

1 **Title: Polymeric nano-encapsulation of curcumin enhances its anti-cancer activity in**  
2 **breast (MDA-MB231) and lung (A549) cancer cells through reduction in expression of**  
3 **HIF-1 $\alpha$  and nuclear p65 (Rel A).**

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8 **Short title:** Nanotechnology enhances anti-cancer efficacy

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29 **Abstract**

30 **Background:** The anti-cancer potential of curcumin, a natural NF $\kappa$ B inhibitor, has been  
31 reported extensively in breast, lung and other cancers. *In vitro* and *in vivo* studies indicate that  
32 the therapeutic efficacy of curcumin is enhanced when formulated in a nanoparticulate carrier.  
33 However, the mechanism of action of curcumin at the molecular level in the hypoxic tumour  
34 micro-environment is not fully understood. Hence, the aim of our study was to investigate the  
35 mechanism of action of curcumin formulated as nanoparticles in *in vitro* models of breast and  
36 lung cancer under a hypoxic micro-environment.

37 **Methods:** Biodegradable poly (lactic-co-glycolic acid) PLGA nanoparticles (NP), loaded with  
38 curcumin (cur-PLGA-NP), were fabricated using a solvent evaporation technique to overcome  
39 solubility issues and to facilitate intracellular curcumin delivery. Cytotoxicity of free **curcumin**  
40 and cur-PLGA-NP were evaluated in MDA-MB-231 and A549 cell lines using migration,  
41 invasion and colony formation assays. All treatments were performed under a hypoxic micro-  
42 environment and whole cell lysates from controls and test groups were used to determine the  
43 expression of HIF-1 $\alpha$  and p65 levels using ELISA assays.

44 **Results:** A ten-fold increase in solubility, three-fold increase in anti-cancer activity and a  
45 significant reduction in the levels of cellular HIF-1 $\alpha$  and nuclear p65 (Rel A) were observed  
46 for cur-PLGA-NP, when compared to free curcumin.

47 **Conclusion:** Our findings indicate that curcumin can **effectively** lower the elevated levels of  
48 HIF-1 $\alpha$  and nuclear p65 (Rel A) in breast and lung cancer cells under a hypoxic tumour micro-  
49 environment when delivered in nanoparticulate form. This applied means of colloidal delivery  
50 could explain the improved anti-cancer efficacy of curcumin and has further potential  
51 applications in enhancing the activity **of anti-cancer agents of low solubility**.

52

53 **Keyword:** nanotechnology, hypoxia, anti-cancer, curcumin, PLGA, intracellular delivery

## 54 **1. Introduction**

55 Curcumin is a well-known anti-cancer agent found in the rhizomes of the perennial herb  
56 *Curcuma longa*. It has a long history of use in traditional medicine, covering a broad spectrum  
57 of conditions, such as cancer, diabetes, inflammation and Alzheimer's disease<sup>1,2</sup>. The FDA  
58 has endorsed its status as a safe, home-based remedy. In more structured and rigorous  
59 evaluations, comprising of more than 65 clinical trials, curcumin's benefit in life-threatening  
60 diseases has been clearly demonstrated<sup>3</sup>. In relation to inflammatory and cancerous disorders,  
61 its therapeutic action is attributed to its ability to module nuclear factor kappa-beta (NFκβ)  
62 transcriptional factor<sup>4,5</sup> and hypoxia inducible factor (HIF)<sup>6,7</sup>. Free curcumin and curcumin  
63 loaded into a nanoparticulate carrier have been reported to be of therapeutic value in almost all  
64 cancer types<sup>8,9,10,11</sup>. However, the exact molecular mechanism of action involved in the  
65 treatment of cancer under a pathophysiological, hypoxic, tumour micro-environment is not  
66 clearly understood. It is known that over activation of NFκβ and HIF pathways is induced by  
67 an hypoxic micro-environment, which has been reported extensively in several tumour types<sup>12</sup>  
68<sup>13,14,15</sup>. This hypoxic micro-environment has been deemed responsible for metabolic adaption  
69 of cancerous cells, which result in tumour development, progression and reduced effectiveness  
70 of cancer therapeutics<sup>14</sup>.

71 Among all cancer types, breast cancer has been reported as the major cause of death  
72 among elderly women in Europe and the United States<sup>16,17</sup>. Paclitaxel, 5-flourouracil,  
73 cyclosporine and doxorubicin are the most commonly prescribed treatment options for breast  
74 and lung cancer<sup>18,19 & 20</sup>. However, these conventional chemotherapeutics result in serious  
75 side-effects, such as constipation, nausea, alopecia, neuronal damage, bone marrow depletion  
76 and heart failure<sup>21,22</sup>. Of similar concern is mortality associated with lung cancer, which is  
77 reported as the major cause of cancer-related death in males in developing countries. This  
78 outcome contributes approximately 18.2% to the total cancer death statistic<sup>23</sup>. Furthermore,

79 tiredness, skin reactions, loss of appetite, neurotoxicity and hair loss are some of the general  
80 side effects associated with conventional therapeutics<sup>24</sup>. Patients suffering from breast and  
81 lung cancers usually have to undergo surgery and radiation-based treatments, in addition to  
82 chemotherapy. Furthermore, these therapies are not site specific and cause extensive damage  
83 to healthy tissue<sup>25</sup>. In addition, these interventions interfere with the menopause and fertility  
84<sup>26</sup>. Hence, there is a pressing need to continue to develop safer and more effective alternatives  
85 for treating neoplastic disease.

86         The therapeutic value of curcumin in the treatment of breast and lung cancer has been  
87 reported in *in vitro*, *in vivo* and clinical studies<sup>27,28</sup>. However, these findings highlight  
88 problematic solubility issues of curcumin, which result in low systemic bioavailability, poor  
89 pharmacokinetic profiles and sub-optimal delivery to target cells<sup>29,30</sup>. Thus, the first objective  
90 of the present work was to use emulsion-based encapsulation methods to formulate a drug  
91 delivery system for curcumin with improved solubility and intra-cellular delivery properties.  
92 Nano-encapsulation of drugs in PLGA matrices has been reported to enhance therapeutic  
93 efficacy and targeting capabilities<sup>31</sup>, protect from degradation<sup>32</sup> and improve aqueous  
94 solubility of poorly water-soluble drugs<sup>33,34</sup>. Since curcumin is practically insoluble in water,  
95 it makes an ideal model drug for our current investigation<sup>35</sup>. Nano-encapsulation is also known  
96 to enhance cellular permeability and retention effects, which are proposed as mechanisms to  
97 target and enhance drug accumulation in solid tumour masses<sup>36</sup>. Since hypoxia plays a critical  
98 role in tumour development and progression, the second objective of our work was to  
99 investigate the molecular mechanism of action of free curcumin and nano-encapsulated  
100 curcumin. Of particular interest was the effect on expression of two master transcriptional  
101 regulators HIF-1 $\alpha$  and p65 (Rel A), which are known to be expressed in response to hypoxia  
102 and are positively associated with almost all cancer types<sup>37,38</sup>.

