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Amino hydroxyapatite/chitosan hybrids reticulated with glutaraldehyde at different pH values and their use for diclofenac removal

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29 Highlights

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31 Amino hydroxyapatite/chitosan/glutaraldehyde hybrids were synthesized

32 The influence of pH on crosslinking was investigated.

33 Hybrids were characterized for structural, thermal and morphologic techniques.

34 Hybrids adsorbed diclofenac from aqueous solution at pH 6.

35 High drug adsorption (125.0 mg mg^{-1}) was obtained for hybrid obtained at pH 3.

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58 ABSTRACT

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60 Diclofenac sodium (DS) is an emergent pollutant and among methods investigated for
61 its removal, adsorption is an appropriated technique. Hydroxyapatite (HA) and chitosan
62 (CS) are biomaterials often used for adsorption. However, both biomaterials are limited
63 due their chemical stability in acidic medium; furthermore, pure HA has poor
64 interaction with diclofenac. Amino bioadsorbents can adsorb DS by different
65 mechanism. In this work, hydroxyapatite was initially organofunctionalized with 3-
66 aminopropyltrimethoxysilane and further used to obtain amino hydroxyapatite/chitosan
67 hybrids by crosslinking with glutaraldehyde. The efficiency of the crosslinking reaction
68 at pH 3, 4, 5 and 6 was investigated at room temperature over 10 min. X-ray diffraction
69 patterns indicated the preservation of the hydroxyapatite phase under all pH conditions.
70 Based on control reaction of the amino hydroxyapatite with glutaraldehyde and its
71 further reduction in sodium borohydride, the formation of C=N moieties was
72 highlighted as the main mechanism of interaction between the aldehyde and amino
73 groups. Therefore, crosslinking with glutaraldehyde was evaluated by infrared, Raman
74 spectroscopy and ^{13}C NMR and suggested contributions of imine formation and also
75 hydrogen bonding. Higher crosslinking was achieved at pH 3 and 4 than at pH 5 and 6,
76 as indicated by CHN analysis and thermogravimetry. Alterations in the surface of the
77 samples were showed by scanning electron microscopy. Rapid diclofenac removal
78 occurred at 30 and 15 min for amino-hydroxyapatite/glutaraldehyde/chitosan obtained
79 at pH 3 and pH 4, respectively. In the equilibrium study, the hybrid obtained at pH 3
80 achieved better adsorption capacity of 125 mg g^{-1} (99% drug removal). Synergism
81 between amino hydroxyapatite and CS crosslinked by glutaraldehyde was
82 demonstrated, and both hybrids obtained at lower pH (3 and 4) presented high
83 efficiency in DS removal from aqueous solution.

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88 Keywords: Amino hydroxyapatite; chitosan; crosslinking; diclofenac; adsorption

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90 **1. Introduction**

91 The increased use of diverse classes of medicines is a growing requirement to
92 ensure a better quality of life for the population (Basheer, 2018; Triebkorn et al.,
93 2015).

94 The consequently enlarged volume of pharmaceutical waste from industries,
95 hospitals and homes is reported to cause disruption in water resources by damaging the
96 quality of drinking water (Yan et al. 2019; Riegger et al. 2018; De Oliveira et al. 2017).
97 Pharmaceutical residues are considered emerging pollutants, and diverse types of these
98 residues have been detected in water, including anti-inflammatories and antibiotics
99 (Bonnefille, Gomez, Courant, Escande, & Fenet, 2018; Sousa, Ribeiro, Barbosa,
100 Pereira, & Silva, 2018).

101 Among pharmaceutical compounds, diclofenac sodium (DS) is one of the main
102 drugs detected in water around the world (Acuña et al., 2015; Park & Lee, 2018;
103 Sathishkumar et al., 2020). It is classified as a nonsteroidal anti-inflammatory drug and
104 is indicated in the relief of all grades of pain and inflammation associated with a wide
105 range of conditions, including arthritic conditions, acute musculoskeletal disorders and
106 other painful conditions resulting from trauma (Lonappan, Rouissi, Kaur, Verma, &
107 Surampalli, 2017; Sathishkumar et al., 2020). Due to its important applications, DS is
108 the twelfth most widely used generic drug in the world. As a result, DS is an important
109 emerging pollutant that is difficult to remove from water (Lessa, Nunes, & Fajardo,
110 2018; Soares, Fernandes, Sacramento, Trindade, & Daniel-da-silva, 2019).

111 Different processes are used for the treatment of polluted water, e.g., chemical
112 precipitation, coagulation-flocculation, ionic exchange and adsorption (Lu & Astruc,
113 2018). Among these processes, adsorption is widely applied, and biosorbents based on
114 natural and sustainable resources are effective in the removal of pharmaceutical

115 pollutants. In this field, biosorbents obtained from biopolymers are particularly
116 promising (Hu et al., 2019).

117 Chitosan (CS) stands out as an excellent biosorbent due to its structure, which
118 consists of N-acetyl-D-glucosamine and D-glucosamine units with free amino and
119 hydroxyl groups that are highly reactive and capable of interacting with various
120 pollutant molecules, such as drugs (Shariatinia & Jalali, 2018; Yang, Li, Huang, Yang,
121 & Li, 2016). In addition, this biopolymer is biodegradable, nontoxic, and derived from a
122 natural and economical raw material obtained mainly from the deacetylation of chitin
123 (Vakili et al., 2018).

124 CS also interacts with inorganic matrices, resulting in composites with improved
125 properties such as enhanced chemical stability (Chatterjee et al., 2018). Among
126 inorganic matrices, calcium phosphates, such as hydroxyapatite, have been applied as
127 good candidates to form new hybrids for environmental applications (Shi, Lv, Wu, &
128 Hou, 2017). Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is used as an adsorbent due to its
129 biocompatibility and its production in large scale by using green, economic and easy
130 processes (Wang et al., 2017). Hydroxyapatite (HA) has reactive functional groups, Ca-
131 OH and P-OH (Harja & Ciobanu, 2018; Hassan & Hrdina, 2018), that allow interactions
132 with several neutral, cationic and anionic species by hydrogen bonding and ion
133 exchange. The adsorptive potential of the HA is improved through chemical
134 modification with biopolymers, among which chitosan is the most investigated polymer
135 for the formation of composites and nanocomposites. CS is used due many actives
136 groups for reaction and adsorption (NH_2 and OH), that are viable to large pollutant
137 removals. Furthermore, like HA, CS is also biocompatible, easily manufactured and
138 also presents possibility to alter its physical texture for use as beads, films and powders
139 that are interesting properties for many applications, including adsorption. The

140 combination of CS with other substrates at the molecular level can improve its use in
141 environmental remediation (Chatterjee et al., 2018; Vakili et al., 2014).

142 Therefore, pure HA/CS composites have been described in the literature (Rogina
143 et al., 2017; Shi et al., 2017) with tentative to describe the interactions between both
144 components. (Y. Li, Liu, Zheng, & Xu, 2013; Sanchez et al., 2018; Sun, Shi, Wang, &
145 Li, 2017; Zima, 2018).

146 In this work, covalent chemical bonding through crosslinking formation between
147 amino apatite and CS was used to obtain a more effective adsorbent for DS removal.
148 Among the crosslink agents, glutaraldehyde is one of the most widely investigated
149 bifunctional agents at pH 6 for the formation of C=N bonds (Ciaccia & Di Stefano,
150 2015; Lal, Arora, & Sharma, 2016). In this study for the first time, the influence of the
151 pH on hybrid formation was systematically investigated and the formation of the
152 hybrids was evidenced by structural, thermal and morphological techniques.

