1 **Antibacterial effectiveness meets improved mechanical properties: Manuka Honey/Gellan**
2 **Gum composite hydrogels for cartilage repair**

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32
33 **Abstract**

34 **Biomaterials for cartilage repair are still far from clinical requirements, even if several studies**
35 **recently focused on this topic. In this respect,** Nature-derived hydrogels are a promising class of
36 scaffolds for **cartilage** tissue engineering, mimicking the native cellular microenvironment.
37 However, they frequently lack mechanical features required for **cartilage ~~certain load-bearing~~**
38 applications and **they** are commonly subjected to infection threat. This work describes the
39 innovative use of Manuka honey as molecular spacer for preparing gellan gum-based composites
40 with intrinsic antibacterial properties and superior compressive Young’s modulus in respect of

several Nature-derived ~~natural polymer-based~~ gels based on chitosan, hyaluronic acid or alginate ~~reported in literature~~. The addition of Manuka honey made hydrogels able to inhibit the proliferation of *S. aureus* and *S. epidermidis* clinical isolates. Furthermore, no cytotoxic effects were detected on human mesenchymal stem cells seeded on the hydrogels. Moreover, chondrogenesis experiments showed a consistent expression of collagen II and high synthesis of GAGs and proteoglycans, thus indicating the formation of cartilage matrix. Overall, these data suggest that the ~~encourage a deeper investigation of the~~ developed smart composites have a great potential as tools for ~~their use in the~~ cartilage tissue engineering ~~regeneration field~~.

Keywords: Manuka honey, Gellan gum, Hydrogel characterization, Mechanical properties, Antibacterial, Cartilage regeneration.

1. Introduction

~~The main challenge that Biomaterials Science is facing during this century consists in the development of multifunctional systems that meet the requirements of clinicians and biomedical engineers. Indeed, ideal biomaterials should combine cytocompatibility with antibacterial features, degradability and proper mechanical performances (Kothiyal & Srinivasan, 2016).~~

Since 1960s, hydrogels are among the most attracting ~~polymeric materials for tissue engineering in this landscape~~, thanks to their high water content and nutrients diffusion, making them ideal candidates as scaffolds for cell colonization and differentiation (Calò & Khutoryanskiy, 2015). These features are particularly useful for cartilage tissue engineering, since a proper substitute is required to compensate the limited self-healing potential of cartilage (Zhang et al., 2017). Hydrogels for cartilage repair are obtained from natural and synthetic biomaterials. Among Nature-derived hydrogels, polysaccharide-based ~~hydrogels~~ ones (e.g. based on gellan gum, alginate and chitosan) are particularly interesting because of their low immunogenic profile, easy handling and low costs (Leone & Barbucci, 2009). Nature-derived hydrogels are more biocompatible as

67 compared to synthetic hydrogels, although their mechanical features still need to be improved (Zhu
68 & Marchant, 2011). To overcome this drawback, several studies describe the functionalization of
69 these polymers with chemical moieties, exploited to support the hydrogel crosslinking (Liu et al.,
70 2017). This approach leads to a moderate improvement of compressive Young's modulus (e.g. up to
71 10-50kPa), still far from the mechanical requirements of cartilaginous tissue (Palumbo et al.,
72 2015). Therefore, the preparation of composite hydrogels is currently being explored and represents
73 one of the most promising available strategies (Yang et al., 2017). In this respect, gellan gum-based
74 composite hydrogels (e.g., blends of gellan gum and polyvinyl alcohol or gelatin) show interesting
75 mechanical properties and bio-printability (Wang et al., 2016; Mouser et al., 2016).

76 In addition, Nature-derived hydrogels have to face the worrying issue of contamination, which
77 could have a dramatic clinical outcome. Indeed, even if surgery-related infections of cartilaginous
78 tissue are uncommon (Stutz et al., 2000), they could cause severe complications (i.e. septic arthritis)
79 mainly due to *Staphylococci* (Wyatt et al., 2017). ~~However, risks of contamination and unsuitable~~
80 ~~mechanical performances limit their exploitation (Zhu & Marchant, 2011). To overcome these~~
81 ~~major drawbacks, several strategies have been explored. Concerning the infections issue, The~~
82 inclusion of antimicrobials into the hydrogel networks is the traditional method to prevent
83 contamination. Recently, the development of hydrogels with intrinsic antimicrobial activity has
84 emerged as an effective alternative. Indeed, hydrogels based on antimicrobial peptides or cationic
85 biopolymers (e.g. chitosan, polycarbonate containing quaternary ammonium groups) actively inhibit
86 bacterial proliferation (Anjum, 2016; Liu et al., 2012).

87 In this scenario, the present work follows a *Nature*-inspired strategy, using **Manuka** honey to test
88 two main hypotheses: 1) the exploitation of **Manuka** honey and its unique viscosity to enhance, ~~at~~
89 ~~the same time,~~ hydrogels' mechanical features 2) the potential to overcome simultaneously
90 contamination risks.

91 Recently, the New Zealand Manuka honey (**MH**), derived from *Leptospermum scoparium*, is very
92 attractive because of the remarkable non-peroxide antibacterial activity of methylglyoxal (MGO, or

