

1 **The effect of erythrosine-B on the structuration of poloxamer 407 and cellulose**
2 **derivative blends: *in silico* modelling supporting experimental studies**

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24 **ABSTRACT**

25 Erythrosine is a dye approved for medical use that has shown promising photodynamic activity,
26 allowing for the inactivation of microorganisms and activity against malignant cells. Despite
27 the great photodynamic potential, erythrosine exhibits hydrophilicity, negatively impacting its
28 action in biological membranes. Therefore, the incorporation of erythrosine in micellar
29 polymeric systems, such as poloxamers, may overcome this limitation. Moreover, using
30 bioadhesive and thermoresponsive polymers to combine *in situ* gelation and bioadhesion may
31 enhance retention of this topically applied drug. In this work, mucoadhesive and
32 thermoresponsive micellar systems were prepared containing erythrosine in two states: the
33 native form (ERI) and the disodium salt (ERIs). The systems were evaluated based on the effect
34 of ERI/ERIs on the micellar structure of the binary polymer mixtures. Optimised combinations
35 of poloxamer 407 (polox407) and mucoadhesive sodium carboxymethylcellulose (NaCMC) or
36 hydroxypropyl methylcellulose (HPMC) were used as micellar systems for ERI or ERIs
37 delivery. The systems were studied with respect to theoretical interactions, qualitative
38 composition, morphology, and micellar properties. *In silico* modelling indicated a higher
39 interaction of the drug with poly(ethylene oxide) (PEO) than poly(propyleneoxide) (PPO)
40 fragments of polox407. Systems containing NaCMC displayed a repulsive effect in the
41 presence of erythrosine, due to the polymer's charge density. Both systems could convert the
42 photosensitizer in its monomeric form, ensuring photodynamic activity. In these mixtures,
43 crystallinity, critical micellar temperature and enthalpy of polox407 micellisation were
44 reduced, and micellar size, evaluated by transmission electron microscopy (TEM), showed low
45 impact of ERI/ERIs in HPMC preparations. Aiming toward photodynamic applications, the
46 findings showed how ERI or ERIs can affect the micellar formation of gels composed of 17.5%
47 (w/w) polox407 and 3% (w/w) HPMC or 1% (w/w) NaCMC, important for understating their
48 behaviour and future utilisation as erythrosine delivery systems.

49 *Keywords:* gel, Pluronic F127, hydroxypropyl methylcellulose, sodium
50 carboxymethylcellulose, erythrosine B, drug delivery.

51

52 **1. Introduction**

53 Erythrosine is a xanthene that has been interest as a photosensitizer (PS) for
54 photodynamic therapy (PDT). Its low toxicity combined with its history of use in dentistry and
55 food products, makes the translation into the clinic easier than many other PS substances [1,2].
56 In PDT, a luminous energy is absorbed by a PS and transferred to oxygen molecules, producing
57 highly reactive cytotoxic species (particularly singlet oxygen $^1\text{O}_2$) [3]. Erythrosine has been
58 reported as dye to detect dental biofilms and has presented promising pharmacological activity
59 in photodynamic inactivation of microorganisms and malignant cells [1,2,4].

60 The successful photodynamic activity is often related to the ability of a PS to present high
61 visible light absorption and high singlet oxygen quantum yield ($\Phi_{\Delta}^1\text{O}_2$), and erythrosine
62 achieves satisfactory values in water (ca 0.62) [3]. The interaction between PS and membrane
63 can have important bearing with the photodynamic activity, since the biological membranes
64 are involved with its mechanism of action [5]. The incorporation of erythrosine in a micellar
65 polymeric system, such as poloxamer-based preparations, could improve the delivery of this
66 drug (log P of ERIs = -0.05 [6] and ERI= 0.46 [7]) to the cells with low changes in
67 pharmacological performance of the drug itself as the native structure is retained. Moreover, it
68 could control the release and improve retention of PS at the desired site, even considering
69 highly humid regions such as the ocular or oral mucosal [8].

70 Poloxamers are a class of ABA triblock copolymer with the structure poly(ethylene
71 oxide)(PEO)-*b*-poly(propylene oxide)(PPO)-*b*-PEO. Poloxamer 407 (polox407) is the most
72 widely used copolymer in the development of thermoresponsive drug delivery systems due to
73 its high performance, safety profile, and low-cost [9]. Poloxamer solutions exhibit critical
74 micellisation temperatures (CMT), heating above which typically results in the formation of a
75 spherical micelle with a hydrophobic PPO core and an hydrophilic PEO shell [10]. In
76 appropriate concentration (above ca 15 %, w/v) and temperature, polox407 exhibits a reversible

77 transition from low concentration liquid to a viscous gel mesophase, a result of the micelles
78 packing into a cubic liquid crystalline structure [11]. This property enables a cool solution to
79 flow and become viscous when in contact with the body temperature, which is an important
80 characteristic for topical formulations [12,13]. Above its CMT, polox407 switches to a face
81 centered cubic structure of spherical core-shell micelles, as determined by small-angle neutron
82 scattering [11]. Such systems present several advantages, alongside *in situ* gelation triggered
83 by the body's heat, the high degree of well-ordered water allows for a smooth texture and the
84 presence of micelles enables the solubilisation of both hydrophobic and hydrophilic drugs [14].

85 The combination of this thermoresponsive polymer with biomacromolecules opens the
86 possibility of combining this *in situ* gelation with other functionality to construct novel
87 nanocarriers in drug delivery systems [15]. The addition of mucoadhesive polymers such as
88 poly(acrylic acid) derivatives [16–20] or cellulose derivatives [21–24] to polox407 systems
89 have been extensively studied. They are able to combine thermoresponsive gelation of
90 polox407 with improved adhesiveness of the polymer additives, however complex non-linear
91 relationships are present in these systems which require careful optimisation [23]. Systems
92 containing polox407 and HPMC or sodium NaCMC as mucoadhesive agents are promising
93 with respect to their rheological, mechanical, micellar, and adhesive characteristics,
94 particularly for topical drug delivery [21–26]. Although the structure of xanthene dyes shows
95 several acid-base groups and that they may present several tautomeric forms, because of its
96 pKa (around 2.35 and 3.79 [27]), erythrosine, at pH 7.0, exists in a predominantly dianionic
97 form. However, considering the differences in log P and solubility of the protonated form (ERI)
98 and its disodium salt (ERIs), changes in physicochemical and photophysical properties are,
99 consequently, expected [27]. This study aimed to evaluate the effect of erythrosine in two
100 different aggregation states (low solubility - ERI and high solubility - ERIs) on the structuration
101 of thermoresponsive micellar systems, composed of polox407 and HPMC or NaCMC for

102 further pharmaceutical and biomedical applications using PDT. Overall, this intends to
103 generate important underpinning knowledge of these formulations for topical PDT.

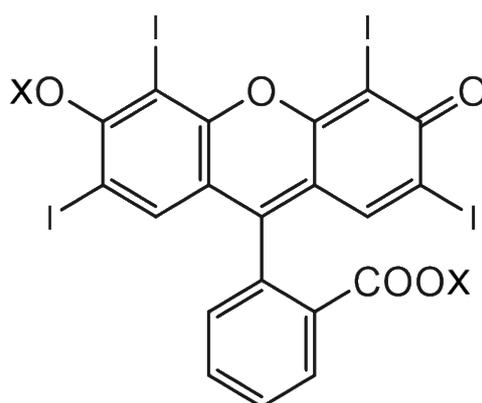
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105 2. Materials and methods

106 2.1. Materials

107 Poloxamer 407 (polox 407), erythrosine B (ERI - MW 835.89 g.mol⁻¹, C₂₀H₈I₄O₅, 95% purity;
108 saturation solubility 0.7 mg.mL⁻¹) [28] and its disodium salt (ERIs - MW 879.86 g.mol⁻¹,
109 C₂₀H₆I₄Na₂O₅, saturation solubility 70 mg.mL⁻¹) [6], mucin (from porcine stomach, type II
110 crude), uric acid, and phosphate buffered tablets (pH 7.4) were purchased from Sigma-Aldrich
111 (Sao Paulo, SP, Brazil). HPMC K100, Methocel[®] (8.1% hydroxypropoxyl 22% methoxyl
112 content) was donated from Colorcon Dow Chemical Company[™] (Dartford, United Kingdom).
113 NaCMC (DS = 0.8-0.95) was purchased from Synth (Diadema, SP, Brazil). Ultra-purified
114 water was obtained in-house using a water purification system (Evoqua Water Technologies,
115 Pittsburgh, PA, USA). All reagents were used without further purification.

116



X= Na (ERIs) or H (ERI)

117

118 **Fig. 1.** Chemical structure of erythrosine in neutral (ERI) or disodium salt form (ERIs)

119

120 2.2. Preparation of systems

121 Systems were prepared by dispersion of 3% (w/w) HPMC or 1% (w/w) NaCMC in
122 purified water, with stirring at room temperature. After the cellulose derivative was completely
123 dispersed, 17.5% (w/w) polox407 was added to the preparation, and the mixture was stored at
124 5 °C, for 48 h, ensuring complete wetting of the poloxamer. The polymeric system was then
125 stirred again to complete dissolution of the remaining polymers. ERI or ERIs were added to
126 the formulation at a level of 1% (w/w), with mechanical stirring, prior to the addition of
127 polymers [2]. Final formulations were kept at 5 °C for at least 24 h prior to further analysis
128 [29–31].