103 We hypothesise that nano-encapsulation of curcumin will enhance its solubility and  
104 facilitate intracellular delivery. These events will result in improved bioavailability and  
105 enhanced anti-cancer activity *via* modulation of the over-activated NFκβ and HIF pathways in  
106 lung and breast cancer cells seen under hypoxic condition. We hypothesise further that there  
107 will be a resultant stabilisation of the tumour, and cessation of cell growth and spread. In this  
108 study, the solvent evaporation method was employed, using sonication to homogenise the  
109 emulsion phases, and poly(vinyl alcohol) (PVA) to stabilise and control particle size <sup>39</sup>.  
110 Optimisations of PLGA and PVA content were carried out to select optimum settings during  
111 the formulation of curcumin-loaded PLGA NP (cur-PLGA-NP). The nanoparticle formulation  
112 exhibiting superior cellular uptake, retention and *in vitro* release was selected for further  
113 assessment of anti-cancer and anti-metastatic activity of curcumin in the highly metastatic  
114 breast (MDA-MB-231) and lung cancer (A549) cell lines.

115

116

## 117 **2. Materials and Methods**

118

### 119 **2.1 Preparation of cur-PLGA NP**

120 Cur-PLGA-NP was formulated using an oil-in-water emulsion technique. PLGA was  
121 dissolved in 2.0 ml chloroform, followed by addition of curcumin and sonication at 55 W for  
122 3 minutes (Branson Sonifier W-350, Danbury, CT, USA). Once dissolution was complete, the  
123 solution was added to an aqueous solution of 1.5% PVA and sonicated again at 55 W for 3  
124 minutes to form the final o/w emulsion. This was stirred overnight and then centrifuged at  
125 16584g for 30 minutes to assist the removal of residual solvents. The nanospheres obtained  
126 were washed three times with deionised distilled water. NP were lyophilised for 72 hours

127 (FreeZone 4.5plus) and stored at 4 °C until further use<sup>40</sup>. Fluorescent NP were prepared by  
128 adding 1µg of Nile Red to PLGA/curcumin solution in ethanol.<sup>40</sup>

129

## 130 **2.2 Physicochemical characterisation**

131

### 132 *2.2.1 Particles size and zeta potential*

133 Particle size and distribution of cur-PLGA-NP were determined using dynamic light scattering  
134 (Zetasizer 5000, Malvern Instruments, Malvern, UK). An aliquot of NP suspension (5 mg ml<sup>-1</sup>),  
135 previously vortex, was diluted in ultrapure water and used to measure mean diameter.  
136 Electrophoretic mobility was used to measure the zeta potential of cur-PLGA-NP.  
137 Conductivity was adjusted using 0.001 M KCl. The mean of three measurements was recorded  
138 <sup>41</sup>.

139

### 140 *2.2.2 Entrapment efficiency*

141 Entrapment efficiency was determined using an indirect method. The concentration of non-  
142 encapsulated curcumin in the supernatant was measured by absorption spectrophotometry at  
143 430 nm and compared to a standard plot<sup>42</sup>. The amount of curcumin encapsulated in NP was  
144 calculated from the difference between the initial amount of curcumin added and the non-  
145 encapsulated curcumin remaining in the external aqueous phase after NP fabrication. All  
146 measurements were recorded in triplicate and the mean of each sample was reported as the  
147 percentage of curcumin entrapment efficiency.

148

149

150

### 151 *2.2.3 In vitro release study*

152 The release of curcumin from PLGA NP was determined in phosphate buffered saline (pH 7.4,  
153 containing 0.1% w/v Tween-80) as a release phase. Briefly, cur-PLGA-NP (5.0 mg) were  
154 suspended in a dialysis compartment (MW cut-off 17 kD) and suspended in 1.0 ml PBS  
155 solution. The sample pouches were incubated at 37 °C in a reciprocal shaking water bath (100  
156 rpm)<sup>35</sup>. Samples were taken during a period of 168 hours at predetermined time intervals.

157

#### 158 *2.2.4 Determination of water solubility*

159 Apparent water solubility of curcumin, formulated as cur-PLGA-NP, was compared with an  
160 equivalent amount of free curcumin **dispersed** in distilled water. The mixtures were stirred for  
161 24 hours. **These solutions were then** centrifuged at 13,450g for 10 minutes and the absorbance  
162 of the supernatant measured using ultraviolet spectroscopy<sup>43</sup>.

163

#### 164 *2.2.5 Particle surface morphology*

165 Surface morphology was characterised using scanning electron microscopy (FEI Quanta 400  
166 FEG, FEI). A sample of NP was mounted on carbon tape and sputter-coated with gold under  
167 vacuum in an argon atmosphere prior to imaging.

168

### 169 **2.3 Culture materials**

170 MDA-MB-231 cells (a metastatic breast cancer cell line) and A549 cells (a metastatic lung  
171 cancer cell line) were maintained as monolayer cultures in Dulbecco's modified Eagle's  
172 medium–high glucose (DMEM-Hi) medium (Gibco BRL, Grand Island, NY) supplemented  
173 with 10% foetal bovine serum (Gibco BRL, Grand Island, NY) and 1% penicillin–streptomycin  
174 (Gibco BRL, Grand Island, NY) at 37 °C in a humidified atmosphere (5% CO<sub>2</sub>).

175 Different concentration of free curcumin and cur-PLGA-NP were dissolved in 20 µl of  
176 DMSO. This solution was then diluted to 2 ml with Optimem<sup>®</sup> media.

177

#### 178 **2.4 Co-localisation of cur-PLGA-NP**

179 Cellular uptake of cur-PLGA-NP was investigated by seeding  $1.0 \times 10^5$  MDA-MB-231 and  
180 A549 cells on chamber slides in 6-well plates. Cells were incubated with Nile Red-labelled  
181 cur-PLGA-NP for 24 hours and fixed with 4% paraformaldehyde for 15 minutes. The nucleus  
182 was stained with  $5.0 \mu\text{g}$  DAPI in 1.0 ml normal media and incubated for 15 minutes<sup>44</sup>. The  
183 fluorescence images were obtained using phase contrast microscopy (Nicole Eclipse E400  
184 Florescence Microscope, Nikon Y-FL, Japan).