93 Unique Manuka Factor). Since 2015, when Manuka honey was defined “a tissue engineering
94 essential ingredient” (Speer, et al., 2015), it was employed as diet supplement or wound dresser **in**
95 **combination with chitosan** (Carter et al., 2016; Sasikala & Durai, 2015). However, at the best of our
96 knowledge, the neat Manuka honey has never been exploited to synthesise implantable hydrogels.
97 In this work, considering **also the** high viscosity **of MH**, it was added, for the first time, as
98 molecular spacer to prepare an innovative gellan gum-based **implantable** hydrogel **for cartilage**
99 **tissue engineering**. The present work explores the intrinsic antibacterial properties of the prepared
100 hydrogels, as well as the improvements in mechanical features. A further enhancement of hydrogels
101 compressive Young’s modulus was also achieved by suitable crosslinkers, such as the cations Mg^{2+}
102 and Ca^{2+} , alone or in combination with the natural Halloysite nanotubes (HNT), added as inorganic
103 filler. Since the percentage of the latter is responsible for the hydrogels compressive strength, the
104 HNT amount was properly chosen to maximise mechanical performances as previously reported
105 (Bonifacio et al., 2017).
106 The characterisation of the prepared hydrogels was achieved by **X-ray Photoelectron Spectroscopy**
107 **(XPS)** and porosity measurements, while the mechanical performances were extensively
108 investigated through compression and stress-relaxation studies. The *in vitro* antibacterial
109 effectiveness of the hydrogels was studied against clinical **multi drug resistant (MDR)** isolates of
110 *S.aureus* and *S.epidermidis*, selected for their pivotal role in cartilage infections. Finally,
111 cytocompatibility was evaluated on human mesenchymal stem cells (hMSCs), successfully induced
112 to differentiate into chondrocytes on the hydrogels surfaces. The reported results showed a great
113 potential of the prepared hydrogels for cartilage repair.

114

115 **2. Materials and methods**

116 **2.1 Materials**

117 Unless otherwise specified, all reagents were supplied by Sigma-Aldrich (Italy). Gellan gum,
118 hereafter named GG (Phytigel™, formula weight 1,000Kg/mol, low acylation degree) was a linear

119 anionic polysaccharide, consisting in the repetition of a tetrasaccharide unit, β -D-glucose- β -D-
120 glucuronic acid- β -D-glucose- α -L-rhamnose, in a 2:1:1 ratio. Halloysite nanotubes
121 ($\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4 \cdot 2\text{H}_2\text{O}$) had a mean diameter of 30-70nm and a mean length of 1-3 μm . CaCl_2 and
122 MgCl_2 salts are of Redi-driTM grade. Medical grade Manuka honey12Plus (MH) (ManukaGuard[®],
123 US) contained 400mg/Kg of MGO. Ultrapure water was obtained by a Milli-Q[®] Integral system
124 (Millipore-Merck, Italy). The TRI Reagent[®] and all the media/supplements for isolation and culture
125 of bacteria and human cells were from SigmaAldrich, while the XTT assay was from
126 LifeTechnologies (Italy). The hanging tissue culture inserts for 24-well plates were from Sarstedt
127 (Italy). The 24-well plates used with the NunclonTM Delta Surface treatment, were supplied by
128 ThermoFisher Scientific (Italy), as well as the 96-well plates. The TaqMan reverse transcription kit
129 was from AppliedBiosystems (Italy).

130 2.2 Hydrogels preparation

131 Hydrogels were obtained as previously reported (Bonifacio et al., 2017) for GG-based hydrogels
132 containing glycerol as molecular spacer. Briefly, GG powder (2%w/v) was dissolved under stirring
133 in 100mL of water at $90 \pm 0.5^\circ\text{C}$, using an ARE Aluminium hot plate stirrer (VELP Scientifica,
134 Italy). After cooling the solution at $60 \pm 0.5^\circ\text{C}$, MH (2%w/v) was added and vigorously stirred.
135 HNT-reinforced samples were obtained mixing the dissolved components with an aqueous
136 suspension of HNT (0.5w/v), previously sonicated in water for 15min. To obtain mechanical
137 stability of the prepared hydrogels, a crosslinking was achieved through the external gelation
138 method (Kaklamani et al., 2014) using as cationic sources CaCl_2 or MgCl_2 solutions under
139 physiological concentrations (0.025%w/v and 0.5%w/v respectively). A list of the hydrogels types
140 prepared in this work is reported in Table 1.

145 Table 1. Nomenclature and nominal composition of the prepared hydrogels. All the %w/v are
 146 expressed in respect to 100mL of water.

Sample Code	GG content (%w/v)	MH content (%w/v)	HNT content (%w/v)
GG-MH	2	2	--
GG-MH Ca	2	2	--
GG-MH Mg	2	2	--
GG-MH-HNT	2	2	0.5
GG-MH-HNT Ca	2	2	0.5
GG-MH-HNT Mg	2	2	0.5

147

148 2.3 Spectroscopic characterisation

149 Spectroscopic characterisation of the hydrogels was carried out by X-ray Photoelectron
 150 Spectroscopy (XPS). Hydrated samples were frozen for 24h at $-20 \pm 0.1^\circ\text{C}$, then freeze-dried at -
 151 $55 \pm 3^\circ\text{C}$ for 48h with an ALPHA1-2/LDPlus (Martin-Christ, Germany). Thus, dried hydrogels were
 152 examined by a scanning microprobe PHI5000VersaProbeII (Physical Electronics, US), equipped
 153 with a monochromatised $\text{AlK}\alpha$ X-ray radiation source. The base pressure in analysis chamber was
 154 10^{-9} mbar. Samples were analysed in High Power mode with an X-ray take-off angle of 45°
 155 (scanned size $\sim 1400 \times 200 \mu\text{m}$). For each specimen, survey scans (Fixed Analyser Transmission
 156 mode, binding energy (BE) range 0-1200eV, pass energy 117.4eV) and high-resolution spectra
 157 (FAT mode, pass energy 29.35eV) were acquired. Data analysis was performed using the MultiPak
 158 software package (version 9.6.1.7). Charge referencing was carried out setting the lower binding
 159 energy C1s photo-peak (i.e., C1s hydrocarbon peak) at 284.8eV. Quantification (atomic percentage,
 160 At.%) was made using normalised peak area. The normalisation of the peak area and comparison of
 161 data from different elements were enabled by correction with empirically derived sensitivity factors,
 162 according to MultiPak library.

