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Enhancement of macrophage uptake via phosphatidylserine-coated acetalated dextran nanoparticles

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33

34 **Abstract**

35 Although vital to the immune system, macrophages can act as reservoirs for
36 pathogens such as tuberculosis and human immunodeficiency virus. Limitations in the
37 treatment of such diseases include targeting therapeutics directly to macrophages and the
38 large systemic dosages needed. The objective of this study is to develop a nanoparticle
39 (NP)-based drug delivery system that can provide targeted delivery into macrophages.
40 Acetalated dextran (Ac-Dex) NP loaded with the lipophilic model compound curcumin
41 (CUR) were synthesized and coated in 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine
42 (DPPS), a phospholipid that induces phagocytosis in macrophages. DPPS-CUR NP were
43 found to release 67.8% of encapsulated CUR within 24 hours at pH 5.35 and exhibited
44 minimal CUR release (6.3%) at pH 7.4. DPPS-CUR NP were uptaken by murine
45 macrophages significantly more than NP without DPPS coating and NP exposure to these
46 macrophages resulted in minimal toxicity to the cells and minimal nitric oxide production.
47 These results suggest that the combination of the DPPS coating and pH-sensitive polymer
48 Ac-Dex can provide a NP delivery system capable of enhanced uptake by macrophages
49 and potential systemic stability to more effectively deliver drugs of interest. As a result,
50 the described DPPS-CUR NP can serve as a viable delivery system for the treatment of
51 macrophage-associated diseases.

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58 **Keywords**

59 Acetalated dextran; 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS); macrophage-
60 associated diseases; targeted cellular uptake; nanoparticles; drug delivery

61

62 **Abbreviations**

63 2-methoxypropene (2-MOP), acetalated dextran (Ac-Dex), curcumin (CUR), cyclic-to-
64 acyclic ration (CAC), dichloromethane (DCM), dimethyl sulfoxide (DMSO), deionized
65 (DI) water, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-
66 glycero-3-phospho(1'-*rac*'glycerol) (DPPG), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-
67 serine (DPPS), differential scanning calorimetry (DSC), encapsulation efficiency (EE),
68 human immunodeficiency virus (HIV), lipopolysaccharide (LPS), nitric oxide (NO),
69 nanoparticles (NP), phosphate buffered saline (PBS), phosphatidylserine (PS), poly(vinyl
70 alcohol) (PVA), poly(lactic-co-glycolic acid) (PLGA), p-toluenesulfonate (PPTS),
71 scanning electron microscopy (SEM), tuberculosis (TB), transmission electron microscopy
72 (TEM), triethylamine (TEA)

73

74 **Introduction**

75 Macrophages are vital in the removal of cellular debris and foreign bodies to
76 maintain homeostasis in the human body [1,2], can colonize in the liver, lungs, spleen,
77 lymph nodes, marrow, or brain, and are critical to the innate immune system [2]. An
78 example of the importance of macrophages is in the removal of apoptotic cells via
79 stimulation of various signals and markers that are overexpressed by presenting cells to
80 initiate identification and engulfment [3]. Although macrophages are involved in the
81 protection and maintenance of the human body, there are scenarios in which these cells can
82 result in more harm than good. Macrophages have the potential to act as reservoirs for
83 infectious pathogens, including those related to two of the most prevalent infectious
84 diseases, tuberculosis (TB) [1,2] and the human immunodeficiency virus (HIV) [1,2]. The
85 commonality amongst these diseases lies in the extensive treatment times and dosing
86 regimens necessary to treat TB and HIV, often resulting in negative side effects. As a result,
87 treatment by way of enhanced delivery to macrophages is of growing interest [1–17].

88 Recent progress made in the development of macrophage targeting systems have
89 been based around receptor-ligand interactions [18]. One common approach is surface
90 functionalization of particle systems with mannose residues to target the mannose receptor
91 CD206), a carbohydrate-recognition domain that is largely expressed on alveolar
92 macrophages [19,20]. In other cases, tumor associated macrophages have been targeted
93 using peptide-based ligands such as rabies virus glycoprotein (RVG), and can be used as
94 carriers for systems encapsulating anti-cancer therapies [21,22]. Other ligands such as 4-
95 SO₄-GalNAc have been used to target other receptors on the surface of phagocytic
96 macrophages to increase targeted uptake [23].

97 Phosphatidylserine (PS) is an anionic phospholipid that is produced and stationed
98 on the inner membrane of healthy cells [3,5,24,25]. Once apoptosis is induced, PS
99 transitions from the inner leaflet to the outer leaflet of the cell membrane [14]. Apoptotic
100 cells produce a signal via PS exposure to stimulate the attraction of macrophages for
101 engulfment of the presenting cells (phagocytosis) via receptor-ligand interactions or
102 identification of PS binding proteins produced by phagocytes [1,3,25,26]. Although it is
103 essential for the phagocytosis signal, there is some debate as to whether sole exposure of
104 PS is sufficient to induce the uptake of apoptotic cells by macrophages *in vitro* [3,25,26].
105 However, particle-based drug delivery systems that utilize PS (specifically, 1,2-
106 dipalmitoyl-*sn*-glycero-3-phospho-L-serine, DPPS) as the particle coating have resulted in
107 enhanced macrophage uptake through the sole presence of PS [3], likely due to the amount
108 of PS presented. Multiple studies centered around treatment of inflammation [7,27], HIV-
109 1 [1,2,6,17], cancer [8,9,28], atherosclerosis [29], and MRI imaging [1,11,16] have used
110 DPPS coatings to increase the uptake of particle-based delivery systems by macrophages
111 or have used PS as a binding target.

112 Acetalated dextran (Ac-Dex) is a biodegradable, biocompatible polymer comprised
113 of the FDA-approved excipient dextran and hydrophobic acetal groups that allow for
114 emulsion-based NP synthesis [30–32]. Unlike commonly used drug carriers such as
115 poly(lactic-co-glycolic acid) (PLGA), the degradation kinetics of Ac-Dex can be easily
116 tuned by altering the ratio of cyclic to acyclic acetal groups by modifying the Ac-Dex
117 synthesis reaction time [33,34]. The acid-sensitivity of Ac-Dex has made it a viable carrier
118 option in applications for cancer [33–35], inflammatory-related conditions [36], vaccines
119 [33,34], and antibiotic delivery [37]. There is a significant difference in degradation and

120 drug release kinetics for Ac-Dex nanoparticle (NP) systems at a pH of 5 (faster release)
121 versus at a pH of 7.4 (slower release) [4,30,31,33–37]. Due to this difference, Ac-Dex NP
122 systems will exhibit slower release and stability during systemic circulation (at pH 7.4)
123 until they are delivered to a site in the body with a lower pH, such as in macrophages,
124 where therapeutics will be readily released.

125 The current study involves the design of a novel therapeutic particle-based drug
126 delivery system consisting of a phosphatidylserine (PS)-coated, polymeric (Ac-Dex), drug-
127 loaded NP designed to enhance uptake into macrophages. The novelty of this formulation
128 lies in the inherent ability of PS to enhance the uptake of the NP into macrophages, while
129 the Ac-Dex core promotes burst release of the cargo directly in the macrophages owing to
130 its acid sensitivity. Curcumin (CUR), a natural product isolated from *Curcuma longa*, was
131 chosen as the model small molecule due to its fluorescent properties, low water solubility,
132 and potential clinical applications [4,5,7,9,13,30,38–42].