185

#### 186 **2.5 Migration assay**

187 A migration assay was performed using an *in vitro* scratch assay, as previously described<sup>45</sup>.  
188 To quantify the results captured from photographic images, ImageJ<sup>®</sup> software was used to  
189 measure the width of the scratch at three different points. The degree of closure over 24 hours  
190 was calculated using the difference of the scratch width between 0 and 24 hours for each  
191 treatment concentration. The degree of scratch closure for each treatment was compared to the  
192 degree of closure in the control cells.

193

#### 194 **2.6 In vitro cytotoxicity**

195 Cytotoxicity assays were performed, as previously reported by Kumar *et al.*<sup>46</sup> with minor  
196 modifications. MDA-MB-231 and A549 cells ( $5.0 \times 10^4$  cells per well in  $500 \mu\text{l}$  media) were  
197 seeded in 24-well plates. The following day, cells were treated with different concentrations  
198 of free curcumin or cur-PLGA-NP suspended in Optimem<sup>®</sup> media. Medium containing  
199 equivalent amounts of blank PLGA NP was used as the control. The cytotoxic effect of 10, 20  
200 and  $30 \mu\text{M}$  of the free curcumin or cur-PLGA-NP was determined every 24 hours using MTT  
201 assay to assess the viability of cancer cells. The treated cells were washed with  $500 \mu\text{L}$  PBS,

202 then 500  $\mu$ L of 15% MTT dye solution in complete media was added to each well. The plates  
203 were incubated at 37°C and 5% CO<sub>2</sub> for an additional 3 hours. The supernatant was discarded  
204 and formazan crystals solubilised by adding 500  $\mu$ L of DMSO. The absorption was measured  
205 at 570 nm (reference wavelength 630 nm) in a microplate reader (Fluostar Omega, BMG Lab  
206 Tech GMBH, Germany). The anti-proliferative effect of different doses of free curcumin or  
207 cur-PLGA-NP treatments was calculated as a percentage of cell growth with respect to the  
208 blank NP controls. The absorbance of the untreated cells was set at 100%. All the experiments  
209 were repeated three times.

210

## 211 **2.7 Invasion assay**

212 The effect of curcumin treatment on invasion of MDA-MB231 and A549 cells was performed  
213 as previously described<sup>47</sup>. Transwell® inserts of 8  $\mu$ m pore size were inserted in 12-well plates  
214 (BD Bioscience, San Jose, CA). 135  $\mu$ l solutions of Matrigel and water (1:1) was placed on  
215 these inserts. Cells ( $4 \times 10^4$ ) were seeded in a suspension of 200  $\mu$ l of serum free media.  
216 Subsequently, 100  $\mu$ l containing 10, 20 and 30  $\mu$ M of the free curcumin or cur-PLGA-NP  
217 suspended in serum free media was added in the insert. The same volume of 100  $\mu$ l containing  
218 equivalent amounts of blank PLGA NP was used as a control. 700  $\mu$ l of DMEM media  
219 containing 10% FBS was added to each well as chemo-attractant. After 24 hours, the media in  
220 both chambers was removed and clean off any remaining cells or Matrigel® layer. The inserts  
221 were placed into another well containing 500  $\mu$ l methanol for 10 minutes for fixation and left  
222 to dry. The membrane was cut and placed with the surface facing upwards in wells of a new  
223 12-well plate. Crystal Violet solution (250  $\mu$ l) was added in the well to stain up the invaded  
224 cells. After drying the membrane 250  $\mu$ l of 70% ethanol was added to membrane and shaken  
225 for 30 minutes. 200  $\mu$ l was pipetted out from this 70% ethanol and absorbance was measured  
226 at 590 nm (Fluostar Omega, BMG Lab Tech GMBH, Germany). The effect of different doses

227 of free curcumin or cur-PLGA-NP treatments was calculated as a percentage of cell invasions  
228 with respect to the blank NP controls. The absorbance of the untreated the absorbance of the  
229 untreated cells was set at 100%. All the experiments were repeated three times.

## 231 **2.8 Colony formation assay**

232 An aliquot of approximately 500 cells (MDA-MB-231 and A549) were seeded in 2.0 ml media  
233 in 6-well plates and allowed stand for 2 days to attach and initiate colony formation <sup>35</sup>. These  
234 cells were treated with different concentrations of 10, 20 and 30  $\mu$ M of free curcumin or cur-  
235 PLGA-NP suspended in Optimem® media over a period of 7 days. The plates were washed  
236 twice with PBS, fixed in chilled methanol, stained with Crystal Violet, washed with water and  
237 air-dried. The number of colonies was counted visually. The % colony formation was  
238 calculated by comparing the number of colonies formed in the test experiment to the number  
239 of colonies formed using blank PLGA NP.

## 241 **2.9 Quantification of HIF-1 $\alpha$ and p65 levels**

242 MDA-MB-231 and A549 cells (treated for 24 hours with 10% oxygen) were treated with (i)  
243 free curcumin, (ii) cur-PLGA-NP and (iii) blank PLGA-NP. Whole cell lysates were prepared  
244 and assayed for HIF-1 $\alpha$  and p65 levels using Invitrogen HIF1A Human ELISA Kit (EHIF1A)  
245 and NF $\kappa$ B p65 (Total) Human ELISA Kit (KHO0371) and used according to the  
246 manufacturer's instructions at 450 nm, using a BioTek optical plate reader. Optical density  
247 was converted to concentration (pg ml<sup>-1</sup>) using the standard calibration curve provided in the  
248 manufacture's protocol.

## 250 **2.10 Statistical analysis**

251 Each experiment was performed in triplicate. Statistical analysis was performed using  
252 GraphPad Prism v5.0. Results are presented as mean +/- standard error of the mean (SEM).  
253 Particle size, zeta potential, entrapment efficiency and values of *in vitro* release profiles were  
254 treated statistically using one-way analysis of variance (ANOVA).  $P < 0.05$  was considered  
255 statistically significant.

