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Title: Multifunctional Nanomedicine Platform for Concurrent Delivery of Chemotherapeutic Drugs and Mild Hyperthermia to Ovarian Cancer Cells

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1 **Multifunctional Nanomedicine Platform for Concurrent Delivery of Chemotherapeutic**  
2 **Drugs and Mild Hyperthermia to Ovarian Cancer Cells**

3  
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23 **Abstract (must not exceed 200 words)**

24 A multifunctional tumor-targeting delivery system was developed and evaluated for an efficient  
25 treatment of drug-resistant ovarian cancer by combinatorial therapeutic modality based on  
26 chemotherapy and mild hyperthermia. The engineered iron oxide nanoparticle (IONPs)-based  
27 nanocarrier served as an efficient delivery vehicle for doxorubicin and provided the ability to  
28 heat cancer cells remotely upon exposure to an alternating magnetic field (AMF). The  
29 nanocarrier was additionally modified with polyethylene glycol and LHRH peptide to improve  
30 its biocompatibility and ability to target tumor cells. The synthesized delivery system has an  
31 average size of 97.1 nm and a zeta potential close to zero, both parameters favorable for  
32 increased stability in biological media and decreased elimination by the immune system. The  
33 nanocarrier demonstrated faster drug release in acidic conditions that mimic the tumor  
34 environment. It was also observed that the LHRH targeted delivery system could effectively  
35 enter drug resistant ovarian cancer cells, and the fate of doxorubicin was tracked with  
36 fluorescence microscope. Mild hyperthermia (40 °C) generated by IONPs under exposure to  
37 AMF synergistically increased the cytotoxicity of doxorubicin delivered by the developed  
38 nanocarrier to cancer cells. Thus, the developed IONPs-based delivery system has high potential  
39 in the effective treatment of ovarian cancer by combinatorial approach.

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43 **Keywords:** Iron oxide nanoparticles; combinatorial treatment; mild hyperthermia; alternating  
44 magnetic field (AMF); doxorubicin; ovarian cancer.

45

## 46 1. Introduction

47 Ovarian cancer is a significant cause of cancer death in women worldwide. The high mortality  
48 rate is attributed to the fact that ovarian cancer is generally detected at an advanced stage when  
49 the tumor has already disseminated at the peritoneal surfaces (Jelovac and Armstrong, 2011;  
50 Lengyel, 2012). Because of the distribution of advanced ovarian cancer, micronodular and  
51 floating tumor colonies cannot be adequately treated by surgery and require extensive  
52 chemotherapy. Although most patients respond effectively to initial chemotherapy, recurrence  
53 occurs in up to 75% cases (Jelovac and Armstrong, 2011; Lengyel, 2012). Patients with recurrent  
54 ovarian cancer ultimately develop resistance to chemotherapy and are generally incurable  
55 (Sehouli et al., 2008). Therefore, there is a critical need to develop novel therapeutic approaches  
56 that can improve the efficacy of conventional chemotherapy in drug-resistant ovarian cancer  
57 cells. In this regard, combinatorial treatment modalities that simultaneously target different  
58 pathways provide a promising direction for overcoming the drug resistance issue and achieving  
59 greater therapeutic outcomes (Kleef et al., 2012; Zhang et al., 2012). Moreover, these therapeutic  
60 approaches can employ lower doses of anticancer agents in order to avoid undesirable side  
61 effects on healthy organs (Zhang et al., 2012).

62 It was demonstrated that the combination of conventional chemotherapy with mild hyperthermia  
63 produces synergistic therapeutic effects on tumor cells and reduces the required effective doses  
64 of the anti-cancer drugs (Kleef et al., 2012; Kulshrestha et al., 2012). Mild hyperthermia is an  
65 adjuvant therapeutic modality to treat cancer by maintaining the temperature of the tumor region  
66 at 40–44 °C (Falk and Issels, 2001; Levi-Polyachenko and Stewart, 2011). At this temperature  
67 range, heat increases the efficacy of different chemotherapeutic drugs and combinatorial  
68 treatment is much more effective than the each of the two treatments applied separately (Pradhan

69 et al., 2010; Wang et al., 2011). However, a critical barrier faced by both conventional  
70 chemotherapy and hyperthermia has been the inability to deliver anticancer drugs and heat to the  
71 tumors in a precise manner. Nonspecific delivery leads to undesired side effects to normal organs  
72 and tissues, and lowers the dosages of heat and anticancer drugs at tumor sites required to kill  
73 cancer cells. Therefore, if both heat and chemotherapeutic drugs can be selectively and  
74 concurrently delivered to the tumorigenic region, the therapeutic efficacy of the combinatorial  
75 treatment is expected to be significantly improved with minimal side effects. Recent studies have  
76 demonstrated the possibility for concurrent delivery of chemotherapy and hyperthermia by the  
77 nanoparticles-based delivery systems (Kulshrestha et al., 2012; Wang et al., 2011). In particular,  
78 magnetic iron oxide nanoparticles (IONPs) offer a great potential as they can be loaded with  
79 anticancer agents and remotely heated with an external alternating magnetic field (AFM) after  
80 localization in cancer tumors (Lee et al., 2011; Pradhan et al., 2010). Moreover, there is an  
81 opportunity to modify IONPs-based delivery systems with cancer cell targeting moieties in order  
82 to improve tumor targeted localization (Fan et al., 2011; Taratula et al., 2011b; Yu et al., 2012a).  
83 There are a number of reports describing the successful development of water soluble iron oxide  
84 nanoparticles for anticancer drug delivery and imaging (Ahmd et al., 2012; Ahsan et al., 2013).  
85 The most widely studied approach to prepare the IONPs-based delivery system for combinatorial  
86 treatment is based on co-encapsulation of both chemotherapeutic drugs and iron oxide  
87 nanoparticles within liposomes, resulting in so called magnetoliposomes. Thus, several recent  
88 reports discuss the preparation and application of magnetoliposomes containing different  
89 chemotherapeutic agents for combinatorial treatment of cancer cells *in vitro* (Kulshrestha et al.,  
90 2012; Pradhan et al., 2010; Wang et al., 2011). For instance, Pradhan et al. successfully  
91 synthesized folate-targeted magnetoliposomes containing doxorubicin (DOX), which

92 demonstrated significant improvement in cancer cell treatment in comparison to drug loaded  
93 non-magnetic liposomes (Pradhan et al., 2010). Although these approaches have shown  
94 favorable results on cultured cancer cells, the resulted magnetoliposomes tend to be large (>300  
95 nm) in size and may have limited application for systemic delivery *in vivo*. A major obstacle for  
96 systemic delivery of large delivery systems is that the reticuloendothelial system (RES) detects  
97 and phagocytosis them, preventing their targeting and therapeutic action. In general, drug  
98 delivery systems that are 10–100 nm in size are considered to be optimal for systemic delivery  
99 whereas particles >200 nm and <10 nm are sequestered by the spleen or removed through renal  
100 clearance, respectively (Alexis et al., 2008; Longmire et al., 2008). Moreover, the current size of  
101 magnetoliposomes would probably be too large to exploit the enhanced permeability and  
102 retention (EPR) effect for targeting delivery systems to invade cancer tumors. Thus, only  
103 liposomes of an average size of approximately 200 nm or less are optimal for the EPR effect and  
104 have a significant chance of encountering the leaky vessels of tumor tissue (Maruyama, 2011).

