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(Article begins on next page)

1 **Ciprofloxacin carrier systems based on hectorite/halloysite**
2 **hybrid hydrogels for potential wound healing applications**

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23 KEYWORDS. Clay minerals, halloysite, hectorite, hybrid hydrogel, wound healing, drug carrier

24

25 ABSTRACT. The design of multifunctional nanomaterials which can help the healing processes
26 of skin, preventing the bacterial infections, is crucial for the development of suitable therapy for
27 the treatment of chronic lesions. The use of clay minerals in wound healing applications is well
28 documented since the prehistoric period and offers several advantages due to their intrinsic
29 properties.

30 Herein, we report the development of ciprofloxacin carrier systems based on hectorite/halloysite
31 (Ht/Hal) hybrid hydrogels for potential wound healing applications. To achieve this objective
32 firstly the ciprofloxacin molecules were loaded onto Hal by a supramolecular and covalent
33 approach. The so obtained fillers were thoroughly investigated by several techniques and at
34 molecular level by means of quantum mechanics calculations along with empirical interatomic
35 potentials. Afterwards the modified Hal were used as filler for Ht hydrogels. The introduction of
36 modified Hal, in hectorite hydrogel, helps the gel formation with an improvement of the
37 rheological properties. The *in vitro* kinetic release from both the fillers and from the hybrid
38 hydrogels was studied both at skin's pH (5.4) and under neutral conditions (pH 7.4); in addition,
39 the factors controlling the ciprofloxacin release process were determined and discussed. Finally,
40 the *in vitro* biocompatibility of the Hal fillers was evaluated by means of cytotoxic assays and laser
41 scanning confocal microscopy on normal human dermal fibroblasts.

42 **INTRODUCTION.** Skin, the biggest organ, represents the natural barrier between the body and
43 the outside world protecting it from the invasion of external microorganisms. When a trauma
44 occurs, the skin tissue is damaged, and it becomes exposed to the external environment. Acute
45 wounds generally heal within few weeks, but if the healing process is somehow impaired

46 chronic wounds occur with a healing time greater than 12 weeks. A delay in wound healing
47 could pose serious and life-threatening medical conditions which could lead to amputation.
48 Bacterial infection is one of the most serious issues that can impair healing (Hu and Xu,
49 2020; Liang et al., 2019).

50 In the last years, the use of clay minerals for application in biomedical field has proven to
51 be advantageous, mostly in tissue engineering since they can enhance cell attachment,
52 proliferation and differentiation (Dawson and Oreffo, 2013; Naumenko et al., 2016), acting
53 as antimicrobials as well (Williams et al., 2011).

54 Hydrogels formed by fibrous clay, have attracted considerable attention since they can
55 promote wound healing process and offer several advantages for topic drug delivery such
56 as prolonged sustained release as well as easy of administration (Sharifzadeh et al., 2020).
57 Furthermore, they are biocompatible and show high swelling capacity, that are crucial for
58 the diffusion of active molecules into the target site (Kevadiya et al., 2014). Hectorite (Ht)
59 is a clay of smectite groups which possesses the ability to form stable hydrogels in aqueous
60 regime. Biocompatibility studies have shown that its hydrogels possess potential wound
61 healing activity (García-Villén et al., 2021).

62 Fluoroquinolone compounds such as ciprofloxacin (Cipro), are widely used as antibiotics
63 to treat skin infections since they are among the most used broad-spectrum antibiotics
64 (Campoli-Richards et al., 1988).

65 However, ciprofloxacin suffers from some inconveniences which limit its clinical use, *i*) Cipro
66 is insoluble in physiological conditions (Korzeniowska et al., 2020) and *ii*) it presents poor
67 permeability across cell membranes (Breda et al., 2009). Unfortunately, there are some
68 limitations in the use of hectorite as carrier for the hydrophobic Cipro (Massaro et al.,

69 2021b), so the introduction of a filler in the hectorite hydrogel, that acts as a carrier, could
70 be advantageous. Recently, the combination, both by a supramolecular and a covalent
71 approach, of two different clay minerals with complementary properties has been
72 investigated. It was found that the obtained hybrid materials showed improved physico-
73 chemical and biological properties compared to pristine components which make them
74 attractive for biological purposes (Massaro et al., 2021b).

75 Halloysite nanotubes (Hal) are an aluminosilicate clay with a predominantly hollow tubular
76 structure and chemically similar to the platy kaolinite ($\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4 \cdot n\text{H}_2\text{O}$). Generally,
77 the length of the tubes is in the range of 0.2–1.5 μm , while their inner and outer diameters
78 are in the ranges of 10–30 nm and 40–70 nm, respectively (Liu et al., 2014). Hal, naturally
79 occurring in huge quantities at low cost, show excellent bio-(Fakhrullina et al., 2015;
80 Kryuchkova et al., 2016; Rozhina et al., 2021; Rozhina et al., 2020) and ecocompatibility
81 (Bellani et al., 2016). Halloysite is positively charged in the inner lumen, which consists
82 mostly of aluminum hydroxide, whereas the external surface, which is silicon dioxide, is
83 negatively charged. However, the charge properties of Hal surfaces are strictly dependent
84 from the pH depending on the protonation/deprotonation equilibria of the surface groups
85 (Bretti et al., 2016). The different surface chemistry allows the selective functionalization
86 at the inner and/or outer side making possible the synthesis of several nanomaterials with
87 hierarchical nanostructure (Massaro et al., 2020b; Peixoto et al., 2021; Stavitskaya et al.,
88 2018). Hal are widely used as enzyme immobilization (Tully et al., 2016), catalysts (Lin et
89 al., 2020; Massaro et al., 2020a; Stavitskaya et al., 2020), environmental remediation
90 (Massaro et al., 2016; Salaa et al., 2020), drug carriers (Massaro et al., 2019b; Massaro et
91 al., 2020c) and delivery systems (Alfieri et al., 2022) and so on (Borrego-Sánchez et al.,

2018; Riela et al., 2021; Santos et al., 2019). Most importantly, Hal are able to penetrate the cellular membrane surrounding the cell nuclei (Gorbachevskii et al., 2021; Lvov et al., 2014). In addition, it has been proved that the modification of the tubes surfaces makes hybrid nanomaterials that penetrate the nucleus membrane, as well (Massaro et al., 2019a). Herein, we report the development of ciprofloxacin carrier systems based on Ht/Hal hybrid hydrogels for potential wound healing applications. Firstly, we studied the ciprofloxacin loading both supramolecular and covalently onto Hal. The fillers obtained were thoroughly characterized both from an experimental point of view and at molecular level by means of quantum mechanics calculations along with empirical interatomic potentials. Then, we explored the possibility of obtaining Ht hydrogels in the presence of modified Hal filler loaded with ciprofloxacin. The mechanical properties of the hydrogels obtained were examined by rheology measurements. The *in vitro* kinetic release from both the fillers and from the hybrid hydrogels was studied both at skin's pH (5.4) and under neutral conditions (pH 7.4); in addition, the factors controlling the ciprofloxacin release process were determined and discussed. Finally, the biocompatibility of the halloysite systems was also evaluated by means of cytotoxic assays and laser scanning confocal microscopy on normal human dermal fibroblasts.

2. MATERIALS AND METHODS.

All chemicals were obtained from Sigma-Aldrich and used as received. Hal-NH₂ was prepared as reported elsewhere.(Massaro et al., 2018b) FT-IR spectra (KBr) were acquired with an Agilent Technologies Cary 630 FT-IR spectrometer. The morphology of the nanomaterials was studied using an ESEM FEI QUANTA 200F microscope with EDX probe. The measurement was carried out in high-vacuum mode ($<6 \times 10^{-4}$ Pa). Before each experiment, the sample was coated with

115 gold in argon (60 s) by means of an Edwards Sputter Coater S150A to avoid charging under an
116 electron beam. Differential scanning calorimetry (DSC) analyses were performed (Mettler Toledo,
117 Columbus, OH, USA) using aluminum crucibles, a 30–400°C temperature range, at a heating rate
118 of 10°C min⁻¹. All the analyses were performed in atmospheric air. X-ray powder diffraction
119 (XRPD) analysis was carried out using a diffractometer (X'Pert Pro model, Malven Panalytical)
120 equipped with a solid-state detector (X'Celerator) and a spinning sample holder. The diffractogram
121 patterns were recorded using random oriented mounts with CuK α radiation, operating at 45 kV
122 and 40 mA, in the range 4–60 °2 θ . Thermogravimetric (TG) analyses were carried out through a
123 Q5000 IR apparatus (TA Instruments) under nitrogen atmosphere (gas flows of 25 and 10 cm³
124 min⁻¹ were employed for the sample and the balance, respectively). The experiments were carried
125 out by heating the sample (ca. 5 mg) to 800°C. The heating rate was 20°C min⁻¹. The rheology
126 measurements were recorded at room temperature on an DHR2 (TA Instruments) oscillatory
127 rheometer using a parallel–plate (20 mm) tool; the sample was placed between the shearing plates
128 of the rheometer. The rheological properties, such as strain sweep and frequency sweep, were
129 recorded three times on three different aliquots of gels. The strain sweeps were performed at an
130 angular frequency of 1 rad s⁻¹ and the frequency sweeps were performed at strains of 1%.