153 Therefore, the aims of this work were to obtain new hybrid bio adsorbents based
154 on amino hydroxyapatite/glutaraldehyde/chitosan at different pH values. The new
155 hybrids were applied to DS removal from aqueous solutions under varied experimental
156 conditions, including the pH, mass of the adsorbent, time and initial drug concentration.
157 The isotherm results were fitted with kinetic and equilibrium models and the
158 interactions between the hybrids and the drug were evaluated.

159

160 **2. Experimental**

161 *2.1 Chemicals*

162 All chemicals were supplied by Sigma Aldrich and used without further
163 purification.

164 For the preparation of HA, calcium chlorite, ammonium phosphate and NaOH were
165 used. Chitosan with an average molecular weight of 190-310 kD and 78% deacetylation
166 degree, glutaraldehyde (Glu, 25%), 3-aminopropyltrimethoxysilane, acetic acid and
167 ammonium hydroxide were used for preparing the hybrids. In the adsorption
168 experiments, diclofenac sodium (CAS 15307-79-6, C₁₄H₁₀Cl₂NNaO₂, pKa 4.1) was
169 used.

170

171 *2.2 Synthesis of silylated hydroxyapatite/chitosan reticulated with glutaraldehyde*

172

173 Initially, the precursor HA was obtained through a coprecipitation (Silva, Alves,
174 dos Santos, Fonseca, & Jaber, 2017). Briefly, 0.033 mol of calcium chloride and 0.056
175 mol of ammonium phosphate were mixed in an aqueous solution at pH 10.

176 The silylation of HA was performed under N₂ atmosphere. First, 3.0 g of HA
177 was reacted with 2.5 mL of 3-aminopropyltrimethoxysilane for 72 h in toluene under
178 reflux. The solid, HA-NH₂, was recovered after filtration and washing with ethanol and
179 water until the pH was neutral and dried at 120 °C.

180 Crosslinking was performed by reacting 1.0 g of the silylated HA-NH₂ and 50
181 mL of a 2.5% Glu solution adjusted to pH 3, 4, 5 or 6 with 1% acetic acid or 1 mol L⁻¹
182 NaOH monitored in a digital pH meter (Digimed) (See Supplementary Material Figure
183 SM1). The system was reacted for 10 min with vigorous stirring at room temperature.
184 The solid changed from white to reddish-brown. CS solution (50 mL) was prepared by

185 dissolving 200 mg of the biopolymer in 1% acetic acid (pH 3, 4, 5 or 6, previously
186 adjusted) and was added to the system. The reaction was maintained for another 20 min
187 and monitored as described. After the addition of the CS solution, the mixture quickly
188 showed gelification (~ 5 min) and became dark reddish-brown, suggesting the formation
189 of the (-C=N-) chromophore (Riegger et al., 2018). The resulting uniform gel was
190 centrifuged, washed in distilled water and dried at 60 °C. All preparations occurred at
191 rigorous pH control at values of 3, 4, 5 or 6 monitored in digital pH meter. Essentially,
192 the pH is a fundamental parameter, as it is important to ensure the protonation of the
193 carbonyl groups and to keep the primary amino groups deprotonated (Antony, Arun, &
194 Manickam, 2019; Poon, Wilson, & Headley, 2014).

195 The obtained hybrids were denoted as HA-NH₂/CS-3, HA-NH₂/CS-4, HA-
196 NH₂/CS-5 and HA-NH₂/CS-6, that are related to precursors and the pH used in the
197 synthesis.

198 As control, hybrid obtained without glutaraldehyde was also prepared at pH 5,
199 aiming to investigate possible interactions in absence of reticulation agent.

200 A second control was synthesized by reaction of the silylated HA with
201 glutaraldehyde without biopolymer at pH 5 at the same conditions and was named as
202 HA-NH₂/Glu.

203 HA-NH₂/Glu also reacted with sodium borohydride in aqueous solutions for 4 h
204 following previous method (Fonseca & Airoidi, 1999). This reaction was used as a third
205 control to reduce C=N moieties previously formed.

206

207 *2.3 Batch adsorption procedure*

208

209 In general, the experiments were carried out in a temperature-controlled Tecnal
210 TE-4200 shaker incubator at a shaking rate of 200 rpm. The experiments were
211 performed starting with 50 mg of each hybrid and 25 mL of a 100 mg L⁻¹ drug solution
212 at controlled pH at 25 °C for 24 h. The influence of the pH on DS removal was
213 monitored by using drug solution at 100 mg L⁻¹ and 50 mg of the adsorbent at fixed
214 initial pH in the range of 6 to 12 for 6 h. pH below 6 was not investigated due to the
215 precipitation of the drug at lower pH than 6. After adsorption, supernatant was separated
216 by centrifugation. The amounts of drug in both the initial and supernatant solutions
217 were quantified at 273 nm by using UV-Vis spectrometry with a TCC-240 model
218 Shimadzu spectrometer. The amount of adsorbed drug was calculated by equation 1:

219

$$q = \frac{(C_i - C_e)V}{m} \quad (1)$$

220

221 where C_i and C_e are the initial and residual drug concentrations, respectively; V is the
222 volume of the drug solution; and m is the mass of the adsorbent.

223 The influences of the dose of the adsorbent, time and initial drug concentrations
224 were also evaluated. All tests were performed in triplicate, and the average value was
225 obtained by statistical analysis.

226

227 *2.4 Kinetic and equilibrium models*

228

229 The experimental data were fitted to kinetic and equilibrium models (see
230 Supplementary material SII).

231

232 *2.5 Characterizations*

233

234 X-ray diffraction patterns were recorded using a XD3A Shimadzu powder X-ray
235 diffractometer with CuK_α radiation ($\lambda = 0.15406$ nm). The XRD patterns were acquired
236 between 3 and 80° (2θ) with a scanning rate of $0.02^\circ \text{ min}^{-1}$ at room temperature, and the
237 diffractometer operated at 40 kV and 30 mA.

238 Fourier transform infrared spectroscopy (FTIR) was performed on a Bomem
239 MB-series MB spectrometer. The samples were prepared in KBr pellets with a
240 concentration of 2% . FT-IR spectra were recorded in the middle IR region (4000 - 400
241 cm^{-1}) with a resolution of 4 cm^{-1} and 30 scans.

242 FT-Raman spectra were recorded on a Bruker RFS/100S model spectrometer
243 fitted with a liquid nitrogen-cooled Ge detector. The 1064 nm line of a $\text{Nd}^{3+}/\text{YAG}$ laser
244 was used at an incident power output of 100 mW. The spectral resolution was 4 cm^{-1} .

245 TG measurements were carried out by a TG I/1600 HT simultaneous thermal
246 analyzer under a nitrogen flow of 50 mL min^{-1} with 10 mg samples and a heating rate of
247 $10 \text{ }^\circ\text{C min}^{-1}$ from room temperature to 1000 $^\circ\text{C}$.

248 The degree of organofunctionalization was determined based on CHN elemental
249 analysis, which was performed on a Perkin-Elmer PE-2400 microelemental analyzer.

250 Zeta potential (ζ) was acquired on a Zetasizer Nano Zs ZS90 model instrument
251 (Malvern Instruments) for isoelectric titration through pH titration. The pH of the
252 solutions was adjusted with 0.100 mol L^{-1} NaOH or 0.500 mol L^{-1} HNO_3 .