105 In order to overcome the above mentioned barriers, down-sizing of the formulated  
106 magnetoliposomes is required. However, this step may result in lower encapsulation efficiency  
107 of iron oxide nanoparticles, which compromises the ability of magnetoliposomes to function as  
108 an efficient hyperthermia source. In addition, while doxorubicin encapsulation within  
109 magnetoliposomes was reported to be relatively high, there is certainly a need to further optimize  
110 magnetic particle incorporation (Pradhan et al., 2010). However, an increase in IONPs loading  
111 may lead to even larger magnetoliposomes.

112 In this work, we created a novel tumor targeted delivery system for combinatorial treatment of  
113 drug resistant ovarian cancer cells. The synthesized delivery system has an average size of 97.1  
114 nm and a zeta potential close to zero, both parameters being favorable for increased stability in

115 biological media and decreased elimination by the immune system. In order to achieve this, we  
116 synthesized superparamagnetic IONPs with a hydrodynamic diameter of 70.8 nm containing  
117 magnetic iron oxide nanocrystals surrounded by three polymer layers (Fig. 1). Due the presence  
118 of a polymer shell, each nanoparticle can be efficiently loaded with DOX and exhibit triggered  
119 drug release in acidic conditions that mimic the cancer tumor environment (Fig. 1A). To enhance  
120 steric stability and extend blood circulation of the delivery system (Alexis et al., 2008; Taratula  
121 et al., 2009), the surface of the drug loaded IONPs was modified with heterobifunctional  
122 polyethylene glycol (PEG) layer (Fig. 1B). Finally, Luteinizing Hormone-Releasing Hormone  
123 (LHRH) peptide, as a ligand to LHRH receptors in human ovarian cancer cells (Taratula et al.,  
124 2009; Zhang et al., 2012), was conjugated to the distal end of PEG for delivery system targeting  
125 specifically to the cancer tumor (Fig. 1C). The developed delivery system efficiently generates  
126 heat in the presence of AMF and synergistically increases cytotoxicity of the delivered DOX in  
127 drug resistant ovarian cancer cells.

## 128 **2. Materials and methods**

### 129 **2.1 Materials**

130 Oleic acid, 1-octadecene, Poly (Maleic Anhydride-*alt*-1-Octadecene) (PMAO, MW=30,000–  
131 50,000 Da), Poly(ethyleneimine) (PEI, MW 25,000 Da), microsized iron (III) oxide, were  
132 obtained from Sigma-Aldrich and used without further purification. Doxorubicin hydrochloride  
133 (DOX) was purchased from Polymed Therapeutics (Houston, TX).  $\alpha$ -Maleimide- $\omega$ -N-  
134 hydroxysuccinimide ester polyethylene glycol (MAL-PEG-NHS) was obtained from NOF  
135 Corporation (White Plains, NY). A synthetic analog of LHRH, Lys6–des-Gly10–Pro9-  
136 ethylamide (Gln–His–Trp–Ser–Tyr–DLys(DCys)–Leu–Arg–Pro–NH–Et) peptide was

137 synthesized by Amersham Peptide Co. (Sunnyvale, CA). Trinitrobenzene sulfonic acid (TNBSA)  
138 and Bicinchoninic Acid (BCA) protein assay kit were obtained from Pierce (Rockford, IL). All  
139 other chemicals were purchased from VWR (Visalia, CA).

## 140 **2.2 Preparation of the delivery system**

### 141 2.2.1 Iron oxide nanoparticles synthesis

142 IONPs were prepared using iron oxide powder as the iron precursor, oleic acid as the ligands,  
143 and 1-octadecene as the solvent according to the previously described procedure (Taratula et al.,  
144 2011b; Yang et al., 2008). Briefly, iron oxide nanocrystals were synthesized in organic solvents  
145 at a high temperature. Typically, microsized iron oxide was mixed with oleic acid, 1-octadecene,  
146 and then heated to 320 °C to produce monodisperse (5–10% size distribution) iron oxide  
147 nanocrystals. The size of the nanoparticles was controlled by reaction time, temperature, and the  
148 iron oxide and oleic acid concentrations. After the reaction was completed, the mixture was  
149 cooled and the iron oxide nanocrystals were precipitated out of 1-octadecene by  
150 chloroform/acetone, and then redispersed in chloroform. These nanocrystals were highly  
151 crystalline and uniform but were not dispersible in water due to the hydrophobic oleic acid  
152 capping layer. For dispersion of iron oxide nanoparticles in water, we modified a previously  
153 published method based on forming micelles through amphiphilic polymer (PMAO) for  
154 transferring iron oxide nanocrystals from organic solvents into water (Yang et al., 2009; Yu et  
155 al., 2006). Prior modification of iron oxide nanocrystals, PMAO was hydrolyzed at 80 °C in 0.5  
156 M NaOH for 24 hrs. The hydrophobic part of this polymer has 18-carbon alkaline side residues  
157 that intercalate and interact hydrophobically with the oleic acid chains that cover the iron oxide  
158 core. The hydrophilic part of the polymer, the carboxylic groups, is exposed to the outermost part

159 of the nanoparticles conferring stability to them (Moros et al., 2010). PMAO modified iron oxide  
160 nanoparticles were added to PEI aqueous solution and the PEI was allowed to adsorb for 20 min  
161 under stirring. The formed nanoparticles were purified by ultracentrifugation and used for further  
162 studies.

### 163 2.2.2 Drug loading into iron oxide nanoparticles

164 Drug loading was achieved by simply mixing DOX and IONPs solutions as previously described  
165 (Fig. 1A) (Yang et al., 2008). DOX was dissolved in water and then added to the IONPs aqueous  
166 solutions at the following weight ratios of DOX to IONPs (iron (Fe) content): (a) 1:1, (b) 1:5 and  
167 (c) 1:10. After rotating at room temperature for 12 hrs, free DOX was separated from the  
168 encapsulated drug using the Microsep 50 k centrifugal device. The following weight ratios of  
169 DOX to IONPs (iron (Fe) content): (a) 1:1 and (b) 1:10 were further employed for cytotoxicity  
170 study and combinatorial treatment experiment, respectively. The amount of DOX encapsulated  
171 into the IONPs was quantified based on UV-visible absorption spectra of IONPs-DOX samples,  
172 with a prominent DOX peak appearing around 460 nm over the IONPs background (UV-1800  
173 spectrophotometer, Shimadzu, Carlsbad, CA). The standard curve was generated by measuring  
174 drug absorption intensity at 460 nm in the standard samples containing different concentrations  
175 of DOX while using a constant concentration of IONPs (Fe content). Drug loading capacity of  
176 IONPs is expressed as the percentage of DOX weight encapsulated into IONPs over the weight  
177 of IONPs (Fe content) (Gou et al., 2010). In addition, drug encapsulation efficiency is  
178 represented as the percentage of DOX weight encapsulated into IONPs over the total weight of  
179 DOX used for drug loading procedure (Gou et al., 2010). The DOX loaded particles will be  
180 further mentioned as IONPs-DOX.

