

1 **Advanced Multi-Targeted Composite Biomaterial Dressing**
2 **for Pain and Infection Control in Chronic Leg Ulcers**

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15 **ABSTRACT**

16 This study aimed to develop advanced biomaterial polysaccharide based dressings to manage
17 pain associated with infected chronic leg ulcers in older adults. Composite carrageenan (CARR)
18 and hyaluronic acid (HA) dressings loaded with lidocaine (LID) and AgNPs were formulated as
19 freeze-dried wafers and functionally characterized for porous microstructure (morphology),
20 mechanical strength, moisture handling properties, swelling, adhesion and lidocaine release.
21 Antimicrobial activity of AgNPs was evaluated (turbidity assay) against *Escherichia coli*,
22 *Pseudomonas aeruginosa* and *Staphylococcus aureus* whilst cell viability studies (MTT) was
23 performed on normal adult human primary epidermal keratinocyte cells. The wafers were soft,
24 flexible and elegant in appearance. HA affected the wafer structure by increasing the resistance
25 to compression but still possessed a balance between toughness and flexibility to withstand
26 normal stresses and prevent damage to newly formed skin tissue respectively. Water uptake was
27 influenced by HA, whilst equilibrium water content and LID release were similar for all the
28 formulations, showing controlled release up to 6 h. AgNPs loaded CARR/HA wafers were
29 effective in inhibiting the growth of both Gram positive and Gram negative bacteria. MTT assay
30 showed evidence that the AgNPs/ LID loaded wafers did not interfere with cell viability and
31 growth. CARR/HA wafers seem to be a promising system to simultaneously deliver LID and
32 AgNPs, directly to infected chronic leg ulcers.

33

34 **KEYWORDS:** Antimicrobial activity; Carrageenan/hyaluronate wafers; Chronic leg ulcers;
35 Lidocaine; Silver nanoparticles; Wound healing

36

37 **1. INTRODUCTION**

38 Pain is very common in leg ulcer patients with about 1% of the Western population
39 estimated to suffer from chronic leg ulcers (CLUs) (O’Meara et al., 2012) with 17-65% of these
40 experiencing severe pain (Philips et al., 1994). Leg ulcers are common in older adults due to risk
41 factors including immobility and venous disease (Simon et al., 2004) and affect up to 2% of the
42 population over 80 years old (Petherick et al., 2013). Pain, together with leaking exudate, odor,
43 restricted mobility and sleep disturbance may be particularly challenging and distressing for
44 patients and have a negative impact on all aspects of daily living, causing depression, anxiety
45 and social isolation (Green et al., 2014). Furthermore, CLUs present a significant socio-
46 economic burden for health systems, with up to 3% of the entire healthcare budget, spent on
47 managing CLUs (Augustin et al., 2014; Posnett et al., 2008).

48 In CLUs, the wound is stuck in a continuous inflammation cycle, which coupled with
49 local factors (e.g. ischemia, infection and maceration) and underlying pathologies (e.g. diabetic
50 neuropathy, peripheral vascular disease), causes background pain. Pain also results from repeated
51 tissue insults caused by physical trauma, but the most common cause of wound pain is during
52 dressing change, debriding, and wound cleansing. Multilayer compression bandaging has been
53 identified as the gold standard in the treatment of venous leg ulcers (Harding et al., 2015), but
54 exudate control is a critical factor for its success. For this reason, health professionals would
55 welcome a dressing that deals with absorption challenges and help pain management,
56 particularly during dressing changes (Jorgensen et al., 2006).

57 Wound infection contributes to pain by triggering a continuous inflammatory response
58 through the release of inflammatory mediators, stimulating the production of enzymes and free
59 radicals, which can cause tissue damage (White, 2009). The consequent pain-related stress leads

60 to reduced immune response to infection and stimulation of pro-inflammatory cytokine
61 production in the wound (Glaser et al., 1999). Therefore, treatment of pain and infection should
62 be equally prioritized (Boateng & Catanzano, 2015). CLUs are particularly at risk of infection
63 due to high microbial bioburden (White et al., 2006) and the inability of leukocytes for
64 phagocytosis and intracellular killing of microorganisms (Oncul et al., 2007). Different
65 antibiotics have been used against wound infections, but the development of resistance has
66 highlighted the need for alternative solutions. Recently, silver nanoparticles (AgNPs) have been
67 recognized as optimal candidates for overcoming wound infections and due to their broad-
68 spectrum antimicrobial characteristics, have been widely used in CLU (Rizzello & Pompa,
69 2014). The use of AgNPs-containing dressings in the treatment of infected wounds has been
70 explored as a way to reduce risk of infection in the wound area and avoid delays in wound
71 closure (Boateng *et al.*, 2008; 2015; Hebeisha *et al.*, 2014). However, as noted earlier, wound
72 infection is also a cause of inflammation and pain so the delivery of AgNPs could be an indirect
73 way to reduce wound pain in CLUs.

74 Natural polysaccharides (e.g. chitosan and cellulose) have been reported by several authors as
75 delivery systems for AgNPs for treating infected wounds (El-Naggar et al., 2016; Ganest et al.,
76 2016; Ding et al., 2017; Singla et al., 2017). Carrageenan (CARR) and hyaluronic acid (HA) are
77 two very promising natural polysaccharides for wound dressing application due their good
78 biocompatibility, structural properties and biological activity (Boateng et al., 2013, 2015). CARR
79 is a natural carbohydrate polymer, extracted from intracellular matrix of red seaweeds, widely
80 used in the food industry for its excellent gelling capacity and the three commercially relevant
81 grades are kappa (κ), iota (ι), and lambda (λ). All CARRs are high-molecular-weight
82 polysaccharides made up of repeating galactose units and 3,6 anhydrogalactose (A), joined by

83 alternating α -1,3 and β -1,4 glycosidic (G) linkages. The main differences between kappa, iota,
84 and lambda CARRs are number and position of the ester sulfate groups on the repeating
85 galactose units and the molecular weight (Cunha & Grenha, 2016). κ -CARR has an ester sulfate
86 content of 25 - 30%, which imparts excellent film and gel forming capacity and together with
87 good biocompatibility, indicate that κ -CARR could be a versatile biomaterial for drug delivery.
88 Furthermore, κ -CARR exhibits immune and blood coagulation activities useful in tissue
89 engineering and wound healing (Cunha & Grenha, 2016; Liu et al., 2015; angestuti & Kim,
90 2014). During heating CARR first exists as random coil but the chains reorganize themselves as
91 double helices when cooled (Cunha and Grenha, 2015). In general, CARR lends itself to a
92 variety of applications to modify drug release and improve drug dissolution, and recently, our
93 group proposed κ -CARR based formulations for buccal mucosa drug delivery (Kianfar et al.,
94 2014; Kianfar et al., 2013), and as dressings for drug delivery to wounds (Boateng et al., 2013,
95 2015).