163

164

165 2.4 Evaluation of pore architecture of hydrogels

166 Hydrogel blocks were fixed overnight (ON) in 4% paraformaldehyde at 4°C and treated as
167 previously reported (Yang et al., 2007). Briefly, hydrogels were rinsed in 5% sucrose in phosphate
168 buffered solution (PBS, pH 7.4) for 1h, then cryoprotected by infiltrating them in PBS with
169 ascending concentrations of sucrose (10%, 12.5%, 15%) at room temperature (~25°C) every 30
170 min, then the hydrogels were stored in 20% sucrose in PBS ON at 4°C. The 20% sucrose solution
171 was discarded and the hydrogels were incubated for 1h at room temperature with solutions in which
172 the ratios of 20% sucrose in PBS and optimal cutting temperature medium (OCT[®], Bio-Optica,
173 Italy) were 2:1, 1:1 and 1:2 v/v. Hydrogels were stored in the last solution ON at 4°C and then
174 infiltrated with fresh OCT[®] at room temperature for 3h to minimize shattering/cracking during the
175 cryosectioning process. Thin cryosections (10µm) were cut with a LeicaCM1950 cryostat, collected
176 on Superfrost Plus[™] microscope slides (ThermoFisher Scientific, Italy) and allowed to air dry.
177 Alternatively, freeze-dried hydrogels (drying procedure in 2.3) were rehydrated in ultrapure water
178 for 24h at 4°C, and infiltrated for 2h with a mixture of PBS/OCT[®] (1:1v/v), then ON in OCT[®] alone
179 at 4°C. Cryosections were obtained and collected as reported above. Sections were observed under
180 transmitted light with a LeicaDM6000B microscope equipped with a 10X planar objective and
181 imaged with a LeicaDFC310FX camera. The analysis of pore Feret's diameter and porosity
182 percentage was performed using ImageJ software. Scaffold porosity was calculated as the ratio
183 between the voids and total surface and expressed as percentage. Calculation was done on six
184 randomly-chosen images for each hydrogel type.

185 2.5 Release test

186 For release tests, three hydrogels of each type were incubated in 5mL phosphate buffer (pH=7.4) at
187 37°C, an aliquot of 0.5mL was collected from each specimen after 24, 48 and 72h and an equal
188 volume of fresh buffer was added. Released calcium and magnesium amounts were determined by
189 the classic direct potentiometric method, exploiting ion selective electrodes (Lvova et al., 2018). To
190 determine MGO release, a derivatisation reaction with o-phenylenediamine (OPD) was carried out ;

191 ~~to obtain the quinoxaline adduct. Samples were incubated for 16h with 2% w/v OPD in phosphate~~
192 ~~buffer 0.5M (pH 6.5)~~ (Adams et al., 2008). The reaction was performed at ~~room temperature~~, in the
193 dark. Samples were examined by an Agilent HPLC (1260Infinity, Agilent Technologies, US)
194 equipped with a 20 μ L Rheodyne injection loop, a Multiple Wavelength detector and a reversed
195 phase column (SynergiTMHydro-RP 4 μ m 150x4.6mm, Phenomenex). An isocratic elution was
196 performed at ~~room temperature~~ (flow rate 0.9mL/min), using mobile phase (Water/ACN 75/25).
197 The column effluent was constantly monitored at 263nm and the OPD derivative of MGO was
198 eluted after 3.50min. ~~The ChemStation software for LC systems (Agilent Technologies) was used~~
199 ~~for data acquisition and processing. MGO quantification was performed comparing the obtained~~
200 ~~peak areas with those detected with the standard compound eluted in the same conditions.~~ A linear
201 response was observed for the standard curve ($R^2=0.995$). Each analysis was performed in triplicate
202 and results were reported as mean $\pm$ standard deviation.

203 2.6 Mechanical characterisation

204 Mechanical properties under compression of the hydrogels (cylinder-shaped samples 1.6cm
205 diameter and 2cm height) were evaluated using a mechanical testing machine (EZ-SX, Shimadzu,
206 Japan). Five specimens for each composition were tested ~~at room temperature (21°C)~~. The
207 crosshead speed was set at 1mm $\cdot$ min⁻¹ and the load was applied while the specimen was compressed
208 until break (~35-40% of original height). Young's compressive moduli (E) were calculated as the
209 slope of the initial linear portion of the stress-strain curve (0-15%). Furthermore, compression tests
210 of rehydrated freeze-dried hydrogels were performed after immersion in PBS for 1h. The resulting
211 Young's compression modulus was calculated following the same procedure reported above. Then,
212 to evaluate stress-relaxation properties, the hydrogels were deformed with impermeable plates to a
213 compressive strain of 10%, with a deformation rate of 50mm $\cdot$ min⁻¹ to approximate an instantaneous
214 deformation. Within 10% compression, the stresses versus strain relations of the gels were almost
215 linear. Subsequently, the strain was held constant for 900s, while the load was recorded as a
216 function of time. The obtained data were analysed using MATLAB R2015a software (Bonifacio et

217 al., 2017). In particular, fitting a third order exponential decay (Equation 1) to the relaxation curves,
218 three relaxation times were acquired. The increase in stress during straining was not included when
219 fitting the relaxation curves.

$$220 \quad \sigma_t = A_1 \cdot e^{-t/\tau_1} + A_2 \cdot e^{-t/\tau_2} + A_3 \cdot e^{-t/\tau_3} + y_0 \quad (1)$$

221 where σ_t is the total stress, while A_1 , A_2 and A_3 are the amplitudes corresponding to the three
222 different relaxation times τ_1 , τ_2 and τ_3 .

223 Finally, to evaluate the gels viscosity, a first order exponential decay (Equation 2) was fitted
224 through the relaxation curves of every measurement.

$$225 \quad \sigma_t = A \cdot e^{-t/\tau} + y_0 \quad (2)$$

226 where σ_t is the total stress, while A is the amplitude corresponding to the relaxation time τ .

227 Moreover, the viscosity (η) was obtained multiplying the relaxation time τ by the linear equilibrium
228 modulus E_{eq} , obtained fitting the initial linear part of the strain curve of the straining protocol and
229 creating a linear fit.

