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1 **Enhanced effects of curcumin encapsulated in polycaprolactone-grafted**
2 **oligocarrageenan nanomicelles, a novel nanoparticle drug delivery system**

3

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20

21 **Abstract**

22 One of the most effective strategies to enhance the bioavailability and the therapeutic
23 effect of hydrophobic drugs is the use of nanocarriers. We have used κ-carrageenan extracted
24 from *Kappaphycus alvarezii* to produce oligocarrageenan via an enzymatic degradation
25 process. Polycaprolactone (PCL) chains were grafted onto the oligocarrageenans using a
26 protection/deprotection technique yielding polycaprolactone-grafted oligocarrageenan. The
27 resulting amphiphilic copolymers formed spherical nanomicelles with a mean size of 187 ± 21
28 nm. Hydrophobic drugs such as curcumin were efficiently encapsulated in the micelles and
29 released within 24 to 72 h in solution. The micelles were non-cytotoxic and facilitated the
30 uptake of curcumin by endothelial EA-hy926 cells. They also increased the anti-inflammatory
31 effect of curcumin in TNF-alpha-induced inflammation experiments. Finally, *in vivo*
32 experiments supported a lack of toxicity in zebrafish and thus the potential use of

33 polycaprolactone-grafted oligocarrageenan to improve the delivery of hydrophobic
34 compounds to different organs, including liver, lung and brain as shown in mice.

35 **Keywords:** graft-copolymer; curcumin; nanomicelles; drug delivery; inflammation;
36 endothelial cells

37

38 1. Introduction

39 It is known that the therapeutic effect of most hydrophobic drugs is lowered due to poor
40 bioavailability (Savjani, Gajjar, & Savjani, 2012; Xu, Ling, & Zhang, 2013). Nanocarriers
41 improve drug efficiency by enhancing their solubility and biodistribution (Shakeel, Ramadan,
42 & Shafiq, 2009). Natural polymer-based nanoparticles such as polysaccharides are favoured
43 due to their nontoxic properties, biodegradability, biocompatible nature and high hydroxyl
44 content allowing further functionalization. Polysaccharides such as oligoagarose, chitosan and
45 alginates from seaweeds have been used to engineer drug delivery devices which have shown
46 sustained release (Bhaw-Luximon, Meeram, Jugdawa, Helbert, & Jhurry, 2011; Cook,
47 Tzortzis, Charalampopoulos, & Khutoryanskiy, 2011; Nesamony, Singh, Nada, Shah, &
48 Kolling, 2012; Cavalli, Leone, Minelli, Fantozzi, & Dianzani, 2014).

49

50 Although, marine polysaccharides are mostly used in **the food and cosmetics industries**,
51 they are largely present in pharmaceutical applications, with an increasing interest in their use
52 as materials for the incorporation of bioactive agents (Cardoso,Pereira, Seca, Pinto, &
53 Silva,2015). Furthermore, **seaweed** polysaccharides have been shown to have enormous
54 potential in the biomedical field (Venkatesan et al., 2015). Amongst them, sulphated
55 polysaccharides represent a group of major interest for their bioactivities resulting from their
56 numerous hydroxyl groups which can act as recognition sites for cells and also from the
57 backbone of their repeat unit which resembles *in vivo* polysaccharides such as hyaluronic
58 acid. Their bioactivities include antioxidant (Barahona, Chandía, Encinas, Matsuhira, &
59 Zúñiga, 2011), anticoagulant (Ciancia, Quintana, & Cerezo, 2010), anticancer (Boopathy &
60 Kandasamy, 2010), antiviral (Bouhlal et al., 2011), anti-allergic (Sang, Ngo, & Kim, 2012)
61 and anti-inflammatory properties (Cumashi et al., 2007).

62

63 Carrageenan, a sulphated marine polysaccharide from red seaweeds, has been explored **for**
64 **its use as a source for** sustained drug delivery nanoparticles. Depending on their degree of
65 sulfation, the position of the sulfate group and the presence of 3-6-anhydro bridges,

66 carrageenans are classified into different families, the three main ones being kappa (κ), iota (ι)
67 and lambda (λ) carrageenans (Figure S1a). The use of carrageenans as drug delivery carriers
68 in the form of carrageenan-based pellets, beads, nanoparticles, microparticles, hydrogels,
69 films and matrices has been investigated (Cunha & Grenha, 2016). A major advantage of
70 polysaccharides including carrageenan is the presence of numerous hydroxyl groups that can
71 be chemically modified to modulate their properties or to target specific cells for drug
72 delivery applications. However, the insolubility of high molar mass carrageenans in common
73 solvents and water renders **their** functionalization difficult. Some of us previously reported on
74 the enzymatic degradation of another marine polysaccharide, namely agarose, into
75 oligoagarose which was then transformed into oligoagarose-g-polycaprolactone amphiphilic
76 micelles and showed drug delivery abilities in preliminary studies with a model molecule,
77 ketoprofen (Bhaw-Luximon et al., 2011). Controlled grafting of polycaprolactone chains was
78 achieved through partial protection-deprotection of the hydroxyl groups on oligoagarose and
79 ring-opening polymerization of ϵ -caprolactone. Chitosan-graft-poly(ϵ -caprolactone)
80 amphiphilic copolymer micelles have also been reported for 5-fluorouracil (5-FU) drug
81 delivery with a release half-time up to 54.46 h and 5-FU comparable **cytotoxic** effect **in** A549
82 cells. (Gu, Le, Lang, & Liu, 2014). Recently, cyclodextrin-g-polyurethane has been prepared
83 using NCO-terminated polyurethane of MW 1400, grafted on cyclodextrin via its hydroxyl
84 groups. The graft copolymer showed sustained release of dexamethasone (40-56%) over 42 h
85 compared to 100% release **within** 2 hours with cyclodextrin alone. This system also showed
86 sustained cytotoxic **effects** on HeLa cells, **inducing** from 25 to 75% **mortality** over 72 h. Thus,
87 grafted polysaccharides can be explored as amphiphilic micelles with a hydrophobic core for
88 drug encapsulation.

89
90 Curcumin (diferuloylmethane) is the main curcuminoid present in turmeric (Figure S1b).
91 This molecule possesses significant anti-inflammatory, antioxidant, anti-carcinogenic, anti-
92 mutagenic, anticoagulant, and anti-infective effects (Mahmood, Zia, Zuber, Salman, &
93 Anjum, 2015). Curcumin also exerts a protective effect against cardiovascular diseases,
94 including atherosclerosis, mainly *via* diverse mechanisms including inhibition of oxidative
95 stress, inflammation, and cell death (He et al., 2015). However, due to its hydrophobic nature,
96 its rapid metabolism and its physicochemical and biological instability, curcumin has a poor
97 bioavailability. To circumvent these difficulties, several approaches have been proposed such
98 as encapsulation in liposomes and polymeric micelles, inclusion complex formation with
99 cyclodextrin or formation of polymer–curcumin conjugates (Mahmood et al., 2015).

100

101 We previously **published** a new process to extract alginates from brown seaweeds
102 (*Sargassum binderi* and *Turbinaria ornata*) and carrageenans from red seaweeds
103 (*Kappaphycus alvarezii* and *Euchema denticulatum*) using ultrasound (Youssouf et al., 2017).
104 In this paper, we report production of the enzymatically-modified κ -carrageenan to produce
105 oligocarrageenan that was then grafted with polycaprolactone chains in order to form
106 spherical nanomicelles allowing vectorization of hydrophobic molecules such as curcumin.
107 The micelles were characterized using Dynamic Light Scattering (DLS) and Scanning and
108 Transmission Electron Microscopy (SEM and TEM). We discuss here the effect of the
109 curcumin-loaded nanomicelles on vascular endothelial cells, EA-hy926, as well as their *in*
110 *vivo* toxicity tested in zebrafish and the biodistribution of the nanomicelles in mice.

111

112 **2. Materials and Methods**

113

114 2.1. Materials

115 Carrageenans were extracted from cultivated red algae *Kappaphycus Alvarezii* received
116 from Ibis Algoculture (Madagascar) as described previously (Youssouf et al., 2017).
117 *Pseudoaltermonas carrageenovora* bacteria were obtained from DSMZ, Germany. For the
118 PCL grafting on carrageenan, all chemical products were purchased from Sigma-Aldrich. D₂O
119 or CDCl₃ used for NMR analysis were from Eurisotop (France).
120 Curcumin was synthesized according to the method previously reported by Pedersen et al.
121 (Pedersen, Rasmussen, & Lawesson, 1985). The chromatogram and the NMR spectrum of the
122 product are presented in Figures S2 and S3.

123 The human endothelial cell line, EA-hy926, was obtained from ATCC and cultured in a
124 Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated Fetal
125 Bovine Serum (FBS), 100 units penicillin/mL, 100 μ g streptomycin/mL and HAT
126 (hypoxanthine 100 μ M; aminopterin 0.4 μ M and thymidine 16 μ M) at 37 °C in a 5% CO₂
127 humidified atmosphere.

128 Three to six months-old adult male and female wild type zebrafish (*Danio rerio*) were
129 purchased from commercial suppliers and maintained under standard conditions of
130 photoperiod (14/10 h light/dark), temperature (28 °C) and oxygenation. They were fed daily
131 with commercially available dry food (TetraMin).