96 Hyaluronic acid (HA) is a long-chain linear glycosaminoglycan consisting of repeating
97 units of (β 1-4)-d-glucuronic acid and (β 1-3)-N-acetyl-d-glucosamine found naturally in the
98 body and acts as a structural component of the extracellular matrix (ECM) and mediator of
99 various cellular functions. HA is involved in each phase of wound healing increasing
100 keratinocyte migration and proliferation and facilitating transport of nutrients and waste products
101 (Dicker et al., 2014; Frenkel, 2012). HA was found to accelerate wound healing in both *in vitro*
102 (Catanzano et al., 2015) and *in vivo* (Foschi et al., 1990; King et al., 1991) models, and
103 accelerated skin wound healing in patients affected by different chronic wounds (Voigt & Driver,
104 2012).

105 The use of local anesthetic in the treatment of chronic wound pain can only give transitory
106 relief in patients that are constantly in pain, whereas the treatment of wound infection alone has
107 no effect on wound acute pain. For these reasons, we hypothesized that a composite
108 polysaccharide dressing comprising CARR and HA, loaded with a local anesthetic and AgNPs
109 could be very beneficial for the patients, acting directly on pain associated with CLUs as well as
110 deal with infection which one of the main causes of chronic inflammatory pain, consequently
111 enhancing wound healing and improving patient well-being. For this purpose, we present an
112 innovative and convenient multi-disciplinary approach based on composite polymeric wafers
113 capable of simultaneous delivery of an anesthetic drug, lidocaine (LID) and AgNPs. Wafers were
114 formulated from gels combining different ratios of CARR and HA, taking advantage of the
115 beneficial and well recognized properties of HA in the wound healing process. Different amounts
116 of LID and AgNPs were tested to evaluate the antimicrobial activity on Gram-negative and
117 Gram-positive bacteria and *in vitro* cytotoxicity in normal adult human primary epidermal
118 keratinocyte cell lines.

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120

121 **2 MATERIALS AND METHODS**

122 **2.1 Materials**

123 Low viscosity κ -carrageenan (Gelcarin GP 812 NF, molecular weight <100 kDa, 25% ester
124 sulfate and 34% 3,6-AG, stable at pH above 3.8) was obtained from FMC Biopolymer Corp
125 (Princeton, NY, USA). Sodium hyaluronate Hyasis[®] (mean molecular weight 0.8 MDa, intrinsic
126 viscosity 15dL/g) was a kind gift from Novozyme (Bagsvaerd, Denmark). Silver nanoparticles
127 (AgNPs) was provided as solution in water (10,000 ppm) from Clusternanotech Ltd. (UK).
128 Acetonitrile (HPLC grade), ethanol (laboratory grade), yeast extract, tryptone, 3-(4,5-
129 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and streptomycin sulphate (STP)
130 were all obtained from Fisher Scientific (Leicestershire, UK). Lidocaine hydrochloride
131 monohydrate (LID), bovine serum albumin (BSA), and all other chemicals were purchased from
132 Sigma Aldrich (Gillingham, UK). Media, sera, and antibiotics for cell cultures were from ATCC
133 (American Type Culture Collection, USA). Deionized ultra-filtered water was used throughout
134 this study.

135

136 **2.2 Preparation of CARR/HA composite wafers**

137 CARR and CARR/HA wafers were prepared by dispersing the polymers in stirred hot
138 water (70°C) until completely dissolved to obtain an aqueous polymeric solution (2% w/v) made
139 of only CARR and blends of CARR with HA in different weight ratios (90/10, 70/30, 50/50,
140 labelled as CARR/HA₁₀, CARR/HA₃₀ and CARR/HA₅₀ respectively. For drug-loaded wafers,
141 LID (10% w/w based on total polymer weight) and AgNPs (100 – 750 μ g/wafer) were dissolved
142 directly in the polymeric solution. Solutions were cast in a 24-well plates (1 ml, well size: 15 mm
143 diameter, 20 mm height) and lyophilized using an automated cycle on a Virtis Advantage XL 70

144 freeze dryer (Biopharma Process Systems, Winchester, UK) to form circular disks. The freeze-
145 drying process involved initially cooling the samples from room temperature to -50°C (8 h) and
146 heated during the primary drying phase (50 mTorr, -25°C) for 24 h to sublimate the ice,
147 followed by 7 h of secondary drying (10mTorr, 20°C) to remove free water. The wafers were
148 removed from the dish and kept in desiccators over calcium chloride at room temperature till
149 required.

150

151 **2.3 Physical and analytical characterization**

152 The surface morphology of the CARR/HA wafers was analyzed using scanning electron
153 microscopy (SEM). Samples were cut into small, thin pieces, placed on Agar Scientific double-
154 sided carbon adhesive tape on Agar Scientific G301 aluminium stub and coated with gold using
155 Sputter Coater (Edwards 188 Sputter Coater S1508) evaporator for 90 - 120 s. Images were
156 obtained using a Hitachi SU8030 (Hitachi High-Technologies, Krefeld, Germany) scanning
157 microscope under low vacuum at 5.0 kV accelerating voltage.

158 The average porosity of wafers was determined by a fluid replacement method. The
159 geometrical volume (V_s) of the wafers ($n = 5$) was calculated by measuring diameter and height,
160 and the pore volume (V_p) was measured by ethanol displacement. The dry wafer was weighed
161 (W_0) and immersed in absolute ethanol at room temperature, and placed in a degasser for 10 min
162 to remove air bubbles. After wiping gently with a filter paper, samples were weighed
163 immediately (W_e). The porosity of the wafers was calculated using equation 1:

164

$$165 \text{ Porosity} = \frac{V_p}{V_s} \times 100 = \frac{W_e - W_0}{\rho_e V_s} \times 100 \quad [\text{Equation 1}]$$

166

167 where ρ_e represents the density of ethanol (0.789 g/cm³).

168 The apparent density (ρ) of the sponge was calculated using equation 2:

169

$$170 \quad \rho = \frac{W_0}{V_s} \quad \text{[Equation 2]}$$

171

172 For water retention rate, wafer was soaked in water for 20 min, carefully removed, gently
173 wiped on tissue paper to remove the excess water and placed in a centrifuge tube equipped with a
174 mesh bottom, centrifuged at 500 rpm for 2 min and the wet weight recorded. An average value of
175 five replicates ($n = 5$) for each sample was taken. Water retention rate (WR) was calculated with
176 equation 3:

177

$$178 \quad WR = \frac{M_h - M_d}{M_d} \times 100 \quad \text{[Equation 3]}$$

179

180 Where M_h is the weight (g) of the wafer after centrifugation, and M_d is the initial dry weight (g).

181 The residual moisture in the wafers was estimated using thermogravimetric analysis
182 (TGA) (Thermal Advantage 2950, TA Instruments, Crawley UK). Samples ($n = 4$) weighing
183 between 3 and 6 mg were placed in a previously tared 70 μ L aluminium crucible and heated at
184 10°C/min from ambient temperature to 200°C under a constant stream of dry nitrogen. The
185 weight loss was plotted against temperature and percentage residual moisture was estimated from
186 the second derivative plot using TA Universal Analysis 2000 software.