253 UV-Vis solid-state spectra were obtained on a Shimadzu spectrometer model
254 UV-2550 in the range of 200 - 900 nm.

255 The morphology of the samples was monitored by scanning electron microscopy
256 performed in an FEI Quanta FEG 250 microscope at an accelerating voltage of 1 - 30 kV.
257 Samples were fixed on carbon tape and coated with Au in a Quorum model Q150R
258 sputter-coater for 35 s at 20 mA by a plasma generated under an argon atmosphere.

259 Specific surface area was determined by using BET equation in the same
260 conditions of previous work (Silva et al., 2017). Samples were degassed at 100 °C prior
261 to analysis.

262

263 **3. Results and Discussion**

264 *3.1 Characterization*

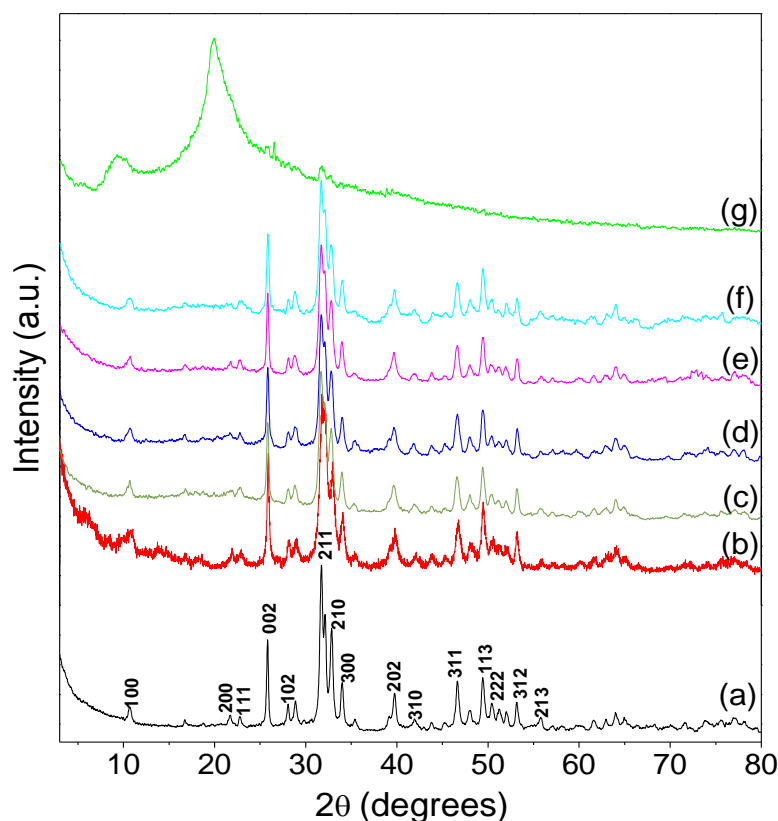
265 *3.1.1 X-ray diffraction*

266

267 The X-ray pattern of the HA (Fig. 1a) exhibited typical reflections at 2θ of 25.8,
268 29.9, 34.0, 39.9, 46.7 and 49.4° indexed to the (002), (300), (202), (130), (222) and
269 (213) diffraction planes, respectively, in agreement with ICDD 09.3204 (Dorozhkin,
270 2012; Szcześ, Hołysz, & Chibowski, 2017). After reaction of HA with the silane, the
271 main reflections were still observed, suggesting the maintenance of the HA phase after
272 silylation (Fig. 1b).

273 The XRD patterns of chitosan presented two broad peaks at 2θ of 9.8° and 19.9°,
274 which were assigned to hydrated regions and coexisting ordered and amorphous
275 regions, respectively (Bayrak, Demirtaş, & Gümüşderelioğlu, 2017; Shi et al., 2017).

276



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278

279 **Figure 1.** X-ray diffractograms for (a) HA, (b) HA-NH₂, (c) HA-NH₂/CS-3, (d) HA-
 280 NH₂/CS-4, (e) HA-NH₂/CS-5, (f) HA-NH₂/CS-6 and (g) CS.

281

282 The crosslinking reaction promotes the interaction between the silylated HA and
 283 chitosan mediated by a bifunctional agent on the molecular scale to generate an
 284 inorganic-organic hybrid (Huang et al., 2011). Therefore, the XRD patterns of the
 285 hybrids predominantly showed characteristics of the inorganic phase at the used w/w
 286 proportion of each phase (15% chitosan and 85% HA-NH₂) for all pH values. No
 287 indication of amorphous phases was observed in the XRD patterns even at pH 3.
 288 Therefore, the crosslinking of calcium phosphate and chitosan did not alter the
 289 inorganic phase in the pH range from 3 to 6.

290 The present results were different from those obtained with a pure
 291 HA/Glu/chitosan composite (Y. Li et al., 2013), where the authors observed CS as

292 preponderant phase in XRD, even for composites prepared with 65% HA by weight in
293 relation to the polymer/HA proportion and different conditions of synthesis including
294 long time of reaction, at least 18 h.

295

296 *3.1.2 Vibrational spectroscopy*

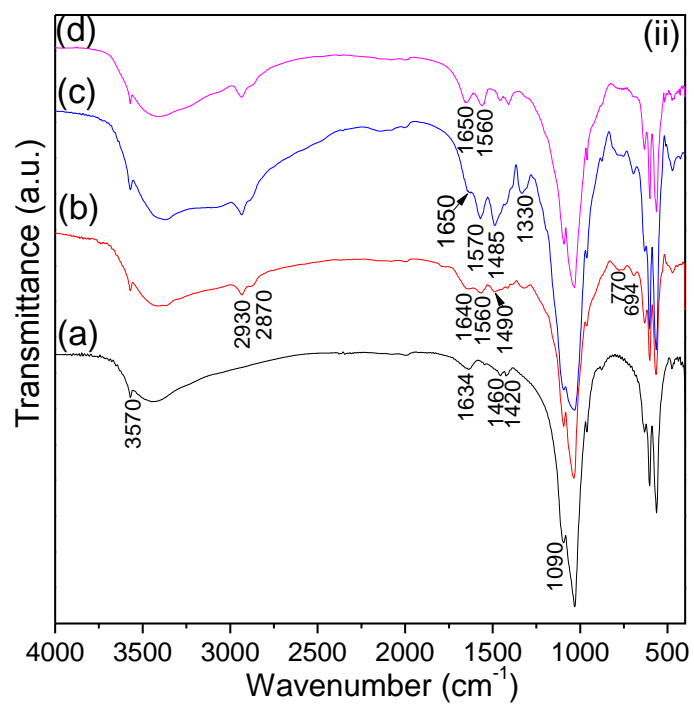
297

298 Initially FTIR spectra of the controls obtained for pure HA-NH₂, HA-NH₂/Glu
299 and HA-NH₂/Glu/reduced were prior analyzed. The FTIR spectrum of the HA showed
300 bands at 3571 cm⁻¹, assigned to O-H stretching of the P-OH group, and at 3443 cm⁻¹ and
301 1640 cm⁻¹ due to OH stretching and bending of water, respectively (Manatunga, De
302 Silva, De Silva, & Ratnaweera, 2016) . Typical bands of the asymmetric and symmetric
303 stretching of PO₄³⁻ and P-O(H) in HPO₄⁻² were observed at 1095-905 cm⁻¹ and 870 cm⁻¹,
304 respectively. Absorptions corresponding to deformations of these same groups were
305 present at 599 and 560 cm⁻¹, respectively (Elliott J.C., 1994; Lin-Vien, Colthup, Fateley,
306 & Grasselli, 1991; Nazeer, Yilgör, & Yilgör, 2017; Rogina et al., 2017; Silva et al.,
307 2017). The organofunctionalization of the HA through silylation resulted in new bands
308 in the spectrum of HA-NH₂ at 2920 and 2850 cm⁻¹ assigned to C-H asymmetric and
309 symmetric stretching and at 1558 cm⁻¹ assigned to NH₂ deformation. After reaction with
310 glutaraldehyde, a broad band centered at 1655 cm⁻¹ was maintained but a new band at
311 1330 cm⁻¹ was assigned to =C-H deformation. Any alteration in bands associated to OH
312 structural and PO₄³⁻ was observed. After reaction of the HA-NH₂/Glu with sodium
313 borohydride, the band associated to =C-H deformation disappeared of the spectrum. The
314 result is consistent with the reaction of primary amine moieties on silylated HA with
315 aldehyde resulting formation of imine, and further reduction of previous imine, resulted

316 in a formation of secondary amine. The sequence of reactions was highlighted based on
317 infrared spectrum by the absence of typical =C-H after imine reduction.

