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1 Microgels based on Infernan, a glycosaminoglycan-mimetic

2 bacterial exopolysaccharide, as BMP-2 delivery systems

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12 Abstract

Bone Morphogenetic Protein (BMP-2) is an osteoinductive growth factor clinically used for 13 bone regeneration. Tuneable sustained strategies for BMP-2 delivery are intensely developed 14 to avoid severe complications related to supraphysiological doses applied. To address this issue, 15 we investigated the ability of the bacterial exopolysaccharide (EPS) called Infernan produced 16 by the deep-sea hydrothermal vent bacterium Alteromonas infernus, exhibiting both 17 18 glycosaminoglycan-mimetic and physical gelling properties, to efficiently bind and release the bioactive BMP-2. Two delivery systems were designed based on BMP-2 retention in either 19 single or complex EPS-based microgels, both manufactured using a microfluidic approach. 20 BMP-2 release kinetics were highly influenced by the ionic strength, affecting both microgel 21 stability and growth factor/EPS binding, appearing essential for BMP-2 bioactivity. The 22 osteogenic activity of human bone-marrow derived mesenchymal stem cells studied in vitro 23

- emphasized that Infernan microgels constitute a promising platform for BMP-2 delivery for
- 25 further *in vivo* bone repair.
- 26

27 Keywords

- 28 Heparin-mimetic, capillary microfluidics, release kinetics, BMP-2 bioactivity, osteogenic
- 29 potential, AFM imaging
- 30

31

1. Introduction

Bone Morphogenetic Proteins (BMPs), belonging to the Transforming Growth Factor- β 32 (TGF- β) superfamily, by inducing cellular processes, such as migration, proliferation, 33 differentiation and adhesion play pivotal roles in the morphogenesis of a variety of tissues and 34 organs (Ebara, 2002; Hogan, 1996). In particular, BMP-2 was shown to be one of the most 35 powerful osteoinductive factor for bone regeneration (Gautschi, Frey, & Zellweger, 2007; 36 37 Kusumoto et al., 1998; Okubo, Bessho, Fujimura, Iizuka, & Miyatake, 2000). BMP-2 actively contributes to the recruitment, proliferation and differentiation of osteoprogenitor cells during 38 the bone healing process and its amount increases immediately after bone trauma (Caetano-39 Lopes et al., 2011; Zhao et al., 2006). In clinics, to achieve an efficient treatment, 40 supraphysiological doses of BMP-2 are applied to overcome the short half-life of the protein 41 42 due to its rapid proteolytic degradation (Poynton & Lane, 2002). An excessive dosage of BMP-2 is however associated with important side effects such as tissue oedema, inflammation and 43 44 heterotopic ossification (Neovius, Lemberger, Docherty Skogh, Hilborn, & Engstrand, 2013; 45 Shields et al., 2006). To overcome these drawbacks, materials with the ability to strongly, but reversibly bind BMP-2 are developed to allow the growth factor sustained release and its 46 localized presentation, which remain crucial for efficient bone repair over long periods of time 47 48 (Hettiaratchi, Miller, Temenoff, Guldberg, & McDevitt, 2014; Hettiaratchi et al., 2020; Kisiel et al., 2013; Krishnan et al., 2017). Since in the natural extracellular matrix, 49 glycosaminoglycans (GAG), negatively charged polysaccharides, are involved in essential 50 cellular processes through growth factor binding (Gandhi & Mancera, 2008; Miller, Goude, 51 McDevitt, & Temenoff, 2014), delivery systems based on GAG have become particularly 52 53 attractive (Caballero Aguilar, Silva, & Moulton, 2019; Subbiah & Guldberg, 2019). Due to its high BMP-2 affinity resulting from its high sulfate content, heparin was shown to enhance the 54 growth factor osteogenic activity by sequestrating BMP-2 on the cell surface and mediating its 55

internalization (Jeon et al., 2008; Takada et al., 2003; Zhao et al., 2006). Heparin can reduce 56 the amount of BMP-2 bound to cell layer or its receptors and maintain BMP-2 in culture 57 medium, prolonging its biological activity (Takada et al., 2003). Heparin was either 58 incorporated into bulk hydrogels based on hyaluronic acid to improve BMP-2 loading and 59 release (Bhakta et al., 2013; Kisiel et al., 2013) or structured into microparticles, which are well 60 adapted for growth factor loading due to their high surface area-to-volume ratio (Hettiaratchi et 61 62 al., 2014; Hettiaratchi et al., 2020; Tellier, Miller, McDevitt, & Temenoff, 2015). Despite several advantages of using heparin, its anti-coagulant properties may induce adverse bleeding 63 complications and its long-term administration can lead to the reduction of bone density and 64 65 the development of osteoporosis (Wolinsky-Friedland, 1995; Muir et al., 1996). Moreover, its 66 animal origin increases the risk of contamination by a non-conventional transmissible agent such as prions or emerging viruses due to a large "species-barrier" (DeAngelis, 2012). 67

68 Recently, a better understanding of the role of GAG has led to the development of GAGmimetics, molecules displaying GAG structural and functional properties (Badri, Williams, 69 Linhardt, & Koffas, 2018). Different classes of GAG-mimetics have been developed mainly 70 based on natural polymers, especially, polysaccharides obtained from different sources, such as 71 72 plant, algae, animals and microorganisms. Amongst microorganisms, bacteria can synthesize 73 extracellular soluble polysaccharides such as exopolysaccharides (EPS), endowed with both unusual structures and diverse biological functions (Delbarre-Ladrat, Singuin, Lebellenger, 74 Zykwinska, & Colliec-Jouault, 2014). The use of EPS producing bacteria is highly 75 76 advantageous over traditional sources used for polysaccharide output: (i) EPS production in bioreactors can be controlled and optimized to obtain high yields, (ii) polysaccharide is 77 produced by bacteria directly into culture medium, any chemical extraction step is necessary to 78 recover it (in contrast to GAG from animal origin), (iii) fermentation process allows a 79 renewable EPS production with no risks due to raw material supply, and both composition and 80

structure of the final polymer are conserved. GAG-mimetics derived from EPS produced by 81 82 deep-sea hydrothermal vent bacteria display biological activities similar to GAG, especially when they present acidic sugars and sulfate groups (Delbarre-Ladrat et al., 2014; Zykwinska et 83 al., 2019a). Alteromonas infernus is a deep-sea hydrothermal vent bacterium producing a 84 naturally slightly sulfated high-molecular weight EPS, called Infernan (GY785 EPS) (Raguénès 85 et al., 1997; Roger, Kervarec, Ratiskol, Colliec-Jouault, & Chevolot, 2004; Makshakova, 86 87 Zykwinska, Cuenot, Colliec-Jouault, & Perez, 2022). In our previous studies, GAG-mimetic activities, including chondrogenic potential, anticoagulant and antimetastatic properties of the 88 native EPS or its highly sulfated low-molecular weight derivatives, EPS DRS_{LMW} (20,000 -89 90 30,000 g/mol and 12 - 14 wt% S) were shown both in vitro and in vivo (Merceron et al., 2012; Heymann et al., 2016; Rederstorff et al., 2017; Colliec-Jouault et al., 2001). For cartilage 91 engineering purposes, the presence of heparin-mimetic EPS DRS_{LMW} was shown to stimulate 92 93 the *in vitro* chondrogenic differentiation of human adipose-derived mesenchymal stem cells, through its physical interaction with Transforming Growth Factor-B1 (TGF-B1) (Merceron et 94 al., 2012). In these previous studies, Infernan and its derivatives were used in their soluble form. 95 However, we have recently shown that the EPS anionic nature confers to the EPS not only its 96 GAG-mimetic properties, *i.e.* growth factor binding ability but also its capacity to form physical 97 gel with divalent cations, such as calcium (Zykwinska et al., 2019b; Makshakova et al., 2022). 98 This GAG-mimetic EPS was thus structured into bioactive physical microgels for TGF-B1 99 100 delivery using capillary microfluidics. Only few studies deal with a growth factor loading within polysaccharide-based microgels using a microfluidic approach (Moshaverinia, Xu, 101 Chen, Akiyama, Snead, & Shi, 2013; Zykwinska et al., 2019b). However, this technique 102 103 presents several advantages over other emulsification techniques, amongst homogenization and ultrasonication. It allows the formation of monodisperse microdroplets of controlled size, 104 without using high energy and temperature conditions (Zhang, Tumarkin, Sullan, Walker, & 105