132 C57Bl6 mice used to study the biodistribution of injected molecules, were purchased from
133 Charles River Laboratories (Saint-Germain-sur-L'Arbresles, France). C57Bl6 mice were
134 maintained at a constant temperature (23±1 °C) and a hygrometry between 55 to 65% under a
135 12-hour light/dark cycle, were permitted free access to food and water and were handled and
136 cared in accordance with guidelines for care and use of laboratory animals of the European
137 Council Directive 2010/63/EU approved by the Ethics Committee of La Reunion, n°114, for
138 Animal Experimentation under the reference APAFIS#9119-2016090515329223v2.

139 2.2. Extraction of κ -carrageenan from *Kappaphycus Alvarezii*

140 To extract carrageenan, algae were first pre-treated with a hydro-alcoholic solution (80%
141 ethanol), and carrageenans were then extracted in hot water (90 °C, pH 7) with ultrasonication
142 (150 W, 15 min). Afterwards, a hot filtration was performed to remove algae residues.
143 Carrageenans were then jellified by lowering the temperature to 4 °C, isolated by filtration,
144 frozen and lyophilized. The resulting carrageenans were composed of κ -carrageenan (68%)
145 and λ -carrageenan (32%) as evidenced by NMR (Youssef et al., 2017).

146 2.3. Production and isolation of κ -carrageenase from *P. carrageenovora*

147 κ -carrageenase was expressed and isolated from *Pseudoaltermonas carrageenovora* after
148 stimulation with a κ -carrageenan solution. The protocol was adapted from that used for the
149 production of λ -carrageenan from the same bacterial strain (Guibet et al., 2007). Bacteria
150 were grown in 1 liter of Marine Broth culture medium in Erlen flasks incubated at 21 °C
151 under shaking. After 3 to 4 h of culture, when the OD_{600nm} reached 0.6, the expression of κ -
152 carrageenase was stimulated with a stock solution of κ -carrageenan dissolved in Tris-HCl 100
153 mM pH=8.5 at a final κ -carrageenan concentration of 0.15% (Mass/Volume of culture). After
154 24 h of culture, bacteria were eliminated by centrifugation (3000 g, 30 min, 4 °C). The
155 supernatant was filtered first through a 0.45 μ m filter and then through a 300 kDa membrane.
156 The enzyme was then concentrated using a 10 kDa centrifugal filter and desalting was
157 performed with a 3 kDa centrifugal filter. The protein content was determined using the
158 Bradford assay.

159 Enzymatic digestion was carried out to obtain oligocarrageenans using κ -carrageenase
160 isolated from *Pseudoaltermonas carrageenovora*. 150 mg of carrageenans were dissolved in a
161 tris-HCl solution (100 mM, pH 8.5) and incubated with κ -carrageenase (75.3 μ g in 1 mL).
162 Different durations of digestion (2 h, 6 h and 24 h) were tested. An ultrafiltration using a 10
163 kDa centricon device was then performed to eliminate the enzyme, non-hydrolysed κ -
164 carrageenan and λ -carrageenan. Oligocarrageenans were then isolated by precipitation with

165 methanol and the product was freeze-dried and analysed by NMR and Size Exclusion
166 Chromatography (SEC).

167 The enzymatic activity was evaluated by measuring the amount of reducing sugar
168 according to the assay described by Kidby and Davidson (Kidby & Davidson, 1973). After
169 hydrolysis, 100 μL of substrate were mixed with 900 μL of ferricyanide solution 1X (300 mg
170 potassium hexacyanoferrate III, 28 g of Na_2CO_3 , 1 mL NaOH 5 M *QS* 1 L). The mixture was
171 boiled for 10 min and the absorbance at 420 nm was measured at room temperature. The
172 control was obtained in the same condition by using a boiled-inactivated enzyme. The method
173 was calibrated using a glucose solution ranging from 0 to 300 $\mu\text{g} \cdot \text{mL}^{-1}$.

174 2.4. Synthesis of polycaprolactone-grafted oligocarrageenan

175 The synthesis of polycaprolactone-grafted oligocarrageenan involved three steps: (i) partial
176 acetylation of the hydroxyl groups on oligocarrageenan; (ii) polymerisation of caprolactone
177 on the partially acetylated oligocarrageenan; (iii) deprotection of the hydroxyl groups. The
178 protocol was adapted from a previous study in which polycaprolactone was grafted onto
179 oligoagarose (Bhaw-Luximon et al., 2009).

180 (i) *Partial acetylation of oligocarrageenan*

181 4 mL of pyridine and 644 μL of acetic anhydride were added to 500 mg of
182 oligocarrageenan. The mixture was placed at room temperature under stirring for 3 h. The
183 reaction was stopped by adding ice to the mixture to hydrolyse unreacted acetic anhydride and
184 partially acetylated-oligocarrageenans were isolated by precipitation using cold methanol. The
185 precipitate was then frozen, lyophilized and characterized by NMR.

186 (ii) *Polymerization of caprolactone*

187 To graft hydrophobic chains onto the oligomers, 200 mg of acetylated-oligocarrageenan
188 were dissolved in 2 mL of toluene and 20 μL of the catalyst tin (II) ethylhexanoate was added.
189 After 2 h of stirring at 40 $^\circ\text{C}$ under a nitrogen atmosphere, 330 mg of ϵ -caprolactone were
190 added and polymerization was allowed to proceed for 20 h at 110 $^\circ\text{C}$. The stability of partially
191 acetylated oligocarrageenan and the absence of depolymerisation in these conditions was
192 confirmed by Thermal Gravimetric Analysis (TGA), Differential Scanning Calorimetry
193 (DSC) and NMR analyses (data not shown). The resulting acetylated-OligoKC-g-PCL was
194 dissolved in chloroform and precipitated in cold methanol. After freezing and lyophilization,
195 the product was characterized by NMR.

196 (iii) *Deprotection of hydroxyl groups from polycaprolactone-grafted acetylated-*
197 *oligocarrageenan*

198 To remove acetyl groups from polycaprolactone-grafted acetylated-oligocarrageenan, the
199 copolymer was dissolved in a solution of THF/methanol (v/v=1/1). A sodium methoxide
200 (NaOCH₃) solution was added drop by drop to reach pH 8 and the mixture was kept under
201 stirring at room temperature. After 4 h, the solution was neutralized with 0.5 M HCl and then
202 underwent vacuum evaporation. The product was resuspended in water under stirring for 1 h.
203 Precipitated PCL was eliminated through a 0.22 μm filter and unreacted caprolactone was
204 removed by dialysis through a 2 kDa membrane. In water, the amphiphilic copolymers,
205 named polycaprolactone-grafted oligocarrageenan, **become** soluble by forming micelles. The
206 product was freeze-dried and then analyzed by NMR.

207 2.5. NMR analysis

208 All NMR analyses were performed on a 600 MHz Avance III Bruker NMR spectrometer
209 equipped with a ¹H/¹⁹F, ¹³C and ¹⁵N cryoprobe. 1D ¹H, 1D ¹³C, 2D COSY and 2D ¹H-¹³C
210 HSQC spectra were recorded in 100% D₂O or CDCl₃ at room temperature and
211 tetramethylsilane was used as reference. ¹H NMR spectra were recorded with 128 scans using
212 a sweep width of 10 ppm. 2D spectra were obtained with 32 scans and a sweep width of 10
213 ppm for ¹H and 120 ppm for ¹³C. In all spectra, the carrier was placed at 4.7 ppm for ¹H and
214 50 ppm for ¹³C.

215 2.6. Size exclusion chromatography

216 Size Exclusion Chromatography (SEC) was performed on a 1260 Infinity GPC/SEC
217 System (Agilent Technologies) with a PSS Suprema column. The sample was passed through
218 a 0.22 μm filter and 400 μL of the solution (1 mg/mL) was injected onto the column. Elution
219 was carried out at a flow rate of 1 mL/min with a 0.5 g/l NaNO₃ solution. A calibration curve
220 was obtained using a mixture of dextrans of various molar weights (180 Da, 4400 Da, 21400
221 Da and 277 000 Da).

222 2.7. Drug loading - Acetone volatilization method

223 Encapsulation of curcumin, rifampicin and Nile Red was performed using the acetone
224 volatilization method. Typically, 500 mg of polycaprolactone-grafted oligocarrageenan and
225 50 mg of drug were dissolved in 10 mL of acetone. Deionized water was added slowly
226 dropwise (250 mL) and the mixture was kept under stirring at room temperature overnight.
227 Acetone was then evaporated at 30 °C and the precipitate corresponding to the non-
228 encapsulated drug was eliminated by passage through a 0.22 μm filter. Dialysis was

229 performed using a membrane with a MWCO of 2000 Da to remove free drugs. Nanomicelles
230 loaded with curcumin, rifampicin or Nile Red were lyophilized. The amount of encapsulated
231 curcumin was determined by fluorescence measurements (Nile Red, excitation wavelength =
232 550 nm – emission wavelength = 630 nm), or by absorbance (at 420 nm for curcumin and 475
233 nm for rifampicin).

234 2.8. *In vitro* release of encapsulated drug

235 10 mg of drug-loaded micelles were dissolved in 5 mL of PBS and subjected to dialysis at
236 37 °C using a Cellu-Sep H1 dialysis membrane (MWCO = 2000 Da) immersed in 100 mL of
237 PBS. The amount of drug released into the external medium was measured either by
238 fluorescence (excitation wavelength = 550 nm; emission wavelength = 630 nm for Nile Red),
239 or by absorbance (at 420 nm for curcumin and 475 nm for rifampicin) over 3 days.