133

134 **Materials and Methods**

135

136 *Materials*

137 Unless stated otherwise, materials were purchased from Sigma Aldrich (St. Louis,
138 MO). 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-
139 3-phospho(1'-*rac*'glycerol) (DPPG), and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine
140 (sodium salt, DPPS) were purchased from Avanti Lipids (Alabaster, AL).

141

142 *Synthesis and Characterization of Acetalated Dextran*

143 Acetalated dextran (Ac-Dex) was synthesized using a previously described method
144 [30,31]. 1 g of lyophilized dextran (9,000-11,000 MW) was dissolved in 10 mL of
145 anhydrous DMSO with 25 mg of p-toluenesulfonate (PPTS) under nitrogen gas. The
146 reaction was carried out for 5 minutes using 5 mL of 2-methoxypropene (2-MOP) and was
147 quenched with 1 mL of triethylamine (TEA). The polymer was then precipitated in basic
148 water (pH 9), filtered, lyophilized, and stored at -20°C. The cyclic-to-acyclic (CAC) ratio
149 of Ac-Dex and acetal coverage were verified using ¹H NMR (Bruker 300 MHz, NMR,
150 MA) as described previously [30,31,37].

151

152 *Synthesis of PVA-Coated Nanoparticles*

153 PVA-coated, CUR-loaded Ac-Dex NP (PVA-CUR NP) were synthesized using a
154 single emulsion/solvent evaporation method [31]. 50 mg of Ac-Dex and 1 mg of CUR were
155 dissolved in 1 mL of DCM. This organic solution was added to 6 mL of 3% PVA (in 1x
156 PBS) and the resulting mixture was emulsified using a probe sonicator attached to an
157 ultrasonic processor at 120 W and 20 kHz (Q500, Qsonica, Newtown, CT) for 1 minute
158 with 1 second on/off pulses. The emulsion was added to 40 mL of 0.3% PVA and was spun
159 for 4 hours to allow for organic solvent evaporation and particle hardening [30]. The
160 spinning solution was centrifuged at 3124 x g for 60 minutes and particles were redispersed
161 in a 0.1% PVA solution and frozen overnight, followed by lyophilization. PVA-coated NP
162 without CUR (PVA-Blank NP) were formulated similarly to PVA-CUR NP by omitting
163 CUR from the organic solutions. NP samples were stored at -20°C.

164

165 *Synthesis of DPPS and DPPC-Coated Ac-Dex NP*

166 PVA-coated NP were coated with DPPS via a film hydration method often used to
167 make liposomal delivery systems [6,7,17,27,43,44]. DPPS was dissolved in 4 mL of
168 chloroform and methanol (9:1 v/v) [9]. The solution was subjected to rotary evaporation
169 using a Heidolph 2 rotary evaporator (Schwabach, Germany) to create a thin film in a 25
170 mL round-bottom flask. The film was dried under vacuum for 1 hour to remove excess
171 solvent, during which the NP were washed with deionized (DI) water to remove excess
172 PVA. The dried film containing the NP was then rehydrated with 4 mL of DI water and
173 this solution was sonicated for 30 minutes, followed by freezing and lyophilization. After
174 lyophilization, the resulting NP were stored at -20°C. The ratio of lipid to NP during the
175 process was 1:5 (w/w). NP coated with DPPC and a small amount of DPPG NP were
176 synthesized using the same method with a 5:1 (w/w) DPPC:DPPG ratio and these NP are
177 referred to as DPPC-NP.

178

179 *Nanoparticle Size and Surface Charge Characterization*

180 Hydrodynamic diameter and surface charge of the NP were evaluated via dynamic
181 light scattering (DLS) and zeta potential analysis, respectively, using a Malvern Nano
182 Zetasizer (Malvern Instruments, Worcestershire, UK). The NP were diluted to 0.25 mg/mL
183 in DI water and were analyzed at 25°C and an angle of 90°.

184

185 *Evaluation of CUR NP Encapsulation Efficiency and Drug Loading*

186 To determine the amount of CUR encapsulated in the NP systems, NP were
187 dissolved in DMSO (1 mg/mL) and the fluorescence of the solutions was analyzed using a
188 SpectraMax M2 Plate Reader (Molecular Devices, Sunnyvale, CA) at an excitation of 420

189 nm and emission of 520 nm. The encapsulation efficiency (EE) and drug loading were
190 calculated using the following equations:

191

$$192 \quad \text{Encapsulation Efficiency (EE)} = \frac{\text{experimental mass of drug in NP}}{\text{theoretical mass of drug in NP}} \times 100\%$$

193

$$194 \quad \text{Drug Loading} = \frac{\text{mass of drug in NP}}{\text{mass of NP}}$$

195

196 *Electron Microscopy Imaging Analysis of Nanoparticles*

197 Images of the NP were taken using a Zeiss Sigma VP Field Emission-Scanning
198 Electron Microscope (FE-SEM) (Germany) for analysis of NP morphology. After NP were
199 suspended in basic water (15 mg/mL), 1-2 drops of this suspension were added to
200 aluminum SEM stubs (TedPella Inc., Redding, CA), and the samples were air dried. Dried
201 samples were then coated with a film of gold/palladium alloy using an Emscope SC400
202 sputter coating system at a 20 μ A for 75 seconds under argon. Images of the NP systems
203 were also captured via a JEOL JEM-2100F transmission electron microscope (TEM,
204 Peabody, MA) for observation of the NP core and outer layers. 1 μ L of 15 mg/mL NP
205 suspension in water were placed on 200 square mesh copper grids (Electron Microscopy
206 Sciences, Hatfield, PA) and air dried prior to imaging.

207

208 *Differential Scanning Calorimetry (DSC) Analysis of Nanoparticles*

209 Thermal phase transitions of the NP systems were analyzed using differential
210 scanning calorimetry (DSC) via a TA Q10 DSC system (TA Instruments, New Castle, DE)

211 connected to an RSC-90 cooling accessory. For dry-state DSC, samples were lyophilized
212 24 hours prior to analysis. 1-3 mg of NP were analyzed at 10°C/min from 0 to 300°C. For
213 wet state analysis, NP were dispersed in DI water (0.1 mM DPPS) and 15 µL of the NP
214 suspension was added to aluminum pans to be analyzed at 2°C/min and from 5 to 65°C.
215 DSC analysis of the raw materials was also performed.

216

217 *PVA Coating Quantification*

218 Quantification of the amount of PVA on the surface of NP was completed using a
219 previously described method [45]. NP samples were dispersed in DI water (1 mg/mL). 400
220 µL of sample solution, 300 µL of iodine solution (1.25 % iodine and 2.5% potassium
221 iodide), and 1.5 mL of 4% boric acid solution were mixed together for 20 minutes at room
222 temperature and 100 rpm. Absorbance of the samples was measured at a wavelength of
223 630 nm.