### 181 2.2.3 Modification of IONPs-DOX with PEG and LHRH

182 The previously published procedure was employed to modify both drug free (IONPs) and DOX  
183 loaded IONPs (IONPs-DOX) with PEG and LHRH (Taratula et al., 2009; Taratula et al., 2011b).  
184 Briefly, the NHS groups on the distal end of heterobifunctional 5 kDa PEG polymer (MAL-  
185 PEG-NHS) were reacted with primary amines on the surfaces of IONPs in 50 mM Phosphate  
186 Buffered Saline (PBS) buffer (pH 7.4) at primary amines to PEG molar ratio of 1:2.5 (Fig. 1B).  
187 The reaction was carried out for 1 hr at room temperature under shaking, following the addition  
188 of LHRH peptide, and then incubation overnight. The molar ratio of MAL-PEG-NHS to LHRH  
189 peptide in the reaction mixture was 1:2. The peptide was covalently conjugated to the distal end  
190 of PEG layer through the maleimide groups on the PEG and the thiol groups in LHRH (Fig. 1C).  
191 After 12 hrs of the reaction, the modified nanoparticles were purified by using Microsep 50 k  
192 centrifugal device. The concentration of amino groups available on the IONPs surface before  
193 PEGylation as well as the decrease in their concentration after the surface modification was  
194 determined by a modified spectrophotometric TNBSA assay as described in our previous report  
195 (Taratula et al., 2009). Determination of the presence of LHRH peptide on the surface of either  
196 IONPs modified with PEG and LHRH peptide (IONPs-PEG-LHRH) or DOX-loaded IONPs  
197 modified with PEG and LHRH peptide (IONPs-DOX-PEG-LHRH) was performed using  
198 Bicinchoninic Acid (BCA) protein assay (Pierce, Rockford, IL) as previously described (Taratula  
199 et al., 2009; Taratula et al., 2011b). The size, morphology and zeta potential of the developed  
200 drug delivery system were evaluated by Transmission electron microscopy (TEM) and Dynamic  
201 Light scattering (DLS) (see details in Supplementary data).

### 202 2.3 Characterization of the delivery system

## 203 2.3.1 Drug release study

204 The drug release profile of DOX from IONPs-DOX-PEG-LHRH was evaluated in PBS at pH 5.5  
205 and 7.4 and 50% human plasma. The drug loaded delivery system was dissolved either in 50%  
206 human plasma or in PBS buffer of appropriate pH and placed in a Float-A-Lyzer dialysis tubes  
207 (molecular weight cutoff of 50 kDa). The dialysis tubes were immersed in 40 mL of the  
208 appropriate solution and incubated at a constant temperature of 37 °C. At fixed time intervals,  
209 200 microliters of the samples were withdrawn from the dialysis tubes to record the absorbance  
210 of DOX at 460 nm as described above. After each absorption measurement, the samples were  
211 returned to the appropriate dialysis tubes for further incubation. The DOX content in the delivery  
212 system at different time points was quantified by using the same approach described above for  
213 the drug loading study. The percentage of drug release at different environments and time points  
214 was calculated as follows:

$$215 \text{ Drug release (\%)} = [DOX]_R/[DOX]_T \times 100,$$

216 where  $[DOX]_R$  is the amount of DOX released at collection time  $t$  and  $[DOX]_T$  is the total amount  
217 of DOX that was encapsulated in the delivery system.

218

## 219 2.3.2 Specific absorption rate measurements

220 Specific absorption rate (SAR) determines the heating ability of magnetic materials in the  
221 presence of an alternating magnetic field (AMF) and can be defined as the amount of heat  
222 generated per unit gram of magnetic material per unit time (Fortin et al., 2007). To measure the  
223 SAR values of IONPs alone and IONPs-DOX-PEG-LHRH, 100  $\mu$ L of each sample (Fe  
224 concentration 0.85 mg/mL) was transferred into a standard 0.5 mL microcentrifuge tube and

225 inserted into the insulating sample holder. The sample holder was then placed in the center of a  
226 5-turn cooper coil (40 mm inner diameter), connected to a radio frequency (RF) generator (MSI  
227 automation, Wichita, KS) that produces AMF with a constant frequency of 393 kHz and an  
228 amplitude of up to 33.5 kA/m. To minimize the influence of heat rising near the coil on the  
229 samples, the coil was cooled with circulating cold water. Within the coil, a jacket, through which  
230 polypropylene based coolant was circulated, additionally provided a thermal barrier to heat  
231 generated directly by the coil. The coolant temperature was adjusted in order to obtain an  
232 equilibrium temperature of 25 °C for all the samples prior to AMF application. The samples  
233 were then exposed to AMF of 33.5 kA/m amplitude and the temperature rise was recorded in 1-s  
234 intervals with a fiber optic probe (Neoptix Inc., QC, Canada) placed in the center of the sample  
235 solution. The SAR values of IONPs and IONPs-DOX-PEG-LHRH were determined from the  
236 initial slope of the time–temperature curve ( $dT/dt$ ), as described previously (Fortin et al., 2007).  
237 SAR was calculated using the following equation:

$$238 \quad \text{SAR} = (CV_s/m) \times (dT/dt),$$

239 where  $C$  is the specific heat capacity of the medium ( $C_{\text{water}} = 4.185 \text{ J g}^{-1} \text{ }^\circ\text{C}^{-1}$ ),  $V_s$  is the sample  
240 volume,  $m$  is the mass of iron in the sample and  $dT/dt$  is the initial slope of the time-dependent  
241 temperature curve.

## 242 **2.4 *In vitro* study**

### 243 2.4.1 Cell line

244 The A2780/AD multidrug resistant human ovarian carcinoma cell line was obtained from T. C.  
245 Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cells were cultured in RPMI 1640  
246 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (VWR, Visalia,

247 CA) and 1.2 mL/100 mL penicillin–streptomycin (Sigma, St. Louis, MO). Cells were grown at  
248 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> (v/v) in air. All experiments were performed on  
249 cells in the exponential growth phase.

