

1 **PEGylated PLGA nanospheres optimized by DoE for ocular administration of**
2 **dexibuprofen – *in vitro*, *ex vivo* and *in vivo* characterization**

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28
29 **ABSTRACT**

30 Dexibuprofen-loaded PEGylated PLGA nanospheres have been developed to improve the
31 biopharmaceutical profile of the anti-inflammatory drug for ocular administration.
32 Dexibuprofen is the active enantiomer of ibuprofen and therefore lower doses may be
33 applied to achieve the same therapeutic level. According to this, two batches of
34 nanospheres of different drug concentrations, 0.5 and 1.0 mg/ml respectively have been
35 developed (the last one corresponding to the therapeutic ibuprofen concentration for
36 inflammatory eye diseases). Both batches were composed of negatively charged
37 nanospheres (-14.1 and -15.9 mV), with a mean particle size below 200 nm, and a high
38 encapsulation efficiency (99%). X-ray, FTIR, and DSC analyses confirmed that the drug
39 was dispersed inside the matrix of the nanospheres. While the *in vitro* release profile was
40 sustained up to 12 hours, the *ex vivo* corneal and scleral permeation profile demonstrated
41 higher drug retention and permeation in the corneal tissue rather than in the sclera. These
42 results were also confirmed by the quantification of dexibuprofen in ocular tissues after
43 the *in vivo* administration of drug-loaded nanospheres. Cell viability studies confirmed
44 that PEGylated-PLGA nanospheres were less cytotoxic than free dexibuprofen in the
45 majority of the tested concentrations. Ocular *in vitro* (HET-CAM test) and *in vivo* (Draize
46 test) tolerance assays demonstrated the non-irritant character of both nanosphere batches.

47 *In vivo* anti-inflammatory effects were evaluated in albino rabbits before and after
48 inflammation induction. Both batches confirmed to be effective to treat and prevent ocular
49 inflammation.

50

51 **Keywords:** Nanospheres; Dexibuprofen; PLGA; PEG; Inflammation; Drug Delivery.

52

53 **Chemical compounds studied in this article**

54 Lactic acid (PubChem CID: 612); Glycolic acid (PubChem CID: 757); Ethylene glycol
55 (PubChem CID: 174); Dexibuprofen (PubChem CID: 39912); Polyvinyl alcohol
56 (PubChem CID: 11199);

57

58 **Abbreviations**

59 NSAIDs, non-steroidal anti-inflammatory drugs; IBU, ibuprofen; DXI, dexibuprofen; GI,
60 gastrointestinal; NPs, nanoparticles; PLGA, poly(lactic-co-glycolic) acid; PEG,
61 poly(ethylene glycol); RES, reticuloendothelial system; NSs, nanospheres; PVA,
62 polyvinyl alcohol; DoE, design of experiments; Z_{av} , average particles size; PI,
63 polydispersity index; ZP, zeta potential; EE, encapsulation efficiency; PCS, photon
64 correlation spectroscopy; HPLC, high performance liquid chromatography; TEM,
65 transmission electron microscopy; DSC, differential scanning calorimetry; XRD, X-Ray
66 diffraction; FTIR, Fourier transformed infrared; PBS, phosphate buffered saline; BR,
67 bicarbonate ringer; CAM, chorioallantoic membrane; SA, sodium arachidonate.

68

69

70 1. Introduction

71
72 Inflammation is a non-specific response of the body against injuries from the external
73 environment, acting as a defense mechanism to isolate and destroy the triggering agent,
74 as well as to repair the damaged tissues. Ocular inflammation is one of the most prevalent
75 diseases in ophthalmology. It can affect any part of the eye or the surrounding tissues.
76 Corticosteroids are commonly used as anti-inflammatory drugs in the treatment of ocular
77 inflammation but they induce serious adverse effects when administered continuously [1].
78 The main alternatives to corticosteroids in the treatment of inflammation are non-steroidal
79 anti-inflammatory drugs (NSAIDs) [2]. In the field of ophthalmology, Ibuprofen (IBU)
80 has been receiving particular attention in recent years due to its anti-inflammatory
81 activity, having however a number of adverse effects that limit its use [3].

82 Rapid elimination of NSAIDs administered as eye drops, results in a pre-corneal drug
83 half-life between 1–3 min. As a consequence, only a very small amount of the drug (1–
84 5% of the dose) actually penetrates the cornea and is able to reach intraocular tissues. On
85 the other hand, drugs administered onto the ocular mucosa are known to suffer absorption
86 via conjunctiva and nasolacrimal duct, easily reaching the systemic circulation [4] and
87 [5]. Drugs, such as ibuprofen, may induce adverse side effects that can be minimized by
88 the use of the active enantiomer – dexibuprofen (DXI), which is twice more potent and
89 has less side effects than the former [6]. Gastric and epigastric pain, nausea and vomiting
90 have been the most frequent side effects reported in randomized clinical trials in patients
91 treated with DXI. Effects of DXI in the central nervous system (CNS) were less common
92 than the use of racemic ibuprofen [7]. The racemic mixture was also responsible for a
93 higher gastric toxicity than the S(+) isomer [6]. Moreover, the safety, tolerability and
94 equivalent efficacy between DXI and the double dose of ibuprofen was confirmed by
95 comparing the oral uptake of both drugs for osteoarthritis treatment in a clinical study [7]
96 and [8].

97 To protect the drug from inactivation by the enzymes present in the tear film or corneal
98 epithelium, to facilitate its transcorneal penetration prolonging its stay in the precorneal
99 area, and to avoid undesired adverse effects, polymeric nanoparticles (NPs) have been
100 proposed. Biodegradable polymers, including poly(lactic-co-glycolic acid) (PLGA)
101 (biopolymer approved by the Food and Drug administration), have been widely used as a
102 biomaterial in medical prostheses and surgical sutures [9]. More recently, PLGA has been
103 used in the development of colloidal carriers for controlled release of drugs, due to its
104 biocompatibility, biodegradability and non-toxicity [10]. Furthermore, compared to
105 natural polymers, these synthetic polymers demonstrate higher reproducibility, are easily
106 formulated and allow the control and prediction of the degradation kinetics [11].

107 Among other, strategies, PEG-coating on PLGA NPs offer several advantages. These are
108 firstly attributed to the enhanced contact time of the particles with the corneal surface by
109 the interaction with the mucus layer of the tear film. NPs interact with the mucus layer of
110 the tear film either by electrostatic, hydrophobic and hydrogen bonding, or by their
111 physical retention in the mucin network [12]. Griffiths et al. [12] demonstrated that such
112 retention in the mucin network is dependent on the hydrophobic surface of the particles,
113 which could be overcome by coating them with PEG. On the other hand, the hydrophobic

114 entrapment could be minimized as long as the nanoparticles were adequately surfaced
115 with such hydrophilic PEG layers and depicted negative electrical charge [12]. Therefore,
116 the accumulation of the NPs in the conjunctival sac, as well as, the ability of the particles
117 to penetrate in the first layers of the corneal epithelium contribute to enhance drugs'
118 bioavailability [13]. In addition, PEGylation contributes to maintain the particles in
119 circulation for a longer time, thus avoiding their recognition by the reticuloendothelial
120 system (RES) [14].

121 In the present work, we report the development of a new formulation for ocular delivery
122 of dexibuprofen (DXI), based on nanospheres (NSs) composed of poly-L-lactic-co-
123 glycolide (PLGA) surrounded by polyethylene glycol (PEG) chains (DXI-PLGA-PEG
124 NSs). The suitability of DXI-PLGA-PEG NSs to treat and prevent ocular inflammation
125 has been demonstrated. Physicochemical properties and drug-polymer interactions were
126 assessed. *In vitro* and *ex vivo* drug release and short-term stability of DXI NSs were
127 studied. DXI quantification after *in vivo* administration was also performed.

