

Dual stimuli-responsive nanocarriers via a facile batch emulsion method for controlled release of Rose Bengal

Original

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31 **1. Introduction**

32 Drug administration *via* the oral route is a highly preferred drug delivery method due to its simple and
33 non-invasive nature. However, the digestive enzymes that are present in the gastrointestinal (GI) tract
34 or the highly acidic environment of the stomach may lead to premature degradation or denaturation of
35 the drug [1]. The variations in the pH of the gastrointestinal (GI) tract (from acidic in the stomach to
36 slightly basic in the small intestine) or the temperature variations around the target tissue pose additional
37 challenges when designing drug delivery systems (DDS) for colonic diseases with drug-specific release
38 kinetics [2].

39 Stimuli-responsive nanocarriers (SRN), which release the drug at the target site, are promising solutions
40 to the challenges faced during oral drug delivery. SRNs with pH and temperature control are recently
41 gaining attention as drug delivery systems that facilitate drug release at high concentrations and
42 sustained release rates at alkaline pH and high-temperature conditions of the colon environment [3], [4].
43 In one study involving SRNS, Kang *et al.* copolymerized N-Isopropylacrylamide (NIPAM) with N-
44 vinylimidazole, to achieve pH and temperature-responsive release of a model protein, bovine serum
45 albumin (BSA), and reported temperature and pH-dependent BSA release with approximately 35% of
46 the BSA being released during the first 4 h [5]. On the other hand, Kim *et al.* reported the synthesis of
47 copolymeric nanocarriers obtained from N-isopropyl acrylamide (NIPAM) and acrylic acid (AA)
48 polymers *via* emulsion polymerization [6]. These dual responsive nanocarriers allowed the control of
49 drug release rates by tuning the temperature and pH, leading to a difference of ~10% in the release
50 amounts between the alkaline and acidic pH conditions. Jin *et al.* also used the same method to
51 copolymerize NIPAM and AA and investigated the release kinetics of ibuprofen under different
52 conditions. By tuning the polymer composition, they achieved a difference of 50% in the drug release
53 amount at acidic and alkaline pH levels [7]. Chung *et al.* performed *in situ* surfactant-free polymerization
54 of NIPAM in chitosan (CS)-poly (acrylic acid) (PAA) micelles and prepared dual responsive
55 nanoparticles with 29% encapsulation efficiency of doxycycline hyclate. The dual responsive SRNs they
56 synthesized showed a considerable difference between the drug release rates at alkaline and acidic pH
57 levels. They obtained about 41% of drug release at the end of 14 days at alkaline pH level, whereas only
58 about 23% of drug was released at acidic pH level [8].

59 Although many researches are available employing PNIPAM as a temperature-responsive polymer,
60 recent studies on the cytotoxicity of PNIPAM due to its degradation into small amide derivatives in the
61 acidic environment raise concerns about its use in nanomedicine, especially in oral drug delivery
62 applications [9]. For this reason, the interest in another temperature-responsive polymer, poly(n-vinyl-
63 caprolactam) (PNVCL), as more biocompatible alternative to PNIPAM in DDS, has recently increased
64 significantly, especially for oral administration where nanoparticles are exposed to highly acidic
65 conditions [10]. Fallon *et al.* reported the preparation of biocompatible pH/temperature-responsive

66 poly(N-vinylcaprolactam-co-itaconic acid) hydrogels by UV polymerization for the oral delivery of
67 acetaminophen [11].

68 In another study, Mundargi *et al.*, prepared poly(N-vinylcaprolactam-co-methacrylic acid)
69 microparticles by free radical polymerization for the oral administration of insulin, and demonstrated
70 pH-responsive release of insulin from the microparticles with an encapsulation efficiency of 52% [12].
71 Medeiros *et al.*, on the other hand, prepared PNVCL and poly (NVCL-co-AA) microparticles using the
72 spray drying method for the oral delivery of ketoprofen and reported pH-triggered release, where 42.6%
73 of ketoprofen was released at the end of 10 days at alkaline pH, whereas this amount was only 11.5% at
74 acidic pH [13]. Rao *et al.* copolymerized NVCL with acrylamidoglycolic acid (AGA) *via* surfactant-
75 assisted batch emulsion polymerization method, to synthesize nanocarriers for the delivery of 5-
76 fluorouracil at gastric and intestinal pH levels and temperatures [14]. They reported release rates of
77 ~60% at pH of 1.2 and ~80% at pH of 7.4 at the end of 12 h, with encapsulation efficiencies < 62%.

78 Although dual responsive SRNs hold potential as drug delivery systems, use of surfactants, multi-step
79 synthesis processes, and low encapsulation efficiencies impede their widespread use. In this study, we
80 demonstrate the synthesis of dual responsive nanocarriers based on a core-shell structure *via* a simple
81 batch emulsion method without the use of any surfactant. The synthesized nanocarriers have a chitosan
82 polyacrylic acid polyelectrolyte complex shell and a poly (n-vinyl caprolactam) core and contain RB as
83 a model drug. Following the fabrication of nanoparticles, characterization studies including size and
84 zeta potential measurements by DLS, morphology analysis by SEM, and chemical characterization by
85 FTIR are performed. Encapsulation efficiency and loading capacity of the nanoparticles and the release
86 profiles at different pH levels and temperatures are determined. Finally, the therapeutic potential of the
87 nanoparticles is investigated on Caco-2 cells in terms of cellular viability and internalization.

88 **2. Experimental**

89 **2.1 Materials**

90 Chitosan (75–85% deacetylated, low molecular weight, CAS no. 9012-76-4), Acrylic Acid (CAS
91 Number: 79-10-7), N- vinyl-caprolactam (CAS Number 2235-00-9), N, N'-Methylenediacrylamide
92 (CAS Number 110-26-9), and Rose Bengal (CAS Number 632-69-9) were purchased from Sigma-
93 Aldrich, USA. Acetic acid (CAS no. 64-19-7) was purchased from Merck, USA. Caco-2 cells (HTB-
94 37™) were purchased from ATCC. Eagle's Minimum Essential Medium (EMEM), heat-inactivated fetal
95 bovine serum (FBS, CAS Number 64742-49-0), penicillin-streptomycin (CAS Number 8025-06-7) were
96 purchased from Gibco. Quant-iT™ PicoGreen® dsDNA Assay Kit and Hoechst were purchased from
97 Invitrogen. WST-1 reagent (CAS Number 150849-52-8) was purchased from Roche. DiO (Vybrant™
98 Multicolor Cell-Labeling Kit, CAS Number 34215-57-1) was purchased from Thermo Fisher Scientific.