240 2.9. Determination of morphology and particle size

241 The particle size and distribution of micelles were recorded on a Dynamic Light Scattering
242 (DLS) particle size analyzer (90 Plus Particle Size Analyzer, Dynamic Light Scattering
243 (Brookhaven Instruments Corporation) or a Zetasizer Nano (Malvern Instruments). The
244 analyses were conducted in aqueous solution after filtration through a 0.22 µm filter to
245 remove free drug/small polymer aggregates. Each analysis was performed at 25 °C in
246 triplicate with an angle detection of 90°. The CMC (Critical Micelle concentration) was
247 determined by DLS analysis of polycaprolactone-grafted oligocarrageenan dissolved in H₂O
248 at concentrations ranging from 0.2 to 2 mg/mL. To obtain the CMC, the logarithm of intensity
249 of the scattered light was plotted as a function of polycaprolactone-grafted oligocarrageenan
250 concentration. The resulting plot can be fitted to two straight lines whose intercept
251 corresponds to the CMC (Topel, Cakir, Budama, & Hoda, 2013 – Figure S4). The
252 morphology of NPs was also examined by transmission electron microscopy (TEM) using a
253 JEOL-1011 TEM instrument (tungsten filament) 100 kV. For TEM measurements, a drop of
254 the nanoparticle solution was placed on a 10 nm thick carbon-coated copper grid. To obtain
255 scanning electron microscopy (SEM) images, samples were mounted on aluminium studs and
256 sputter-coated with gold/palladium for 120 s. To obtain scanning electron microscopy (SEM)
257 images, samples were mounted on aluminium studs and sputter-coated with gold/palladium
258 for 120 s. The micelles were then imaged by a TESCAN VEGA 3 LMU field emission
259 scanning electron microscope at 10.0 kV.

260

261 2.10. Cytotoxicity of loaded polycaprolactone-grafted oligocarrageenan micelles

262 Cell toxicity of curcumin-loaded and empty micelles was evaluated by the MTT assay
263 using EA-hy96 endothelial cells. Cells were seeded in 96-well plates in 200 μL of complete
264 medium to obtain a concentration of 50,000 cells per well and incubated at 37 °C for 24 h.
265 The medium in each well was then removed and replaced with 200 μL of fresh medium
266 containing either empty micelles, curcumin-loaded micelles or free curcumin. Free curcumin
267 was dissolved in DMSO at concentrations ranging from 0 to 20 μM , empty micelles and
268 curcumin-loaded micelles were diluted in culture medium at concentrations ranging from 0.1
269 $\mu\text{g}/\text{mL}$ to 1000 $\mu\text{g}/\text{mL}$. After 48 h, the supernatant was removed and an MTT solution
270 (100 μL , 0.5 mg/mL) was added to each well and the cells were incubated for 4 h at 37 °C.
271 The supernatant was then eliminated and 100 μL of DMSO were added to each well to
272 dissolve the formazan crystals. The absorbance at 570 nm was measured with a microplate
273 reader (Tecan, Infinite M200Pro).

274 2.11. Cellular uptake of micelles by fluorescence microscopy

275 EA-hy926 cells were cultured to about 70% confluency in 12-well microplates containing
276 cover slips with 2 mL of complete medium. After 24 h, the culture medium was replaced with
277 fresh medium containing curcumin or curcumin-loaded micelles at a curcumin concentration
278 of 15 μM and incubated at 37 °C for 4 h, 6 h, 8 h, 16 h or 24 h. After incubation, the culture
279 medium was removed, and the cells were washed three times with PBS to remove micelles or
280 free curcumin that were not incorporated into the cells. EA-hy926 cells were then fixed using
281 4% paraformaldehyde in PBS (10 min) at room temperature, incubated with DAPI
282 (200 ng/mL) for 20 min at room temperature and visualized using an Eclipse 80i fluorescence
283 microscope (Nikon, France) equipped with a Hamamatsu ORCA-ER digital camera (Life
284 Sciences, Japan). Quantification was then performed by determining the mean curcumin
285 fluorescence of 90 cells for each condition in three independent experiments using ImageJ
286 software.

287 2.12. Quantification of TNF- α -induced secretion of IL-6 and MCP-1 by EA-hy926 cells

288 The protective effect of curcumin on EA-hy926 cells was evaluated. Cells were seeded in
289 6-well microplates containing 2 mL of culture medium and cultured to confluency (24 h). The
290 medium was then replaced with solutions containing empty micelles, curcumin-loaded
291 micelles or free curcumin respectively. Two concentrations of curcumin were tested, namely
292 5 μM and 15 μM . After 24 h, the medium was removed, and cells were stimulated with TNF-
293 α (tumor necrosis factor-alpha) overnight, diluted in culture medium (200 μL , 10 ng/mL). The

294 supernatants were then subjected to ELISA (e.Biosciences, France) to measure the secretion
295 of inflammatory factors (IL-6 and MCP-1). ELISA was performed according to the
296 manufacturer's instructions.

297 2.13. Preliminary safety evaluation in zebrafish

298 To assess the *in vivo* toxicity of blank micelles, curcumin-loaded micelles and free
299 curcumin, animals were injected intraperitoneally with these one of these respective solutions.
300 Briefly, fish were deeply anesthetized with 0.02% tricaine and received a single
301 intraperitoneal injection of blank micelles (300 mg/kg; n=12), free curcumin (195 μ M; n=12),
302 curcumin-loaded micelles (300 mg/kg equivalent to 195 μ M of curcumin; n=12) or PBS
303 (n=8). The viability of the three groups of zebrafish was then observed for 7 days. Zebrafish
304 behaviour, stress and/or suffering were carefully monitored in order to eventually euthanize
305 any suffering animals. No striking warning signs (stress, behavioural changes...) indicating
306 that the treatments induced animal suffering were observed. All experiments were conducted
307 in accordance with the French and European Community Guidelines for the Use of Animals
308 in Research (86/609/EEC and 2010/63/EU) and approved by the local Ethics Committee for
309 animal experimentation (APAFIS#9984-2016111814324578).

310 2.14. Biodistribution of free vs encapsulated Nile red in mice

311 **Mice** (12 weeks old, approximately 25 g) were randomly assigned to 1 of 2 groups (free
312 Nile red and encapsulated Nile red): n=3 per group, 2 females and 1 male.

313 To study the biodistribution, free (225 μ g) **or** encapsulated Nile red (100 mg/kg
314 equivalent to 225 μ g of Nile red) were injected via the tail vein using a 30-gauge catheter.
315 Retro-orbital 140 μ L blood sampling was performed 1 h, 2 h and 4 h after administration,
316 under anaesthesia (isoflurane, 4% induction and 1.5% maintenance in air). Mice were then
317 sacrificed and tissues (brain, liver, spleen, and kidney) were collected after perfusion with
318 saline buffer. Nile Red was then extracted from tissues with a mixture of ethyl
319 acetate/propanol (9:1) as described by Kim et al. (2011). Extracted Nile Red was then
320 quantified by fluorescence (excitation wavelength = 550 nm – emission wavelength =
321 630 nm).

322 2.15. Statistical analysis

323 All statistical analyses were carried out using GraphPad Prism 5 software. Analysis of
324 variance (ANOVA) was performed to compare all data. Significant differences were
325 highlighted using a post-hoc Tukey test.

326

327 **3. Results and Discussion**

328

329 3.1 Synthesis of polycaprolactone-grafted oligocarrageenan

330 The synthesis of polycaprolactone-grafted oligocarrageenan was performed using a method
331 described by Bhaw-Luximon et al., 2009. to obtain oligoagarose-graft-PCL starting from
332 oligoagarose. Carrageenan was selectively degraded into oligocarrageenans using a κ -
333 carrageenase enzyme isolated from *Pseudoaltermonas carrageenovora* followed by partial
334 acetylation of the oligocarrageenan hydroxyl groups. The remaining free hydroxyl groups
335 were then used to copolymerize ϵ -caprolactone. Finally, acetyl groups were removed to yield
336 amphiphilic polycaprolactone-grafted oligocarrageenan which can self-assemble into micelles
337 in aqueous solution.

338 *Enzymatic degradation of κ -carrageenan to oligocarrageenan*

339 κ -carrageenase (EC3.2.1.83) is a member of the glycoside hydrolase 16 family (GH16).
340 This enzyme hydrolyses β -(1 \rightarrow 4) linkage with retention of the anomeric configuration (Yao,
341 Wang, Gao, Jin, & Wu, 2013; Knutsen et al., 2001; Sun et al., 2014). It can be isolated from
342 the cell free culture medium of the bacteria *Pseudoaltermonas carrageenovora* (Guibet et al.,
343 2007). κ -carrageenase was produced from a carrageenan-stimulated culture of
344 *Pseudoaltermonas carrageenovora* and the enzyme was recovered from the bacterial culture
345 medium. A reducing ends measurement was performed to check the activity of the enzyme
346 (Figure S5A). The successful degradation can be measured by the increasing amount of
347 reducing sugar during the degradation. The control reaction performed using boiled-
348 inactivated enzyme showed no increase in reducing sugar level. Degradation products of κ -
349 carrageenan were further characterized using gel permeation chromatography with a dextran
350 calibration curve (Figure S5B). At 2 h, **most** of the κ -carrageenan was converted into
351 octamers (DP8). After 24 h of degradation, hexamers (DP6) and dimers (corresponding to
352 neo- κ -carrabiose) could be identified. A previous study on oligoagarose-*g*-polycaprolactone
353 showed that oligosaccharide chains of between 8 and 15 repeat units were best suited for
354 functionalization to form micelles (Bhaw-Luximon et al., 2009, 2011). Thus, to obtain a good
355 yield of oligocarrageenan with DP8, hydrolysis of κ -carrageenan was performed for 6 h and
356 oligocarrageenan was then separated from the enzyme and unreacted κ -carrageenan by
357 ultrafiltration.