128

129 **2. Materials and methods**

130

131 **2.1. Materials**

132 Diblock copolymer PLGA-PEG 5% Resomer[®] was obtained from Evonik Corporation
133 (Birmingham, USA) and the active compound *S*-(+)-Ibuprofen (dexibuprofen) was
134 purchased from Amadis Chemical (Hangzhou, China). Polyvinyl alcohol (PVA) and
135 acetone were purchased from Sigma-Aldrich (Madrid, Spain) and Fisher Scientific
136 (Pittsburgh, USA), respectively. Reagents for cell culture were obtained from Gibco
137 (Alfagene, Portugal). Alamar Blue, from Invitrogen Alfagene[®] (Portugal), was used for
138 cell viability estimation. Water filtered through Millipore MilliQ system was used for all
139 the experiments and all the other reagents were of analytical grade.

140

141 **2.2. Methods**

142

143 **2.2.1. Nanospheres preparation**

144 NSs were prepared by solvent displacement method described elsewhere [15]. Briefly,
145 the co-polymer PLGA-PEG and the drug (DXI) were firstly dissolved in acetone. This
146 organic phase was added dropwise, under moderate stirring, into 10 ml of an aqueous
147 solution of PVA (0.33-1.17 %) adjusted to the desired pH (3.2-4.8). After that, acetone
148 was evaporated under reduced pressure and the resulting particles were ultracentrifuged,
149 at 15000 r.p.m. for 20 min, in order to remove excess of PVA.

150

151 **2.2.2. Optimization of nanospheres parameters**

152 Design of experiments (DoE) is frequently used to plan research because it provides
153 maximum information, whilst requiring a minimal number of experiments [16]. A central
154 composite factorial design was developed to analyze the effect of independent variables
155 (pH, DXI and PVA concentrations) on the dependent variables (average particle size

156 (Z_{av}), polydispersity index (PI), zeta potential (ZP) and encapsulation efficiency (EE).
 157 The amount of polymer was kept constant for all the assays (90 mg).
 158 According to the composite design matrix generated by Statgraphics Plus 5.1 software, a
 159 total of 16 experiments (8 factorial points, 6 axial points and two replicated center points)
 160 were required. The experimental responses were the result of the individual influence and
 161 the interactions of the three independent variables, as shown in Table 1. The responses
 162 were therefore modeled through the full second-order polynomial equation shown in
 163 equation 1/:

$$164 Y_u = \beta_0 + \beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \beta_3 \cdot X_3 + \beta_{11} \cdot X_1^2 + \beta_{22} \cdot X_2^2 + \beta_{33} \cdot X_3^2 + \beta_{12} \cdot X_1 \cdot X_2 + \beta_{13} \cdot X_1 \cdot X_3 + \beta_{23} \cdot X_2 \cdot X_3$$

165 /1/

166 where Y_u is the measured response, β_0 to $\beta_{2,3}$ are the regression coefficients and X_1 , X_2
 167 and X_3 are the studied factors. The effect and the significance level of the factors were
 168 evaluated by analysis of variance (ANOVA) [17].

170 **Table 1.** Values of the experimental factors according to the matrix designed by $2^3 + star$
 171 central composite rotatable factorial design parameters and measured responses.

172

	pH		C DXI		C PVA		Z_{av} (nm)	PI	ZP (mV)	EE (%)
	Coded level	pH	Coded level	(mg/ml)	Coded level	(%)				
Factorial points										
F1	-1	3.5	-1	0.50	-1	0.50	221.4 ± 0.5	0.082 ± 0.023	-4.17 ± 0.29	99.79
F2	1	4.5	-1	0.50	-1	0.50	219.3 ± 6.6	0.050 ± 0.033	- 11.9 ± 0.19	90.80
F3	-1	3.5	1	1.50	-1	0.50	225.5 ± 3.2	0.072 ± 0.027	- 3.16 ± 0.28	99.20
F4	1	4.5	1	1.50	-1	0.50	201.1 ± 4.6	0.048 ± 0.018	- 5.13 ± 0.55	87.65
F5	-1	3.5	-1	0.50	1	1.00	216.5 ± 2.9	0.068 ± 0.026	- 3.24 ± 0.93	90.72
F6	1	4.5	-1	0.50	1	1.00	216.6 ± 2.1	0.049 ± 0.007	- 5.65 ± 0.26	85.52
F7	-1	3.5	1	1.50	1	1.00	219.0 ± 3.8	0.065 ± 0.009	- 3.53 ± 0.17	89.77
F8	1	4.5	1	1.50	1	1.00	213.3 ± 1.2	0.048 ± 0.029	- 3.15 ± 0.53	89.82
Axial points										
F9	1.68	4.8	0	1.00	0	0.75	229.2 ± 0.7	0.054 ± 0.018	-2.75 ± 0.17	89.87
F10	-1.68	3.2	0	1.00	0	0.75	205.8 ± 2.3	0.063 ± 0.013	-2.53 ± 0.44	92.36
F11	0	4.0	1.68	1.84	0	0.75	223.5 ± 0.6	0.036 ± 0.018	-4.38 ± 0.25	90.90
F12	0	4.0	-1.68	0.16	0	0.75	223.4 ± 1.6	0.052 ± 0.022	- 6.84 ± 0.27	99.10
F13	0	4.0	0	1.00	1.68	1.17	220.4 ± 0.3	0.036 ± 0.007	- 5.73 ± 0.13	98.90
F14	0	4.0	0	1.00	-1.68	0.33	203.9 ± 0.4	0.072 ± 0.024	- 8.18 ± 0.23	97.84
Center points										
F15	0	4.0	0	1.00	0	0.75	220.3 ± 6.6	0.046 ± 0.023	-6.01 ± 0.16	90.51
F16	0	4.0	0	1.00	0	0.75	217.3 ± 2.9	0.043 ± 0.030	- 6.56 ± 0.93	85.45

173

174

2.2.3. Physicochemical characterization

175 Z_{av} and PI of NSs were determined by photon correlation spectroscopy (PCS) (after 1:10
 176 dilution) with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 25 °C using
 177 disposable quartz cells and (Malvern Instruments).

178 NSs surface charge, measured as zeta potential (ZP), was evaluated by using laser-
179 Doppler electrophoresis with M3 PALS system in Zetasizer Nano ZS. ZP indirectly
180 indicates the rate of aggregation of particles. A greater ZP (in absolute value) would
181 induce less aggregation due to repulsion forces between the particles. To calculate this,
182 the Henry equation was used /2/:

$$183 \quad \mu_E = \frac{\epsilon Z P f(K a)}{6 \pi \eta} \quad /2/$$

184 where μ_E is the electrophoretic mobility, ϵ is the dielectric constant of the medium, ZP is
185 the zeta potential, η is the viscosity of the medium, K is the Debye-Hückel parameter
186 and f (Ka) is a correction factor that takes into account the thickness of the electrical
187 double layer (1/K) and particle diameter (a). The unit of K is a reciprocal length.