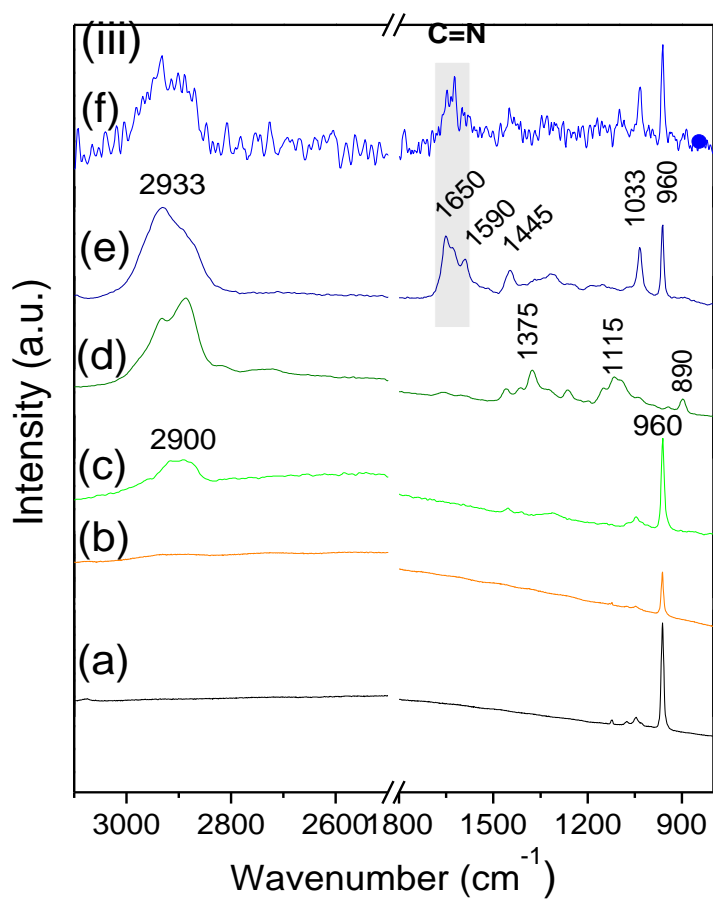
318 For chitosan, a characteristic broad band centered at 3430 cm^{-1} was assigned to
319 O-H and N-H stretching of free hydroxyl and amino groups and was also related to
320 hydrogen bonding in the polymer (Modrzejewska et al. 2015; Salehi, Daraei and Arabi
321 Shamsabadi 2016; Venkatesan et al. 2016). A C-H stretching was observed in the 2935
322 -2870 cm^{-1} region. Additional bands at 1661 cm^{-1} were attributed to C=O stretching of
323 the amide, vibrations of the NH_2 group and deformation of water (Kyzas, Bikiaris and
324 Lazaridis 2008). The NH_2 deformation peak normally appears at 1577 cm^{-1} ; however, it
325 overlaps with the amide II band near 1580 cm^{-1} . Therefore, the absorption at 1424 cm^{-1}
326 was assigned to the amide II band. Other absorption peaks were observed at 1150 cm^{-1} ,
327 due to C-O-C asymmetric stretching, and at 1080 and 1030 cm^{-1} , attributable to CO
328 stretching. This set of absorption peaks was associated with the chitosan structure (Jó et
329 al. 2017; Deepthi et al. 2016; Leceta et al. 2013).

330 For pure HA/CS hybrid obtained without glutaraldehyde, infrared spectrum
331 presented the band at 1640 cm^{-1} , assigned to N-H bending of the CS and also OH
332 deformation of water, which shifted for lower wavenumber compared to the initial band
333 of CS at 1664 cm^{-1} . Any alteration in the position in the OH stretching of the structural
334 hydroxyl of the HA (band at 3571 cm^{-1}) was observed as displayed in Table SM1 and
335 only N-H deformation at 1550 cm^{-1} was detected. Bands of phosphate occurs at same
336 region of C-O-C asymmetric stretching of the chitosan, and they can be overlapping in
337 the hybrids. Therefore, change in the N-H bending can be an indication of interaction
338 between HA/CS via hydrogen bonding. This result suggested weak interaction between
339 HA and CS.



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348 **Figure 2.** Infrared spectra of (i) (a) CS, (b) HA, (c) HA/CS, (d) HA-NH₂/CS-3, (e) HA-
349 NH₂/CS-4, (f) HA-NH₂/CS-5 and (g) HA-NH₂/CS-6, (ii) (a) HA, (b) HA-NH₂, (c) HA-
350 NH₂-GLU, (d) HA-NH₂-GLU-NaB and (iii) Raman spectra of (a) HA, (b) CS, (c)
351 HA/CS (d) HA-NH₂, (e) HA-NH₂/CS-3, (f) HA-NH₂/CS-4, (g) HA-NH₂/CS-5 and (h)
352 HA-NH₂/CS-6.

353

354 For hybrids the shift of the band from at 1655 cm⁻¹ was associated to the
355 presence of an interaction between HA-NH₂ and CS. A possible mechanism of HA-
356 NH₂ and CS crosslinking involves amino groups and formation of C=N bond between
357 the amine groups of HA-NH₂ and CS and the carbonyl of Glu. For the hybrids (Fig.
358 2i), the band at 1655 cm⁻¹ can be assigned to C=N stretching, suggesting the presence of
359 an imine group (Atak et al., 2017; Ramachandran, Nandhakumar, & Dhanaraju, 2011).
360 Other studies suggested that C=N stretching is located in the 1660 – 1630 cm⁻¹ region
361 (Frick, Ambrosi, Pollo, & Tessaro, 2018; Poon et al., 2014). However, this attribution
362 is not definite considering that the band overlap with the C=O stretching and NH₂
363 deformation of CS at 1664 cm⁻¹. Similar results were obtained for chitosan crosslinker
364 with Glu (Frick et al., 2018; Pratt, Wilson, & Kozinski, 2013). The band at 1560 cm⁻¹
365 corresponds to C=C and/or NH bending (Liu, Thormann, Claesson, & Tyrode, 2014).
366 Change in the NH₂ deformation initially at 1577 cm⁻¹ in HA-NH₂ and CS, which
367 shifted to 1560 cm⁻¹ in the hybrids can also be associated to unreacted NH₃⁺, offering
368 adsorption sites for DS (Pratt et al., 2013; Shi et al., 2017).

369 Furthermore, in the HA-NH₂ sample, the bands associated to OH stretching and
370 PO₄³⁻ didn't shift, suggesting that these latter groups of the HA-NH₂ did not interacted
371 with CS.