#### 250 2.4.2 Cytotoxicity study

251 The cellular cytotoxicity of all studied formulations was assessed using a modified Calcein AM  
252 cell viability assay (Fisher Scientific Inc.). Briefly, cancer cells were seeded into 96-well  
253 microtiter plates at the density of  $10 \times 10^3$  cells/well and allowed to grow for 24 hrs at 37 °C.  
254 Then the culture medium was discarded and the cells were treated for 24 hrs with 200 µL of  
255 medium containing different concentrations of the following formulations: (1) control (fresh  
256 media), (2) IONPs (Fe content from 100 µg/mL to 0.78 µg/mL), (3) IONPs-PEG-LHRH (Fe  
257 content from 100 µg/mL to 0.78 µg/mL), (4) free DOX (drug concentration from 15 µg/mL to  
258 0.058 µg/mL), and (5) IONPs-DOX-PEG-LHRH (DOX concentration from 15 µg/mL to 0.058  
259 µg/mL). After treatment, the cells were rinsed with Dulbecco's Phosphate-Buffered Saline  
260 (DPBS) buffer and incubated for 1 hr with 200 µL of freshly prepared Calcein AM solution (10  
261 µM in DPBS buffer). Fluorescence was measured using a multiwell plate reader (Synergy HT,  
262 BioTek Instruments, Winooski, VT) with a 485 nm excitation and a 528 nm emission filters. On  
263 the basis of these measurements, cellular viability was calculated for each concentration of the  
264 formulation tested. The 50% inhibitory concentration (IC<sub>50</sub>) was determined as the drug  
265 concentration that resulted in a 50% reduction in cell viability.

#### 266 2.4.3 Cellular internalization

267 Prior to the visualization, A2780/AD cells were plated in 6-well tissue culture plate at the density  
268 of  $10 \times 10^3$  cells/well and cultured for 24 hrs. The medium was then replaced by a suspension of

269 (1) IONPs-DOX, (2) IONPs-DOX-PEG, and (3) IONPs-DOX-PEG-LHRH in the culture media  
270 at the concentration of doxorubicin of 5  $\mu\text{g}/\text{mL}$  and the cells were incubated with the studied  
271 formulations for 10 hrs. In addition, to visualize the intracellular accumulations of DOX at  
272 different time points, A2780/AD cells were incubated with (1) free DOX and (2) IONPs-DOX-  
273 PEG-LHRH for 3 and 24 hrs and their nuclei were stained with 4', 6-diamidino-2-phenylindole  
274 (DAPI) for 30 min at 37  $^{\circ}\text{C}$  as previously described (Taratula et al., 2011a). Cellular  
275 internalization of the studied formulations was analyzed by a fluorescence microscope (Leica  
276 Microsystems Inc., Buffalo Grove, IL).

#### 277 2.4.4 Quantification of intracellular iron content

278 The ferrozine-based colorimetric assay was used to estimate the intracellular iron (Fe) content in  
279 A2780/AD cells after treatment with the developed delivery system as previously reported (see  
280 details in Supplementary data) (Basel et al., 2012).

#### 281 2.4.5 *In vitro* evaluation of hyperthermia and combinatorial treatment

282 Prior evaluation of the developed delivery system for hyperthermia applications, A2780/AD cells  
283 were plated in T-25 cell culture flasks and grown to 80% of confluence. Subsequently, cells were  
284 incubated for 12 hrs with drug free delivery system (IONPs-PEG-LHRH) dispersed in 6 mL of  
285 cell culture media (15  $\mu\text{g Fe}/\text{mL}$ ). Cells loaded with the delivery system were washed with  
286 DPBS in order to remove loose IONPs-PEG-LHRH, detached by using 0.25% trypsin/EDTA and  
287 resuspended in cell culture media prior to counting. Subsequent to counting, a portion of the cell  
288 suspension containing  $5 \times 10^6$  cells was centrifuged at 1000 rpm for 5 min to form the cell pellet.  
289 The formed cell pellets were maintained in a constant volume of 0.1 mL of culture media in a  
290 standard 0.5 mL microcentrifuge tube before and during treatment. Samples were then positioned

291 in the center of a 5-turn cooper coil as described above for the SAR evaluation experiment. The  
292 water jacket inside of the copper coil was maintained at 37 °C, and the samples were allowed to  
293 equilibrate to this temperature before exposure to AMF. The cell pellets were exposed to AMF at  
294 fixed 393 kHz frequency and the temperature changes were measured in 1-s intervals by placing  
295 a fiber optic temperature probe (Neoptix Inc., QC, Canada) inside of the pellets. Once the cell  
296 pellets reached the targeted temperatures (40 °C or 44 °C), the power of the RF generator was  
297 adjusted in order to maintain these temperatures for 30 min. After AMF exposure, the cells were  
298 cooled to 37 °C, washed with DPBS buffer, resuspended in media and seeded in a 96-well plate  
299 at a density of  $10 \times 10^3$  cells/well and were cultured for another 48 hrs. Finally, the cell viability  
300 was assessed using a modified Calcein AM assay as described earlier for the cytotoxicity study.

301 In addition, three control groups were employed in the current study to evaluate efficacy of  
302 hyperthermia and combinatorial treatments. These controls were (1) untreated cells, (2) cells  
303 treated with IONPs-PEG-LHRH only and (3) untreated cells exposed to AMF only. Thus, pellets  
304 consisting of (1) untreated cells and (2) cells treated with IONPs-PEG-LHRH were placed in the  
305 copper coil, allowed to equilibrate to 37 °C using heat generated by the water jacket and  
306 maintained under these conditions for 30 min with AMF power off. Finally, for magnetic field  
307 only treatments, the pellets of untreated cells were exposed to AMF at the maximum strength for  
308 30 min. The cells from all the control groups were further cultured and evaluated with the cell  
309 viability assay as described above.

310 The cells were treated in the same manner for the chemotherapy only and combinatorial  
311 treatment modality (chemotherapy and 30 min of hyperthermia at 40 °C), except that cells in T-  
312 25 flask were incubated with the drug loaded delivery system (IONPs-DOX-PEG-LHRH, 1.0 µg  
313 DOX/mL and 15 µg Fe/mL) for 12 hrs. Finally, the cell pellets designated to combinatorial

314 treatment experiment were additionally exposed to AMF in order to generate temperature of 40  
315 °C for a 30 min period.

316 The combined effect of the combinatorial treatment was evaluated by Valeriote's method as  
317 described previously (Pradhan et al., 2010). With (A), (B) and (A+B) representing the percentage  
318 of cell viability for treatments A (hyperthermia) and B (chemotherapy) and A+B (combinatorial  
319 treatment). Combined effects were defined as follows: synergistic,  $(A+B) < (A) \times (B) / 100$ ;  
320 additive,  $(A+B) = (A) \times (B) / 100$ ; sub-additive,  $(A) \times (B) / 100 < (A+B) < (A)$ , if  $(A) < (B)$ , interference,  
321  $(A) < (A+B) < (B)$ , if  $(A) < (B)$ , antagonistic,  $(B) < (A+B)$ , if  $(A) < (B)$ .