131 **2.1. Loading of ciprofloxacin into Hal lumen (Hal/Cipro)**

132 To a dispersion of Hal in MeOH (5 mL), 1 mL of a solution 10⁻² M of ciprofloxacin in HCl 0.1
133 N was added. The suspension was sonicated for 5 min, at an ultrasound power of 200 W and at
134 25°C and then was evacuated for 3 cycles. The suspension was left under stirring for 18 h at room
135 temperature. After this time, the powder was washed with water and then dried at 60 °C.

136 **2.2. Covalent grafting of Cipro onto Hal (Hal-Cipro)**

137 Ciprofloxacin (50 mg, 0.15 mmol) was suspended in CH₂Cl₂ (10 mL), and N,N-
138 dicyclohexylcarbodiimide (DCC) (35 mg, 0.15 mmol) was added. The suspension was stirred
139 under an argon atmosphere at room temperature for 10 min. Then, Hal–NH₂ (100 mg) was quickly
140 added. The mixture was stirred for 48 h. Then, the solvent was removed by filtration; the powder
141 was then rinsed successively with H₂O and CH₂Cl₂ and finally dried at 80°C under vacuum.

142 **2.3. Preparation of Ht hydrogels.**

143 Pure gels were prepared by weighing into a screw-capped sample vial (diameter 2.5 cm) the
144 amount of hectorite (100 mg) and solvent (~ 1 g). The mixture was first dispersed for 5 minutes
145 with ultrasound irradiation and left at room temperature until a gel was obtained.

146 **2.4. Preparation of Ht hybrid hydrogels.**

147 Hybrid hydrogels were prepared by weighing into a screw-capped sample vial (diameter 2.5 cm)
148 the amount of Ht (100 mg), modified Hal (5 mg) or Cipro (5 mg) and solvent (~ 1 g). The mixture
149 was first dispersed for 5 minutes with ultrasound irradiation and subsequently left at room
150 temperature until a gel was obtained.

151 **2.5. Models**

152 The adsorption complex models were created from the atomic coordinates of a slice of a halloysite
153 nanotube from previous work (Guimarães et al., 2010) with the stoichiometry Al₂Si₂O₅(OH)₄.
154 Periodic boundary conditions of this nanotube were used to generate a periodical crystal structure,
155 and the cell parameters of the structure was described previously (Borrego-Sánchez et al., 2017;
156 Carazo et al., 2017). The unit cell of the nanotube of halloysite has an internal layer of aluminium
157 hydroxide octahedral, with an internal diameter of 27 Å, joined to an external layer of silicon oxide
158 tetrahedra. This structure is a great model to reproduce the interactions at molecular level of the
159 adsorption process. The unit cell of halloysite has the formula Al₇₆Si₇₆O₁₉₀(OH)₁₅₂ with 646 atoms.

160 To carry out the adsorption of ciprofloxacin drug, a $1 \times 1 \times 2$ supercell of halloysite was generated
161 with the formula $\text{Al}_{152}\text{Si}_{152}\text{O}_{380}(\text{OH})_{304}$ and with 1292 atoms. The ciprofloxacin drug was taken
162 from crystallographic data (Turel et al., 1997). A molecule was extracted from the crystal to study
163 its adsorption in the halloysite $1 \times 1 \times 4$ supercell.

164 **2.6. Molecular modeling methodology**

165 The optimization of the unit cell of halloysite nanotube structure was performed with quantum
166 mechanical calculations by using Density Functional Theory (DFT) with CASTEP code of the
167 Materials Studio package (BIOVIA, 2016). The functionals used were the Perdew–Burke–
168 Ernzerhof (PBE) correlation exchange one in the generalized gradient approximation (GGA). On-
169 the-fly generated (OTFG) ultrasoft pseudopotentials were used with Koelling-Harmon relativistic
170 treatment (Vanderbilt, 1990), and the cut off energy of the calculation was 300 eV (BIOVIA,
171 2016). After the optimization of the halloysite unit cell, the $1 \times 1 \times 2$ supercell was created to study
172 the adsorption of the drug. The ciprofloxacin molecule was optimized with the Compass Force
173 Field (FF) by using the Forcite program that have provided good results in previous studies
174 (Borrego-Sánchez et al., 2017; Borrego-Sánchez et al., 2016) (BIOVIA, 2016). For non-bonding
175 interactions, the coulomb and van de Waals interactions were calculated by the Ewald and atom-
176 based methods, respectively, with a cut-off of 18.5 Å. The methanol molecule geometry was
177 optimized using the same methodology that the drug. Different conformations of the optimized
178 ciprofloxacin drug and different relative orientations between the drug and the clay were randomly
179 explored, both inside the halloysite nanotube and on the external surface of the clay. For this
180 purpose, Monte Carlo methods using the Compass FF with Adsorption Locator module was used
181 (BIOVIA, 2016). The more stable drug-clay complexes were selected when the ciprofloxacin is
182 adsorbed on the inner surface and on the outer surface of the halloysite nanotube. Later, in both

183 selected adsorption models, a calculation was performed to fill the model with previously
184 optimized methanol molecules using the Compass FF with Amorphous Cell module (BIOVIA,
185 2016). In this way, the methanol with a density of 0.792 g/cm^3 filled the adsorption complexes
186 (500 molecules of methanol), as it happened experimentally. These complexes were optimized
187 fixing the structure of the clay, except the hydrogen atoms of the inner surface of halloysite with
188 a cut-off of 18.5 \AA . The adsorption energies of these complexes were compared according to the
189 equation $\Delta E_{\text{ads}} = (E_{\text{drug}} - E_{\text{drug/clay}}) - (E_{\text{drug}} + E_{\text{clay}})$. For this, the energy of each optimized adsorption
190 complex was calculated, as well as the drug and the clay with the solvent of the optimized
191 complexes were isolated and the energies were calculated, with an energy calculation with the
192 Compass FF, the Forcite program and a cut-off of 18.5 \AA (BIOVIA, 2016).

193 **2.7. Kinetic Release**

194 The release of Cipro from Hal both in Hal/Cipro and Hal-Cipro was done as follows: 20 mg of the
195 sample were dispersed in 1 mL of dissolution medium (phosphate buffers pH 7.4 and 5.5) and
196 transferred into a sealed dialysis membrane (Medicell International Ltd MWCO 12-14000 with a
197 diameter of 21.5 mm). Subsequently the membrane was put in a round bottom flask containing 9
198 mL of the release medium at 37°C and stirred. At fixed time, 1 mL of the release medium has been
199 withdrawn and analyzed by UV-*vis* measurements. To ensure sink conditions, 1 mL of fresh
200 solution has been used to replace the collected one.

201 **2.8. Cipro release from gel matrix.**

202 Hybrid hydrogels obtained at 3 wt % of Ht and 5 wt% of modified Hal were prepared, as discussed
203 above, in a total volume of 3 mL. 3 mL of the phosphate buffer pH 5.5 or pH 7.4 were casted on
204 gel matrix. The release kinetic was carried out at 37°C . At fixed intervals of time, 750 μL of
205 supernatant solution were taken out to be spectrophotometrically analyzed controlling Cipro peak

206 at 320 nm, and simultaneously refilled with other 750 μ L of the same release medium pre-warmed
207 at 37°C.

208 Total amounts of drug released (F_t) were calculated as follows:

$$209 \quad F_t = V_m C_t + \sum_{i=0}^{t-1} V_a C_i \quad (\text{Eq. 4})$$

210 where V_m and C_t are the volume and the concentration of the drug at time t . V_a is the volume of
211 the sample withdrawn and C_i is the drug concentration at time i ($i < t$).