99 Paraformaldehyde (PFA, CAS Number 30525-89-4) and Phalloidin-Atto 488 were purchased from
100 Sigma-Aldrich.

101 **2.2 Preparation of Multiresponsive Nanoparticles**

102 Surfactant free batch emulsion polymerization technique was used in the synthesis of the
103 CS/PAA/PNVCL nanoparticles. A proper amount of n-vinyl caprolactam (0.11 g) was dissolved in 20ml
104 ultrapure double distilled water. After complete dissolution, 0.11 g acrylic acid and 0.25g chitosan were
105 added to this solution. Sodium bicarbonate buffer (0.065 g) was used to maintain a constant pH value
106 of the reaction mixture preventing hydrolysis of n-vinyl-caprolactam under acidic conditions.[15] The
107 reaction mixture was placed in a reflux system and purged with nitrogen for 30 minutes. The temperature
108 was adjusted to 80°C after purging and KPS solution (0.041 g in 5 ml) was injected to the system as the
109 initiator for surfactant-free polymerization of n-vinyl-caprolactam (NVCL) and acrylic acid (AA) in the
110 presence of chitosan. The solution became milky after 10 minutes of initiation. Polymerization was
111 carried for 5 h, and the resulting solution was filtered (0.45 µm) then centrifugated at 40.000 rpm, 4°C
112 for 45 min.

113 **2.3 Preparation of Rose Bengal (RB) Loaded Multiresponsive Nanoparticles**

114 A diffusion-based drug loading technique was used to obtain drug-loaded nanoparticles. Briefly, a stock
115 solution of blank nanoparticles was diluted 10 times and incubated in aqueous RB solution (0.25 mg/ml)
116 for 72h at room temperature then centrifugated for 45 min at 40000 rpm and 4°C in order to remove the
117 free RB molecules and calculate the encapsulation efficiency through the supernatant.

118 **2.4 Characterization of the Multiresponsive Nanoparticles**

119 Hydrodynamic size, dispersity, and zeta potential values were measured using ZetaSizer Nano ZS
120 (Malvern Instruments, UK) instrument, which contains a 4.0 mV Helium-Neon laser (633 nm). Size
121 analyses were performed between 25°C to 45°C and at pH levels ranging from 3.5 to 6.5.

122 Chemical characterization of the particles was performed using Fourier-Transform Infrared
123 Spectroscopy (Thermo Scientific, Nicolet, iS10, USA). UV-Vis spectroscopy was utilized in order to
124 evaluate the encapsulation efficiency and the loading capacity of the nanoparticles and to determine the
125 release profiles at different pH levels and temperatures.

126 The size and morphology of the synthesized nanoparticles were additionally assessed by a field-emission
127 scanning electron microscope (Zeiss Leo Supra 35VP SEM-FEG, Germany) at a 3 kV operating voltage.
128 10ul of the nanoparticles were pipetted on a piece of the silicon wafer and dried for 5 h at room
129 temperature. The dried samples were coated with Au-Pd using a sputter coater (Cressington 108, UK)
130 at 40 mA for 120 s. The SEM images were obtained by the secondary electron (SE) detector. On the

131 other hand, 3 μ l of stock solution was pipetted on a transmission electron microscopy (TEM) grid, and
132 analysis was performed at 200 kV using the device (JEMARM200, JEOL, Japan).

133 **2.5 Drug Release Studies of the Multiresponsive Nanoparticles**

134 For the release studies, RB-loaded nanoparticles were placed in dialysis capsules with a cellulose
135 membrane of 12-14 kDa. The capsules were placed in beakers containing 50 ml of PBS at pH=3.0, 5.0
136 and 7.4 and incubated in shaking incubators at 25°C and 40°C. The pH values were varied within the
137 range of 3 - 7.4 which are close to the pH variations in the gastrointestinal tracts to study the stability
138 and response of the nanoparticles at these pH values. Temperature values, on the other hand, were
139 chosen as 25 and 40°C to observe the temperature response of pNVCL which has the the LCST
140 temperature at 32°C.

141 The measurements were taken periodically over a time span of 120 h. 1 ml of release medium was
142 removed from the beakers and RB concentration was determined via UV-Vis analysis. The percent drug
143 release was then calculated using Eq. (1).

144

$$145 \quad \text{Release}(\%) = \frac{\text{Released Amount of Drug}}{\text{Total Amount of Drug}} \times 100 \quad (1)$$

146 **2.6 Cell culture**

147 Caco-2 cells (ATCC® HTB-37™) were cultured in proliferation condition using Eagle's Minimum
148 Essential Medium (EMEM, Gibco) supplemented with 20% heat-inactivated fetal bovine serum (FBS,
149 Gibco) and 1% penicillin-streptomycin (100 IU/ml of penicillin and 100 μ g/ml of streptomycin, Gibco),
150 at 37°C in an atmosphere of 5% CO₂.

151 **2.7 Cellular viability**

152 Cellular viability was assessed using either the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen)
153 and the WST-1 assay (Roche). For both experiments, Caco-2 cells were seeded at 10000 cells/cm²
154 density in a 96-well plate (Corning) and let adhere for 24 h. Cells were treated with increasing
155 concentrations of free RB (0.00, 0.85, 1.70, 3.40, 8.50 and 17.00 μ g/ml), CS/PAA/PNVCL nanoparticles
156 (0, 25, 50, 100, 250 and 500 μ g/ml), and RB-loaded CS/PAA/PNVCL nanoparticles (0, 25, 50, 100, 250
157 and 500 μ g/ml respectively corresponding to 0.00, 0.85, 1.70, 3.40, 8.50 and 17.00 μ g/ml of RB) and
158 incubated for 24 and 72 h. After the treatment, cells were washed in Dulbecco's phosphate buffered
159 saline (DPBS). Regarding the Quant-iT™ PicoGreen® dsDNA Assay, cells were suspended in 100 μ l
160 of Milli-Q water, then subjected to four cycles of freeze/thaw (from -80°C to 37°C), to allow cellular
161 lysis and dsDNA release. Quant-iT™ PicoGreen® dsDNA assay was carried out by mixing PicoGreen®
162 reagent, buffer, and cell lysate in Corning Costar® 96-well black polystyrene plates following the

163 manufacturer's instructions. Fluorescence was measured with a Victor X3 Multilabel Plate Reader (λ_{ex}
164 485 nm, λ_{em} 535 nm). WST-1 assay was carried out by suspending the cells with 300 μl of phenol red-
165 free complete medium added with the WST-1 reagent (1:20 dilution), then 30 min of incubation at 37°C.
166 Finally, absorbance at 450 nm was measured, again using a Victor X3 Multilabel Plate Reader. Values
167 were expressed in % with respect to untreated controls.