372 The Raman spectrum of HA-NH₂ (Fig. 2ii) showed a band at 2900 cm⁻¹
373 associated with CH₂ asymmetric stretching, suggesting the successful silylation of HA.
374 The band at 960 cm⁻¹ was assigned to P-O symmetric stretching of PO₄³⁻ (Lin -Vien et
375 al., 1991).

376 The Raman spectrum of pure chitosan presented bands at 1375 cm⁻¹ (CH₂
377 deformation), 1115 cm⁻¹ C-N stretching in C-2 (Mahaninia & Wilson, 2017) and 890
378 cm⁻¹, C-C stretching of the pyranose monomers and C-O-C symmetric stretching of the
379 ether groups (Lin-Vien et al., 1991).

380 For the hybrids, a CH₂ stretching was observed at 2933 cm⁻¹, possibly due to the
381 presence of new groups. Furthermore, the presence of the Schiff base was confirmed by
382 the new band at 1650 cm⁻¹, assigned to C=N stretching. Antisymmetric stretching was
383 observed at C=C at 1590 cm⁻¹ (Liu et al., 2014) and CH₂ deformation at 1445 cm⁻¹. For
384 pure HA/CS spectrum, only absorptions of the HA were present and absence of the
385 band at 1650 cm⁻¹, is a strong evidence of the absence of the C=N moieties.

386 3.1.3 ¹³C NMR

387

388 ¹³C NMR spectrum of the organofunctionalized hydroxyapatite (Fig. 3a)
389 exhibited three characteristic peaks at 11, 23 and 43 ppm, associated to carbons in the 3-
390 aminopropyl, which confirmed the silylation of the HA (Goonasekera, Jack, Cooper-
391 White, & Grøndahl, 2013). The peak at 164 ppm was attributed to carbonate (Pinto et
392 al., 2011).

393 ¹³C NMR spectrum of the chitosan (Fig. 3f) presented peaks at 105, 83, 75 and
394 58 ppm assigned to C₁, C₄, C_{3,5}, C_{2,6} of the polymer (Almeida et al., 2018; Rui et al.,
395 2017). The peak at 169 ppm was assigned to C₇ (Monteiro & Airoidi, 1999) and 23 ppm
396 is associated the incomplete deacetylation of the chitin (Almeida et al., 2018).

397 For the hybrids, ^{13}C NMR spectra (Fig. 3a-d) showed peaks of both silylated HA
398 and CS and broader signal at 170-180 ppm, which can be associated to overlapping of
399 the the signal of carbon in $\text{C}=\text{N}$ and $\text{C}=\text{O}$, in concordance with Raman and infrared
400 spectroscopies and also previous studies about reticulated chitosan (Monteiro & Airoldi,
401 1999; Ziegler-Borowska, Chelminiak, Kaczmarek, & Kaczmarek-Kędziera, 2016).
402 However, ^{13}C NMR spectra presented new two signals at 128 and 143 ppm and were
403 assigned to $\text{C}=\text{C}$ (Monteiro & Airoldi, 1999). The latter group indicated the tautomeric
404 species of the imine groups in acid medium (Nick, Stuart, Jonathan, Nick, & Stuart,
405 2012).

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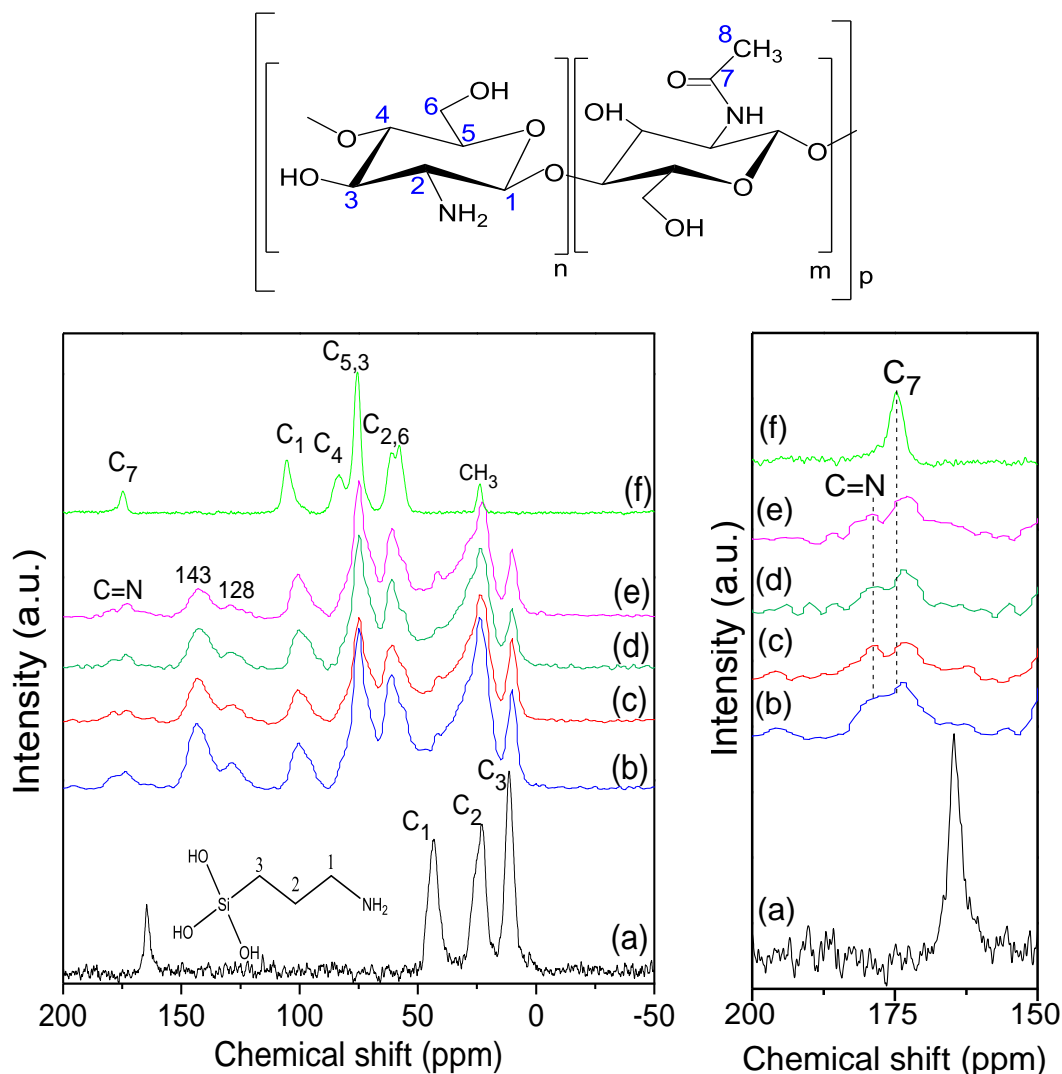
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Figure 3. ¹³C NMR spectra of (a) HA-NH₂, (b) HA-NH₂/CS-3, (c) HA-NH₂/CS-4, (d) HA-NH₂/CS-5 (e) HA-NH₂/CS-6 and (f) CS. *signal at 165 ppm is assigned to carbonate impurity in the pristine HA.

3.1.4 CHN elemental analysis and mechanism of crosslinking

431

432 The quantification of the organic groups in the hybrids was based on CHN
433 elemental analysis (Table 1). For pristine HA, a low content of carbon was detected,
434 possibly due the presence of carbonate. The quantity of nitrogen in HA-NH₂ indicated
435 the presence of 1.80 mmol g⁻¹ organic anchored moieties on the HA surface.

