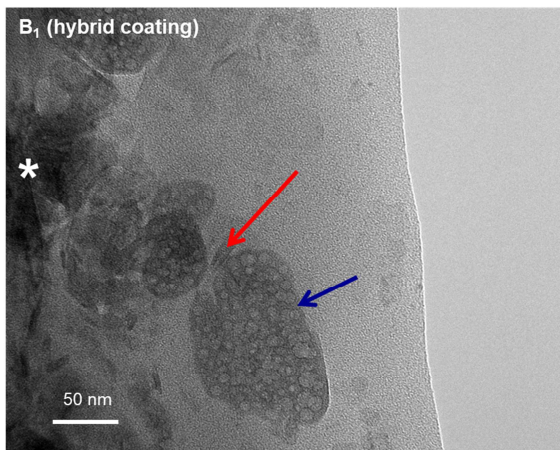
275 Representative top views of hybrid-coated polymeric collagen membranes, observed by FEG-
276 SEM, showed the presence of mineral structures with both granular and rod shapes (Figure
277 1A). In contrast to inorganic coating, hybrid coating-derived rods were wrapped in
278 biopolymers film (Figure 1A₂ red arrow) and exhibited a smaller size ($\approx 8.5 \times 4 \mu\text{m}$ *versus* 18
279 $\times 7 \mu\text{m}$). A deeper characterization of the crystal structure was performed by HR-TEM

280 combined with electron diffraction (ED). Both coatings displayed a nanostructured network of
281 interconnecting elliptical-shaped particles (Figure 1B). However, difference in coatings
282 particles shake-up was noticed; highlighting the presence of multiple clustered crystals
283 embedded in an amorphous matrix in case of the hybrid coating (Figure 1B₁). The growth of
284 crystals within a confined environment provided by the amorphous organic film hampered the
285 quantification of their size. Inorganic coating-derived crystals exhibited a diameter of about
286 10-20 nm (Figure 1B₃). In addition to the difference in particles shake-up, HR-TEM revealed
287 the presence of crystalline structures suggesting the presence of both octacalcium phosphate
288 and hydroxyapatite in hybrid coating whereas inorganic coating derived crystals exhibited
289 hydroxyapatite phase as evidenced by ED. Hybrid coating displayed ($\bar{1}\bar{7}1$), (109), (301) and
290 (113) corresponding to the respective inter-planar distance of 0.25 and 0.21 nm, whereas
291 inorganic coating showed (131) with a characteristic spacing of 0.21 nm (Figure 1B₂ and 1B₄)
292 (according to joint committee on powder diffraction standards (JCPDS[®]) n°26-1065 and
293 American mineralogist structure (AMS) data n°15). Collagen gap junction and mineral
294 deposition, usually observed during *in vitro* biomineralization, in the presence of collagen(22)
295 were not observed in our case. This is probably due to the collagen membrane features.
296 Although HR-TEM combined with electron diffraction is currently considered as the “gold
297 standard” technique for the determination of crystal structure for any given mineral, recent
298 report showed a great instability of both amorphous calcium phosphate and octacalcium
299 phosphate under beam, making this investigation inaccurate(23).