168 **2.8 Cellular internalization**

169 DiO-stained CS/PAA/PNVCL Nanoparticles were prepared: 10 μM of DiO (Vybrant™ Multicolor Cell-
170 Labeling Kit, Thermo Fisher Scientific) were added to 1 ml of Milli-Q water containing 5 mg/ml of
171 nanoparticles, and then stirred for 2 h at room temperature. Finally, the mixture was washed four times
172 with Milli-Q water through centrifugation at 16602 g.

173 Cellular internalization was evaluated both with flow cytometry and with confocal microscopy imaging.
174 For flow cytometry purposes, Caco-2 cells were seeded at 10000 cells/cm² density in a 24-well plate
175 (Corning) and let adhere for 24 h. Therefore, cells were treated with 100 $\mu\text{g}/\text{ml}$ of DiO-stained
176 CS/PAA/PNVCL nanoparticles and incubated for 24, 48, or 72 h. Finally, cells were washed in DPBS,
177 detached from the wells, then analyzed with a CytoFLEX platform (Beckman Coulter, λ_{ex} 488 nm,
178 FITC λ_{em} 525/40 nm). Regarding confocal microscopy experiments, Caco-2 cells were seeded at 10000
179 cells/cm² density in WillCo-dish® and let adhere for 24 h. Then, cells were treated with 100 $\mu\text{g}/\text{ml}$ of
180 DiO-stained CS/PAA/PNVCL nanoparticles and incubated for 24, 48, or 72 h. After the incubation cells,
181 were washed in DPBS, fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich in DPBS) at 4°C for 20
182 min, and washed twice with DPBS. Cytoskeleton and nuclei of fixed cells were stained respectively
183 with 2.5 $\mu\text{g}/\text{ml}$ of TRITC-phalloidin (Sigma) and 5 $\mu\text{g}/\text{mL}$ of Hoechst (Invitrogen), following standard
184 staining protocols. Confocal microscopy images were acquired with a C2s system (Nikon) with a 60 \times
185 oil immersion objective.

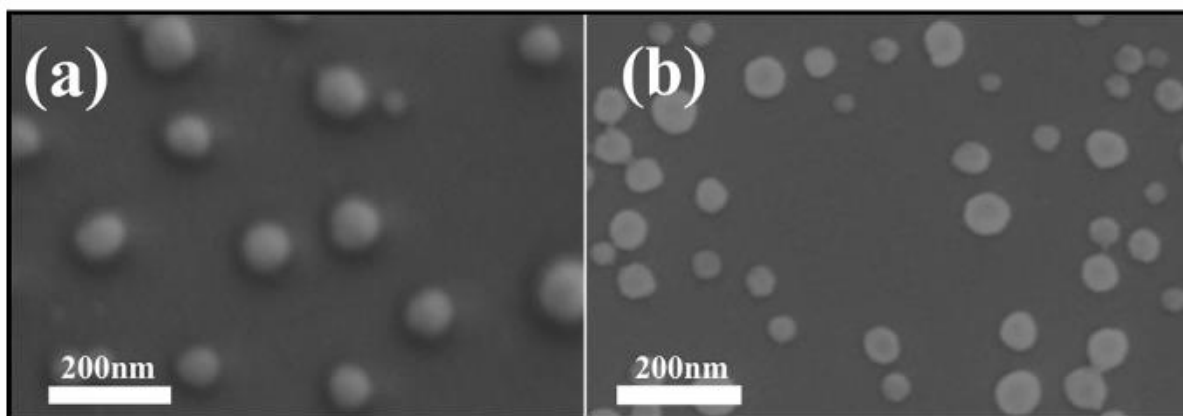
186 **2.9 Statistical analysis**

187 The normality of data distributions was verified with the Shapiro-Wilk normality test. Normally
188 distributed data were analyzed *via* ANOVA, other data *via* Kruskal-Wallis followed by Wilcoxon
189 signed-rank test. Each experiment has been performed in triplicate ($n=3$), if not differently indicated.

190 **3. Results and Discussion**

191 **3.1 Characterization of Multiresponsive Nanoparticles**

192 Figure 1 shows the SEM images of the blank (a) and drug-loaded (b) nanoparticles. For both blank and
193 loaded nanoparticles, the average diameters were below 100 nm with uniform size distribution.
194 Furthermore, comparing the SEM images it was observed that diffusion-based loading of the RB did
195 not have a significant impact on the size and uniformity of the nanoparticles.



196

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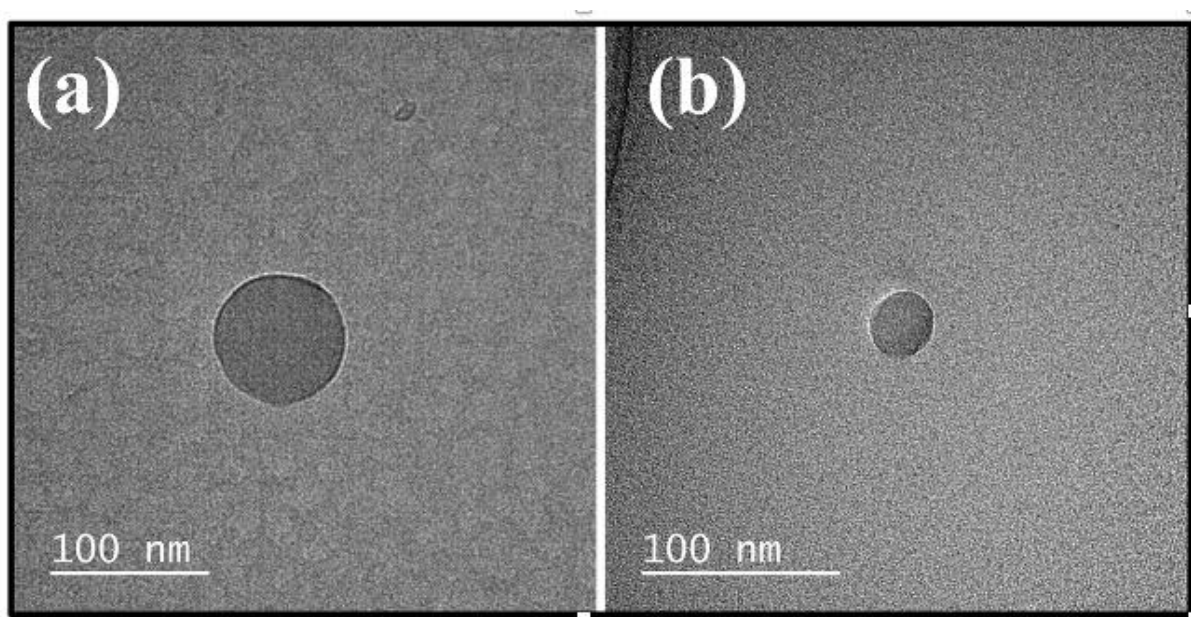
Fig. 1. SEM images of (a) blank and (b) loaded nanoparticles

198 For further analysis of the nanoparticle shape and structure, TEM analyses were performed. Figure 2

199 shows the TEM images of the (a) blank and (b) loaded nanoparticles. The TEM images confirmed the

200 size and shape of the nanoparticles determined by the SEM analyses. In addition, the dark, outer shell

201 observed in TEM images can be attributed to the chitosan-polyacrylic acid polyelectrolyte complex.