436

437 **Table 1.** CHN elemental analysis of HA-NH₂, CS and their bionanohybrids obtained by
438 *crosslinking* with Glu and the specific surface area results (SA) for the hybrids.

439

Sample	%C	%H	%N	Q _C (mmol g ⁻¹)	Q _N (mmol g ⁻¹)	C/N	SA (m ² g ⁻¹)
HA-NH ₂	6.50	1.90	2.50	5.41	1.80	3.03	13
HA-NH ₂ /CS-3	22.63	3.94	2.55	18.86	1.82	10.35	24
HA-NH ₂ /CS-4	21.46	3.76	2.10	17.88	1.49	11.92	34
HA-NH ₂ /CS-5	18.20	3.19	1.78	15.15	1.27	11.93	45
HA-NH ₂ /CS-6	19.56	3.50	2.11	16.56	1.50	11.04	50

440 *Q is the amount of carbon (C) or nitrogen (N).

441

442

443 For pure chitosan, high CHN contents were observed, and the data were
444 consistent with the chitosan composition. For all composites, high organic contents
445 were observed, and the CHN quantities increased for reactions at lower pH compared to
446 reactions at higher pH. Small differences (~1.2-1.4%) were observed between pH 3 and
447 4 and pH 5 and 6. Considering the nitrogen percentages, the quantity of amino groups
448 was greater for HA-NH₂/CS-3 and HA-NH₂/CS-4 than for the other samples.

449 Similar behavior was observed for the crosslinking of chitosan with Glu (Lal,
450 Arora and Sharma 2016). The lower C/N mol ratio observed for HA-NH₂/CS-3 can be
451 related to the presence of unreacted and protonated amino groups. This behavior
452 suggests that the formation of the imine at pH 3.0 is not effective because of the
453 protonation of the NH₂ groups in more acidic medium and the consequently lower
454 probability of C=N formation. On the other hand, more favorable conditions for the
455 formation of the Schiff base occurred at pH 4 to 5 due the presence of unprotonated
456 NH₂ groups and protonated carbonyl groups, which are reactive species in the formation
457 of C=N bonds (Antony et al., 2019; Kildeeva, Perminov, Vladimirov, Novikov, &
458 Mikhailov, 2009).

459 Based on specific surface area results and the amount of nitrogen in the solids
460 (Table 1), different density of amino groups were observed: for example, for HA-
461 NH₂/CS-3 and HA-NH₂/CS-4, the values were 0.076 and 0.044 mmol of N per m²,
462 while 0.03 of N per m² for other two hybrids. This aspect can influence the performance
463 of the solids during the drug adsorption.

464 These results are in accordance with the vibrational spectroscopy and ¹³C NMR
465 results and suggest the occurrence of bonding among HA-NH₂, Glu, and CS. Based on
466 the characterization results, the mechanism of crosslinking (Figure SM2) and the
467 structure of the hybrids are proposed in Figure 4. The proposed mechanism considers
468 that Glu species at acid pH are the monomeric molecule (Kildeeva et al., 2009;
469 Migneault, Dartiguenave, Bertrand, & Waldron, 2004).

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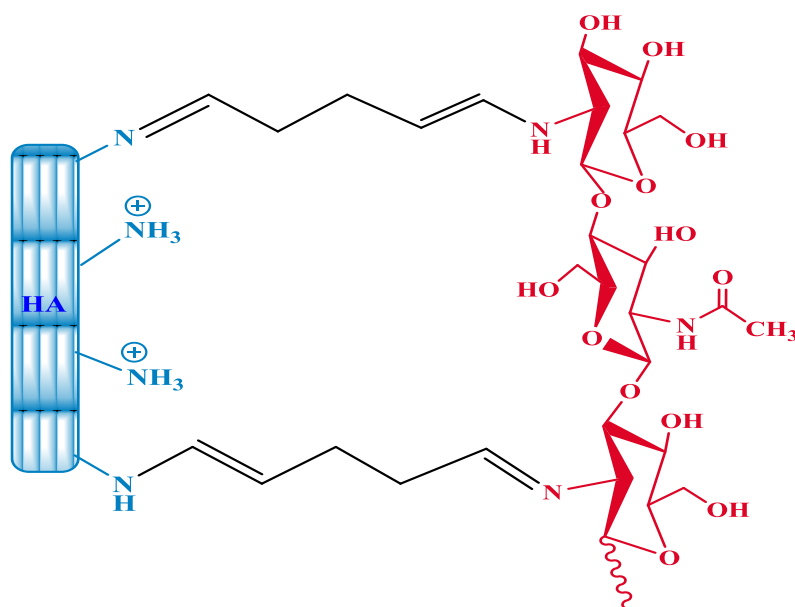
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478 **Figure 4.** Structure of the amino-hydroxyapatite reticulated glutaraldehyde chitosan
479 hybrids. Impurity of carbonate in apatite was not considered.

480

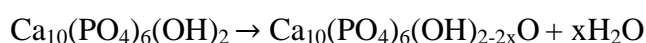
481 3.1.6 Thermal analysis

482

483 Thermogravimetric curves (TG/DTG) of hydroxyapatite, chitosan and their
484 hybrids are shown in Figure SM3. The thermal degradation of the silanized
485 hydroxyapatite presented three stages of 3.9%, 12.2% and 0.8% mass loss, which were
486 attributed to the loss of water adsorbed at 25-185 °C, decomposition of the silane
487 covalently bonded to the inorganic matrix in the range of 185 to 635 °C (Lung et al.
488 2016; Silva et al. 2006), and hydroxyapatite decomposition, respectively (Costa et al.
489 2009; Adolfsson et al. 2004; Locardi et al. 1993).

490

491



492

493 Pristine chitosan showed a 7% initial mass loss, which was associated with the
494 loss of adsorbed water (Lal et al., 2016). Subsequent events showed 38 and 13.4%
495 thermal degradation and were attributed to the degradation of the biopolymer by
496 depolymerization of the chains and decomposition of the pyranose rings followed by
497 dehydration, deamination and finally ring-opening reactions (Wanjuan et al., 2015,
498 Zawadzki and Kaczmarek, 2010).

499 Hybrids presented similar thermal degradation profiles influenced by the pH
500 during the synthesis. All thermogravimetric assignments are listed in Table SM2.

501 Three mass loss events are identified in all hybrids. The first one (20 - 131 °C)
502 was associated with the elimination of the adsorbed water (Ruphuy et al., 2016). The
503 second in the 131 - 354° C range and third stages at 346 - 642 °C corresponded to the
504 thermal decomposition of the organic part of the hybrids. In addition, the third event
505 occurred in the temperature range for the crosslinking reaction with glutaraldehyde
506 (Mohamed and Wilson, 2016). A similar result was described for HA/CS hybrids
507 obtained with 17 and 23% weight/weight chitosan (Zima et al., 2018).

508 The residual percentages were 54.8%, 61.4%, 67.2% and 63,9% for the HA-
509 NH₂/CS-3, HA-NH₂/CS-4, HA-NH₂/CS-5 and HA-NH₂/CS-6 solids, respectively, and
510 were associated with the inorganic part of the hybrids.

511 The order of thermal stability was HA.NH₂/CS-5 > HA.NH₂/CS-6 >
512 HA.NH₂/CS-4 > HA.NH₂/CS-3. Therefore, the system obtained at pH 3 presented lower
513 thermal stability than the other hybrids, which was associated with pH 3 disfavoring
514 formation of the imine, as indicated by the CHN analysis and other studies in the
515 literature, since crosslinking results in thermally stable structures (Antony et al., 2019;

516 Baldino, Concilio, Cardea, De Marco, & Reverchon, 2015). However, the thermal
517 stability of the HA-NH₂/CS-3 solid was better than that of pristine chitosan.