358 *Acetylation of oligocarrageenan*

359 Partial acetylation of hydroxyl groups was performed with acetic anhydride in the presence
360 of pyridine at room temperature. The degree of acetylation was assessed using ^1H NMR using
361 the peak corresponding to the acetyl function at 2.13 ppm (Figure 1A). The degree of
362 acetylation was expected to be in the range 30-70% to obtain a good balance between the
363 hydrophilic oligocarrageenan chain and the hydrophobic PCL to be added in the next stage
364 (Bhaw-Luximon et al., 2009). ^1H NMR spectra indicated an acetylation of 60% using the
365 integration values of protons at 5.04 ppm and 4.6 ppm (corresponding to anomeric H in
366 oligocarrageenan) and at 2.13 ppm (acetyl groups).

367 *Partially acetylated polycaprolactone-grafted oligocarrageenan*

368 Partially acetylated oligocarrageenan was used as a macroinitiator to polymerize ϵ -
369 caprolactone in the presence of tin(II) octanoate (Bhaw-Luximon et al., 2009). Different
370 lengths of polycaprolactone were obtained by varying the monomer to initiator ratio. ^1H
371 NMR spectra of the resulting products showed characteristic signals due to the PCL unit
372 (Figure 1B). Assignments of the polycaprolactone moiety were completed using information
373 from 1D ^{13}C , 2D COSY and ^1H - ^{13}C HSQC spectra (Figure S6). The peaks at 3.99 and 2.23
374 ppm were assigned to $-\text{CH}_2\text{-O-C=O}$ protons and $-\text{CH}_2\text{-C=O}$ protons respectively. $-\text{CH}_2\text{-CH}_2\text{-}$
375 CH_2 proton groups were detected at 1.58 and $\text{CH}_2\text{-CH}_2\text{-CH}_2$ protons at 1.31 ppm. The NMR
376 spectra confirmed the expected copolymer structure. Copolymers with 10 caprolactone units
377 were selected for further studies.

378 *Polycaprolactone-grafted oligocarrageenan*

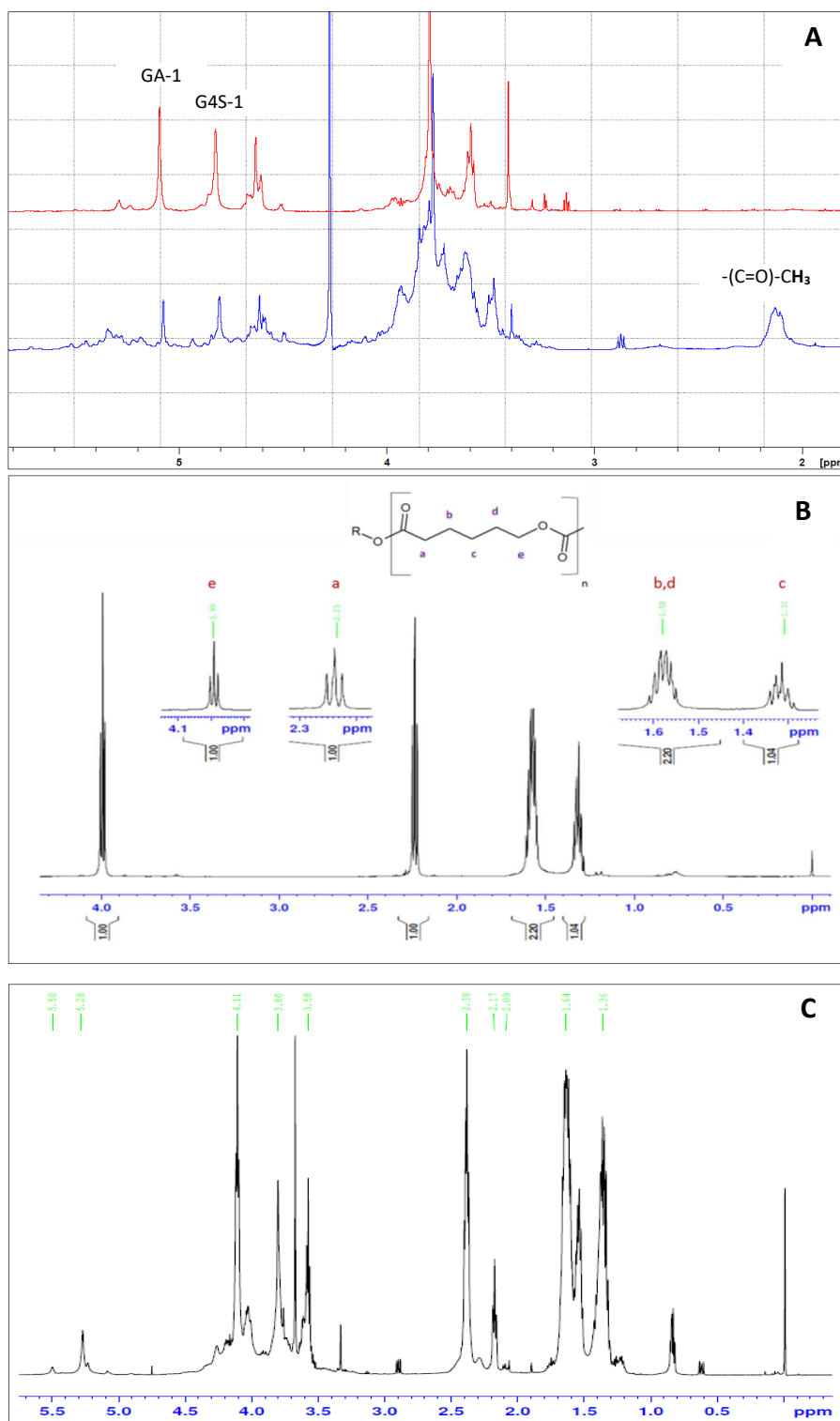
379 Removal of acetyl groups was performed under mild conditions to avoid cleavage of the
380 grafted PCL chains. The ^1H NMR spectrum (Figure 1C) of the product showed PCL signals
381 between 1.3 and 4 ppm and oligocarrageenan signals from 3 to 5.5 ppm. The peak
382 corresponding to acetyl proton at 2.09 ppm was not detected after this last step indicating that
383 complete deprotection of hydroxyl groups was achieved. No change was found in the number
384 of caprolactone repeat units.

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400 **Figure 1:** (A) ¹H NMR spectra of oligocarrageenan (red spectrum) and acetylated-oligocarrageenan (blue spectrum) obtained
401 in D₂O. Acetyl-group protons were detected at 2.13 ppm. GA-1 and G4S correspond to anomeric protons of the repeat unit
402 of κ-carrageenan. (B) ¹H NMR spectrum of acetylated-oligocarrageenan-graft-PCL obtained in CDCl₃. a, b c, d and e
403 correspond to caprolactone protons. R represent the partially acetylated-oligocarrageen chain. (C) ¹H NMR spectrum of
404 oligocarrageenan-graft-PCL obtained in D₂O. Signals corresponding to oligocarrageenan (between 3 ppm and 5.5 ppm) and
405 caprolactone (between 1.3 ppm and 4 ppm) are both observed.

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407 **3.2 Characterization of micelles and drug loading-release**

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409 The amphiphilic copolymer self-assembled in water to form micelles with an average size
410 of 187 ± 21 nm (Z-Average), as determined by dynamic light scattering (DLS) (Figure 2A.i).
411 The CMC estimated by DLS was $4 \cdot 10^{-5}$ M. This value was similar to **that** of $2.5 \cdot 10^{-5}$ M of
412 oligoagarose-g-PCL nanomicelles developed by Bhaw-Luximon et al. (2011).

413 TEM images of the micelles showed a spherical morphology with a diameter in the range
414 of 100-150 nm (Figure 2B). The size of micelles appears generally smaller than the value
415 determined by DLS, probably because of the dry state of samples in TEM measurements. This
416 phenomenon has been reported by several other authors (Bordallo, Rieumont, Tiera, Gómez,
417 & Lazzari, 2015; Wang et al., 2015). Similarly, a previous study on oligoagarose-graft-PCL
418 resulted in a particle diameter of 20 nm by DLS and 12 nm by TEM (Bhaw-Luximon et al.,
419 2011). SEM images also showed a uniform spherical morphology with a diameter in the
420 range of 75-100 nm (Figure 2 C).

421 Encapsulation of hydrophobic molecules namely curcumin, rifampicin and Nile Red was
422 performed using the acetone volatilisation method. After encapsulation a slight decrease in
423 particle size was noted with a Z-Average value of $177,2 \pm 1.2$ nm (Figure 2A.ii). This could
424 be explained by the high affinity between the hydrophobic micellar core and the hydrophobic
425 molecule. The spherical nature of Nile red-loaded and curcumin-loaded micelles was
426 confirmed by TEM and SEM images (Figure 2B and 2C).