188 The reported values correspond to the mean \pm SD of at least three different batches of
189 each formulation. [18].

190

191 **2.2.4. Evaluation of the encapsulation efficiency**

192 The EE of DXI in the NSs was determined indirectly by measuring the concentration of
193 the free drug in the dispersion medium. The non-encapsulated DXI was separated by a
194 filtration/centrifugation technique (1:10 dilution) by using an Ultracell-100K (AmiconR
195 Ultra; Millipore Corporation, Massachusetts) centrifugal filter devices at 4 °C and 700 g
196 for 30 min (Heraeus, Multifuge 3 L-R, Centrifuge. Osterode, Germany). The EE was
197 calculated using equation /3/:

$$198 \quad EE (\%) = \frac{\text{total amount of DXI-free DXI}}{\text{total amount of DXI}} \times 100 \quad /3/$$

199 Samples were evaluated by high performance liquid chromatography (HPLC), as
200 described elsewhere [19]. Briefly, samples were quantified using HPLC Waters 2695
201 separation module and a Kromasil® C₁₈ column (5 μ m, 150 x 4.6 mm) with a mobile phase
202 of methanol/ phosphoric acid 0.05 % (80:20) at a flow rate of 1 ml/min and a wavelength
203 of 220 nm. Standards were prepared in methanol:water (90:10) from a stock solution of
204 500 μ g/ml (50-0.5 μ g/ml). Data was processed using Empower 3® Software.

205

206 **2.2.5. Nanospheres characterization and interaction studies**

207 NSs were diluted (1:5) and a morphological study was carried out by transmission
208 electron microscopy (TEM) on a Jeol 1010. To visualize the NSs, copper grids were
209 activated with UV light and samples were placed on the grid surface. Negative staining
210 was performed with uranyl acetate (2%).

211 X-ray diffraction (XRD) was used to analyze the state (amorphous or crystalline) of the
212 samples (centrifuged NSs or formulation compounds). Compounds were sandwiched
213 between polyester films and exposed to CuK α radiation (45 kV, 40 mA, $\lambda = 1.5418 \text{ \AA}$) in
214 the range (2 θ) from 2° to 60° with a step size of 0.026° and a measuring time of 200 s per
215 step.

216 Fourier transform infrared (FTIR) spectra of different samples (NSs formulations or
217 compounds separately) were obtained using a Thermo Scientific Nicolet iZ10 with an
218 ATR diamond and DTGS detector. The scanning range was 525–4000 cm^{-1} .
219 Thermograms were obtained on a Mettler TA 4000 system (Greifensee, Switzerland)
220 equipped with a DSC 25 cell. Temperature was calibrated by the melting transition point
221 of indium prior to sample analysis. All samples were weighed (Mettler M3 Microbalance)
222 directly in perforated aluminum pans and heated under a nitrogen flow at a rate of 10
223 $^{\circ}\text{C}/\text{min}$ (25–125 $^{\circ}\text{C}$).

224
225

2.2.6. Determination of the *in vitro* release profile

226 One of the main goals of drug release from the polymer matrix is the possibility to provide
227 an extended release profile over time. *In vitro* release was evaluated using a bulk-
228 equilibrium reverse dialysis bag technique [20]. This technique is based on the dispersion
229 of the colloidal suspension in the dialysis medium accomplishing sink conditions [21].
230 The release medium was composed of a buffer solution (PBS 0.1 M, pH 7.4). 16 dialysis
231 sacs containing 1 ml of PBS were previously immersed into the release medium. The
232 dialysis sacs were equilibrated with the dissolution medium a few hours prior to the
233 experiments. A volume of 15 ml of free drug in PBS or NSs was added to 285 ml of the
234 dissolution medium. The assay was carried out in triplicate comparing the free drug in
235 PBS against NSs formulations. Release kinetic experiments were performed at a fixed
236 temperature of 32 $^{\circ}\text{C}$ (ocular surface temperature) under constant magnetic stirring
237 ($n=6/\text{group}$). At predetermined time intervals, the dialysis sacs were withdrawn from the
238 stirred release solution and the volume was replaced by 1 ml of PBS. The content of the
239 sacs at each time point was evaluated and data were adjusted to the most common kinetic
240 models [18].

241
242

2.2.7. *Ex vivo* corneal and scleral permeation study

243 *Ex vivo* corneal and scleral permeation experiments were carried out with New Zealand
244 rabbits (male, weighting 2.5–3.0 kg), under veterinary supervision, and according to the
245 Ethics Committee of Animals Experimentation from the University of Barcelona (CEEA-
246 UB). The rabbits were anesthetized with intramuscular administration of ketamine HCl
247 (35 mg/kg) and xylazine (5 mg/kg) and euthanized by an overdose of sodium
248 pentobarbital (100 mg/kg) administered through marginal ear vein under deep anesthesia.
249 The cornea and sclera were excised and immediately transported to the laboratory in
250 artificial tear solution. The assay was done using Franz diffusion cells and the tissue was
251 fixed between the donor and receptor compartment. The area available for permeation
252 was 0.64 cm^2 . The receptor compartment was filled with freshly prepared Bicarbonate
253 Ringer's (BR) solution. This compartment was kept at 32 and 37 \pm 0.5 $^{\circ}\text{C}$ for corneal and
254 scleral permeation, respectively, and stirred continuously. A volume of 1 mL of F (A)
255 NSs or 0.5 mg/ml of DXI was placed in the donor compartment and covered to avoid
256 evaporation. A volume of 300 μl was withdrawn from the receptor compartment at fixed
257 times and replaced by an equivalent volume of fresh BR solution at the same temperature.

258 The cumulative DXI amount permeated was calculated, at each time point, from DXI
259 amount in the receiving medium and plotted as function time (min) [22].

260 At the end of the study, the cornea was used to determine the amount of drug retained.
261 The tissue was cleaned using a 0.05% solution of sodium lauryl sulfate and washed with
262 distilled water, weighed and treated with methanol under sonication during 30 min using
263 an ultrasound bath. The amount of DXI permeated and retained through the cornea was
264 determined.

265 Results are reported as the median \pm SD of six replicates for the amount of DXI permeated
266 and retained on each tissue, respectively [22].

267 Lag time, T_L (h), values were calculated by plotting the cumulative DXI permeating the
268 cornea versus time, determining x-intercept by linear regression analysis. The corneal
269 permeability coefficient K_p (cm/h), partition coefficient P_1 (cm) and diffusion coefficient
270 P_2 (h^{-1}) were calculated from the following equations:

$$271 K_p = P_1 \times P_2 \quad /4/$$

$$272 P_1 = \frac{J}{A \times C_0 \times P_2} \quad /5/$$

$$273 P_2 = \frac{1}{6 \times T_L} \quad /6/$$

274 where C_0 is the initial concentration of drug in the donor compartment, A (0.64 cm^2) is
275 the exposed corneal surface [22].

276

277 **2.2.8. Short-term stability**

278 NSs stability at 4, 25 and 38 °C was assessed by light backscattering by means of a
279 Turbiscan[®] Lab. For this purpose, a glass measurement cell was filled with the sample for
280 each temperature. The light source, pulsed near infrared light-emitting diode LED ($\lambda=880$
281 nm), was received by a backscattering detector at an angle of 45° from the incident beam.
282 Backscattering data were acquired once a month for 24 h, at 1 h intervals. In addition to
283 this technique, NSs Z_{av} , PI and ZP were also measured monthly. Temperature studies
284 were carried out by duplicate and visual observation of the samples was undertaken.