Kumacheva, 2007; Marquis, Davy, Cathala, Fang, & Renard, 2015; Zykwinska et al., 2016). 106 107 Microdroplets are solidified to form gelled microparticles or microgels by chemical, photochemical or physical methods directly into the microfluidic device or in the collecting 108 bath. Contrary to other emulsification techniques, where the encapsulation of active 109 compounds, such as growth factors, is ensured by their adsorption inside microdroplets through 110 their diffusion from solution (Hettiaratchi et al., 2014; Hettiaratchi et al., 2020; Tellier et al., 111 112 2015), microfluidic offers a unique possibility to formulate microdroplets and encapsulate the totality of the active compound in only one step process. 113

In this context, the objective of the present study was to further exploit physico-chemical 114 115 (gelling) and biological (GAG-mimetic) properties of Infernan and its derivatives to develop EPS-based microgels appropriate for bone healing purposes. Although the chondrogenic 116 potential of Infernan and its derivatives was clearly shown in our previous studies, their 117 118 osteogenic activity was not completely explored. However, this point remains crucial for further development of efficient treatment for subchondral bone repair. To better understand the impact 119 of the polysaccharide sulfate content on the growth factor binding and release kinetics, BMP-2 120 was either directly loaded into microgels based on a slightly sulfated medium-molecular weight 121 122 EPS derivative (EPS DR_{MMW}) or firstly complexed with highly sulfated low-molecular weight 123 derivative (EPS DRS_{LMW}), exhibiting heparin-mimetic properties, prior to its loading into EPS DR_{MMW} microgels (Figure 1). Both microgels were generated using a capillary microfluidic 124 approach. BMP-2 release from single (EPS DR_{MMW} + BMP-2) and complex (EPS DR_{MMW} + 125 126 EPS DRS_{LMW}/BMP-2) microgels was then assessed in media with different ionic strengths. Bioactivity of growth factor released from microgels was then evaluated by stimulating murine 127 osteoblastic MC3T3-E1 cells. Finally, the effect of BMP-2 released from complex microgels 128 on the *in vitro* osteogenic activity of human bone-marrow derived mesenchymal stem cells 129 (hBM-MSC) was assessed by measuring the alkaline phosphatase (ALP) activity and matrix 130

mineralization ability. The main hypothesis of this study was related to the ability of Infernanbased microgels to efficiently bind and then release bioactive BMP-2 that can further induce
expected cellular responses.



134 Figure 1. (A) Schematic representation of preparation of BMP-2 loaded single (EPS DR_{MMW} + BMP-2)

and complex (EPS DR_{MMW} + EPS $DRS_{LMW}/BMP-2$) microgels based on Infernan (GY785 EPS) by capillary microfluidic approach.

137 2. Materials and Methods

138 2.1. Production of the native Infernan (GY785 EPS) by A. infernus fermentation

139 The production of the EPS was previously described (Raguénès et al., 1997). Briefly, A.

140 *infernus* was cultured at 25°C in Zobell medium composed of yeast extract (1 g/L), tryptone (4

- 141 g/L) and aquarium salts (33.3 g/L) at pH 7.4 in a 30 L fermenter (Techfors 30 L INFORS,
- 142 Switzerland). Glucose at 30 g/L was added as a carbon source. After 48 h of fermentation, the
- culture medium was centrifuged at 9000 g for 45 min, the supernatant containing soluble EPS
- 144 was ultrafiltrated on a 100 kDa cut-off membrane and freeze-dried.

145

147 2.2. Preparation of EPS derivatives, EPS DR and EPS DRS, and their physico-chemical 148 characterizations.

EPS derivatives were prepared, as previously described (Chopin et al., 2015). Briefly, the native 149 EPS (2.5 g) solubilized in water (350 mL) was depolymerized at 60°C for 45 min using 150 hydrogen peroxide added dropwise. After overnight reduction by sodium borohydride and 151 purification on Chelex® 20 resin, the solution containing depolymerized EPS (EPS DR) was 152 ultrafiltrated on a 10 kDa cut-off membrane and freeze-dried. To obtain homogeneous fractions 153 of EPS DR with low polydispersity, a predominant population of polysaccharide chains with a 154 155 narrow molecular weight distribution was selected by a gel filtration chromatography on either Superdex® 30 (EPS DR_{LMW} of 20,000 g/mol) or Sephacryl S-100 HR (EPS DR_{MMW} of 260,000 156 g/mol) (Cytiva), using an AKTA FPLC system coupled with a refractometric detector (Hitachi 157 158 L2490). Samples eluted with water were pooled and freeze-dried.

Highly sulfated low-molecular weight derivative (EPS DRS_{LMW}) was obtained by a chemical
oversulfation of EPS DR_{LMW} of 20,000 g/mol, as descried earlier (Zykwinska *et al.*, 2019b;
Chopin *et al.*, 2015). Briefly, EPS DR_{LMW} (50 mg) in its pyridinium salt form was firstly
solubilized in anhydrous DMF (100 mL) at 45°C for 2 h under continuous stirring and then
sulfated for the next 2 h at 45°C in the presence of SO₃.Py (250 mg). The final aqueous solution
(pH 7) was dialyzed against water for three days prior to be freeze-dried.

165 Monosaccharide composition of the native EPS and EPS DR was determined according to 166 Kamerling *et al.* (1975) method, modified by Montreuil *et al.* (1986). EPS DRS_{LMW} could not 167 be analyzed properly due to its high sulfate content, which prevents from correct derivatization 168 and formation of the per-O-trimethylsilyl methyl glycosides. Briefly, samples were hydrolyzed 169 using MeOH/HCl at 100°C for 4 h. Myo-inositol was used as internal standard. The methyl 170 glycosides thus obtained were then converted to trimethylsilyl derivatives using *N*,*O*-171 bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA:TMCS) 99:1. Gas 172 chromatography (GC-FID, Agilent Technologies 6890N) was used for separation and173 quantification of the per-O-trimethylsilyl methyl glycosides formed.