187 A Fourier transform infrared spectrometer (FT-IR) equipped with a ZnSe attenuated total
188 reflectance (ATR) crystal accessory (Nicolet 8700 FTIR spectrometer, Thermo Fisher Scientific,
189 Surrey, UK) was used to investigate interaction between CARR and HA during formulation and

190 presence of unmodified HA. Wafers and starting materials, were placed on the ATR crystal and
191 pressed with a pressure clamp to allow optimal contact between the material and the crystal and
192 FT-IR spectra were collected over 4000–400 cm^{-1} using a resolution of 1 cm^{-1} .

193 **2.4 Swelling studies**

194 The percentage swelling ratio was determined by placing the wafers in simulated wound fluid
195 (SWF, CaCl 0.02 M, NaCl 0.4 M, Trizma base 0.4 M, BSA 2% w/v). The initial weight of each
196 wafer was recorded and completely immersed in 5 mL of SWF (37°C). Samples were carefully
197 taken out, excess SWF removed by carefully blotting with tissue paper, weighed and re-
198 immersed in SWF. Weights were recorded at intervals of 1 h up to 6 h and every 24 h from then
199 onwards until equilibrium was established and SWF replaced after every weight measurement.
200 The percentage swelling ratio ($SR\%$) was calculated using equation 4:

201

$$202 \quad SR\% = \frac{W - W_0}{W_0} \times 100 \quad \text{[Equation 4]}$$

203

204 Where W is the mass of the swollen sample and W_0 is the mass of the initial dry sample. The
205 equilibrium water content (EWC) per cent was calculated using equation 5:

206

$$207 \quad EWC (\%) = \frac{W_e - W_0}{W_e} \times 100 \quad \text{[Equation 5]}$$

208

209 Where W_e is the mass of the swollen sample at equilibrium.

210

211 **2.5 Mechanical hardness and *in vitro* wound adhesive properties**

212 Mechanical properties (resistance to deformation and ease of recovery) of the freeze-dried wafers
213 were investigated by compressing on a Texture Analyzer (TA) (Stable Microsystems Ltd.,
214 Surrey, UK) equipped with 5 kg load cell and Texture Exponent-32[®] software program. A 6 mm
215 cylindrical stainless steel probe was used in compression mode. Wafers ($n = 5$) were compressed
216 at three different locations to a depth of 2 mm at a speed of 1 mm/s using a trigger force of 0.001
217 N to determine the effects of HA on the resistance to deformation ('hardness').

218 *In vitro* wound adhesion was performed using the same apparatus but in tension mode.
219 Samples ($n = 5$) were attached to a 75 mm diameter probe using double-sided adhesive tape. A
220 6.67% w/v gelatin solution was allowed to set as solid gel in a Petri dish (diameter 88 mm) and
221 0.5 mL of SWF spread over the surface to simulate a moist wound (Boateng et al., 2013, 2015).
222 The probe, lined with wafer, was set to approach the model wound surface with the following
223 conditions: pre-test speed - 0.5 mm/s; test speed - 0.5 mm/s; post-test speed - 1.0 mm/s; applied
224 force - 0.01 N; contact time - 60.0 s; trigger type - auto; trigger force - 0.05 N and return distance
225 of 10.0 mm (Pawar et al., 2014). The maximum force required to detach the wafer on the upper
226 probe (stickiness) from the model wound surface, known as the peak adhesive force (PAF), total
227 work of adhesion (WOA) represented by the area under the force versus distance curve, and
228 cohesiveness, defined as the distance travelled by wafer till detached, were calculated using the
229 Texture Exponent-32[®] software.

230

231 **2.6 *In vitro* LID release**

232 *In vitro* release of LID from CARR and CARR/HA wafers ($n = 3$) was evaluated in SWF
233 at 37°C under gentle shaking and SWF (11 mL) was in contact with the wafers only by their
234 lower surface. The SWF was prepared without BSA to avoid blocking of the HPLC column. At

235 set times, 1.0 mL of release medium was withdrawn, replaced by the same amount of fresh SWF
236 and analyzed. Concentration of LID within the wafers and amount released at each time point
237 was determined by HPLC using an Agilent 1200 HPLC system (Agilent Technologies, Cheshire,
238 UK) equipped with a Chemstation[®] software program. The stationary phase was a Gemini C18
239 column (250 mm x 4.6 mm, 300 Å) (Phenomenex, USA), mobile phase comprised 5% acetic
240 acid in water (pH 3.4) and acetonitrile in the ratio of 80:20 (v/v) at flow rate of 1.0 mL/min,
241 injection volume was 20 µL and detection wavelength at 262 nm. The linearity of the response
242 was verified over a LID concentration range of 5 - 500 µg/ml ($R^2 = 0.997$).

243

244 **2.7 *In vitro* cytotoxicity**

245 Cell viability studies were performed on normal adult human primary epidermal
246 keratinocyte cells (ATCC PCS-200-011). The cells were cultured in cell culture flasks using a
247 complete culture medium consisting of dermal cell basal medium (ATCC[®] PCS-200-030) plus
248 one keratinocyte growth kit (ATCC[®] PCS-200-040) containing bovine pituitary extract (BPE), rh
249 TGF α , L-glutamine, hydrocortisone hemisuccinate, insulin, epinephrine and apotransferrin and
250 supplemented with 10 IU/mL of penicillin, and 10 µg/mL of streptomycin. Cultures were
251 maintained in humidified atmosphere of 95% air and 5% CO₂ at 37°C. The MTT (3-(4,5-
252 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was used to
253 evaluate the cell viability when in contact with the wafers (International Standardization
254 Organization, 1992) (details in supplementary information S1).

255

256 **2.8 Antimicrobial assay**

257 The antimicrobial activity of AgNPs-loaded wafers was tested on *E. coli* (ATCC 25922),
258 *P. aeruginosa* (ATCC 10145) and *S. aureus* (ATCC 29213). Wafers containing 100 µg
259 (CARR/AgNPs₁₀₀), 250 µg (CARR/AgNPs₂₅₀), 500 µg (CARR/AgNPs₅₀₀), and 750 µg
260 (CARR/AgNPs₇₅₀) each of AgNPs were used. Bacterial suspensions were prepared by taking 2-3
261 fresh colonies grown on Luria Bertani (LB) agar plates and suspended in sterile LB broth with no
262 sodium chloride (Tryptone 10 g/L, yeast extract 5 g/L). The turbidity was adjusted to 0.5
263 McFarland standard ($\sim 10^8$ cfu/mL) and then diluted to 10^6 cfu/mL (representing highly infected
264 chronic wounds) with fresh LB broth. Equally weighed samples were placed in sterile plastic
265 tubes, submerged with 10 mL of bacterial suspension, and incubated at 37°C in a shaking
266 incubator (100 rpm/min). Absorbance (A) at 580 nm of the bacterial suspension incubated with
267 samples ($n = 3$) at different time points (6, 24 and 48 h) were noted and difference in turbidity
268 was visually analyzed. 400 µL of streptomycin sulfate solution (1 mg/mL), and unloaded wafers
269 were used as positive and negative controls respectively.