518 Based on TG results, the hybrids with higher organic contents were the ones
519 prepared at pH 3 and 4, and were in concordance with CHN results. This result
520 indicated that unreacted NH₂ groups are free for drug interaction.

521 *3.1.5 Scanning electron microscopy*

522

523 All nanocomposites presented a uniform surface without polymeric or inorganic
524 aggregates, indicating the homogeneous dispersion of each component in the hybrids
525 and particles with irregular shapes and rough surface (Fig. SM4).

526

527

528 *3.2 Adsorption of diclofenac sodium*

529

530 *3.2.1 Influence of the composition and Zeta potential measurements*

531

532 The influence of pH on drug adsorption was investigated at pH 6 -12 for all
533 hybrids (Fig. SM5i). For pH below 6, precipitation of the drug was detected. Better drug
534 adsorptions were obtained at lower investigated pH, following the Zeta potential results.
535 Zeta potential measurements (Fig. SM5ii) suggested that increasing the pH during
536 synthesis influenced the isoelectric point, which was 9.3, 8.5, 7.1 and 6.55 for the
537 hybrids-obtained at pH 3, 4, 5 and 6, respectively. At pH values higher than the zeta
538 potential, the surfaces of the hybrids are negatively charged, which would tend to cause
539 electrostatic repulsion between the hybrids and DS. Therefore, pH 6 was selected for
540 adsorption studies.

541 A positive and higher Zeta potential was obtained for HA-NH₂/CS-3 in relation
542 to the other hybrids. Considering that the pH in the removal test was 6, both HA-

543 NH₂/CS-3 and HA-NH₂/CS-4 are positively charged at this pH, and therefore, both
544 surfaces are favorable for DS removal, as its pK_a is 4.1 (De Oliveira et al., 2017; Hiew
545 et al., 2019). Thus, a better interaction with the anionic drug was established by the
546 hybrids with greater cationic character—in this case, HA-NH₂/CS-3.

547 The zeta potential measurements for HA-NH₂/CS-3 were not possible below pH
548 4 because the system was not stabilized. This finding is an indication that the population
549 of imine and unreacted amino groups is dependent on the experimental conditions used
550 to obtain the hybrids at different pH (Kamari, Ngah, Chong, & Cheah, 2009; M. G. Li,
551 Cheng, & Yan, 2007).

552 Accordingly, (See Fig. SM5i and Table SM3), HA-NH₂/CS-3 hybrid presented a
553 high drug removal capacity of 45 mg g⁻¹ (95%), while for the HA-NH₂/CS-6 hybrid, the
554 value was 35 mg g⁻¹ (77%). All values detected for the composites were higher than
555 those detected for pure (3.4 mg g⁻¹) and silylated HA (11.9 mg g⁻¹) and CS (22.4 mg g⁻¹).
556 The results can be related to the surface charge of the various hybrids, as when the
557 crosslinking reaction is conducted in an acidic medium, not only increased imine
558 formation but also protonation of the unreacted amino groups occurs.

559

560 3.2.2 Influence of the adsorbent dose

561

562 Test investigating the influence of the adsorbent dose were performed on the
563 composites with higher removal efficiencies, namely, HA-NH₂/CS-3 and HA-NH₂/CS-4
564 (Fig. SM6). The results indicated an increase in the amount of drug removed until an
565 equilibrium was reached above 50 mg of the hybrid, and 95% removal (50.5 mg g⁻¹)
566 was achieved by HA-NH₂/CS-3.

567 HA-NH₂/CS-4 did not show a variation in drug removal efficiency, and the
568 values were approximately the same (91%) for adsorbent doses between 25 and 100 mg.

569

570 3.2.3 Kinetic study

571

572 The effect of time on drug removal (Fig. SM7) indicated that an equilibrium was
573 reached at 30 and 15 min and that the maximum removal was 52 mg and 45 mg g⁻¹ for
574 HA-NH₂/CS-3 and HA-NH₂/CS-4, respectively.

575 Different equilibrium times were observed for DS adsorption on different studies
576 on the literature. For example, Feng et al. (2018) obtained 10 mg L⁻¹ DS removal with
577 graphene-chitosan composites in 1 h. Soares, Fernandes, Sacramento, Trindade, &
578 Daniel-da-silva, (2019) observed 100 mg L⁻¹ DS removal with magnetite coated with
579 chitosan in 5 h at pH 6.

580 Results of the data were fitted kinetic models are presented in Table 2. Both
581 systems were well fitted with a second-order kinetic model, as indicated by the
582 correlation coefficient and small difference between the theoretical and experimental
583 adsorption capacities.

584 **Table 2.** Kinetic and equilibrium parameters obtained from nonlinear fitting of the data
585 with the pseudo-first-order, pseudo-second-order and Elovich equations for the
586 adsorption of DS on HA-NH₂/CS-3 and HA-NH₂/CS-4 at 25 °C, pH 6 and 100 mg L⁻¹
587 and according to the nonlinear Langmuir model.

Models	Sample
Kinetic models	
HA-NH ₂ /CS-3	HA-NH ₂ /CS-4

$q_{e(\text{exp})}$ (mg g ⁻¹)	51.9	45.2
Pseudo-first-order		
$q_{e(\text{teor.})}$ (mg g ⁻¹)	50.5±0.7	45.2±0.1
K_1 (g min ⁻¹)	1.0±0.2	1.4±0.1
R^2	0.9756	0.9991
SD (mg g ⁻¹)	2.3	0.4
Pseudo-second-order		
$q_{e(\text{teor.})}$ (mg g ⁻¹)	51.9±0.5	45.5±0.1
k_2 (10 ⁻² g mg ⁻¹ min ⁻¹)	4.182±0.67	0.139±0.01
R^2	0.9931	0.9999
SD (mg g ⁻¹)	1.2	0.1
Elovich		
α (mg g ⁻¹ min ⁻¹)	(16.10±6.10) 10 ⁹	(2.07±36.0) 10 ³³
β (g mg ⁻¹)	0.5±0.1	1.8±0.4
R^2	0.9919	0.9981
SD (mg g ⁻¹)	1.3	0.5
Equilibrium model		
Langmuir		
$q_{e(\text{exp})}$ (mg g ⁻¹)	125.0	65.7
$q_{\text{máx}}$ (mg g ⁻¹)	139.5 ± 4.0	74.1 ± 1.0
K_L (10 ⁻² L mg ⁻¹)	4.8 ± 0.4	4.2 ± 0.2
R^2	0.9899	0.9976
SD	4.72	1.24

