PEGylated and poloxamer-modified chitosan nanoparticles incorporating a lysine-based surfactant for pH-triggered doxorubicin release

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ABSTRACT

The growing demand for efficient chemotherapy in many cancers requires novel approaches in target-delivery technologies. Nanomaterials with pH-responsive behavior appear to have potential ability to selectively release the encapsulated molecules by sensing the acidic tumor microenvironment or the low pH found in endosomes. Likewise, polyethylene glycol (PEG)- and poloxamer-modified nanocarriers have been gaining attention regarding their potential to improve the effectiveness of cancer therapy. In this context, DOX-loaded pH-responsive nanoparticles (NPs) modified with PEG or poloxamer were prepared and the effects of these modifiers were evaluated on the overall characteristics of these nanostructures. Chitosan and tripolyphosphate were selected to form NPs by the interaction of oppositely charged compounds. A pH-sensitive lysine-based amphiphile (77KS) was used as a bioactive adjuvant. The strong dependence of 77KS ionization with pH makes this compound an interesting candidate to be used for the design of pH-sensitive devices. The physicochemical characterization of all NPs has been performed, and it was shown that the presence of 77KS clearly promotes a pH-triggered DOX release. Accelerated and continuous release patterns of DOX from CS-NPs under acidic conditions were observed regardless of the presence of PEG or poloxamer. Moreover, photodegradation studies have indicated that the lyophilization of NPs improved DOX stability under UVA radiation. Finally, cytotoxicity experiments have shown the ability of DOX-loaded CS-NPs to kill HeLa tumor cells. Hence, the overall results suggest that these pH-responsive CS-NPs are highly potent delivery systems to target tumor and intracellular environments, rendering them promising DOX carrier systems for cancer therapy.

Keywords: chitosan nanoparticles; doxorubicin; *in vitro* release; *in vitro* cytotoxicity; lysine-based surfactant; pH-sensitivity

1. Introduction

52 Doxorubicin (DOX) is an anthracycline antibiotic commonly used as a chemotherapeutic agent

53 [1]. Due to its broad-spectrum of antitumor activity, it has been incorporated into several nano-

54 sized materials, including pH-responsive microgels [2], temperature-responsive micelles [3],

55 liposomes [4] and polymeric nanoparticles (NPs) [5,6]. DOX antineoplastic effects can occur

56 by different mechanisms, such as free radical generation, which is well associated with the

57 cardiotoxicity of anthracyclines [7]. Drug delivery systems have been gaining attention in recent

58 years as a promising approach to improve cancer treatment and prevent toxicity in healthy

59 tissues. It is noteworthy that by adding different modifiers, these systems can be designed for

60 cancer cell-specific targeting as well as for biological, chemical, or physical stimulus response

61 [8,9].

62   Considering that endosomal pH ($\sim$ 6.5 to 5.5) [10] and the tumor extracellular pH (pH_e

63 $\sim$ 6.6) are notably lower than those of normal tissue (pH $\sim$ 7.4) [11], pH-sensitive devices have

64 been widely researched as drug delivery strategies for cancerous diseases [9]. In this context,

65 our group has paid special attention to a bioactive amino acid-based surfactant derived from

66 N^α,N^ε-dioctanoyl lysine with an inorganic sodium counterion (77KS), which in previous studies

67 shown pH-responsive properties and low cytotoxicity [12-14]. Therefore, here we selected

68 77KS as an adjuvant with potential ability to promote the pH-triggered DOX release in the

69 tumor microenvironment and endosomal compartments (Fig. 1).

70   Chitosan (CS) is a nontoxic, biocompatible and biodegradable polymer that has been

71 emerging as one of the most promising delivery vehicles for cancer chemotherapy [15].

72 Chitosan has been widely used for the development of DOX-loaded NPs by simple and mild

73 preparation techniques [5,16-18]. CS-NPs modified by polyethylene glycol (PEG) are explored

74 due to the ability of this hydrophilic polymer to prolong the circulation time of nanocarriers in

75 the blood stream. This mechanism allows NP accumulation in the tumor region via enhanced

76 permeability and retention (EPR) effect, which, in turn, increases tumor exposure to the

3

encapsulated drug [19-22]. Likewise, Pluronic block copolymers (or non-proprietary name "poloxamer") have been studied as biological response modifiers. They are amphiphilic synthetic polymers with tumor-sensitizing activity in multidrug resistant (MDR) cells, which is especially attributed to the inhibition of P-glycoprotein [23]. For this reason, it has been reported that the association of DOX to poloxamer-based formulations potentiates the drug activity against non-MDR and, especially, MDR tumor cells [24-26].

Therefore, the aim of the present study was to prepare PEGylated and poloxamer-modified CS-NPs incorporating a lysine-based surfactant as a pH-responsive bioactive adjuvant. The NPs were well characterized and the mathematical modeling of pH-triggered DOX release profiles was discussed. NP suspensions and lyophilized samples were analyzed regarding their stability at low temperature and under UVA radiation. Finally, in order to gain preliminary insights into the role of the modifiers on the antitumor activity of NPs, the cytotoxicity of free and entrapped drug was assessed by an *in vitro* cell-based assay.

2. Materials and methods

2.1. Materials

Chitosan (CS) of low molecular weight (deacetylation degree, 75-85%; viscosity, 20-300 cP according to the data sheet of the manufacturer), pentasodium tripolyphosphate (TPP), polyethylene glycol methyl ether (mPEG, M_n = 5,000), poloxamer 188 solution (10%, w/v) and 2,5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl) and tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents for cell culture were from Vitrocell (Campinas, SP, Brazil). Doxorubicin (DOX, state purity 98.32%) was obtained from Zibo Ocean International Trade (Zibo, Shangdong, P.R., China). Acetonitrile and glacial acetic acid were purchased from Tedia (Fairfield, USA). All other solvents and reagents were of analytical grade.

2.2. Surfactant included in the nanoparticles

An anionic amino acid-based surfactant derived from $N^{\alpha},N^{\varepsilon}$-dioctanoyl lysine and with an inorganic sodium counterion (77KS) was included in the NP formulation. The surfactant chemical structure is formed by two alkyl chains (each with eight carbon atoms) bound to the amino acid lysine. It has a molecular weight of 421.5 g/mol and a critical micellar concentration (CMC) of 3 x 10^3 µg/ml [27,28]. This surfactant was synthesized as described elsewhere [29].

2.3. Preparation of nanoparticles

CS-NPs were spontaneously formed by ionotropic gelation process, according to the methodology first described by Calvo et al. [30]. DOX stock solution was prepared in ultrapure water in order to give a final concentration of 2.0 mg/ml. Chitosan at 1.0 mg/ml was dissolved in a 1.0% (v/v) acetic acid aqueous solution under magnetic stirring for 2 h, and pH was adjusted to 5.5 with 10 M NaOH [31]. A mixed solution of the cross-linker TPP and the surfactant 77KS was prepared in ultra-pure water at 2.0 mg/ml and 0.5 mg/ml, respectively. Initially, DOX stock solution was added to 5 ml of CS solution (CS:DOX ratio 5:0.5, w/w) and maintained under magnetic stirring (1000 rpm) for 10 min. Then, 1 ml of a premixed TPP:77KS solution (ratio equal 2:0.5, w/w) was added drop-wise into the CS:DOX solution. NPs (DOX-CS-NPs) were formed spontaneously and the gelation process was carried out under constant magnetic stirring for 20 min at room temperature.

In order to obtain PEGylated DOX-CS-NPs (PEG-DOX-CS-NPs), a mixed solution of CS and PEG (at 1 mg/ml and 10 mg/ml, respectively) was prepared in 1.0% (v/v) acetic acid. To 5 ml of this solution, DOX stock solution was added and stirred for 10 min (CS:PEG:DOX ratio 5:50:0.5, w/w/w). Afterwards, 1 ml of TPP:77KS (2:0.5, w/w) was added drop-wise and stirred for 20 min.