224

225 *Phospholipid Content Quantification*

226 Phospholipid quantification was carried out using Stewart's method [46]. Briefly,
227 2.7 g ferric chloride hexahydrate and 3 g of ammonium thiocyanate was dissolved in 100
228 mL of distilled water. NP samples were dissolved in chloroform (1 mg/mL) and were
229 mixed with the ammonium ferrothiocyanate solution via vortexing for 5 minutes at a 2:1
230 (v/v) ratio. After mixing, the solution was allowed to separate, and the phospholipid content
231 in the chloroform portion was measured via UV-vis spectroscopy at 488 nm (DPPC/G) and
232 452 nm (DPPS).

233

234 *In Vitro Drug Release from CUR NP*

235 Release of CUR from the NP was carried out using a previously established
236 centrifugation technique [30]. 1 mg/mL NP samples were dispersed in PBS (pH 7.4) or
237 sodium acetate buffer (pH 5.35) supplemented using 0.2% Tween® 80 to enhance CUR
238 solubility. The suspensions were incubated at 37°C and 100 rpm and at predetermined time
239 points the suspensions were removed and centrifuged at 23,102 x g for 15 minutes. 200 µL
240 of the supernatant was collected and frozen at -20°C and 200 µL of fresh medium was
241 added to the particle solution prior to re-dispersion and re-incubation. Release samples
242 were mixed with DMSO (1:1 by volume) prior to fluorescence spectroscopy analysis at an
243 excitation of 420 nm and emission of 520 nm [30].

244

245 *Cell Culture*

246 Murine macrophage cells (RAW 264.7) and A549 human adenocarcinoma cells
247 obtained from American Type Culture Collection (ATCC, Manassas, VA) were used for
248 cell culture studies. The cells were maintained at 37°C and 5% CO₂ in Dulbecco's modified
249 Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml
250 penicillin, 100 µg/ml streptomycin, Fungizone® (0.5 µg amphotericin B, 0.41 µg/mL
251 sodium deoxycholate), and 1 mM sodium pyruvate.

252

253 *In Vitro Cytotoxicity Analysis of NP*

254 The cytotoxic effect of the formulated NP on RAW 264.7 macrophages was
255 determined using a resazurin assay. Cells were seeded in 96-well plate at 5,000 cells/well
256 and incubated overnight at 37 °C and were then exposed to varying concentrations of PVA-

257 CUR and DPPS-CUR NP (0.001 to 0.2 mg/ml). Untreated cells were used as negative
258 controls. After 48 hours, resazurin solution (60 μ M) was added to the cells and incubated
259 for 3 hours. The fluorescence intensity of resorufin produced by viable cells was detected
260 at 544 nm (excitation) and 590 nm (emission) using BioTek Cytation 3 plate reader. The
261 relative viability of each sample was calculated by:

262

$$263 \quad \text{Relative Viability} = \frac{\text{Sample Fluorescence Intensity}}{\text{Control Fluorescence Intensity}} \times 100$$

264

265 *Nitrite Analysis*

266 A Griess assay was performed to determine nitric oxide (NO) production by
267 macrophages. Following 48-hour incubation with varying concentrations of NP (CUR-
268 loaded and blank), 96-well plates containing the samples were centrifuged and 50 μ L of
269 the resulting supernatant was removed from each well. Griess reagents were added per
270 manufacturer's instructions and the absorbance was measured at 550 nm. LPS (500
271 ng/mL), was used to promote NO production as a control.

272

273 *In Vitro Cellular Uptake Via Spectroscopy and Confocal Microscopy*

274 Cellular uptake of CUR-loaded NP by RAW 264.7 and A549 cells was observed
275 using a Cytation 3 image reader (BioTek, Winooski, VT). Cells (7,500 cells/well) were
276 seeded in a 96-well plate and incubated overnight at 37 $^{\circ}$ C. Cells were then incubated with
277 0.1 mg/ml of NP solutions and equal concentration of CUR for 1 and 3 hours. After
278 incubation, the media was removed, and the cells were washed three times with 200 mM
279 glycine to remove any unbound NP that were not taken up by the cells. For quantification

280 of cellular uptake, the fluorescence of the CUR within the cells was analyzed via
281 fluorescence spectroscopy at 420 nm (excitation) and 520 nm (emission).

282 RAW 264.7 and A549 cells were seeded into 35 mm glass-bottom petri dishes at a
283 concentration of 500,000 cells per dish and allowed to grow overnight. DPPS-CUR NP,
284 DPPC-CUR NP, or PVA-CUR NP were suspended and bath sonicated in cell culture
285 medium at 0.1 mg/mL and were then incubated with cells for 1 or 3 hours. Cells were
286 washed 3x in PBS and incubated in fresh media containing CellMask Deep Red
287 (Invitrogen) at 1 μ L/mL media for 10 minutes. Cells were then fixed with 4%
288 paraformaldehyde for 10 minutes and rinsed 3x with PBS. Fresh PBS was added and cells
289 were immediately imaged using a Nikon Eclipse Ti2 inverted confocal fluorescent
290 microscope.

291

292 *Statistical Analysis*

293 All measurements were performed in at least triplicate. Statistical differences for *in*
294 *vitro* cellular studies was determined using one-way or two-way ANOVA with Tukey's
295 multiple comparisons test (GraphPad Prism version 7). For cumulative drug release,
296 Student's *t*-Test was used to determine statistical differences. A p-value of < 0.05 or lower
297 was considered as statistically significant. Values are presented as mean \pm standard
298 deviation.

299

300 **Results**

301

302 *Characterization of Ac-Dex Polymer and Nanoparticles*

303 Analysis of the acetal coverage and cyclic-to-acyclic (CAC) ratio of Ac-Dex
304 following its synthesis was confirmed via NMR. The acetal coverage of Ac-Dex was 73%,
305 whereas the CAC ratio was 45%. PVA-coated NP were synthesized using the emulsion
306 method to create NP with an Ac-Dex polymer core and PVA coating both with and without
307 curcumin loading (PVA-CUR NP and PVA-Blank NP, respectively). As seen in **Table 1**,
308 PVA-coated NP were 260-275 nm in diameter, exhibited polydispersity index (PDI) values
309 of 0.04, and had nearly neutral surface charges (less than -3 mV), as seen from the zeta
310 potential values.

311 After the synthesis of PVA-coated NP, the particles were coated with DPPS using
312 a thin film hydration method to form DPPS-coated NP, both with and without CUR loading
313 (DPPS-CUR NP and DPPS-Blank NP, respectively). The diameters for both the blank and
314 CUR-loaded DPPS NP increased by approximately 75 nm in comparison to the PVA-
315 coated NP. The PDI values were low for both DPPS NP systems (less than 0.21), however,
316 there was a substantial decrease in zeta potential, from -3 mV (PVA-coated NP) to -40 mV
317 (DPPS-coated NP) for both blank and CUR-loaded NP systems.

318 The encapsulation efficiency (EE) and drug loading (by mass) of CUR-loaded NP
319 was analyzed to quantify the amount of CUR loaded in the particles. Both PVA- and DPPS-
320 coated NP exhibited EE values around 25% whereas the drug loading was 7.1 and 5.2 μg
321 CUR/mg NP for PVA-CUR NP and DPPS-CUR NP, respectively.