Weight-average molecular weight of the native EPS and its derivatives (EPS DR and EPS DRS)
was determined by High-Performance Size-Exclusion Chromatography (HPSEC, Prominence
Shimadzu) coupled with a multiangle light scattering (MALS, Dawn Heleos-II, Wyatt
Technology) and a differential refractive index (RI) (Optilab Wyatt technology) detectors.
Samples were eluted with 0.1 M ammonium acetate. The molecular weight was calculated using
a refractive index increment dn/dc of 0.145 used for polysaccharides.

The linked ester sulfate group content of the native EPS and its derivatives (EPS DR and EPS DRS) was determined using High-Performance Anion-Exchange Chromatography (HPAEC)
(Dionex DX-500) by calculating the difference between the total sulfur content in the hydrolyzed sample and in the non-treated sample (free sulfur), as previously described by Chopin *et al.* (2015).

185 2.3. Single and complex microgel formation using capillary microfluidics

Single microgels (EPS DR_{MMW} + BMP-2) were prepared by mixing 1.5 mL of EPS DR_{MMW} 186 (260,000 g/mol) aqueous solution (final concentration of 30 mg/mL) with 500 µL of 187 188 recombinant human BMP-2 (CHOcells, PeproTech, Neuilly-sur-Seine, France) at 34 µg/mL in water. To prepare complex microgels (EPS DR_{MMW} + EPS DRS_{LMW}/BMP-2), 500 µL of EPS 189 DRS_{LMW} (31,000 g/mol) aqueous solution at 3.4 mg/mL was mixed with 500 µL of BMP-2 at 190 34 µg/mL in water and incubated for 1 h at 37°C under gentle stirring. EPS DRS_{LMW}/BMP-2 191 complex solution was then added to 1 mL of EPS DR_{MMW} aqueous solution at the final 192 193 concentration of 30 mg/mL. To structure EPS DR_{MMW} into microgels, a home-made capillary microfluidic co-flow device was developed. The dispersed phase containing either EPS DR_{MMW} 194 + BMP-2 or EPS DR_{MMW} + EPS DRS_{LMW}/BMP-2 aqueous solution was delivered through a 195 silica tube with interior diameter of 75 µm inserted into chromatography tee fitting. The 196

continuous phase, a sunflower seed oil (Fluka), was delivered through a Fluorinated Ethylene 197 198 Propylene (FEP) 1/16" tube (ID 750 µm, OD 1.57 mm) (Cluzeau Info Labo, France) perpendicularly to the tee fitting. The dispersed phase co-flowed in a FEP 1/16" tube (ID 750 199 µm, OD 1.57 mm) containing the continuous oil phase. The following flow rates were applied 200 to produce microdroplets: dispersed phase at 2 µL/min and continuous phase at 120 µL/min. 201 The flow rates were controlled using Microfluidic Flow Control System MFCSTM-EZ with 202 203 flow-rate platform (Fluigent). The device was run for 30 min per sample, allowing to prepare single and complex microgels with the final concentrations of BMP-2 and EPS DRS_{LMW} of 204 around 500 ng/mL and 50 µg/mL (in 1 mL), respectively. Microgels were recovered in 205 206 collecting bath containing 10 mL of 360 mM CaCl₂. After overnight storage at 4°C, microgel 207 suspensions were washed three times with either 2 mM or 180 mM CaCl₂ aqueous solutions prior to release experiments started immediately after washing step. 208

209 2.4. In vitro BMP-2 release from microgels and stability assessment of microgels

210 In vitro release study was performed in three different conditions: Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher Scientific), 2 mM and 180 mM CaCl₂ aqueous solutions at 211 37°C. Firstly, BMP-2 release was performed in DMEM containing Bovine Serum Albumin 212 213 (BSA) at 1%. Single and complex microgel suspensions after washing step in 2 mM CaCl₂ were either directly incubated in DMEM/BSA 1% (non-equilibrated microgels) or firstly equilibrated 214 in 1 mL of 2 mM CaCl₂ for another 24h at 4°C and then incubated in 1 mL of DMEM/BSA 1% 215 (equilibrated microgels). For the release study in CaCl₂ solutions, single and complex microgels 216 were either directly incubated in 1 mL of 180 mM CaCl₂/BSA 1% after washing step in the 217 218 same solution or firstly equilibrated in 1 mL of 2 mM CaCl₂ for 24h at 4°C followed by the release experiment in 1 mL of 2 mM CaCl₂/BSA 1%. All samples were incubated at 37°C under 219 continuous shaking for 23 or 27 days. At different incubation times, 500 µL of the supernatant 220 were removed after centrifugation step (5 min, 2000 g) and replaced by a fresh buffer (500 μ L). 221

Release experiments were performed in duplicate for each condition and each time from two 222 223 independent encapsulation experiments. The recovered supernatants were stored at -20°C prior to analyses. The amount of BMP-2 released from microgels during (i) the overnight storage in 224 360 mM CaCl₂ at 4°C, (*ii*) the equilibration step in 2 mM CaCl₂ for 24h at 4°C and (*iii*) the 225 release experiments run for 23 or 27 days at 37°C was determined using ELISA Duoset® assay 226 227 kit (DY355, R&D Systems – Bio-Techne). The standard curves were obtained in an appropriate 228 buffer, 2 mM CaCl₂, 180 mM CaCl₂ or DMEM. In addition, the standard curves using BMP-2 mixed with EPS DRS_{LMW} at 1, 5 and 50 μ g/mL in DMEM were prepared. 229

In parallel to the release experiments, the stability of microgels was assessed in each condition
tested by measuring the microgel size every day using an optical microscope (Optika, Italy).
Scanning Electron Microscopy (SEM) observations on freeze-dried microgels were performed
using JEOL 7600F instrument (JEOL, US).

234

235 **2.5. Surface Plasmon Resonance (SPR).**

SPR experiments were carried out on a Biacore 3000 instrument (Cytiva). BMP-2 (CHOcells, 236 PeproTech, Neuilly-sur-Seine, France) was covalently immobilized to the dextran matrix of a 237 CM5 sensor chip (Biacore), as recommended by the manufacturer at a flow rate of 5 μ L/min. 238 239 Binding assays of EPS DR_{LMW} (20,000 g/mol), EPS DRS_{LMW} (31,000 g/mol) and heparin (15,000 g/mol, H4784 Sigma) were performed in 10 mM HEPES at pH 7.4 containing 150 mM 240 NaCl and 0.005% P2O surfactant (HBS-P buffer, Biacore) with dissociation monitored for 15 241 242 min. Regeneration was achieved with NaOH (4.5 mM/L) after each cycle. The resulting sensorgrams were fitted using BiaEval 4.1 software (Biacore). 243

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247 **2.6.** Atomic Force Microscopy (AFM) imaging.