A: FEG-SEM



B: TEM/HR-TEM

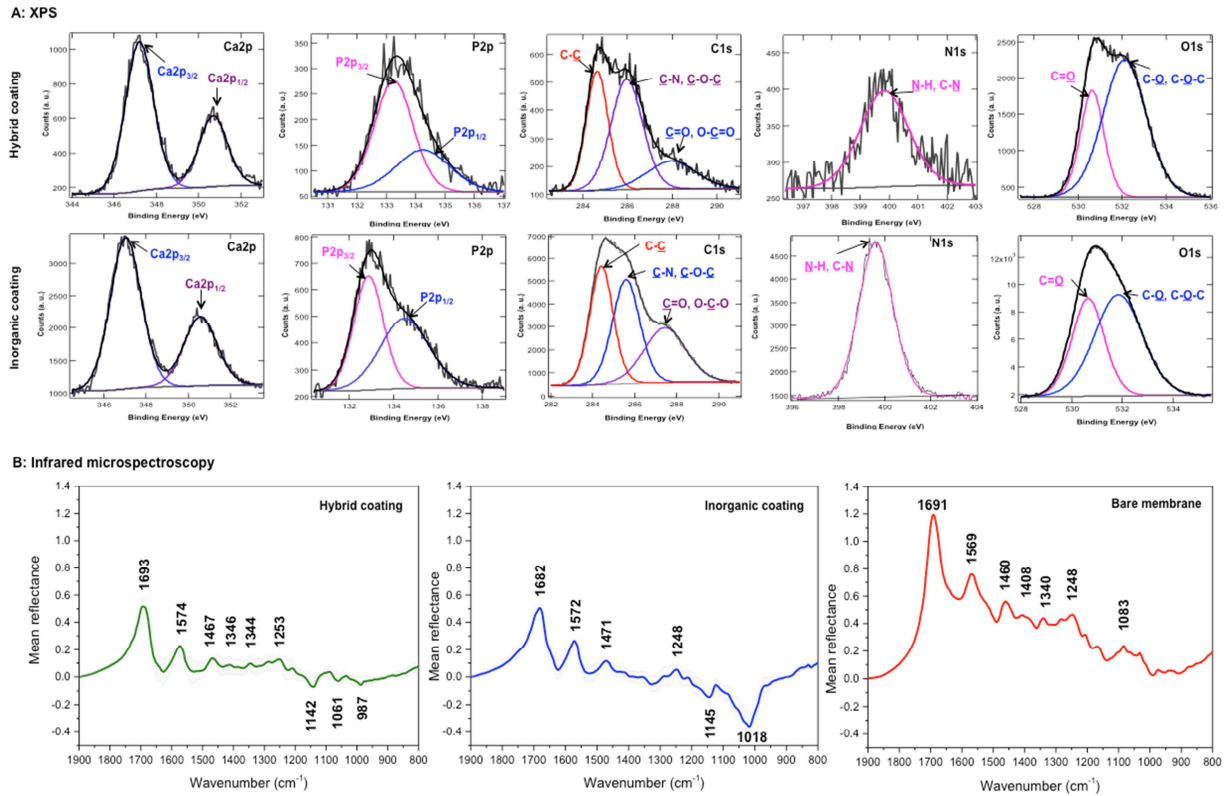


301 **Figure 1:** Surface morphology of hybrid and inorganic-coated membranes. A: Field Emission
302 Gun-Scanning Electron Microscopy (FEG-SEM, scale bars = 10 and 1 μm) and B:
303 Transmission Electron Microscopy (TEM) and High-Resolution (HR)-TEM (scale bars = 50
304 and 10 nm). Green and yellow squares show rod and granular shapes, respectively. Red and
305 blue arrows indicate biopolymers film and minerals, respectively, whereas the stars highlight
306 the polymeric collagen based membrane.