322

323 *Morphological Analysis of NP Via Electron Microscopy*

324 Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)
325 were used to analyze the morphology and structure of PVA- and DPPS-coated NP loaded

326 with CUR. SEM micrographs indicated that PVA-CUR NP presented spherical, smooth
327 morphology and were homogenous with respect to in size (**Figure 1, top**). TEM images
328 allowed for the visualization of a thin layer around the Ac-Dex polymer core, which likely
329 corresponds to the PVA coating. DPPS-CUR NP were fairly monodisperse with spherical,
330 smooth morphology as seen via SEM imaging (**Figure 1, bottom**). TEM micrographs
331 show a coating on the Ac-Dex NP core, likely indicating the DPPS coating on the NP.

332

333 *Coating Quantification*

334 The amount of PVA and total phospholipids present in the NP formulations were
335 quantified and are presented in **Figure 2**. There was significantly less PVA present on the
336 DPPS and DPPC-NP in comparison to the PVA-NP ($p < 0.001$ and $p < 0.0001$,
337 respectively). In addition, significantly more phospholipid was present in DPPS and
338 DPPC-NP in comparison to PVA-NP ($p < 0.01$).

339

340 *Differential Scanning Calorimetry (DSC) Thermal Analysis*

341 **Figure 3** shows the thermograms of the prepared NP and their corresponding raw
342 components in their dry states. An endothermic peak at 90°C was present, indicating a
343 bilayer phase transition for the DPPS-coated NP, which is slightly lower than the
344 endothermic phase transition temperature of raw DPPS at 99°C. An endothermic peak was
345 present at 187°C for raw PVA, whereas a broadened endothermic peak was present at
346 195°C for PVA-coated NP. Both raw CUR and Ac-Dex exhibited endothermic peaks at
347 177°C, signifying their melting points [30,31,37]. These prominent endothermic peaks
348 were not present in the formulated NP. The shift from 177°C to a broad peak at 195°C for

349 PVA-coated NP could be due to the interaction between Ac-Dex and PVA. DPPS-coated
350 NP exhibited a broad endothermic peak at 165°C, potentially due to the removal of the
351 PVA and the interaction between the Ac-Dex and DPPS. For wet-state DSC analysis of the
352 formulated NP, there were no measurable peaks present for the temperature range used;
353 curvature corresponded to the evaporation of water.

354

355 *In Vitro Drug Release from NP*

356 The release of CUR from the particles was analyzed at two pH values, including
357 pH 7.4 to simulate normal physiological pH and pH 5.35 to approximate the pH in
358 macrophages [4]. Results in **Figure 4** shows that there was no significant difference in the
359 cumulative release of CUR from PVA-coated versus DPPS-coated NP at pH 7.4 after 24
360 hours (5.2% vs 6.3%), signifying that the NP coating played no appreciable role in affecting
361 CUR release at this pH. However, significantly more CUR was released at pH 5.35 for
362 both DPPS- and PVA-coated particles (67.8% and 88.8% release at pH 5.35, respectively)
363 in comparison to pH 7.4 ($p < 0.001$), demonstrating the sensitivity of Ac-Dex to acidic
364 conditions. Furthermore, when comparing DPPS- and PVA-coated NP at pH 5.35,
365 significantly more CUR was released from NP with a PVA coating, indicating the potential
366 influence of the DPPS layer in delaying the release of the cargo ($p < 0.05$).

367

368 *In Vitro Cytotoxicity Assay*

369 The impact of the described NP systems on macrophage toxicity was evaluated and
370 the results are presented in **Figure 5**. These results indicate that the cells exhibited relative
371 viabilities similar to the control (media only) after 48 hours of exposure to the NP systems.

372 Increasing the concentration of NP imparted no significant change in the viability of cells
373 for PVA-Blank NP, DPPS-Blank NP, or PVA-CUR NP for the concentrations tested.
374 However, for DPPS-CUR NP there was a slight but statistically significant decrease in the
375 viability of cells at 0.2 mg/ml in comparison to the control ($p < 0.05$).

376

377 *Nitrite Analysis*

378 Following 48-hour incubation of NP samples on cells, CUR-loaded and blank
379 (control) NP groups produced significantly lower NO in comparison to LPS ($p < 0.0001$)
380 (**Figure 6**). The NP groups were the same as the control group (media only) statistically,
381 indicating little to no production of NO [47].

382

383 *In Vitro Cellular Uptake*

384 The cellular uptake of CUR-loaded NP was evaluated in RAW 264.7 macrophages
385 and A549 adenocarcinoma cells for up to 3 hours as shown in **Figure 6**. Exploiting the
386 fluorescent properties of CUR, the presence of CUR-loaded NP within cells can easily be
387 detected via fluorescence imaging and spectroscopy. After 1 hour of exposure, significantly
388 more DPPS-coated NP were uptaken by macrophages and A549 cells in comparison to
389 PVA-coated NP, DPPC-coated NP, and raw CUR ($p < 0.0001$). For both cell types and all
390 formulations, uptake was not statistically different between 1 and 3-hour exposures. In
391 addition, significantly more NP were uptaken in RAW macrophages in comparison to
392 A549 cells for all NP formulations. Confocal microscopy confirmed fluorescence
393 spectroscopy quantification, showing higher fluorescence in macrophages in comparison
394 to A549 cells, indicating increased NP uptake. In addition, more NP were uptaken in DPPS-

395 coated NP in comparison to DPPC- and PVA-coated systems in RAW and A549 cells.
396 Images indicate that CUR-loaded NP were primarily uptaken into the cytoplasm of
397 macrophages, especially for DPPS-CUR NP. On the other hand, there were minimal NP
398 located in the cytoplasm of A549 cells; instead the NP were more readily located in the
399 cellular membranes.

400

401 **Discussion**

402

403 *Nanoparticle Design and Analysis*

404 The use of Ac-Dex as a biodegradable NP core for drug delivery can allow for a
405 system with tunable and/or triggered degradation [32]. For the described NP systems, the
406 acetal coverage of Ac-Dex was similar to previous studies [30,31,37], whereas the CAC
407 ratio was slightly lower. The resulting Ac-Dex was favorable for the given application as
408 the adequate acetal coverage imparts hydrophobicity to Ac-Dex, allowing for NP
409 formulation and the lower CAC ratio will allow for faster drug release at acidic pH
410 [30,31,35,37].

411 The increase in diameter and PDI from PVA-coated NP to DPPS-coated NP is
412 likely due to the presence of the DPPS coating around the Ac-Dex core. It has been shown
413 that an increase in NP diameter can lead to an increase in the uptake of NP into
414 macrophages, where particles 200 nm in diameter or greater are often subjected to a greater
415 amount of macrophage uptake [48–50]. For this study, larger NP were desired and
416 successfully formed (up to 350 nm for DPPS-coated NP), which was advantageous since
417 increased macrophage uptake was an objective [4]. While DPPS-coated NP exhibited

418 higher PDI values than PVA-coated NP, these values were still low, indicating that all of
419 the formulated NP systems were homogenous with respect to size.

420 The surface charge of the NP, as indicated by zeta potential, decreased significantly
421 (from -3 to -40 mV) upon coating with DPPS. The negative surface charge on the DPPS-
422 loaded NP is indicative of the polar head of DPPS being exposed to the outer environment,
423 similar to how apoptotic cells present DPPS to the outer leaflet of cells prior to engulfment
424 by macrophages [6,28]. The highly negatively surface charge is significantly lower than
425 the -25 mV threshold of being functionally negative, indicating that the NP are less likely
426 to aggregate due to electrostatic repulsion [51,52]. It should be noted that DPPC-coated NP
427 were also lower than the -25 mV threshold, indicating a functionally negative charge. In
428 addition, there were no differences in size, PDI value, or zeta potential upon the loading of
429 CUR on the NP core, indicating that the loading of the model drug had no significant effect
430 on the particle systems with respect to these characteristics.