212 **2.9. Cytotoxicity Test (MTT assay)**

213 NHDF were seeded in 96-well plates (0.35×10^5 cell/well in 200 μ L/well) and incubated for 24 h
214 at 37°C in a humidified atmosphere containing 5% CO_2 (CO_2 Incubator, PBI International, Milan,
215 I). After 24h, the medium was removed, cells were washed once with 200 μ L/well PBS (phosphate
216 saline buffer, Sigma-Aldrich, I), and then treated for 24 h. Four different concentrations of
217 ciprofloxacin for each nanomaterial were evaluated (5, 10, 25 and 100 μ M). These were compared
218 with the free drug diluted at the same concentrations in growth medium (drug stock solution in 0.1
219 N HCl to allow drug solubilization). Eight replicates for each sample were performed. After 24 h
220 of treatment, samples were withdrawn, fibroblasts washed once with PBS and exposed for 3 h to
221 50 μ l MTT solution (2.5 mg/mL solubilized in DMEM w/o red phenol) diluted in 100 μ L of
222 DMEM. Then, the reagent was removed, cells washed and 100 μ L DMSO were pipetted in each
223 well. The absorbance at 570 nm, with 690 nm as reference wavelength, was immediately read by
224 means of an ELISA plate reader (FLUOstar Omega - BMG LabTech, G). Cell viability was
225 calculated as percentage ratio between the absorbance of each sample and the absorbance of
226 control, cell substrates in growth medium, GM.

227 **2.10. Cell morphology**

228 Cell morphology was evaluated using CLSM analysis. Briefly, cover slides with $\varnothing=13$ mm were
229 placed on the bottom of the wells of a 24-well plate and 10×10^5 cells/well (400 μL /well) were
230 seeded. After 24 h, cells were incubated for further 24 h with Cipro loaded nanomaterials and free
231 Cipro (concentration 10 μM). Cells were fixed with 3% (w/v) glutaraldehyde for 2 h at room
232 temperature. Cellular substrates were washed twice in PBS and cytoskeleton was stained with 150
233 μL (50 $\mu\text{g}/\text{mL}$) phalloindin-Atto 488 (Sigma-Aldrich, I) incubated for 40 min at room temperature,
234 in the dark. After two-time washing with PBS, cell nuclei were stained with 100 μL of Iodide
235 Propidium, (25 $\mu\text{g}/\text{mL}$) for 2 min in the dark, then samples were placed on a microscope slide and
236 imaged using a Confocal Laser Scanning Microscope (CLSM, Leica TCS SP2, Leica
237 Microsystems, I) using $\lambda_{\text{ex}} = 535\text{nm}$ and $\lambda_{\text{em}} = 617$ nm for Iodide Propidium and $\lambda_{\text{ex}} = 501$ nm and
238 $\lambda_{\text{em}} = 523$ nm for phalloindin-Atto 488. The acquired images were processed with the software
239 associated with the microscope (Leica Microsystem, LASX, CMS, GmbH, I).

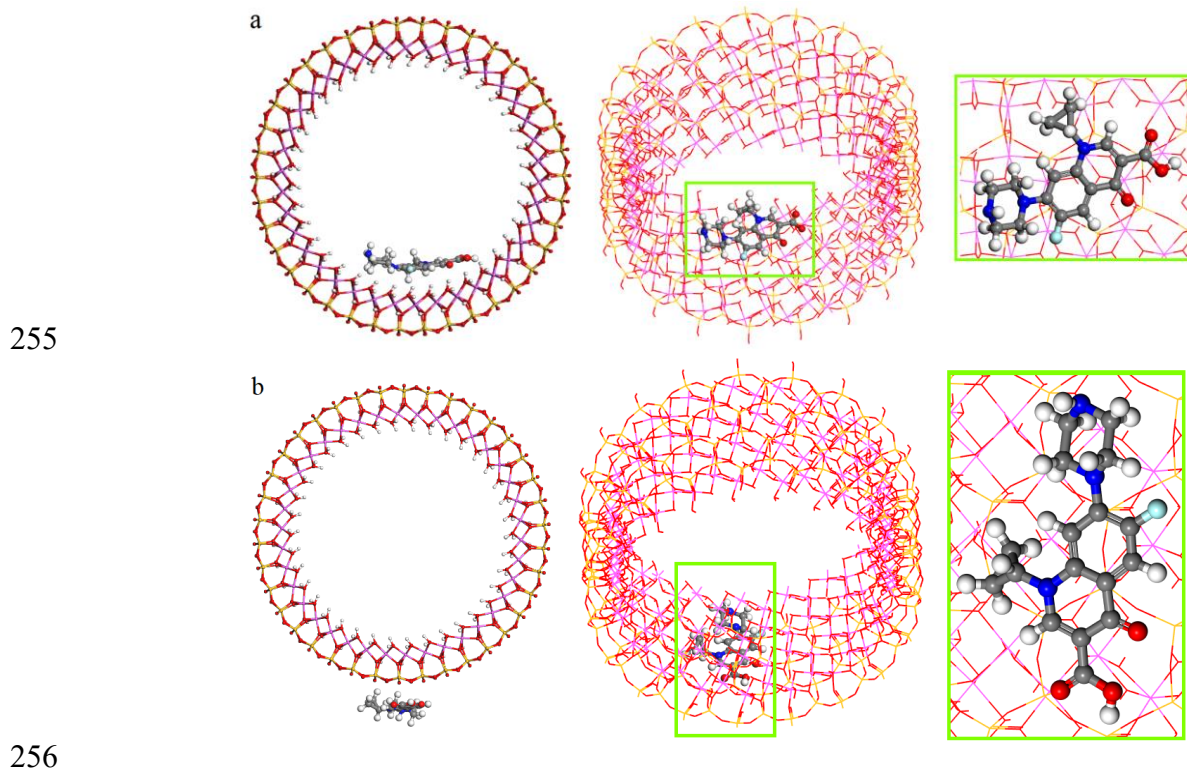
240 **3. RESULTS AND DISCUSSION.**

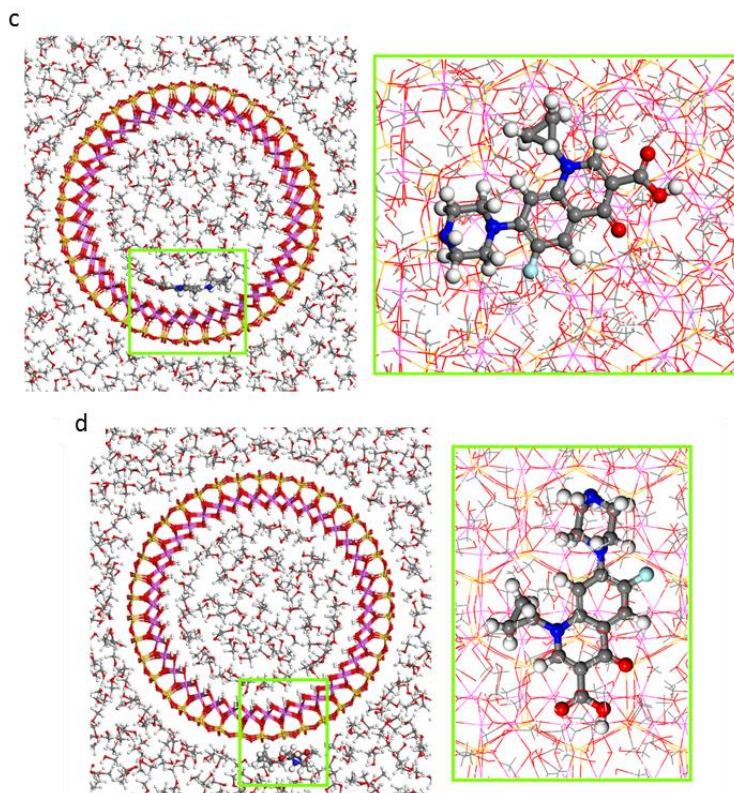
241 **3.1. Loading of Cipro on Hal: supramolecular binding (Hal/Cipro)**

242 Ciprofloxacin is an insoluble drug in water. To build optimal ciprofloxacin carrier systems based
243 on HT/Hal hybrid hydrogels with high entrapment efficiency and high stability of the drug in
244 water, we first performed preliminary investigation on the drug adsorption ability of Hal by of
245 quantum mechanics calculations along with classical mechanics calculations and by the
246 construction of the adsorption isotherms.

247 To investigate the nature of the ciprofloxacin adsorption on Hal we performed some molecular
248 modelling simulations,(Prishchenko et al., 2018) considering both the internal and external Hal
249 surface and methanol (MeOH) as solvent.

250 Monte Carlo methods using the Compass Force Field (FF) were performed to explore the more
251 stable halloysite/Cipro interaction on the two surfaces of the halloysite nanotube model (on the
252 inner and on the outer surfaces). The most stable model, with ciprofloxacin absorbed on the inner
253 clay surface, was selected (Figure 1a). In the same way, the most stable model when the drug is
254 adsorbed on the external surface was also selected (Figure 1b).