248 For AFM imaging, BMP-2 was prepared at 5 µg/mL in water. Solutions of EPS DRS_{LMW}/BMP-2 complexes and of EPS DR_{MMW} containing EPS DRS_{LMW}/BMP-2 complexes prepared for 249 microfluidic encapsulation were observed after 5-fold and 200-fold dilution in water, 250 respectively. Five µL of each solution were deposited onto freshly cleaved mica surface and 251 dried at 20°C. AFM images were recorded using a NanoWizard® Atomic Force Microscope 252 253 (JPK, Germany) in intermittent contact mode in air at room temperature. A standard rectangular cantilever (Nanosensors NCL-W) with a free resonance frequency of 170 kHz and a spring 254 constant of 40 N/m was used. AFM tip with a radius curvature of ~5 nm was cleaned by UV-255 256 ozone treatment prior to AFM observations. Each sample was imaged on three different zones. 257 JPK Data Processing software (JPK, Germany) was used for image processing and height measurements (N = 50 per sample). 258

259 2.7. Size distribution of BMP-2, EPS DRS and EPS DRS/BMP-2 complexes by dynamic 260 light scattering (DLS) measurements

A Zetasizer Nano Series (Malvern Instrument, UK) was used to determine the hydrodynamic 261 diameter of soluble BMP-2 (500 ng/mL), soluble EPS DRS_{LMW} (50 µg/mL) and EPS 262 263 DRS_{LMW}/BMP-2 complexes, as prepared for microfluidic encapsulation. Additionally to the control samples, the supernatants recovered during the incubation of complex microgels in 264 either 2 mM or 180 mM CaCl₂ for 48h were analyzed. Solutions were analyzed at 20°C in a 265 backscattering configuration at 173° for 120 s. The hydrodynamic diameter was measured in 266 triplicate for each sample. A refractive index of 0.145 was used to determine the volume-size 267 distribution. 268

269

271 **2.8. BMP-2 bioactivity assay.**

Bioactivity of BMP-2 released from microgels was assessed by measuring activation of Smads 272 1,5,8 signaling pathway after stimulation of murine MC3T3-E1 Subclone 4 (CRL-2593TM from 273 274 ATCC) with the supernatants from release experiments, as described previously (Guicheux et al., 2003). MC3T3-E1 cells were cultured in α -MEM supplemented with 10% Fetal Bovine 275 Serum (FBS, Dominique Dutscher) and 1% glutamine and 1% Penicillin/Streptomycin (P/S, 276 Thermo Fisher Scientific) at 37°C and 5% CO₂. Cells were seeded on six-well plates at 40,000 277 cells/cm² and proliferated until reaching 80% confluence. To synchronize the cells, the medium 278 was changed to 0.5% FBS 12h before stimulation. 279

MC3T3-E1 were stimulated with the supernatants containing BMP-2 released from complex 280 281 microgels (EPS DR_{MMW} + EPS DRS_{LMW}/BMP-2) in DMEM or in 2 mM CaCl₂. Soluble BMP-2 (sBMP-2) (100 ng/mL), soluble EPS DRS_{LMW} (50 μ g/mL) and EPS DRS_{LMW}/BMP-2 282 complex (final concentration of 50 µg/mL EPS DRS_{LMW} and 500 ng/mL BMP-2) were used as 283 controls. Release supernatants recovered at different time points: day D0 (4h or 5 h), D1, D2, 284 D5, D7, D14, D21 were incubated with MC3T3-E1 for 1 h at 37°C. Medium was then removed 285 286 and cells were snapped freezed in liquid nitrogen. Proteins were then extracted on ice using homemade RIPA buffer and assayed with the Pierce BCATM Protein Assay Kit (23225, Thermo 287 Fisher Scientific). For Western Blot experiments, after migration on AnykD Criterion[™] Midi 288 TGX[™] Stain-Free precast gels (Bio-Rad), proteins were transferred onto Trans-Blot Turbo 289 Midi PVDF transfer Packs (Bio-Rad) using the Transblot® Turbo[™] Transfer System (Bio-290 Rad). Total Smad 1 and Phosphorylated-Smad 1,5,8 were analyzed using specific antibodies 291 292 for cells stimulated with BMP-2, as described previously (Guicheux et al., 2003). All primary antibodies were revealed by an anti-rabbit secondary antibody (Cell Signalling) and the 293 SuperSignalTMWest Dura substrate for Smad 1 or Femto substrate for Phosphorylated-Smad 294 1,5,8 (ThermoFisher Scientific) using the ChemiDocTM MP Imaging System (Bio-Rad) and the 295

Image Lab software. Bioactivity assay was performed in duplicate using equilibrated complexmicrogels from two encapsulation experiments.

298 2.9. Effect of BMP-2 released from complex microgels on the *in vitro* osteogenic activity 299 of hBM-MSC.

The in vitro osteogenic activity of hBM-MSC (451Z012.1 from PromoCell) in the presence or 300 absence of BMP-2 released from equilibrated complex microgels was studied by measuring 301 302 ALP activity and Alizarin red staining. hBM-MSC cells were seeded on 24-well plates at 10,000 cells per cm² in 500 µL of DMEM supplemented with 10% FBS and 1% 303 304 Penicillin/Streptomycin (P/S, ThermoFisher Scientific) at 37°C and 5% CO₂. After 24h, medium was switched to osteogenic differentiation medium (ODM) composed of DMEM 305 supplemented with 10% FBS, 1% P/S, 10 mM β-glycerophosphate, 50 μM ascorbic acid and 306 dexamethasone. Control medium was composed of DMEM supplemented with 10% FBS and 307 308 1% P/S. Suspension of equilibrated complex microgels loaded with BMP-2 (EPS DR_{MMW} + EPS DRS_{LMW}/BMP-2) was deposited (200 µL in DMEM) on Transwell inserts with an 8 µm 309 pore polycarbonate membrane (Corning, 3422, Kennebunk ME, USA) placed above 24-well 310 plate with hBM-MSC. In parallel, unloaded complex microgels (EPS DR_{MMW} + EPS DRS_{LMW}), 311 unloaded complex microgels supplemented with soluble BMP-2 (100 ng/mL) (EPS DR_{MMW} + 312 313 EPS DRS_{LMW} + sBMP-2), soluble BMP-2 (100 ng/mL) and DMEM were used as controls. Transwell experiments (two independent experiments performed in duplicate) were carried out 314 315 for either 7 or 16 days in ODM changed every three days. Soluble sBMP-2 was supplemented every three days. ALP activity of hBM-MSC stimulated by BMP-2 from transwell experiments 316 was measured according to the Bessey-Lowry enzymological method (Bessey, Lowry, & 317 Brock, 1942). Cells were washed twice with PBS and then dry frozen at -80°C. ALP activity in 318 319 cell lysate was measured by Alkaline Phosphatase Substrate kit (Biorad 172-1063) using p-

nitrophenyl phosphate (pNPP). ALP activity was normalized by a total protein content 320 321 determined using the Pierce BCA[™] Protein Assay Kit (23225, Thermo Fisher Scientific). Alizarin red staining was performed after 16 days of culture, as described by Lavenus et al. 322 (2011). As before, the mineralization was determined in both standard and osteogenic media. 323 The culture medium was discarded, confluent cell layers were washed with PBS and staining 324 with alizarin red S (2%, pH 4.2, Merck) was performed for 2 min. After aspiration of the 325 326 overflow, cells were washed three times with water. Staining layers were visualized by optical microscopy (Nikon Instruments Inc.). 327