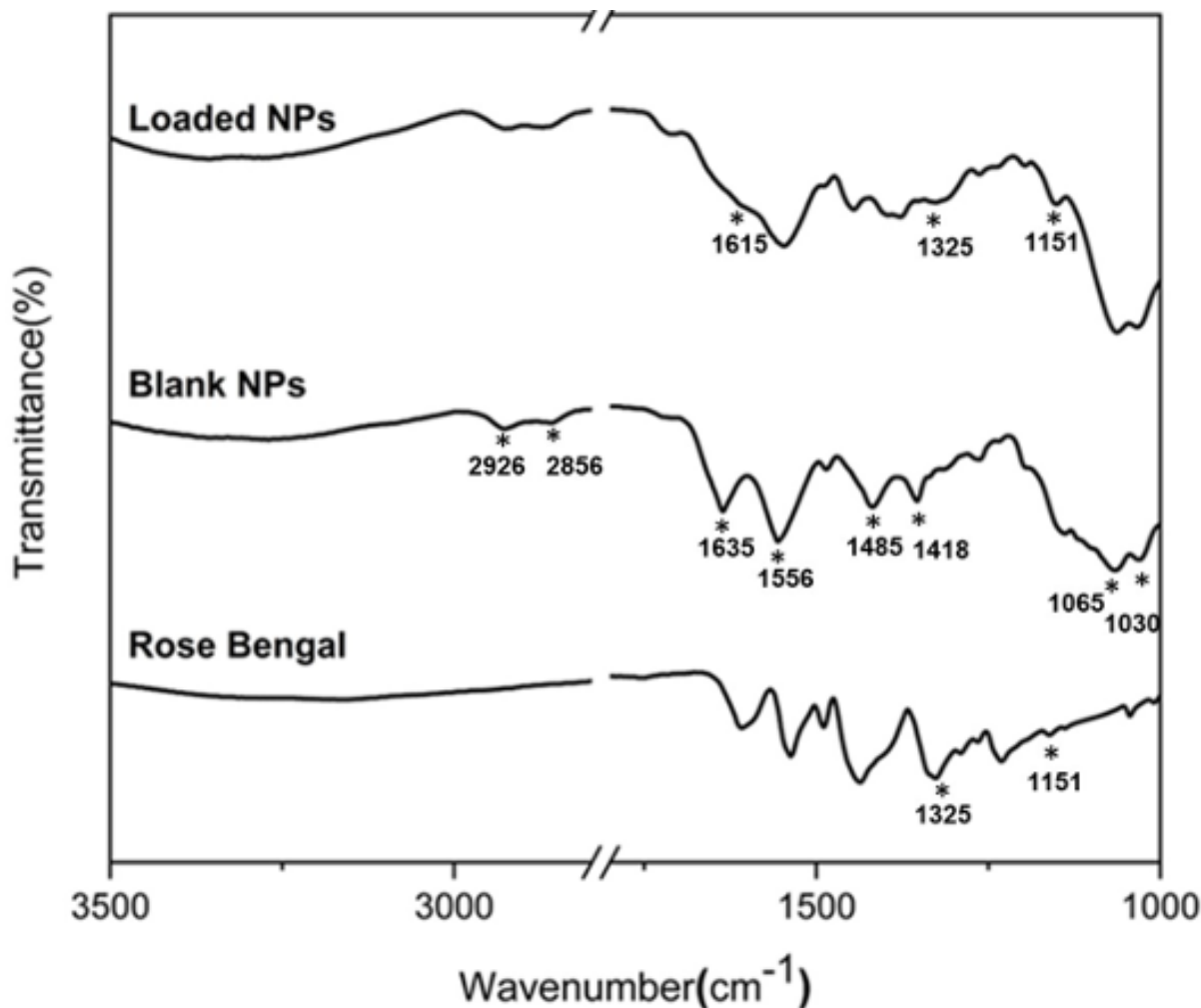


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203

Fig. 2. TEM Images of Blank (a) and Loaded(b) Nanoparticles

204



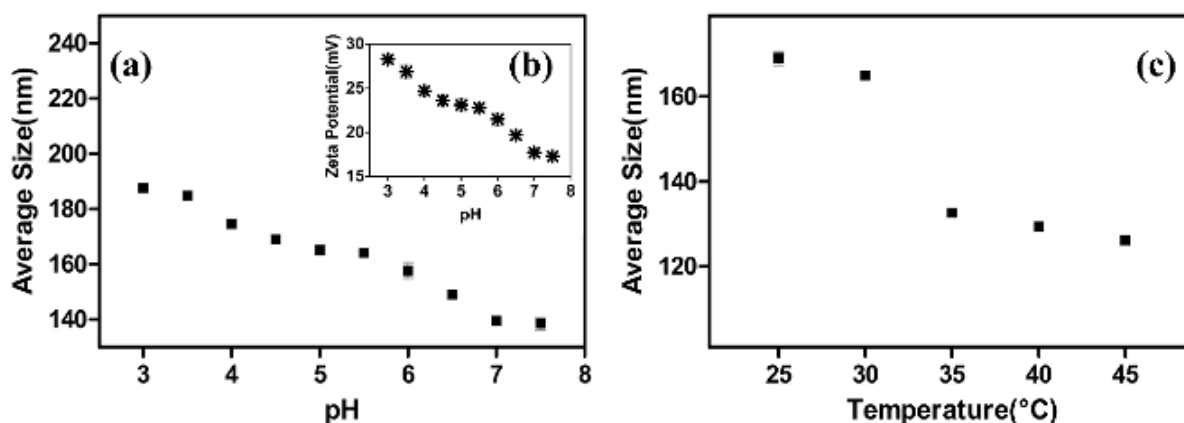
205

206 **Fig. 3.** FTIR Spectra of RB, Blank Nanoparticles, and RB Loaded Nanoparticles