270

271 **2.9 Statistical analyses**

272 Statistical analyses were undertaken using GraphPad Prism[®], version 6.00 (GraphPad Software,
273 La Jolla California USA, www.graphpad.com) and data compared using a Student's t-test and a
274 one-way ANOVA where relevant, with a Bonferroni post-test (parametric methods).

275

276 **3 RESULTS**

277 **3.1 Formulation development**

278 Freeze drying is a simple way to produce porous sponge-like dressings, useful in medium to
279 high exuding wounds (Matthews et al., 2005). Composite CARR/HA wafers possessed a smooth

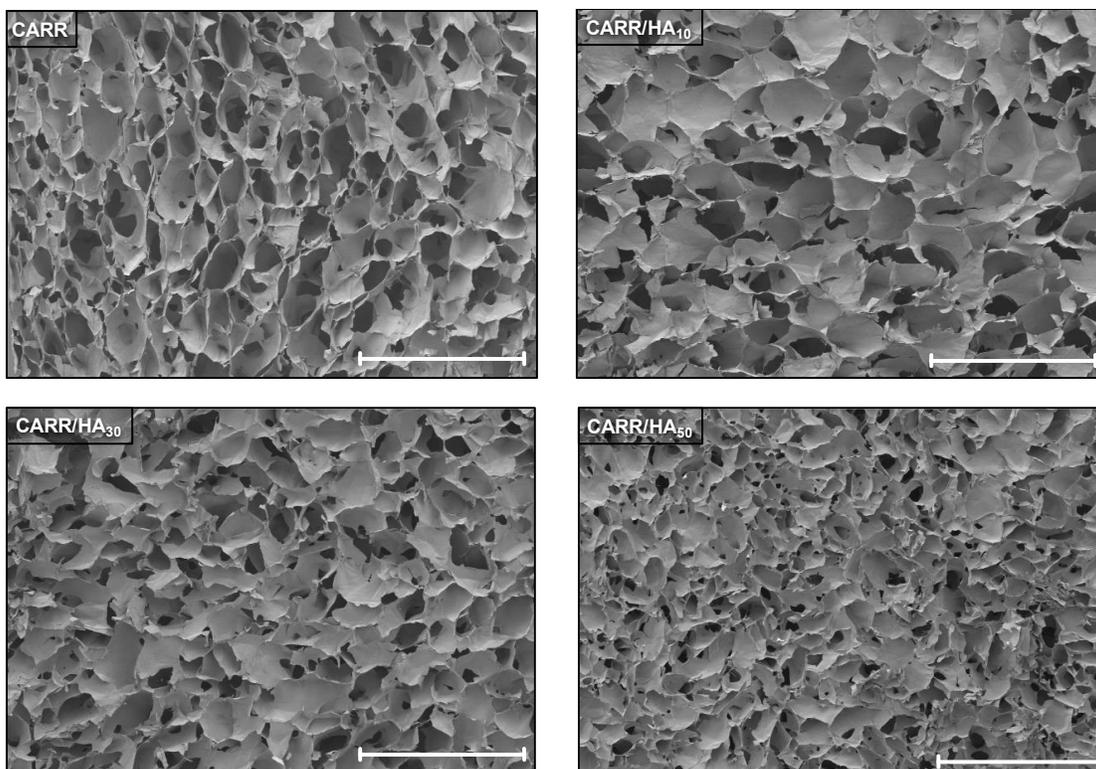
280 surface with uniform mass, texture and toughness. All formulations were elegant with no visible
281 cracks but the balance between flexibility and toughness changed depending on HA
282 concentration.

283

284 **3.2 Wafer characterization**

285 Representative SEM images of wafers are shown in Figure 1, showing porous
286 interconnecting network of polymeric strands having irregular and hexagonal shaped pores with
287 thin strands. CARR wafers formed an interconnecting network, with an average pore diameter of
288 50 μm and an open cell structure with hexagonal shaped pores. Addition of HA resulted in
289 smaller pore sizes with an average pore diameter between 10 and 30 μm . HA has an important
290 role in porosity development and wafers with higher HA amount showed relatively smaller sized
291 pores and a more irregular matrix structure. Such changes in microstructure can affect other
292 physical properties such as ‘hardness’ and hydration (Pawar et al., 2014).

293



294

295 **Figure 1:** Representative SEM micrographs of CARR and CARR/HA wafers showing differences in porous
 296 microstructure with different amounts of HA (x40, scale bar = 1mm).
 297

298 The porosity and water handling properties for the various wafers are summarized in
 299 Table 1. CARR wafers with more open and ordered pores and solid internal structure, had a
 300 water absorption > 3700 g water per 100 g of wafer, however addition of HA caused variation in
 301 all the physical properties. Both the porosity values, and apparent density of the wafers were
 302 influenced by HA, which made them more porous and less dense, therefore decreasing the water
 303 absorption. The water retention capacity was highest for CARR/HA₅₀ and generally higher in
 304 CARR/HA wafers compared to CARR wafer, due to the higher hydrophilic character of HA
 305 (Tool, 2004) which also resulted in slightly higher residual moisture content for CARR/HA
 306 wafers ranging from $17.00 \pm 0.57\%$ (CARR wafer) to $18.50 \pm 0.28\%$ (CARR/HA₅₀ wafer).
 307 Figure S2 (supplementary data) shows the FTIR spectrum (A) and assignment of the main
 308 absorption bands (B) of the raw materials and CARR/HA wafers at different ratios of HA.