328 **3. Results and discussion**

329 **3.1. Native EPS and its derivatives.**

Native EPS synthesized by A. infernus is a naturally slightly sulfated highly branched anionic 330 heteropolysaccharide with a complex repeating unit composed of nine osidic residues 331 (Raguénès et al., 1997; Roger et al., 2004). Its main chain is composed of glucose (Glc), 332 galacturonic acid (GalA) and galactose (Gal), covalently linked in the sequence: \rightarrow 4)- β -D-333 Glcp- $(1\rightarrow 4)$ - α -D-GalpA- $(1\rightarrow 4)$ - α -D-Galp- $(1\rightarrow . GalA residue is substituted at O-2 by one$ 334 sulfate group and at O-3 by a short side chain constituted of two glucuronic acids (GlcA), Gal 335 and Glc linked in the sequence: β -D-Glcp-(1 \rightarrow 6)- α -D-Galp-(1 \rightarrow 4)- β -D-GlcpA-(1 \rightarrow 4)- β -D-336 GlcpA- $(1 \rightarrow . In addition, the two GlcA of the side chain are each substituted by a terminal Glc$ 337 (Roger et al., 2004). The presence of three consecutive uronic acids per repeating unit and one 338 sulfate group confers to Infernan its gelling properties in the presence of divalent cations, such 339 as calcium (Zykwinska et al., 2019b; Makshakova et al., 2022). It was previously shown that 340 341 by decreasing the molecular weight of the native EPS (~2,000,000 g/mol) to intermediate molecular weight derivatives, physical gelation with calcium ions was favoured. Herein, 342 slightly sulfated medium-molecular weight EPS DR_{MMW} of ~260,000 g/mol was prepared by a 343

free-radical depolymerization of the native EPS. The depolymerization reaction had no major 344 345 impact on the polysaccharide structure (Table 1). Besides decreasing the molecular weight, the depolymerization process was previously shown to efficiently decrease the amount of the 346 lipopolysaccharides (LPS) present within the EPS produced by A. infernus, being a Gram-347 negative bacterium (Grivaud-Le Du et al., 2017). Indeed, the endotoxin level decreased from 348 72,500 EU/mg for the native high molecular weight EPS to 135 EU/mg for the low-molecular 349 350 weight derivative of 30,000 g/mol. Biochemical and chemical analyses of the LPS extracted from A. infernus membranes have shown the lack of the typical LPS architecture (absence of 351 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo), glucosamine (GlcN), and phosphorylated 352 353 monosaccharides), such as that known for Escherichia coli (Grivaud-Le Du et al., 2017). This suggests that the outer membrane of A. infernus is most likely composed of peculiar glycolipids, 354 which did not induce toxic effects, as previously observed in our in vivo studies (Heymann et 355 356 al., 2016; Rederstorff et al., 2017).

Table 1. Monosaccharide composition (wt%), sulfur content (wt%) and weight average molecular
weight, Mw (g/mol) of the native slightly sulfated high-molecular weight EPS, slightly sulfated medium
molecular weight derivative, EPS DR_{MMW}, slightly sulfated low-molecular weight derivative, EPS
DR_{LMW} and highly sulfated low-molecular weight derivative, EPS DRS_{LMW}.

	Monosaccharide composition (wt%)							S	Mw
	Rha	Fuc	Man	Gal	Glc	GlcA	GalA	(wt%)	(g/mol)
Native EPS	2.2	1.5	4.2	10.4	18.5	12.7	7.6	3.2	2,000,000
EPS DR _{MMW}	1.1	0.7	2.5	8.8	12.8	15.7	9.2	3.2	260,000
EPS DR _{LMW}	0	0	0	12.2	11.9	9.8	7.3	3.3	20,000
EPS DRS _{LMW}	nd	nd	nd	nd	nd	nd	nd	14.0	31,000

Rha: rhamnose, Fuc: fucose, Man: mannose, Gal: galactose, Glc: glucose, GlcA: glucuronic acid, GalA:galacturonic acid.

363 nd: non-determined

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365

367 3.2. Single and complex microgel formation by capillary microfluidics.

368 In the following step, EPS DR_{MMW} was formulated in BMP-2 loaded microgels using a homemade capillary microfluidic co-flow device (Figure 1). To obtain single microgels (EPS 369 DR_{MMW} + BMP-2), BMP-2 was directly solubilized in EPS DR_{MMW} aqueous solution and 370 formulated into microgels. In the case of complex microgels (EPS DR_{MMW} + EPS 371 DRS_{LMW}/BMP-2), BMP-2 was firstly incubated for 1h at 37°C with highly sulfated low-372 373 molecular weight derivative, EPS DRS_{LMW} (31,000 g/mol and 14 wt% S) obtained after oversulfation of low-molecular weight derivative, EPS DR_{LMW} (20,000 g/mol and 3.3 wt% S) 374 (Table 1). EPS DRS_{LMW}/BMP-2 complex was then incorporated within EPS DR_{MMW} aqueous 375 376 solution, which was further structured in complex microgels. Single and complex microgels 377 with similar sizes of 463 μ m ± 63 μ m in diameter were collected in 360 mM CaCl₂ bath by applying the same experimental conditions (the same flows of disperse and continuous phases) 378 379 (Figure 2A). No major differences in morphologies were noticed between single and complex microgels, as revealed by SEM (Figure 2B). In both cases, a porous structure was clearly 380 observed. 381



Figure 2. (A) Optical microcopy image of complex microgels collected in 360 mM CaCl₂ bath. (B) SEM
image of freeze-dried complex microgels.

The complexation of positively charged BMP-2 (pI \sim 9) with negatively charged EPS DRS_{LMW} before growth factor loading inside microgels may further preserve the growth factor from degradation and improve its half-life and bioactivity. SPR analyses revealed very high binding

affinity of BMP-2 for EPS DRS_{LMW} with dissociation constant Kd of 0.63 nM, compared to 387 388 that obtained for EPS DR_{LMW} with Kd of 0.1 µM. Growth factor affinity for EPS DRS_{LMW} was even higher than for unfractionated heparin (15,000 g/mol) with Kd of 8.29 nM for similar 389 sulfur content (12-14 wt% S). To reveal the ability of EPS DRS_{LMW} to bind BMP-2 and to form 390 assemblies, AFM imaging was used (Figure 3). Free BMP-2 in water was compared to BMP-2 391 mixed with EPS DRS_{LMW} (EPS DRS_{LMW}/BMP-2 complex) and to EPS DRS_{LMW}/BMP-2 392 393 complex incorporated in EPS DR_{MMW} solution, as used for microfluidic encapsulation. Figure 3A shows BMP-2 nanoparticles with diameters varying from 2 to 10 nm (height measurements), 394 which are consistent with the size of the protein homodimer of 70 Å x 35 Å x 30 Å, the 395 396 biologically active form (Scheufler, Sebald, & Hülsmeyer, 1999). When BMP-2 was mixed with the highly-sulfated EPS derivative to prepare EPS DRS_{LMW}/BMP-2 complexes for 397 microfluidic encapsulation, only larger nanoparticles of 10 nm to 30 nm in diameter were 398 399 observed, indicating the absence of free BMP-2 (Figure 3B). It appeared that upon incubation, positively charged BMP-2 co-assembled with negatively charged EPS DRS_{LMW}, leading to 400 nanoparticles of higher diameters. Incorporation of these complexes into EPS DR_{MMW} solution 401 prior to microfluidic encapsulation, caused further nanoparticle assembly, since an increase in 402 403 measured diameters was noted. Indeed, the nanoparticle assembly diameters varied from 15 nm 404 to 60 nm (Figure 3C). The ability to form nanoassemblies between BMP-2 and EPS DRS_{LMW} may have a considerable impact on both growth factor bioactivity and biodisponibility. In our 405 previous study, only highly sulfated EPS derivative was shown to stimulate in vitro hATSC 406 407 chondrogenic differentiation in the presence of TGF- β 1, probably through electrostatic interactions with the growth factor (Merceron et al., 2012). Non-covalent binding of TGF-β1 408 409 to EPS DRS_{LMW} enhanced the growth factor bioactivity, in comparison to a slightly sulfated EPS DR_{LMW} (Merceron et al., 2012; Zykwinska et al., 2019b). It was shown by AFM that the 410

411 positively charged TGF- β 1 also formed nanoassemblies with the negatively charged EPS

412 DRS_{LMW} (Zykwinska *et al.*, 2018).



413

Figure 3. AFM height images $(2 \ \mu m \ x \ 2 \ \mu m)$ and height distribution of (A) BMP-2, (B) EPS DRS_{LMW}/BMP-2 complexes and (C) EPS DR_{MMW} with EPS DRS_{LMW}/BMP-2 complexes. All sample solutions in water were dried on mica surface at 20°C prior to imaging in intermittent contact mode in air.