431 CUR was encapsulated in the NP for two purposes: 1) to provide a model small
432 molecule that is easy to encapsulate owing to its hydrophobicity and 2) the inherent
433 fluorescence of CUR within the NP allows for easy fluorescence imaging and detection.
434 The encapsulation efficiency (EE) of CUR in the NP was lower than previously reported
435 values, which have ranged from 50-88% [7,9,38]. However, since CUR was used as a
436 model small molecule, the EE values were satisfactory for this study. Upon coating the NP
437 with DPPS, CUR loading decreased slightly but the encapsulation efficiency stayed the
438 same, indicating that the DPPS coating did not significantly affect CUR loading.

439 The SEM and TEM micrographs indicate that the particles are spherical in shape
440 and monodisperse, which correlates to the PDI values for the systems. The DPPS-CUR NP

441 seem to have slightly more coating on the NP surfaces, which could be due to the increased
442 presence of DPPS.

443

444 *Coating Quantification*

445 DPPS was selected to coat the nanoparticles owing to its ability to stimulate an
446 “eat-me” signal on apoptotic cells [1,3,11,16,17]. For cell studies, DPPC-NP were
447 produced as a control lipid system. Initially, the NP systems were coated with PVA because
448 it is one of the most widely used polymers in the pharmaceutical industry [45] and has been
449 extensively used as a NP coating agent due to the steric effects PVA coating imparts on
450 NP, inhibiting aggregation. During the synthesis of phospholipid-coated NP, a significant
451 portion of the PVA was washed away prior to phospholipid coating, since there is
452 significantly less PVA present on phospholipid-coated NP in comparison to PVA NP
453 (**Figure 2**). It is also likely that the phospholipid coating shielded the remaining PVA upon
454 adsorption to the NP surface, as indicated by the low mass presence of PVA on
455 phospholipid-coated NP the high mass presence of total phospholipid on the NP, and the
456 changes in surface charge (**Figure 2**).

457

458 *Thermal Analysis of Nanoparticle Formulations*

459 Dry-state DSC thermograms indicated the presence of DPPS in the DPPS-coated
460 NP via the bilayer phase transition peak that occurred at 90°C for the NP, which is slightly
461 lower than the raw DPPS endothermic peak present at 99°C. This transition signifies the
462 presence of bilayer formation of the phospholipid around the Ac-Dex polymer core in its
463 dry state. The decrease in the transition temperature is likely due to the interaction of the

464 multiple components in the formulation. As the transition temperatures of the NP
465 formulations were just below or well above 100°C, they are likely to be stable in the dry
466 state and can be easily reconstituted before use [53]. The endothermic peak that raw CUR
467 exhibited at 177°C was not observed in CUR-loaded NP. According to our previous
468 research (data not shown), this is likely due a limitation in DSC detection with relation to
469 the actual amount of CUR in the samples.

470 In studies using liposomal formulations, DSC is used to assess the transition
471 temperature(s) in the systems in order to determine the presence and state of bilayers and
472 the differences associated between blank liposomes and modified/drug-loaded systems
473 [43,44]. For wet-state analysis (**Figure 3C**), there was no endothermic peak present around
474 50-60°C, which is the temperature at which the gel/crystalline phase transition occurs for
475 PS bilayers in aqueous solutions [24,54]. Such a phenomenon could have occurred based
476 on the preparation method used, as described previously. In certain cases, self-assembly of
477 polymer-lipid structures can occur via hydrophobic interaction between the lipid tails and
478 a polymeric core simultaneously with hydrophilic interactions between the polar head and
479 external environment [55]. Due to the concentration dependency of DSC, it is more likely
480 that the DPPS concentration may have been too low to be detectable by thermal analysis,
481 like that of the CUR detection limit mentioned previously. It should be noted that the
482 curvature of the thermograms in **Figure 3C** corresponds to the evaporation of water, which
483 is not shown in the thermogram. Overall, the results indicate the presence of DPPS on the
484 surface of the NP, further strengthening what has been previously described with respect
485 to successful DPPS coating of the described systems.

486

487 *Curcumin Release from Nanoparticles*

488 The release from CUR from PVA- and DPPS-coated NP was evaluated to
489 demonstrate the ability of the system to provide sustained release of a therapeutic agent
490 and to show the acid-sensitive nature of Ac-Dex. Ac-Dex is known to degrade more quickly
491 in acidic environments [32]. In a previous study, Ac-Dex particles released 60% of their
492 camptothecin payload after 7 days [35] and 15% of rapamycin after 10 days [4] at pH of
493 7.4. Comparatively, at pH values of 5 or lower, Ac-Dex particles released 100% of their
494 content in as little as 24 hours [4,30,33]. For this study, at pH 5.35, 9.3% of CUR was
495 initially released from DPPS-CUR NP and 29.2% was released after 1 hour, followed by a
496 sustained release of cargo of 67.8% after 24 hours, when equilibrium is reached. At pH 7.4,
497 both NP systems exhibited minimal CUR release, demonstrating the stability of Ac-Dex in
498 neutral pH. However, both systems exhibited triggered release at pH 5.35, exhibiting a
499 significantly higher total release in comparison to their pH 7.4 counterparts ($p < 0.001$).
500 Overall, these data indicate a more stable NP formulation at pH 7.4 and the potential for
501 triggered release at pH 5.35, corresponding to stability in circulation and burst release in
502 macrophages, respectively.

503

504 *Cytotoxic Analysis and Macrophage Uptake of Nanoparticles*

505 To assess the *in vitro* cytotoxicity of the NP formulations on RAW 264.7
506 macrophages, the cells were exposed to the systems for 48 hours. It was observed that the
507 NP systems were not toxic to the cells, however, a slight, but significant, decrease in
508 viability of RAW 264.7 cells for DPPS-CUR NP at 0.2 mg/ml was observed (**Figure 5**).
509 This decrease in viability was minimal and could easily be overcome by using lower

510 concentrations of the particles in future studies. Overall, these results indicate the safety of
511 the described NP for a time period longer than necessary for NP uptake into macrophages,
512 indicating that they are safe to use in the intended fashion.

513 Nanomaterials may provoke immune and inflammation responses following
514 interactions with multiple biological entities [49]. To assess inflammatory and immune
515 response stimulation, NO production was measured using a Griess assay, where LPS was
516 used as a positive control to stimulate NO production in RAW macrophages. It was
517 observed that NP at varying concentrations stimulated significantly less NO production in
518 comparison to LPS and resulted in NO concentrations comparable to that of the control
519 (media only). Results indicated that blank NP caused NO production at concentrations
520 similar to that of the CUR-loaded NP. These data indicate both blank and CUR-loaded
521 formulations produced minimal NO and that CUR did not play an active role in the
522 inhibition of NO production.