124   Poloxamer-modified DOX-CS-NPs (Polox-DOX-CS-NPs) were obtained by adding

125 0.5% (w/v) of poloxamer to 5 ml of a 1 mg/ml CS solution. Next, DOX stock solution was

126 added to give a final ratio of CS:Poloxamer:DOX 5:25:0.5 (w/w/w). Finally, 1 ml of TPP:77KS

127 (2:0.5, w/w) was added drop-wise under vigorous magnetic stirring for 20 min.

128   Unloaded NPs were prepared similarly for each formulation, thus omitting the drug. All

129 procedures involving DOX were conducted in a low incidence of light. The resulting DOX-

130 loaded NPs were purified by dialysis for 1 h in distilled water (dialysis bag - Sigma-Aldrich,

131 14,000 MWCO), in order to remove the non-encapsulated drug and non-incorporated

132 constituents.

133 *2.4. Characterization of nanoparticles*

134 The mean hydrodynamic diameter and the polydispersity index (PDI) of the NPs were

135 determined by dynamic light scattering (DLS) using a Malvern Zetasizer ZS (Malvern

136 Instruments, Malvern, UK), without any dilution of the samples. The zeta potential (ZP) values

137 of the NPs were assessed by determining electrophoretic mobility using the same equipment

138 after dilution of the formulations in 10 mM NaCl aqueous solution (1:10 volume per volume).

139 Each measurement was performed using at least three sets of ten runs at 25°C. The pH

140 measurements were verified directly in the NP suspensions, using a calibrated potentiometer

141 (UB-10; Denver Instrument, Bohemia, NY, USA), at room temperature. Finally, the spectral

142 properties of the drug were assessed before its encapsulation and also after extraction from the

143 NP structure. This assay was performed in order to verify the stability of DOX after entrapment

144 into the NP matrix. Experiments were performed on a double-beam UV-Vis spectrophotometer

145 (Shimadzu, Japan) model UV−1800, with a fixed slit width (2 nm) and a 10 mm quartz cell was

146 used to obtain spectrum and absorbance measurements. The diluent optimized was water pH

147 3.0, acidified with glacial acetic acid.

148   *2.5. Drug encapsulation efficiency*

149   The quantitative analyses were performed by a reversed-phase liquid chromatography (RP-LC)

150   method that was previously validated according to international guidelines and proved to be

151   specific, linear, precise, accurate and robust (unpublished data). Chromatographic analyses were

152   carried out on a LC 1260 Agilent Technologies system (Agilent Technologies, Santa Clara, CA,

153   USA), using a Waters XBridgeTM C18 column (250 mm x 4.6 mm I.D., 5μm), with a mobile

154   phase consisting of 90% (v/v) acetonitrile in water and water pH 3.0, acidified with glacial acetic

155   acid (33:67, v/v) and UV detection set at 254 nm. Data analysis was performed with EZChrom

156   software program (version A.01.05). Total drug content was achieved by dilution of the NP

157   suspensions in methanol (1:1, v/v) followed by sonication for 15 min, which allowed total drug

158   extraction from the NP matrix. The resulting solution was diluted to the suitable concentration

159   and   analyzed   by   RP-LC.   The   drug   association   efficiency   was   determined   by

160   ultrafiltration/centrifugation technique using Amicon Ultra-0.5 Centrifugal Filters (10,000 Da

161   MWCO, Millipore). An amount of the non-purified NP suspension was placed into this device

162   and submitted to 10,000 rpm for 20 min in a Sigma 2-16P Centrifuge (Sigma, Germany). The

163   encapsulation efficiency (EE%) was calculated as the difference between total and free DOX

164   concentrations determined in the NP suspension (total drug content) and in the ultrafiltrate,

165   respectively, using the mentioned analytical method.

166   *2.6. pH-dependent in vitro DOX release*

167   *In vitro* release assessments of DOX from the different CS-NPs were performed using the

168   dialysis method. An aliquot of the NPs (1 ml) was placed into a dialysis bag (Sigma-Aldrich,

169   14,000 MWCO), which was immersed in 50 ml of phosphate buffered saline (PBS) at 37ºC and

170   kept under gentle magnetic stirring (100 rpm) for 24 h. This process was carried out, separately,

171   in PBS at pH 7.4, 6.6 and 5.4. At specific time intervals, an aliquot of 2 ml of the external

172 medium was withdrawn and filtered through a 0.45-μm membrane. Thereafter, equal volume of

173 fresh buffer was added to maintain the sink conditions and constant volume. The release of the

174 free drug was also investigated in the same way. The released amount of DOX in each scheduled

175 time was estimated by the RP-LC method described in the previous section (*section 2.5*), using

176 analytical curves obtained with the release medium (PBS at pH 7.4, 6.6 or 5.4) as diluents. The

177 cumulative release percentage (CR%) of DOX was determined from the following equation (Eq.

178 (1)):

179 $CR\% = (M_t/M_i)\ 100$ (1)

180 where M_t and M_i are the amount of drug released at the time t and the initial amount of drug

181 encapsulated in the NPs, respectively. The *in vitro* release studies were carried out in triplicate.

182   For understanding the pH-sensitivity behavior of NPs, swelling studies were performed

183 by soaking lyophilized NPs into PBS pH 7.4, 6.6 and 5.4 at room temperature and under gentle

184 shake. Hydrodynamic diameter was measured after 3 h incubation.

185 *2.7. Mathematical modeling of drug release profiles*

186 Monoexponential (Eq. (2)) and biexponential (Eq. (3)) mathematical models as well as the

187 Korsmeyer-Peppas model (Eq. (4)) were used to analyze DOX *in vitro* release profile

188 (MicroMath® Scientist version 2.01, USA). The model that best fit the drug release profile was

189 selected according to the model selection criteria (MSC), correlation coefficient (*r*), and

190 graphical adjustment. The release kinetic rate constants are *k* (for monoexponential), k_1 and k_2

191 (for biexponential). C_0, *a* and *b* are the initial concentration for mono- and biexponential models,

192 respectively [32,33]. Finally, the DOX release mechanism was investigated by fitting 60% of

193 the initial amount of drug released from CS-NPs to the Korsmeyer-Peppas model. In its

194 corresponding equation, *n* is the exponent that characterizes the release mechanism and *a* is a

195 constant comprising the structural and geometric characteristics of the carrier [34-36].

196     $$C = C_0 e^{-kt} \tag{2}$$

197     $$C = a\, e^{-k_1 t} + b\, e^{-k_2 t} \tag{3}$$

198     $$ft = a\, t^n \tag{4}$$

199   *2.8. Lyophilization of nanoparticles*

200   NP suspensions DOX-CS-NPs, PEG-DOX-CS-NPs and Polox-DOX-CS-NPs were subjected to

201   the lyophilization process to obtain dried formulations (L-DOX-CS-NPs, L-PEG-DOX-CS-NPs

202   and L-Polox-DOX-CS-NPs, respectively). To avoid possible particle aggregation, glycerol

203   (10%, w/v), mannitol (1%, w/v) and lactose (1, 5 and 10%, w/v) were tested for their

204   cryoprotectant efficiency. Cryoprotectants were dissolved in the entire volume of NPs under

205   magnetic stirring for 20 min. Then, these mixtures were frozen at -20°C for 48 h. The water was

206   removed from frozen NPs by sublimation under vacuum for 48 h using a bench top lyophilizer

207   (Liotop L101, Liobras, São Carlos, Brazil). As required, lyophilized products were redispersed

208   with ultra-pure water by magnetic stirring for 10 min. The macroscopic appearance,

209   physicochemical properties and EE% were evaluated.

210   *2.9. Fourier Transform Infrared Spectroscopy (FT-IR) analysis*

211   In order to investigate the interactions between the drug and NP matrix, FT-IR spectra of dried

212   NPs, pure DOX, CS and 77KS raw materials were recorded using compressed KBr disk method

213   with a FT-IR spectrophotometer (Bruker Tensor 27, Bruker Optik, Ettlingen, Germany).