256

257

## 258 **3. Results and Discussion**

259

### 260 **3.1 Effect of PLGA concentration**

261 Three batches nanoparticles (F1, F2 and F3) were formulated with a different amount of PLGA  
262 and an equal amount of curcumin, as shown in the table I. The effect of increasing the quantity  
263 of polymer for the formulation of cur-PLGA-NP on its physiochemical properties is shown in  
264 Figure 1. It was found that particle size increased significantly from 265 nm to 606 nm (P-  
265 value  $< 0.0001$ ) as the quantity of PLGA increased (Figure 1A). This increase in size is due to  
266 increase in the viscosity of polymer solution and formation of larger disperses droplets<sup>41,48</sup>. It  
267 was also observed that increasing the amount of polymer increased the zeta potential  
268 significantly from  $-13.7$  mV (F1) to  $-3.1$  mV (Figure 1B; P-value  $< 0.01$ ). This could be due  
269 to the accumulation of ionised carboxylic groups on the nanoparticles as result of an increase  
270 of PLGA. Entrapment efficiency increased significantly, as the ratio of PLGA increased  
271 (Figure 1C). Similar results were reported by Derman *et al.*<sup>49</sup>. It is proposed that the increase  
272 in PLGA in the organic phase increases the viscosity and reduces diffusivity to the water phase  
273<sup>41, 50</sup>. The data in Figure 1D show the release profile for curcumin from three different

274 formulations. All three formulations show a similar release trend with a burst effect after 24  
275 hours.

276

### 277 **3.2 Solubility of cur-PLGA-NP and free curcumin**

278 The apparent water solubility of curcumin in both free and nano-encapsulated form was  
279 determined by UV spectroscopy at 430 nm. The vials in Figure 2A give a visual representation  
280 of the enhanced solubility of curcumin when formulated as cur-PLGA-NP when compared to  
281 free curcumin in water. It is evident that cur-PLGA-NP produce a uniform dispersion, whereas  
282 free curcumin was sparingly soluble in water. The solubility of free curcumin was found to be  
283  $0.935 \mu\text{mol}$ , whereas ostensible solubility of cur-PLGA-NP (curcumin encapsulated in NP)  
284 was  $9.535 \mu\text{mol}$ . These results indicate an approximately ten-fold increase in aqueous  
285 solubility of cur-PLGA-NP as compared to free curcumin.

286

### 287 **3.3 Scanning Electron Microscopy**

288 Figure 3 shows SEM images of F1, F2, and F3 formulated with 30 mg, 60 mg and 90 mg of  
289 polymer with equal amounts of curcumin and PVA. F1 exhibits a monodisperse appearance,  
290 with the smallest particle size. SEM images of cur-PLGA-NP suggest that an increase in the  
291 polymer (PLGA) concentration not only leads to an increase in particle size but also results in  
292 non-spherical particles. Thus, **F1 batch was selected for *in vitro* analysis based on its smaller**  
293 **particle size.**

294

### 295 **3.4 Cellular uptake**

296 The nuclei of MDA-MB-231 and A549 cells were stained with DAPI and Nile Red-tagged cur-  
297 PLGA-NP were observed under fluorescent microscopy. Cellular uptake of cur-PLGA-NP  
298 labelled with Nile Red, in MDA-MB-231 and A549 cells, were visualised by overlaying images

299 obtained by fluorescent microscopy. Nile Red encapsulated cur-PLGA-NP can be seen within  
300 the MDA-MB-231 and A549 cells as depicted in the Figure 4A and 4B. The result indicates  
301 that formulation F1 NP **may be taken up by the cells. However, since the cell boundaries are**  
302 **not seen clearly this result cannot be confirmed.**

303

### 304 **3.5 Migration assay**

305 Migration of cancer cells from the primary tumour site to other sites is a predominant feature  
306 of metastatic breast and lung cancer cells <sup>51</sup>. Therefore, in this work, a scratch assay was  
307 conducted on both cell lines to investigate doses response of free curcumin and cur-PLGA-NP  
308 on the migration of MDA-MB-231 (Figure 5A) and A549 (Figure 5B) cells. Figure 5 shows  
309 the graphical quantification of the degree of closure in breast and lung cancer cell lines relative  
310 to the blank NP controls. Treatment with free curcumin resulted in higher degree of wound  
311 closure for all concentrations in both cell lines. Treatment with cur-PLGA-NP resulted in  
312 significant reduction in the degree of wound closure compared to the free curcumin after 72  
313 hours at similar concentrations of curcumin (Figure 5A and B). These results demonstrated  
314 that cur-PLGA-NP have the superior anti-migration capability when compared to free  
315 curcumin.

316

### 317 **3.6 Invasion assay**

318 An invasion assay was conducted to investigate the effect of free curcumin and cur-PLGA-NP  
319 on the invasion capability of MDA-MB-231 (Figure 6A) and A549 (Figure 6B) cells.  
320 Dissemination of tumour cells from the primary site to distant locations starts with cell  
321 detachment followed by the local invasion of the normal tissues adjacent to the tumour which  
322 then can infiltration through the lymphatic drainage system <sup>51</sup>. Treatment with cur-PLGA-NP  
323 resulted in a significant decrease in cell invasion compared to free curcumin for all

324 concentrations in both cell lines as shown in Figure 6. These results suggest that Cur-PLGA-  
325 NP can significantly inhibit the invasion capability of invasive lung and breast cancer cells  
326 when compared to free curcumin. This enhanced anti-invasive effect imparted to curcumin by  
327 nano-encapsulation is critical to inhibit metastasis of highly invasive breast and lung cancer  
328 cells.

329

### 330 **3.7 Colony formation assay**

331 Colony formation assay has been widely to study the long-term anti-cancer activity of  
332 curcumin<sup>35</sup>. The number of colonies formed after 7 days was quantified for MDA-MB-231  
333 (Figure 7A) and A549 (Figure 7B) cells treated with blank PLGA-NP as control, along with  
334 three different concentrations of free curcumin and cur-PLGA-NP. Treatment with cur-PLGA-  
335 NP resulted in significantly lower numbers of colonies compared to free curcumin, as shown  
336 Figure 7A and B.

337

### 338 **3.8 Assessment of HIF-1 $\alpha$ and p65 levels**

339 Having assessed the physical parameters, aqueous solubility, intracellular uptake and anti-  
340 cancer potential of cur-PLGA-NP versus free curcumin, we need to investigate the molecular  
341 mechanism for improved anti-cancer potential of curcumin when formulated as PLGA  
342 nanoparticles. Recent research indicates that metastatic human cancer tumours are hypoxic  
343 and these cells could have elevated levels HIF-1 $\alpha$  and NF $\kappa$ B/p65 (Rel A) which may result in  
344 tumour development and progression of cancer<sup>52 53-56</sup>. Hence, to investigate if curcumin  
345 treatment has an effect on these transcription factors, we used whole cell lysates from  
346 metastatic breast (MDA-MB-231) and lung (A549) cancer cells treated with blank NP, cur-  
347 PLGA-NP and free curcumin under hypoxic condition (10% O<sub>2</sub>) to quantify the levels of HIF-  
348 1 $\alpha$  and p65 (Rel A) using ELISA assay. The results from the ELISA assay indicated that free