257
 258 **Figure 1.** Adsorption models of the ciprofloxacin drug adsorbed on the internal (a-c) and external (b-d)
 259 surfaces of halloysite nanotube without or with methanol as solvent optimized with Monte Carlo method
 260 and Compass FF, views from (001) and (100) planes. The atoms of silicon, aluminium, hydrogen, carbon,
 261 nitrogen, oxygen, and fluorine are presented in yellow, pink, white, grey, blue, red, and cyan, respectively.
 262

263 In the most stable adsorption model (Figure 1), the ciprofloxacin inside the halloysite was oriented
 264 in a perpendicular direction with respect to the c axis of the nanotube (Figure 1a). On the contrary,
 265 the drug on the external surface of the clay adopted a parallel position with respect to the c axis
 266 (Figure 1b).

267 Subsequently, the optimization of the adsorption models was performed filling previously each
 268 model with 500 optimized molecules of methanol by the Compass FF (Figure 1c-d). In these
 269 calculations, the structure of the halloysite previously optimized with Density Functional Theory
 270 (DFT) was fixed (Borrego-Sánchez et al., 2018), except the hydrogen atoms of the inner surface
 271 of the halloysite. In both cases, the ciprofloxacin maintained the same conformation that the
 272 adsorption complexes without solvent as it can be seen in Figure 1c-d.

273 In the adsorption model where the ciprofloxacin was adsorbed on the inner surface of the clay
274 (Figure 1c), the mainly interaction between the drug and halloysite were hydrogen bonds, between
275 the oxygen of the carbonyl groups and the hydrogens of the internal surface of the halloysite with
276 $d(\text{COc}\dots\text{HOAl}) = 1.93\text{-}2.00 \text{ \AA}$, and between the nitrogen of the ciprofloxacin and the hydrogen of
277 the internal surface of the halloysite with $d(\text{Nc}\dots\text{HOAl}) = 2.43 \text{ \AA}$. Also, hydrogens bonds between
278 the drug and methanol solvent were found, specifically between the carbonyl O atoms of the drug
279 and the hydroxyl H atom of methanol with $d(\text{COc}\dots\text{HOMe}) = 1.78\text{-}1.80 \text{ \AA}$, and between the
280 hydrogens of the ciprofloxacin molecule and the oxygen of the hydroxyl group of methanol with
281 $d(\text{Hc}\dots\text{OHMe}) = 1.86\text{-}1.96 \text{ \AA}$. Moreover, electrostatic interactions were showed between the C
282 atoms of the ciprofloxacin and the hydrogens of the internal surface of the halloysite with
283 $d(\text{Cc}\dots\text{HOAl}) = 2.35\text{-}2.51 \text{ \AA}$, and between the hydrogens of the drug and the oxygens of the
284 halloysite with $d(\text{Hc}\dots\text{OHAL}) = 2.24\text{-}2.34 \text{ \AA}$.

285 In the adsorption model where the ciprofloxacin was adsorbed on the outer surface of the halloysite
286 nanotube (Figure 1d), hydrogen bonds were found only between the drug and the methanol
287 molecules, in particular between the nitrogen of the amine group of the drug and the hydrogens of
288 methanol with $d(\text{HNc}\dots\text{HOMe}) = 1.85 \text{ \AA}$, and between the hydrogens of the ciprofloxacin and the
289 oxygen of methanol with $d(\text{Hc}\dots\text{OHMe}) = 1.80\text{-}2.07 \text{ \AA}$. Additionally, hydrogen bonds were
290 showed between the fluorine atom and the hydrogen atom of methanol $d(\text{Fc}\dots\text{HOMe}) = 2.22 \text{ \AA}$,
291 and between the oxygen of the carbonyl group of the drug and the hydrogen of methanol with
292 $d(\text{COc}\dots\text{HOMe}) = 1.86 \text{ \AA}$. Furthermore, electrostatic interactions were observed such as between
293 the hydrogens of the drug and the oxygens of the external surface of halloysite with $d(\text{Hc}\dots\text{OSi})$
294 $= 2.47\text{-}2.49 \text{ \AA}$.

295 Lastly, the adsorption energies of both models were compared according to the equation $\Delta E_{\text{ads}} =$
296 $(E_{\text{drug/clay complex}}) - (E_{\text{drug}} + E_{\text{clay}})$. The results showed that both adsorption models presented a
297 negative adsorption energy. Therefore, the adsorption of the drug is favorable both on the internal
298 and external surface of the halloysite nanotube. Comparing both adsorption complexes, the most
299 stable is one in which the ciprofloxacin molecule is adsorbed on the inner surface of the halloysite.
300 Specifically, the energy of adsorption of the complex with the drug on the internal surface of the
301 clay is 14.5 kcal/mol more stable than when ciprofloxacin is adsorbed on the external surface of
302 the nanotube.

303 Adsorption studies in different media (see SI) confirmed that halloysite nanotubes showed the
304 highest adsorption capacity towards Cipro in MeOH, in agreement with the computational results.
305 In these conditions, where the drug is insoluble, the main effect should be a hydrophobic effect,
306 which maximize the interaction with the Hal lumen, increasing the adsorption efficiency.

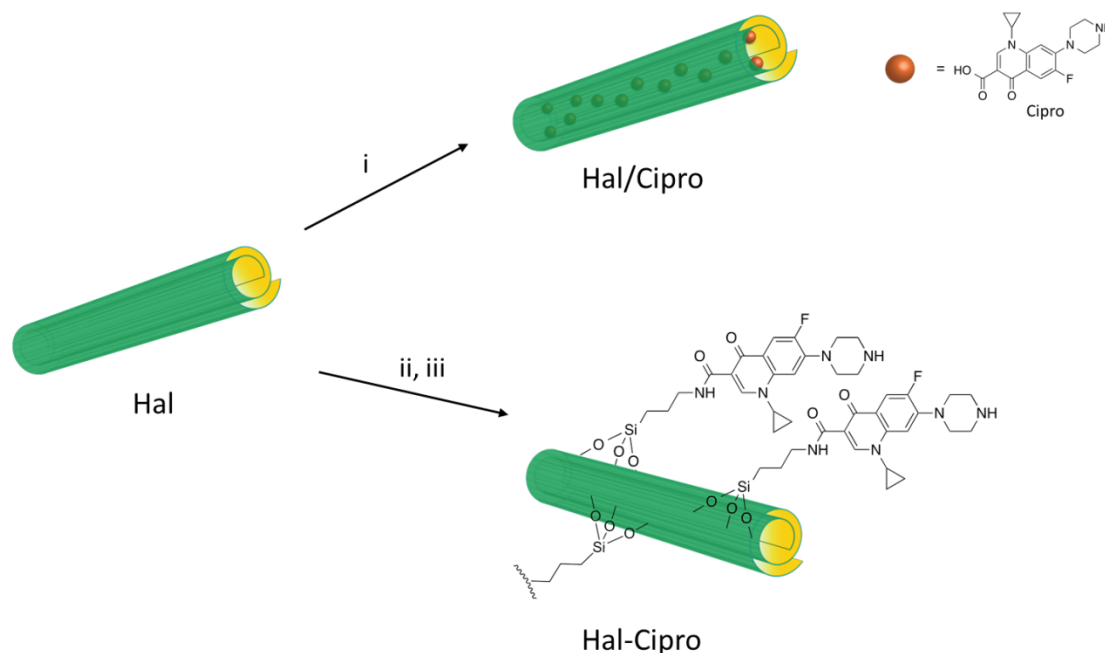
307 Based on the above results, loading of ciprofloxacin into pristine Hal was carried out by vacuum
308 cycling of a Hal methanolic suspension in a ciprofloxacin acidic solution (HCl 0.1 N). This cycle
309 was repeated several times to obtain the highest loading efficiency (Figure 2). After loading, the
310 Hal/Cipro filler was washed several times with methanol to remove free drug molecules. The
311 loading of ciprofloxacin molecules loaded into the Hal carrier, estimated by TGA, was ca. 2.6
312 wt%.

313 According to the densities of the pure components (2.53 and 1.5 g cm^{-3} for Hal and ciprofloxacin,
314 respectively) 2.6 wt% of Cipro corresponds to a volume loading of 4.4% in the Hal/Cipro filler.

315 **3.2. Loading of Cipro on Hal: covalent grafting (Hal-Cipro).**

316 To obtain a pH-sensitive carrier system for wound healing and to improve the drug loading onto
317 Hal, the ciprofloxacin molecules were also covalently grafted on Hal external surface (Figure 2).

318 In details, the synthesis of ciprofloxacin modified clays was carried out by N,N-
319 dicyclohexylcarbodiimide-catalyzed amide condensation of Cipro units to Hal-NH₂ (0.5 mmol/g
320 loading of -NH₂ groups) by forming an amide bond that afforded hybrid materials with a loading
321 of ca. 16 ± 0.1 wt% (0.48 mmol/g of ciprofloxacin molecules) estimated by TGA, with respect to
322 starting material.