307

308 To gain more structural information, XPS and IR micro-spectroscopy were performed on both
309 hybrid-coated and inorganic-coated polymeric membranes. Different biological calcium
310 phosphate phases are known including hydroxyapatite, dicalcium phosphate dehydrate and
311 octacalcium phosphate(24). XPS allows identifying the chemical composition on the extreme
312 surface. Besides the prominent peaks corresponding to biopolymers (*i.e.* collagen, chitosan
313 and hyaluronic acid) as C_{1s} , N_{1s} and also the O_{1s} , general XPS spectra showed calcium
314 phosphate characteristic peaks, namely Ca_{2p} , P_{2p} and O_{1s} (Figure 2A). Although both coatings
315 exhibited globally similar profiles, Ca/P atomic ratios were about 1.24 for the hybrid and 1.44
316 for the inorganic coating, which could be attributed to octacalcium phosphate and calcium-
317 deficient hydroxyapatite, respectively. These values are consistent with the composition of the
318 mineral phase observed during bone tissue formation, where the octacalcium phosphate phase
319 is more abundant in the newly formed tissue and evolves toward a more crystalline
320 carbonated hydroxyapatite phase, characteristics of mature bone matrix(25). Furthermore, *in*
321 *vitro* biomineralization studies have demonstrated that the formation of hydroxyapatite as a
322 final phase occurs through the conversion of an amorphous precursor phase into octacalcium
323 phosphate *via* a cluster-growth mechanism(26). Many XPS studies reported that Ca/P atomic
324 ratios of bone derived mineral (*i.e.* calcium-deficient hydroxyapatite) are lower than
325 stoichiometric hydroxyapatite value (about 1.6), whereas O/Ca ratios are higher(27). In our
326 case, the important contribution of biopolymers hindered the determination of a reliable O/Ca
327 atomic ratio.

328 IR microspectrometry allows obtaining average spectra of both coatings. A slight variation in
329 collagen bands intensities of the bare membrane was observed in comparison to known
330 collagen peaks(28), probably due to the topography leading to weak variations of the focal
331 point (Figure 2B). Collagen amide bands on both coated membranes appeared different in
332 terms of shape and intensity (with a decrease of about 60%), comparing to bare collagen
333 membrane; confirming the coating deposit. Data for main vibration bands from micro-infrared
334 are compiled in Table 1 (Figure SI-1). Regarding chitosan and hyaluronic acid, their
335 contributions are weak and overlap with hydroxyapatite, collagen and water peaks(9)(29).
336 Thus, infrared analysis was focused on the area of spectra related to inorganic compounds
337 ($1200-950\text{ cm}^{-1}$). This latter showed unexpected negative peaks, probably due to specific
338 reflections of the IR beam on the uneven surface and to the poor crystalline state of mineral as
339 previously described(30)(31). Inorganic-coated membranes exhibited bands assigned to
340 mainly poorly crystalized carbonated hydroxyapatite. In contrast, hybrid-coated polymeric
341 membrane showed several bands mainly assigned to dicalcium phosphate dehydrate(32).
342 Raman spectra recorded on these samples were consistent with micro-infrared spectra,
343 showing the same phosphate compounds (data not shown). Unsuccessful detection of poorly
344 crystalized hydroxyapatite phase on the hybrid-coated polymeric membrane could be due to
345 the uneven surface and/or to the shake-up of clustered crystals embedded within an
346 amorphous matrix as shown in TEM experiments. In addition, XPS experiments revealed
347 higher Ca/C and P/C ratios of respectively 26% and 31% for the hybrid-coated polymeric
348 membranes in comparison to inorganic-coated ones, probably due the weak presence of
349 carbonates.



350

351 **Figure 2:** Chemical composition of hybrid and inorganic-coated membranes. A: X-rays
 352 photoelectron spectroscopy (XPS) of hybrid-coated polymeric collagen based membrane
 353 (upper line) and inorganic-coated polymeric collagen based membrane (lower line). B:
 354 Average spectra and standard deviation calculated from 18-recorded spectra obtained by
 355 Infrared micro-spectroscopy in reflectance mode, indicating the main peaks of dicalcium
 356 phosphate dehydrate for the hybrid-coated polymeric collagen based membrane and
 357 carbonated hydroxyapatite for the inorganic-coated polymeric collagen based membrane.