129

130 *2.3. Interaction studies supported by theoretical modelling*

131 *2.3.1. Obtaining the association isotherm*

132 The interaction capacity of ERIs with polox407, polox407/NaCMC and
133 polox407/HPMC micelles were performed by titration of aliquots of a stock solution of each
134 system. The concentration of the solutions into the cuvette ranged from 0 to 1.4 % w/v for
135 polox407, and from 0 to 0.12 % w/v for NaCMC or HPMC (keeping NaCMC or
136 HPMC/polox407 ratio fixed at 0.07 at each addition) in a 5.0×10^{-7} mol.L⁻¹ of ERIs. All
137 solutions were prepared in McIlvaine buffer (0.10 mol.L⁻¹; pH 7.4) [32,33]. The interaction
138 was monitored, at 35 °C, by acquiring the fluorescence emission spectra after the addition of
139 polox407 or copolymer blends. The excitation used was 500 nm, reading from 520 nm to 750
140 nm. The absorbance at excitation wavelength was less than 0.05 to avoid internal filter errors.

141

142 *2.3.2. In silico modelling*

143 Molecular modelling studies of ERIs, ERIs/NaCMC, ERIs/HPMC,
144 ERIs/NaCMC/polox407 and ERIs/HPMC/polox407, ERIs/PEO and ERIs/PPO were

145 performed in Orca 4.0 program [34] optimised in vacuum, employing Hartree-Fock (HF)
 146 method with implementations for long range interactions (HF-3c), methodology developed to
 147 obtain the most stable geometric structure in macromolecular systems [35]. The advanced
 148 molecular editor Avogadro program version 1.1.1 (University of Pittsburgh, Department of
 149 Chemistry, Pittsburgh, PA, USA.) was applied for graphical visualisation of the structures [36].

150

151 3.2.3. Determination of complexation energy between copolymers fragments and ERIs 152 tautomers

153 For the determinations of the complexation energy (ΔE_{Comp}) formed between ERIs and
 154 the polymers studied, NaCMC (-NaCMC₂-), HPMC (-HPMC₃-) and proportional copolymer
 155 fragments of PEO (-PEO₁₂-), PPO (-PPO₁₂-) and polox407 (-(PEO)₅-(PPO)₃-(PEO)₅-) are
 156 described in Eq. 1 for interactions between two components and Eq. 2 considering three
 157 elements interaction [37].

$$158 \Delta E_{Comp} = E_{ERIs+polox407/Polymers/PEO/PPO} - (E_{ERIs} + E_{polox407/Polymers/PEO/PPO}) \quad (1)$$

159

$$160 \Delta E_{Comp} = E_{ERIs+polox407+Polymers} - (E_{ERIs} + E_{Polymers} + E_{polox407/PEO/PPO}) \quad (2)$$

161

162 where, $E_{ERIs/polox407/Polymers/PEO/PPO}$ are the total electronic energy for the optimised
 163 structures, considering the complex formed between ERIs, NaCMC and HPMC polymers, as
 164 well as, the copolymers fragments of PEO, PPO and polox407 respectively. E_{ERIs} , $E_{Polymers}$,
 165 and $E_{polox407/PEO/PPO}$ are the individual electronic energy of the polymers and copolymers
 166 fragments respectively used in the complexation process.

167

168 *2.4. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)*

169 The infrared spectra were obtained by means of an Attenuated Total Reflectance (ATR)
170 technique using an ATR-FTIR Nicolet iZ10 instrument (Thermo Fisher Scientific, EUA). All
171 measurements were taken at room temperature (25 °C) on the zinc selenide (ZnSe) ATR crystal.
172 The spectra were recorded over the range 4000-600 cm⁻¹, at 4 cm⁻¹ resolution and represented
173 by an average of 64 scans. The spectrum of the clean and dry ZnSe ATR crystal in ambient
174 atmosphere was used as background for infrared measurement [38].

175

176 *2.5. Differential scanning calorimetry (DSC)*

177 Ca 35 mg of each formulation was placed in aluminum pans and hermetically sealed.
178 The DSC was performed in a DSC Q20 (TA Instruments®, Surrey, United Kingdom) at a
179 heating rate of 5 °C.min⁻¹ between 5 and 40 °C, under a nitrogen atmosphere. The CMT was
180 determined from the associated endothermic peak in the DSC thermograms of the formulations,
181 which occurred between 10 and 20 °C [24]. At heating rate of 5 °C/min between 0 to 400 °C,
182 the polox407 crystallinity was calculated using DSC thermograms as the ratio of the measured
183 polymer crystallisation enthalpy to the product of the polymer weight fraction and the
184 crystallisation enthalpy of completely crystallized polox407 [39].

185

186 *2.6. Scanning electron microscopy (SEM)*

187 Samples were subjected to instant freezing using liquid nitrogen at -80 °C for 20 min.
188 Frozen samples were then lyophilized for 48 h. Segments of the dried samples were carefully
189 deposited on stubs containing double-sided adhesive carbon tape. The samples were metallised
190 by the deposition of a thin layer of gold and evaluated by a SS550 Superscan Scanning Electron
191 Microscopy (Shimadzu, Tokyo, Japan).

192

193 2.7. *Transmission electron microscopy (TEM)*

194 The TEM analysis was performed using a JEM-1400 Transmission Electron
195 Microscope (JEOL, Tokyo, Japan), with an accelerating voltage of 120 kV. Samples were
196 diluted 50-fold, and then negatively stained with 2% (w/v) uranyl acetate solution for
197 observation. Samples were prepared at 37 °C to study the micelle formation. The measurements
198 of the micelles by TEM were expressed as arithmetic mean and standard deviation of 250
199 micelles of each system.

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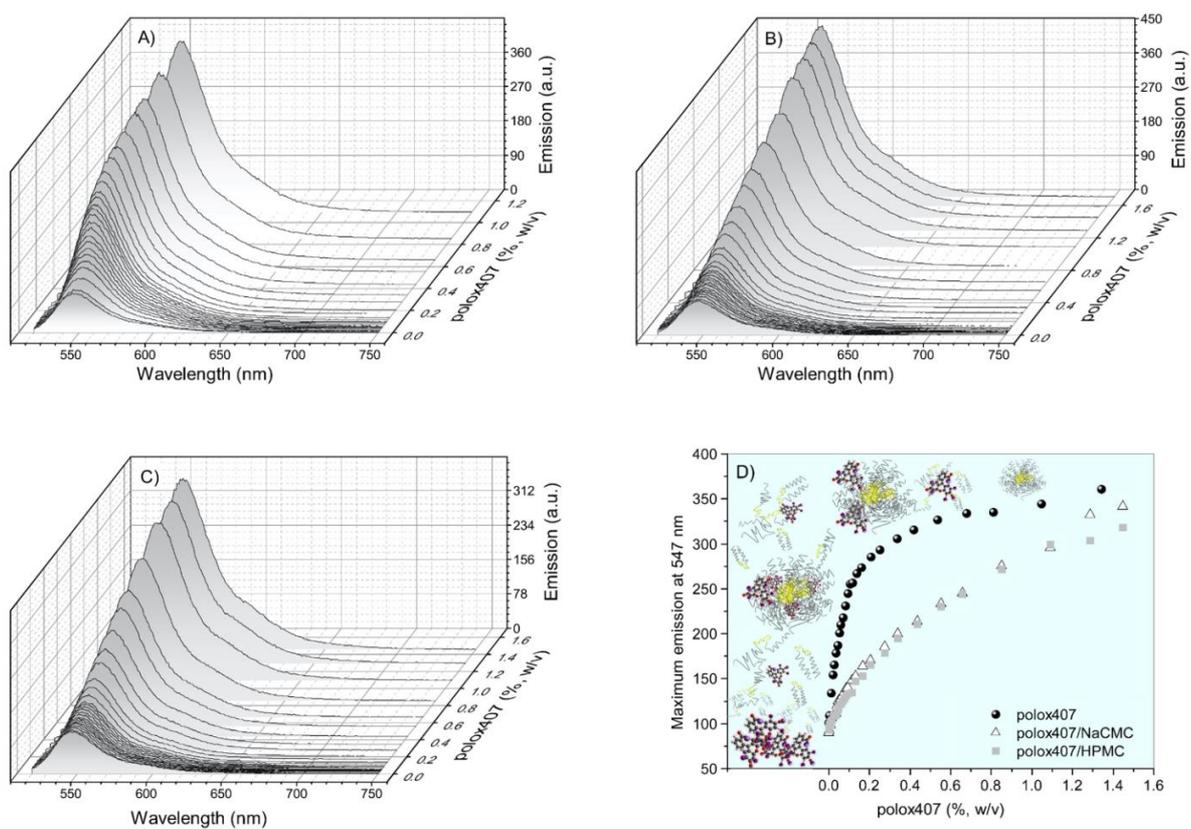
201 3. Results and discussion

202 3.1. *Interaction studies supported by theoretical modelling*

203 Xanthene photosensitizers, such as ERI/ERIs, show several acid-base groups. Thus, the
204 pH of solution it is present in may determine physical-chemical properties [27]. The carboxylic
205 group commonly shows higher acidity than the phenolic group; however, some inversion is
206 expected depending on the structure of the studied compound. Although there are plenty of
207 tautomeric structures for each proteolytic form, considering a pKa around 2.35 and 3.79
208 described in the literature, both ERI and ERIs predominantly exist in their dianionic form at
209 pH of 7.0 [27]. Considering the high solubility of ERIs in its complete dianionic state, this form
210 was theoretically modelled with each polymer of the systems, improving the comprehension
211 of the structure of the hydrogels.