309

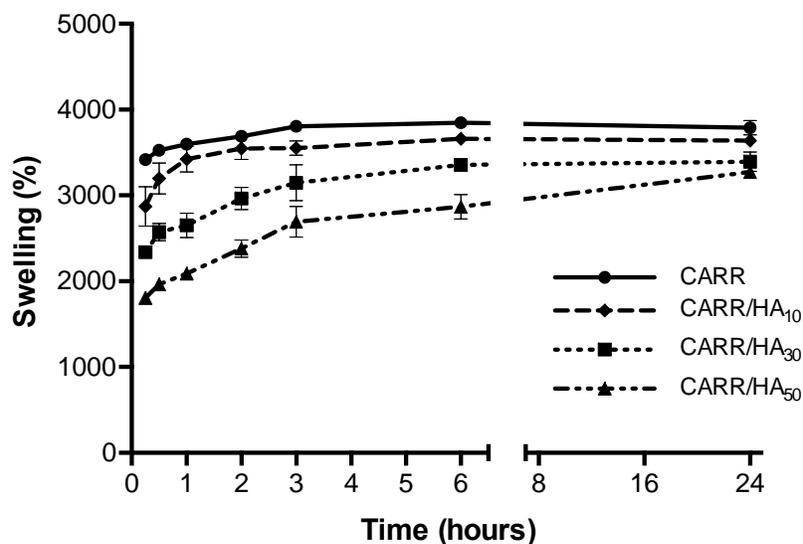
Table 1: Comparison of physical properties between different composite CARR/HA wafers

	Porosity (%)	Apparent density (mg/cm³)	Water absorption (%)	Water retention (%)	EWC (%)	Moisture content (%)
CARR	92.25 ± 4.53	26.78 ± 1.51	3782.7 ± 86.9	96.91 ± 0.53	97.40 ± 0.09	17.00 ± 0.57
CARR/HA ₅₀	85.00 ± 2.62	30.32 ± 1.25	3640.2 ± 39.1	98.88 ± 0.57	97.33 ± 0.03	18.50 ± 0.28
CARR/HA ₃₀	87.95 ± 3.55	29.69 ± 1.75	3394.1 ± 112.5	97.61 ± 1.70	97.04 ± 0.21	17.10 ± 0.44
CARR/HA ₁₀	89.06 ± 2.98	30.55 ± 0.60	3270.7 ± 70.8	95.65 ± 1.33	97.03 ± 0.08	16.73 ± 0.29

310

311 **3.3 Swelling studies**

312 Figure 2 showed differences in swelling capacity depending on the proportion of HA. The
 313 percentage swelling increased with time but the total capacity was lower in the presence of HA
 314 which was pronounced after 30 min, where the water uptake was 3528 ± 61% and 1966 ± 27% for
 315 CARR and CARR/HA₅₀ respectively and statistically significant ($p < 0.05$). The curves show that
 316 after 6 h the maximum swelling was achieved for all the formulations except CARR/HA₅₀, which
 317 continued to absorb water until 24 h. After 6 h, CARR wafers showed a significantly ($p < 0.05$)
 318 higher swelling capacity compared to CARR/HA₃₀ and CARR/HA₅₀ with a maximum of 3847 ±
 319 30%, whilst increase in HA caused a decrease in the swelling with CARR/HA₅₀ showing a
 320 maximum value of 2867 ± 143%. After 6 h no significant differences are found in swelling between
 321 CARR and CARR/HA₁₀ wafers.



322

323 **Figure 2:** Swelling profiles of CARR and CARR/HA wafers ($n = 3, \pm SD$). After 6 h CARR wafers have a significantly
 324 ($p < 0.05$) higher swelling capacity vs CARR/HA₃₀ and CARR/HA₅₀ while the difference between CARR and
 325 CARR/HA₁₀ was not significant. Moreover, no differences were noted in case of LID and AgNPs loaded wafers.

326

327

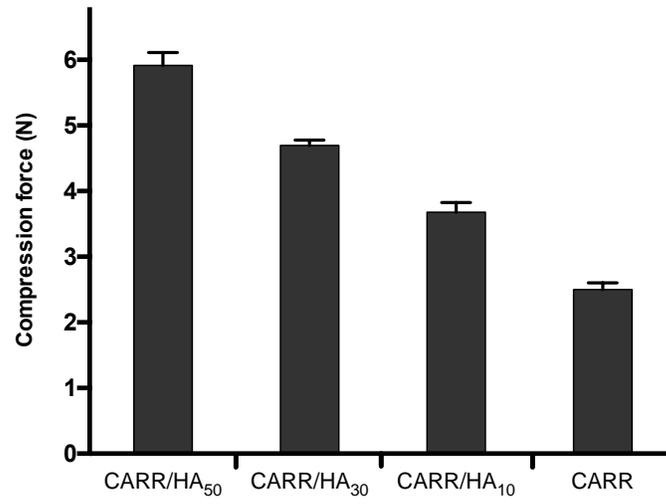
328 3.4 Mechanical hardness and in vitro mucoadhesive properties.

329 Figure 3A shows the changes in ‘hardness’ for the wafers with increasing concentration of
 330 HA which suggests increased ‘hardness’ (decreased flexibility), which could affect rate of
 331 hydration, swelling and mucoadhesion performance. Figure 3B shows the adhesion properties of
 332 CARR and composite CARR/HA wafers represented by PAF, WOA and cohesiveness. The CARR
 333 wafers had a higher PAF (0.740 ± 0.070 N) compared to the CARR/HA₅₀ (0.355 ± 0.057 N), with
 334 a decreasing trend proportional to the presence of HA. The same trend was observed for WOA and
 335 cohesiveness. These three parameters are directly influenced by the physicochemical properties of
 336 the wafer such as the pore size distribution and the consequent hydration capacity (Pawar et al.,
 337 2014).

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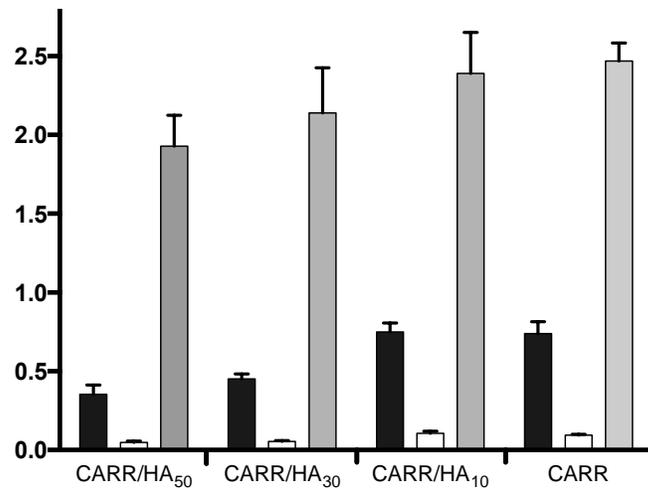
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340 A)



341

342 B)



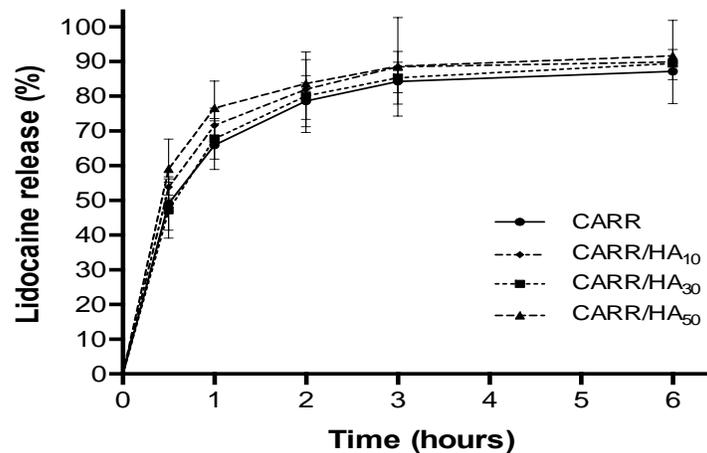
343 ■ PAF (N) □ WOA (N/mm) ■ Cohesiveness (mm)

344

345 **Figure 3:** A) Resistance to deformation ('hardness') of CARR or CARR/HA wafers compressed at three different
346 locations ($n = 5, \pm SD$). B) Adhesion profiles showing PAF, WOA and cohesiveness of CARR and CARR/HA wafers
347 ($n = 5, \pm SD$). Ordinate definition for the different parameters was reported in the figure legend.