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370 **Table 1:** Assignment of the main vibrational bands obtained by IR microspectroscopy (4000 -
 371 800 cm⁻¹) on hybrid- and inorganic-coated polymeric collagen based membranes in
 372 comparison to bare membrane(28)

| Infrared Wavenumber (cm ⁻¹) | | | Assignment | Associated compound |
|---|-------------------|---------------|--|----------------------------------|
| Hybrid coating | Inorganic coating | Bare membrane | | |
| | ~3630 | | vOH | Non-associated water |
| 3538 | 3547 | | vOH | Weakly associated water |
| 3488 | 3487 | ~3450 | vOH | Water, COL |
| 3369 | 3355 | 3347 | vNH | COL |
| 3091 | | 3081 | vCH, aromatics | |
| ~2970 | | 2964 | vaCH ₃ | |
| | | 2934 | vaCH ₂ | |
| 2881 | | 2877 | vsCH ₃ | |
| ~2130 | ~2125 | ~2130 | OH and δOH combination | Water |
| 1693 | 1682 | 1691 | Amide I (vC=O) | COL |
| 1643 (sh) | 1641 (sh) | 1640 (sh) | δH ₂ O | Water |
| 1574 | 1572 | 1569 | Amide II (vC-N, δNH) | COL |
| 1467 | 1471 | 1460 | δCH _n | |
| | 1421 | | δCO ₃ (v3) | Carbonate |
| 1411 | | 1408 | vCOO ⁻ | COL |
| 1384 | | 1389 (sh) | δCH ₃ | |
| | 1355 | | δCO ₃ (v3) | Carbonate |
| 1344 | | 1340 | Amide III, ωCH ₂ , δCH ₃ | COL |
| 1285 | 1286 | 1283 | Amide III, ωCH ₂ , δCH ₃ | |
| 1253 | 1248 | 1248 | Amide III, ωCH ₂ , δCH ₃ | |
| 1209 | 1212 | 1207 | Amide III | |
| 1142 (neg.) | 1146 (neg.) | | | DCPD |
| | | 1083 | vC-O | COL (carbohydrate moiety) |
| 1061 (neg.) | 1060 (neg.) | | | DCPD |
| | 1036 (sh, neg.) | 1032 | vC-O | COL (carbohydrate moiety) HAp |
| | 1017 (neg.) | | | HAp |
| 987 (neg.) | | | | DCPD |

373 Key: v: stretching, δ: bending, ω: wagging, a: antisymmetric, s: symmetric, sh: shoulder, DCPD: dicalcium
 374 phosphate dehydrate, COL: collagen, HAp: Hydroxyapatite.

375

376 Functionalized polymeric GBR membranes must possess adequate strength and elastic
 377 modulus to allow easy handling for their placement at the defect site and to avoid collapse
 378 under intraoral forces(33). Elastic modulus is the measure of a material stiffness, where a high
 379 elastic modulus reflects a stiff material while a low elastic modulus reflects a flexible one.

380 Blending of collagen with ceramics leads to enhancement of its mechanical properties(34). On
 381 the other hand, amalgamation with biopolymers confers toughness and flexibility to strong
 382 and hard calcium phosphate minerals(34). Thus, elastic moduli of hybrid- and inorganic-
 383 coated polymeric collagen based membranes were investigated by dynamic mechanical
 384 analysis (DMA) at 1 Hz under dry and wet conditions. Elastic moduli values of the
 385 membranes were higher in dry conditions, compared to wet conditions. Under wet condition,
 386 predicting the mechanical properties of the membrane *in vivo*, data in Table 2 indicate that the
 387 elastic moduli of hybrid- and inorganic-coated polymeric collagen based membrane,
 388 compared to bare membrane, were improved by more than 50% and 100% (Table 2).
 389 Moreover, a high flexibility and good handling of the hybrid-coated membrane were observed
 390 (Figure SI-2), which is useful for the tight attachment to curved surfaces in the context of
 391 GBR applications. Thus by incorporating calcium phosphate, chitosan and hyaluronic acid,
 392 we were able to functionalize dental polymeric collagen based collagen medical device and to
 393 improve its mechanical properties without affecting its flexibility and handling.