349 curcumin had no significant effect on the levels of HIF-1 $\alpha$  and p65 (Rel A) when compared to  
350 blank NP. However, there was a significant reduction in the levels of HIF-1 $\alpha$  (Figure 8: Panel  
351 A) and p65 (Rel A) (Figure 8: Panel B) in both breast and lung cancer cells treated with cur-  
352 PLGA-NP (Figure 8; (P< 0.001 – 0.01). These findings indicate that curcumin can suppress  
353 the cellular levels of HIF-1 $\alpha$  and p65 (Rel A) only when delivered using a colloidal carrier.  
354 **Metastatic breast (MDA-MB-231) and lung (A549) cancer cells show basal levels of HIF-1 $\alpha$**   
355 **and NF $\kappa$ B/p65 (Rel A) under normal (21%) atmospheric O<sub>2</sub> level (Supplementary Figure 1).**

#### 4. Conclusions

Curcumin has received considerable attention as a potent anti-cancer<sup>57</sup> and anti-inflammatory agent<sup>5</sup>. However, it is not currently used as a clinical medicine for treating inflammation or cancer. Results from our work have demonstrated the effect of increasing amount of PLGA on particle size, zeta potential, entrapment efficiency and *in vitro* release. These findings are in agreement with previously published data<sup>58</sup>. Figure 1 shows that increase in PLGA amount increases the particle size, zeta potential and entrapment efficiency significantly. However, the percentage release decreased with increases in PLGA concentration in the nanoparticle formulation. This may be due to increase in the viscosity of polymer solution. We also observed a ten-fold increase in curcumin solubility when formulated as PLGA nanoparticles (Figure 2) this increased solubility can increase the bioavailability of curcumin and intracellular delivery<sup>59</sup>. SEM images from the batches F1, F2 and F3, as shown in Figure 3, demonstrated that increase in PLGA amount not only leads to increases in particle size, but also distorts the spherical structure of the nanoparticles. Our work indicates that curcumin uptake by cancer cells is achieved when it is formulated as PLGA nanoparticles (Figure 4). This evidence of cellular uptake of curcumin nanoparticles forms the basis of our further findings indicating reduced migration (Figure 5), and **proliferation (Supplementary Figure 2)** ability decreased invasion power (Figure 6), with reduction in colony formation (Figure 7) of the breast and lung cancer cells. Similar findings related to the anti-cancer potential of curcumin and curcumin nanoparticles have been reported by several researchers<sup>60-67</sup>. However, there is no study to date reporting the action of curcumin and curcumin nanoparticles on elevated levels HIF-1 $\alpha$  and p65 (Rel A) in breast and lung cancer cells under tumour hypoxic condition.

The most important findings of our current work indicate that nano-encapsulation of curcumin is capable of downregulating the expression of HIF-1 $\alpha$  and p65 (Rel A) levels in

breast and lung cancer cells when exposed to hypoxia (10% O<sub>2</sub>) (Figure 8). This finding suggests that the increased therapeutic value of the natural anti-cancer agent curcumin could be via suppression of over activated HIF and NFκβ pathways, which are predominately responsible for adaptation of hypoxic tumours resulting in the development and spread of cancer in our body. Hence, the findings from our work conclude that nanotechnology-based drug delivery system could be an effective tool to enhance the anti-cancer activity of several natural and/or novel anti-cancer compounds, which are currently not in clinical use due to their low solubility and/or poor bioavailability. Future work will involve functionalising these PLGA-NP using salic acid to selectively target cancer tumour cells in *in-vivo* models of lung and breast cancer.

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## Figure captions

### **Figure 1. Effect of polymer amount on particle size, zeta potential, encapsulation efficiency and *in-vitro* release**

The particle size of nanoparticles increases significantly as the polymer amount increases from 30 mg to 90 mg (A). Zeta potential decreases significantly (p-value < 0.002) upon increasing the polymer amount from 30 mg to 90 mg (B). Entrapment efficiency increases with increase in PLGA amount (C). The release profile depicts initial burst release after 24 hours. Nanoparticles formulated using 60 mg PLGA shows the highest percentage release of curcumin (D). Values are mean  $\pm$  SEM (n = 3). \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001 compared with 30 mg PLGA.  $\Delta$ p < 0.05 and  $\Delta\Delta\Delta$ p < 0.001 compared with 60 mg of PLGA.

### **Figure 2. Water solubility of cur-PLGA-NP, free curcumin and UV-spectra of cur-PLGA-NP and free curcumin.**

The vials in Figure A shows the solubility of cur-PLGA-NP and free curcumin respectively. The free curcumin precipitates and settles down whereas cur-PLGA-NP shows uniform dispersion throughout the vial. Figure B shows the absorption spectra of cur-PLGA-NP and free curcumin. cur-PLGA-NP shows the greater area under the curve as compared to free curcumin in Figure B.

### **Figure 3. Scanning Electron Microscopy of cur-PLGA-NP F1, F2, and F3 containing 30 mg, 60 mg and 90 mg of PLGA polymer respectively.**

Scanning electron microscopic images of F1, F2 and F3 batches containing 30 mg, 60 mg and 90 mg of PLGA demonstrate an increase in particle size. F1 has smaller and uniform particle size as compared to F2 and F3. SEM images confirm that increase in polymer amount leads to increase in particle size and distortion of particle shape.

**Figure 4. Cellular Uptake of cur-PLGA-NP in MDA-MB231.**

Cellular localisation of Nile red-coated cur-PLGA-NP were observed in MDA-MB231 (A) and A549 (B) and visualised by overlapping under fluorescent microscopy.

**Figure 5. Effect of cur-PLGA-NP and free curcumin on the migration of MDA-MB231 and A549 cell lines.**

The effect of cur-PLGA-NP and free curcumin on migration capacity of MDA-MB231 (A) and A549 (B) cell lines were evaluated. Cur-PLGA-NP shows significant reduction in migration capability of both cell lines as compared to free curcumin. Values are mean  $\pm$  SEM with n=3. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared with control (blank NP).  $\Delta$ P<0.05,  $\Delta\Delta$  P<0.01,  $\Delta\Delta\Delta$ P<0.001 compared with the same dose of free curcumin.

**Figure 6. Effect of cur-PLGA-NP and free curcumin on invasion of MDA-MB231 and A549 cell lines.**

The effect of cur-PLGA-NP and free curcumin on invasion capability of MDA-MB231 (A) and A549 (B) cell lines was observed. Cur-PLGA-NP shows a significant reduction in invasion capability of both cell lines as compared to free curcumin. Values are mean  $\pm$  SEM with n=3. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared with control (blank NP).  $\Delta$ P<0.05,  $\Delta\Delta$  P<0.01,  $\Delta\Delta\Delta$ P<0.001 compared with the same dose of free curcumin.