214   Spectral acquisition was carried out from 4000 to 400 cm^{-1} range.

215   *2.10. Stability studies of nanoparticles*

216   NP suspensions (DOX-CS-NPs, PEG-DOX-CS-NPs and Polox-DOX-CS-NPs) and the

217   lyophilized formulations (L-DOX-CS-NPs and L-PEG-DOX-CS-NPs) were studied for their

218 stability in low temperature (2 – 8°C). Experiments were conducted over 8 weeks. Lyophilized

219 samples were first placed inside a desiccator containing silica and then exposed to low

220 temperature whilst protected from light. Analyses were carried out on the first day of the study,

221 and subsequently after 2, 4 and 8 weeks. In each time point, all samples were evaluated for

222 particle size, PDI, ZP and drug content (total drug amount (%) in regard to freshly prepared

223 formulations).

224 Additionally, photostability studies were carried out to assess whether suspensions

225 and/or lyophilized formulations were able to protect the drug after exposure to UVA radiation.

226 An aliquot of DOX solution or DOX-loaded NPs was put separately into transparent capped

227 cuvettes (Brand®, UV-Cuvettes micro) and placed into a mirrored chamber with approximately

228 1,350 W h/m^2 incident UVA radiation [37]. On the other hand, an amount of the lyophilized

229 formulations were weighed and well distributed in Petri dishes. The drug concentration was

230 measured in different schedule times (0, 2, 8, 24 and 48 h) by the validated RP-LC method.

231 Zero, first and second order graphics were delineated and the one with the best fit was

232 considered to establish the kinetic order.

233 *2.11. Cytotoxicity assays*

234 The *in vitro* antitumor activity of unloaded-CS-NPs, DOX-loaded CS-NPs and free DOX was

235 determined against HeLa cell line (human epithelial cervical cancer), which was cultured in

236 DMEM medium (4.5 g/l glucose) supplemented with 10% (v/v) FBS, at 37ºC in a 5% CO_2

237 atmosphere. HeLa cells were seeded into 96-well cell culture plates at a density of 7.5 x 10^4

238 cells/ml. Cells were incubated for 24 h under 5% CO_2 at 37ºC and afterwards, the medium was

239 replaced with 100 μl of fresh medium containing the treatments. Free DOX as well as DOX-

240 loaded CS-NPs were assayed at 1 and 10 μg/ml DOX concentration, while unloaded CS-NPs

241 were assessed at 50 and 200 μg/ml. Following 24 h incubation, the medium was removed and

242   100 µl of MTT in PBS (5 mg/ml) diluted 1:10 in medium without FBS was added to the cells

243   and incubated for 3 h. Finally, the MTT containing medium was removed and 100 µl of DMSO

244   was added to each well in order to dissolve the purple formazan product. After shaking, the

245   absorbance of the resulting solution was measured using a SpectraMax M2 (Molecular Devices,

246   Sunnyvale, CA, USA) microplate reader at 550 nm. Cell viability was calculated as the

247   percentage of tetrazolium salt reduced by viable cells in each sample. The untreated cell control

248   (cells with medium only) was taken as 100% viability.

249   *2.12. Statistical analyses*

250   Results are expressed as mean ± standard error (SE) or mean ± standard deviation (SD) of three

251   independent experiments, and statistical analyses were performed using one-way analysis of

252   variance (ANOVA) to determine the differences between the datasets, followed by Tukey's

253   post-hoc test for multiple comparisons, using SPSS® software (SPSS Inc., Chicago, IL, USA).

254    $p < 0.05$ and $p < 0.01$ indicated significant and highly significant differences, respectively.

255   **3. Results and discussion**

256   In this study, NPs encapsulating DOX were prepared by combination of the standard ionotropic

257   gelation method [30] and the inclusion of procedures deliberated by our research group.

258   Therefore, novel pH-responsive CS-NPs were obtained using a mild and solvent-free process

259   for efficient drug loading [38]. CS is widely regarded as being a non-toxic and biologically

260   compatible polymer, with great medical potential [39]. Once dissolved in acetic acid aqueous

261   solution, the amino groups of CS are protonated (NH_3^+) and available to interact with the

262   negatively charged TPP ($P_3O_{10}^{5-}$) to spontaneously form the NPs [40,41]. With the aim to find

263   the suitable CS:TPP ratio (w/w), different TPP concentrations were tested since the size and

264   PDI of NPs depended on the amount of TPP in the formulation. The first condition tested was

265   CS:TPP (5:1, w/w), but the ratio CS:TPP (5:2, w/w) was chose since it presented the smallest

11

266 size and PDI value. This behavior can be attributed to the greater interaction of CS positive

267 charges with increasing amount of negative charges of the polyanion TPP [42]. These results

268 are in agreement with the study reported by Gan et al. [43], in which a linear decrease of size

269 with decreasing CS to TPP weight ratio was observed. Furthermore, it is worth pointing out that

270 by increasing the amount of negative charges into the formulation matrix, the free positive

271 charges of CS were reduced. This lower protonation diminishes the repulsion between CS and

272 DOX (also positively charged), which, in turn, increases the drug encapsulation efficiency.

273   The surfactant 77KS was selected as a bioactive adjuvant in the NP formulation based

274 on previous studies, which showed its pH-sensitive activity along with improved kinetics in the

275 endosomal pH range and low cytotoxic potential [12,13]. Moreover, it was already

276 demonstrated that the inclusion of another amphiphile from the same family (77KL, with lithium

277 counterion) in the composition of polymeric NPs improved their *in vitro* antitumor activity and

278 also gave them a pH-responsive behavior [44]. The surfactant 77KS was included into the NPs

279 at a concentration below its CMC, indicating that it is present in the formulations in the

280 monomer form. Different concentrations of the surfactant were tested, ranging from

281 CS:TPP:77KS 5:2:0.1 to 5:2:1 (w/w/w), with 0.1 increase amount of 77KS each time. By having

282 the concentration ratio of 77KS higher than 5:2:0.5, a flocculation of the NPs took place. In

283 contrast, concentrations between 0.1 and 0.5 provided satisfactory results. Therefore, the ratio

284 5:2:0.5 (w/w/w) of CS:TPP:77KS was chosen and maintained for all formulations.

285   The process to prepare the NPs was optimized to be simple and fast. Firstly, positive

286 charges (DOX and CS) were mixed [5,17] and, a premixed solution of the negatively charged

287 compounds (TPP and 77KS) was added drop-wise, leading to spontaneous formation of the

288 colloidal system. It is known that the polyanion TPP has multiple charged functional groups,

289 which makes it able to interact with both DOX and CS, resulting in shielding and electrostatic

290 interactions [17]. The pH of CS solution was set at 5.5, in which about 90% of the amino groups

291   of CS (pKa = 6.5) are protonated [45]. Likewise, DOX (pKa = 8.2) possess an amino sugar

292   moiety also protonated at this pH [46], which allowed competitive binding of DOX to the

293   negatively charged cross-linking agent (TPP) while forming the NPs.

294   The PEGylation of nanomaterials was shown not only to diminish clearance of the

295   loaded drug, but also to provide enhanced tumor targeting ability due to the prolongation of

296   plasma circulation time [47]. PEGylated DOX-CS-NPs were prepared from CS and PEG joint

297   solubilization prior to gelation process, where a CS/PEG network is formed by cross-linking

298   between hydroxyl groups of PEG and amino groups of CS [48]. Likewise, it is known that block

299   copolymers, such as the poloxamers, are biological response modifiers with potential ability to

300   modulate drug resistance in MDR cancer cells. Therefore, here poloxamer-modified DOX-CS-

301   NPs were prepared upon the addition of TPP:77KS into CS:Poloxamer:DOX solution [49].

302   Different concentrations of poloxamer were tested (0.2%, 0.5% and 1%, w/v), and the

303   intermediate one (0.5%) was chose with acceptable physicochemical characteristics. It was

304   previously reported that micelles containing block copolymers at 0.25 and 2% (w/v), in which

305   DOX is also non-covalently incorporated, exhibited greater efficacy than free DOX in *in vitro*

306   and *in vivo* tumor models [50].