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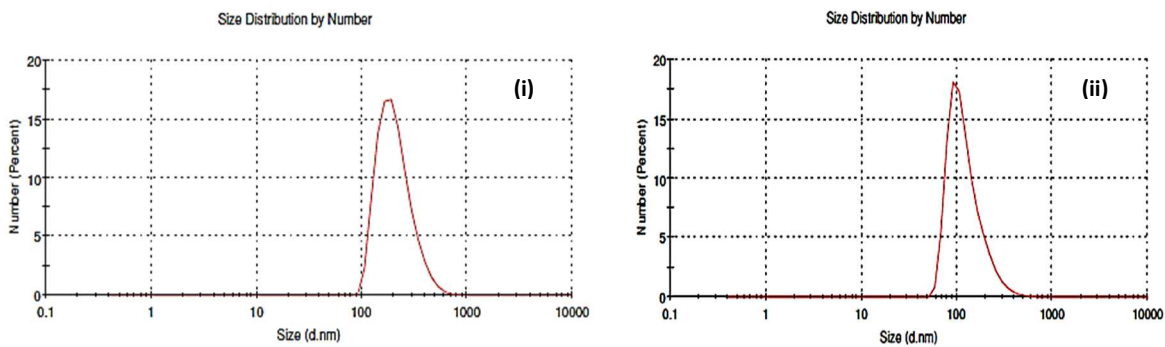
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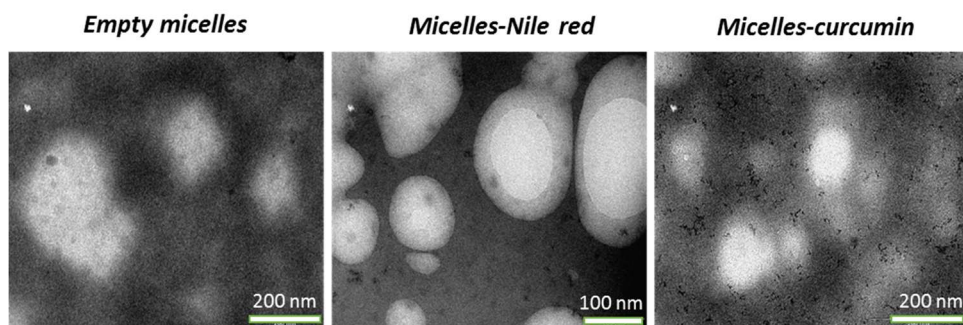


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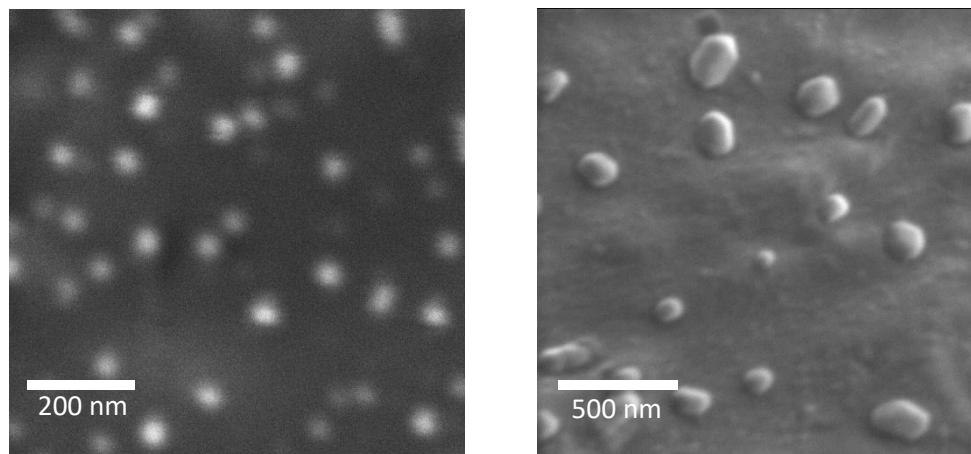


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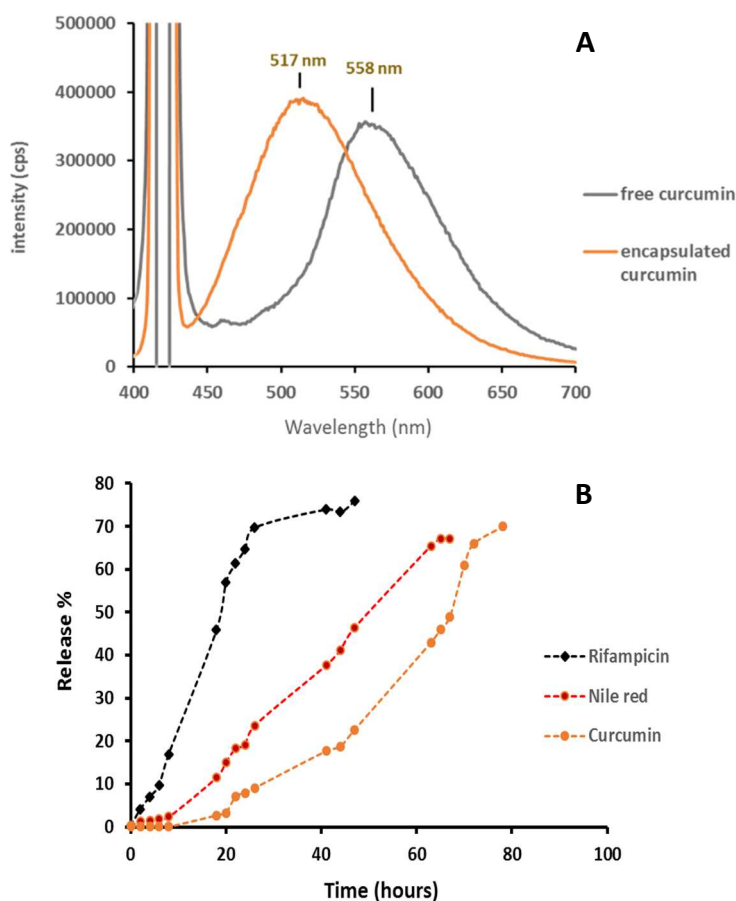
448 **Figure 2: Characterization of oligocarrageenan-graft-PCL:** (A) Size distribution of empty micelles (i) and curcumin-
 449 loaded micelles (ii) by Dynamic Light Scattering (DLS); (B) TEM images of empty micelles, Nile red loaded micelles and
 450 curcumin-loaded micelles at a concentration of 0.5 or 1 mg/mL; (C) SEM images of empty and curcumin loaded micelles.

451

452 Fluorescence analysis of curcumin showed a maximum emission at 558 nm for free
 453 curcumin and 517 nm for curcumin-loaded micelles (Figure 3A), which reflected the different
 454 chemical **environments** of curcumin. The encapsulation efficiency was determined using UV
 455 (curcumin, rifampicin) or fluorescence (Nile Red) measurements. A calibration curve allowed
 456 determination of the amount of encapsulated drug. The amount of encapsulated drug (w/w)
 457 was 10% for curcumin, 17% for rifampicin and 9% for Nile red.

458 The kinetics of hydrophobic molecule release from the micelles was studied in PBS at 37
459 °C (Figure 3B). Curcumin and Nile red were released over a longer period (with 65% released
460 respectively after 72 and 60 h) than rifampicin which showed 70% release after 24 h. The
461 time of release could depend on the affinity between the micelle core and the encapsulated
462 molecule as shown previously by Jeetah et al. (Jeetah, Bhaw-Luximon, & Jhurry, 2013). The
463 drug release probably proceeded via a combined erosion/diffusion mechanism due to the slow
464 hydrolytic degradation rate of the polycaprolactone core. For instance, non-enzymatic
465 degradation of PVP-b-PCL micelles is quite slow under neutral conditions and fast in acidic
466 or basic media (Hu, Jiang, Chen, Wu, & Jiang, 2010).

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472 **Figure 3:** (A) Characterization of free curcumin and encapsulated curcumin by fluorescence. Excitation was performed at
473 420 nm, and the maximum emission wavelength of free curcumin and curcumin-loaded micelle was detected at 558 nm and
474 517 nm respectively. (B) Kinetics of release of curcumin, Nile red and rifampicin from OligoKC-g-PCL micelles. The
475 concentrations of lipophilic molecule released in the medium during time was measured either by fluorescence (excitation
476 wavelength = 550 nm – emission wavelength = 630 nm) for Nile Red, or by absorbance (at 420 nm for curcumin and 475 nm
477 for rifampicin).