230 2.7 Antibacterial Activity Evaluation

231 2.7.1 Bacterial growth conditions

232 Bacterial strains were clinical isolates tested for their multi-drug resistance (MDR) by the Novara
233 Maggiore Hospital, Clinical Microbiology Unit. All the specimens were obtained after patient's
234 informed consent and in full accordance with the Declaration of Helsinki. A single colony of
235 *S.aureus* and *S.epidermidis* MDR strains from an ON culture onto differential and selective
236 Mannitol Salt and Trypticase Soy (TSB) Agar plates was resuspended in 9mL of Luria Bertani
237 broth (LB) and incubated at 37°C for 18h at 200rpm. Then, cultures were diluted 1:10 in LB and
238 incubated at 37°C for 3h (Lag phase) to achieve the logarithmic growth phase. Finally, a fresh
239 culture was prepared for each experiment diluting bacteria until a concentration of 1×10^5 cells/mL
240 (o.d. at 600nm~0.001) (Cochis et al., 2016).

241

242

243 2.7.2 Biofilm formation

244 To test antibacterial properties, hydrogel specimens were manufactured as 16mm diameter, 3mm
245 thickness disks; these were gently handled by sterile surgical tweezers and placed onto the bottom
246 of ~~in a~~ 24-well plates (without fixing the samples). Then, specimens were ~~and~~ sterilised by UV-
247 irradiation for 30min/side. Sterile samples were covered with 1mL of LB containing 1×10^5 cells/mL.
248 Samples were incubated for 90min at 37°C under agitation at 2g (adhesion phase). Then,
249 supernatants containing floating planktonic cells (separation phase) were removed and the
250 specimens gently washed with 1mL of 1X PBS. Finally, each specimen was rinsed with 1mL of
251 fresh LB and incubated for 24, 48 and 72h at 37°C for biofilm culture.

252 2.7.3 Bacterial viability

253 A metabolic analysis, assay based on 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-
254 carboxanilide assay (XTT) was performed to assess bacterial growth ability after 24, 48 and 72h of
255 direct contact with the hydrogels compared to polystyrene. Briefly, 100µL of XTT solution
256 (0.3mg/mL in acetone containing 0.1M menadione) were added to each well and incubated at 37°C
257 for 5h in the dark. Then, 50µL were collected, centrifuged for 2min at 480g to remove debris and
258 the o.d. was evaluated spectrophotometrically at 450nm using a VICTOR Multilabel Plate Reader
259 (Perkin Elmer, US). ~~The Bacteria~~ viability ~~of bacteria~~ onto polystyrene was considered as control.

260 2.8 In vitro Biological tests

261 2.8.1 Eukaryotic cell culture conditions

262 Human mesenchymal stem cells (hMSCs) purchased from the American Type Culture Collection
263 (adipose-derived, ATCC-PCS-500-011) were cultured at 37°C, 5% CO₂ in basal medium intended
264 as ~~low~~-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% foetal
265 bovine serum (FBS) and 5ng/mL of human fibroblast growth factor-2 (hFGF-2). Cells were
266 cultured until 70-80% confluency, detached by trypsin-EDTA and used between passages II-IV, to
267 ensure undifferentiated state.

268

269 2.8.2 MSCs cytocompatibility

270 Hydrogels' cytocompatibility was investigated *in vitro* by direct and indirect assays (~~Fig.6A-B~~) to
271 detect the possible presence of toxic compounds onto the hydrogel surfaces or released into the
272 medium, respectively. For the direct assay, specimens were ~~manufactured as 16mm diameter, 3mm~~
273 ~~thickness disks and~~ placed into a 24-well plate ~~by sterile tweezers for UV sterilization as prior~~
274 ~~described in 2.7.2. Then, -and~~ 2×10^4 cells were seeded ~~directly~~ onto each sample surface ~~in order to~~
275 ~~achieve a direct contact between cells and specimens' surface (Fig.6A).~~ Cells were diluted into 1mL
276 of basal medium (intended as low-glucose DMEM supplemented with 5% FBS and 5ng/mL hFGF-
277 2) and dropwise deposited onto specimens' surface. For the indirect assay, 2×10^4 cells were seeded
278 in the polystyrene bottom of a 24-wells plate and cultured in basal medium, with the test hydrogel
279 suspended in a porous insert on the top of the same well to obtain a continuous release without a
280 direct contact with cells (~~Fig.6B~~). For both experiments, cell viability was evaluated after 1, 3, 5
281 and 7days by the XTT assay. At each time-point, medium was ~~completely~~ removed and replaced
282 with 1mL/specimen of ~~by~~ XTT solution (3mg/mL ~~prepared~~ in basal medium). The plates were
283 incubated at 37°C for 4h in the dark, then 100μL were collected from each test well and transferred
284 to a 96-well plate. The o.d. was evaluated spectrophotometrically at 450nm by a VICTOR
285 Multilabel Plate Reader (Perkin Elmer, US). Cells cultured onto polystyrene were considered as
286 control (100% viability) to normalise the results (Cochis et al., 2017).

287 2.8.3 Chondrogenesis

288 MSCs were directly seeded on hydrogels surface and cultured for 48h in basal medium to allow
289 adhesion and cell spread. Then, basal medium was replaced with chondrogenic medium (high
290 glucose DMEM 4.5g/L with 10% ITS+1 Premix Tissue Culture Supplement, dexamethasone
291 10^{-7} M, ascorbate-2-phosphate 1μM, 1% sodium pyruvate and TGF-β1 10ng/mL) and cells were
292 cultured for 21days. MSCs cultured onto polystyrene in the same conditions ~~onto polystyrene~~ were
293 used as positive ~~considered as~~ control considering polystyrene as the gold standard material for
294 stem cells cultivation and differentiation.

295 2.8.4 Chondrogenesis analysis

296 After 21days of culture, hMSCs chondrogenesis was evaluated by real-time **Polymerase Chain**
 297 **Reaction (PCR)** and histology. Cells were digested for 5min with TRI reagent[®] and RNA was
 298 reverse transcribed with TaqMan reverse transcription kit using random hexamers. For real-time
 299 PCR, TaqMan Gene Expression Assays was used in a GeneAmp7500Real-Time PCR System
 300 (Applied Biosystems, USA). Data were normalised towards the endogenous control gene (18S
 301 rRNA) and reported as fold expression. Chondrogenic collagen-II (COLII), aggrecan (ACAN) and
 302 collagen-I (COLI) were analysed (Table 2). For histological analysis, cells were fixed onto the
 303 specimens surfaces by a sucrose-formaldehyde solution (50:50, 10min at RT) and stained with
 304 Safranin-O or Alcian blue (Cochis et al., 2017).