324 **Figure 2.** Schematic representation of the synthesis of Hal/Cipro and Hal-Cipro nanomaterials. Synthetic
325 route: (i) ciprofloxacin, MeOH, vacuum, 18 h, r.t., (ii) APTES, toluene, reflux, 48 h, (iii) ciprofloxacin,
326 DCC, CH₂Cl₂, r.t., 3 d.

327 3.3. Physico-chemical characterization of Hal/Cipro and Hal-Cipro fillers.

329 Hal/Cipro and Hal-Cipro fillers were characterized by FT-IR spectroscopy and thermogravimetric
330 analysis (TGA). The spectroscopic study showed that both nanomaterials present, in their FT-IR
331 spectra (see SI) the typical Hal vibration stretching bands (Massaro et al., 2018a). The FT-IR
332 spectrum of Hal/Cipro shows, beside the Hal bands, some stretching bands of ciprofloxacin (Figure
333 S.1b). In particular, the band at ca. 1539 cm⁻¹ related to the C=O stretching of quinolone moiety

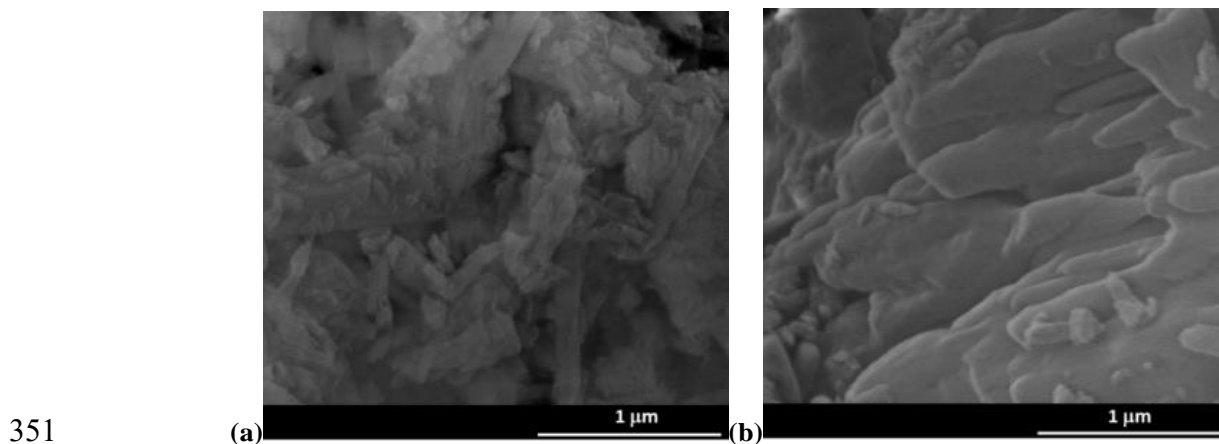
334 and the bands around 1483 and 1435 cm^{-1} due to the stretching of the C–N groups. On the contrary,
335 the FT-IR spectrum of the ciprofloxacin covalently linked to the Hal external surface is quite
336 different. Indeed, in this case, beside the typical vibration bands, new peaks are present which
337 confirm the successful linkage.

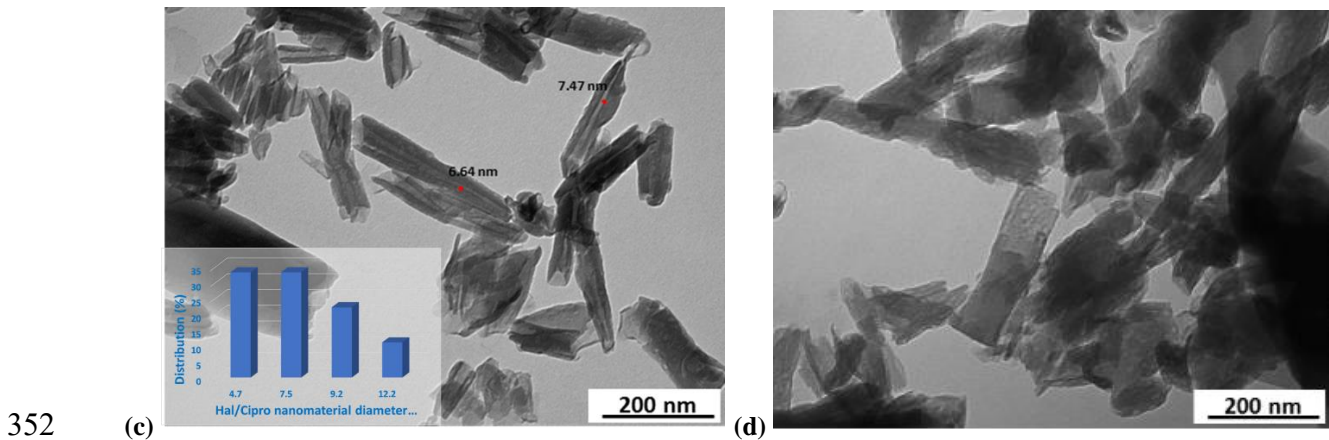
338 Specifically, it is possible to observe the band at ca. 3320 cm^{-1} related to stretching of the N–H
339 group of the amide bond and the band at ca. 1612 cm^{-1} which superimposed the typical band of
340 Hal, due to the stretching of the amide C=O.

341 In addition, the stronger intensities of the ciprofloxacin bands in the Hal-Cipro compared to those
342 in Hal/Cipro confirm the high loading obtained after the covalent grafting.

343 As stated above the ciprofloxacin amount loaded onto Hal fillers was estimated by TGA. TGA
344 data highlighted that the Cipro content is much larger in Hal-Cipro that also shows a larger
345 hydration with respect to Hal/Cipro. The mass loss due to Cipro degradation starts at ca. 250°C in
346 both cases.

347 The morphology of the two different nanomaterials was imaged by SEM and TEM investigations
348 (Figure 3). After the ciprofloxacin loading, the characteristic lengths, and the tubular shape of Hal
349 are preserved in the sample (Figures 3a and S.3); on the contrary, the grafting of Cipro molecules
350 on the external Hal surface causes a change in the morphology (Figure 3b).





353 **Figure 3.** (a-b) SEM and (c-d) TEM images of (a, c) Hal/Cipro (the inset shows the Hal/Cipro diameter size
 354 distribution (n = 20)) and (b, d) Hal-Cipro fillers.

355
 356 In this case, we found rather compact structures, where the tubes are not observable, probably due
 357 to the large amount of ciprofloxacin grafted onto the HNTs external surface, which interact each
 358 other by π - π and hydrogen bonding interactions.

359 Furthermore, TEM images confirm the different morphology of Hal/Cipro and Hal-Cipro fillers.
 360 In the TEM image of Hal/Cipro filler (Figure 3c) the lumen is not apparent in all its length (see
 361 red mark in Figure 3c) and exhibits a decreased diameter in comparison to pristine Hal (Massaro
 362 et al., 2018a); i.e., from 14.9 nm to ca. 7.3 ± 2.6 nm. On the contrary, in Hal-Cipro filler (Figure
 363 3d) the external Hal surface seems to be rougher and less defined with respect to those of pristine
 364 nanotubes (Figure S.3), indicating the presence of organic matters.

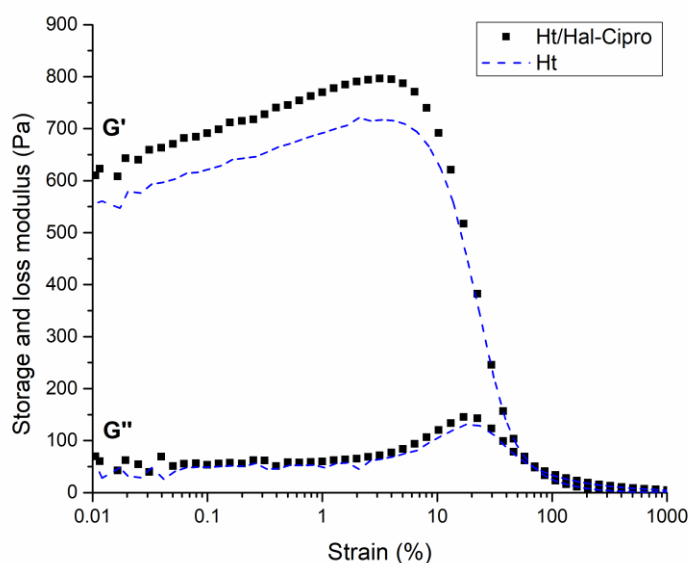
365 3.4. Study of gel properties

366 As known hectorite can form stable hydrogels in aqueous media due to the formation of
 367 delaminated dispersions by the self-assembling of its nanodisk via face-edge aggregation.
 368 Furthermore, it is reported that the introduction of pristine halloysite filler, in Ht hydrogel, helps
 369 the gel formation with an improvement of the rheological properties (Massaro et al., 2019a).
 370 Therefore, herein we studied the effect of the covalent modified Hal on the rheological properties

371 of Ht hydrogels. To perform these studies a series of different hydrogels were prepared by varying
372 the concentration of gelator (from 2 wt% to 10 wt%) and filler (from 2 wt% to 7 wt%). The
373 experimental data showed that the Ht/Hal hydrogels more stable were form at concentration of 5
374 wt% of both gelator and filler.