307   *3.1. Characterization and EE% of nanoparticles*

308   Following the preparation procedure, the stability of the drug after its encapsulation was

309   assessed through the spectral analysis, as shown in Fig. 2. The UV-Vis spectrum of the drug

310   extracted from NPs was similar to that obtained for DOX in free solution, which proved the

311   integrity of DOX molecule after its entrapment into the NP matrix. Moreover, as summarized

312   in Table 1, DOX-loaded and unloaded NPs were characterized for particle size, PDI, ZP and

313   pH. The average particle size analysis is a common characterization method, which allows the

314   understanding of their dispersion and aggregation, as well as helping to predict their possible

315   biodistribution. The size of unloaded NPs was in the range of 170 to 211 nm. Increasing

316   diameters were noticed when DOX was added, indicating the retention of the drug. Likewise,

317   the mean diameter of PEGylated NPs increased with respect to unmodified NPs, which is a good

318   indicator of PEG incorporation into the NP structure [22]. Here, it can be stated that PEG was

319   incorporated into the colloidal gel system via hydrogen bonding between the oxygen atom of

320   PEG and amino groups of CS. This interaction is weak, which makes the structure of the

321   PEGylated NPs looser and, consequently, increases their mean diameter [20]. Conversely,

322   poloxamer-modified NPs presented smaller mean diameter than those PEG-modified NPs. This

323   is due to the stabilizer power of poloxamer, fact that leads to a rigid arrangement of particles

324   with less water uptake [49]. Additionally, all CS-based NPs formed systems with narrow size

325   distribution with PDI values lower than 0.24. The ZP values of the NPs in the range of 21 to 25

326   mV indicate a positively charged surface owing to the cationic amino groups of CS. Likewise,

327   when DOX was present, the electric charge remained positive and no considerable changes were

328   noted.

329   DOX-loaded NPs displayed high EE% and the mean values obtained for all formulations

330   were constantly around 65%. These results are in agreement with those found elsewhere [22,51],

331   and allow us to state that the drug was entrapped into the polymeric network regardless of

332   modifications made in NPs. Indeed, different amounts of drug loading were tested and discussed

333   based on EE% capacity. By increasing DOX concentration from 80 to 154 µg/ml, the DOX

334   EE% decreased from 66.50% ± 2.68 to 51.09% ± 2.88. Similar results were found elsewhere

335   [17,18,52], pointing out that a larger amount of drug does not mean any increase in

336   encapsulation efficiency. As a limited number of functional groups is available for electrostatic

337   interactions with the drug in the NP matrix, the increase in the amount of drug added to the

338   formulation could have resulted in a decrease in drug entrapment efficiency. Finally, it is worthy

339   mentioning that NPs without 77KS showed the highest mean EE% value. This behavior could

be attributed to the assembling of a consistent CS/TPP network with greater amount of TPP molecules and, thus, of remaining negative charges that allow DOX association. When 77KS (with only one negatively charged group) binds to CS, no free negative charge remains available to interact with DOX, therefore leading to diminished EE%. However, it is important to highlight that when 77KS was incorporated, we achieved higher EE% values than previous studies that reported DOX EE% values in the order of 47% for PLGA NPs and 20% for CS-based NPs [5,53].

3.2. In vitro DOX release

Taking advantage of the acidic pH_e (6.5 − 7.2) found in the tumor environment compared to the normal tissues [11,54], pH-sensitive NPs have been developed to achieve accelerated drug release at the tumor site. In this context, the *in vitro* drug release profiles of DOX-CS-NPs, PEG-DOX-CS-NPs and Polox-DOX-CS-NPs were studied in PBS buffer mediums at pH 7.4, 6.6 and 5.4 at 37 ± 2°C (Fig. 3).

When 77KS was first studied, it demonstrated pH-dependent membrane-lytic activity on hemolysis assay, with significant increase at pH 5.4; although with no pharmaceutical applications up to this time [13]. Here, this surfactant was incorporated into DOX-loaded CS-based NPs and, as can be seen in Fig. 3A, it was clearly demonstrated that the pH-dependent release pattern of these nanostructures was as evident as was for CS-NPs without 77KS (Fig. 3D). In acidic environment, the release rate was accelerated; with 97 and 100% of DOX released at pH 6.6 and 5.4 after 6 h, respectively, while only 71% of drug release was reached at pH 7.4. The cumulative release amount of DOX at pH 6.6 and 5.4 was in general significantly faster (p < 0.05) than at pH 7.4. A control experiment using free DOX was also carried out under similar conditions and almost total drug release was reached after 6 h.

363   The release of PEG-DOX-CS-NPs was also studied at different pH values, wherein at

364   acidic conditions the release was noticeably accelerated with 100% of the DOX available in

365   both pH 6.6 and 5.4 mediums after only 4 h (Fig. 3B). These results demonstrate that PEG did

366   not inhibit drug release at acidic conditions, which is particularly important in order to maintain

367   the improved drug delivery in the tumor microenvironment and intracellular compartments.

368   Unexpectedly, DOX release from PEGylated NPs was not delayed at physiological pH in

369   comparison with those NPs without PEG (~75 and 76% DOX released at 24 h, respectively).

370   This behavior appears to be attributed to the formation of a semi-interpenetrating network

371   between CS and PEG [48] and not to the assembly of a PEG shell around the NPs.

372   Among the three formulations, Polox-DOX-CS-NPs was the one that presented faster

373   release rate: release amount of DOX reached 100% after 3 h, 5 h and 8 h at pH 5.4, 6.6 and 7.4,

374   respectively (Fig. 3C). This behavior may be explained by the hydrophilic pattern of poloxamer

375   that consequently forms a porous structure in the surface of the DOX-CS-NPs [55]. Poloxamers

376   are reported to be pore-forming agents and drug-releasing enhancers [56], which corroborated

377   our results. At this point there is no significant difference among the release rates at each pH (p

378   > 0.05), which may be justified by the faster release achieved at physiological conditions.

379   The release mechanisms from CS-based NPs have been reported to be desorption of the

380   drug from the surface, diffusion of the drug through pores, and degradation of the polymeric

381   matrix [43]. In the swelling experiments, a considerable increase of particle size was noticed

382   with a decrease of the buffer pH from 7.4 and 6.6 to 5.4 (178.9 nm, 173.6 nm and 309.7 nm,

383   respectively). At lower pH value, the protonation of the amino groups of CS is promoted,

384   leading to an increase of electric density and repulsion force between cross-linked CS chains

385   [57]. This mechanism allows the medium to penetrate into the nanoparticulate system,

386   consequently increasing the mean hydrodynamic size [58]. This pH-sensitive swelling behavior,

387   in turn, could be one of the mechanisms underlying the faster diffusion of DOX from NPs,

especially in acidic environments with pH as low as 5.4. On the other hand, the lack of swelling at pH 6.6 is probably attributed to the diminishing CS protonation in this condition, suggesting that the repulsion forces are not enough to induce NP swelling and, thus, other mechanisms are involved in the accelerated drug release.