305

306

307 Table 2. Primers and Probes Summary.

Gene	Abbreviation	Forward (5'-3')	Reverse (5'-3')	Probe (5'FAM/3'TAMRA)
Collagen type-I	COL 1	CCC TGG AAA GAA TGG AGA TGA T	ACT GAA ACC TCT GTG TCC CTT CA	CGG GCA ATC CTC GAG CAC CCT
Collagen type-II	COL 2	GGC AAT AGC AGG TTC ACG TAC A	GAT AAC AGT CTT GCC CCA CTT ACC	CCT GAA GGA TGG CTG CAC GAA ACA TAC
Aggrecan	ACAN	AGT CCT CAA GCC TCC TGT ACT CA	CGG GAA GTG GCG GTA ACA	CCG GAA TGG AAA CGT GAA TCA GAA TCA ACT
Gene	Abbreviation	Applied Biosystems Serial number		
ribosomal 18S	18S	4310893E		

308

309 2.9 Statistical analysis

310 Mechanical characterisation was studied on five replicates/~~of each~~ sample type, while biological
 311 experiments were performed in triplicate. Statistical analysis of data was performed using the

312 Statistical Package for Social Sciences (SPSSv.20.0, IBM, US). Data were compared by ANOVA,
313 followed by Tukey's test. The significance level was set at $p < 0.05$. The Student's *t*-test for unpaired
314 data was used ~~only~~ for porosity measurements ~~only~~.

315

316 **3. Results and discussion**

317 *3.1 XPS characterisation*

318 The hydrogels elemental composition was studied by XPS analysis (Fig.1A). As expected from GG
319 and MH composition, the main elements detected were carbon and oxygen. Indeed, GG consists in
320 a tetrasaccharide unit (see section 2.1), repeated to form long linear chains (Bacelaret al., 2016).
321 Similarly, the content of total reducing sugars in MH (mainly glucose and fructose) was >75%
322 (Visweswara Rao et al., 2016), while a wide range of phenolic and flavonoids compounds
323 represented the minor MH constituents. Magnesium and calcium elements, deriving from
324 crosslinking, were also detected. Beyond the signals clearly ascribable to the polymeric matrix, the
325 molecular spacer and crosslinkers, the samples enriched with HNT additionally displayed silicon
326 and aluminium signals in the characteristic 1:1 stoichiometry.

A

	Atomic percentage (%) calculated by XPS analysis					
Sample Type	C1s	O1s	Al2p	Si2p	Ca2p	Mg2p
GG-MH Ca	63.7	36.0	-	-	0.3	-
GG-MH-HNT Ca	79.1	20.3	0.2	0.2	0.2	-
GG-MH Mg	67.8	31.9	-	-	-	0.3
GG-MH-HNT Mg	74.4	24.9	0.3	0.2	-	0.2

327

328 Figure 1. XPS characterization of the prepared hydrogels. Relative atomic percentages calculated
 329 for each sample type (A). XPS curve fitting of high resolution C1s spectra of the relevant samples
 330 (B).

331 In our previous work, when HNT were incorporated in a gellan gum-glycerol matrix, a decrease in
 332 silicon and aluminium binding energies was detected (Bonifacio et al., 2017), suggesting the
 333 hydrogen bond formation between nanoclays and the polymeric network. In this study, the binding
 334 energies of silicon and aluminium signals, 102.8 and 74.2eV respectively, did not show any
 335 difference when HNT were included in all the hydrogel matrices. Therefore, no interactions
 336 between HNT and the organic network occurred.

337 An accurate curve fitting of the C1s spectra (Fig.1B) revealed the overlapping of GG and MH
 338 contributions, making impossible an unequivocal interpretation. On the other hand, considering the
 339 binding energies of carboxylic acid groups, it was evident that, in the case of GG-MH-HNT Mg,
 340 this contribution fell at 289.0 ± 0.1 eV, whereas for the other samples it fell at 288.5 ± 0.1 eV. This

341 result indicated a poor coordination between the cation and the GG carboxylic acid groups for GG-
342 MH-HNT Mg system.

343 *3.2 Evaluation of pore architecture of hydrogels.*

344 The ultrastructure of hydrogels was investigated on cryosections. Since the water content in the
345 samples was high, we adapted a previously reported infiltration method to completely and gradually
346 replace water with freezing medium before cryosectioning (Yang et al., 2007). Fig.2 showed
347 micrographs of each hydrogel type before (A-D) and after freeze-drying (E-H). Void spaces are
348 present in all hydrogels, likely representing cross-section areas of gel pores. HNT were easily
349 detectable as darker particulate scattered in the hydrogel matrix (Fig.2B, D, F and H). The shape of
350 the voids appeared more regular and round in Fig.2A-B compared with Fig.2C-D, regardless of the
351 presence of HNT. Concerning the freeze-dried hydrogels, a different organization of voids, with
352 larger voids surrounded by much smaller ones in all hydrogel matrices was observed (Fig.2E-H).
353 Fig.2I summarises the results of the analysis of mean Feret's diameter of the pore and the
354 percentage of porosity. Statistical analysis, on Feret's diameter, found no significant difference in
355 A_vsB and C_vsD but a significant difference in A_vsC and B_vsD (both $p < 0.0001$) with the voids of
356 Mg²⁺-crosslinked hydrogels significantly smaller than Ca²⁺-crosslinked ones. Concerning freeze-
357 dried hydrogels, there was no significant difference in G_vsH. Statistically significant differences
358 were obtained in E_vsF ($p < 0.01$), E_vsG and F_vsH (both $p < 0.05$). The porosity of all samples
359 (between 41 and 75%) may influence the mechanical behaviour of each hydrogel prepared. It has to
360 be underlined that hydrated hydrogels did not show a porous internal structure (data not shown). As
361 they cannot undergo the procedure of paraffin embedding, thus cryosectioning, preceded by the
362 substitution of the water in the matrix, is the only method to cut and observe ultrathin slices.
363 Freezing the samples at -20°C before cutting and air drying of the sections may mimic a
364 conventional procedure of freeze-drying. After conventional freeze-dehydration protocol, the
365 hydrogels were also studied. In both conditions, we observed a change of micro-architecture,
366 shifting from a homogeneous to a porous structure with pore diameter depending both on the drying

367 protocol and the crosslinker used. This observation is fundamental as it suggests that, playing on
 368 different parameters (e.g. freeze-frying, crosslinker), a wide range of GG hydrogels suitable for
 369 different needs of regenerative medicine could be obtained. In all cases, the pore size and
 370 distribution are optimal for chondrocyte differentiation and cartilage regeneration.