394 **Table 2:** Dynamic mechanical analysis under wet condition

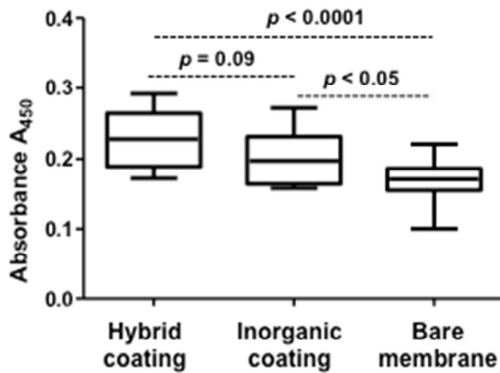
| | Hybrid coating | Inorganic coating | Bare membrane |
|----------|----------------|-------------------|---------------|
| E' (MPa) | 1.8 ± 0.3 | 2.5 ± 0.2 | 1.2 ± 0.2 |

395
 396 Recently, it was demonstrated that GBR membrane acts as a bioactive compartment, rather
 397 than merely a passive barrier. *In vivo*, GBR membrane attracts cells of different phenotypes,
 398 which sequentially express and secrete factors and signals favouring bone regeneration and
 399 remodelling (BMP-2, IL-6, b-FGF and TGF-β) and vascularization (VEGF)(35).
 400 Mesenchymal stem cells (MSCs) are a heterogeneous population, which exert their
 401 therapeutic effects by differentiating into osteoblasts and/or by secreting local soluble factors

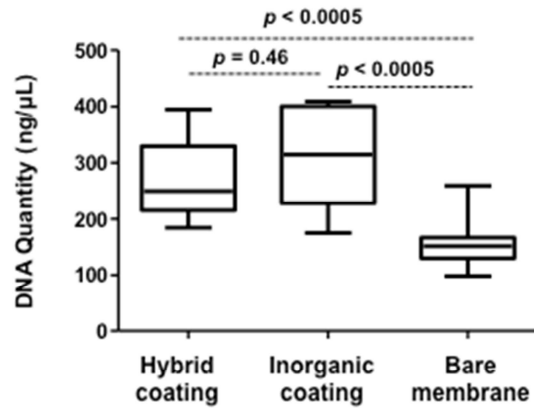
402 required for bone regeneration. These soluble factors include interleukin (IL) -6 and -
403 8, basic fibroblast growth factor (b-FGF), vascular endothelial growth factor (VEGF), bone
404 morphogenic protein-2 (BMP)-2 and osteoprotegerin (OPG)(36)(37). Together, these secreted
405 factors are known to dampen the inflammatory response, inhibit bone resorption and promote
406 endothelial and osteoblast activities. Thus, it is necessary to check whether the membrane is
407 able to support MSCs proliferation and function. Human MSCs were seeded and
408 cytocompatibility of coated membranes was firstly assessed by WST-1 proliferation assay.
409 Results showed that MSCs were metabolically active in contact with hybrid-coated
410 membranes and both controls (inorganic-coated and bare membranes). An exponential
411 increase in absorbance values from day 2 to 7 was observed for hybrid- and inorganic-coated
412 polymeric collagen based membranes and a linear increase for bare membrane (Figure SI-3).
413 In addition to WST-1 results showing an increase in metabolic activity of 23% (resp. 6%) for
414 hybrid (resp. inorganic) coatings, DNA quantification at day 7 confirmed a higher MSCs
415 content on both coated membranes (Figure 3A-B).

416 The differentiation of MSCs and the subsequent formation of bone are guided by several
417 micro-environmental signals. We recently demonstrated that biophysical features of bone
418 mimetic-coated glass (*i.e.* roughness and stiffness around 270 nm and 2 GPa, respectively),
419 through mechanobiological signalling, carry away dramatic changes on MSCs morphology
420 with polygonal cells exhibiting perpendicularly oriented F-actin fibres gathering around the
421 nuclei, signature of osteoblastic commitment(18). In the present study, stained cytoskeleton
422 showed a well elongated and flattened morphology where adhered MSCs displayed F-actin
423 stress fibres parallel to the longitudinal cell axis whatever the studied condition (Figure 3C).
424 Morphological studies highlighted MSCs fibroblastic shape, suggesting an uncommitted cell
425 state.