207

208 FTIR analyses were performed for the chemical characterization of the nanoparticles. The spectra of
 209 blank nanoparticles, pure RB, and RB-loaded nanoparticles are shown in Figure 3. In the spectrum of
 210 blank nanoparticles, the presence of chitosan was confirmed through the peaks at 1635 cm^{-1} , 1065 cm^{-1}
 211 cm^{-1} , and 1030 cm^{-1} which correspond to the amino group, C3-OH, and C6-OH respectively. The carboxylic
 212 acid compounds of the polyacrylic acid in the structure were detected at 1556 cm^{-1} and 1418 cm^{-1} . The
 213 characteristic peaks corresponding to the aliphatic CH stretching of PNVCL appear at 2926 cm^{-1} and
 214 2856 cm^{-1} , also the peak at 1485 cm^{-1} corresponds to the C-N stretching in the ring [15]. Formation of
 215 polyelectrolyte complex between the positively charged chitosan and negatively charged polyacrylic
 216 acid moieties was confirmed through the NH_3^+ absorption of chitosan at 1635 cm^{-1} and the symmetric
 217 and asymmetric COO^- absorption of polyacrylic acid at 1556 cm^{-1} and 1418 cm^{-1} , respectively. The FTIR
 218 analyses confirm that the carboxylic acid groups of polyacrylic acid are deprotonated during
 219 polymerization, turning into the anionic COO^- groups which then interact with the protonated groups of
 220 chitosan [16], [17]. The FTIR spectra of the loaded nanoparticles show the RB characteristic peaks at
 221 1325 cm^{-1} and 1151 cm^{-1} , which correspond to the C=C stretching and C-H bending of RB, respectively.

222 In addition, the peak appearing at 1615 cm^{-1} (C=O stretching) in the spectrum of the loaded nanoparticles
223 confirms the presence of the RB [18].



224
225 **Fig. 4.** (a) Average size and Zeta Potential Values of Blank nanoparticles at different pH levels at a
226 constant temperature of 25°C , (b) zeta potential of Blank nanoparticles at different pH levels at a
227 constant temperature of 25°C (c) average size of blank nanoparticles at different temperature levels
228 ($\text{pH}=4.5$). Standard deviation values of the results of average size and zeta potential measurements are
229 in the range of 0.32 to 0.81 for each data point

230 Figure 4 (a) shows that at 25°C , the average size of the nanoparticles decreases from $187.7 \pm 1.2\text{ nm}$ at
231 $\text{pH}=3.0$ to $138.5 \pm 2.3\text{ nm}$ at $\text{pH}=7.5$. The zeta potential values also show a similar trend as pH increases.
232 The change in size and zeta potential values with pH can be related to the changes in the ionization of
233 chitosan ($\text{pK}_a=6.0\text{-}6.5$) and polyacrylic acid ($\text{pK}_a=4.25\text{-}4.5$) in the polyelectrolyte nanoparticle
234 complex.

235 At the pH levels below 4.0, the amino groups of the chitosan are well protonated, resulting in the
236 swelling of the chitosan shell which leads to the larger nanoparticle sizes measured. At pH levels
237 between 4.0 and 6.0, the polyacrylic acid in the core is ionized, and the electrostatic interaction between
238 the negatively charged carboxylic acid groups of the polyacrylic acid and positively charged amino
239 groups of the chitosan results in a decrease in the nanoparticle size. As pH increases, chitosan chains
240 start losing their charge and above pH 6.0, the chitosan chains collapse, leading to smaller nanoparticle
241 sizes. These changes in ionization degrees of the pH-responsive polymers are also confirmed by the zeta
242 potential values of the nanoparticles at different pH levels. The nanoparticles have $28.3 \pm 0.6\text{ mV}$ zeta
243 potential at pH 3.0 whereas the zeta potential of the nanoparticles is $17.3 \pm 0.6\text{ mV}$ at pH 7.5. The decrease
244 in the zeta potential of the nanoparticles can be attributed to both the ionization of the polyacrylic acid
245 and the deprotonation of chitosan with increasing pH.

246 The temperature dependence of the nanoparticles is due to the presence of the temperature-responsive
247 poly (n vinyl caprolactam) (pNVCL) in the core. At pH of 4.5, the average size of the nanoparticles
248 decreases from $169.0 \pm 1.7\text{ nm}$ at 25°C to $126.2 \pm 0.1\text{ nm}$ at 45°C (Fig 4c), as a result of the collapsing of

249 the pNVCL chains at temperatures above LCST of 32°C. As seen in Fig 4c. around the LCST of 32°C,
250 the sizes of the nanoparticles decrease from 165.0±0.1 nm at 30°C to 132.7±0.8 nm at 35°C.

251 Following the dynamic light scattering-based characterization studies of blank nanoparticles in terms of
252 pH and temperature responsiveness, diffusion-based RB loading was done for 72 hours. During loading,
253 the medium was maintained at a pH value of 4.5, where the chitosan chains are protonated through the
254 NH₃⁺ groups and can interact well with the COO⁻ groups of RB. Furthermore, diffusion of RB molecules
255 towards the core is facilitated at this pH where the polyacrylic acid chains are mostly neutral.
256 Furthermore, the medium temperature was kept at room temperature during loading in order to operate
257 below the LCST of pNVCL to improve the loading of the hydrophilic RB. After loading, the average
258 size of the nanoparticles was measured as 163.0±0.9 nm with a 23.6±0.5 mV zeta potential value,
259 indicating that the loading of the RB did not cause a significant increase in the nanoparticle size.

260 3.2 Drug Release Profile and Kinetic Analysis

261 Encapsulation efficiency of the RB-loaded nanoparticles is calculated as 93.66% ±1.55 using Eq. (2),
262 where the amount of the free drug is determined from the UV-Vis measurements.

$$263 \quad \textit{Encapsulation Efficiency} (\%) = \frac{\textit{Total Drug Amount} - \textit{Free Drug Amount}}{\textit{Total Drug Amount}} \times 100 \quad (2)$$