It is worth mentioning that besides the swelling mechanism of CS, DOX may have an improved solubility and, TPP, a reduced ionization in acidic environments [17,57]. This later condition may result in NP network destabilization and thus faster drug delivery, which could be the basis for the pH-responsive drug release observed for the NPs without 77KS (Fig. 3D). Considering that either CS-NPs with or without 77KS displayed a pH-dependent release behavior, it can be evidenced that the pH-responsive nature of CS itself appears to play the dominant role. However, 77KS appears to delay the release at pH 7.4, which is quite important in order to achieve a target drug release at the tumor site. Therefore, it can be stated that 77KS has a synergic effect with CS to give to the NPs the pH-responsive behavior. Moreover, it is noteworthy that another study performed by our research group evidenced that only the NPs incorporating 77KS showed pH-sensitive membrane-lytic activity (unpublished data), which also proves the important role of 77KS to improve the pH-sensitivity of the NPs. The ionization of 77KS is expected to be reduced in an acidic environment [13], which in turn would also contribute for the destabilization of the NP structure due to the reduced amount of available anionic charges that interact electrostatically with CS. This process would retain the drug at physiological conditions and facilitate the drug release as the pH decreases to 6.6 and 5.4.

The increased release at pH 6.6 and 5.4 shows that drug delivery appears to be triggered at tumor extracellular pH_e, as well as at the acidic environment of endosomes. Moreover, the low DOX release at normal physiological conditions may reduce the side effects that can occur during cancer treatment. Altogether, these results support the idea that these nanocarriers are a

412 potential design to be used as a pH-sensitive system to improve the drug availability on tumor

413 microenvironment and intracellular compartments.

414 *3.3. Mathematical modeling*

415 The data obtained from *in vitro* release studies were used to calculate values of release constants

416 and release exponents with the aim to help understanding the mathematics of release profiles

417 (Table 2). According to the values of the correlation coefficients (r) and MSC, the data for all

418 NPs suspensions at pH 7.4 fit better to the biexponential equation ($r > 0.99$). At this condition,

419 the DOX release showed an initial burst release (k_1), continued by a steady-state release (k_2).

420 These two phases can be explained by the initial drug release from NP surface (drug adsorbed

421 or entrapped in surface layer), followed by buffer penetration into NPs and drug diffusion

422 through the swollen rubbery matrix [58]. Moreover, according to the results for a and b

423 parameters, approximately 68% of the drug was in Polox-DOX-CS-NPs and only 31% was

424 superficially adsorbed on this nanostructure. Conversely, PEG-DOX-CS-NPs and DOX-CS-

425 NPs had about 25% encapsulated and 75% adsorbed on NP surface. When the mathematical

426 modeling was performed for pH 6.6 and 5.4, a good fit was observed using the monoexponential

427 model, with constant rates (k) in the following ranking order: PEG-DOX-CS-NPs > Polox-

428 DOX-CS-NPs > DOX-CS-NPs.

429 In the Korsmeyer-Peppas model, high correlation coefficient was obtained ($r > 0.99$ for

430 NPs and $r > 0.98$ for free DOX). The values of release exponent (n) between 0.43 and 0.85 for

431 DOX-CS-NPs (release medium at pH 7.4, 6.6 and 5.4, with $n = 0.6836$, 0.4608 and 0.5235,

432 respectively) indicate a non-Fickian-type release mechanism, i.e., the phenomena responsible

433 for the DOX release are drug diffusion process from the NPs coupled to relaxation of the

434 polymeric chains [59]. A non-Fickian model also was found for PEG-DOX-CS-NPs at pH 7.4

435 ($n = 0.5010$) and Polox-DOX-CS-NPs at pH 7.4 and pH 5.4 ($n = 0.4836$ and 0.6638,

respectively). The same mechanism transport was identified for the release of rivastigmine from CS-based nanoparticles for brain targeting [60]. When the release data of PEG-DOX-CS-NPs at pH 6.6 and 5.4 mediums were analyzed, $n < 0.43$ was obtained and, therefore, the release mechanism was Fickian, suggesting that the release is a consequential effect of only DOX amount diffused from the nanostructure. The same occurred for Polox-DOX-CS-NPs at pH 6.6. Fickian release mechanism was also presented to an anticancer drug loaded into CS-NPs [57]. Finally, $n = 0.2276$ was obtained for non-encapsulated DOX, indicating that its release profile is diffusion-controlled. Altogether, our results demonstrated the remarkable contribution of the relaxational process of the polymeric matrix for DOX release at pH 7.4, which may justify the slower drug release under physiological conditions.

3.4. Lyophilization of nanoparticles

Nanoparticulate systems for drug delivery have been subjected to lyophilization in order to overcome their instabilities [61]. Herein, NP suspensions were lyophilized by freeze drying with lactose, mannitol or glycerol as cryoprotectants, which are important adjuvants with the ability to protect NP suspensions from the stresses generated during the lyophilization process, i.e. freezing and desiccation [62]. When mannitol and glycerol were tested as protectants, the obtained result was not satisfactory since the redispersion procedure showed a strong tendency to form aggregates. For the sake of choosing between 1, 5 and 10% lactose, the major criteria evaluated were the yield, drug content and redispersibility index (ratio between the size after lyophilization and before lyophilization). Satisfactory values were achieved for 10% lactose (~92%, ~93% and 1.10, respectively). Moreover, only 10% lactose was able to produce a clear suspension, without any visible precipitates (Table 1). Sugars are suitable protective agents, acting by hydrogen bonding and maintaining the solute in a pseudo hydrated state during the

459   dehydration step, which thus protects the NP structure from damage in dehydration and

460   rehydration process [63].

461   *3.5. FT-IR analysis*

462   FT-IR analyses were performed in order to support the CS:TPP cross-link as proof of NP

463   formation, as well as to confirm the grafting of 77KS, PEG and poloxamer on the surface of

464   NPs (Fig. 4 and 5). Fig. 4B represents the FT-IR spectrum of CS. The characteristic absorption

465   peak at 3384 cm^{-1}, representing the presence of OH- groups, indicates that CS is partially

466   deacetylated. [64]. Peaks at 2850 to 2920 cm^{-1} show the stretching band of methylene in CS

467   structure. Moreover, for CS-NPs (Fig. 4C; 5B, C and D), the amino band is shifted from 1652.5

468   to ~1570 cm^{-1}, confirming that amino groups of CS were involved in the cross-linking by

469   phosphate (TPP) [49]. This shifting was confirmed by analyzing the spectrum of unloaded CS-

470   NPs (data not showed). Another peak that can be observed in CS-NPs spectra (Fig. 4C; 5B, C

471   and D) is at 1202 cm^{-1}, corresponding to P=O stretching of the TPP [64]. Pure DOX spectrum

472   (Fig. 4A) shows peaks at 2933 (C-H), 1730 (C-O), 1617 and 1582 (N-H), 1413 (C-C) and 1072

473   cm^{-1} (C-O). In DOX-CS-NPs spectra (Fig. 4C; 5B, C and D), these peaks are also presented as

474   shifted to 2900 (C-H), 1642 and 1572 (N-H), 1415(C-C) and 1031 cm^{-1} (C-O). Thus, these

475   results indicate that DOX was loaded into CS-NPs [18]. Absorption peaks associated to PEG

476   can be seen at 784 and 897 cm^{-1}, suggesting that PEG grafting was successfully achieved in

477   PEG-DOX-CS-NPs (Fig. 5D) [21]. Likewise, for Polox-DOX-CS-NPs (Fig. 4C), a stretching

478   band from 2860 to 2950 cm^{-1} confirms the incorporation of poloxamer 188. The same strong

479   peak appears for pure poloxamer, which represents the stretching vibrational band of methylene

480   group [49,65]. Finally, for 77KS, two strong bands at 1550 cm^{-1} and 1414 cm^{-1} represents the

481   carboxylate ion present in the molecule (Fig. 5A) [66]. The peak at ~1414 cm^{-1} remains as a

482   strong band and evidences the incorporation of 77KS in CS-NPs (Fig. 5B and D). For DOX-

3.6. Stability studies of nanoparticles

NP suspensions and NPs after lyophilization were submitted to stability studies for a storage period of 8 weeks at $2 - 8°C$. Particle size, PDI, ZP and drug content were evaluated in each scheduled time. After two weeks storage, all samples presented a tendency to aggregate. The parameters evaluated that prove this fact are particle size (> 600 nm) and PDI (> 0.3), suggesting an increase in the number of larger particles and a decrease in the narrow size distribution of the suspension. These results were not unexpected, as it was previously reported that CS microparticles showed reduced ZP and enhanced particle size after 28 days storage [67]. Factors to explain the size evolution during time storage are swelling, particle aggregation and interaction of free polymer chains with the particle network [63]. On the other hand, NP suspensions presented no considerable variations for drug content, which remained around 99% during storage time. However, the lyophilized NPs displayed a slight decrease in the drug content after 1-month storage. Altogether, the results obtained in these preliminary studies indicated that further studies must be conducted in this field in order to improve the stability of the design formulations.