523 Since the purpose of this study was to formulate acid-sensitive NP capable of
524 enhanced macrophage uptake, the ability of DPPS-coated NP to be uptaken by
525 macrophages was evaluated, with A549 cells acting as control tissue. PVA and DPPC-
526 coated NP were used as other coatings for comparison to DPPS. Several environmental
527 and physicochemical factors are known to influence the cellular uptake of NP into
528 macrophages. The preferential uptake of DPPS-CUR NP by macrophages in comparison
529 to PVA-CUR NP and DPPC-CUR NP is likely due to the DPPS coating on the Ac-Dex
530 polymeric core. Confocal microscopy in tandem with fluorescence quantification of CUR-
531 loaded NP within the cells confirm the uptake of DPPS-CUR-NP and localization to the
532 cytoplasm of macrophages. The presence of DPPS on the cellular surface is known to cause

533 an “eat-me” signal in cells that are transitioning toward an apoptotic state, causing
534 macrophages to identify and phagocytose the dying cells [1–3,7,11]. Therefore, in terms of
535 recognition, the DPPS coating on the Ac-Dex NP was identified by the macrophages,
536 which produced the necessary phagocytotic action, resulting in cellular uptake of the
537 particles [1,6,7,16,25,27]. In most cases, apoptosis is initiated by the presence DPPS and
538 other signaling pathways such as receptor-ligand interactions or identification of PS
539 binding proteins produced by phagocytes. However, there is a debate as to whether DPPS
540 recognition by itself is sufficient for the uptake of apoptotic cells by phagocytotic cells
541 [3,25]. In the case of nanoparticles, it has been shown that a DPPS coating alone is
542 sufficient for uptake, as seen in this current study and otherwise [7,16,17].

543 Another consideration for cellular uptake of the described particles is in terms of
544 their physicochemical characteristics. Stimulation of phagocytosis of nanoparticle-based
545 delivery systems via macrophages is also dependent on the size and shape of the particles.
546 Since the DPPS-CUR NP were greater than 300 nm in diameter, it is likely that these NP
547 were phagocytosed not only because of the DPPS layer, but also because of their size
548 [4,48,56]. It is well known that surface charge can play a role in biological interactions.
549 Interestingly, surface charge did not play a role in the uptake of the NP, as shown by the
550 similar charges of DPPC and DPPS-coated NP that produced significantly different uptake
551 quantities [48,51,52]. This phenomenon could also explain the minimal phagocytosis of
552 the PVA-coated NP and confirm the effect of DPPS on the uptake of the NP. Overall, the
553 results indicate the successful uptake of DPPS-coated NP into macrophages, indicating that
554 these systems could be used for the treatment of a multitude of diseases involving infected
555 cells.

556

557 **Conclusions**

558 Macrophages can harbor infectious agents that lead to potentially fatal diseases
559 such as TB and HIV. Due to this, treatment of these diseases is challenging and therefore,
560 the goal of this study was to design a delivery system that would enhance the uptake of
561 nanoparticles into macrophages, allowing for targeted delivery, a decrease of systemic side
562 effects, and decreased treatment times. Results showed that both PVA-coated and DPPS-
563 coated NP were monodisperse and that there was a significant increase in size upon DPPS
564 coating. The surface charge of the DPPS NP was -40 mV, indicating that DPPS was present
565 on the surface of the NP. This was further confirmed via PVA quantification, which
566 indicated minimal amounts of PVA on the DPPS NP. DSC results confirmed the presence
567 of a DPPS bilayer on the NP surface and the likely stability of the NP during synthesis and
568 storage conditions. CUR-loaded NP successfully encapsulated CUR and this agent was
569 more quickly released in acidic conditions in comparison to neutral pH owing to the acid
570 sensitivity of Ac-Dex. In addition, CUR-loaded NP exhibited sustained release of CUR.
571 PVA-coated NP were not readily phagocytosed by macrophages, whereas DPPS-coated
572 NP were phagocytosed within 1 hour of exposure, showing the physiological relevance of
573 the DPPS coating for the enhanced delivery to macrophages. These data show that the
574 particles can potentially deliver therapeutic agents throughout the body with fewer
575 concerns of systemic drug exposure, and when exposed to macrophages, the particles are
576 capable of being phagocytosed relatively quickly. Therefore, the combination of Ac-Dex
577 and DPPS can be a viable option for targeted delivery for macrophage-associated diseases.
578

579 **Conflicts of Interest**

580 The authors have no conflicts of interest to report for this study.

581

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599 **Tables and Figures**

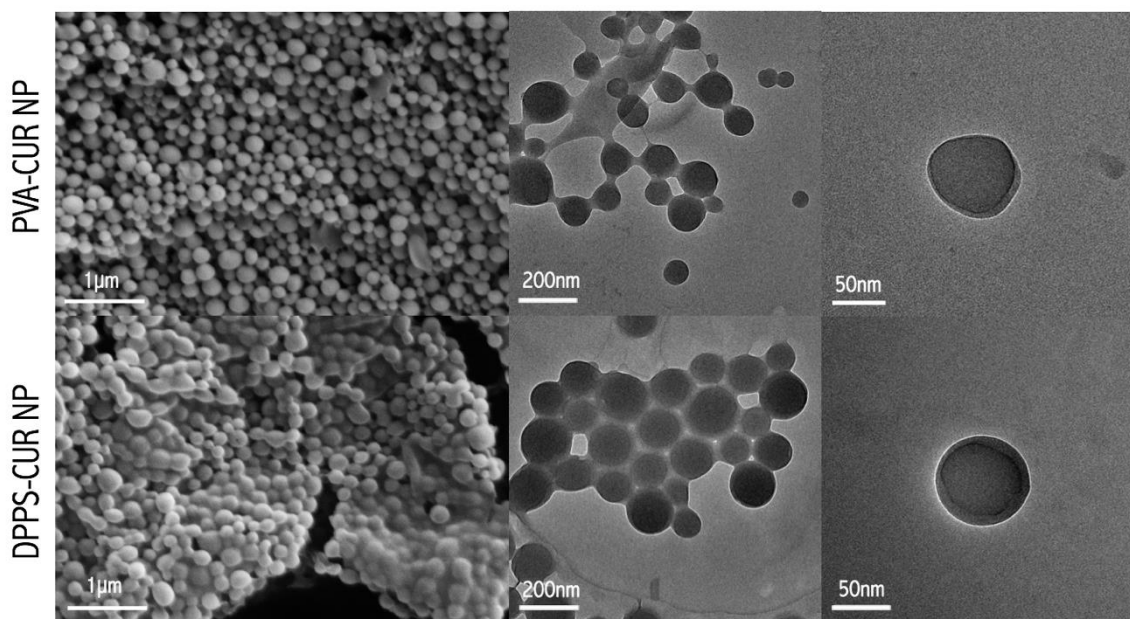
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601 **Table 1.** Diameter, polydispersity index (PDI), surface charge via zeta potential,
 602 curcumin encapsulation efficiency, and drug loading of nanoparticle systems.