212 The evaluation of the interaction and monomerisation of a PS compound in a micellar
213 nanostructured system presents elevated relevance to predict formulation functionality in
214 photodynamic therapy. The studies were carried out by obtaining the ERIs-micelle association
215 isotherm (Fig. 2), achieved by monitoring the recovery of ERIs fluorescence by the effect of

216 gradual increase in polymer concentration. The high spectral overlap of monomers and small
 217 aggregates formed makes electronic absorption studies unviable. Thus, the fluorescence
 218 emission technique was selected due to its high sensitivity for detecting small aggregates,
 219 similarly to the reported by Pellosi et al using the same PS [32,40]. The results were
 220 complemented with molecular modelling, which provided important correlations when
 221 employing the HF-3C method for structural optimisation.



222

223 **Fig. 2.** Interaction studies, at 35 °C, obtained by increasing the polymer (polox407, NaCMC
 224 and HPMC) concentration on the ERIs in buffer solution (pH= 7.25): (A) polox407; (B)
 225 polox407/NaCMC, (C) polox407/HPMC and (D) Isotherms of association. Graphs are
 226 presented as ERIs emission vs. polox407 concentration. [NaCMC] = [HPMC] = 0 to 0.12 %
 227 w/v; [ERI] = 5.0×10^{-7} mol.L⁻¹ (λ_{exc} = 500 nm, and monochromator slits excitation/emission
 228 of 10/5, nm/nm).

229

230 Fig. 2A-C show low intensity of fluorescence emission of ERIs in an aqueous
231 environment. This behaviour can be justified by the hydrophobic force of the carbon chain of
232 PS, and its self-aggregated state (aggregates of small extension) in this solvent. It is known that
233 self-aggregate are non-fluorescent species, and that collisions between PS and water molecules
234 deactivate its excited state by non-radioactive processes (internal conversion) [41–43].
235 However, as titration occurs, the polymer concentration increases and micelles of polox407 are
236 formed after reaching the CMC for each system (values ranging from 0.0169 to 0.022 % w/v
237 at 37 °C) [44]. When systems undergo micellisation, they are able to recover ERIs fluorescence
238 intensity to a limit, where, theoretically, all drug molecules would be in their monomeric form
239 (Fig. 2D). Moreover, as already described in the literature, alongside the increase of the
240 fluorescence intensity, a bathochromic shift occurs (Fig. 2A-C). Thus, the peak of maximum
241 emission changes from 547 nm to 556 nm, with measurements increasement of 9 nm in each
242 case [45]. The spectral variations may suggest changes in the chemical environment, as ERIs
243 partitions from water to the micellar microenvironment of reduced polarity [32]. The ERIs-
244 water intermolecular interactions, that stabilize the PS fundamental state, are reduced with
245 micellar incorporation. Thus, a reduction in the energy gap between fundamental and excited
246 state produces the bathochromic shift observed [46].

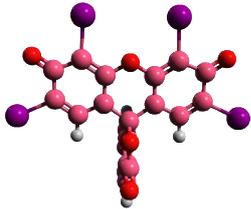
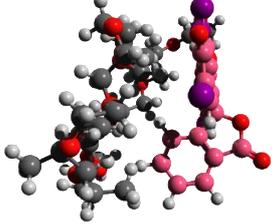
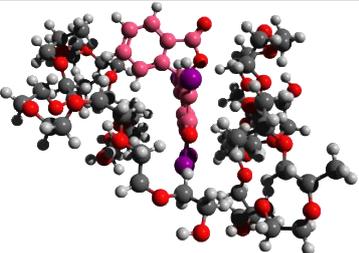
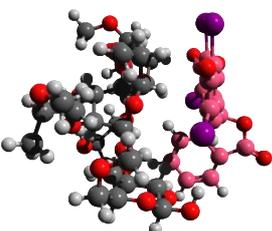
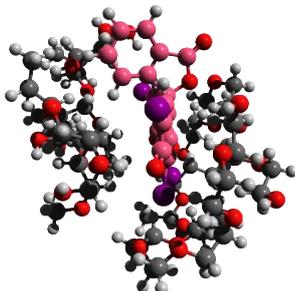
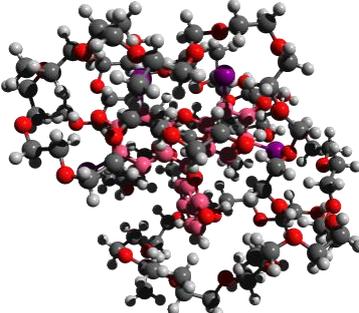
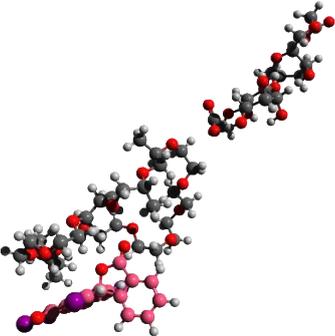
247 The results of computational modelling, displayed in Table 1, exhibit some tendency
248 for ERIs partition into the polox407 micelles due to the favourable complexation energy
249 ($\Delta E_{Comp} = -44.47805379 \text{ kcal.mol}^{-1}$). The increase in the number of polox407 unimers makes
250 the process even more favourable to ERIs incorporation ($\Delta E_{Comp} = -70.29447533 \text{ kcal.mol}^{-1}$),
251 reflecting the effect of the higher concentration of surfactant in this dynamic. Additionally,
252 Table 2 demonstrates increased interaction of ERIs with PEO oligomers ($\Delta E_{Comp} = -$
253 $74.15897585 \text{ kcal.mol}^{-1}$) if compared to PPO ones ($\Delta E_{Comp} = -15.2446224 \text{ kcal.mol}^{-1}$). The

254 results agree with the literature, which have shown the most efficient packing of this PS into
255 the hydrated region of the micelle (PEO moieties). By Stern-Volmer constant it has been
256 confirmed as well, with values of $K_{sv} = 0.68 \text{ L.mol}^{-1}$ for ERIs [7]. Therefore, low values of this
257 constant suggests ERIs is placed mainly in PEO region of the micelle [32].

258

259 **Table 1**

260 Total electronic energy for the optimised structures of erythrosine (ERIs) and polox407,
261 NaCMC, HPMC or their mixture calculated at the HF-3c level of theory.

ERIs	ERIs/1polox407
<i>HF-3c/hartrees</i> = -2314.46546376715	<i>HF-3c/hartrees</i> = -4481.59083
^a $\Delta E_{\text{Comp}}/\text{kcal mol}^{-1}$ = -1452350.223	^a $\Delta E_{\text{Comp}}/\text{kcal mol}^{-1}$ = -44.47805379
	
ERIs/2polox407	ERIs/1HPMC
<i>HF-3c/hartrees</i> = -6648.720389	<i>HF-3c/hartrees</i> = -4736.864382
^a $\Delta E_{\text{Comp}}/\text{kcal mol}^{-1}$ = -70.29447533	^a $\Delta E_{\text{Comp}}/\text{kcal mol}^{-1}$ = -44.83596244
	
ERIs/1HPMC/1polox407	ERIs/1HPMC/2polox407
<i>HF-3c/hartrees</i> = -6903.987973	<i>HF-3c/hartrees</i> = -7928.622291
^a $\Delta E_{\text{Comp}}/\text{kcal mol}^{-1}$ = -88.20016883	^a $\Delta E_{\text{Comp}}/\text{kcal mol}^{-1}$ = -2002883.549
	
ERIs/1NaCMC/1polox407	ERIs/1NaCMC
<i>HF-3c/hartrees</i> = -5070.410046	<i>HF-3c/hartrees</i> = -4045.82797
^a $\Delta E_{\text{Comp}}/\text{kcal mol}^{-1}$ = 716929.8552	^a $\Delta E_{\text{Comp}}/\text{kcal mol}^{-1}$ = 16.99276609
	

262

263 1 hartree = 627.5095 kcal.mol⁻¹.

264 ^aFor the calculations of $\Delta E_{\text{Comp}}/\text{kcal mol}^{-1}$ were used the values from HF-3c/hartrees.