348 **3.5 *In vitro* LID release**

349 The LID release profiles for CARR and CARR/HA wafers ($n = 3$) are shown in Figure 4,
350 showing controlled release over 6 h. Wafers with HA appear to release the drug more rapidly
351 initially, though the differences do not appear marked.
352



353

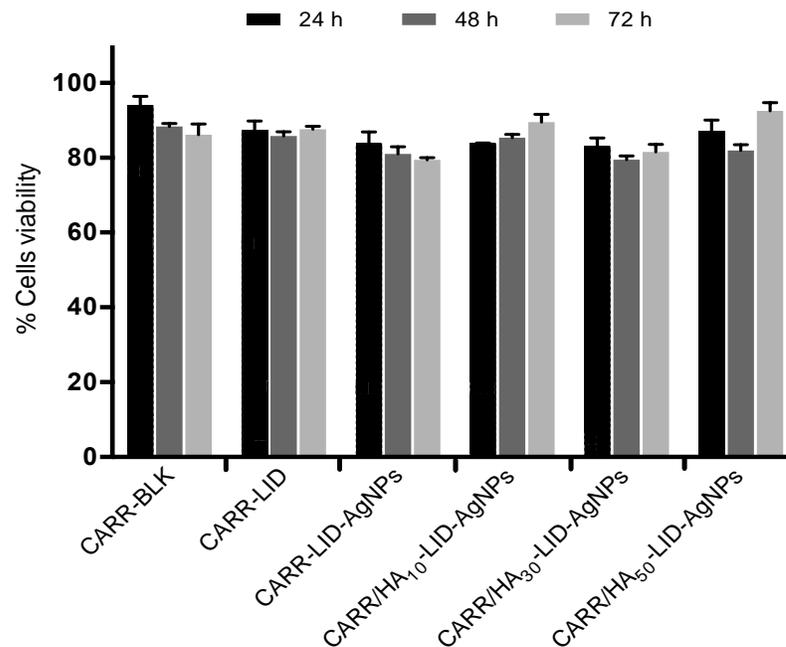
354 **Figure 4:** *In vitro* LID cumulative release ($n = 3 \pm$ SD) profiles from optimized drug loaded
355 wafers containing different proportions of CARR/HA.
356

357

358 **3.6 *In vitro* cytotoxicity**

359 Figure 5 shows the effect of the wafers on keratinocyte cells with all formulations showing
360 a good toxicity profile with a not significant reduction in viability in all the samples if compared
361 with the CARR- BLK wafer. In the range of concentrations studied the presence of both LID and
362 AgNPs seems not to influence the cells viability if compared to the positive control, triton-x, which
363 showed less than 10% cell viability (data not shown). Though the results show that there is a time
364 dependent mortality at 72 h below 90%, this is acceptable because the recommended guidelines
365 for *in vitro* cytotoxicity for medical devices and delivery systems such as wound dressings (DIN

366 EN ISO 10993-5) specifies that such materials can be deemed non-cytotoxic for $\geq 70\%$ cell
 367 viability after exposure (Moritz et al., 2014, Cerchiara et al., 2017). Therefore, the results obtained
 368 in the current study show that all the formulated dressings are generally safe.



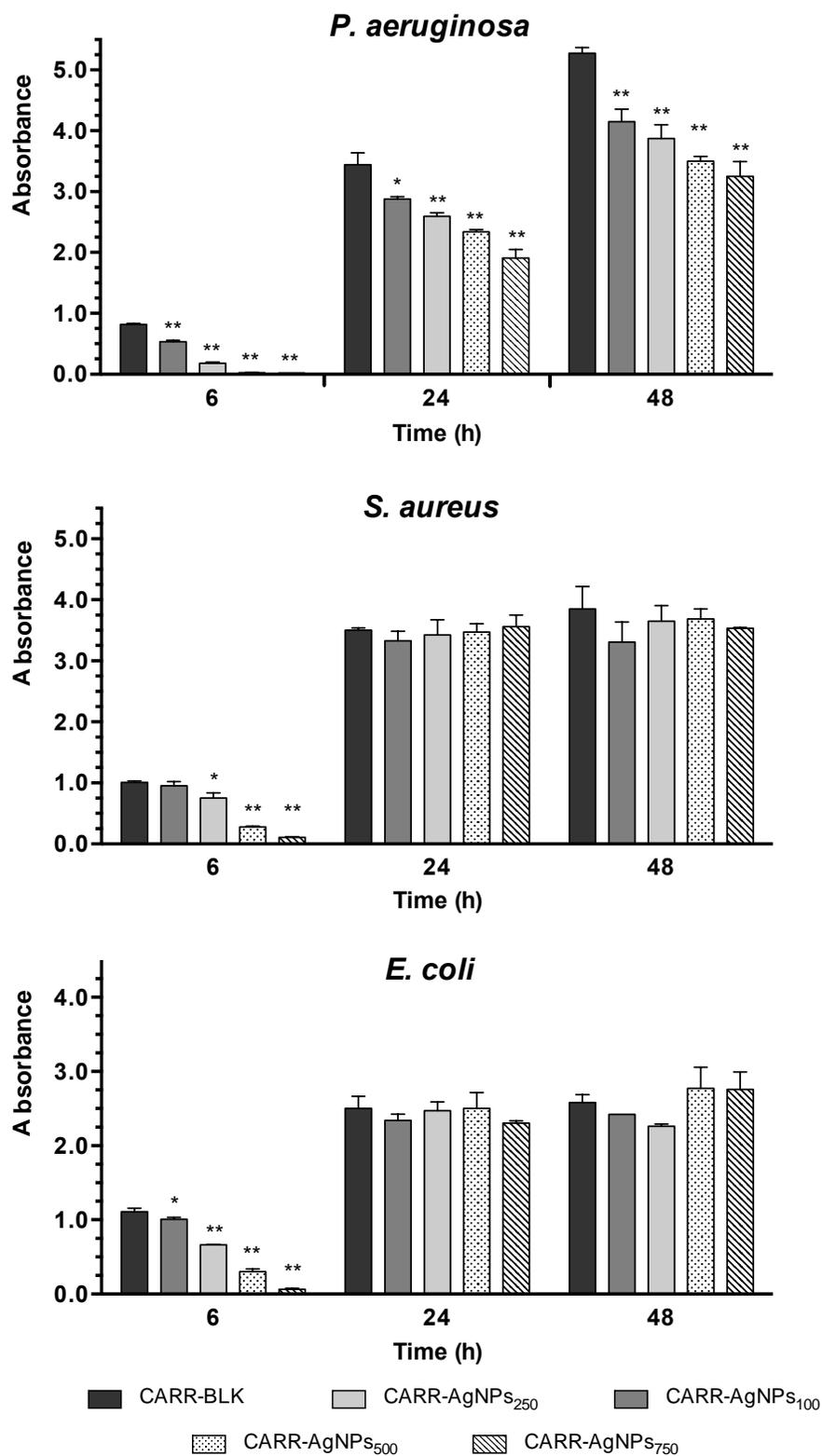
369
 370 **Figure 5.** Effect of CARR/HA wafers on cells viability of normal adult human primary epidermal keratinocytes.
 371 HaCaT have been seeded at 10,000 cells per well in a 96-well flat-bottomed culture plates and cultured with the
 372 extraction media for 24, 48 and 72 h. Bars represent the mean \pm standard deviation of three independent experiments
 373 ($n = 3 \pm SD$).
 374