With the aim to study the ability of the nanosystems to protect the encapsulated drug from photodegradation, DOX water solution, as well as DOX-CS-NPs and PEG-DOX-CS-NPs in both suspension and lyophilized states were exposed to UVA radiation. DOX water solution followed a first kinetic order ($r = 0.9857$), with half-live ($t_{1/2}$) $= 9.15$ h. Likewise, the degradation profiles of DOX into DOX-CS-NPs and PEG-DOX-CS-NPs were according to a first ($r = 0.9374$) and second kinetic order ($r = 0.9818$), with $t_{1/2} = 4.17$ h and 5.57 h, respectively. These

21

507 findings of $t_{1/2}$, therefore, revealed that the nanostructured systems were not able to protect DOX

508 from the UVA radiation during the entire study period. In contrast, the lyophilized samples L-

509 DOX-CS-NPs and L-PEG-DOX-CS-NPs followed a second kinetic degradation order ($r =$

510 0.9975 and 0.9950, respectively) and presented encouraging results about $t_{1/2}$. L-DOX-CS-NPs

511 and L-PEG-DOX-CS-NPs demonstrated $t_{1/2}$ values 15- and 7.5-fold greater (62.5 h and 41.67 h)

512 compared to their suspension forms, respectively, suggesting an improvement on photostability

513 of dry solid forms.

514 *3.7. Cytotoxicity assays*

515 *In vitro* assays are very attractive due to ethical aspects and for being a rapid and effective

516 pathway to assess toxicological responses of new nanotechnologies before going to *in vivo*

517 studies. Therefore, here we performed a preliminary study on the potential antitumor activity of

518 the pH-responsive DOX-loaded NPs using an *in vitro* cell model. The cytotoxic responses of

519 unloaded CS-NPs, DOX-loaded CS-NPs and free DOX were evaluated against HeLa tumor

520 cells using MTT viability assay. A dose-dependent effect for all formulations tested can be seen

521 in Fig. 6. The results obtained with DOX-loaded NPs were compared to those with free DOX

522 in order to ensure that the drug encapsulation improves or at least maintains the cytotoxic effects

523 of DOX. The *in vitro* antitumor activity of modified and unmodified DOX-loaded NPs was

524 generally higher than that of free DOX at both tested concentrations. Finally, the cell viability

525 was higher than 85% at both tested concentrations of unloaded CS-NPs, indicating that the

526 surfactant 77KS did not promote significant cytotoxic effects [12].

527 **4. Conclusions**

528 In this work, we prepared and characterized PEGylated and poloxamer-modified DOX-CS-NPs

529 incorporating the pH-sensitive lysine-based surfactant 77KS. NPs showed nanoscale size with

530 relatively high EE%, whereas an improvement on DOX photostability was noticed when NPs

531 were into dry solid forms. All formulations displayed pH-triggered DOX release and can be

532 stated as switching nanodevices in release kinetics, ranging from slow drug delivery while

533 circulating (pH 7.4) to rapid release kinetics once target sites have been reached (pH 6.6 to 5.4).

534 Finally, cytotoxicity experiments showed the ability of DOX-loaded CS-NPs to kill HeLa tumor

535 cells. However, further studies in MDR cancer cells are needed to enhance our knowledge

536 regarding the role of poloxamer together with 77KS in the sensitization of tumor cells.

537 Altogether, our findings suggested that the pH-responsive DOX-loaded CS-NPs developed here

538 could be potential stimulus-responsive drug delivery systems to target cancer cells by triggering

539 the acidic tumor microenvironment as well as endosomal compartments.

540 **Conflict of interest statement**

541 The authors state that they have no conflict of interest.

542 **Acknowledgments**

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688

689 **Figure captions:**

690 **Fig. 1.** Design of pH-responsive DOX-loaded CS-NPs to facilitate target drug release at the

691 tumor site.

692 **Fig. 2.** UV-Vis absorption spectra of the DOX extracted from NPs (A) and DOX aqueous

693 solution (B).

694 **Fig. 3.** pH-dependent *in vitro* cumulative DOX release from NPs in PBS buffer at pH 7.4, 6.6

695 and 5.4. (A) DOX-CS-NPs, (B) PEG-DOX-CS-NPs, (C) Polox-DOX-CS-NPs and (D) DOX-

696 CS-NPs without 77KS. Results are expressed as the mean ± SE of three independent

697 experiments. Statistical analyses were performed using ANOVA followed by Tukey's multiple

698 comparison test. [a] Significant difference from PBS pH 7.4 ($p < 0.05$), [b] highly significant

699 difference from PBS pH 7.4 ($p < 0.01$).

700 **Fig. 4.** FT-IR spectra of pure DOX (A), CS raw material (B), Polox-DOX-CS-NPS (C) and

701 Poloxamer 188 (D).

702 **Fig. 5.** FT-IR spectra of 77KS (A), DOX-CS-NPs (B), DOX-CS-NPs without 77KS (C) and

703 PEG-DOX-CS-NPs (D).

704 **Fig. 6.** *In vitro* antitumor activity of unloaded-CS-NPs, free DOX and DOX-loaded CS-NPs in

705 HeLa cell line.

706

707 **Table 1.** Characterization of unloaded and DOX-loaded CS-NPs with or without 77KS. The
708 lyophilized NPs (L-NPs) were analyzed after redispersion in ultra-pure water.