285

286 **2.2.9. Cytotoxicity assay**

287 Alamar blue assay was carried out in order to investigate the possible toxicity of the
288 developed NSs in comparison with free DXI. To perform this assay Y-79 (human
289 retinoblastoma) cell line acquired from Cell Lines Service (CLS, Eppelheim, Germany)
290 was used. Y-79 cells were maintained in RPMI-1640, supplemented with 10% (v/v) fetal
291 bovine serum (FBS), 2 mM L-glutamine, and antibiotics (100 U/ml penicillin and 100 μg
292 mL^{-1} of streptomycin) at 37 °C under an atmosphere of 5% CO_2 /95% air with controlled
293 humidity (Binder chamber). Cells were centrifuged, re-suspended in FBS-free culture
294 media, counted and seeded, after appropriate dilution, at 1×10^5 cells/ml, in poly-L-lysine
295 pre-coated 96-well plates (100 μl /well). For this study, dilutions of NSs in FBS-free
296 culture media (namely F(A) and F(B), see NSs optimization section), as well as their
297 corresponding free drug were carried out and added to cells 24 h after seeding (100

298 $\mu\text{L}/\text{well}$). Cell viability was assayed with Alamar Blue (AB, Alfacel, Invitrogen,
299 Portugal), 24 or 48 h after exposure to test compounds, by addition of 100 $\mu\text{L}/\text{well}$ of AB
300 solution, 10% (v/v) diluted in FBS-free media, preceded by removal of test solutions. The
301 AB absorbance was determined at λ of 570 nm (reduced form) and 620 nm (oxidized
302 form) after 4 h of cell contact. Data were analyzed by calculating the percentage of
303 Alamar blue reduction (according to the manufacture recommendations) and expressed
304 as percentage of control (untreated), as reported before [23].

305

306

2.2.10. Ocular tolerance assays: HET-CAM and Draize irritation test

307 To assess the potential risk of ocular irritation caused by NSs, ocular tolerance test by *in*
308 *vivo* and *in vitro* methods were carried out.

309 To study the ocular tolerance *in vitro* the HETCAM[®] test was developed as described in
310 the INVITTOX n^o 15 protocol [24]. This test is based on the observation of the irritant
311 effects (bleeding, vasoconstriction and coagulation) in the chorioallantoic membrane
312 (CAM) of an embryonated egg (10 days) induced by application of 300 μL of the studied
313 formulation, during the first 5 minutes [25]. In the experimental procedure, fertilized and
314 incubated eggs during 10 days were used. These eggs (from the farm G.A.L.L.S.A.,
315 Tarragona, Spain) were maintained at a temperature of $12 \pm 1^\circ\text{C}$ for at least 24 hours
316 before placing them in the incubator with controlled temperature (37.8°C) and humidity
317 (50-60 %) during the incubation days. A series of controls were performed: SDS 1%
318 (positive control for slow irritation), 0.1 N NaOH (positive control for fast irritation),
319 NaCl 0.9% (negative control). Data were analyzed as the media \pm SD of the time at which
320 the injury occurred (n=6/group). Scores of irritation potential can be grouped into four
321 categories (see Table A.1 of Supplementary Material) [26].

322 *In vivo* ocular tolerance assays were performed using primary eye irritation test of Draize
323 et al. [22] using New Zealand albino male rabbits of 2.5 kg middle weight from San
324 Bernardo farm (Navarra). This test was performed according to the Ethical Committee
325 for Animal Experimentation of the UB and current legislation (Decret 214/97, Gencat).
326 The sample was placed in the conjunctival sac of the right eye and a gentle massage was
327 applied to assure the proper sample circulation through the eye. The appearance of
328 irritation was observed at the time of administration and after 1 hour, using the left eye as
329 a negative control (n=6/group). The evaluation was performed by direct observation of
330 the anterior segment of the eye, noting the possible injury of the conjunctiva
331 (inflammation, chemosis, redness or oozing), iris and cornea (opacity and affected
332 surface) (for detailed punctuation see Table A.2 of Supplementary Material). Ocular
333 irritation index (OII) was evaluated according to the observed injuries (Table A.1 on
334 Supplementary material).

335

336

337

2.2.11. Inflammatory activity assay

338

339 Corneal inflammatory activity of the developed formulations was assessed *in vivo*
340 (n=6/group). Ocular inflammation was induced administering 50 µl of sodium
341 arachidonate (SA) 0.5% (w/v) dissolved in PBS (pH 7.4). Inflammation was quantified
342 using a slit lamp at various times, according to a modified Draize scoring system [26].
343 The sum of the inflammation score is expressed by the mean ± SD (detailed punctuations
344 can be found in Table A.2 of supplementary Material).

345 To assess inflammation prevention, free drug and DXI NSs were instilled (50 µl) in the
346 conjunctival sac, 30 min before induction of ocular inflammation. In order to test
347 inflammation treatment, ocular inflammation was induced and after 30 min, either NSs
348 or free DXI in saline serum were applied.

349

350 **2.2.12. Ocular drug bioavailability**

351

352 In order to achieve steady-state concentrations, DXI NSs were administered to New
353 Zealand rabbits (n=6), every 8 hours for two weeks . A volume of 50 µl of each
354 formulation was administered and, at the end of the experiments, animals were scarified
355 and drug amount was quantified in vitreous humor and aqueous humor. Retained DXI on
356 cornea and sclera were also measured. [19].

357

358 **2.2.13. Statistical analysis**

359 All of the data are presented as the mean ± S.D. Two-way ANOVA followed by Tukey
360 post hoc test was used for multi-group comparison. Student's *t*-test was used for two-
361 group comparisons. Statistical significance was set at $p < 0.05$. GraphPad Prism V6.0
362 InStat (GraphPad Software Inc., San Diego, CA, EE.UU.) was used to carry out the
363 analysis.

364

365 **3. Results and discussion**

366

367 **3.1. Nanospheres optimization**

368 The results obtained from the central composite factorial design are shown in Table 1.
369 EE is greatly influenced by pH (Figure 1a) and decreases at alkaline media. Therefore, a
370 low pH value would have to be chosen. Moreover, this acidic media would contribute to
371 obtain a monodisperse population, as the alkaline pH values were shown to increase PI
372 (Figure 1b). However, acidic pH contributes to sample instability by decreasing the ZP
373 in absolute values (Figure A.1 of Supplementary Material). In order to obtain a balance
374 between the long-term stability of the particles and the physicochemical NSs parameters, a
375 pH of 3.5 was selected (F1, Table 1).

376

377 The increase of DXI concentration in the formulations did not have a significant effect
378 on the EE, suggesting that the tested concentrations did not reach polymer-loading
379 capacity. Further studies with F1 particles were carried out, leading to a high EE (99 %)
380 using 45 mg of PLGA-PEG. Drug loading capacity depends on the physicochemical

381 properties of the molecule as well as of the nanoparticle polymer, and also on the
 382 manufacturing process for these nanoparticle systems [27]. In our case, despite the small
 383 drug concentration in F1 (0.5 mg/ml), some authors have suggested that DXI is more
 384 effective than the racemic counterpart (ibuprofen) in a ratio 1:0.5 [3]. Thus, this
 385 concentration would theoretically be enough to treat corneal inflammation. A second
 386 formulation, containing an identical drug/polymer ratio but twice the amount of the drug
 387 (1 mg/ml), was also developed and characterized for storage stability, inflammation and
 388 irritation assays. Both formulations have been studied: F(A) for NSs containing DXI 0.5
 389 mg/ml, and 45 mg of polymer and F(B) for NSs containing 1 mg/ml DXI and 90 mg of
 390 polymer.

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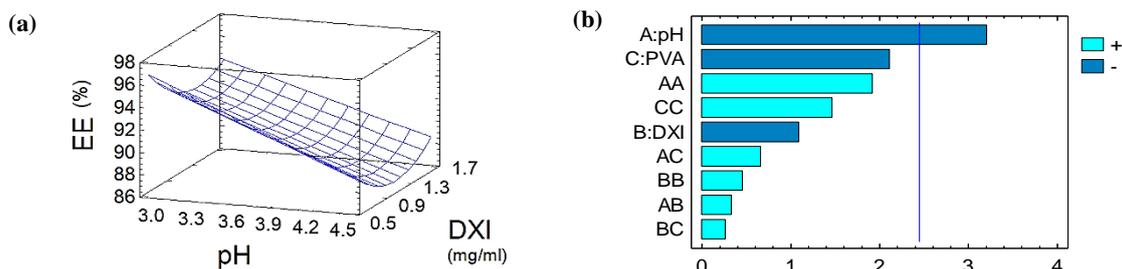
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Figure 1. Optimization of DXI NSs. (a) EE (%) surface response at a fix PVA concentration (0.75%), (b) PI surface response at a fix DXI concentration (0.5 mg/ml).