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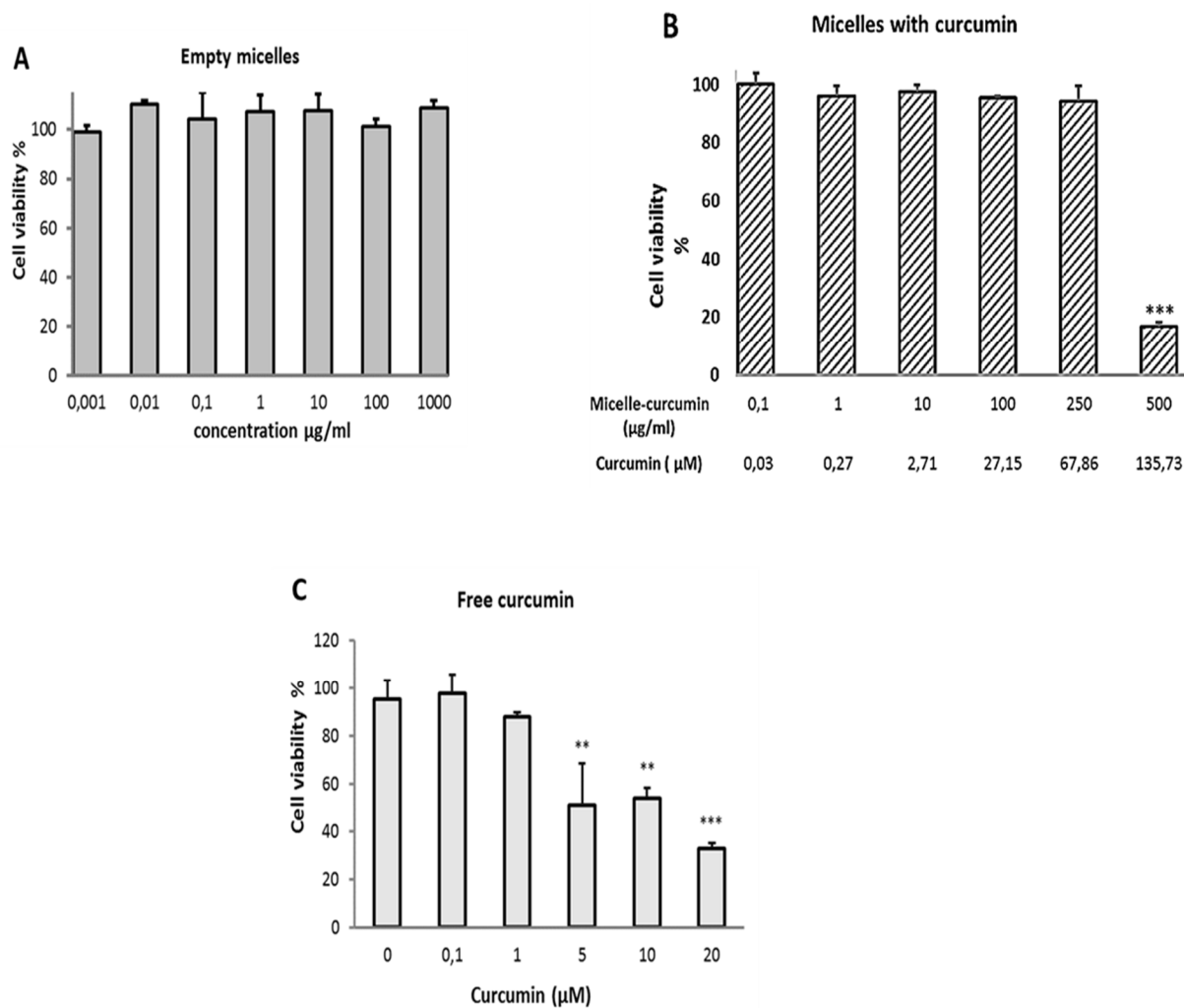
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481 3.3. Toxicity study of polycaprolactone-grafted oligocarrageenan

482 To determine the relative toxicity of polycaprolactone-grafted oligocarrageenan on
483 endothelial cells an MTT assay was used. Even if the MTT assay is not a direct measurement
484 of viability, the quantification of mitochondrial activity is, in most cases, proportional to the
485 number of cells and thus provides an indirect assessment of the toxicity. The empty micelles
486 did not affect cell viability at concentrations up to 1000 $\mu\text{g/mL}$ (Figure 4A). When the
487 micelles were loaded with curcumin, cell viability was not affected up to a concentration of
488 250 $\mu\text{g/mL}$, corresponding to 67 μM of encapsulated curcumin. However, a significant
489 decrease in viable cell number was observed at 500 $\mu\text{g/mL}$ (Figure 4B). The cytotoxic effect
490 of curcumin is well documented. Free curcumin at concentrations above 5 μM affected cell
491 viability (Figure 4C) as previously described by Kam et al. (2015). Thus, curcumin-loaded
492 polycaprolactone-grafted oligocarrageenan limited the cytotoxicity of curcumin. This could
493 be explained by a progressive and sustained release of curcumin from the micelles. The MTT
494 assay was performed after 48 h incubation whereas cell-free drug release kinetics indicated
495 23% release at 48 h (Figure 3 B) corresponding to an estimated released curcumin
496 concentration of 16 μM at the highest non-cytotoxic micelle concentration (250 $\mu\text{g/mL}$
497 micelle/curcumin). Both release kinetics and uptake of micelles may impact the bio-
498 availability of intracellular curcumin. Furthermore, the cytotoxicity of released curcumin may
499 be limited by cell biotransformation involving chemical or enzymatic processes (Heger, van
500 Golen, Broekgaarden, & Michel, 2013).

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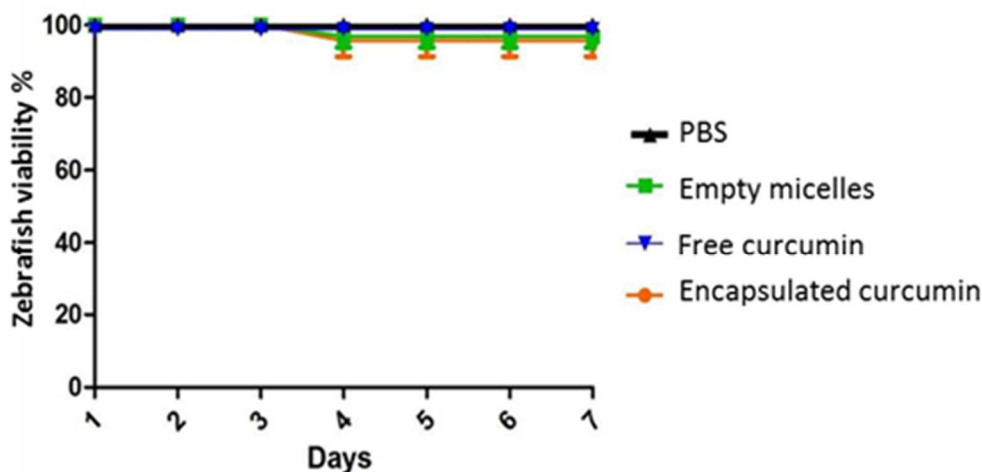
503 **Figure 4:** *In vitro* toxicity study of empty micelles and micelles loaded with curcumin. Cell viability of EA.hy926 cell was
 504 measured using MTT assay after treatment with empty micelles (A), curcumin-loaded micelles (B) or curcumin (C) for 48
 505 h. *** (p < 0.01) significant difference compared with the control without treatment.

506

507 The zebrafish is well-recognized to be a suitable vertebrate model for toxicity
 508 assessment (Hill, Teraoka, Heideman, & Peterson, 2005; Nishimura et al., 2016).
 509 Consequently, we used zebrafish as an *in vivo* model to evaluate the acute toxicity of our
 510 nanomicelles (Figure 5). A previous study conducted in rats used intraperitoneal injection
 511 of curcumin (100 mg/kg to 300 mg/kg) and demonstrated beneficial effects of this
 512 polyphenol after stroke (Thiyagarajan & Sharma, 2004). We thus used a maximum
 513 concentration of 300 mg/kg of blank (vehicle) or curcumin-loaded micelles. After being
 514 deeply anesthetized, the fish were injected intraperitoneally with a single dose of empty
 515 micelles (300 mg/kg; n=12), curcumin-loaded micelles (300 mg/kg equivalent to 195 µM of
 516 curcumin; n=12), curcumin (5.5 mg/kg; 195 µM; n=12) or PBS (n=8). The viability of
 517 zebrafish was monitored over 7 days with no significant difference in survival rates

518 between the treated groups. Furthermore, the respective treatments did not result in any
519 striking impairment of zebrafish behaviour, stress, locomotor activity or food intake.

520 Taken together, these results demonstrate the absence of visible toxic effects of the blank
521 and curcumin-loaded micelles on the viability of adult zebrafish and on their main
522 behaviour (stress, locomotor activity, and food intake) compared to PBS-injected ones. This
523 reinforces the idea that polycaprolactone-grafted oligocarrageenan micelles are safe
524 nanocarriers.