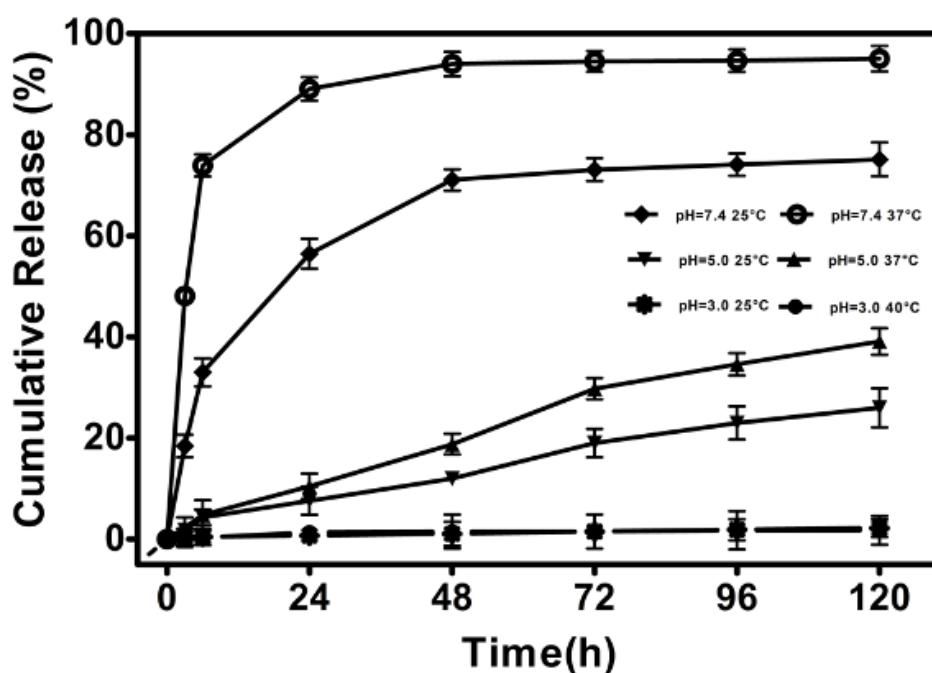
264 The loading capacity of the RB-loaded nanoparticles is calculated as 4.93% ±1.5 using Eq. 3, where the
265 encapsulated drug amount is determined from Eq.2

$$266 \quad \textit{Loading Capacity} (\%) = \frac{\textit{Encapsulated Drug Amount}}{\textit{Total Nanoparticle Weight}} \times 100 \quad (3)$$

267 The results of the RB release studies from the loaded nanoparticles at different pH and temperature
268 values are presented in Figure 5. At the acidic pH value of 3.0, only 2.19% and 1.77 % of RB was
269 released at 25°C and 40°C, respectively, at the end of 120 hours. As the pH is increased from 5.0 to 7.4,
270 the amount of RB released increases from 26% to 75.11% at 25°C and from 39.11% to 95.02% at 40°C,
271 respectively. In addition to the cumulative drug release percentage, the pH of the medium also affected
272 the release rates at the early stages. Comparing the release profiles at pH 5.0 and 7.4, at pH of 5.0, the
273 drug release showed a linear behavior, indicative of a sustained release. On the other hand, at a higher
274 pH value of 7.4, burst release of RB was observed, indicating the presence of a dominant pH-dependent
275 mechanism. Considering the core-shell structure and the pH-dependent nature of the constituent
276 polymers, the burst release observed at high pH is presumably due to the collapsing of the polymer
277 chains which push the RB molecules out. Another factor is the electrostatic interaction between the pH-
278 dependent moieties in the structure and the RB molecule. The nanoparticle structure is mainly a
279 polyelectrolyte complex of negatively charged poly (acrylic acid) and positively charged chitosan
280 chains. In acidic conditions, RB, which has a carboxylic acid group linked to one of its aromatic rings,
281 can interact with the positively charged amino groups of chitosan. As pH increases, this interaction

282 becomes weaker as chitosan is deprotonated. On the other hand, formation of the negatively charged -
283 COO⁻ groups due to the deprotonation of the poly (acrylic acid) triggers the burst release of the RB
284 molecules because of the electrostatic repulsion.

285 Under constant pH, increasing the temperature also increased total amount of RB released, though the
286 effect of the temperature on the release kinetics was observed to be less significant than that of the pH.
287 At the end of 120 h, 26% of RB was released at 25°C as opposed to 39.11% at 40°C at a constant pH of
288 5.0. Similarly, at pH of 7.4, 75.11% of RB was released at 25°C and 95.02% was released at 40°C.
289 Temperature dependence was not observed at pH of 3.0, due to the negligible RB release at this low pH
290 environment. The temperature dependence observed at pH 5.0 and 7.4 can be attributed to the
291 conformational change in the pNVCL chains above LCST, leading to the transition from the hydrophilic
292 to hydrophobic states. The increased hydrophobic nature of the polymer results in shrinking of the core
293 structure which in turn, accelerates the drug release. This behavior is observed both at alkali and acidic
294 pH levels. Ultimately, the consequence of the stimuli-responsive release of RB can be seen in the
295 photograph of dialysis capsules after 72 h at 40°C, in the supplementary data.



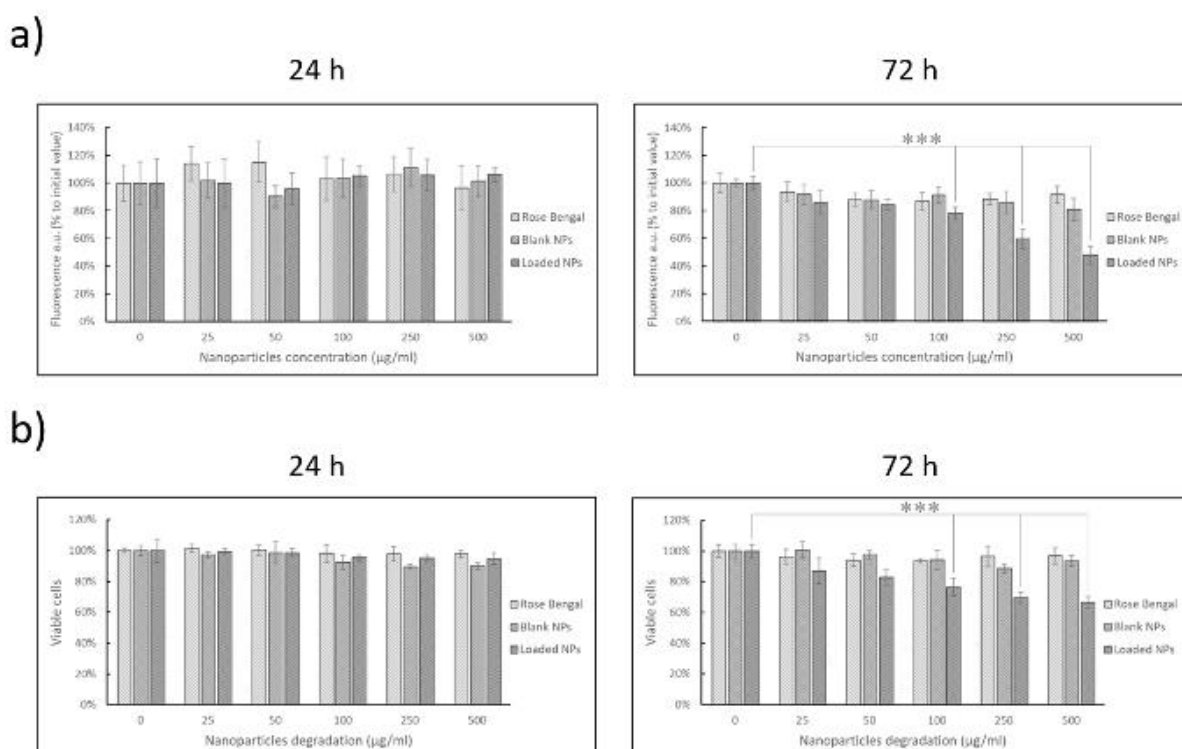
296
297 **Fig. 5.** Release Profiles of Nanoparticles in different temperature and pH levels. Standard deviation
298 values of the release results are in the range of 0.14 to 0.55 for each data point