## 322 2.5 Statistical analysis

323 Data were analyzed using descriptive statistics, single-factor analysis of variance (ANOVA), and  
324 presented as mean values  $\pm$  standard deviation (SD) from three to eight independent  
325 measurements. The comparison among groups was performed by the independent sample  
326 student's *t*-test. The difference between variants was considered significant if  $P < 0.05$ .

## 327 3. Results and discussion

### 328 3.1 Preparation and characterization of iron oxide nanoparticles

329 In order to develop the tumor-targeted delivery system for concurrent delivery of  
330 chemotherapeutic drugs and nanoheaters, water soluble IONPs covered with three polymer  
331 layers such as oleic acid, PMAO and PEI (Fig. 1A) have been synthesized according to the  
332 previously published procedure (Taratula et al., 2011b; Yang et al., 2008). The prepared IONPs  
333 contain spherical iron oxide cores of 28.5 nm in diameter with relatively uniform size  
334 distribution (Fig. 2A and Fig. S1A). The as-synthesized iron oxide cores were hydrophobic  
335 owing to an oleate coating. To provide water solubility, oleic acid coated iron oxide crystals

336 were modified with two polymers in tandem such as the monolayers of amphiphilic PMAO and  
337 hydrophilic PEI. The prepared IONPs are very stable in most buffer solutions in the pH range of  
338 5-10 and can survive autoclaving process (121 °C for 30 min). Analysis of negatively stained  
339 TEM images revealed that the developed nanoparticles with three polymer layers had a diameter  
340 of  $34.2 \pm 2.1$  nm and the thickness of polymer layers around the iron oxide cores was  
341 approximately 3.5 nm (Fig. 2 A, B and Table 1). Concurrently, the DLS study demonstrated that  
342 a hydrodynamic size of the same IONPs nanoparticles was  $70.8 \pm 0.2$  nm (Table 1, Fig. S2A),  
343 which is about 35 nm larger than the IONPs diameter measured by TEM. The observed  
344 discrepancy between TEM and DLS measurements is not related to the aggregation of the  
345 nanoparticles in solution. Thus, a low polydispersity index (PDI) of  $0.141 \pm 0.010$ , obtained from  
346 DLS measurement, indicates stability and a narrow size distribution of IONPs in solution.  
347 Moreover, TEM images further confirmed the presence of non-aggregated nanoparticles with  
348 relatively uniform size distribution (Fig. 2A and C). The discrepancy between the obtained  
349 results is related to the fact that TEM and DLS are different techniques and TEM provides more  
350 accurate size measurement. It worth mentioning that DLS yields a larger average size by  
351 measuring the hydrodynamic diameter of the particle including the solvation layers, while the  
352 polymer layers around the iron core could potentially dehydrate and shrink during TEM sample  
353 preparation resulting in lower nanoparticle diameter. The zeta potential of the prepared IONPs  
354 was highly positive  $+38.0 \pm 1.8$ mV, which is attributed to the presence of protonated amine  
355 groups in the structure of PEI monolayer (Table 1). The TNBSA assay further demonstrated that  
356 1 mg of iron oxide nanoparticles (Fe content) contain 5.4  $\mu$ mole of primary amine groups, which  
357 could be employed for further modification of the developed IONPs with PEG and cell targeting  
358 peptide. Furthermore, the presence of the PEI layer on IONPs surface provides a mechanism for

359 the developed delivery system to escape the endosome/lysosome through the proton sponge  
360 effect (Yezhelyev et al., 2008). Finally, the magnetic property of the developed IONPs was  
361 investigated using a vibrating sample magnetometer at room temperature. The results revealed  
362 that the coercivity for IONPs was close to zero, indicating that most particles have  
363 superparamagnetic behavior (Fig. S2B) (Lee et al., 2011).

### 364 **3.2 Drug loading**

365 Encapsulation of DOX into iron oxide nanoparticles offers a chance to alter the  
366 pharmacokinetics and tissue distribution profile in favor of tumor specific accumulation, and  
367 thus minimize cytotoxicity of the chemotherapeutic agent to the healthy organs (Peng et al.,  
368 2008). Moreover, several reports indicated that encapsulating drugs into iron oxide nanoparticles  
369 is also a promising way for overcoming multidrug resistance (Kievit et al., 2011). Two main  
370 strategies to incorporate DOX into iron oxide nanoparticles have been widely employed: (1)  
371 conjugation of the drug to the nanoparticles surface via labile chemical bonds (Yang et al., 2010)  
372 and (2) physical loading of DOX in the polymer surface layer (Quan et al., 2011; Yang et al.,  
373 2008). Because DOX may lose therapeutic efficacy in a conjugated form, the loading of  
374 anticancer agent through covalent binding could be a limited approach. Therefore, our drug  
375 loading strategy takes advantage of the availability of a drug reservoir formed by three polymer  
376 layers on the developed IONPs surfaces. We found that DOX can be efficiently incorporated into  
377 the polymer layers on IONPs surfaces by simply mixing the IONPs with the appropriate amount  
378 of the drug.

379 Thus, both drug loading capacity and drug encapsulation efficiency at a DOX to IONPs (Fe)  
380 ratio of 1 mg DOX: 1 mg of Fe were equal to 38.0% w/w, respectively. In addition, the loaded  
381 drug amount achieved at the above mentioned DOX to IONPs (Fe) ratio was evaluated to be

382 0.538 mmol per 1 g of IONPs-PEG-LHRH. Moreover, we also demonstrated an ability to control  
383 drug loading capacity by changing a DOX to IONPs weight ratio. For instance, a DOX to IONPs  
384 ratio of 0.1 mg DOX: 1 mg of Fe decreased drug loading capacity to 6.6% w/w. In general, drug  
385 loading capacity showed a strong dependence on DOX concentration during the drug loading  
386 process and decreases as the DOX: IONPs ratio is reduced (Fig. S3A). These data revealed a fact  
387 that doses of both chemotherapeutic drugs and IONPs can be controlled during concurrent  
388 delivery to the cancer cells by the developed delivery system in order to achieve the desired  
389 therapeutic outcome. Further, we examined the hydrodynamic size and zeta potential of the DOX  
390 loaded IONPs (Table 1). Our result demonstrated that the resulting iron oxide nanoparticles  
391 loaded with DOX are  $86.4 \pm 0.7$  nm in diameter with uniform size distribution (PDI  $0.129 \pm$   
392  $0.012$ ), which is larger than IONPs alone ( $70.8 \pm 0.2$  nm, Table 1 and Fig. S2A). TEM analysis  
393 further confirmed an increase in diameter of the DOX loaded nanoparticles ( $37.5 \pm 2.1$  nm, Table  
394 1 and Fig. S1B) in comparison to non-modified IONPs ( $34.2 \pm 2.1$  nm, Table 1 and Fig. 2B). An  
395 increase in the size of nanoparticles can be attributed to the swelling of the polymer coating on  
396 the surface of the nanoparticles upon DOX encapsulation. The observed result is in a good  
397 agreement with previous studies that demonstrated that the size of human serum albumin coated  
398 iron oxide nanoparticles increased by about 20 nm after DOX encapsulation (Quan et al., 2011).  
399 In contrast, the zeta potential values of the prepared IONPs ( $+38.0 \pm 1.8$ ) were not affected by  
400 the encapsulation of DOX ( $+42.6 \pm 0.7$ , Table 1). These data indicate that DOX is not physically  
401 adsorbed on IONPs and rather encapsulated into the polymer coating of iron oxide nanoparticles.