371 Figure 2. Bright field micrographs of gellan gum scaffold hydrogels cryosections.
 372 Upper panels (A-D) refer to hydrated gels with different composition. Lower panels (E-H) refer
 373 to freeze-dried hydrogels with the same composition. Magnification bars = 100μm. (I) reports a
 374 detailed measurement of hydrogels pores.
 375 In particular, the GG-MH-HNT Mg hydrogel showed a mean pore diameter (257±48μm) and
 376 porosity percentage (75%), that seems to better approach the required standard of an optimal
 377 scaffold for cartilage regeneration (Tanaka et al., 2010).

378
 379 *3.3 Release test*

380 Cationic release was studied in the early stages of conditioning in PBS (24, 48 and 72h) (Fig.3A-B).
 381 For calcium release, no significant differences were observed between GG-MH Ca and GG-MH-
 382 HNT Ca at each time-point (Fig.3A). Conversely, the presence of HNT delayed magnesium ions
 383 release (*p<0.05) (Fig.3B), which might be explained by the formation of transient bonds,
 384 encouraged by the higher mobility of Mg^{2+} , compared to Ca^{2+} , in the polymeric network.

385 Figure 3. Calcium (A) and magnesium (B) release from the prepared hydrogels, with or without
 386 HNT, after 24, 48 and 72h. (C) Methylglyoxal release from the same samples. Statistically
 387 significant differences ($p<0.05$) are indicated by the *.
 388 This hypothesis is also supported by mechanical test (section 3.4). Furthermore, the release of MGO
 389 from hydrogels was also evaluated by HPLC analysis, since in literature this molecule seems to
 390 have a remarkable influence on MH antibacterial performances (Stephens et al., 2010). The results
 391 of MGO release are shown in Fig.3C. After 24h of incubation, no significant differences were
 392 observed between the four crosslinked hydrogel types and the two non-crosslinked hydrogels.
 393 However, after 48 and 72h, the amount of MGO released from non-crosslinked samples was

394 remarkably higher than that of the other hydrogel types (* $p < 0.05$). Therefore, it could be
395 hypothesised that Ca^{2+} and Mg^{2+} could play a pivotal role hindering MGO release. Conversely,
396 HNT presence did not alter MGO release.

397 *3.4 Mechanical characterisation*

398 The mechanical properties of all the hydrogels were evaluated by static compression tests. As an
399 example, Fig.4A shows the stress-strain curve obtained compressing a GG-MH-HNT Mg sample at
400 a strain of 0-35%. For each sample, compression Young's modulus (E) was calculated from the
401 relevant stress-strain curves (Fig.4C). Interestingly, all the samples showed high values of E
402 ($> 100 \text{ kPa}$) compared with other natural hydrogels for soft tissue and/or cartilage regeneration (Peak
403 et al., 2013). Only HNT-reinforced gels showed a dramatic increase of E value compared with the
404 non-reinforced samples. Moreover, in this study, the compressive moduli of the prepared hydrogels
405 were also higher ($\sim 25\text{-}30\%$) than those of similar systems recently described (Bonifacio et al.,
406 2017).

407 Thus, it could be hypothesised that MH, herein exploited for the first time as molecular spacer for
408 hydrogel manufacturing, played a key role in the enhancement of the compressive moduli of the
409 prepared systems. Furthermore, MH presence made the hydrogels more flexible and easy to handle.
410 Concerning the impact of HNT addition on mechanical features, the inorganic filler did not
411 significantly change the compressive moduli of the gels crosslinked with Ca^{2+} (GG-MH Ca
412 $116 \pm 5 \text{ kPa}$ vs GG-MH-HNT Ca $108 \pm 10 \text{ kPa}$). On the other hand, HNT presence dramatically
413 improved the stiffness of Mg^{2+} -crosslinked samples ($102 \pm 9 \text{ kPa}$ for GG-MH Mg vs $143 \pm 8 \text{ kPa}$ for
414 GG-MH-HNT Mg). This mechanical reinforcement could be attributed to the different gelification
415 mechanisms introduced by Mg^{2+} and Ca^{2+} ions. In this respect, Donati and co-workers reported that
416 Mg^{2+} act as diffusively bound ions rather than strongly site-bounded ions such as Ca^{2+} (Donati et
417 al., 2009). This behaviour may allow a different mobility of the nanofillers in the polymeric
418 network. Therefore, in Mg^{2+} -crosslinked hydrogels, HNT may orient and align under compression
419 stress, establishing temporary bonds between polymer chains (without the formation of permanent