375 Figure 4 shows the effect of increasing strain amplitude on the storage and loss modulus of the
376 hectorite hydrogels (Ht and Ht/Hal-Cipro, respectively). The behavior of the studied gels was very
377 similar. Both pristine hectorite hydrogels and the hybrid one (Ht/Hal-Cipro) showed strain-induced
378 gel–sol transitions with initial critical high-modulus gel at low strains transforming to a low
379 modulus liquid at high strains. Pristine hectorite hydrogels were quite strong at 5 wt % with G'
380 ($\gamma \rightarrow 0$) ~ 550 Pa, increasing to ~ 600 Pa by the presence of Hal. Storage and loss moduli crossed
381 at $\sim 8\%$ strain in both pristine and hybrid hydrogels. Our results are similar to those observed by
382 Annemieke et al. (ten Brinke et al., 2007, 2008), with hectorite hydrogels in presence of small
383 amounts of rod-like particles of boehmite.

384 Accordingly, the increase in the linear region of the G' curves of our hydrogels can be explained
385 by the addition of a more strain-sensitive component (rod-shape particles of Hal) resulting in the
386 observed enhancement of the hydrogel structure.



387
 388 **Figure 4.** Amplitude sweeps for the Ht/Hal-Cipro hybrid hydrogel compared to pristine hectorite one at the
 389 same solid concentration.
 390

391 **3.5. Kinetic Release**

392 The kinetic release of Cipro molecules from both Hal fillers and from the hybrid hydrogels was
 393 performed both at skin's pH (5.4) and under neutral conditions (pH 7.4).

394 **3.5.1. Ciprofloxacin release from Hal fillers.**

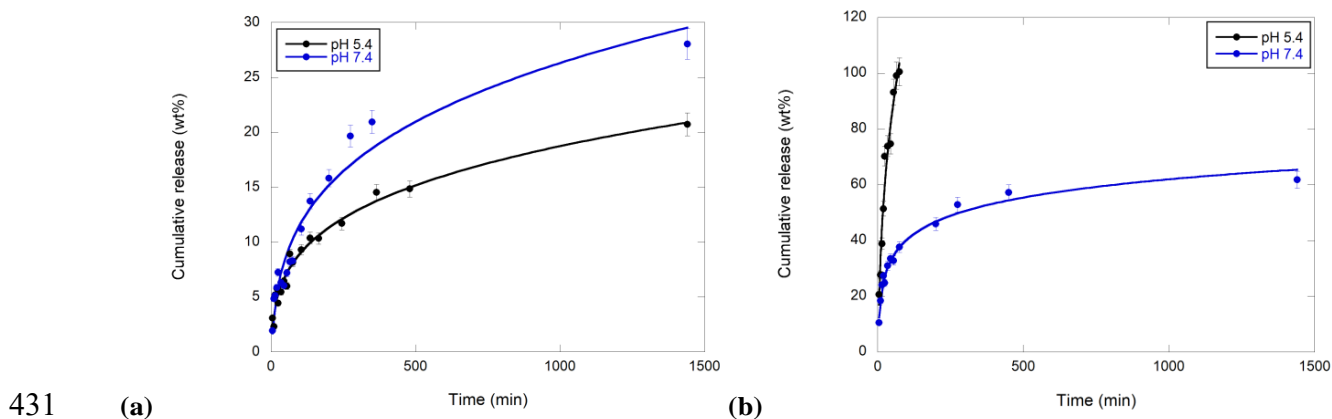
395 As far as is regarding the ciprofloxacin release from the Hal fillers we found that at pH 5.4 (Figure
 396 5) a sustained release of Cipro from the supramolecular Hal/Cipro filler (Figure 5a) was achieved,
 397 with ca. 20 wt% of the total amount of drug molecules loaded released in 24 h. Different behaviour
 398 was obtained in the case of Hal-Cipro, where the drug is grafted onto Hal surface by a pH sensitive
 399 bond. In this case, after the hydrolysis of amide bonds, the ciprofloxacin is quantitatively released
 400 within 75 min (Figure 5b).

401 At pH 7.4 (Figure 5), both systems exhibited sustained release of Cipro with an initial rapid-release
 402 phase, followed by a gradually slower release pattern. In particular, the Cipro showed a slower

403 release from Hal/Cipro nanomaterial, where only 30% of the total amount of drug molecules
404 loaded into Hal lumen is released after 24 h (Figure 5a); on the contrary, ca. 60% of Cipro of the
405 total amount of Cipro grafted onto halloysite is released in the case of Hal-Cipro (Figure 5b).
406 It is noteworthy that in the same conditions the free drug spread through the dialysis membrane
407 almost totally within few minutes (data not shown) indicating that release of Cipro from solution
408 through the dialysis membrane is a fast process according to that already reported for other
409 biologically active molecules (Massaro et al., 2021a).

410 The kinetic data obtained were analyzed by a first order, double exponential (DEM) and
411 Korsmeyer–Peppas models to deep investigate the release behavior of Cipro from the two different
412 fillers. The results showed that at pH 5.4 the release of ciprofloxacin from Hal-Cipro filler follows
413 the first order model ($M_{\infty} = 109 \pm 8$ wt%; $k = 0.029 \pm 0.008$ min⁻¹, $R^2 = 0.9712$) indicating that after
414 the cleavage of the amide bond, the diffusion of the molecule occurs through the dialysis
415 membrane. Conversely, the release of ciprofloxacin from Hal/Cipro is ruled by a Fickian
416 mechanism, therefore following a Power fit model ($k = 4.4 \pm 2.9$ min⁻¹; $n = 0.24 \pm 0.07$, $R^2 =$
417 0.9683). At pH 7.4, the release of Cipro from Hal/Cipro nanomaterial follows a first order model,
418 in agreement with a slow diffusion from Hal lumen ($k = 0.0037 \pm 0.0003$ min⁻¹), whereas the Cipro
419 release from Hal-Cipro is better fitted by a DEM model. According to the literature, the DEM
420 describes a mechanism consisting of two parallel reactions involving two distinguishable species.
421 Based on these findings, we hypothesized that might exist favorable π – π interactions between the
422 Cipro molecules grafted onto the external Hal and some free drug molecules which did not take
423 part in the amide condensation. Due to the strong supramolecular interactions, these free molecules
424 were not removed during the work-up of the reaction (Figure 5c). Therefore, it is possible to
425 suppose a faster release of Cipro because of the diffusion of Cipro molecules supramolecular

426 interacting ($k_I = 0.10 \pm 0.01 \text{ min}^{-1}$) and a slow release of the covalently linked ones, due to the
427 slow hydrolysis of amide bond in neutral conditions ($k_I = 0.0046 \pm 0.0006 \text{ min}^{-1}$). By the fitting
428 of the kinetic data, we calculated the amount of ciprofloxacin supramolecular interacting onto Hal
429 corresponding to ca. 4 wt% in comparison to the total ciprofloxacin loading (16 wt%) in the Hal-
430 Cipro.