### 402 **3.3 Development and characterization of targeted delivery system**

403 The size and surface charge are important physiochemical parameters in the development of  
404 nanoparticles-based delivery systems. Thus, the positive charge on the surface promotes

405 nanoparticles aggregation in the blood stream, due to electrostatic association with negatively  
406 charged serum proteins and results in rapid uptake of drug carriers by the reticuloendothelial  
407 system (Alexis et al., 2008). Moreover, positively charged nanoparticles are more toxic than  
408 neutral counterparts due to more pronounced disruption of cellular membrane integrity (Taratula  
409 et al., 2009). To reduce nonspecific binding with blood components and minimize cytotoxicity  
410 due to the presence of positively charged amines groups, DOX loaded IONPs were PEGylated  
411 using an excess of heterobifunctional PEG polymer (MAL-PEG-NHS) (Fig. 1B). The employed  
412 5 kDa PEG contains an amine reactive NHS ester and thiol reactive maleimide (MAL) group on  
413 the opposite sides and allows for a further modification of IONPs in a layer-by-layer fashion.  
414 The PEGylation of IONPs-DOX was carried out by the coupling of linear MAL-PEG-NHS to the  
415 amino groups on surface of nanoparticles, introduced by PEI polymer. The availability of the  
416 primary amines on the IONPs before PEGylation as well as the decrease in their concentration  
417 after modification has been estimated by the TNBSA assay. Thus the obtained data reveal a 95%  
418 decrease of  $\text{NH}_2$  concentration on the surface of the nanoparticles after PEGylation. Moreover,  
419 conjugation of heterobifunctional PEG to the positively charged primary amine groups on the  
420 DOX loaded IONPs surface resulted in the reduction of zeta potential from  $+42.6 \pm 0.7$  mV to  
421  $+4.9 \pm 0.9$  mV (Table 1). As was expected, PEGylation significantly minimized the cytotoxicity  
422 of the drug free IONPs (Fig. S3B). Thus, the modified drug free nanoparticles do not  
423 significantly compromise viability of A2780/AD multidrug resistant human ovarian carcinoma  
424 cells in the studied concentration range of 0.8-100  $\mu\text{g}/\text{mL}$  (Fig.5A and Fig. S3B), indicating  
425 biocompatibility of the developed drug delivery system. On the other hand, non-modified iron  
426 oxide nanoparticles reduced viability of the cells more than 50% at the concentrations higher  
427 than 50  $\mu\text{g}/\text{mL}$  (Fig. S3B). Furthermore, positively charged iron oxide nanoparticles showed

428 strong aggregation in the cell medium containing 10% of fetal bovine serum (Fig. 3A). In the  
429 case of the PEGylated nanoparticles, there was no aggregation observed in the serum-  
430 supplemented medium (Fig. 3C and E), indicating that the PEG layer can prevent aggregation of  
431 the delivery system induced by serum proteins. In order to confirm the obtained result, both non-  
432 modified (IONPs-DOX) and PEGylated delivery systems (IONPs-DOX-PEG-LHRH) were  
433 dispersed in 50% human plasma and incubated at 37 °C for 24 hrs. The obtained data revealed  
434 that PEGylation significantly improved stability of DOX-loaded IONPs nanoparticles in human  
435 plasma. Thus the hydrodynamic diameter of non-modified delivery systems (IONPs-DOX)  
436 recorded 24 hrs after incubation was significantly increased from  $70.8 \pm 0.2$  nm to  $1488.0 \pm 38.0$   
437 nm, while the size of PEGylated IONPs changed only from  $97.1 \pm 1.0$  nm to  $118.9 \pm 0.7$  nm,  
438 respectively (Fig. S4). The tendency of non-modified particles to form aggregates might be  
439 attributed to stronger electrostatic interaction between plasma proteins and IONPs which have  
440 higher surface charges ( $+42.6 \pm 0.7$  mV) as compared to PEGylated drug delivery systems ( $+4.9$   
441  $\pm 0.9$  mV). Another key issue in the development of drug delivery systems is the rapid  
442 elimination of nanoparticles from the blood stream, which is attributed to their recognition by  
443 macrophages of the mononuclear phagocyte system. Therefore, the uptake of nanoparticles by  
444 murine macrophages (RAW264.7) was investigated with the aim of evaluating the capability of  
445 nanoparticles to reduce phagocytosis. The intracellular iron content measurement indicated that  
446 the uptake of PEG-modified nanoparticles (0.54 pg/cell) into macrophage cells was three times  
447 lower than that of unmodified nanoparticles (1.62 pg/cell, Fig. S5). The obtained result is  
448 consistent with an earlier report indicating that PEGylation prevents the nanoparticles from  
449 agglomeration and increases their resistance to protein adsorption (Zhang et al., 2002).

450 According to the previous reports, while improving stability and reducing cytotoxicity  
451 PEGylation usually limits cellular internalization of the nanoparticle-based delivery systems  
452 (Taratula et al., 2009). Thus, the neutral surface charge of PEGylated drug delivery systems  
453 reduces their interactions with a negatively charged cell membrane when compared with a  
454 positively charged non-modified delivery system (Taratula et al., 2009). Fluorescence  
455 microscopy studies revealed that, despite strong aggregations, the non-modified IONPs-DOX  
456 were still capable of delivering DOX into A2780/AD human cancer cells (Fig. 3A and B). In  
457 contrast, the PEGylated IONPs-DOX showed significantly lower internalization efficiency by  
458 the cells under the same experimental conditions (Fig. 3C and D). According to the cell uptake  
459 study (Fig. S6), non-modified nanoparticles (IONPs-DOX) were internalized in A2780/AD cells  
460 four times more efficiently than their PEGylated counterparts. In general, this positive feature of  
461 the surface modification results in the minimization of non-specific cellular internalization and  
462 thus reducing side effects of the delivery systems on healthy organs (Taratula et al., 2009). To  
463 achieve targeted delivery to the cancer cells and enhance cellular internalization, the  
464 modification of sterically stabilized carriers with cell targeting ligands is usually used (Pradhan  
465 et al., 2010; Taratula et al., 2009; Zhang et al., 2012). Recently, we successfully employed a  
466 modified peptide synthetic analog of LHRH decapeptide as a targeting moiety to tumors  
467 overexpressing LHRH receptors, including ovarian cancer tumors (Taratula et al., 2009; Zhang  
468 et al., 2012). In order to conjugate a targeting moiety (LHRH peptide) to the IONPs-based  
469 delivery system, the maleimide group at the distal end of the PEG-chain was coupled to the thiol  
470 group presented by cysteine residue in the modified LHRH sequence. The presence of the LHRH  
471 peptide on the complex surface was confirmed by BCA protein assay according to the  
472 manufacturer's protocol. Thus, results of fluorescence microscopy and cell uptake studies