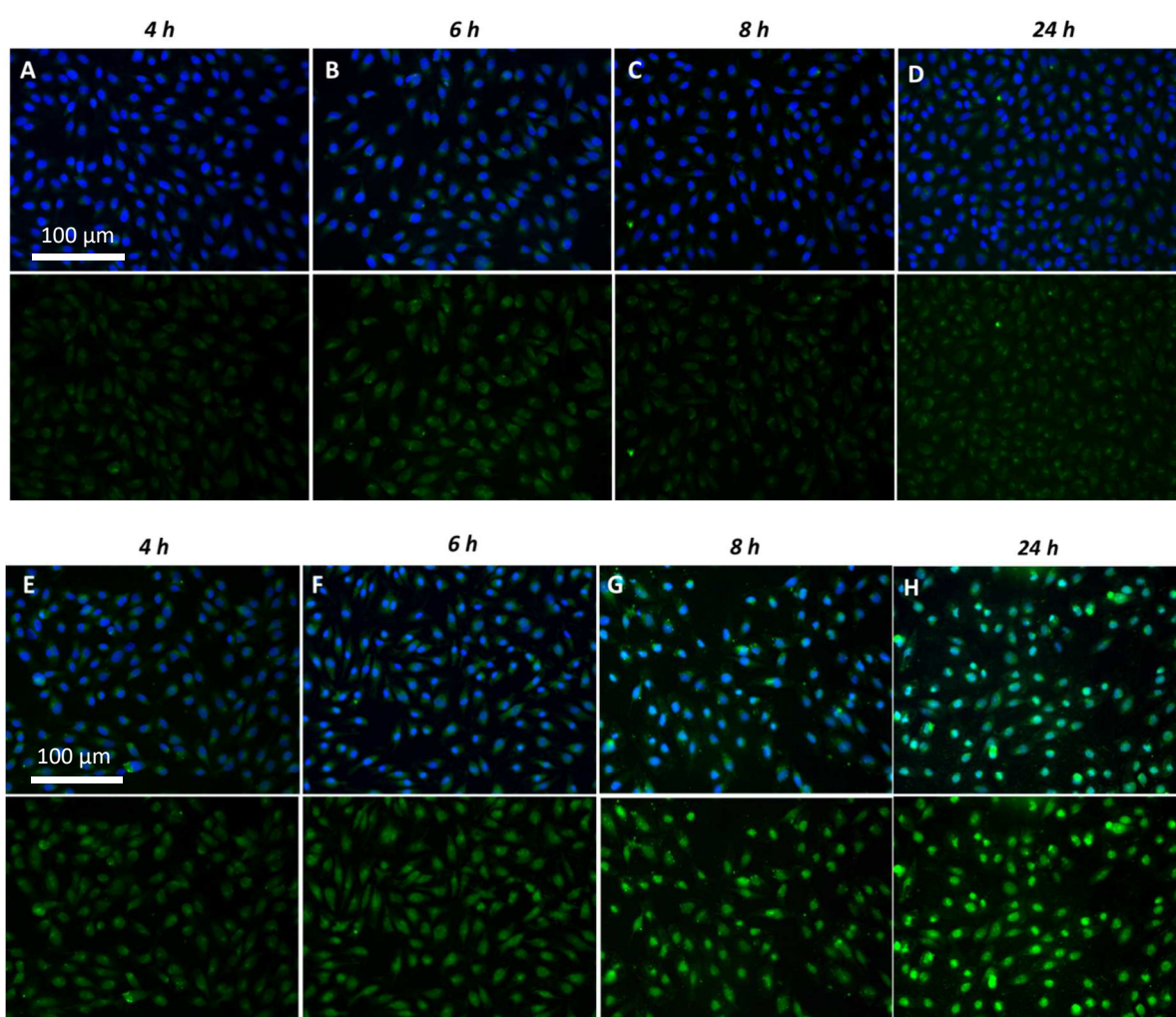
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526 **Figure 5:** Effect of intraperitoneal injection of empty micelles (n=12), curcumin, encapsulated curcumin (n=12), Free
527 curcumin (n=12) and PBS (n=8) on zebrafish viability. Zebrafish received an injection of 300 mg/kg of empty micelles,
528 curcumin-loaded micelles, free curcumin or PBS and the viability was observed for 7 days, without any significant change
529 between groups.

530

531 3.4. Cellular uptake of curcumin by EA.hy926 cells

532 The cellular uptake of OligoKC-g-PCL was investigated using endothelial EA-hy926 cells
533 treated with free curcumin or curcumin-loaded micelles and stained with DAPI. As the
534 treatment with free curcumin during 48 h showed a decrease in cell viability, the cell uptake
535 kinetics were monitored from 4 h to 24 h. Treated cells were then visualized using
536 fluorescence microscopy. Free curcumin could not be taken up effectively by the cells as
537 demonstrated by low intensity of curcumin fluorescence (green colour) in cultured cells
538 (Figure 6 A-D, first row). Curcumin-loaded micelles showed an increase in cell fluorescence
539 intensity associated with curcumin from 6 to 24 h of incubation (Figure 6 E-H, first row).
540 These results indicate that polycaprolactone-grafted oligocarrageenan micelles facilitated the
541 uptake of curcumin into the endothelial cells. Fluorescence quantification confirmed these
542 observations by showing a significant enhancement of encapsulated-curcumin incorporation

543 inside cells compared to free curcumin (Figure S7). As described by by Sahay, Alakhova, &
544 Kabanov (2008), endocytosis is the main mechanism involved in the internalization of
545 nanometric-sized particles by cells. Polymeric micelles can be taken up by cells using specific
546 mechanisms such as clathrin- or caveolae-mediated endocytosis. Furthermore, Bartczak et al.
547 (2012) showed that spherical particles **achieved** the highest cellular uptake by human
548 endothelial cells compared to other shapes, such as rod shapes or hollow particles. Further
549 study will help to determine the endocytosis pathway implicated in the uptake of our micelles.
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553 **Figure 6:** EA.h926 cellular uptake of free curcumin (A-D) and curcumin-loaded micelles (E-H). The incorporation was
554 studied between 4 h and 24 h with free or encapsulated curcumin at 15 μM. For each condition, the overlay of DAPI
555 staining (blue, nuclear) with curcumin fluorescence (green) is shown in the first row and curcumin fluorescence only in the
556 second row.

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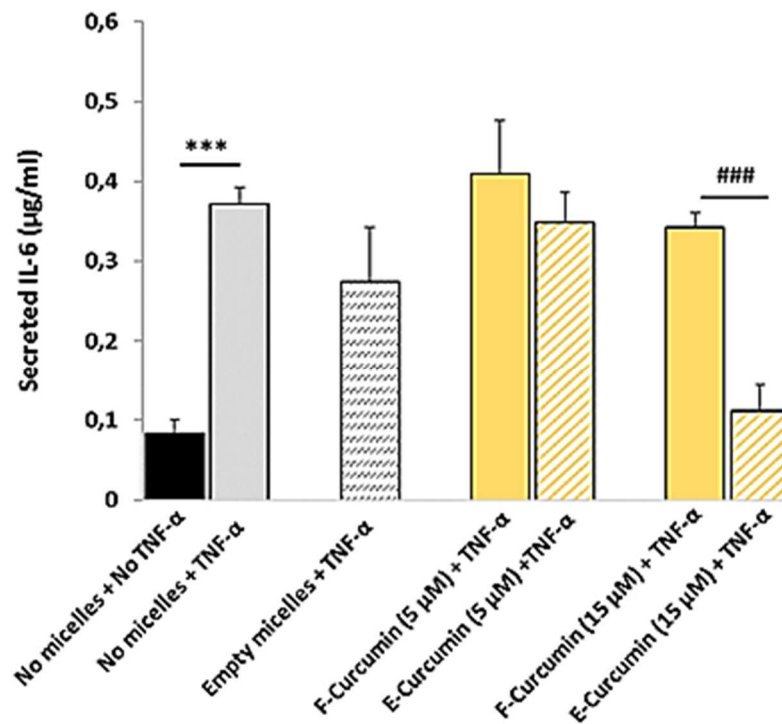
560 **3.5. Effect of curcumin-loaded polycaprolactone-grafted oligocarrageenan on**
561 **inflammation**

562 TNF-alpha was added to the endothelial cells to mimic inflammatory conditions. TNF-
563 alpha induces the secretion of inflammatory factors such as IL-6 and MCP-1 (Scarpini et al.,
564 1999; Cella, Engering, Pinet, Pieters, & Lanzavecchia, 1997). The effects of free curcumin
565 and curcumin-loaded OligoKC-g-PCL were investigated on endothelial cells in inflammatory
566 conditions. Empty polycaprolactone-grafted oligocarrageenan was used as control. Free or
567 encapsulated curcumin at 5 μ M did not modify the secretion of IL-6. At a higher dose of
568 15 μ M, the encapsulated curcumin inhibited almost completely the TNF-induced IL-6
569 production, while the free curcumin induced no significant effect compared to control (Figure
570 7A). For the chemokine MCP-1, the dose of 5 μ M of free or encapsulated curcumin had a
571 similar inhibitory effect. The 15 μ M concentration induced a greater inhibition for the
572 encapsulated curcumin relative to free curcumin, reaching levels similar to those of non-
573 stimulated cells (Figure 7B). The inhibitory effect of curcumin on TNF-alpha-stimulated cells
574 was thus significantly enhanced when curcumin was encapsulated into polycaprolactone-
575 grafted oligocarrageenan which is associated with its enhanced cellular uptake.

576 These results are in agreement with other studies using vectorized curcumin. Liposome-
577 loaded curcumin induced a reduction in IL-1 β and TNF- α secretion by lipopolysaccharide
578 (LPS)-stimulated macrophages, compared to free curcumin (Basnet, Hussain, Tho, & Skalko-
579 Basnet, 2012). In another study performed in LPS-injected mice, curcumin-loaded exosomes
580 showed better anti-inflammatory effects than free curcumin (Sun et al., 2010).

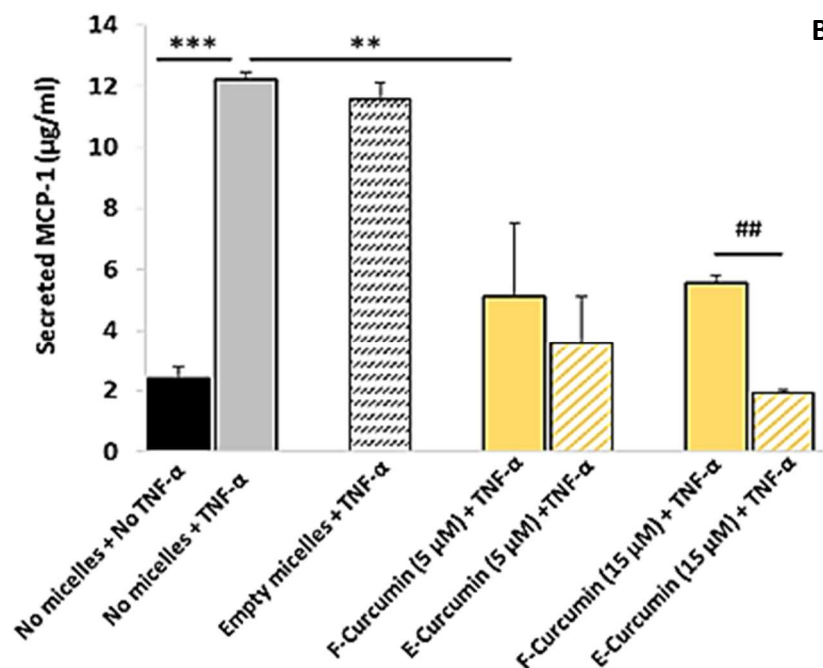
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597 **Figure 7:** Effect of free curcumin (F-curcumin) and encapsulated curcumin (E-curcumin) on the production of IL-6 (A)
 598 and MCP-1 (B) by EA.h926 stimulated by TNF-alpha. Cells were treated for 24 h with free or encapsulated curcumin at
 599 5 µM and 15 µM and then stimulated by TNF-α (10 ng/mL) overnight. *p < 0.05, **p < 0.01, ***p < 0.001 compared to
 600 TNF-α stimulated group without micelles (No micelles + TNF-α) and ##p < 0.01 compared to cells treated with Free-
 601 curcumin at 15 µM.