	Diameter (nm)	PDI	Zeta Potential (mV)	Encapsulation Efficiency (%)	Drug loading (μg drug/mg particle)
PVA-Blank NP	262.6 ± 1.9	0.04 ± 0.00	-2.2 ± 3.5	---	---
PVA-CUR NP	272.6 ± 10.4	0.04 ± 0.01	-3.0 ± 0.6	25.1 ± 0.1	7.1 ± 1.8
DPPS-Blank NP	335.6 ± 3.8	0.21 ± 0.43	-40.4 ± 3.3	---	---
DPPS-CUR NP	350.5 ± 16.9	0.13 ± 0.65	-40.6 ± 1.3	24.5 ± 0.1	5.2 ± 0.7

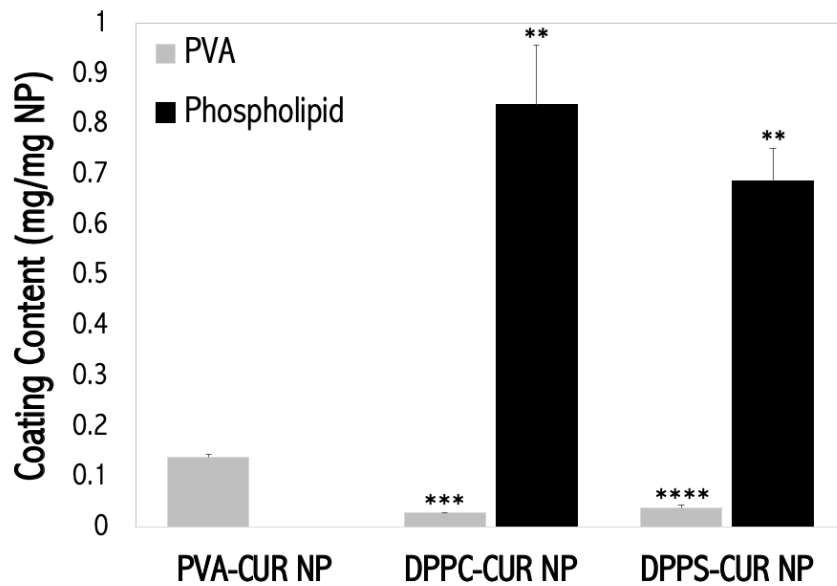
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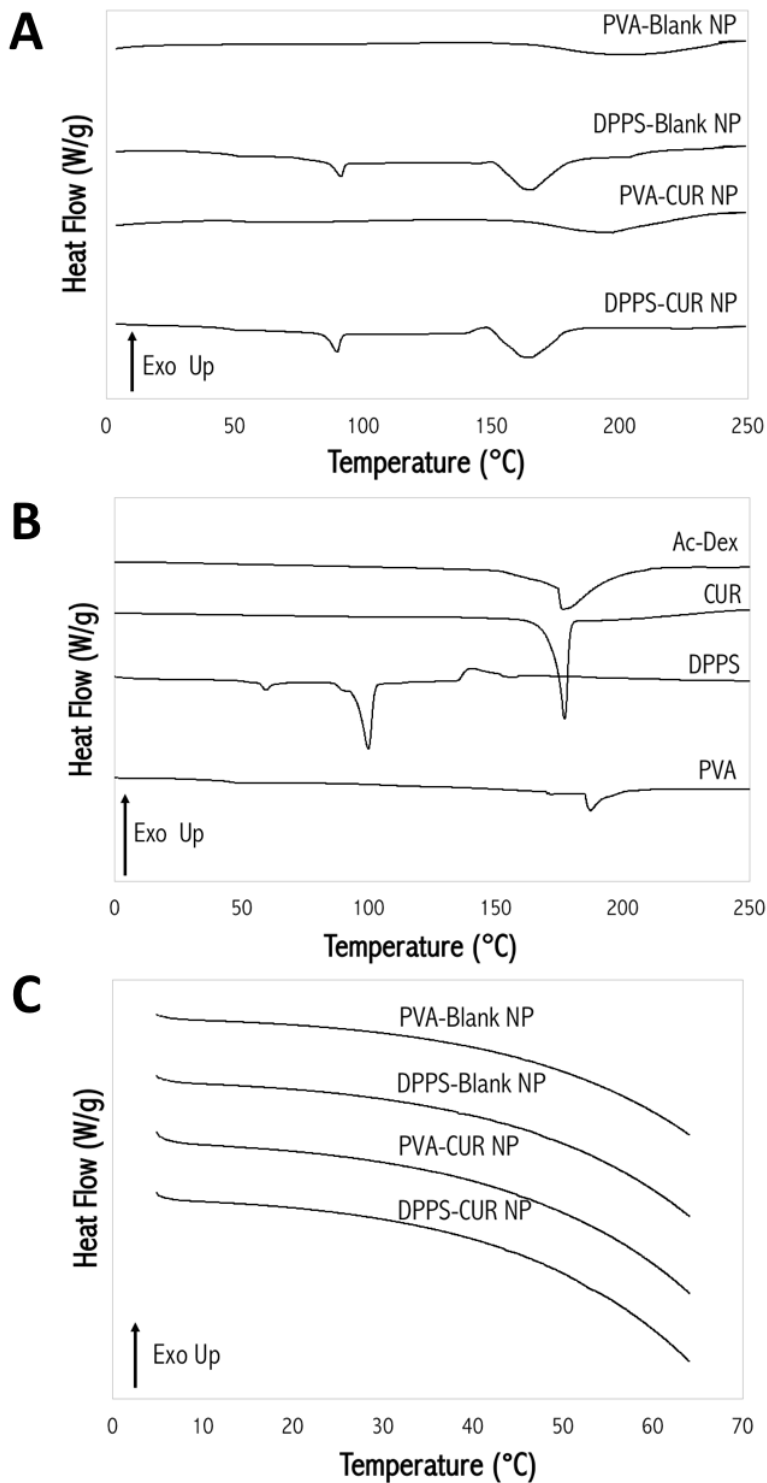
605 **Figure 1.** Representative SEM (left) and TEM (middle, right) micrographs of PVA- and
 606 DPPS-coated, CUR-loaded nanoparticles (PVA-CUR NP and PVA-DPPS NP,
 607 respectively).

608



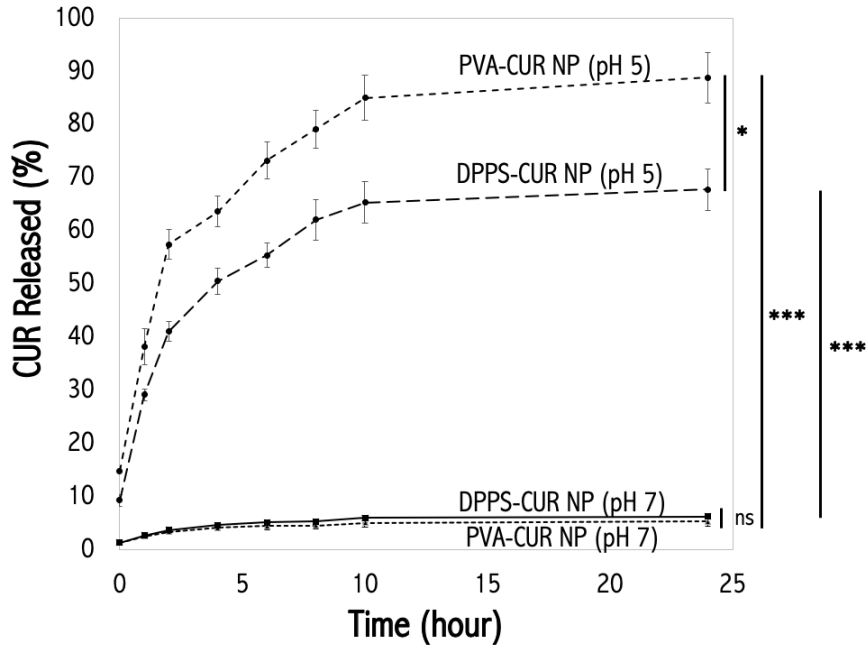
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610 **Figure 2.** Quantification of the amount of poly(vinyl alcohol) (PVA) and total
 611 phospholipid in PVA-CUR, DPPC-CUR, and DPPS-CUR nanoparticles (NP) (* $p < 0.05$,
 612 ** $p < 0.01$, *** $p < 0.001$ in comparison to PVA-CUR NP).