473 demonstrated an increase in the cellular internalization of the LHRH targeted delivery systems as  
474 compared to PEGylated ones without LHRH targeting moieties (Fig. 3D, F and Fig. S6). Thus,  
475 IONPs-DOX-PEG-LHRH were taken up three times more efficiently than IONPs-DOX-PEG in  
476 A2780/AD cells. To verify the specific targeting of the LHRH receptor by IONPs-DOX-PEG-  
477 LHRH, A2780/AD cells were pre-incubated with the free LHRH peptide prior to the cell uptake  
478 study. The obtained result revealed that IONPs-DOX-PEG-LHRH were internalized in the cells  
479 two times less efficiently than under normal conditions (without cell pre-incubation and with free  
480 LHRH), indicating that the interaction between the LHRH-modified delivery system and cancer  
481 cells was competitively inhibited by free LHRH in culture medium. Finally, the hydrodynamic  
482 size of the resulting LHRH-PEG-DOX-IONPs was evaluated to be  $97.1 \pm 1.0$  nm (PDI  $0.218 \pm$   
483  $0.037$ ), which is larger than DOX-IONPs alone ( $86.4 \pm 0.7$  nm, Table 1 and Fig.S2A). Moreover,  
484 TEM analysis also demonstrated an increase in diameter of the LHRH-targeted delivery system  
485 ( $43.5 \pm 2.8$  nm) in comparison to non-modified DOX-loaded nanoparticles ( $37.5 \pm 2.1$  nm, Table  
486 1 and Fig. 2C and D). The increase in the size of the DOX loaded IONPs after modification  
487 could be explained by the presence of the PEG layer and LHRH peptides on the surface of the  
488 delivery system as previously demonstrated (Taratula et al., 2009). According to the previous  
489 study by Yu et al, the size of PEGylated iron oxide nanoparticles is a stronger determinant of  
490 non-specific uptake by macrophages (Yu et al., 2012b). Thus, the final delivery systems have a  
491 hydrodynamic size within the range of 10-100 nm to reduce elimination by the kidneys ( $<10$  nm)  
492 and recognition by macrophages cells ( $>100$ nm) (Alexis et al., 2008; Longmire et al., 2008).

### 493 **3.4 Intracellular DOX localization and anticancer effect**

494 The intracellular localization of DOX plays an important role in its anticancer activity (MacKay  
495 et al., 2009; Shi et al., 2009). Therefore, localization of DOX in the A2780/AD ovarian

496 carcinoma cells after incubation with either free drug or drug loaded into LHRH targeted IONPs  
497 was evaluated by fluorescence microscopy. We found that initially DOX delivered by IONPs  
498 was predominantly distributed in the cytoplasm after 3 hrs of incubation (Fig. 4A and B), but  
499 later also diffused in the cell nuclei (Fig. 4C and D), where it is expected to exert a therapeutic  
500 effect. In contrast, free DOX incubated with A2780/AD cells was only localized in the nuclei  
501 (Fig. 4E and F), which is consistent with the results of previous studies (MacKay et al., 2009).  
502 Our data confirm a well-known fact, that free DOX permeates cellular and nuclear membranes  
503 by passive diffusion and tend to rapidly accumulate in the cell nuclei. Conversely, DOX loaded  
504 nanoparticles are expected to be taken up by cancer cells via endocytosis, followed by  
505 endosomal/lysosomal escape, drug release in the cytoplasm and subsequent drug diffusion to the  
506 nucleus. These processes are much slower than passive drug diffusion *in vitro* and would result  
507 in slow delivery of DOX encapsulated into IONPs to the cell nuclei (MacKay et al., 2009; Shi et  
508 al., 2009). Thus, cell uptake study revealed that after the same period of time (3 hrs) free DOX  
509 was internalized in A2780/AD cells two times more efficiently than DOX encapsulated in the  
510 developed delivery system (Fig. S7). In fact, this is one common reason why DOX delivered by  
511 nanocarriers usually have lower *in vitro* activity than the original molecular drug (MacKay et al.,  
512 2009; Shi et al., 2009). As expected, we observed that the encapsulation of DOX into IONPs  
513 decreased anticancer activity of the drug particularly at the higher concentrations ( $>1\mu\text{g/mL}$ )  
514 (Fig. 5A), supporting the differences in cellular internalization route and intracellular localization  
515 pattern. The half maximal inhibitory concentration ( $\text{IC}_{50}$ ) for free DOX against A2780/AD was  
516  $1.9\ \mu\text{g/mL}$  while this value for DOX loaded into the delivery system was  $10.5\ \mu\text{g/mL}$ ,  
517 respectively. The detected cellular toxicity of free DOX against A2780/AD cell line is in a good  
518 agreement with previously published data, which revealed that  $\text{IC}_{50}$  for free DOX against