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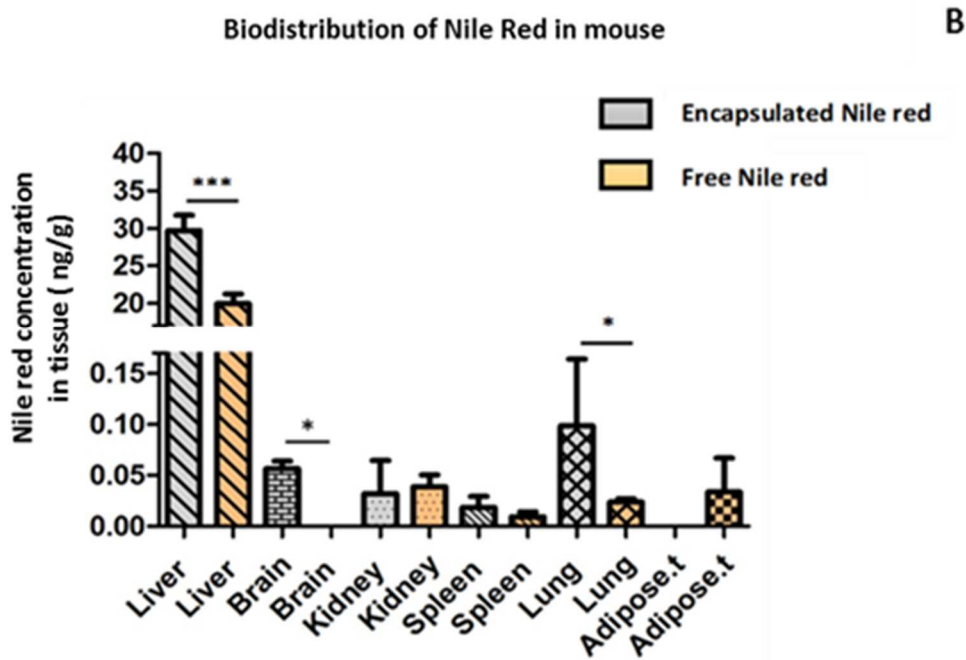
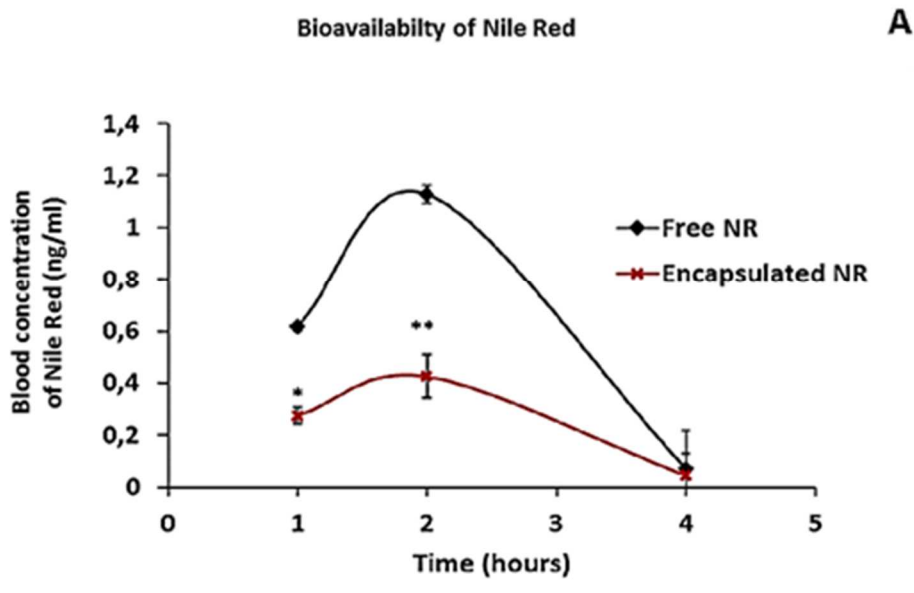
603 **3.6 Biodistribution in mouse**

604 An *in vivo* biodistribution study was performed using micelles loaded with Nile red
605 (100 mg/kg equivalent to 225 µg of Nile red), due to its greater fluorescence intensity and less
606 complex metabolization than curcumin. The micelles were injected into C57/B16 mice.

607
608 The plasma concentration of Nile red was measured at 1 h, 2 h and 4 h after injection. As
609 shown in Figure 8A, free Nile red gave higher levels in the blood than **did** encapsulated Nile
610 red. At 1 h, free Nile red concentration was 2.3-fold higher **than** micelle-loaded-Nile red. This
611 difference was exacerbated between 1 and 2 hours after injection. At 4 h, both free and
612 encapsulated Nile red had been completely cleared from the blood (distributed into organs or
613 eliminated from the body).

614
615 Biodistribution of free Nile red and encapsulated Nile red in various organs was then
616 studied in these mice. For this, tissue homogenates were prepared for liver, brain, kidneys,
617 spleen, and adipose tissue, 4 h after injection. The maximal concentration of Nile red was
618 observed in the liver which is the principal organ involved in the metabolism of exogenous
619 molecules (Figure 8B). In the other organs, fluorescence intensity was comparatively weak,
620 but results showed that micelles improved the incorporation of Nile red into the brain and the
621 lung. In the adipose tissue, only free Nile red was detected, probably due to its lipophilic
622 nature. It is interesting to note that scientific reports demonstrate that curcumin has a high
623 therapeutic ability for treating hepatic disorders (Nabavi, Daglia, Moghaddam, Habtemariam,
624 & Nabavi, 2014).

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629 **Figure 8:** Biodistribution of free and encapsulated Nile red in C57/B16 mice studied after intravenous injection. To
 630 measure the bioavailability of Nile Red, retro-orbital sampling was performed at 1h, 2h and 4h. Nile red was then extracted
 631 from the blood and quantified by fluorescence (A). Mice were sacrificed after 4h and Nile red in organs extracted and
 632 quantified to study the biodistribution (B). Excitation of Nile Red was performed at 550 nm and emission was observed at
 633 630 nm. Both experiments were performed in triplicate (n=3).

634

635 4. Conclusion

636 Oligocarrageenans (DP=8) produced by selective enzymatic hydrolysis of carrageenans
 637 were successfully functionalized via grafting of polycaprolactone chains. The graft copolymer

638 self-assembled into spherical nanomicelles of diameter in the range of 150-200 nm.
639 Hydrophobic molecules were successfully encapsulated and released in a sustained manner.
640 Empty polycaprolactone-grafted oligocarrageenan micelles were not cytotoxic to zebrafish or
641 endothelial cells and they increased the delivery of curcumin into the cells as evidenced by
642 fluorescence microscopy. The nanomicelles also potentiate the anti-inflammatory activity of
643 curcumin in TNF-stimulated endothelial cells as demonstrated by the decreased secreted
644 levels of inflammatory factors IL-6 and MCP-1.

645 Our *in vivo* study showed that the nanomicelles improved the distribution of Nile red to
646 organs. In particular, the nanomicelles enhanced the incorporation of this hydrophobic
647 molecule into the liver but also the brain. This suggests that polycaprolactone-grafted
648 oligocarrageenan could promote the delivery of hydrophobic therapeutic molecules across the
649 blood-brain barrier-(~~BBB~~). Further studies are required to determine the mechanisms for the
650 delivery of encapsulated molecules to the brain parenchyma.

651 These first *in vivo* experiments are promising and now pave the way for further *in vivo*
652 studies. In addition, the presence of free hydroxyl groups and galactose end groups on the
653 micelles offers the possibility to graft specific sequences like antibodies and thus to target
654 tissues. The ability of the micelles to encapsulate **larger** molecules, such as rifampicin, also
655 **opens up the possibility of** dual molecule encapsulation.

656

657 **Supporting Information, SI**

658 **Figure S1:** Chemical structure of the three main classes of κ -carrageenan and curcumin.

659 **Figure S2:** ^1H and ^{13}C NMR spectra of curcumin.

660 **Figure S3:** HPLC analysis of curcumin.

661 **Figure S4:** Determination of CMC (critical micelle concentration) of oligocarrageenan-g-
662 PCL by dynamic light scattering.

663 **Figure S5:** Hydrolysis of κ -carrageenan using κ -carrageenase isolated from
664 *Pseudoaltermonas carrageenovora*.

665 **Figure S6:** 600 MHz NMR COSY, ^1H - ^{13}C HSQC and 1D ^{13}C spectra of oligocarrageenan-
666 graft-PCL recorded in D_2O at 25 °C.

667 **Figure S7:** Fluorescence quantification of free (F-curcumin) or encapsulated-curcumin (E-
668 curcumin) incorporated in Eahy926 endothelial cells between 4 and 24 h.

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