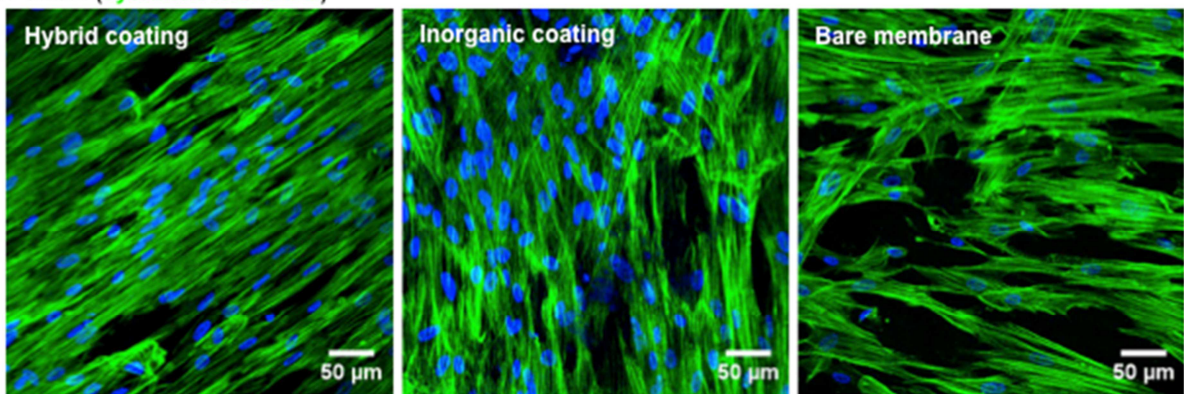
A: WST-1



B: DNA quantification



C: CLSM (cytoskeleton/nuclei)