613

614 **Figure 3.** Differential scanning calorimetry (DSC) thermograms of: (A) formulated
 615 nanoparticles and (B) the raw components that make up the nanoparticle systems in their
 616 dry state, and (C) formulated nanoparticles in their wet state.

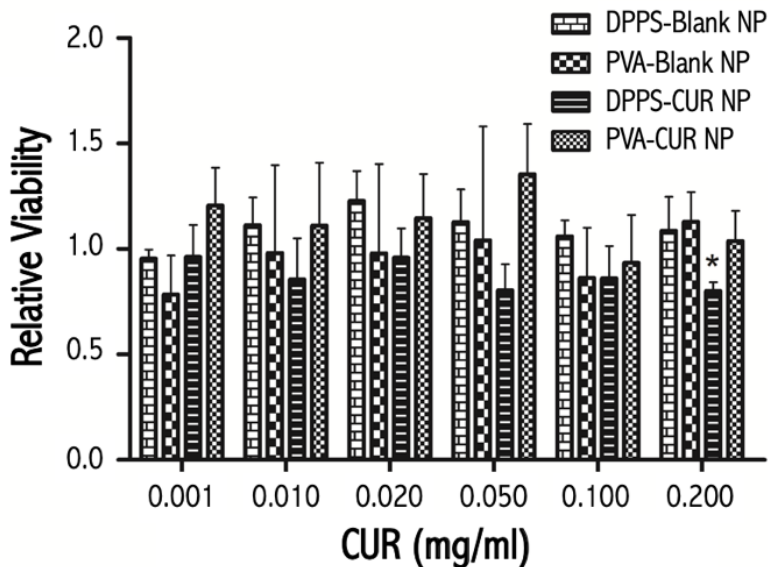


617

618 **Figure 4.** Cumulative release of curcumin (CUR) from nanoparticle systems at pH 7 and

619 pH 5 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = non-significant).

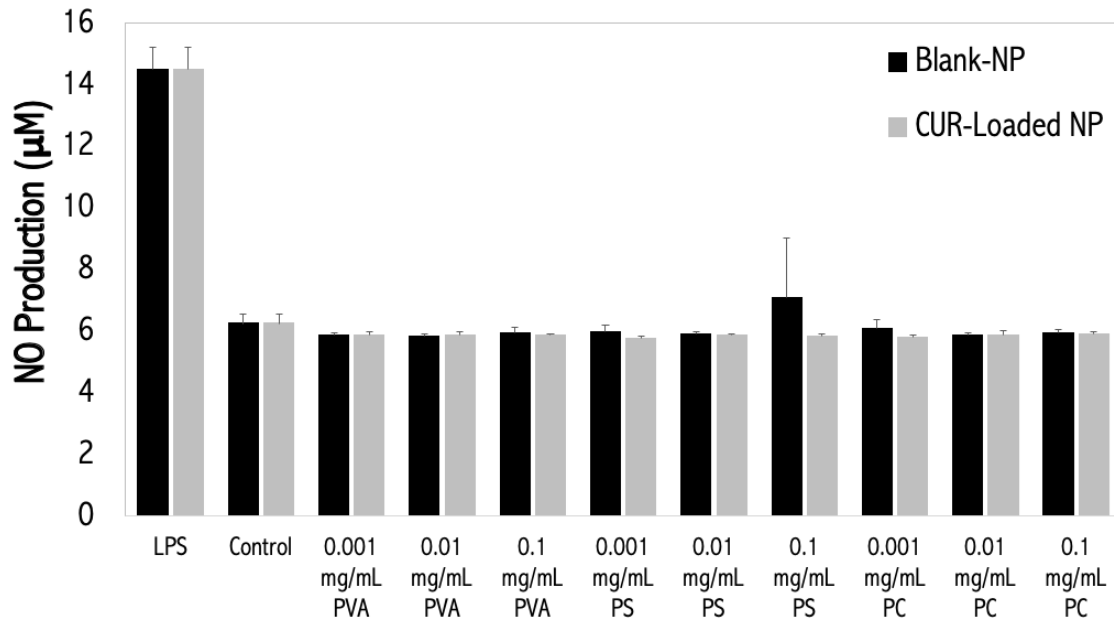
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622 **Figure 5.** Cytotoxicity analysis (relative viability) of nanoparticle systems on RAW

623 264.7 macrophages after 48 hours of exposure compared to control (* $p < 0.05$).



624

625 **Figure 6.** Nitric oxide (NO) production from RAW 264.7 macrophages incubated for 48
 626 hours with varying concentrations of nanoparticle formulations or LPS. No statistical
 627 significance was observed between the groups and control group (no treatment) and all
 628 were statistically lower than the LPS sample ($p < 0.0001$).

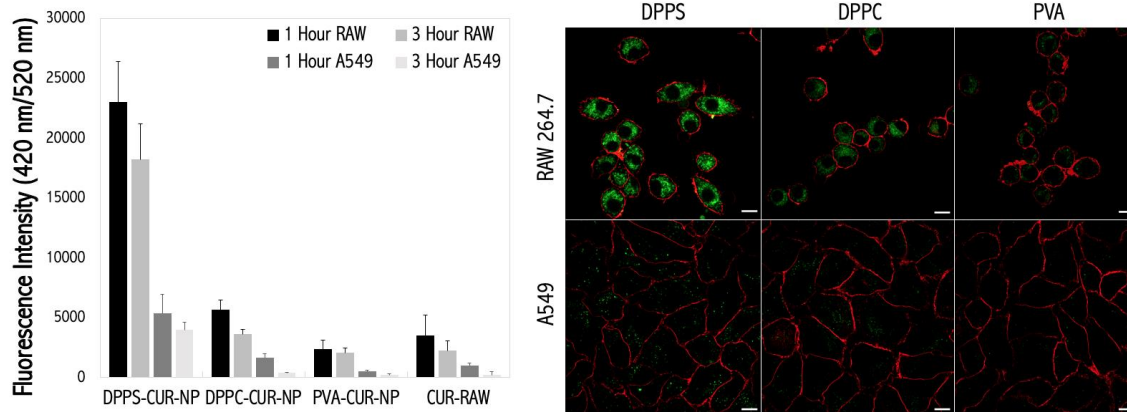
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635 **Figure 7.** (Left) Fluorescence spectroscopy analysis of nanoparticle uptake into cells and

636 (Right) representative confocal images of nanoparticle uptake by RAW 264.7 macrophages

637 and A549 cells (scale bar = 10 μm).

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