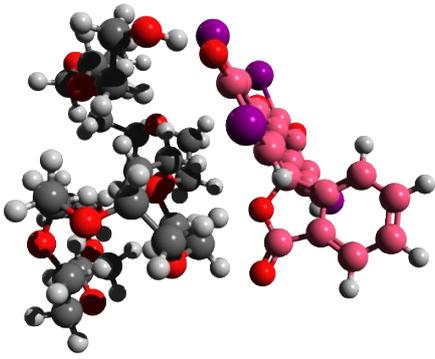
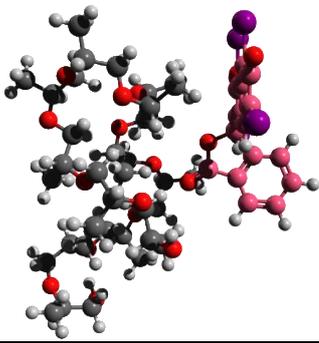
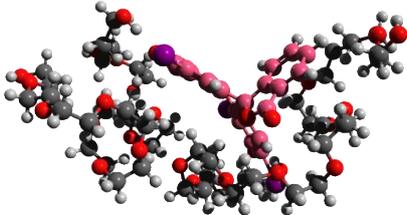
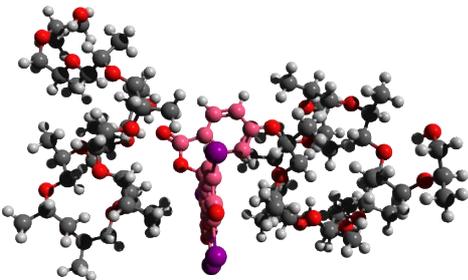
265

266 For the titration with polox407 solution (Fig. 2D), the saturation in fluorescence
267 intensity was reached around 0.8 % (w/v). However, the ERIs binding isotherms presented
268 reduced angles for the polox407/NaCMC and polox407/HPMC systems (Fig. 2D), without
269 reaching complete saturation in the concentration range of surfactant evaluated. This behaviour
270 can be justified by the higher hydration of the PEO segments when polox407 is mixed with
271 cellulose derivative, in agreement with previously determined gelation [23] and CMT [24] for
272 systems without drug. Thus, hydrated microenvironments may suppress the fluorescence of
273 ERIs, besides changing its preferential microenvironment for accommodation. Evaluation
274 involving titrations with NaCMC and HPMC, in the absence of polox407, were also performed.
275 In these cases, a small interaction of ERIs with NaCMC polymeric systems was verified, with
276 the slope of the association isotherm more inclined with HPMPC (Fig. S1). This effect is
277 associated with NaCMC's negative charge in physiological pH, which repels the electronic
278 density of the same nature for ERIs in this condition. Table 1 shows the preferable interaction
279 of ERIs with HPMC ($\Delta E_{Comp} = -44.83596244 \text{ kcal.mol}^{-1}$). As observed through the analysis,
280 there is some separation degree between ERIs and NaCMC molecules ($\Delta E_{Comp} =$
281 $16.99276609/\text{kcal.mol}^{-1}$). The complexation energy of the systems containing
282 ERIs/HPMC/polox407 presented negative energy ($\Delta E_{Comp} = -88.20016883/\text{kcal.mol}^{-1}$), which
283 highly decreases in the presence of two molecules of polox407 ($\Delta E_{Comp} = -$
284 $2002883.549/\text{kcal.mol}^{-1}$), indicating a minor impact of this mucoadhesive polymer on
285 polox407 structuration in comparison to NaCMC, in presence of ERIs. On the other hand,
286 systems composed of ERIs/NaCMC/polox407 may present higher solvation likely related to
287 space promoted by the repulsion between charged species ($\Delta E_{Comp} \text{ ERIs/NaCMC} > 0$). For
288 instance, similar data have already been reported in the literature, ensuring low monomerisation
289 capacity of ERIs in the presence of other anionic biomimetic systems, such as Sodium Dodecyl
290 Sulphate (SDS) micelles [32].

291

292 **Table 2**

293 Total electronic energy for the optimised structures of erythrosine (ERIs) and fragments PEO
 294 and PPO of polox407 calculated at the HF-3c level of theory.

ERIs/1PEO	ERIs/1PPO
<i>HF-3c/hartrees</i> = -4213.291184	<i>HF-3c/hartrees</i> = -4678.76299
^a $\Delta E_{\text{Comp}} / \text{kcal mol}^{-1}$ = -53.86684692	^a $\Delta E_{\text{Comp}} / \text{kcal mol}^{-1}$ = -28.19099173
	
ERIs/2PEO	ERIs/2PPO
<i>HF-3c/hartrees</i> = -6112.102021	<i>HF-3c/hartrees</i> = -7043.062538
^a $\Delta E_{\text{Comp}} / \text{kcal mol}^{-1}$ = -74.15897585	^a $\Delta E_{\text{Comp}} / \text{kcal mol}^{-1}$ = -15.2446224
	

295

296 1 hartree = 627.5095 kcal.mol⁻¹297 ^aFor the calculations of $\Delta E_{\text{Comp}} / \text{kcal mol}^{-1}$ were used the values from HF-3c/hartrees.

298

299 Although some repulsion was found for NaCMC systems at molecular level, all the
 300 binary systems showed satisfactory interaction, ensuring the monomerisation and
 301 incorporation of ERIs in the micellar microenvironment. Studies at molecular level proved that
 302 the platform combining polox407/NaCMC and polox407/HPMC presents sufficient
 303 requirements to ensure adequate performance in PDT.

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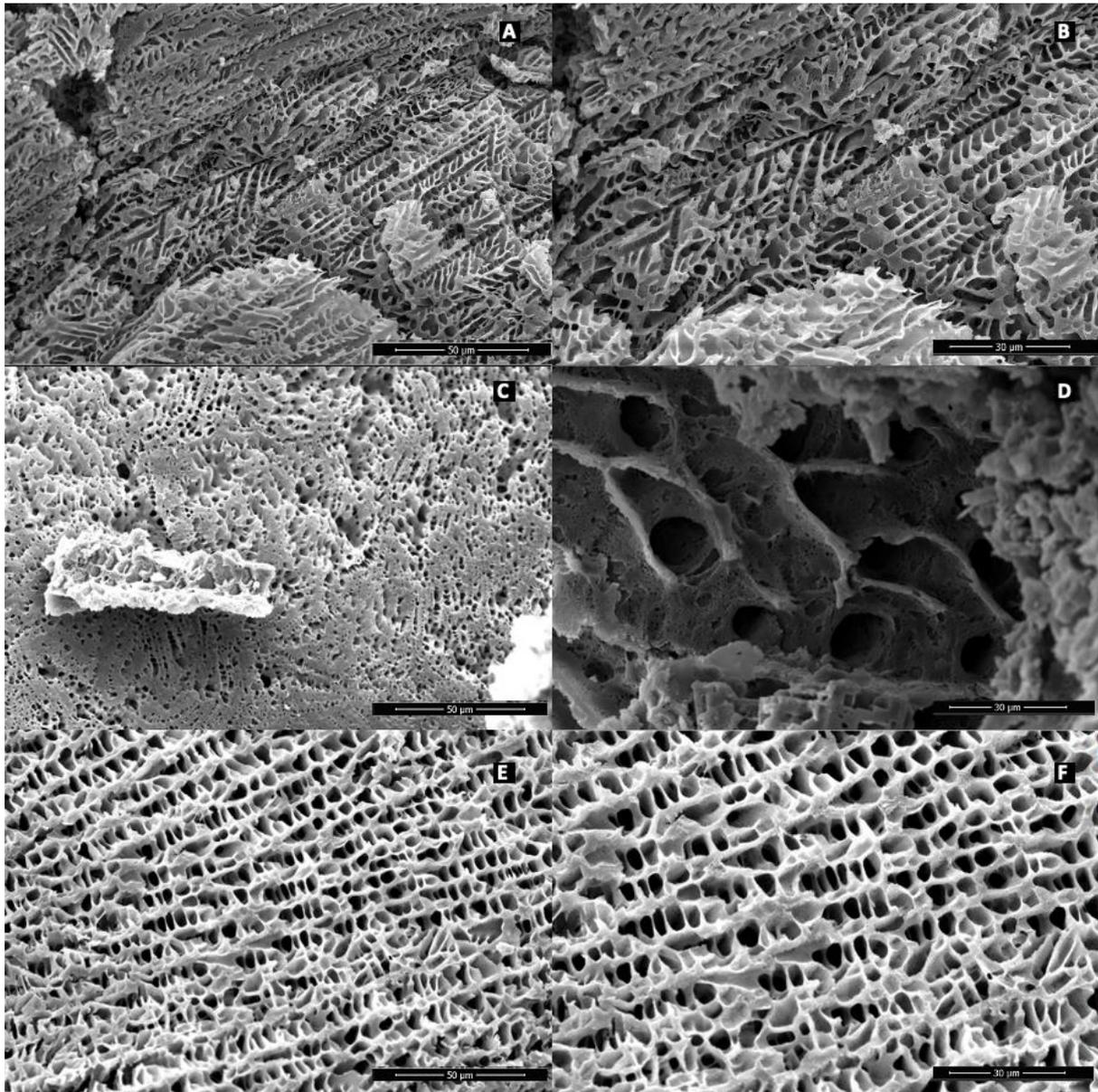
305 *3.2. Composition and morphological characterisation*

306 Polymeric systems' morphological analysis can provide a comprehension of their
307 structure and organisation, aiding mechanistic understanding of the rheological
308 characterisation [23]. Fig. 3 and 4 display SEM images of systems containing polox407 and
309 HPMC or NaCMC in presence or absence of ERI or ERIs (at magnifications 2000 and 3000x).