**Figure 7. Effect of cur-PLGA-NP and free curcumin on colony formation ability of MDA-MB231 and A549 cell lines.**

Cur-PLGA-NP significantly reduces the colony formation ability of both MDA-MB231 (A) and A549 (B) cell lines. Cur-PLGA-NP shows a significant reduction in colony formation capability of both cell lines as compared to free curcumin. Values are mean  $\pm$  SEM with n=3. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared with control (blank NP).  $\Delta$ P<0.05,  $\Delta\Delta$  P<0.01,  $\Delta\Delta\Delta$ P<0.001 compared with the same dose of free curcumin.

**Figure 8. Effect of cur-PLGA-NP and free curcumin on expression of cellular levels of HIF-1 $\alpha$  and p65 (Rel A).**

Cur-PLGA-NP significantly reduces the levels of HIF-1 $\alpha$  (A) and p65 (Rel A) (B) in both MDA-MB231 and A549 cell lines. Values are mean  $\pm$  SEM with n=4. \*\*P<0.01, \*\*\*P<0.001 compared with blank NP.  $\Delta\Delta$  P<0.01,  $\Delta\Delta\Delta$ P<0.001 compared with the same dose of free curcumin treatment for 24 hours.

**Supplementary Figure 1. The level of HIF-1 $\alpha$  and p65 in MDA-MB-231 and A549 at normal atmospheric (21% O<sub>2</sub>) level.**

**Supplementary Figure 2. Cytotoxicity of cur-PLGA-NP and raw curcumin in MDA-MB231 and A549 cell lines**

Cytotoxicity was performed on MDA-MB231 (A) and A549 (B) cell lines.  $40 \times 10^4$  cells were seeded on 24-well plates and transfected with 10 $\mu$ M, 20 $\mu$ M and 30 $\mu$ M of raw curcumin and cur-PLGA-NP. The absorbance was measured after 24 hours, 48 hours, 72 hours and 96 hours. It is evident from the figures that cur-PLGA-NP have higher cellular viability and less cytotoxicity as compare to raw curcumin. Values are mean  $\pm$  SEM (n = 3).

Figure 1.

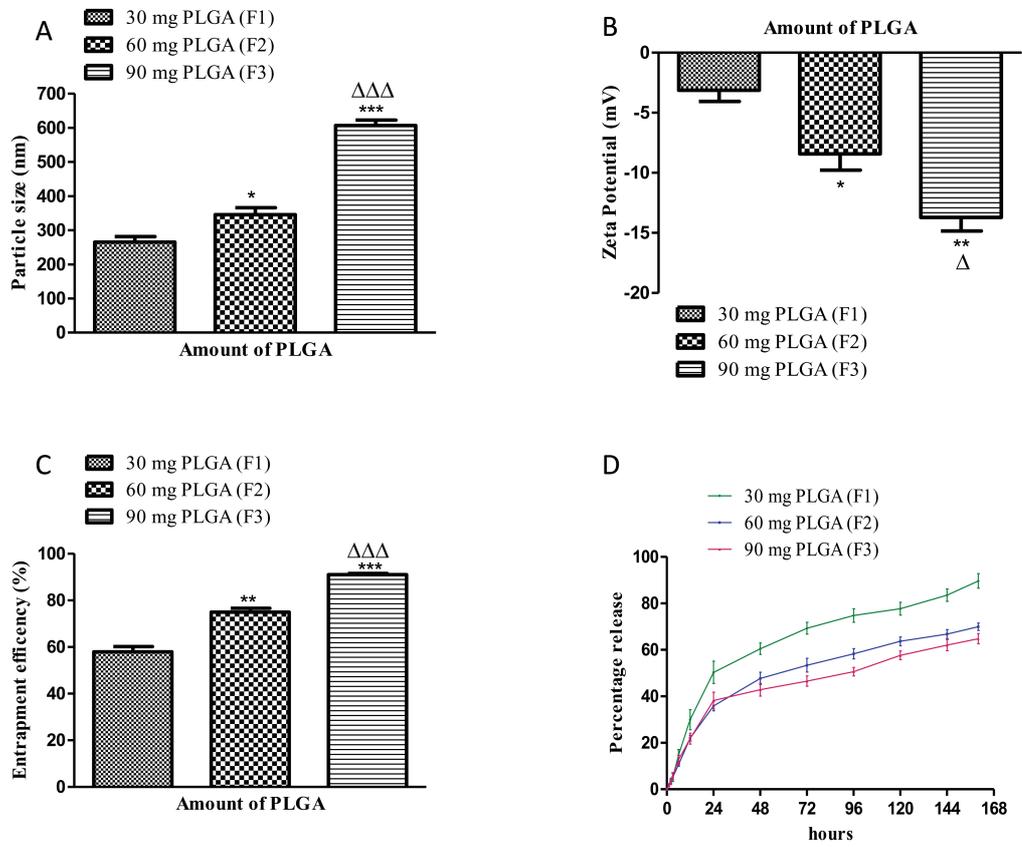
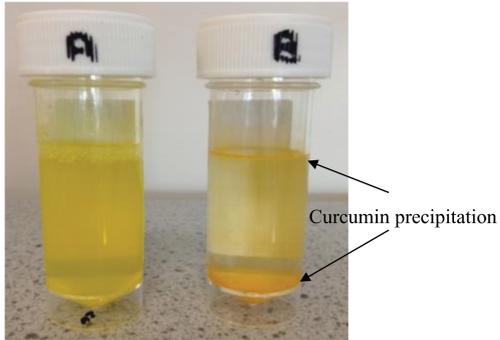


Figure 2.

A



B

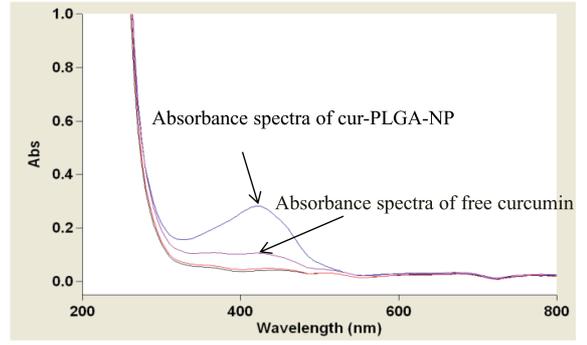


Figure 3.