418 **3.3.** *In vitro* **BMP-2** release from single and complex microgels.

BMP-2 release was firstly followed under *in vitro* conditions by incubating single or complex microgels in cell culture medium (DMEM). Since DMEM is particularly rich in inorganic salts, the effect of salt concentration on BMP-2 release from microgels was further assessed at two different ionic strengths, either at physiological calcium concentration in 2 mM CaCl₂ or at high ionic strength in 180 mM CaCl₂. Considering that the microfluidic approach allows to encapsulate the totality of BMP-2 during microdroplet formulation and by taking into account the run time for the microgel production, the flow rate of the dispersed phase and the growth factor concentration, it can be estimated that around 500 ng/mL of BMP-2 were encapsulated within both single and complex microgels per tube used for each release experiment performed in 1 mL. The amount of BMP-2 released from both single and complex microgels measured by ELISA during their overnight storage in 360 mM CaCl₂ at 4°C as well as during their equilibration in 2 mM CaCl₂ for 24h at 4°C was very low (< 2 ng/mL). The amount determined was systematically added to each release experiment at day 0.

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433 *3.3.1. BMP-2 release from single and complex microgels in DMEM.*

434 The profiles obtained for BMP-2 released from single and complex microgels upon incubation 435 in DMEM are presented on Figure 4A. Low growth factor release was observed from single microgels (EPS DR_{MMW} + BMP-2), as only 8.4 ng of BMP-2 were quantified in the supernatant 436 437 after 3 days of incubation and no evolution was further observed. Weak growth factor binding to single microgels may result in a rapid BMP-2 degradation leading to undetectable protein. 438 In contrast, different profiles were observed for BMP-2 loaded complex microgels, depending 439 if they were equilibrated in 2 mM CaCl₂ for 24 h before release in DMEM or not. Equilibrated 440 441 complex microgels exhibited fast growth factor release kinetic with a burst release of 29 ng of 442 BMP-2 for the first 5 h, followed by a rapid increase in growth factor concentration during the first 2 days (27 ng of BMP-2 per day) up to 83 ng. Afterwards, the amount of growth factor 443 increased only slightly to reach 103 ng at 7 days with a rate of 4 ng per day. No increase in 444 445 BMP-2 released was further observed. In comparison, the release kinetic from non-equilibrated complex microgels was considerably slower. Lower burst release of 11.5 ng of BMP-2 during 446 447 the first 4 h was firstly observed, followed by a sustained growth factor release up to 65.8 ng of BMP-2 for 15 days with a rate of 3.6 ng per day. Then, slower release rate of 0.5 ng of BMP-2 448 per day was still observed to reach 72 ng of BMP-2 released during 27 days. Considering that 449

the initial amount of the growth factor encapsulated within microgels was of 500 ng/mL, the
total release of BMP-2 in equilibrated and non-equilibrated complex microgels was of 20.6%
and 14.4%, respectively.

Greater and faster BMP-2 diffusion from equilibrated complex microgels, observed especially 453 during the first hours, is due to the mechanical weakness of the physical microgel network, 454 resulting from the equilibration step. In this step, microgels collected in 360 mM CaCl₂ were 455 incubated in 2 mM CaCl₂ for 24h at 4°C to decrease the calcium concentration to a 456 physiological calcium concentration present in DMEM. During this ionic concentration change, 457 the density of cross-links between polysaccharide chains mediated by calcium ions decreased, 458 459 leading to a looser network compared to the initial network formed in 360 mM CaCl₂. At this step, a weak swelling of ~15% was observed, as microgel diameter increased from 463 μ m \pm 460 63 μ m to 535 μ m ± 53 μ m. As microgels swelled only weakly, the release of BMP-2 from the 461 microgels during this step was very low, as measured by ELISA assay (< 2 ng/mL). The 462 463 difference in the network cross-linking density between equilibrated and non-equilibrated microgels clearly impacted the release profiles measured in DMEM at 37°C. Indeed, the 464 presence of inorganic salts (e.g. 110 mM sodium chloride) provoked a faster network loosening 465 466 resulting from calcium exchange in equilibrated microgels compared to non-equilibrated ones. A rapid swelling of both complex microgels was observed within the first 24 h, where the 467 microgel diameter increased up to $719 \pm 53 \,\mu\text{m}$ and $824 \,\mu\text{m} \pm 85 \,\mu\text{m}$ for non-equilibrated and 468 equilibrated complex microgels, respectively. After 5 days of incubation in DMEM, mainly 469 microgel debris were observed. 470

471

472 *3.3.2. BMP-2* release from single and complex microgels at two ionic strengths.

The effect of salt concentration on BMP-2 release from single and complex microgels was
further studied in CaCl₂ aqueous solutions at two different ionic strengths, either 2 mM or 180

mM (Figure 4B). At high ionic strength (180 mM CaCl₂), single microgels displayed only weak 475 476 BMP-2 release with a plateau value of 9 ng rapidly reached within the first 3 days, as already 477 observed in DMEM. It appeared once again that single microgels are not suitable for BMP-2 release. Indeed, slightly sulfated EPS DR_{MMW} used for microgel formation is unable to 478 efficiently protect BMP-2 from degradation. Similar results were reported for partially 479 desulfated heparin microparticles, where BMP-2 release was shown to be dependent on 480 481 sulfation pattern, with the lowest release of detectable growth factor observed from more desulfated heparin microparticles (Tellier et al., 2015). However, upon incubation of complex 482 microgels in 180 mM CaCl₂, a sustained release of BMP-2 was observed with the initial burst 483 484 release of 5.8 ng of BMP-2 for the first 4 h. Then, a progressive release of BMP-2 was observed for 18 days, with the rate of 3.7 ng per day, to reach a plateau value of 73 ng of BMP-2 released. 485 The BMP-2 release kinetic at high ionic strength was very similar to that observed in DMEM 486 487 for non-equilibrated complex microgels with the total release of 14.6% of BMP-2 initially loaded. Interestingly, when complex microgels were incubated at low ionic strength (2 mM 488 CaCl₂), very low growth factor release was observed, as only 3.8 ng of BMP-2 were quantified 489 in the supernatant for 23 days. During the release experiments performed in CaCl₂ solutions at 490 37° C, complex microgels progressively swelled to reach the diameters of $902 \,\mu\text{m} \pm 82 \,\mu\text{m}$ and 491 492 $723 \,\mu\text{m} \pm 74 \,\mu\text{m}$ in 2 mM and 180 mM CaCl₂, respectively, at 14 days of incubation. Beyond 14 days, microgels started to dissolve. It was noted that in the case of CaCl₂ solutions, the 493 incubation at 37°C induced swelling and final dissolution of microgels, while they remained 494 495 almost unchanged during their storage at 4°C for one month.