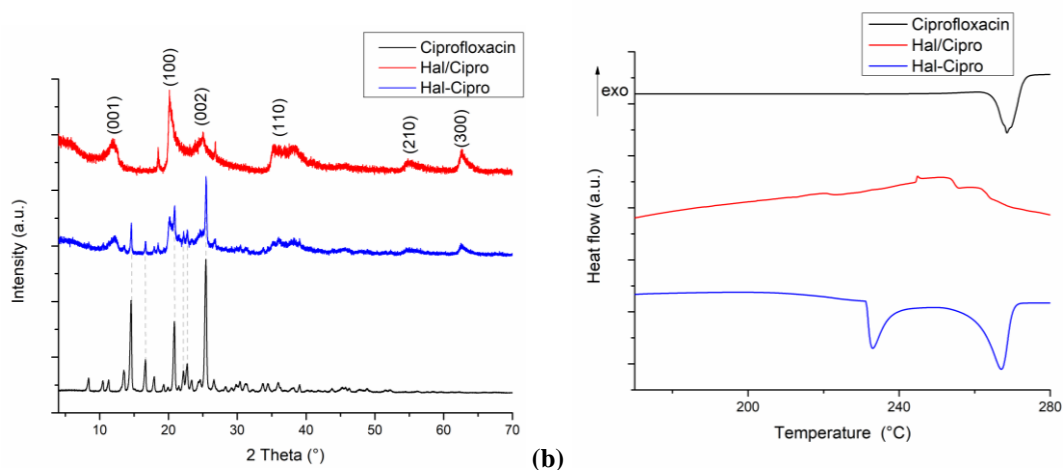
432
433 **Figure 5.** Kinetic release of ciprofloxacin from (a) Hal/Cipro and (b) Hal-Cipro nanomaterials in phosphate
434 buffer (0.05 M) solution at pH 5.4 and pH 7.4, at 37°C; (c) cartoon representation of the interaction existing
435 between “free” ciprofloxacin and Hal-Cipro filler.
436

437 To prove this hypothesis, we performed some XRD and DSC measurements (Figure 6). In Figure
438 6a are reported the XRD spectra of ciprofloxacin, Hal/Cipro, and Hal-Cipro.

439 All nanomaterials showed the typical reflections of Hal (Aguzzi et al., 2019), namely the
440 reflections at 2θ 12° , 20° , 25° , 35° , 54° and 62° corresponding to the planes (001), (100), (002), (100),
441 (210) and (300) respectively, matching with the JCPD card no. 00-029-1487. After the covalent
442 linkage of the Cipro on the clay, all reflection peaks remain unchanged indicating that no clay
443 structural variation occurs, confirming that the covalent linkage occurs only on the Hal external
444 surface without intercalation. In addition, typical XRD features of ciprofloxacin are clearly
445 observable in the fillers. Thus, XRD profile of Hal-Cipro confirmed the presence of some Cipro
446 molecules recrystallized on the Hal surfaces (sharp peaks in the range of 10° – 40° confirms the
447 presence of the crystalline form of Cipro). On the contrary, the XRD profile of Hal/Cipro is almost
448 consistent with the XRD feature of pristine Hal. This evidence indicates that the interaction
449 between Hal and ciprofloxacin and that the whole process of preparation does not disturb the
450 structure of Hal and once again no exfoliation of the clay occurs.

451 In Figure 6b the DSC thermograms of Cipro (free drug), Hal/Cipro and Hal-Cipro fillers are
452 showed. The DSC profile of Cipro exhibited a sharp endothermic peak due to the melting of the
453 drug molecules, in the boundary 264 – 270°C (centered at 268.6°C). As it reported, pristine Hal
454 shows a wide endothermic phenomenon, in the range 50 – 120°C , ascribed to the dehydration of
455 physisorbed water and interlayer water, followed by another endothermic band (270 – 320°C)
456 (Aguzzi et al., 2019). The Hal/Cipro filler showed the typical peaks in the thermograms related to
457 the endothermic processes of Hal. In addition, some minor Cipro degradation events were observed
458 at ca. 160°C , 225°C and 255°C . These results demonstrated that Cipro molecules are not in its
459 crystalline form after loading into Hal lumen (Silva et al., 2020). On the contrary, the DSC

460 thermogram of Hal-Cipro filler evidenced the presence of two significant endothermic peaks
461 centered at ca. 232 and 267°C. This finding further confirms the remaining unreacted Cipro
462 recrystallized on Hal external surface.



463 (a) (b)
464 **Figure 6.** (a) XRD patterns of ciprofloxacin, Hal/Cipro and Hal-Cipro fillers and (b) DSC profiles of ciprofloxacin,
465 Hal/Cipro and Hal-Cipro fillers.

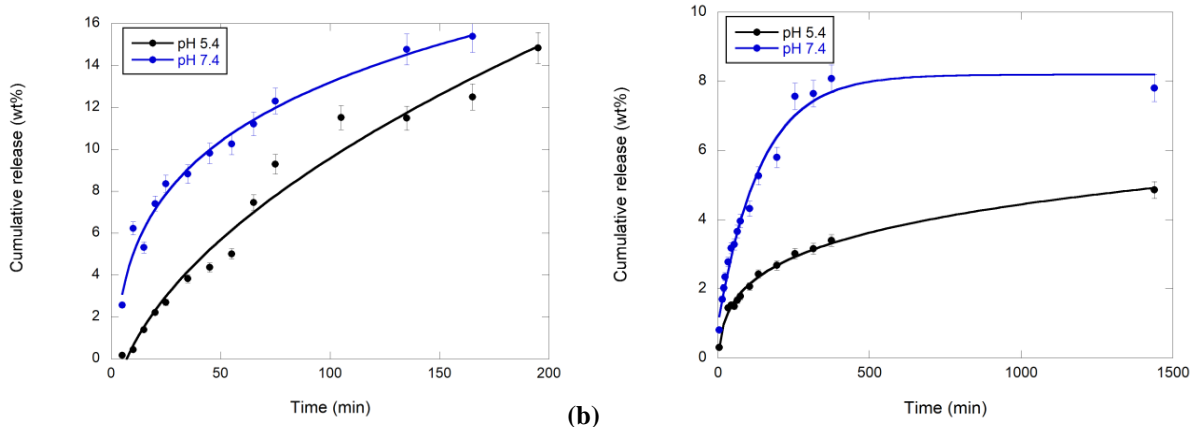
466 3.5.2. Ciprofloxacin release from the hybrid hydrogels

467 The release of Cipro from the hybrid hydrogels was also studied to verify if incorporation of Hal
468 into the gel matrix could induce a time-controlled drug release process at at skin's pH (5.4) and
469 under neutral conditions (pH 7.4). To make a proper comparison, we also prepared a Ht hydrogel
470 in the presence of ciprofloxacin alone (5 wt% of ciprofloxacin with respect to Ht). In this case, no
471 gel formation occurred even after 48 h (Figure S.5). It could be probable that ciprofloxacin
472 interacts by cation exchange with the Ht interlayers, as already found with similar inorganic
473 excipients, disturbing the gelation process (Wang et al., 2011; Wu et al., 2013). This result
474 confirms the crucial role played by the halloysite fillers in the Ht matrix as carrier for the sustained
475 release of drugs.

476 The trends of cumulative Cipro release from the hybrid hydrogels as a function of time are
477 displayed in Figure 7. In the case of Hal/Cipro filler, at both pH values, a similar release pattern

478 was observed. We found a slow release of the drug from the hybrid hydrogels for the first 200 min
479 (Figure 7a), afterwards, we observed the dissolution of the gel matrix. In this case, since there is
480 not the influence of the gel matrix, it was assumed that the Cipro kinetic release should be very
481 similar to that from pristine halloysite filler (see above). Conversely, the introduction of Hal-Cipro
482 filler in the Ht hydrogel led a slower release pattern of the Cipro without no relevant differences
483 between the two media investigated. Also in this case the ciprofloxacin covalently grafted onto the
484 external surface of halloysite could interact once again by cation exchange with the Ht interlayers
485 and this, could hamper the drug release as already reported for similar clays (Chen et al., 2015).
486 The small amount of ciprofloxacin released from the gel corresponded to the drug molecules
487 recrystallized on Hal external surface, which spread through the hydrogel (Figure 7b). Cipro
488 molecules released from the Hal and then sorbed into the Ht interlayer will not easily exchanged
489 by cations. Interestingly, permanence of Cipro in the gel is consider a positive matter, as it has
490 been demonstrated that Cipro loaded in similar nanoclays vehicles (montmorillonite) significantly
491 inhibited bacteria growth compared to free drug molecules (Chen et al., 2015). Furthermore, since
492 frequent medical treatment or manipulation over a wounded area could impair/interrupt new tissue
493 formation, a sustained release of antibiotic could potentially reduce the number of applications
494 needed over time and, allow lesions to heal faster.

495 In this case, the experimental data are better fitted by a Higuchi model ($k = 0.43 \pm 0.01 \text{ min}^{-1}$, R^2
496 $= 0.9897$) for kinetic data obtained at pH 7.4, whereas the Cipro release from the Ht/Hal hybrid
497 hydrogel follows the Power fit model at pH 5.4 ($k = 1.7 \pm 0.4 \text{ min}^{-1}$, $n = 0.19 \pm 0.02$, $R^2 = 0.9945$).



498 (a) (b)

499 **Figure 7.** Kinetic release of ciprofloxacin from (a) Ht/Hal/Cipro hybrid hydrogel and (b) Ht/Hal-Cipro hybrid
500 hydrogel phosphate buffer (0.05 M) solution at pH 5.4 and pH 7.4, at 37°C.