310 In general, all formulations demonstrated clear network structure with differing pore
311 size and quantity. Although heterogeneous, their morphology was well-defined, with
312 conformation mainly attributed to the interactions between polar groups of micellar copolymer
313 and cellulose derivatives [47]. Formulations containing polox407 and HPMC showed a
314 lamellar layout in this dry state, which was retained into the presence of both ERI and ERIs
315 (Fig. 3). ERI systems demonstrated amorphous morphology in comparison to ERIs, which may
316 be given by its higher hydrophobicity, solubility balance, and self-aggregation ability [3],
317 quoted by its amphiphilic log P 0.46 [7]. Among HPMC formulations, HPMC-ERIs was able
318 to form numerous porous in comparison to the others. Since ERIs presents large solubility in
319 water due to the predominance of the carboxylate form, it may establish strong ion-dipole
320 interactions with water.

321

322



323

324 **Fig. 3.** Scanning electron microscopy (SEM) images of binary polymeric formulations
 325 containing 17.5% (w/w) poloxamer 407 and 3% (w/w) hydroxypropyl methylcellulose, at
 326 2000x magnitude (A) and 3000x magnitude (B), with 1% (w/w) erythrosine 95% purity (C and
 327 D) or 1% (w/w) disodium salt erythrosine (E and F).

328

329 Formulations containing polox407 and NaCMC demonstrated a more porous
 330 morphology. With a reduction of size accompanied by an increase in pore number, induced by
 331 the addition of ERI or ERIs (Fig. 4). The addition of ERI or ERIs changed the organisation of

332 the system, which may be confirmed by computational modelling, where the complexation
333 energy become extensively high ($\Delta E_{\text{Comp}}/\text{kcal.mol}^{-1}$ of ERIs/NaCMC/1polox407 =
334 716929.8552) when compared with the complexation energy of the same conditions of the
335 system without ERI/ERIs ($\Delta E_{\text{Comp}}/\text{kcal.mol}^{-1}$ of NaCMC/1polox407 = -32.71768652 [24]). The
336 presence of drug may affect the micelle structure or the crystallisation of water during freezing.
337 It may also induce the reduction of crystallinity of the polymers, providing morphological
338 differences [13].

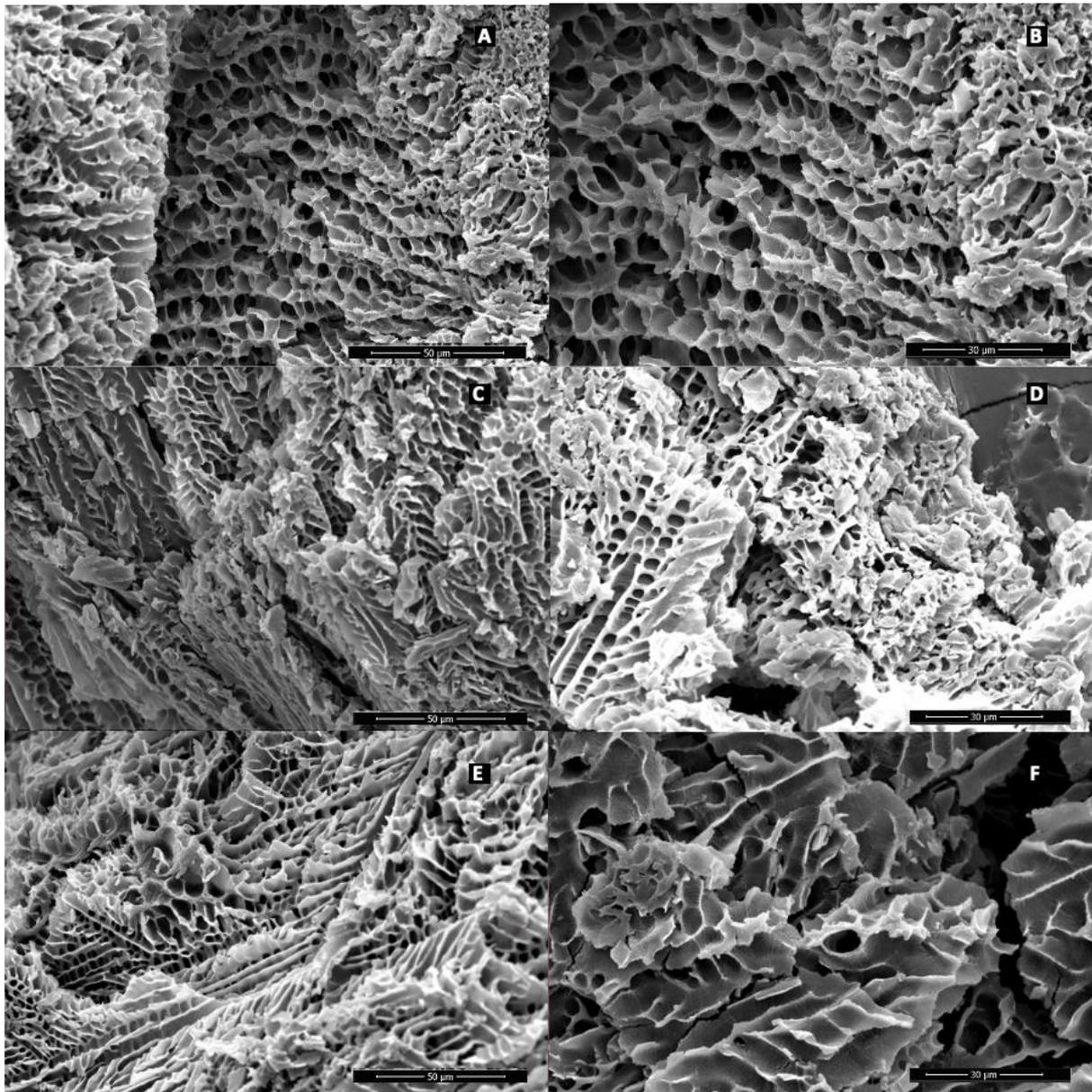
339 Dried formulations were also characterised by ATR-FTIR and DSC, as shown in Fig.
340 5 and 6. The polox407 FTIR spectrum presented bands at 3420, 2870, and 1096 cm^{-1} attributed
341 to the -OH stretching, C-H aliphatic stretching, and C-O stretching [13,48], respectively. The
342 band at 1645 cm^{-1} is attributed to the -OH bending. NaCMC FTIR spectrum showed bands at
343 1589 and 1414 cm^{-1} attributed to -COO^- symmetric and asymmetric stretching, respectively
344 [49]. Bands at 1322, 1103, and 1096 cm^{-1} are ascribed to C-H stretching symmetric [50],
345 primary, and secondary alcohols, respectively. In polox407/NaCMC FTIR spectrum (Fig. 5A),
346 a band shifted at 1597 cm^{-1} is observed in relation to that observed in the polox407 spectrum
347 (1645 cm^{-1}), suggesting H-bonds between both polymers. Moreover, in the polox407/NaCMC
348 FTIR spectrum, polox407 profile prevails due to the majority presence of this polymer into the
349 system.

350 Fig. 5B displays HPMC FTIR spectrum, where bands around 2870 cm^{-1} is linked to the
351 C-H stretching vibration [48]. Bands at 1375, 1108, and 1043 cm^{-1} are attributed to the C-H
352 aliphatic stretching, primary and secondary alcohol, respectively. The slight broadening
353 observed at 2870 cm^{-1} , when polox407 is in presence of HPMC into polox407/HPMC gel may
354 indicate C-H/C-H hydrophobic interactions between the polymeric chains. Furthermore, also

355 in polox407/HPMC spectrum, polox407 exhibited predominant bands due to its increased
356 concentration into the preparation.