299 300 3.3 *In vitro* Studies

301 Dual responsive nanoparticles' interaction with cells was first analyzed by evaluating effects on Caco-2
302 cells viability. Cells were treated with increasing concentrations of free RB (0.00, 0.85, 1.70, 3.40, 8.50
303 and 17.00 µg/ml), blank nanoparticles (0, 25, 50, 100, 250 and 500 µg/ml), and RB-loaded nanoparticles
304 (0, 25, 50, 100, 250 and 500 µg/ml respectively corresponding to 0.00, 0.85, 1.70, 3.40, 8.50 and 17.00

305 $\mu\text{g/ml}$ of RB), and analyzed via both PicoGreen[®] and WST-1 assay, after 24 and 72 h of treatment. The
 306 results show that encapsulation of RB inside nanoparticles promotes a cytotoxic effect which is not
 307 observed with the free drug (Figure 6). Both analyses show a statistically significant ($p < 0.001$) decrease
 308 in cell viability after 72 h of treatment with a concentration of RB-loaded nanoparticles of 100 $\mu\text{g/ml}$ or
 309 more. In particular, PicoGreen assay results show a statistically significant ($p < 0.001$) 52.2% decrement
 310 in viability after 72 h of treatment with the highest tested concentration of RB-loaded nanoparticles,
 311 compared to the cells treated with free RB.

312 In earlier studies reported by Qin *et al.* and by Koevary *et al.*, where the effectiveness of RB was tested
 313 on colon cancer cells with immunotherapeutic purposes and on various cell types, respectively, a
 314 statistically significant reduction ($p < 0.001$) of cell viability is achieved with free RB concentrations of
 315 higher than 48 $\mu\text{g/ml}$ [19][20]. In our work, we obtained comparable results with 14 times lower
 316 quantities of RB encapsulated within the nanoparticles. In particular, by comparing our results with
 317 those obtained on colon cancer cells in the work of Qin *et al.*, we obtained a statistically significant
 318 reduction ($p < 0.001$) of cell viability using a 28 times lower quantity of RB loaded inside the
 319 nanoparticles. This significant reduction in cell viability, for the same amount of RB supplied, could be
 320 due to its encapsulation within the nanoparticles, which due to their lipophilic nature, could have
 321 increased the internalization of RB by treated cells.

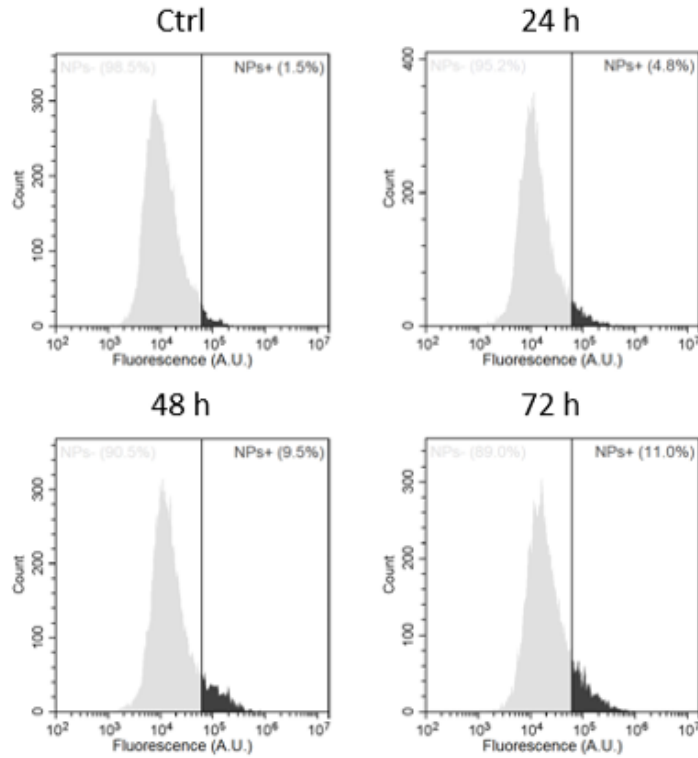


322
 323 **Fig. 6.** Viability analysis on Caco-2 cells performed with increasing concentration of free RB,
 324 CS/PAA/PNVCL nanoparticles, and RB-loaded nanoparticles, at 24 h and 72 h. In a) results from
 325 PicoGreen assay, in b) results of WST-1 assay (***) $p < 0.001$ ($n=6$).

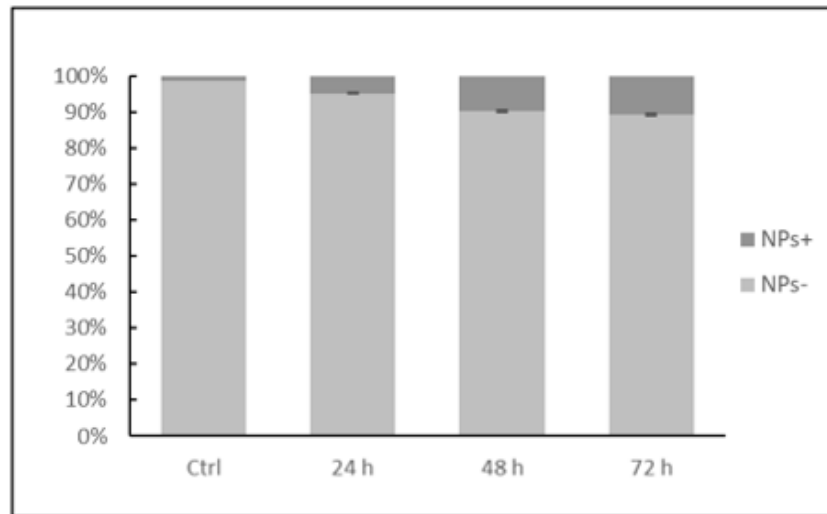
326 To assess cellular internalization, fluorescently labeled CS/PAA/PNVCL nanoparticles were exploited.
327 Cells were treated with 100 $\mu\text{g/ml}$ of nanoparticles for 24, 48, and 72 h, and then analyzed. Flow
328 cytometry analysis showed $4.8\% \pm 0.2\%$ of fluorescence-positive cells after 24 h, a result that increased
329 up to $11.0 \pm 0.8\%$ of fluorescence-positive cells after 72 h of treatment (Figure 7), showing a time-
330 dependent internalization of these nanoparticles by Caco-2 cells. Confocal microscopy imaging
331 confirms the trend observed via flow cytometry, highlighting a perinuclear localization of
332 CS/PAA/PNVCL nanoparticles (Figure 8). Both techniques showed how the encapsulation of RB in
333 these structures can overcome the cytosolic internalization problem of the administration of free RB in
334 acetate form[21].