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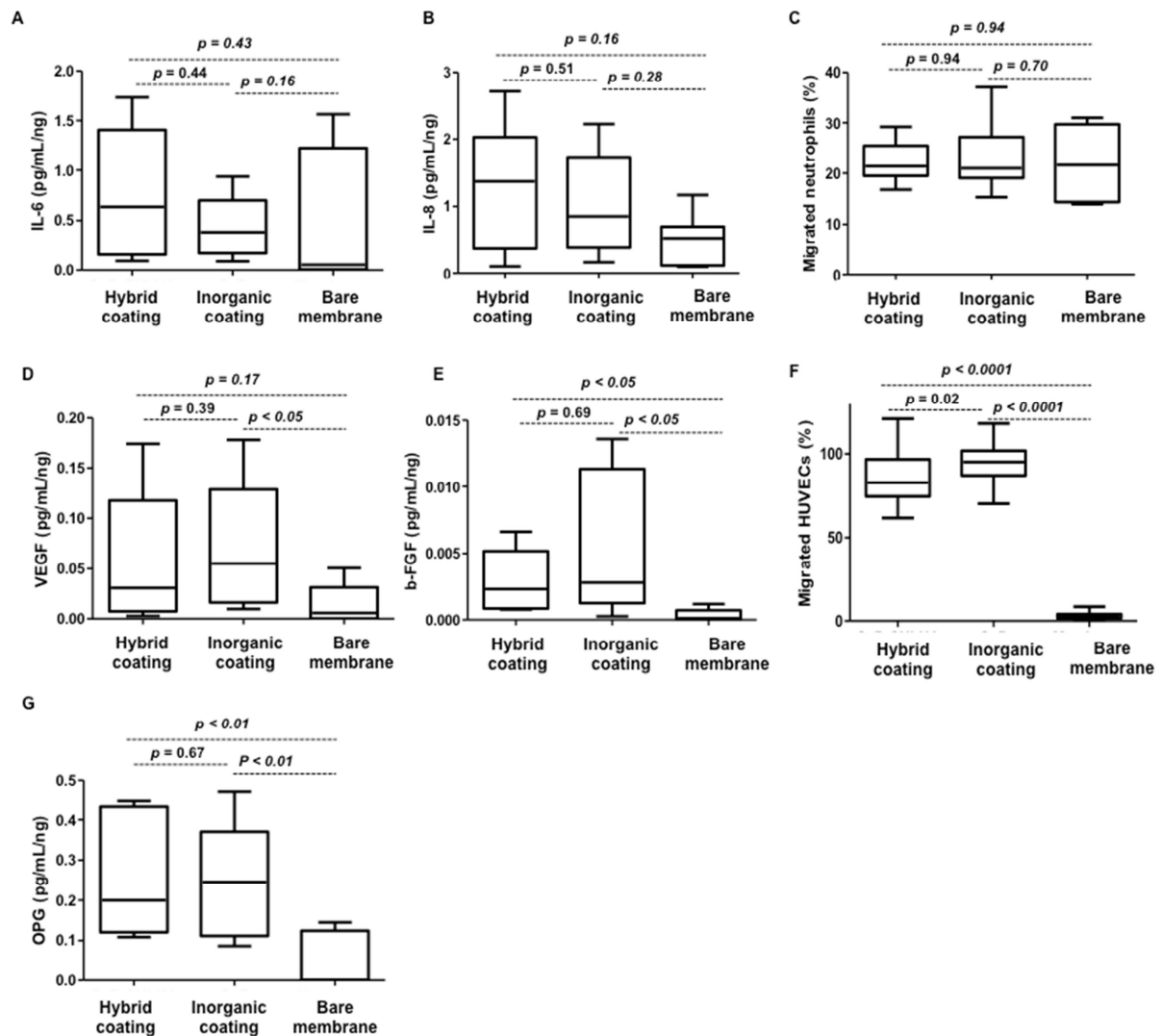
427 **Figure 3:** Biocompatibility of hybrid- and inorganic-coated polymeric collagen based
428 membranes. A: WST-1 proliferation assay, B: DNA quantification and C: Confocal laser
429 scanning microscopy (CLSM) fluorescent images of F-actin cytoskeleton (green colour) and
430 nuclei (blue colour), confirming the higher cell proliferation on both coatings. (n=6, Mann &
431 Whitney test, scale bars = 50 μm).

432

433 After a week of culture on coated and uncoated membranes, soluble factors secreted by MSCs
434 were quantified by Enzyme-Linked Immunosorbent Assay (ELISA). BMP-2, described as a
435 potent osteogenic factor secreted by bone cells, was not detected in supernatants in all tested
436 conditions (Figure 4). However, we noticed a modest increase in IL-6 and IL-8 secretion by
437 MSCs cultured on both coated membranes in comparison to bare membrane (Figure 4A and
438 4B). Pro-inflammatory cytokines TNF-α and IL-1β, known to harm bone regeneration, were
439 below the detection limit whatever the investigated conditions. Although IL-6 and IL-8 are
440 currently described as pro-inflammatory cytokines, a lack of exacerbated inflammatory

441 mediators release suggests that both hybrid- and inorganic-coated polymeric collagen based
442 membranes offer a non-harmful environment for MSCs(19). Furthermore, IL-6 produced by
443 MSCs is described to maintain their “stemness” and can also have an anti-inflammatory effect
444 by limiting inflammatory infiltrates as neutrophils(38)(39). On the other hand, the basic
445 biological role of IL-8 is attracting and activating neutrophils. Boyden-migration assays
446 showed about 20% of neutrophils recruitment by MSCs conditioned media whatever the
447 culture condition (Figure 4C), confirming then the neutral role of both coatings. Attractively,
448 these inflammatory mediators are recently reported to be involved in natural regenerative
449 process along with a great potential in bone repair treatment, through the regulation of
450 osteoblasts and osteoclasts differentiation as well as angiogenesis(33)(40).