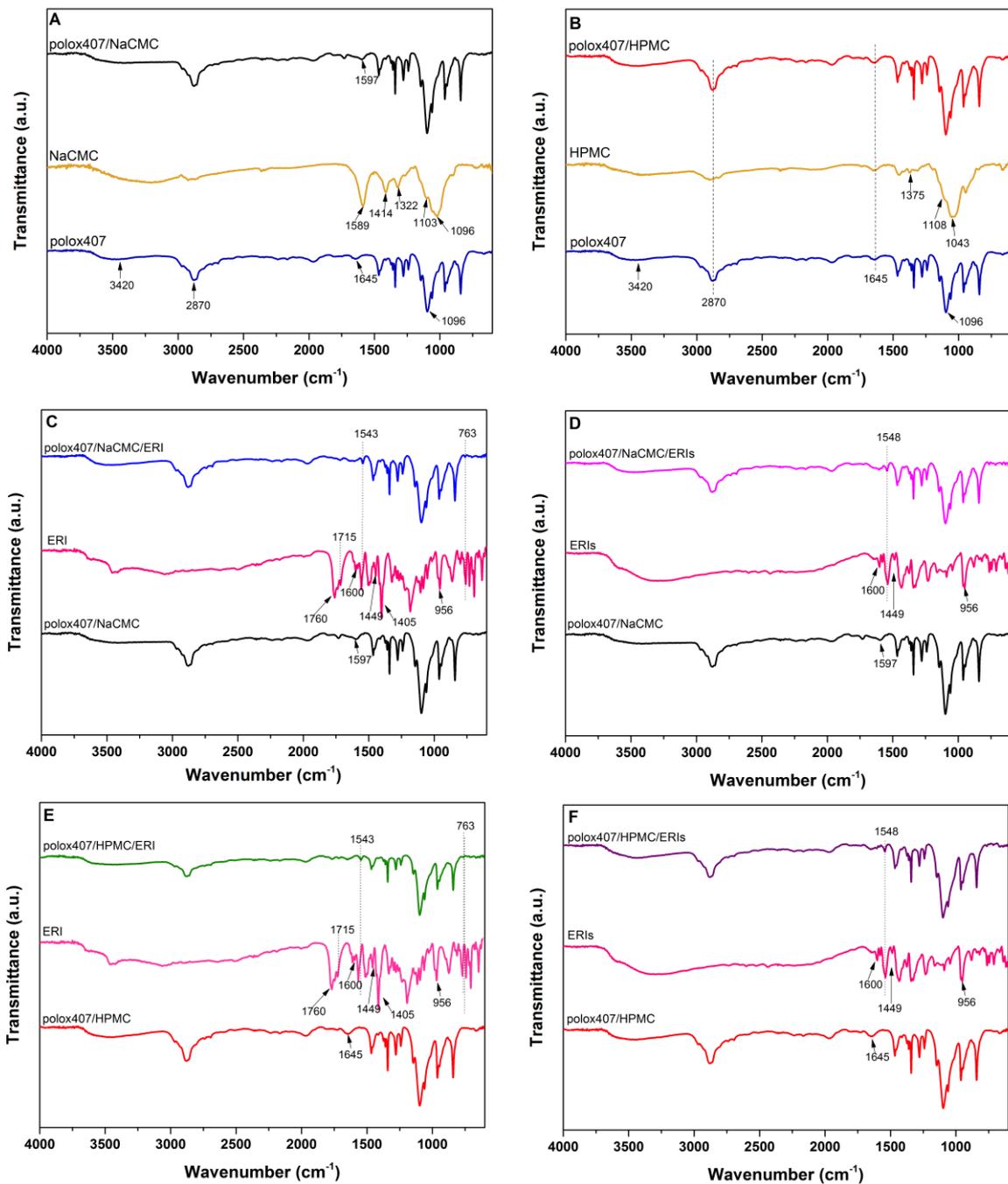
357 Fig. 5C-F presents the ERI and ERIs spectra and formulation containing both PS. The
358 ERI FTIR spectrum present bands at 1600, 1543, and 1449 cm^{-1} attributed to the benzene rings
359 stretch [51]. Meanwhile, bands at 956 and 763 cm^{-1} , were assigned to the C=C-H and aromatic
360 ring's angular deformation, respectively. Bands at 1760, 1715, and 1405 cm^{-1} , in ERI spectrum,
361 were attributed to the carboxylic acid C=O stretching, C=O stretching of conjugate acid, and
362 -OH bending of carboxylic acid, respectively. Although ERIs FTIR spectrum (Fig. 5C and D)
363 exhibited similar bands to that observed in the ERI FTIR spectrum, ERIs did not display the
364 bands at 1760, 1715, and 1405 cm^{-1} due to the disodium salt form of this PS. Indeed, as
365 observed in the FTIR spectra, ERI or ERIs were incorporated in the formulations.

366 .



367

368 **Fig. 4.** Scanning electron microscopy (SEM) images of binary polymeric formulations
369 containing 17.5% (w/w) poloxamer 407 and 1% (w/w) sodium carboxymethylcellulose at
370 2000x magnitude (A) and 3000x magnitude (B), with 1% (w/w) erythrosine 95% purity (C and
371 D) or 1% (w/w) disodium salt erythrosine (E and F).



372

373 **Fig. 5.** Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)
 374 spectra of the binary polymeric formulations of 17.5% (w/w) poloxamer 407 (polox407) and
 375 3% (w/w) hydroxypropyl methylcellulose (HPMC, B, E and F) or 1% (w/w) sodium
 376 carboxymethylcellulose (NaCMC, A, C and D) without or with erythrosine 95% purity (ERI,
 377 E and F) or disodium salt erythrosine (ERIs, C and D).

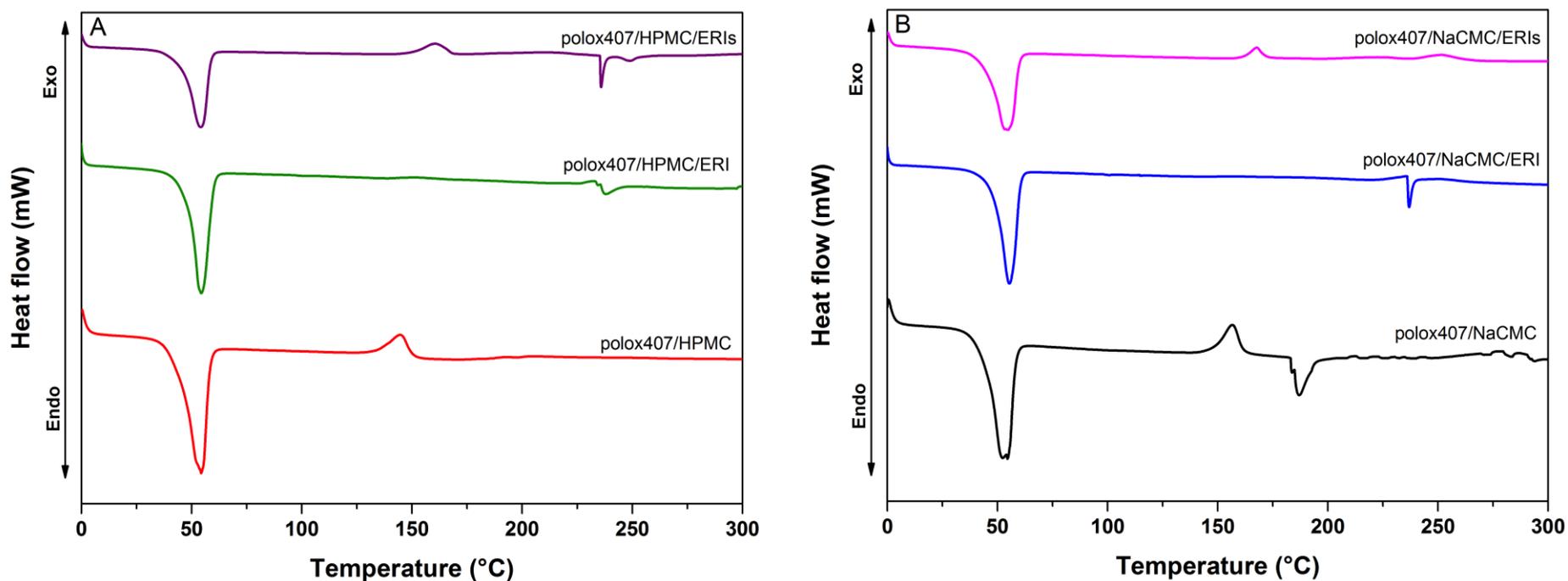
378 DSC thermograms of the dried formulations with or without drug are displayed in Fig.
379 6. The formulations show an endothermic peak at 55.2 °C, which is associated with the
380 polox407 copolymer melting point [24,52,53], revealing the presence of polox407 in its
381 crystalline state when in the presence of both cellulose derivatives. The enthalpy associated
382 with endotherm melting transition decreased in the presence of the drugs (Table 3), whereas
383 the peak position remained approximately unaltered. The addition of cellulose derivatives
384 plasticize polox407, since a decrease in its crystallinity occurs [24]. This could be related to
385 interaction between both polymers [54], which was already detected by rheology [23].
386 Comparing polox407 crystallinity into the mixture with and without drug (Table 3), there is a
387 reduction of this property when ERIs is added on it, while ERI promoted an increase of it. The
388 presence of ERIs seems to disturb the formation of the ordered crystalline structure of polox407
389 chains in both systems. Meanwhile, ERI facilitated an ordered association of the copolymer
390 molecules. Additionally, the exothermic peak observed near to 150 °C for the preparation
391 without drug and containing ERIs, was not exhibited for samples with ERI, indicating the
392 favourable organisation of polox407 crystallinity may result in a unique crystalline form.