375 3.7 Antibacterial assays

376 Significant difference ($*p < 0.05$; $**p < 0.01$) in the absorbance value at 580 nm was observed
 377 with *S. aureus*, *E. coli* and *P. aeruginosa* after 6 h of incubation for all the samples tested (Figure
 378 6). This activity was strictly related to AgNPs concentration. The activity profile of all the wafers
 379 on *P. aeruginosa* was quite different compared to the other bacterial species used in this
 380 experiment, since the antimicrobial activity lasted for at least 48 h. Difference in turbidity after 24
 381 h can be clearly visualized from the photograph of the test tubes containing CARR-AgNPs₅₀₀
 382 (supplementary data, Figure S4). Furthermore, even though some authors have found that LID has
 383 some antibacterial action against *E. coli*, *S. aureus*, and *P. aeruginosa* (Aydin et al., 2001) this

384 activity was not noticeable in initial trial observations with starting materials which suggested that
385 the LID present in the wafers did not show any recordable antibacterial activity. The wafers were
386 loaded with 10% LID (w/w based on total polymer weight) which was equivalent to 0.2% w/w
387 and this loading was chosen to be comparable with a high loading commercially available cream.
388 This dose of LID in our wafers was significantly lower than those tested in Aydin et al, (2001),
389 (5%, 2% and 1%) in solution, with only the 5 and 2% solutions showing broad antibacterial
390 activity. In addition, the LID used in the Aydin et al (2001) study was in solution and therefore in
391 direct immediate contact with the bacteria cells whiles the LID in our wafers will not be released
392 immediately due to the need for initial wafer hydration and subsequent diffusion from the swollen
393 dressing into the medium to allow antibacterial inhibition. Therefore we attributed all the
394 antimicrobial activity to the AgNPs, though future study investigating possible synergistic
395 antibacterial activity of both LID and AgNPs will be interesting, given the potential to reduce
396 possible toxicity by using lower doses of AgNPs.

397



398

399 **Figure 6:** Absorbance values (on ordinate axis) of *P. aeruginosa*, *E. coli* *S. aureus* after 6, 24 and 48 h of incubation
 400 with wafers loaded with different amounts of AgNPs. Significant differences in absorbance in comparison to control
 401 are indicated with asterisks (* $p < 0.05$; ** $p < 0.01$). Data are means of three independent experiments ($n = 3 \pm SD$).

402 **4 DISCUSSION**

403 Pain management in wound healing represents a challenge for physicians and nurses faced with
404 an increased number of patients affected by different pathologies. Wound dressings are the main
405 devices employed in these therapies and their correct use is an important factor that influences
406 the therapeutic outcome. The simultaneous delivery of a local anesthetic (LID) and an
407 antimicrobial agent (AgNPs) directly to the wound site can be an effective way to respectively
408 target pain itself directly and indirectly on infections, one of the main causes of wound pain due
409 to prolonging the inflammatory phase. Furthermore, the importance of HA in wound repair and
410 tissue regeneration is now considered a critical biomaterial affecting wound healing phases
411 (Frenkel, 2012) and its integration into advanced dressings represents a versatile approach to
412 promote wound healing that can be easily translated into a clinical setting (Catanzano et al.,
413 2015).

414 κ -carrageenan has been employed as films and wafers for wound healing (Boateng et al.,
415 2013; Pawar et al., 2014; Pawar et al., 2013). For our purpose, CARR and composite CARR/HA
416 wafers with different HA content were successfully prepared by freeze-drying. FT-IR
417 spectroscopy was used to confirm the absence of chemical interaction between CARR and HA
418 during the wafer formulation (Figure S2 in supplementary data). No reaction occurred between
419 CARR and HA as there was no significant new peak observed for the composite wafers,
420 suggesting that the two polymers were readily compatible within the composite wafer matrix.
421 The characteristic peak of pure HA appears almost at the same position in the FT-IR spectra of
422 CARR/HA wafers, despite the changes in peak intensity attributed to different amounts of HA
423 used. From a microscopic point of view, all the wafers were porous with an interconnecting
424 network, but micrographs from the SEM shows the effect of HA on the pore shape. CARR/HA

425 wafers pores are smaller with reduced depth since the HA partially fills the gaps between the
426 CARR chains, which also explains the reduction in porosity. This effect of HA on porosity was
427 also confirmed by evaluating the wafer recovery after deformation at different compression
428 depths (Supplementary data, Figure S3).

429 During wound dressing formulation, pore size and depth should be optimized as they
430 could significantly affect dressing performance in terms of hydration capacity, adhesion,
431 swelling and drug release (Kianfar et al., 2014). Furthermore, a uniform and solid porous
432 structure of crosslinked sponges should “lock” the water molecules and prevent easy runoff of
433 exudate. Attaining a dry state after lyophilization is essential to reduce molecular mobility and
434 hence, increased shelf life. Since no product melt-back was observed, the robustness of the
435 freeze-drying cycle is depicted, with the primary drying stage removing all the loose water. The
436 residual moisture content was derived from the loss in weight and represent bound water that
437 remains after the freeze-drying process. The swelling capacity is an essential characteristic, as
438 CLUs generally produce significant amounts of exudate that should be quickly absorbed and
439 retained over a prolonged period. The ability to absorb fluids and retain moisture without leaking
440 is essential for application on suppurating wounds, where high water retention capacity is
441 required (Boateng et al., 2008). During wound healing, moisture reactive dressings are able to
442 maintain a moist wound environment whilst at the same time avoiding the accumulation of
443 excess exudate on the wound, which can slow down wound healing and cause skin maceration
444 (Boateng & Catanzano, 2015). The water absorption kinetics of the wafers developed in this
445 study was mainly related to the composition of HA, despite the hydrophilic and polyanionic
446 characteristics of both polymers. This behavior was recently reported (Catanzano et al., 2017)
447 and can be attributed to the HA chain steric hindrance and increased internal micro-viscosity

448 within the cross-linked network that opposes the osmotic force, which drives water molecules
449 into the matrix. This is interesting, as the rate and duration of swelling, determines the ability of
450 the dressing to control drug release over a prolonged period, without the need for regular
451 dressing change (King et al., 1991).