Figure 4. (A) BMP-2 cumulative release from non-equilibrated single microgels (EPS $DR_{MMW} + BMP-$ 2), equilibrated (2 mM CaCl₂, 24h, 4°C) and non-equilibrated complex microgels (EPS $DR_{MMW} + EPS$ DRS_{LMW}/BMP-2) upon incubation in DMEM at 37°C for 23 or 27 days. (B) BMP-2 cumulative release from single microgels (EPS $DR_{MMW} + BMP-2$) and complex microgels (EPS $DR_{MMW} + EPS$ DRS_{LMW}/BMP-2) upon incubation in either 2 mM CaCl₂ or 180 mM CaCl₂ at 37°C for 23 or 27 days. Release experiments were performed in duplicate for each condition and each time from two independent encapsulation experiments.

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The difference in BMP-2 release kinetics from complex microgels incubated at low and high 505 506 ionic strengths results most likely from the fact that at high salt concentration the screening of 507 charges decreased the binding strength between the positively charged BMP-2 and negatively charged EPS DRS_{LMW}, favouring their dissociation. Similar results were reported for lysozyme 508 509 released from microparticles based on methacrylated hyaluronic acid and chondroitin sulfate at different ionic strengths (Schuurmans et al., 2018). By dissociating from the highly sulfated 510 derivative, soluble BMP-2 can then be quantified by ELISA assay. Indeed, tight complexation 511 512 of BMP-2 with EPS DRS_{LMW}, even at polysaccharide concentrations higher than 5 µg/mL, prevented its appropriate quantification most likely through inhibition of binding between 513 BMP-2 and ELISA antibodies. Similar release profiles obtained in 180 mM CaCl₂ and DMEM 514

515 (Figure 4A and 4B) suggest that in DMEM, particularly rich in inorganic salts, BMP-2 also 516 dissociated from EPS DRS_{LMW} . It was further observed that only 14% to 20% of the initially 517 loaded growth factor was quantified, which could be due to incomplete BMP-2 dissociation 518 from EPS DRS_{LMW} . Similar results were also reported by Hettiaratchi *et al.* (2014), where less 519 than 20% of the BMP-2 initially loaded was released from heparin microparticles, suggesting 520 that the majority of the growth factor was retained inside the microparticles.

521 Although BMP-2 unbinding can appear inside microgels, it can also be thought that EPS DRS_{LMW}/BMP-2 complex may diffuse outside the microgels and then dissociate or not, 522 depending on the ionic strength applied. To explore this hypothesis, the supernatants recovered 523 524 during BMP-2 release experiments at low and high ionic strengths were analyzed using 525 dynamic light scattering (DLS) and compared to control samples (BMP-2, EPS DRS_{LMW} and EPS DRS_{LMW}/BMP-2 complex in water) (Figure 5). Two populations with hydrodynamic 526 527 diameters of 3.1 nm and 8.7 nm were measured for free BMP-2, which are consistent with the size of the protein homodimer and the diameters of nanoparticles measured by AFM (Figure 528 3A). For EPS DRS_{LMW}, a predominant population of 28 nm was observed together with larger 529 aggregates of 295 nm in diameter, resulting most likely from further chain-chain associations. 530 531 When BMP-2 was complexed with EPS DRS_{LMW} in water, two populations were observed with 532 38 nm and 342 nm in diameter, corresponding to EPS DRS_{LMW}/BMP-2 complex and EPS DRS_{LMW}, respectively. No peak of free BMP-2 was observed. 533

In the supernatant recovered during BMP-2 release from complex microgels at high ionic strength (180 mM CaCl₂), the presence of soluble BMP-2 was noticed with the peak at 7.5 nm, followed by a second peak at 24.4 nm corresponding to soluble EPS DRS_{LMW} and/or EPS DRS_{LMW}/BMP-2 complex. The high amplitude of the first peak indicates the presence of free BMP-2, suggesting dissociation of EPS DRS_{LMW}/BMP-2 complex. The low amplitude of the second peak relative to EPS DRS_{LMW} alone and/or EPS DRS_{LMW}/BMP-2 complex suggests that

this dissociation may already take place inside microgels. In contrast, in the supernatant 540 recovered during BMP-2 release from complex microgels at low ionic strength (2 mM CaCl₂), 541 only one large peak at 18.2 nm was observed, which could be attributed to EPS DRS_{LMW}/BMP-542 2 complex and EPS DRS_{LMW}, while free BMP-2 was not observed. Therefore, it comes out that 543 at low ionic strength EPS DRS_{LMW}/BMP-2 complexes diffuse outside the microgels and they 544 do not dissociate at this low salt content. Diffusion of these complexes is facilitated by a looser 545 microgel network formed in 2 mM CaCl₂. Hydrodynamic diameters of BMP-2 and EPS 546 DRS_{LMW}/BMP-2 complexes measured by DLS in solution are in good agreement with AFM 547 data presented on Figure 3. 548



Figure 5. Hydrodynamic diameter of soluble BMP-2 (500 ng/mL), soluble EPS DRS_{LMW} (50 µg/mL) and EPS DRS_{LMW}/BMP -2 complexes in water as well as the supernatants recovered during the incubation of complex microgels in either 2 mM or 180 mM CaCl₂ for 48h.

552 **3.4. BMP-2 bioactivity.**

To assess the bioactivity of BMP-2 released from complex microgels in either DMEM or 2 mM CaCl₂, the phosphorylation of Smads 1,5,8 intracellular signaling proteins was investigated. Indeed, Smad 1 and the closely related Smads 5 and 8, specifically mediate BMP-2 responses, including the osteoblastic differentiation of precursor cell lines (Guicheux *et al.*, 2003;

Nishimura et al., 1998; Yamamoto et al., 1997). MC3T3-E1 cells were stimulated with the 557 supernatants recovered after incubation of equilibrated complex microgels in either DMEM or 558 2 mM CaCl₂. It was clearly observed that BMP-2 maintained its biological activity, as 559 evidenced by the activation of phosphorylation of Smads 1,5,8 (Figure 6). For BMP-2 released 560 from complex microgels incubated in DMEM, a strong phosphorylation was observed over the 561 entire first week and lower but still detectable activation after 14 and 21 days. Very similar 562 563 results were observed in 2 mM CaCl₂ with a detectable activation measured up to 14 days. It appeared that BMP-2 complexation with EPS DRS_{LMW} did not preclude the appropriate binding 564 of the BMP-2 to cell surface type I and II serine/threonine kinase receptors. The presence of 565 566 this highly sulfated derivative seems essential to preserve the growth factor bioactivity for long-567 term period, as already shown for TGF-β1 (Merceron *et al.*, 2012; Zykwinska *et al.*, 2019b).



Figure 6. Bioactivity assessment of BMP-2 released from equilibrated complex microgels (EPS DR_{MMW} + EPS DRS_{LMW}/BMP-2) upon incubation in DMEM or 2 mM CaCl₂ at 37 °C for 21 days. Smad 1 and P-Smad 1,5,8 were detected by Western Blot using specific antibodies. Native BMP-2 (100 ng/mL), EPS DRS_{LMW} (50 μ g/mL) and EPS DRS_{LMW}/BMP-2 (EPS DRS_{LMW} and BMP-2 final concentrations of 50 μ g/mL and 500 ng/mL, respectively) were used as internal controls.