393 **Table 3**

394 Differential scanning calorimetry (DSC) crystallinity of pure poloxamer 407 (polox407) polymer and mixtures composed of 17.5% (w/w) polox407
 395 and 1% (w/w) sodium carboxymethylcellulose (NaCMC) or 3% (w/w) hydroxypropyl methylcellulose (HPMC), without or with 1% (w/w) ERI
 396 or ERIs.

Formulation	Cellulose derivative fraction	Drug fraction	polox407 weight fraction	Enthalpy (J/g)	polox407 crystallinity (%)	Crystallinity reduction (%)
polox407	0.000	0.000	1.000	112.20	100.00	0.00
polox407/HPMC	0.146	0.000	0.854	84.65	88.38	11.62
polox407/HPMC/ERI	0.146	0.010	0.814	87.69	96.02	3.98
polox407/HPMC/ERIs	0.146	0.010	0.814	82.06	89.85	10.15
polox407/NaCMC	0.054	0.000	0.946	75.74	71.36	28.64
polox407/NaCMC/ERI	0.054	0.010	0.897	88.73	88.12	11.88
polox407/NaCMC/ERIs	0.054	0.010	0.897	84.13	83.55	16.44

397



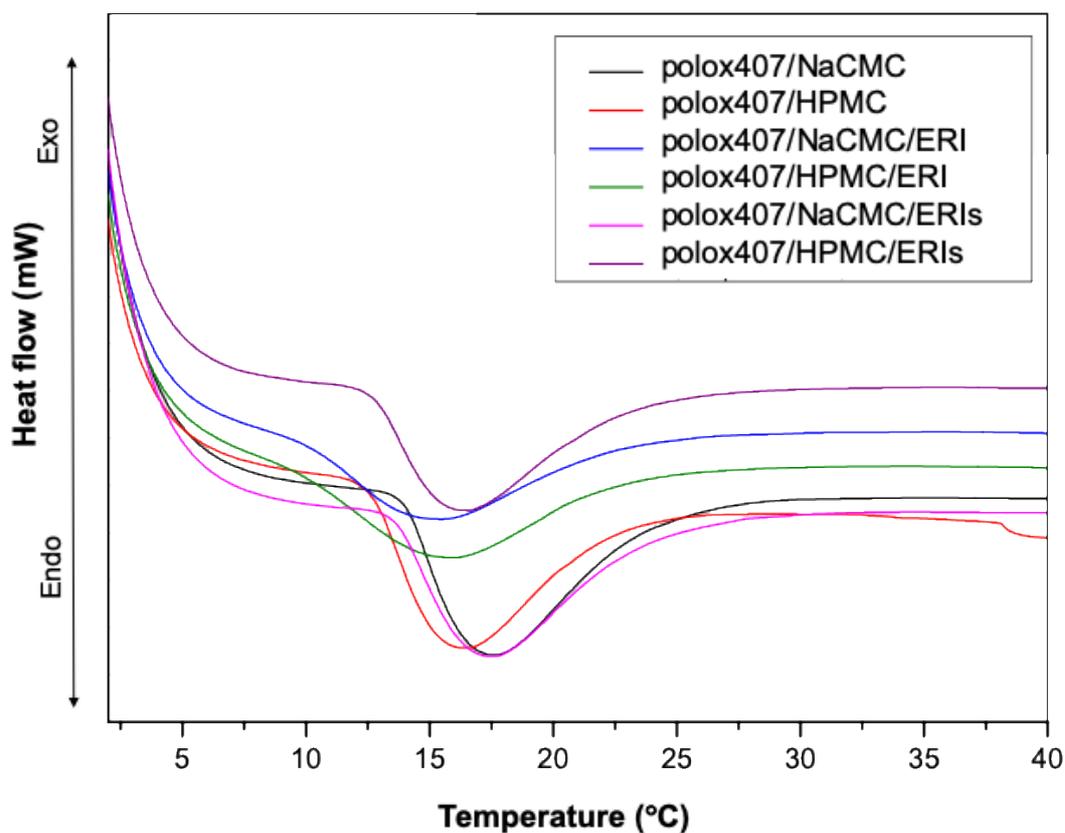
398

399 **Fig. 6.** Differential scanning calorimetry (DSC) thermograms of dried binary polymeric systems containing (A) poloxamer 407 (polox407) and
 400 hydroxypropyl methylcellulose (HPMC) or (B) sodium carboxymethylcellulose (NaCMC) without or with erythrosine 95% purity (ERI) or
 401 disodium salt erythrosine (ERIs).

402

403 *3.3. Micellar characterisation*

404 The DSC analysis of the gels allows for the characterisation of nanostructured systems,
405 providing detailed information regarding enthalpy associated with thermally-induced events
406 [55]. It may be used alongside the study of self-assembly behaviour of polox407, since micellar
407 domain formation is a crucial step for gelation of systems containing this thermoresponsive
408 polymer [56]. Therefore, the CMT of the systems with or without drug was determined by DSC
409 (Fig. 7). The endothermic peaks demonstrate the desolvation of hydrophobic PPO block of
410 polox407 with increasing temperature. As this phenomenon is responsible for micelle
411 formation, the peak of this calorimetric event may be considered as the CMT of the
412 preparations [57]. As reported in the literature, the CMT of HPMC/polox407 was found around
413 16.7 °C, whilst NaCMC/polox407 preparations exhibited a CMT of 17.8 °C [24]. HPMC
414 preparations with ERI presented a CMT of 16.0 °C, while ERIs preparations demonstrated
415 CMT at 16.4 °C. NaCMC systems with ERI or ERIs demonstrated CMTs of 15.4 °C and 17.5
416 °C, respectively.



417

418 **Fig. 7.** Differential scanning calorimetry (DSC) thermograms of binary polymeric hydrogels
 419 composed of poloxamer 407 (polox407) and hydroxypropyl methylcellulose (HPMC) or
 420 sodium carboxymethylcellulose (NaCMC) without or without of 1% (w/w) erythrosine 95%
 421 purity (ERI) or 1% (w/w) disodium salt erythrosine (ERIs).

422

423 The addition of drug decreased the CMT of both polymeric systems, which can be
 424 mechanistically interpreted through several viewpoints. In part, the presence of co-solute alters
 425 the amount of water available to solvate polox407 chains. ERI reduced this temperature to a
 426 greater extent than ERIs, with this effect more pronounced in NaCMC preparations. This
 427 agrees with computational analysis, which found that the NaCMC/polox407 system containing
 428 ERI has high solvation, since there is a large distance between the charged species (ΔE_{Comp}
 429 ERI/NaCMC > 0). Whilst a negative entropy contribution is driving force for the micellisation

430 of polox407 block copolymer [58], direct interactions between cellulose derivatives and
 431 copolymer may contribute with changes in either thermodynamic parameters, altering CMT
 432 [24]. The literature reports a CMT for pure 20% (w/w) polox407 dispersions at about 12 °C
 433 [56,59], and an enthalpy of micellisation of 25.5 ± 2 J/g for polox407 [60,61]. Compared to a
 434 polox407 solution, both cellulose derivatives reduced the CMT and enthalpy of micellisation
 435 of polox407 (Table 4), reflecting a decline in the energy consumed for PPO dehydration [62].
 436 Although mixtures between ERI and polox407 have been reported with relative low bonding
 437 ability, with the dye being located in PEO region [3], the results suggest that hydrophobic
 438 interactions between PPO blocks and aliphatic backbone of drug and cellulose derivatives may
 439 promote PPO nanophase separation, requiring less energy for dehydration [62]. ERI
 440 demonstrated significant reduction of enthalpy which is likely related to its relatively high
 441 hydrophobicity and therefore reduced interaction with water or, perhaps, a tendency of
 442 interaction with PPO, in comparison to ERIs [24,63,64]. For instance, the literature reports, by
 443 differences between erythrosine pKa in water and in polox407 solution, that its carboxylic
 444 group establishes interactions with PEO groups while phenyl ring is accommodated in PPO
 445 inner region [65].

446

447 **Table 4**

448 Micellisation enthalpy of hydrogels containing 17.5% (w/w) polox407 and 1% (w/w) sodium
 449 carboxymethylcellulose (NaCMC) or 3% (w/w) hydroxypropyl methylcellulose (HPMC),
 450 without or with 1% (w/w) ERI or ERIs obtained by DSC.