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3.2. Nanospheres characterization and interaction studies

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TEM images (Figure A.2, Supplementary Material) reveal that the optimized NSs showed spherical shape without signals of aggregation phenomena. The mean NSs size was similar to that obtained by PCS (< 200 nm).

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In order to study interactions between drug and NSs, spectroscopic analysis and DSC studies were carried out.

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XRD profiles of DXI (Figure 2a) show sharp intense peaks of crystallinity whereas the polymer diffracted an amorphous pattern. PVA exhibits a peak at $2\theta = 20^\circ$ due to its

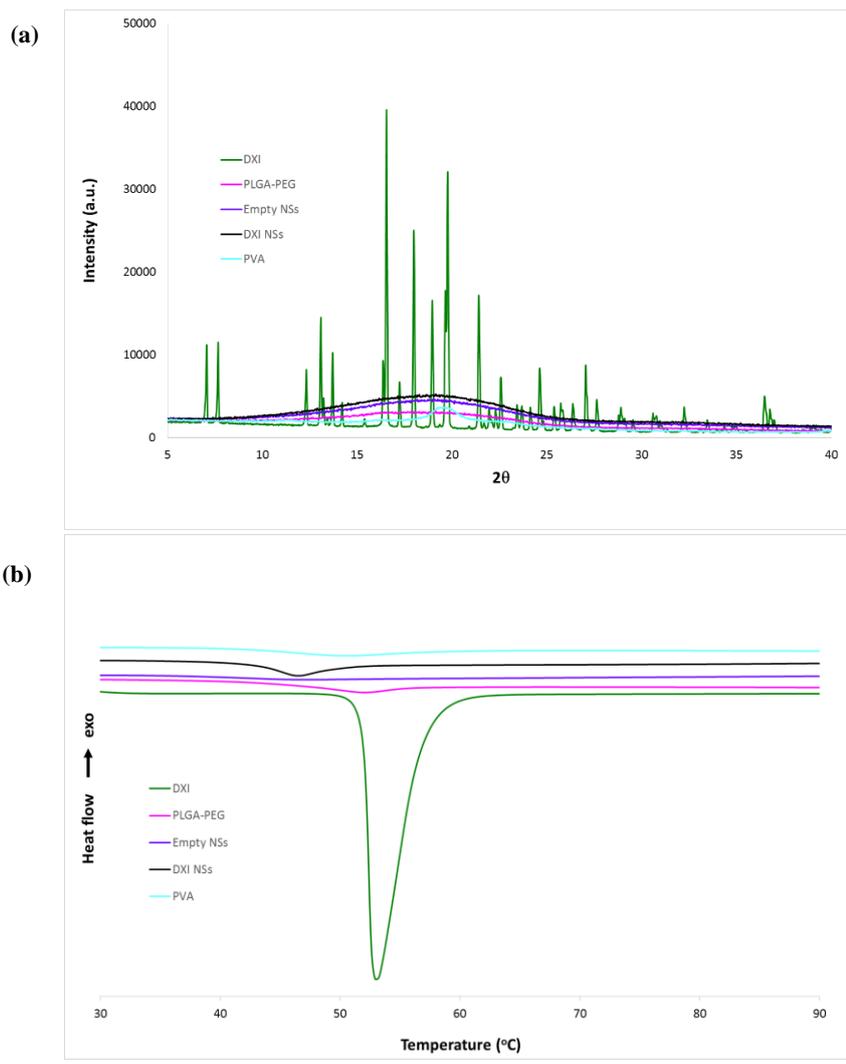
426 semi-crystalline state. Empty NSs, drug loaded NSs and PLGA-PEG present similar
427 profiles. The peaks corresponding to the drug were not detected in DXI NSs. This may
428 indicate that the drug was present mainly in the dissolved state (molecular dispersion)
429 [30].

430 FTIR analysis was used to study the interactions between the drug and polymer. There
431 was no evidence of strong bonds between DXI and PLGA-PEG or between NSs and the
432 polymer (Figure A.3, Supplementary Material). DXI presents a peak at 1697 cm^{-1} due to
433 C=O stretching, some small peaks corresponding to C-C stretching (1403 , 1461 and 1504
434 cm^{-1}) and C-O (1277 cm^{-1}) and finally a peak at 777 cm^{-1} corresponding to OH bending
435 [31]. PLGA-PEG exhibits intense bands at 2907 and 2950 cm^{-1} corresponding to the C-H
436 stretching, also present in NSs formulations. An intense peak at 1743 cm^{-1} is shown by
437 the polymer and the developed formulations, this corresponds to the C=O stretching
438 vibration of the carbonyl groups present in the two monomers that form the polymer
439 matrix. Bands obtained 1077 , 1199 and 1305 cm^{-1} in the developed formulations and in
440 the PLGA-PEG profile were attributed to stretching vibrations of the OH group [32]. The
441 pattern displayed by both empty and drug loaded NSs correspond to the polymer bands,
442 but their absorbance was probably increased due to the DXI present in the DXI-PLGA-
443 PEG NSs. It is worth to remark that neither empty nor DXI NSs showed the characteristic
444 peak corresponding to PVA (at 3300 cm^{-1}), indicating an effective reduction of the
445 surfactant amount by centrifugation process [15].

446

447 DSC profiles of DXI (Figure 2b) show a sharp endotherm corresponding with its melting
448 transition characterized by a $\Delta H=86.35\text{J/g}$ and a $T_{\text{max}}=53.06\text{ }^{\circ}\text{C}$, which were not detected
449 in DXI-PLGA-PEG NSs [31]. This fact suggests that DXI formulated in PLGA-PEG NSs
450 are in an amorphous or disordered crystalline phase of a molecular dispersion or a solid
451 solution state in the polymer matrix [26]. These results are in accordance to those obtained
452 by other authors [4]. The polymer presented the onset of the glass transition (T_g) at 43.50
453 $^{\circ}\text{C}$, whereas the NSs presented the onset at $42.50\text{ }^{\circ}\text{C}$, due to drug-polymer interaction. The
454 slight decrease of NSs T_g against polymer T_g has been attributed to the effect of the acidic
455 drug due to weak interactions with PLGA [33] and [34]. PVA showed a peak at 193.55
456 $^{\circ}\text{C}$ which was not present in the developed formulations (data not shown).

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Figure 2. Physical characterization of DXI-PLGA-PEG NSs, empty PLGA-PEG NSs and NSs compound separately. **(a)** X-Ray diffraction patterns, **(b)** Differential scanning calorimetry.