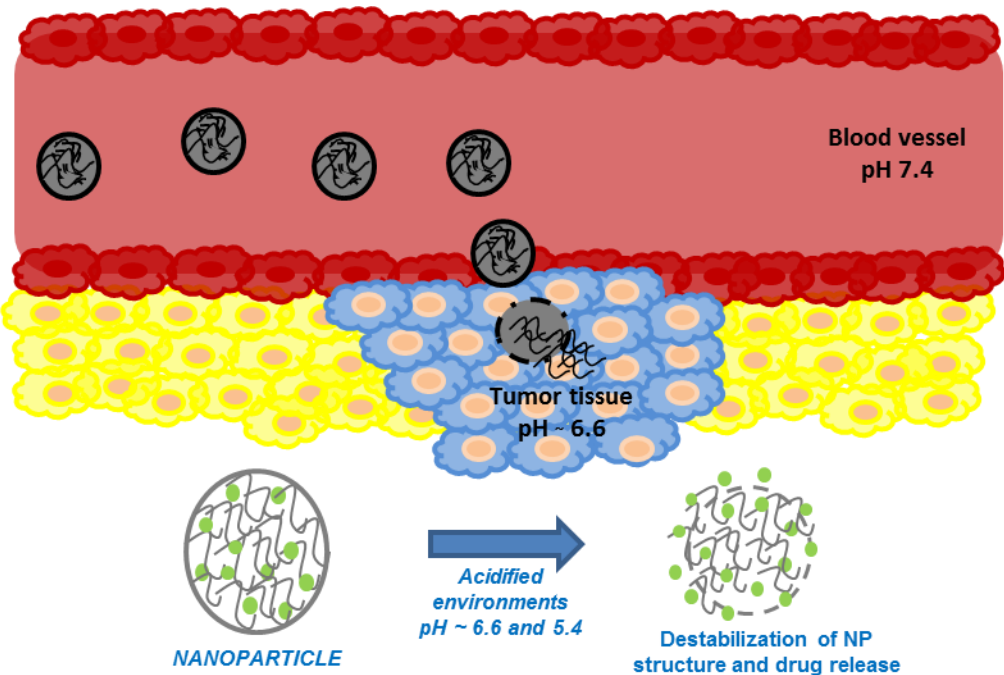| Sample	Particle size (nm) ± SD*	Polydispersity index ± SD*	Zeta potential (mV) ± SD*	pH	EE% ± SD*
CS-NPs (CS:TPP)	170.30 ± 0.84	0.19 ± 0.02	25.20 ± 1.87	5.66	-
DOX-CS-NPs (CS:TPP)	190.35 ± 1.70	0.22 ± 0.01	21.90 ± 1.12	5.70	75.54 ± 4.98
CS-NPs	176.77 ± 1.79	0.20 ± 0.02	24.00 ± 1.82	5.66	-
DOX-CS-NPs	197.50 ± 2.30	0.22 ± 0.01	21.70 ± 0.81	5.72	66.50 ± 2.68
PEG-CS-NPs	211.10 ± 1.55	0.24 ± 0.01	23.30 ± 1.96	4.68	-
PEG-DOX-CS-NPs	226.40 ± 2.33	0.23 ± 0.01	23.65 ± 1.06	5.19	66.32 ± 3.54
Polox-CS-NPs	184.50 ± 2.00	0.21 ± 0.02	22.05 ± 0.91	5.48	-
Polox-DOX-CS-NPs	209.70 ± 1.35	0.22 ± 0.03	21.00 ± 0.85	5.60	62.21 ± 2.88
L-DOX-CS-NPs	217.45 ± 4.49	0.33 ± 0.02	12.40 ± 0.15	6.14	67.42 ± 10.85
L-PEG-DOX-CS-NPs	491.60 ± 32.38	0.73 ± 0.09	20.45 ± 0.78	5.91	65.32 ± 3.18
L-Polox-DOX-CS-NPs	252.80 ± 7.46	0.40 ± 0.03	17.50 ± 0.93	5.98	61.27 ± 2.28

709

710

Table 2. Observed rate constants, correlation coefficients, MSC and half-lives ($t_{1/2}$) obtained by mathematical modeling of DOX release from the different NPs when immersed in PBS buffer at pH 7.4, 6.6 and 5.4. Results are expressed as mean ± standard deviation (SD) of three experiments.

	pH medium	DOX-CS-NPs	PEG-DOX-CS-NPs	Polox-DOX-CS-NPs
Biexponential				
r		0.99 ± 0.01	1.00 ± 0.01	1.00 ± 0.01
MSC		3.96 ± 0.36	4.28 ± 0.25	4.17 ± 0.45
k_1 (h^{-1})		0.44 ± 0.05	0.67 ± 0.07	2.84 ± 1.25
$t_{1/2}$ k_1 (h^{-1})	7.4	1.58 ± 0.47	1.02 ± 0.29	0.24 ± 0.09
k_2 (h^{-1})		0.002 ± 0.01	0.01 ± 0.01	0.36 ± 0.36
$t_{1/2}$ k_2 (h^{-1})		407.64 ± 33.76	93.64 ± 9.12	1.91 ± 0.38
a		0.74 ± 0.04	0.70 ± 0.03	0.31 ± 0.08
b		0.23 ± 0.04	0.26 ± 0.02	0.68 ± 0.08
Monoexponential				
r		0.99 ± 0.01	0.99 ± 0.01	0.98 ± 0.01
MSC	6.6	3.74 ± 0.32	3.46 ± 0.63	3.13 ± 0.35
k (h^{-1})		0.64 ± 0.04	1.23 ± 0.08	1.05 ± 0.08
$t_{1/2}$ (h^{-1})		1.07 ± 0.05	0.56 ± 0.03	0.65 ± 0.14
r		1.00 ± 0.01	0.99 ± 0.01	1.00 ± 0.00
MSC	5.4	4.68 ± 0.29	3.31 ± 0.31	5.07 ± 0.25
k (h^{-1})		0.76 ± 0.03	0.98 ± 0.07	0.91 ± 0.03
$t_{1/2}$ (h^{-1})		0.90 ± 0.10	0.71 ± 0.20	0.76 ± 0.19