573 3.5. Effect of BMP-2 released from complex microgels on the *in vitro* osteogenic activity
574 of hBM-MSC.

In order to address whether BMP-2 released from complex microgels may be able to stimulate the *in vitro* osteogenic activity of hBM-MSC, ALP activity and matrix mineralization were investigated. Indeed, ALP plays a key role in bone matrix mineralization by osteoblasts and is thus involved in bone formation (Hessle *et al.*, 2002). For this study, hBM-MSC were

physically separated from the BMP-2 loaded equilibrated complex microgels (EPS DR_{MMW} + 579 580 EPS DRS_{LMW}/BMP-2) by a transwell membrane and cultured for 7 and 16 days (Figure 7A). In parallel, non-treated cells (NT), unloaded complex microgels (EPS DR_{MMW} + EPS DRS_{LMW}), 581 unloaded complex microgels supplemented with soluble BMP-2 (100 ng/mL) (EPS DR_{MMW} + 582 EPS DRS_{LMW} + sBMP-2) and soluble BMP-2 (100 ng/mL) were used as controls. Soluble 583 BMP-2 was supplemented every three days. No statistical difference in ALP activity was found 584 585 between BMP-2 loaded microgels, unloaded complex microgels and those supplemented with soluble BMP-2, and sBMP-2 (Figure 7B). The mineralization was effective in all four groups 586 as underlined by the red alizarin coloration compared to NT (Figure 7C). 587

588 Although, BMP-2 loaded complex microgels induced ALP activity in similar manner than 589 sBMP-2, the EPS-based microgels able to deliver bioactive BMP-2 to the surrounding microenvironment over several days present an undeniable advantage over exogeneous 590 591 repeated supplementations of the growth factor (added every three days), clearly incompatible with in vivo treatments (Hettiaratchi et al., 2020). Interestingly, unloaded complex microgels 592 also displayed a positive impact on the ALP activity. This can result from the progressive 593 swelling and dissolution of microgels, as observed during release experiments in DMEM, 594 595 liberating both EPS DR_{MMW} and EPS DRS_{LMW}. Soluble EPS derivatives could then diffuse 596 through transwell membrane and bind cell-secreted growth factors, including BMP-2, thus favoring cell signaling through growth factor/receptor interactions. No inhibitory effect related 597 to the presence of a heparin-mimetic EPS DRS_{LMW} was noticed. In contrast, an inhibitory effect 598 599 of heparin toward BMP-2-dependent mineralization has already been reported in osteoblast cells (Bramono et al., 2012; Hausser & Brenner, 2004). Indeed, studies investigating the co-600 601 delivery of BMP-2 with soluble heparin have demonstrated either stimulatory or inhibitory effects on BMP-2 mediated osteogenesis (Kanzaki et al., 2008, 2011; Ratanavaraporn & 602 Tabata, 2012; Zhao et al., 2006). Heparin was shown to be able to bind not only to BMP-2 but 603

also to its receptor, leading to a strong suppression of the expression of several genes well 604 605 known as essential factors in osteoblast differentiation (Runx2, osterix, ALP, osteocalcin) and inhibition of the signaling pathways of Smads and p38 MAPK of MC3T3 (Kanzaki et al., 2008). 606 607 In contrast, in long-term cultures, heparin was shown to enhance the BMP-2 mediated bioactivity by inhibiting binding to the growth factor of its antagonists (noggin, follistatin) and 608 609 inhibitory Smads 6, 7 (Kanzaki et al., 2011). It appears that depending on heparin molecular 610 weight, its sulfation pattern, its concentration and its presentation either in soluble or structured form, opposite effects can be observed. Indeed, other studies on heparin structured in 611 microparticles for BMP-2 delivery have shown an increase in ALP activity compared to soluble 612 613 BMP-2 or co-delivered with soluble heparin (Hettiaratchi et al., 2014). Heparin-mimetic EPS DRS_{LMW} used in our present study seems to exhibit stimulatory effects. Due to its lower 614 anticoagulant properties compared to low-molecular weight heparin or unfractionated heparin 615 616 (2.5 and 6.5 times, respectively) (Colliec-Jouault et al., 2001), this derivative may represent an interesting alternative to heparin. Supplementary studies will be conducted to get further insight 617 into the osteogenic activity of BMP-2 loaded microgels. The impact of complex microgels on 618 osteogenic gene expression will be investigated in direct contact with cells to better match the 619 620 in vivo conditions. Finally, for an effective regenerative therapy, combination of these 621 microgels with an appropriate scaffold, e.g. injectable hydrogel will also be considered.



Figure 7. (A) Schematic representation of transwell experiment with the hBM-MSC incubated with
BMP-2 loaded complex microgels and control samples: no treatment (NT), unloaded complex
microgels, unloaded complex microgels supplemented with soluble BMP-2 (sBMP-2) and soluble
BMP-2 (sBMP-2). (B) ALP activity of hBM-MSC incubated with BMP-2 loaded complex microgels
and control samples. No significant differences were found between the different conditions. (C)
Mineralization of hBM-MSC pellets revealed by Alizarin red staining.

632 **4. Conclusions**

633 In the present study, a bacterial EPS displaying both GAG-mimetic and physical gelling properties was formulated into microgels for delivery of BMP-2, clinically used to promote 634 635 bone formation. BMP-2 loading in single microgels based only on a slightly sulfated EPS DR_{MMW} did not provide a sustained release profile. In contrast, BMP-2 complexation with a 636 highly sulfated low-molecular weight heparin-mimetic EPS DRS_{LMW} and its subsequent 637 incorporation within microgels led to a progressive growth factor release. Release kinetics 638 performed at low and high ionic strengths allowed to better understand the impact of salt 639 concentration on growth factor release. At high ionic strength, such as in a cell culture medium, 640 BMP-2 partly dissociated from EPS DRS_{LMW}, whereas at low ionic strength, BMP-2 was still 641 tightly bound to the highly sulfated derivative. It was further shown that EPS DRS_{LMW}/BMP-2 642 complexes diffuse outside the microgels and that bound BMP-2 can also activate Smad 643 signaling pathways. The presence of this highly sulfated heparin-mimetic derivative was 644 645 essential to preserve the growth factor bioactivity for long-time period. BMP-2 loaded complex 646 microgels were then explored for their pro-osteogenic properties by measuring both ALP 647 activity and matrix mineralization induced by BMP-2 released from microgels during transwell experiments. Although BMP-2 loaded complex microgels induced ALP activity and induced 648 matrix mineralization in similar manner to control samples, the EPS-based microgels able to 649 deliver bioactive BMP-2 to the surrounding microenvironment over several days constitute an 650 undeniable advantage over exogeneous repeated supplementations of the growth factor, clearly 651 incompatible with therapeutic treatment. Taken together, the results obtained confirmed the 652 initial hypothesis and emphasized that Infernan-based microgels constitute a promising 653 654 platform for BMP-2 delivery. Further in vitro and in vivo studies will be performed to explore their bone healing potential. 655

657 **Declarations of interest:** The authors declare no conflict of interest.

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