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3.3. *In vitro* drug release

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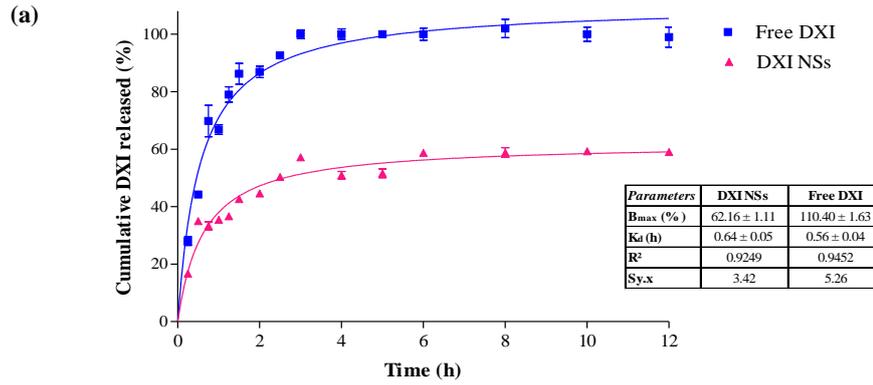
The release profiles of free DXI and DXI loaded NSs are shown in Figure 3a. As expected, free DXI showed faster release kinetics than the drug-loading particles. After three hours, the free drug achieved 100% release, whereas after 12 hours the NSs have released 55 % of the initial amount [35]. This assay confirms that NSs could release the drug at a faster rate during the first 3 hours followed by a slower diffusion (Figure 3a, triangle symbols), which would assure a prolonged effect by a slower drug release. Some authors describe that the drug can be released from PLGA matrix either via diffusion, polymer erosion or by a combination of both mechanisms. But if drug diffusion is faster than matrix degradation, drug release occurs mainly by diffusion [4] and [26]. In our case, a burst effect was observed, due to the fraction of DXI, which is absorbed or weakly bounded to the large surface area of the NSs. The second part of the profile corresponds to a sustained release behavior, where the loaded DXI slowly diffuses from the polymeric matrix to the

477 release medium. In order to ascertain the kinetic model that better fits for DXI release,
478 data were adjusted to the most common kinetic models [36]. The most appropriate release
479 profile corresponds with a hyperbola equation. NSs K_d was higher than the free drug, this
480 confirms the slower DXI release from the particle matrix. These results indicate that the
481 developed formulations could offer a prolonged release of DXI from the polymeric matrix
482 where it is dispersed [26].

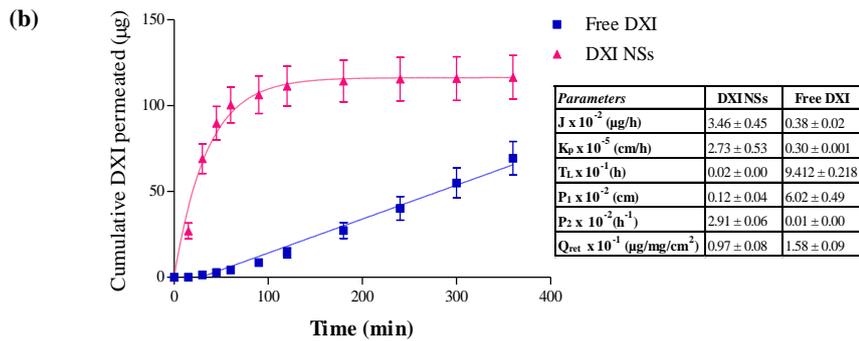
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484 **3.4. *Ex vivo* corneal and scleral permeation study**

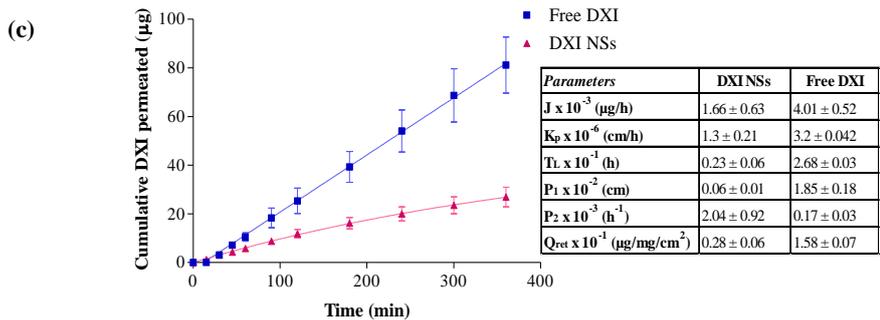
485 An *ex vivo* corneal and scleral permeation study, comparing NSs formulation with the
486 free DXI, was carried out for 6 hours. Results and permeation parameters are summarized
487 in Figure 3b and 3c. J and K_p values in the cornea and the sclera are both similar in free
488 DXI, whereas DXI NSs present high corneal permeation and accumulation in the cornea
489 than in the sclera. This fact could be useful due to drug effect on the cornea and aqueous
490 humor. Moreover, the amount of drug released through the cornea was higher in DXI NSs
491 than in free DXI and the opposite effect was found on the sclera. This study shows that
492 DXI NSs may deliver the drug effectively to the specified area by releasing DXI slowly
493 across the corneal tissue which would be useful in the treatment of inflammatory process
494 such as that induced by cataract surgery. T_L corresponding with DXI NSs on the cornea
495 and sclera are smaller than free DXI; thus displaying a sustained release achieved by the
496 NSs on the studied tissues.



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Figure 3. Release profiles of free DXI against DXI-PLGA-PEG NSs (n=6/group).
 (a) *In vitro* release, (b) *Ex vivo* corneal permeation, (c) *Ex vivo* scleral permeation

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3.5. Short-term stability

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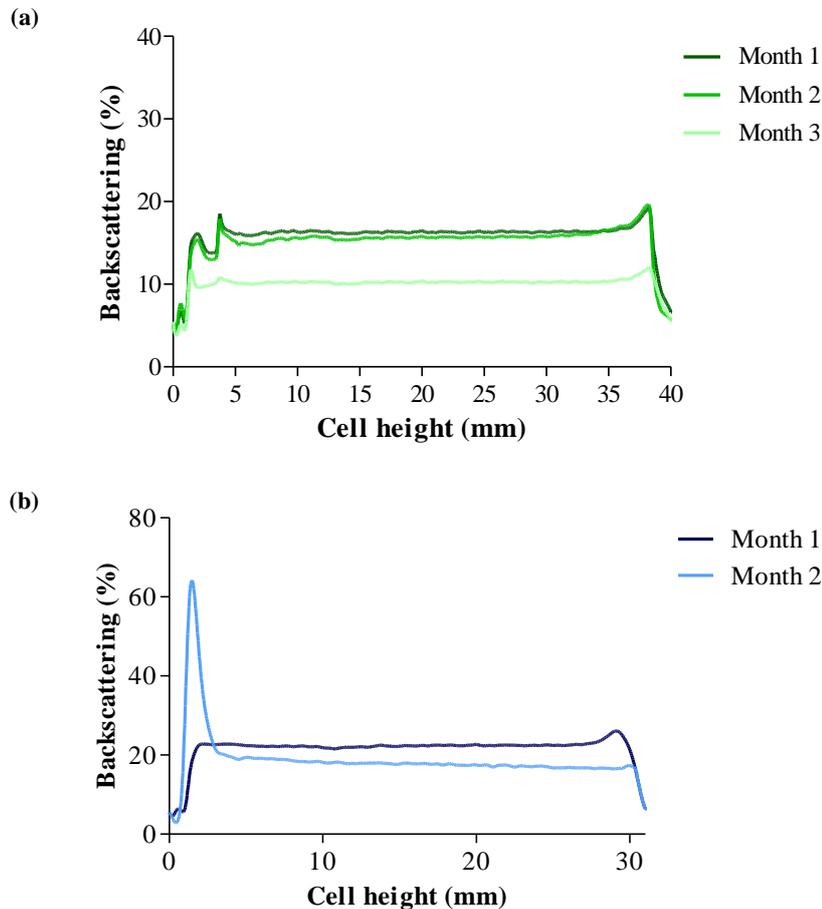
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Figure 4 shows the evolution of the backscattering (BS) profile of DXI loaded NSs during the first months of storage. The above graphs show instability, affecting the homogeneity of the sample (fluctuations in BS signals, lower than 10%) in the third month of storage for F(A). The particles showed a significant decrease of the surface charge in the third month, in accordance with backscattering results (Table A.4, Supplementary Material). Due to the aggregation phenomena, F(B) was shown to be unstable at the end of the second month of storage (Fig. 4b), decreasing the ZP and increasing particle size. The limited stability of polymeric NPs in aqueous suspension is well known and these results confirm that in order to improve long-term stability, the removal of water from the solution (either by freeze drying or spray drying) is necessary [22]. Having into account

516 the fact that the expiration date of collyria is limited to one month after opening the bottle,
517 the stability of freeze dried samples, reconstituted before application, would be more
518 suitable for ocular administration.
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Figure 4. DXI-PLGA-PEG NSs backscattering profile. (a) F(A), (b) F(B).