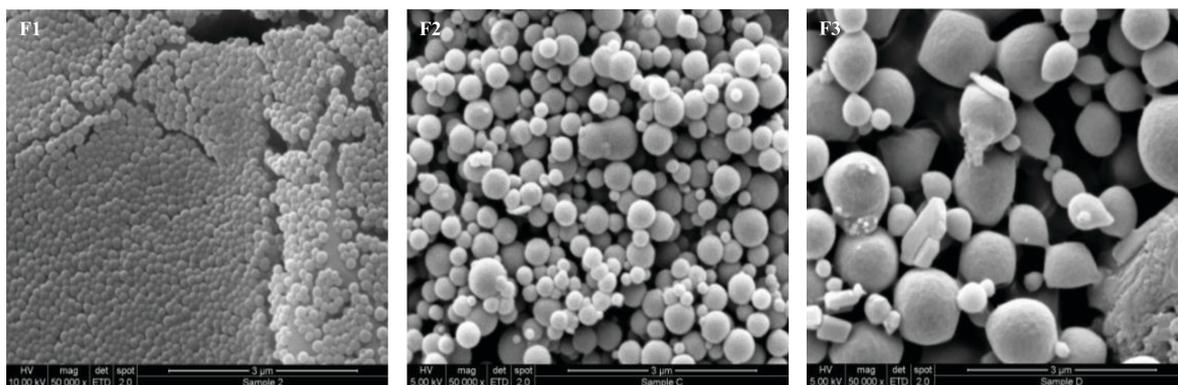


Figure 4.

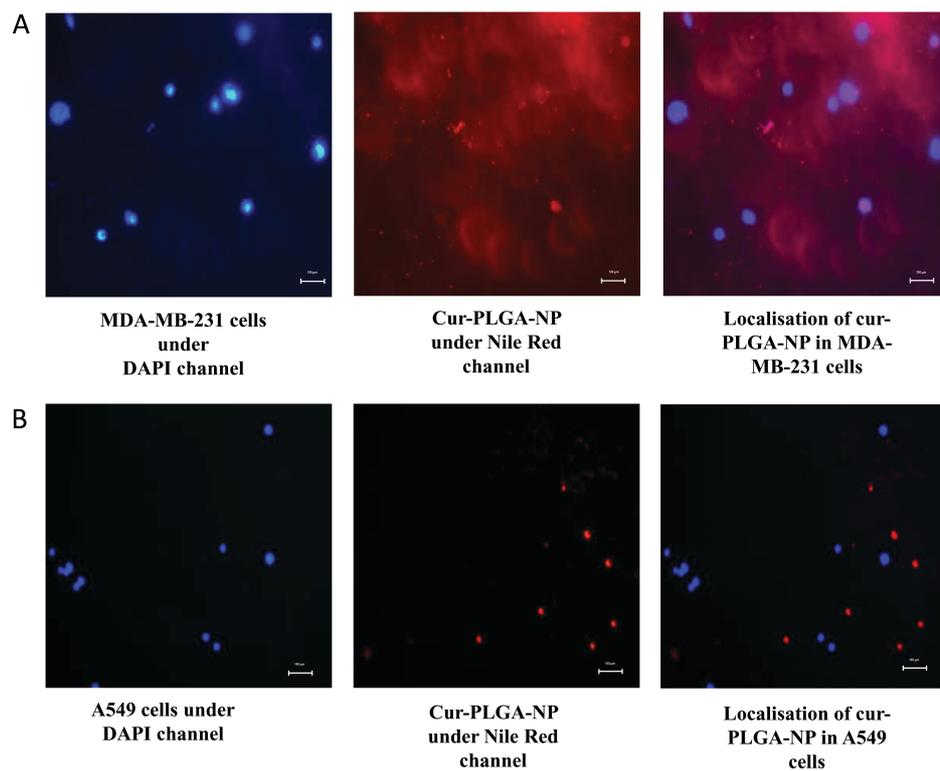


Figure 5.

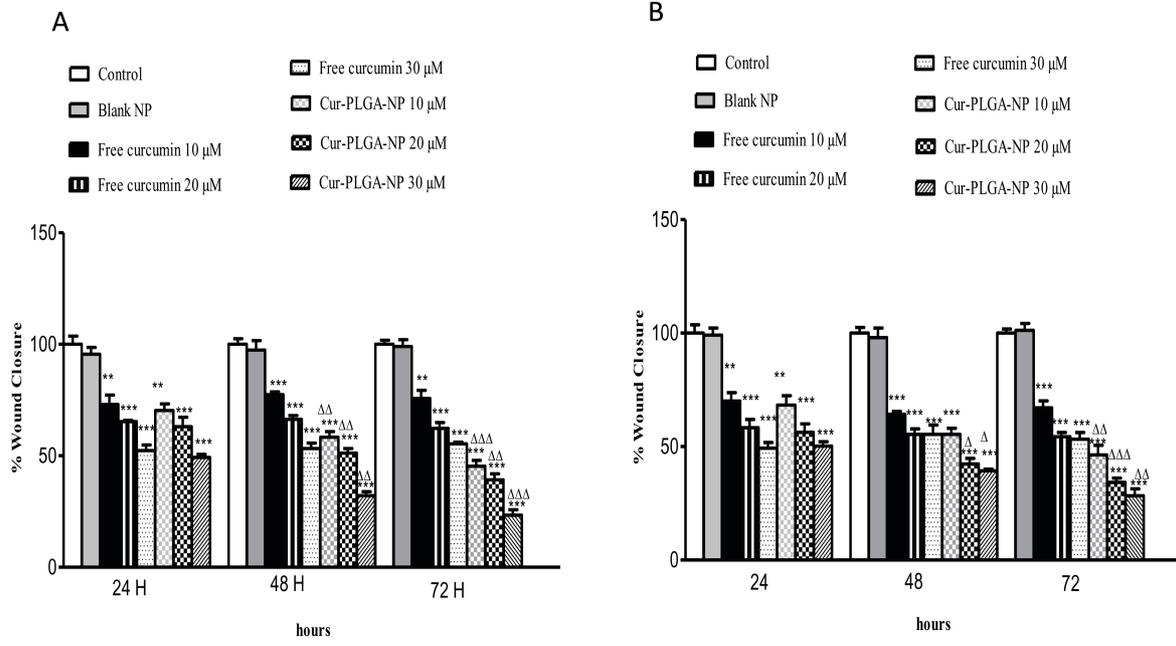


Figure 6.