hydrogen bonds, as revealed by XPS analysis), thereby creating a local region of enhanced strength. Conversely, in presence of Ca^{2+} , HNT may become less mobile, preventing the occurrence of the energy dissipation mechanism hypothesised for Mg^{2+} (Shah et al., 2005). Furthermore, as described in section 3.2, Mg^{2+} -crosslinked hydrogels were characterised by a lower volumetric porosity than Ca^{2+} -crosslinked ones. As largely described in literature, the porosity is inversely proportional to scaffolds compression moduli. Therefore, both parameters should be carefully considered in *in vivo* cell enrichments because, when the mechanical strength is sufficient to maintain the pore structure upon implantation, the pore volume determines the number of recruited cells (Kim et al., 2014). Finally, the hydrogels mechanical properties were evaluated after rehydration of freeze-dried samples in PBS for 1h. A non-statistically significant difference in the calculated moduli (compared with the values of the hydrated one) was observed (ranging from 12 to 15%), that indicates a high mechanical stability and consistency of the hydrogels. In addition to static compression tests, hydrogels viscoelasticity behaviour was studied by relaxation experiments, in which the load decreased at fixed strain in response with time. It is noteworthy to compare the stress-relaxation behaviour of the prepared hydrogels with that of native tissues, since it affects loads transfer, as well as nutrients diffusion (Marijnissen et al., 2002). Fig.4B shows the typical stress relaxation curve obtained after 900s, reporting the relaxation times and viscosity values. A similar response was observed for all the samples: a fast decrease in stress for few seconds after the step strain, followed by a slow relaxation time. Wagenseil et al. (2003) proposed the generalised Maxwell model, consisting in three relaxation times ($\tau_1=1-10\text{s}$, $\tau_2=10-100\text{s}$ and $\tau_3>1000\text{s}$) for modelling soft biological tissues to evaluate their viscoelastic behaviour. The calculated relaxation times (τ_1 7-9s, τ_2 70-80s and τ_3 480-650s) were similar to the values reported in literature for polymeric gels (Wagenseil, et al., 2003). These relaxation times are related with the calculated equilibrium modulus, ranging from 72kPa (GG-MH Ca) to 98kPa (GG-MH-HNT Mg). These values are in accordance with those reported for native cartilage, that vary with depth from the articular surface

(Nguyen et al., 2012). Finally, HNT addition did not significantly influence the viscosity of the hydrogels (Fig.4C).

Figure 4. Mechanical characterization of the prepared hydrogels. (A) Stress-strain curve obtained by compression test. (B) stress-relaxation curve. (C) The table reports the Elastic moduli (E), relaxation times (τ_1 , τ_2 and τ_3) and viscosities (η) relevant to each hydrogel type.

3.6 Antibacterial Activity

Most of the studies on innovative biomaterials for cartilage repair lack of antibacterial active compounds, likely because arthroscopy-related infections are nowadays considered as very low-risk episodes, affecting only 0.09 to 0.4% of patients undergoing surgery (Bert et al., 2007). However, infections can determine septic arthritis, leading to potentially devastating post-operative complications (Balato et al., 2017). Several case reports showed that the most commonly identified pathogens were *Staphylococci* (Wyatt et al., 2017). Therefore, the prepared hydrogels were tested against clinical isolates of *S. aureus* (coagulase positive) and *S. epidermidis* (coagulase negative), as representative pathogens responsible for joint arthroscopy infections. To this respect, one of the strengths of this work is to exploit MH not only as innovative molecular spacer, but also as antibacterial agent. MH contains a large amount of MGO, which is considered among the most

462 effective natural antibacterial compounds, due to its non-peroxide activity (Adams et al., 2008). MH
 463 contains 38-725mg/kg of MGO (Karygianni et al., 2016) while ordinary honey only 1.6-135mg/kg.
 464 To give an example of MGO effectiveness, a minimum inhibitory concentration for *S. aureus* was
 465 observed at 1.1mM of MGO (Mavric et al., 2008). Considering these assumptions, the tested
 466 hydrogels were infected with *S. aureus* and *S. epidermidis* for 24, 48 and 72h, to verify changes in
 467 bacterial viability (section 2.8.2). Overall, the tested hydrogels were significantly effective in
 468 reducing *S. aureus* biofilm viability at each time-point in comparison with polystyrene control
 469 (Fig.5A, * $p < 0.05$). Particularly, GG-MH Mg samples resulted effective regardless of the presence
 470 of HNT, at each tested time-point. Conversely, the introduction of HNT significantly enhanced the
 471 antibacterial activity of Ca-crosslinked specimens (Fig.5A, $\$p < 0.05$). Furthermore, all hydrogel
 472 types reduced *S. aureus* viability more than 60% compared to control and GG-MH-HNT Ca gels
 473 determined a viability reduction of more than 90% after 72h. Concerning *S. epidermidis*, all tested
 474 hydrogels significantly reduced bacterial viability compared to controls at each time-point (Fig.5B
 475 * $p < 0.05$), with the only exception of GG-MH Mg after 24h (likely due to the high standard
 476 deviation). As observed for *S. aureus*, HNT increased the antibacterial activity of Ca-crosslinked
 477 hydrogels. However, for *S. epidermidis*, significant differences were noticed only after 24h
 478 ($p < 0.05, \$$). In agreement with the results obtained with *S. aureus*, bacterial viability was overall
 479 reduced more than 60% after 72h. The raise of the antibacterial effect during the 24-72hs
 480 observation time (Fig.5) was in line with Ca(II) and Mg(II) increased cumulative release from the
 481 analysed hydrogels as depicted in Fig.3A-B.
 482 To this regard, as evidenced by Xie et al. (2016), although both the two alkaline-earth metal Ca(II)
 483 and Mg(II) ions are fundamental for many living organisms, they can also increase the
 484 antimicrobial activity of MH/GG composite hydrogels. Indeed, they are also necessary to
 485 selectively destabilize and destroy cytoplasmic membranes of MDR or wt *S. aureus* strains. This
 486 membrane-destabilization event likely occurs through their binding to cardiolipin, *S. aureus*' major

lipid component also present in *S. epidermidis* (Luo et al, 2018) thus converting to negative its originally zero-intrinsic curvature.

Figure 5. Antibacterial activity of the hydrogels against *S. aureus* (A) and *S. epidermidis* (B). Biofilm viability after 24, 48 and 72 h. At each time point, all the tested specimens were effective in reducing biofilm viability in a significant manner in comparison with untreated controls ($p<0.05$, indicated by the *). Bars represent means and standard deviations.