335

a)



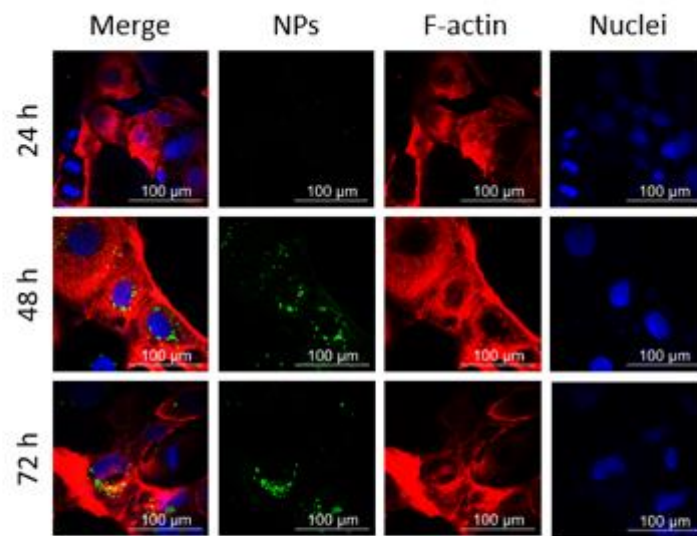
b)



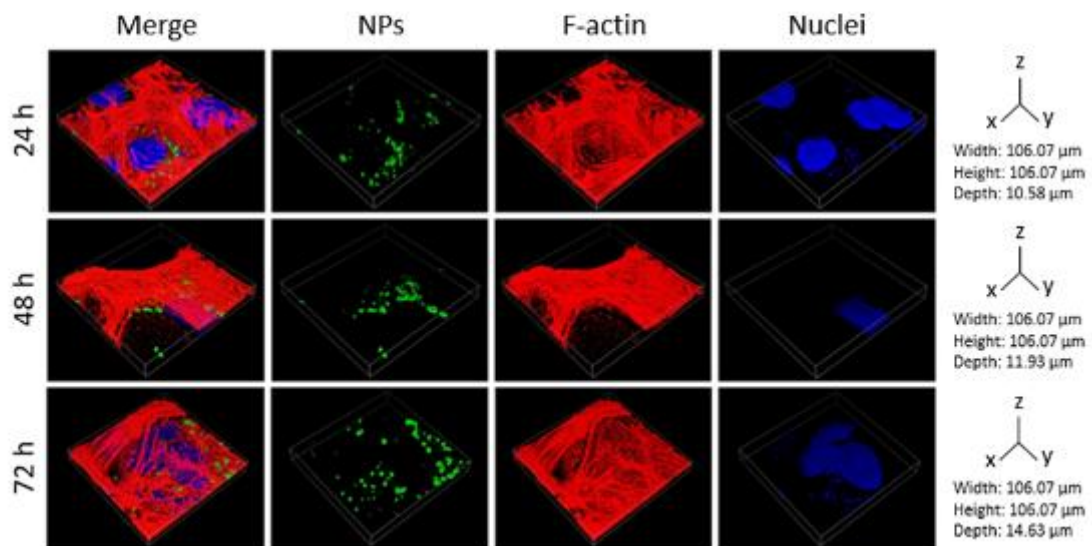
336

337 **Fig. 7.** Flow cytometry analysis of Caco-2 cells incubated with 100 $\mu\text{g/ml}$ of DiO-stained nanoparticles
338 at different time points (24, 48, and 72 h). In dark grey nanoparticles-positive cells (NPs+), in light grey
339 nanoparticles-negative cells (NPs-). a) Representative flow cytometry plots showing fluorescence levels
340 of cells in different experimental conditions. (b) Percentages of NPs+ and NPs- cells for each
341 experimental condition ($n=3$).

a)



b)



342

343 **Fig. 8.** Representative confocal images of Caco-2 cells incubated with 100 µg/ml of DiO-stained
344 nanoparticles at different time points (24, 48, and 72 h, nanoparticles in green, F-actin in red, nuclei in
345 blue): a) single Z-stacks and b) 3D rendering.

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351 **4. Conclusion**

352 Stimuli-responsive nanocarriers (SRNs) composed of a pH-responsive chitosan / polyacrylic acid
353 complex shell and a temperature-responsive poly (n-vinyl caprolactam) core, were synthesized *via* a
354 facile batch emulsion method without the use of any surfactants. Fast release of the RB was achieved at
355 pH 7.4, with more than 70% of the drug released within the first 10 h, whereas at pH 5.0 sustained
356 release with a release percentage of approximately 10% was achieved at the end of 24 h. Furthermore,
357 the therapeutic potential of RB-loaded SRNs against colon cancer cells (Caco-2 cell line) was
358 investigated, and it was observed that RB-loaded nanoparticles displayed considerable cytotoxicity with
359 respect to free RB, suggesting that the SRNs promote the cellular uptake of the RB. The pH and
360 temperature-dependent release behavior of the nanocarriers, in addition to the improved drug uptake by
361 the cells, make these SRNs promising candidates for the treatment of colon cancer *via* oral drug delivery.

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363 **Credit Author Contribution Statement**

364 **ACE:** Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing –
365 original draft. **AC:** Methodology, Investigation, Formal analysis, Visualization, Writing – original draft.
366 **MB, EA, BSS:** Methodology, Formal analysis. **GC:** Supervision, Resources, Writing – review and
367 editing. **GC, GOI:** Conceptualization, Supervision, Project administration, Resources, Validation,
368 Writing – review and editing

369

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373

374 **Declaration of Competing Interest**

375 All authors declare that they do not have any financial interests or personal relationships that could
376 influence the work reported in this paper.

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APPENDIX A. SUPPLEMENTARY MATERIAL

Dual Stimuli-Responsive Nanocarriers via a Facile Batch Emulsion Method for Controlled Release of Rose Bengal

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Fig. S1. The photograph of drug release capsules at pH=7.4(left) and pH=5.0 (right) after 72h at 40°C