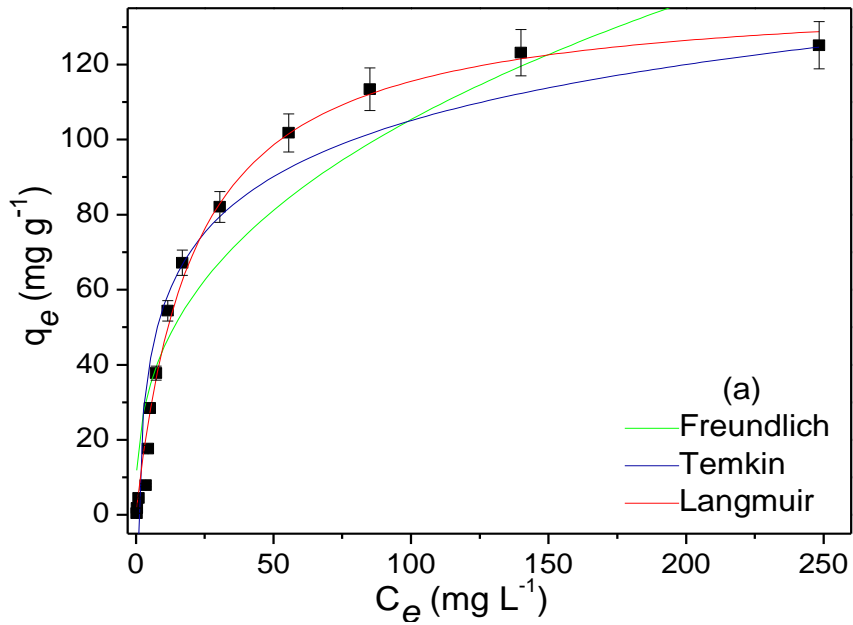
588 3.2.4 Influence of initial DS concentration

589

590 The influence of the initial DS concentration was evaluated in the range of 1 –
591 500 mg L⁻¹ in the equilibrium time and pH 6 (Fig. 5). The adsorption was more
592 pronounced at the lower DS concentrations than at the higher DS concentration until an
593 equilibrium was reached at 140 mg L⁻¹, showing a maximum adsorption of 125 mg of
594 adsorbed DS per gram of HA-NH₂/CS-3. The same behavior was observed for HA-
595 NH₂/CS-4 with a maximum adsorption of 65.7 mg g⁻¹, which was half of the value

596 observed for HA-NH₂/CS-3. This behavior can be associated with the lower quantity of
597 organic moieties on HA-NH₂/CS-4 than on HA-NH₂/CS-3, as indicated by the CHN
598 data.

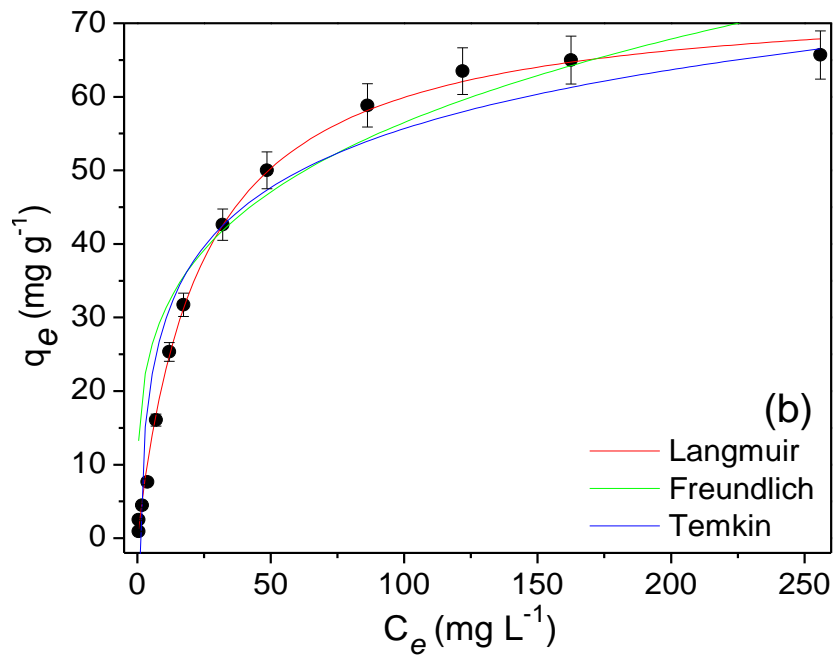
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606 **Figure 7.** Isotherms and their nonlinear fitting with equilibrium models for DS
607 adsorption by (a) HA-NH₂/CS-3 and (b) HA-NH₂/CS-4.

608

609 The removal data were fitted with Langmuir model, as summarized in Table 2.

610

611 Both systems were well fitted by the Langmuir model, showing a correlation
612 coefficients greater than 0.98, although the SD between the experimental and theoretical
613 maximum q was lower for HA-NH₂/CS-4 than for HA-NH₂/CS-3.

614 Compared with those from other studies, these adsorption results were promising
615 (Table SM4). The synthesized hybrids presented higher adsorption capacities than other
616 modified chitosan materials. Furthermore, lower adsorption for the precursors, HA-NH₂
617 (11.91 mg g⁻¹) and CS (22.43 mg g⁻¹), illustrates the synergism between the
618 constituents in the hybrids and improvement of the drug adsorption.

619

620 *3.2.5 Mechanism of the interaction of drug/ hybrid*

621 Infrared spectra of the hybrids after drug removal (Fig. SM6) indicated new
622 bands associated with drug were observed at 1310, 1581 and 1507 cm⁻¹, assigned to
623 C=O and C=C stretching (Kaur & Datta, 2014; Kumar et al., 2018).

624 The mechanism of the interaction between DS and the hybrids was suggested
625 based on the characteristics of the modified surface and the drug in solution. At pH 6.0,
626 the anionic form of the DS molecule (deprotonated species) is predominant, considering
627 that its pKa is 4.1 (Hu et al., 2019; Lessa et al., 2018; Lonappan et al., 2017).

628 As indicated by the zeta potential, the surfaces are positively charged at pH 6;
629 therefore, electrostatic interactions between the charged species can be the principal
630 mechanism of interaction (Fig. SM7). The same behavior was proposed for chitosan

631 reticulated with Glu (Riegger et al. 2018) and for CS reticulated with genipin
632 (Lonappan et al., 2018). Other mechanisms acting in synergy with electrostatic
633 interactions and promoting the adsorption efficiency can be present, i.e., hydrogen
634 bonding through the free polar groups -OH and -NH₂ and van der Waals forces (Liang
635 et al., 2019; Soares et al., 2019). In addition, the crosslinking of chitosan also breaks the
636 hydrogen bonds between adjacent units of the polymer, which may result in partial
637 breaking of the chitosan structure and the release of additional free polar groups (OH),
638 thereby promoting the removal of DS via the hydroxyl sites of chitosan (Wilson & Xue,
639 2013).

640

641

642 **4. Conclusion**

643

644 Silylated HA/Glu/CS hybrids were synthesized at pH 3, 4, 5 and 6. Control
645 reaction of amino hydroxyapatite and glutaraldehyde following by reduction indicated
646 that C=N formation is the main mechanism of interaction between amino modified HA
647 and aldehyde. The XRD, FTIR, Raman, ¹³C NMR and CHN analysis results confirmed
648 the formation of hybrid possibly by interaction between the amine groups present in HA
649 and chitosan and the aldehyde groups present in the bifunctional agent, resulting in a
650 new inorganic-organic hybrid. The difference between all hybrids relies on the amount
651 of organic parts as indicated in CHN analysis and thermogravimetry.

652 The hybrid HA-NH₂/CS-3 presented higher cationic character than the hybrid
653 HA-NH₂/CS-4, and the difference in surface charge was likely responsible for the
654 different adsorptive properties of the materials for DS. HA-NH₂/CS-3 presented better
655 drug adsorption performance than HA-NH₂/CS-4 at pH 6 and the maximum removal
656 capacity obtained was 125 and 65.7 mg g⁻¹, respectively.

657 This work demonstrates that HA/chitosan hybrids were obtained and functioned
658 as promising biosorbents for the remediation of effluents contaminated with DS.

659

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661

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666

667 **References**

668

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