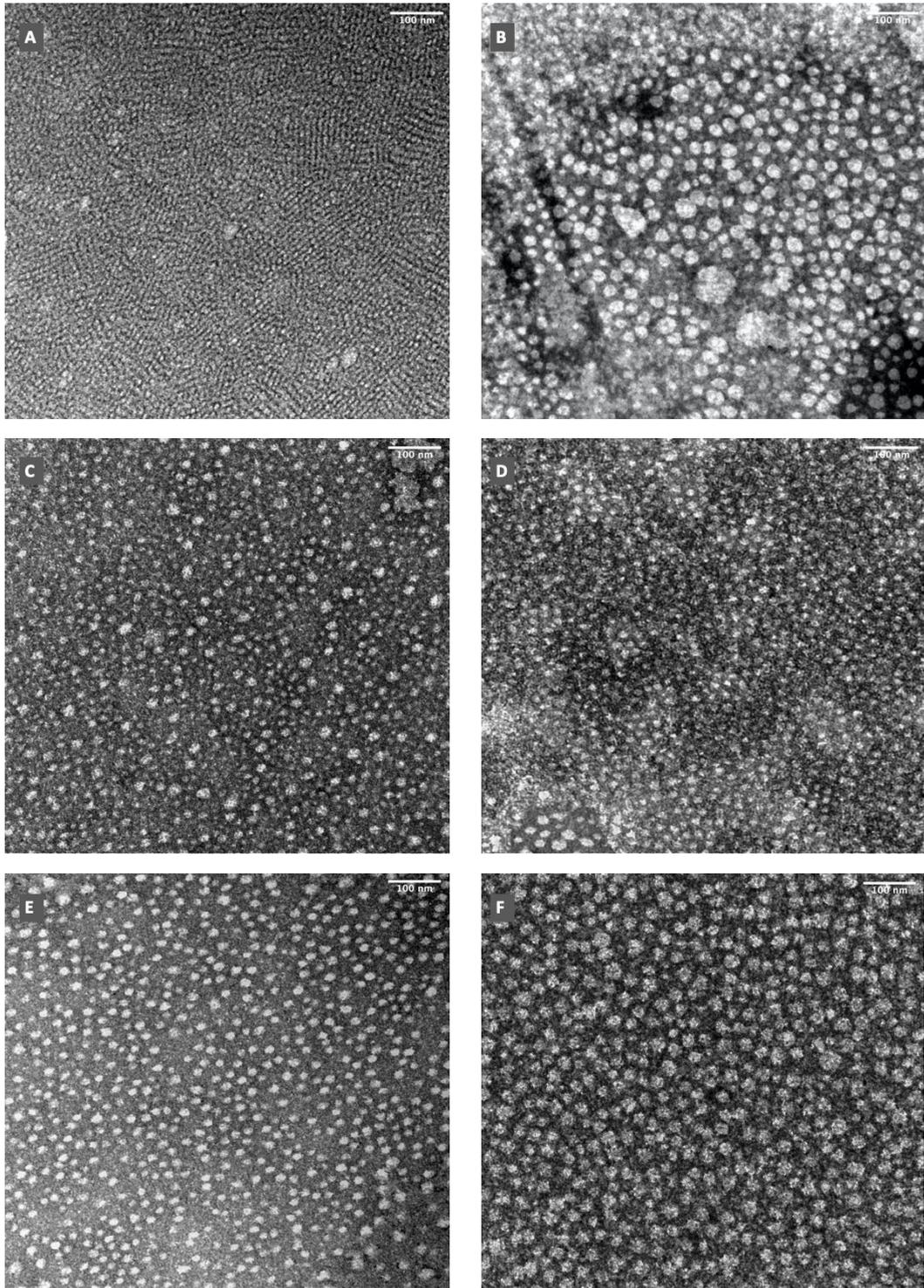
Formulations	Concentration (%, w/w)	T _{onset} (°C)	T _{peak} (°C)	Micellisation enthalpy (J/g of polox407)
polox407/HPMC ^a	17.5/3	12.5	16.7	17.91

polox407/HPMC/ERI	17.5/3/1	10.7	16.1	10.53
polox407/HPMC/ERIs	17.5/3/1	12.8	16.2	13.65
polox407/NaCMC ^a	17.5/1	14.0	17.7	15.53
polox407/NaCMC/ERI	17.5/1/1	10.8	15.6	10.29
polox407/NaCMC/ERIs	17.5/1/1	13.7	17.5	17.69

451 ^a[24]

452

453



454

455 **Fig. 8.** Transmission electron microscopy (TEM) images of formulations comprising
456 poloxamer 407 and hydroxypropyl methylcellulose (A) with erythrosine 95% purity (C) or
457 disodium salt erythrosine (E) and poloxamer 407 and sodium carboxymethylcellulose (B) with
458 erythrosine 95% purity (D) or disodium salt erythrosine (F) at 37 °C (A). Original
459 magnification x100,000.

460 TEM micrographs allow for the determination of micelle size and shape by direct
461 observation. Images of all six systems, dried at 37 °C, are displayed in Fig. 8. The individual
462 organisation with spherical shape was observed, in line with the literature [24,66], reflecting
463 the micellar organisation of polox407 and its triblock structure [66]. HPMC system without
464 drug had a micelle diameter of 13.4 ± 2.3 nm. The measurements show the absence of
465 significant changes in the size among HPMC formulations, agreeing with theoretical
466 modelling, which demonstrated a minor impact on polox407 structuration. Meanwhile,
467 NaCMC system without drug had an average diameter of 25.5 ± 4.5 nm. With ERIs, expressive
468 changes was not observed, with average diameter of 21.5 ± 9.8 nm. However, the ERI
469 incorporation showed a trend to reduced values, with micelles diameter close to 14.4 ± 7.3 nm.
470 Overall, the images revealed a high heterogeneity of size in NaCMC preparations, which
471 negatively impacts micellar packing, as observed by DSC and *in silico* modelling of the
472 hydrogels [56].

473 The location of xanthene dyes by fluorescence quenching experiments have been
474 reported with some preference of ERIs to be positioned in PEO segments of polox407, when
475 in its raw dispersion [3], agreeing with the *in silico* model. This justifies the small contraction
476 tendency observed for the ERIs system, since micellar clusters and heterogeneity of size are
477 frequently linked to changes in the shell [56]. However, the trend to reduction exhibited for
478 ERI in NaCMC preparations, may be indicative of stronger interaction with PPO fragment
479 (drug molecules in the aggregate state due to the solubility equilibrium) driving collapse of
480 the micelle core (in accordance with CMT measurements). Although ERI has a relatively
481 amphiphilic profile (log P 0.46) [7], its backbone and self-aggregation in water [27] may favour
482 its interaction with PPO core, reducing micelle size of NaCMC system in comparison to the
483 raw blend. For instance, the literature reports the presence of hydrophobic drug molecules,

484 such as naproxen and indomethacin in polox407 solution decreases its micellar size and
485 aggregation number [67,68].

486 Although most of literature report a pKa around 2.35 for the carboxylic group of ERI
487 and 3.79 for the phenolic group, some authors have found different values [27]. Erythrosine
488 pKa may change depending on the chemical environment the dye is placed. For instance,
489 Freitas et al. demonstrated the pKa of erythrosine increases in polox407 solutions with pKa
490 inversion observed ($pK_{aOH} < pK_{aCOOH}$), giving values of 6.54 for the carboxylic group and
491 2.17 for phenolic groups [65]. That is linked to the presence of oxyethylene groups in the
492 external portion of the micelle, which are able to attract positive charges to its surface,
493 increasing pKa values, since negative electrostatic micelles would repel the dianionic form of
494 the dye [69,70]. Therefore, the differences found through these outcomes, comparing systems
495 containing ERI or ERIs, can have a bearing at some level, with possible modification in the
496 composition of predominant protolytic forms of ERI/ERIs as a function of the solubility
497 balance and changes in pka values (due to the interaction of the drug with polox407/NaCMC
498 and polox407/HPMC system). When incorporated to the polymer mixture studied, oxyethylene
499 groups and cellulose derivatives can change the deprotonation equilibrium of phenolic and
500 carboxylic groups of the PS, avoiding repulsion effects. The presence of ERI and ERIs in these
501 mucoadhesive and thermoresponsive systems may foster a predominance of the neutral form
502 of this dye by increasing their pKa, mainly in NaCMC gel, which presents increased negative
503 electrostatic density. Additionally, ERI that presents both an ionization equilibrium and a
504 solubility equilibrium, may major exist in its monoanionic form, while ERIs is in the dianionic
505 one. Hence, the consideration of deprotonation equilibria may not follow the typical behaviour
506 assumed by the Henderson-Hasselbalch equation which assumes that the chemical species is
507 dilute in an aqueous environment [71].

508

509 **4. Conclusion**

510 Polymeric blends composed of polox407 and HPMC or NaCMC were developed and
511 their molecular structuration characterised for systems containing ERI or ERIs. Interaction
512 studies demonstrated when the systems undergo micellisation drug molecules are converted
513 into their monomeric form. By *in silico* study, ERIs has shown higher interaction with PEO
514 than PPO. Moreover, systems composed of NaCMC and ERIs presented a repulsion effect due
515 to its increased charge density, with polox407 structuration less impacted by the presence of
516 HPMC. Morphological analysis evidenced the micelles had well-defined spherical structures,
517 consistent with the native polox407. Calorimetry showed a reduction of polox407 crystallinity,
518 CMT and enthalpy of micellisation when mixed with the cellulose derivatives, ERI or ERIs.
519 Micellar size was evaluated by TEM, with NaCMC system reducing its micellar size, mainly
520 in presence of ERI, while for HPMC significant changes were not observed. This retention of
521 structure is crucial where encapsulation in micellar nanoparticles is commensurate to drug
522 solubilisation, targeting, and cellular internalisation. Aiming toward photodynamic
523 applications, these findings represent a rationale for understanding how the two states of the
524 PS affect the polymeric blends and their micelle formation.

525

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535

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