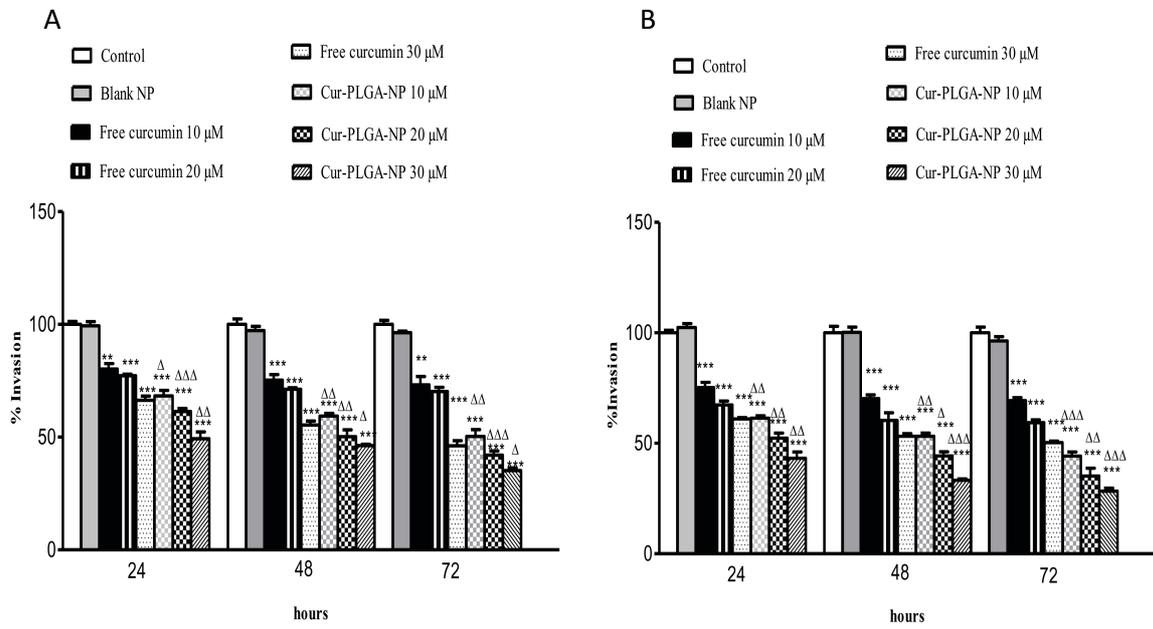


Figure 7.

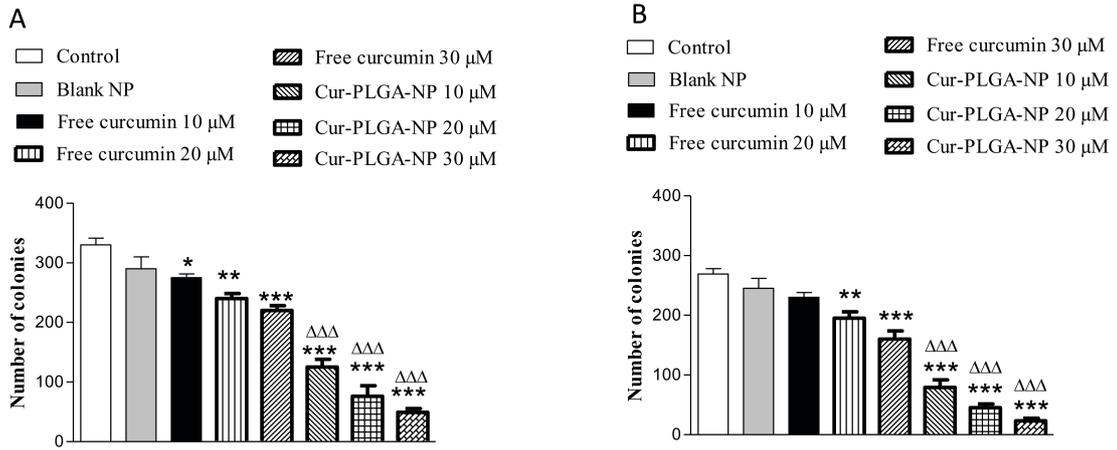
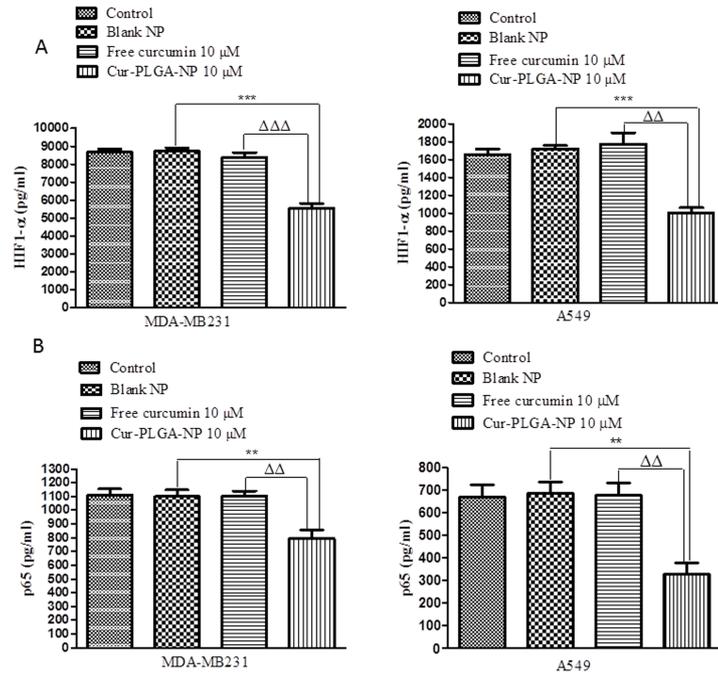
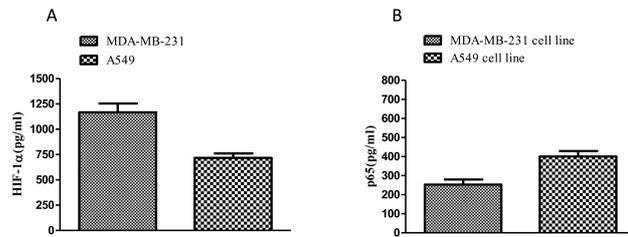


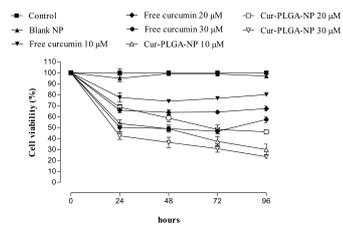
Figure 8.



Supplementary Figure 1.



Supplementary Figure 2.



**Table 1:** Process variables for curcumin loaded NPs and corresponding identifiers.

| Formulation code | Amount of drug (mg) | Amount of polymer (mg) |
|------------------|---------------------|------------------------|
| F1               | 20                  | 30                     |
| F2               | 20                  | 60                     |
| F3               | 20                  | 90                     |