3.7 Preliminary biocompatibility evaluations

3.7.1 Cytotoxicity

GG has been already described as a cytocompatible material for different purposes and in combination with other molecules (Koivisto et al., 2017), besides MH has been widely used in medicine. Moreover, according to the present literature, the free amount of the Ca^{2+} (Bronner 2001) and Mg^{2+} (Romani, 2011) ions detected in the release test were not sufficiently large to determine significant variations in cells homeostasis. However, at the best of our knowledge, this is the first time that GG is combined with MH and HNT at the same time. Therefore, considering that these

502 composite materials are aimed for tissue engineering, their cytocompatibility was firstly evaluated
503 towards hMSCs, normally recruited after tissue damage. During the direct assay, hMSCs viability
504 was evaluated after 1, 3, 5 and 7 days of culture onto the hydrogels surfaces (Fig.6C).
505 Overall, each hydrogel type allowed cell growth, as a general increase in o.d. was detected for all
506 samples over time. No significant differences in terms of viability of cells cultured on the four
507 hydrogel types were observed, with values always higher than 80%. Thus, beyond their antibacterial
508 effectiveness, the prepared hydrogels are suitable to host hMSCs colonization, as other hydrogels
509 proposed for cartilage repair (e.g. fibrin glue (Deponti et al., 2013), hyaluronic acid (Ha et al., 2015)
510 and methylcellulose (Cochis et al., 2017). Furthermore, the indirect assay aimed at the study of the
511 potential biomaterial toxicity, overcoming the effect due to the lack of adhesive signals, which is
512 common on polysaccharide-based hydrogels (Cochis et al., 2017). None of the hydrogel types
513 negatively affected hMSCs viability, which was not significantly different between all samples and
514 controls at each time-point (Fig.6D, $p>0.05$). Moreover, the viability of cells cultured on the gels
515 was always higher than 90% compared to controls. These findings are very encouraging, as the
516 investigated systems were effective in killing bacteria, but not toxic towards hMSCs.

517

518 Figure 6. Cytocompatibility evaluation. Direct cytocompatibility assay **schematized in A**. Stem cells
 519 viability was evaluated after 1, 3, 5 and 7 days after direct seeding onto specimens' surface (C). No
 520 significant differences were noticed between the four hydrogel types. Bars represent means and
 521 standard deviations. Indirect cytocompatibility assay (**schematized in B**). Stem cells viability at 1, 3,
 522 5 and 7 days (D). No significant differences were noticed comparing all the hydrogels with
 523 untreated controls ($p>0.05$). Normalized percentage viability was calculated considering untreated
 524 controls as 100%.

525 3.8 Chondrogenesis evaluation

526 3.8.1 Gene expression

527 After the validation of the *in vitro* cytocompatibility, chondrogenesis experiments were performed
 528 to test the hydrogels ability to support chondro-like matrix formation for further cartilage
 529 regeneration. **All the produced materials were investigated in order to verify whether single ions or**
 530 **nanotubes-enriched composites could have represented an interference element during**
 531 **chondrogenesis in comparison with the same proceeding performed using the gold-standard**

532 polystyrene surface. Real-time PCR results are reported in Fig.7A-D. Chondrogenic genes COLII
 533 and ACAN were successfully expressed (Fig.7A-B) and no significant differences were observed
 534 between samples and control in terms of fold expression ($p>0.05$). The low expression of the
 535 osteogenic COLI gene (Fig.7B) confirmed that the cells correctly differentiated towards a cartilage-
 536 like matrix, as indicated by the positive COLII:COLI ratio (Fig.7D).

537
 538 Figure 7. Chondrogenesis evaluation. hMSCs cultivated onto specimens' surfaces successfully
 539 expressed chondrogenic genes collagen type II and aggrecan (A,C). No significant differences were
 540 detected between samples and control in terms of expression ratio ($p>0.05$). Accordingly, cells
 541 expressed low levels of the osteogenic gene collagen type I (B) in comparison with collagen type II,
 542 as shown in (D). PCR results were confirmed by histology (E). Scale bars in (E): 50µm.
 543 This is an important confirmation of the cartilage-like matrix formation, since it is normally
 544 composed of 90% collagen-II and 10% other collagens (I-IV-V-VI-IX), that are very useful to
 545 stabilise the collagen-II fibril network (Fox et al., 2009). Moreover, according to real-time PCR
 546 results, cells seeded on hydrogels surfaces were positive for both Safranin-O and Alcian blue

547 (Fig.7E), confirming a massive presence of GAGs and proteoglycans in the secreted extracellular
548 matrix (Cochis et al., 2017).

549

550 **4. Conclusions and future perspectives**

551 This work confirmed the interesting features of the prepared Manuka honey-gellan gum hydrogels:
552 1) intrinsic antibacterial activity, evaluated against two MDR *Staphylococci* strains 2) suitable
553 mechanical properties, with compressive moduli (from 116 up to 143kPa) even better than those of
554 other natural hydrogels (from 10 to 60kPa) already described in literature (Song et al., 2015;
555 Palumbo et al., 2015). Therefore, the composite hydrogels herein described, combining for the first
556 time Manuka honey and gellan gum, provide useful mechanical and antibacterial features for
557 cartilage repair. Moreover, no cytotoxic effect of the hydrogels was observed when human
558 mesenchymal stem cells were seeded on the gel surfaces. In addition, after 21 days of culture,
559 chondrogenic differentiation occurred on hydrogels without differences with the control substrate.
560 Interestingly, all the gels showed a microscopic porous internal architecture upon drying, thus
561 paving the way for future studies in which cells will be allowed to colonise the inside of the
562 hydrogels by fluidic stimulation. Overall, the satisfying mechanical and biological data encourage a
563 deeper investigation of the developed hydrogels, ~~in order to exploit them~~ that have the potential to
564 become implantable tools for cartilage repair.

565

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573

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Highlights

- Composite hydrogels based on gellan gum and Manuka honey have been prepared.
- The addition of halloysite nanotubes improves gel compressive modulus up to 143kPa.
- Hydrogels inhibit the proliferation of *S. aureus* and *epidermidis* clinical isolates.
- Chondrogenesis of human stem cells seeded on the gels was successfully achieved.
- The developed gel composites could be an innovative tool for cartilage repair.