451 MSCs cultured on both hybrid- and inorganic-coated polymeric collagen based membranes
452 released VEGF and b-FGF in a different manner compared to those cultured on bare
453 membrane. VEGF release was moderately enhanced on hybrid- and significantly enhanced on
454 inorganic-coating compared to bare membrane (Figure 4D). In contrast, b-FGF was
455 significantly increased on both hybrid- and inorganic-coated polymeric collagen based
456 membranes compared to bare membrane (Figure 4E). VEGF and b-FGF are known to
457 contribute to vascularization(40). This latter was further confirmed through attraction of
458 human umbilical vein endothelial cells (HUVECs) by MSCs conditioned media. Transwell
459 chemotaxis assay showed that MSCs on hybrid- and inorganic-coated polymeric collagen
460 based membranes enhanced HUVECs recruitment with about 85% and 97%, respectively
461 (Figure 4F). MSCs on bare collagen membrane recruited only 3%. These results highlighted a
462 superior angiogenic chemotaxis effect of MSCs cultured on hybrid- and inorganic-coated
463 polymeric collagen based membranes.



464

465 **Figure 4:** Paracrine activities of MSCs cultured on hybrid- and inorganic-coated polymeric
 466 collagen based membranes. A, B, D, E, G: ELISA quantification assay normalized to DNA
 467 content, C and F: Boyden and transwell migration assays of neutrophils and human umbilical
 468 vein endothelial cells (HUVECs) against MSCs conditioned media, respectively. Migrated
 469 neutrophils using IL-8 (200 ng/mL) and migrated HUVECs using endothelial growth medium
 470 were considered as 100%. (n=6, Mann & Whitney test).

471

472

473 The lack of BMP-2, as mentioned above, could be explained by the absence of osteoblast
 474 differentiation on both coated membranes. However, endothelial cells, in response to VEGF,
 475 are able to produce BMP-2, stimulating osteoblast recruitment/differentiation and promoting
 476 fracture healing(41)(42). Thus, despite a lack of MSCs commitment into osteoblastic lineage,
 477 our results suggest that hybrid- and inorganic-coated membranes could provide a local

478 environment favouring vascularization and subsequently bone formation. The receptor
479 activator of nuclear factor kappa-B ligand (RANKL) is a soluble factor which
480 induces osteoclasts (bone-resorbing cells) differentiation and activation through its binding
481 to RANK. RANKL was not detected in supernatants whatever the studied conditions. Acting
482 as a decoy receptor, OPG can prevent bone resorption by limiting RANKL/RANK
483 interactions(43). OPG, constitutively produced by MSCs, was significantly increased on both
484 hybrid and inorganic-coated membranes compared to bare membrane (Figure 4G). Taking
485 together, these results suggest that both coatings could provide a local environment that
486 blocks bone resorption.

487 **3. Conclusion**

488 We successfully functionalized the bone-facing side of polymeric collagen based membrane
489 with brushite, octacalcium phosphate and hydroxyapatite combined with chitosan and
490 hyaluronic acid biopolymers. The hybrid coating increased the membrane stiffness by 50%
491 without affecting its flexibility and handling. The structural, chemical and mechanical features
492 of the hybrid-coated polymeric collagen based membrane supported MSCs adhesion and
493 function. Although no MSCs commitment into osteoblast lineage was observed after 7 days of
494 culture, improvement in MSCs paracrine activities was achieved by promoting the secretion
495 of inflammatory cytokines and pro-angiogenic growth factors, required for bone regeneration.
496 The straightforward manufacturing process suggests that hybrid coating has a great potential
497 for timely clinical translation in bone-soft tissue interface regeneration fields, including
498 dentistry, craniofacial surgery, and orthopaedics.

499 **Supporting Information:** Supporting Information is available from ScienceDirect Online
500 Library or from the author.

501 **Conflicts of Interest:** The authors declare no competing financial interest.

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