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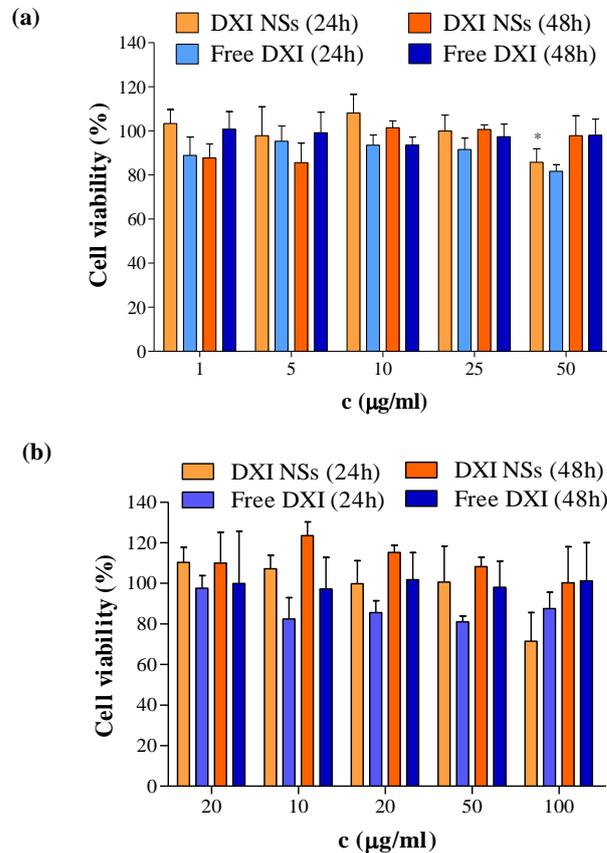
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3.6. Cytotoxicity assay

Evaluation of cell viability is important to ensure the safety of the developed NSs and avoid cell cytotoxicity. Our results demonstrate that F(A) NSs are safer, in the first 24 h, than the free drug, in all the tested concentrations (Figure 5a). NSs with a concentration of 50 $\mu\text{g/ml}$ slightly decreased cell viability (15% decrease). Although cell viability of the free DXI was lower than the obtained with the F(A) NSs at 24 h, both exhibit cell viability above 80 %. After 48 h, cells exposed to free DXI showed more than 90 % survival, probably due to DXI metabolism by the cytochrome P450. This could be due to metabolite formation, namely 2-[4-(2-hydroxy-2- methylpropyl)phenyl] propionic acid and 2-[3-(2-carboxypropyl)phenyl] propionic acid within 48 h of contact, which are not toxic for the cell [37].

The results from cell viability studies corresponding to F(B) NSs are shown in Figure 5b. Cells exposed to the concentration of 100 $\mu\text{g/ml}$ of NSs showed 70 % cell viability in the

539 first 24 h, whereas at 48 h the same concentration did not show cytotoxic effects,
 540 attributed to a drug degradation mechanism. Regarding the other concentrations, NSs were
 541 safe and produced higher rates of cell survival than the free drug. No statistically
 542 significant differences were detected when comparing free drug and the NSs for F(A) and
 543 F(B).
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547 **Figure 5.** Alamar Blue cytotoxicity of DXI NSs against free DXI. (a) F(A) DXI-PLGA-
 548 PEG NSs at different concentrations, (b) F(B) DXI-PLGA-PEG NSs at different
 549 concentrations.

550 Values are expressed as mean \pm SD; * $p < 0.05$, significantly lower than the same
 551 formulation at different time of exposure.

552

553

3.7. *In vitro* ocular tolerance

554 *In vitro* ocular tolerance was studied using the HET-CAM test. An addition of 0.9 %
 555 saline solution to the healthy membranes produced no visual response over a five minutes
 556 period. In contrast, 1M NaOH produced severe, hemorrhage, which increased over five
 557 minutes grading this solution as severe irritant. Application of 300 μ l of the samples
 558 (F(A), F(B) or PBS solution containing 0.5 or 1 mg/ml DXI) into the chorioallantoic
 559 membrane, revealed optimal ocular tolerance in the first 5 minutes of application (Figure
 560 A.4, Supplementary Material) [38]. OII for all tested samples show a non-irritant reaction
 561 (Table A.5, Supplementary Material). These results are in accordance to those obtained
 562 by other authors loading NSAIDs into PLGA NSs for ocular applications [15] and [39].

563

564 **3.8. *In vivo* ocular tolerance**

565

566 A single *in vitro* test could not properly mimic the entire situation *in vivo*, therefore,
567 tolerance assays in male albino rabbits were performed. The OII obtained for both F(A)
568 and F(B) and for free DXI was null (Figure A.5, Supplementary material), being the NSs
569 classified as non-irritant (Table A.5, Supplementary Material). These results are in
570 agreement to those obtained with the HET-CAM test, confirming the suitability of the *in*
571 *vitro* method and the non-irritant properties of the developed formulations for ocular
572 administration [26], [38] and [40].

573

574 **3.9. Inhibition of the inflammation**

575

576 Two studies were performed to determine the anti-inflammatory efficacy of the
577 developed NSs, in order to confirm their usefulness for preventing and treating
578 inflammation.

579 F(A) NSs prevent inflammation showing significant differences regarding positive
580 control within the first 30 minutes after SA administration ($p < 0.01$) (Figure 6a). As
581 described elsewhere, this correlates with the amount of drug retained in the cornea [15].
582 In addition, free DXI at 0.5 mg/ml also prevents inflammation compared to the control
583 which shows significant differences after 1 hour of testing. This demonstrates that
584 reduced DXI doses are an effective therapeutic agent for ocular inflammation. In addition,
585 encapsulation in polymeric NSs, increases drug effect. These results show that F(A)
586 would be adequate to prevent ocular inflammation. F(B) also significantly reduced
587 inflammation significantly after 30 minutes of application ($p < 0.001$). In general, PLGA-
588 PEG nanoparticles enhance ocular bioavailability of drugs due the especial behaviour of
589 PEG that facilitates the drug-mucin interactions [12] and [41].