717 Fig 1

NANOPARTICLE

Acidified environments pH ~ 6.6 and 5.4

Destabilization of NP structure and drug release

Blood vessel pH 7.4

Tumor tissue pH ~ 6.6

718

719

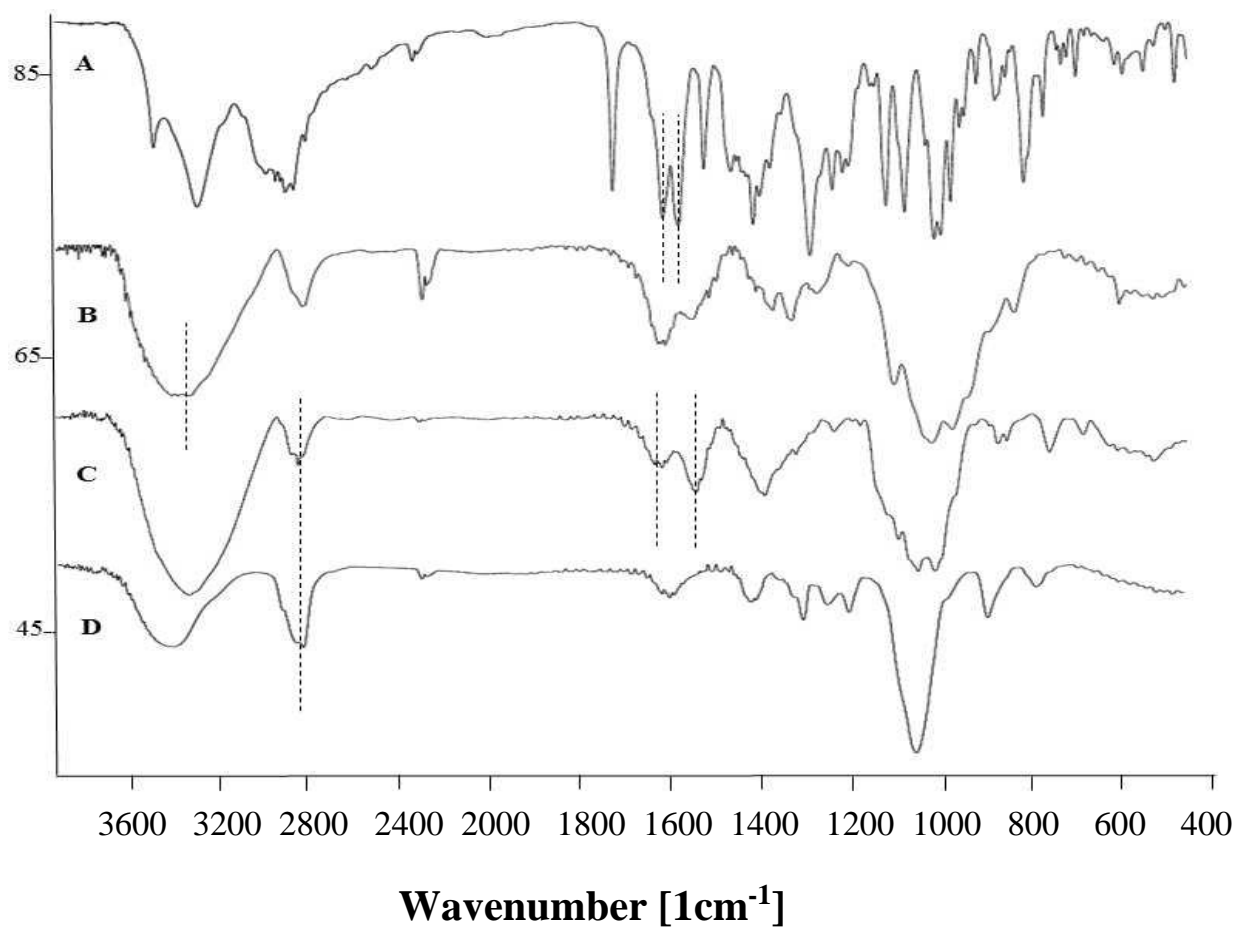720 Fig 2
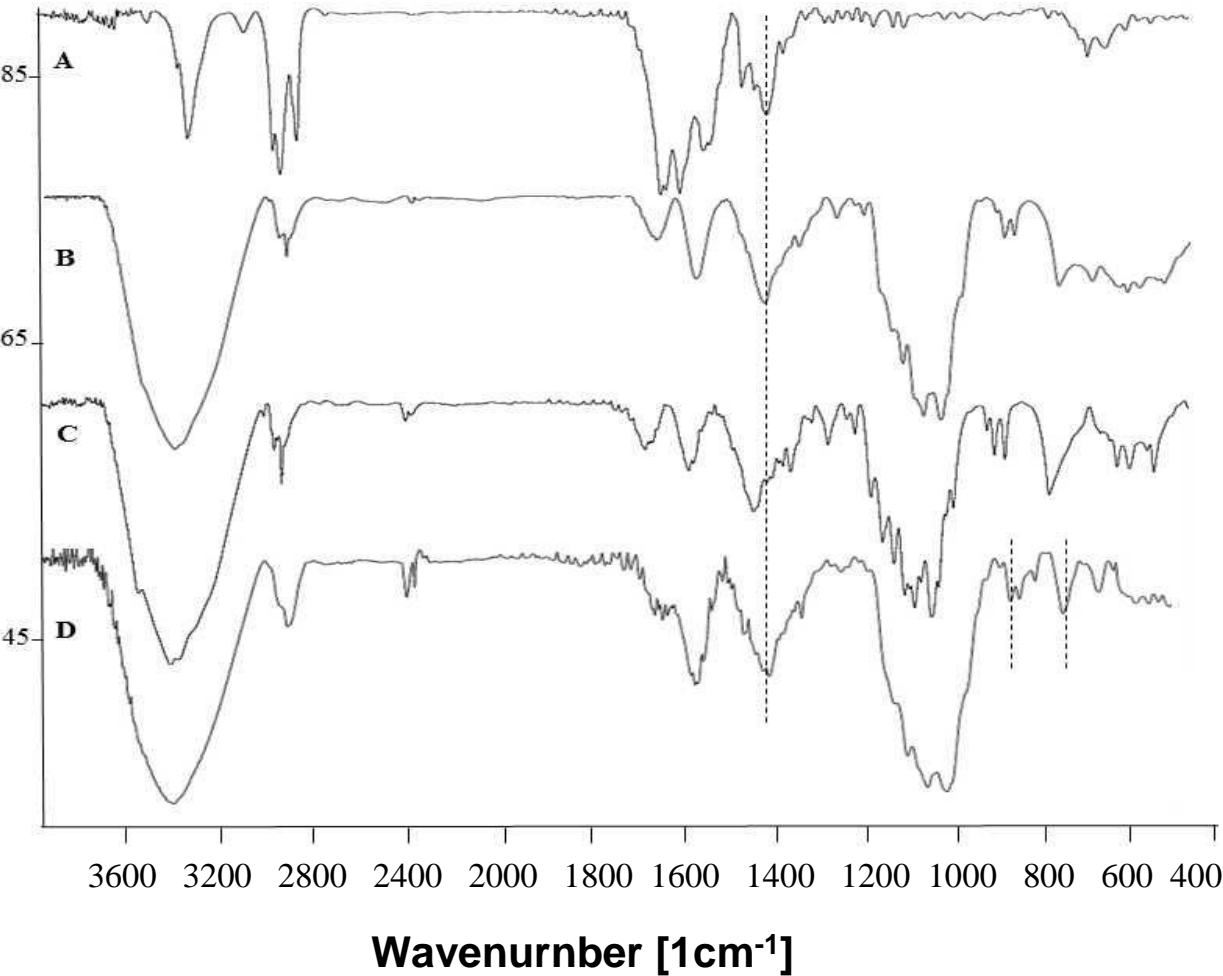

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722

723 Fig 3

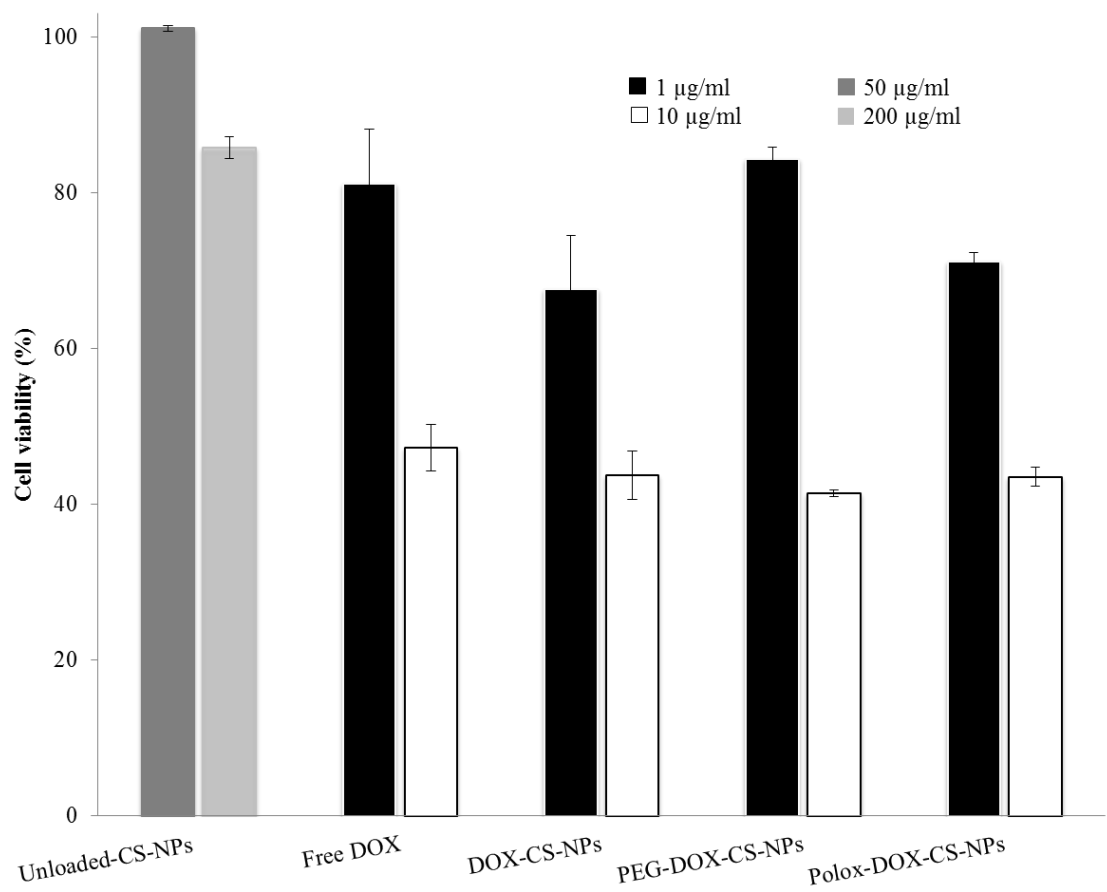

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725

726   Fig. 4

727

728   3600 3200 2800 2400 2000 1800 1600 1400 1200 1000 800   600   400

729   **Wavenumber [1cm⁻¹]**

730

731 Fig 5

732

Wavenurnber [1cm⁻¹]

733

734

735　Fig 6

736

737