501 3.6. Cytocompatibility

502 It is reported that Ht hydrogels did not possess cytotoxic effects on normal human dermal
503 fibroblasts within 24 h (García-Villén et al., 2021). Thus, we studied the effects on the same cell
504 lines of the Hal fillers obtained in this study.

505 Figure 8 reports the % viability of fibroblasts after 24 h contact with Hal-Cipro and Hal/Cipro
506 fillers at different concentrations compared to ciprofloxacin (free drug) as reference and the
507 control, GM, corresponding to standards growth conditions. Pristine Hal did not show any
508 cytotoxicity in the concentration range investigated (Sandri et al., 2020).

509 A dose-dependent and statistically significant decrease in cell viability was particularly evident for
510 cells treated with Cipro as free drug. Besides the increasing amount of the free quinolone used (Shi
511 et al., 2018), the viability reduction could be related to the cell sensitivity to the acidic pH of Cipro
512 sample (drug stock solution in 0.1 N HCl to allow drug solubilization).

513 Hal/Cipro causes a statistical viability reduction at concentrations higher than 25 μ M. Despite this,
514 as shown from the results, the sample containing Cipro at 100 μ M lead to 60% cell viability higher
515 than that of the free drug at the same concentration (40%), suggesting no additional cytotoxic

516 effects. When cells were exposed to covalent halloysite-ciprofloxacin composites (Hal-Cipro), no-
517 statistically dose-dependent cytotoxicity occurred with a 90% viability up to 25 μ M.

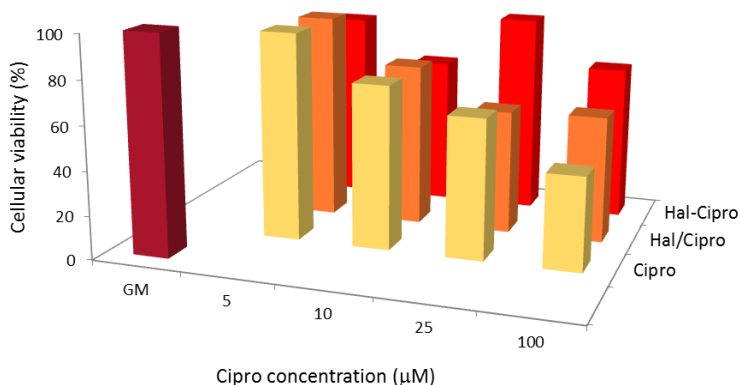
518 This could be related to the different release kinetic from Hal at pH 7.4 (pH of growth medium).

519 Hal-Cipro is characterized by a cytocompatibility strongly influenced by drug concentration in the
520 nanomaterial since about 60% of the drug is released in the medium, while Hal/Cipro shows a less
521 marked influence of concentration on cytocompatibility, and this could be due to a lower drug
522 release (about 20% in 24 h).

523 In Figure 9, Confocal Laser Scanning Microscopy (CLSM) photographs of fibroblasts treated with
524 10 μ M of free ciprofloxacin and ciprofloxacin loaded carriers are reported.

525 The fusiform shape of fibroblasts was totally preserved and no differences among each sample
526 occurred, confirming the sample biocompatibility at concentration used. Although it was not
527 possible to localize the drug moiety and to study the Hal and drug cell uptake, the results confirm
528 a positive cell-sample interaction. F-actin filaments of the cytoskeleton are well organized
529 (Phalloidin FITC staining in green), and the nuclei (Iodine Propidium staining in red) are normal.

530

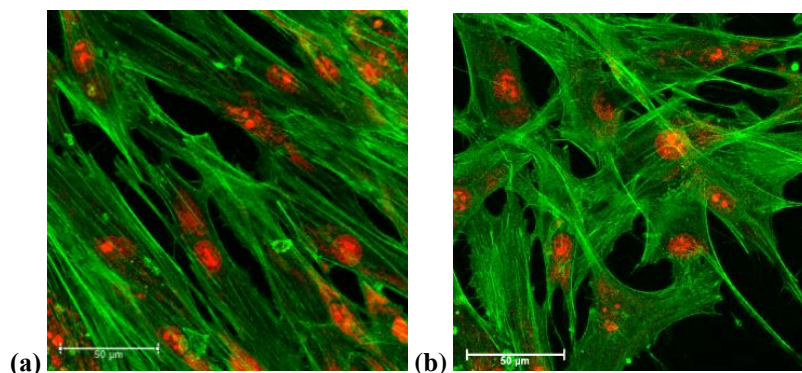


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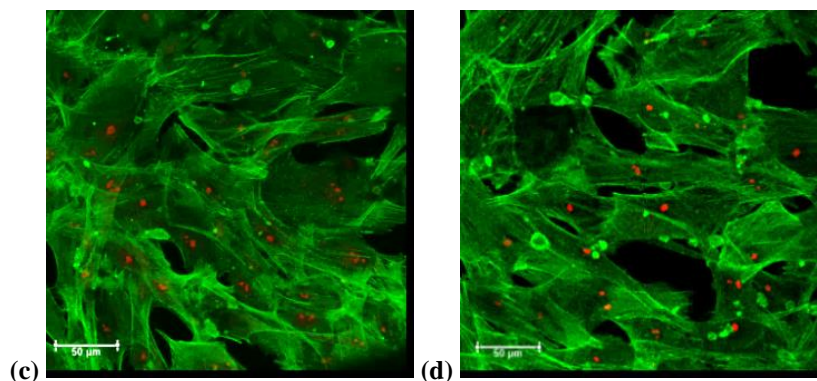
532 **Figure 8.** % viability of fibroblasts after 24 h contact with Hal-Cipro and Hal/Cipro samples at different
533 concentrations of ciprofloxacin in the nanomaterials compared to ciprofloxacin (free drug) as reference and the
534 control, GM, corresponding to standards growth conditions (means values \pm es; n=8) (see ESI for error bars).

535

536



537



538 **Figure 9.** CLSM images of fibroblasts after 24 h culture in (a) GM (standard growth conditions); (b) Cipro; (c)

539 Hal/Cipro and (d) Hal-Cipro, Cipro concentration 10 µM.

540 CONCLUSIONS

541 In summary, in this paper, we pointed our attention to the development of ad hoc covalently
542 modified halloysite as filler for Ht hydrogels. The introduction of such fillers into the gel matrix
543 indeed, was crucial to achieve a sustained release of ciprofloxacin for potential wound healing
544 applications.

545 To achieve this objective, firstly, we studied the ciprofloxacin loading onto Hal both by a
546 supramolecular and covalent approach (Hal/Cipro and Hal-Cipro, respectively). The interaction
547 between ciprofloxacin and halloysite was thoroughly investigated by several techniques and at
548 molecular level by means of quantum mechanics calculations along with empirical interatomic
549 potentials. Both fillers were characterized by FT-IR spectroscopy and TGA measurements which
550 confirmed the successful loading and grafting of ciprofloxacin. The different morphologies of the

551 two fillers were imaged by SEM and TEM investigations. Rheological measurements highlighted
552 that the introduction of modified Hal into the gel matrix improved its properties helping the gel
553 formation.

554 Kinetic release experiments of the drug from Hal fillers at at skin's pH (5.4) and under neutral
555 conditions (pH 7.4), showed that the release is strictly dependent to the pH.. Conversely the
556 ciprofloxacin molecules were slowly released from the gel matrix at both pH, which could be
557 important for further applications as transdermal systems.

558 Finally, both MTT test and CLSM investigations proved the absence of any relevant cytotoxic
559 effects of the synthesized fillers on normal human fibroblast cell lines.

560 In conclusion, in view of the positive *in vitro* cytocompatibility and *wound healing* effects reported
561 in the literature for both hectorite and halloysite future work will be devoted to further *in vitro* and
562 *in vivo* studies to assess the feasibility of the novel systems to enhance wound healing and to
563 effectively prevent bacterial for the treatment of chronic skin lesions.

564 **Supporting Information.** Ciprofloxacin adsorption studies onto Hal, FT-IR spectra and TGA
565 analysis, TEM and SEM images of pristine Hal and Ht, UV-*vis* spectra of ciprofloxacin released,
566 % viability of fibroblasts after 24 h contact with Cipro, Hal-Cipro and Hal/Cipro.

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574 **Author Contributions**

575 C.V.I and S.R. conceptualization, project administration, supervision, writing – review & editing,
576 M.M. and A.B.-S. writing – original draft, M.M., A.B.S., R.S.E., G.C., F.G.-V., S.G., G.L., D.M.,
577 C.I.S.-D., G.S. formal analysis and investigation.

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