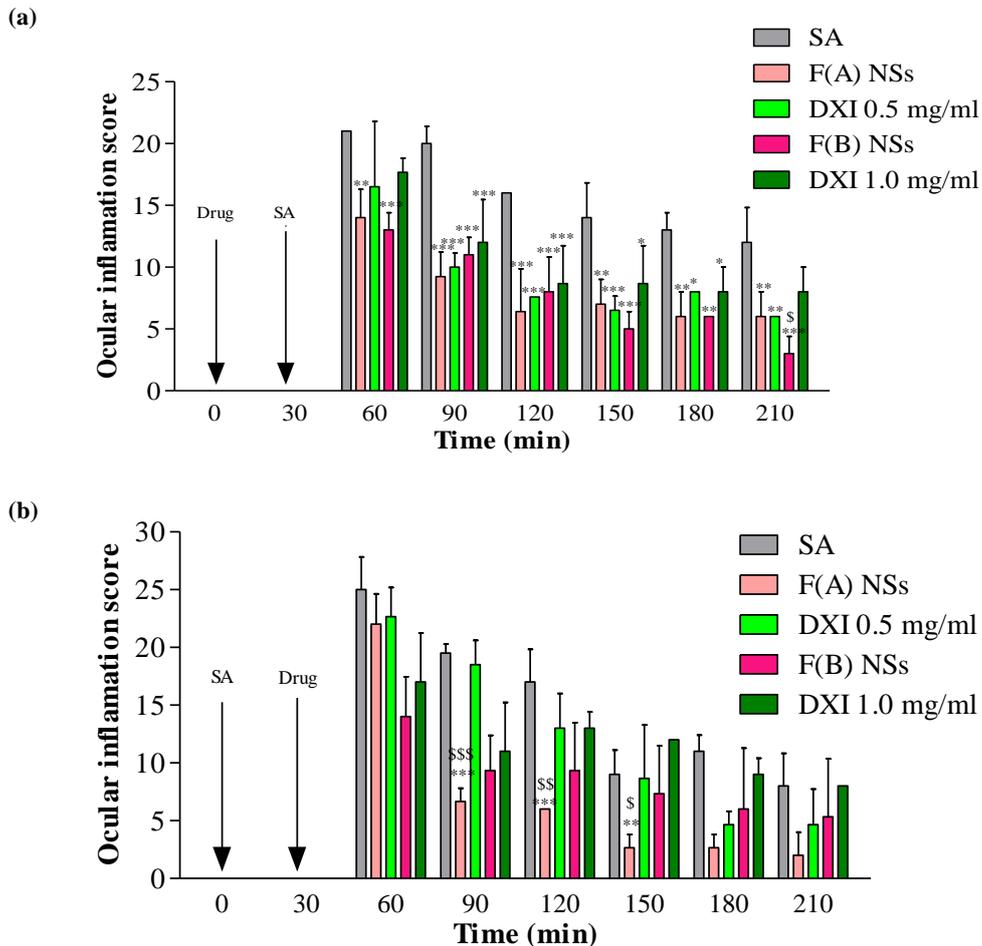
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591 Conjunctival inflammation with significant hyperemia was induced by SA after 30
592 minutes of exposure. At this time, the drug was applied to the conjunctival sac and degree
593 of inflammation was measured accurately. Formulations containing 1 mg/ml of DXI,
594 F(B) and the corresponding free drug, reduced the inflammation faster (Figure 6b),
595 making it adequate for high rates of inflammation which need an emergency treatment.
596 However, F(A) demonstrated to reduce the inflammatory response more effectively than
597 the control after 1 hour of application ($p < 0.001$).

598 The differences observed in degrees of inflammation and the prevention and treatment of
599 this pathology could be related to the different absorption of NSs in healthy and inflamed
600 tissues. This could be because the instillation of SA prior to the administration of
601 developed formulations lead to enhanced lacrimation increasing precorneal loss and
602 clearance of NSs [42]. F(A) would be adequate for the prevention of inflammatory
603 injuries (e.g. cataract surgery) reducing inflammation and probably provides less adverse
604 systemic effects than free drug or F(B) NSs [42].

605 The results obtained in this study are in accordance with other literature data, such as
606 studies carried out by Buccolo et al [43] and also Musumeci et al [29] with melatonin-

607 loaded PLGA-PEG, that show a higher prolonged lowering of IOP in rabbits for
 608 melatonin-PLGA-PEG nanoparticles than obtained without drug encapsulation. In these
 609 systems mucoadhesion can be related to the PEG crown which allows a better and longer
 610 interaction between the nanocarrier and the eye [44]. Similar results were obtained for
 611 acyclovir-loaded PEG-PLA nanospheres [45].
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614

615 **Figure 6.** Comparison of ocular anti-inflammatory efficacy of F(A), F(B) and the free
 616 DXI. **(a)** Inflammation treatment, **(b)** inflammation prevention.
 617 Values are expressed as mean \pm SD; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ significantly
 618 lower than the inflammatory effect induced by SA; \$ $p < 0.05$, \$\$ $p < 0.01$ and \$\$\$ $p < 0.001$
 619 significantly lower than the inflammatory effect induced by the corresponding free drug.
 620
 621

622 Globally, F(A) would be a sufficient treatment in the prevention of inflammation and the
 623 treatment of medium-low inflammation pathologies. In the case of rescue treatment, both
 624 slight free DXI at 1 mg/ml and F(B) would be suitable, representing the NSs an
 625 improvement in reducing corneal inflammation levels. The NSs improvement during the
 626 first hour could be due to NSs corneal preference. As demonstrated by the ocular
 627 permeation studies, free DXI is distributed and retained in the cornea and the sclera
 628 indistinctively, whereas DXI NSs provide higher drug levels on the cornea, as well a
 629 higher drug penetration to achieve aqueous humor.

630

631 **3.10. Ocular drug bioavailability**

632 In order to elucidate NSs amount into eye structures, F(A) was administered *in vivo* and
633 DXI amount was quantified 2 hours after the last administration. DXI amount in the
634 cornea (3.08 µg/ml) was higher in comparison to every other tissue, including the sclera
635 (1.28 µg/ml). These results are in accordance to those obtained in *ex vivo* corneal and
636 scleral permeation study. As reported by other authors [43], a certain amount of drug was
637 also measured in the aqueous humor (in our case, 0.32 µg/ml), but no DXI was found in
638 the vitreous humor. These results demonstrate that DXI NSs remained retained in the first
639 structures of the eye and released the drug slowly to inner tissues such as aqueous humor.

640 Moreover, it has been demonstrated that with small amount of drug, the active enantiomer
641 loaded within NSs achieves an effective ocular anti-inflammatory activity, thus probably
642 leading to a potential reduction in adverse effects.

643

644 **4. Conclusions**

645

646 Ocular administration for the treatment of pathological eye tissues offers the advantage
647 of delivering the drug directly to the site of action whilst providing high drug
648 concentration. In this study PLGA-PEG NSs were developed for topical delivery of DXI.
649 The DoE approach shows that pH was one of the most influential parameters on the
650 preparation of the nanoparticles. The optimized formulations of NSs were shown to be
651 monodisperse (PI < 0.1), with a mean particle size smaller than 200 nm, with a negative
652 surface charge and high EE. DSC studies showed that DXI was distributed as a molecular
653 dispersion inside the polymeric matrix. XRD showed evidence of the drug loaded within
654 the NSs. FTIR studies showed that there was no evidence of chemical interaction or strong
655 bond formation between the NSs compounds. F(A) NSs showed stability at 25 °C for
656 three months, whereas F(B) NSs showed a sedimentation process in the second month
657 probably due to an increase in polymer and drug concentration that, in addition,
658 contributed to their interactions. DXI *in vitro* release from the polymeric matrix was
659 slower than the release of free drug. *Ex vivo* and *in vivo* studies confirmed that NSs
660 permeate better through corneal tissue than free DXI. The opposite effect was observed for
661 the sclera, thus confirming that NSs were appropriate for the treatment of corneal
662 inflammation. Cytotoxicity studies show that NSs do not reduce significantly cell
663 viability with respect to the free drug. Both presented high survival percentages. HET-
664 CAM assay results correlate with Draize test, both showing good ocular tolerance for the
665 developed colloidal systems. *In vivo* assays with F(A) showed therapeutic effects on
666 prevention and inflammation treatment.

667 Our study demonstrates the advantages of using DXI-loaded PLGA nanospheres coated
668 with PEG for prophylaxis of eye inflammation and/or for the treatment of non-severe
669 inflammatory processes. The results obtained from the pharmacokinetic studies confirm
670 the capacity of the developed PLGA-PEG NSs to achieve a sustained release of DXI,
671 therefore reducing its systemic absorption and associated side effects.

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673

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