452 The ideal dressing is required to be tough to allow ease of application without breaking
453 but should be soft and flexible to avoid possible contact irritation on the wound and the
454 destruction of newly formed skin tissue (Boateng et al., 2010). The increase in ‘hardness’ with
455 the increasing HA could affect swelling and mucoadhesion performance as ‘hardness’ is
456 indicative of wafer density in the internal matrix which affects the rate of water ingress,
457 hydration, impacting on swelling and adhesion (King et al., 1991; Boateng et al., 2010).

458 The use of local anesthetics in wound management is controversial due to their reported
459 delaying effects on wound healing mainly attributable to a reduction in collagen synthesis
460 (Chvapil et al., 1979). However, as demonstrated by Drucked *et al.*, local infiltration of LID does
461 not substantially alter wound healing as they observed no differences in the morphology and
462 mechanical properties of the wounds (Drucker et al., 1998). Furthermore, LID has a lower
463 incidence of allergic reactions than the ester-type anesthetics such as procaine and tetracaine
464 (Popescu & Salcido, 2004). Though solid lipid microparticles (Albertini et al., 2013) or
465 electrospun scaffolds (Thakur et al., 2008) have been proposed for controlled delivery of LID to
466 the wound site, they are difficult for large scale production and in clinical settings. The purpose
467 of this work was the development of a simple, economical but effective advanced multi-
468 functional composite dressing with LID directly dispersed in the polymeric matrix. Drug release
469 from biodegradable polymeric matrices is strictly correlated to water ingress into the device (Fu
470 & Kao, 2010). For wound dressings, drug release is driven by the exudate first hydrating the

471 polymer to cause swelling, dissolving and releasing the drug by gradual diffusion through the
472 swollen gel to the wound site. HA caused a significant ($p < 0.05$) decrease in water uptake but
473 had minimal influence on LID release. The drug release was sustained up to 6 h in all the
474 formulations, which implies that the drug release is mainly determined by the CARR, however
475 HA is still a vital dressing component, given its known wound healing properties. Furthermore,
476 enough LID was released from all the formulations (about 50%) in the first hour of dissolution
477 study. This suggests that the dressing could potentially manage pain within the first 60 minutes
478 of application which is important to quickly relieve the severe acute pain, common in leg ulcers
479 especially during dressing change, wound cleansing and new dressing application. However,
480 further *in vivo* studies are needed to confirm the real pain relieving and wound healing activities
481 of these dressings

482 Keratinocytes are the major cellular component of the epidermis and are directly involved
483 in re-epithelialization (Pastar et al., 2014) and provide a reliable *in vitro* model of re-
484 epithelialization phase in wound healing (Sivamani et al., 2007). For the toxicity studies the
485 higher concentration of AgNPs (750 $\mu\text{g/wafer}$) was chosen among all the formulations prepared
486 and the keratinocyte viability was comparable to that achieved with the blank wafer. All
487 formulations showed a similar viability profile and the addition of both LID and AgNPs in the
488 range of concentrations studied did not have any effect on cell viability. On the basis of toxicity
489 data, the AgNPs maximum amount in the hydrogels was fixed at 750 $\mu\text{g/wafer}$ in all the
490 subsequent experiments.

491 Wound colonization by bacteria is common in chronic wounds and in some cases, low
492 levels of bacteria seems to be beneficial to the wound healing process (Edwards & Harding,
493 2004). The progression from colonization to infection depends on the bacterial count, host

494 immune response, number of different species present, virulence of the organisms and
495 synergistic interactions between the different species (Edwards & Harding, 2004). AgNPs are
496 well established as antibacterial and dependent on physicochemical properties of the
497 nanoparticles, particularly the surface characteristics and particle size. For this study, very small
498 AgNPs with a narrow size distribution (mean diameter 0.8 nm) and a low polydispersity index
499 (0.110) were used. Several studies have reported the size dependent activity of AgNPs (Rizello
500 & Pompa, 2014) with small AgNPs (< 20 nm) inducing stronger bactericidal effect compared to
501 larger ones due to larger area-to-volume ratio and higher total surface area for membrane
502 interaction. The antimicrobial properties of AgNPs loaded wafers were tested on the most
503 common microorganisms present in CLUs (Ramani et al., 1991), and as expected the
504 antibacterial activity was strictly related to AgNPs concentration. The AgNP loaded wafers were
505 able to kill all the bacteria tested after 6 h of contact time while a different antimicrobial activity
506 was detected between Gram-positive and Gram-negative bacteria after 24 and 48 h, possibly due
507 the structural differences between the two classes. However, the Gram-negative *E. coli* showed a
508 lower susceptibility after 24 and 48h to CARR-AgNPs wafers than the other Gram-negative
509 strain (*P. aeruginosa*). Apart from the composition of the outer cell layers, AgNPs antibacterial
510 activity may also be related to the features of the individual bacterial species, and some strain-
511 specific variation in MICs and MBCs was observed for *E. coli* (Ruparelia et al., 2008).

512

513 **5 CONCLUSIONS**

514 In the current study, we hypothesized that composite CARR/HA based wafers, loaded with LID
515 and AgNPs could potentially address pain associated with CLUs as well as deal with infection
516 which is one of the main causes of chronic inflammatory pain and this was confirmed from the

517 results. We have demonstrated how lyophilized wafers can be designed to obtain a simple,
518 economical but effective multi-targeted composite wound dressing useful for pain management
519 in CLUs. CARR/HA wafers with various HA contents showed a porous nature with an
520 interconnecting network and pore size related to the amount of HA which was also confirmed by
521 texture analysis and swelling studies. Texture analysis also confirmed the good handling
522 properties that together with ease of preparation are essential to provide a rapid and effective
523 treatment for CLUs in hospitalized patients. The fast drug release and the effective antimicrobial
524 activity confirm that the association of an analgesic drug (LID) with an antimicrobial compound
525 (AgNPs) could further increase the effectiveness of this dressing in pain and infection
526 management. Finally, CARR/HA wafers seems to be a very promising system for the treatment
527 of wound pain, however, further studies are needed to evaluate the *in vivo* wound healing
528 activities of the dressings.

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