519 A2780/AD cell line was  $\sim 4 \mu\text{M}$  ( $\sim 2.1 \mu\text{g/mL}$ ) (Savla et al., 2011). Thus, A2780/AD ovarian  
520 cancer cells were more sensitive to free DOX, which is likely due to free drug rapidly diffusing  
521 to the nucleus *in vitro*, whereas IONPs-DOX have to be trafficked through the cell before the  
522 drug is released and can reach the nucleus to intercalate DNA (MacKay et al., 2009). A similar  
523 behavior was previously described in the other studies, where DOX encapsulation in the  
524 nanoparticles-based delivery systems were less cytotoxic than free DOX under cell culture  
525 conditions due to longer time required for drug nuclear transportation (MacKay et al., 2009; Shi  
526 et al., 2009). Despite this behavior, the ultimate therapeutic outcome of DOX encapsulated in the  
527 delivery system is positive because the delivery system reduces the amount of available drug in  
528 the extracellular environment, and potentially diminishes unwanted side-target effects on healthy  
529 organs *in vivo* and inhibits multi drug resistance as compared to free DOX (MacKay et al.,  
530 2009). Therefore, another critical aspect for the development of an efficient drug delivery system  
531 is the ability of the drug to be predominantly released from the carrier in the cancer tumors and  
532 not in the blood stream. To assess the potential of the developed delivery system as a drug carrier  
533 capable of triggered drug release, the release profiles of DOX from IONPs-DOX-PEG-LHRH  
534 were evaluated at  $37^\circ\text{C}$  in PBS buffer at pH 5.5 (pH of cancer tumor and endosomes) and 7.4  
535 (pH of blood plasma) and human plasma (Hruby et al., 2005). The difference in release kinetics  
536 at pH 5.5 and 7.4 indicated that the pH strongly influenced DOX release from the developed  
537 drug delivery system, and the DOX release is more pronounced at pH 5.5 than at pH 7.4 (Fig.  
538 5B). An initial burst release of 44.7% was detected within 2 hrs of IONPs-DOX-PEG-LHRH  
539 incubation at pH 5.5. Further incubation of the delivery system for 72 hrs under the same  
540 experimental conditions resulted in 91.1% of total drug release. In contrast, only 17.6% of the  
541 total DOX was gradually released at pH 7.4 after 72 hrs of incubation. A similar drug release

542 pattern has been observed after incubation of the drug delivery system in 50% human plasma and  
543 resulted in 16.5% of total DOX release within 72 hrs. The obtained result is in good agreement  
544 with the previously published reports, which demonstrated that DOX exhibit pH dependent  
545 release from iron oxide nanoparticles modified with two polymer layers in tandem such as oleic  
546 acid and amphiphilic polymer containing carboxylic groups (Savla et al., 2011; Yang et al.,  
547 2008; Zou et al., 2010). There are two main reasons for the observed DOX behavior: (1) the  
548 protonation of the primary amine of DOX molecules under acidic pH which dramatically  
549 increased the solubility of the drug in an aqueous solution, and (2) the weakened interaction  
550 between DOX and the partially neutralized carboxyl groups of amphiphilic polymers under  
551 acidic pH (Zou et al., 2010). Such drug release behavior is important for an efficient and  
552 nontoxic drug delivery system because it ensures that there is low drug release at the  
553 physiological pH 7.4 during the transport of the drug in the blood stream, while there is fast or  
554 controlled drug release at acidic pH on arriving at the targeted tumor cells (Hruby et al., 2005;  
555 MacKay et al., 2009; Yang et al., 2008).

### 556 **3.5 Heating properties of the delivery system: in vitro hyperthermia**

557 Exposing iron oxide nanoparticles to AMF at a specified frequency can produce hyperthermia  
558 suitable for cancer treatment, and the efficiency of IONPs to generate heat is measured by the  
559 specific absorption rate (SAR) (Lee et al., 2011). Because the presence of coatings can  
560 compromise heating efficiencies of iron oxide nanoparticles, which in turn determine the  
561 efficacy of the treatment, the effect of drug loading and surface modification on the heat  
562 generation was evaluated based on SAR values of free IONPs and IONPs-DOX-PEG-LHR (Lee  
563 et al., 2011; Yuan et al., 2012). According to our data, loading with DOX and following coating  
564 with both PEG and LHRH layers did not significantly reduce the heating properties of the

565 synthesized IONPs. The heating behavior of both non-modified IONPs and LHRH-PEG-DOX-  
566 IONPs of concentration 0.85 mg/mL subjected to AMF at the magnetic field frequency of 393  
567 KHz and amplitude of 33.5 kA/m is shown in Figure 6A. The samples exhibited a similar heating  
568 rate reaching 50 °C in 7.5 min and 8.3 min for IONPs and IONPs-DOX-PEG-LHRH,  
569 respectively. The SAR values were calculated to be 289 W/g and 271 W/g for IONPs and PEG-  
570 LHRH-DOX-IONPs, respectively. The decrease in the SAR value for IONPs-DOX-PEG-LHRH  
571 is in good agreement with the magnetization study (Fig. S2B), which showed that drug loading  
572 and surface modification slightly decrease magnetic moments of IONPs. The obtained high SAR  
573 values indicate that the developed delivery system can be efficient for hyperthermia therapy.  
574 To evaluate the suitability of the developed delivery system for hyperthermic applications we  
575 tested its capacity to generate heat intracellularly. For hyperthermia experiments cells were  
576 incubated with both drug free (IONPs-PEG-LHRH) and DOX loaded (IONPs-DOX-PEG-  
577 LHRH) delivery systems for 12 hrs at an iron concentration of 15µg/mL. This was followed by  
578 cells trypsinization, washing and centrifugation at a constant speed to form pellets consisting of 5  
579 x 10<sup>6</sup> cells. The cell pellets were placed in the coil to generate hyperthermia under exposure to  
580 AMF at constant magnetic field frequency of 393 kHz and maximum allowed amplitude of 33.5  
581 kA/m. Our experimental design is based on previous studies that indicate that a maximum  
582 temperature change in a single cell transfected with iron oxide nanoparticles and exposed to  
583 AMF is negligible (Hedayati et al., 2012). Therefore, a collection of nanoparticle-containing  
584 cells into pellets would be necessary to achieve therapeutic levels of hyperthermia. Moreover,  
585 the hyperthermia generation efficiency also depends on the amount of iron oxide nanoparticles  
586 internalized by the cells (Hedayati et al., 2012). Thus, the amount of internalized iron in the  
587 cancer cells under these experimental conditions was quantified by using ferrozine assay as

588 previously described (Basel et al., 2012). Both DOX loaded and drug free delivery systems  
589 entered cancer cells with similar efficiency, yielding a measured iron concentration of  
590 approximately 14.9 pg/cell and 11.1 pg/cell for IONPs-PEG-LHRH and IONPs-DOX-PEG-  
591 LHRH, respectively. After exposure of the formed cell pellets with the above mentioned  
592 nanoparticles loading to AMF, the maximum achieved temperature of cell pellets was as high as  
593 44 °C. An example of a measured cell pellet temperature profile is represented in Figure 6B.  
594 Thus, with a starting temperature of 37 °C, the nanoparticles loaded A2780/AD cells exhibit  
595 rapid heating upon exposure to AMF, crossing 40 °C in 1.5 min and reaching 44 °C in 11.2 min.  
596 The achieved cellular temperature can be maintained during extended periods of time in the  
597 presence of AMF. However, switching off the AMF resulted in the rapid cooling of cells to 37  
598 °C in 5 min. It appears realistic to heat remotely non accessible small tumors or disseminated  
599 metastases via intravenously injected tumor targeted IONPs.

600 The optimum temperature for most mild hyperthermia applications is in the 40–44 °C range  
601 ( $T < 40$  °C causes limited effect,  $T < 44$  °C results in thermoablation), and requires durations of  
602 30–60 min (Falk and Issels, 2001; Levi-Polyachenko and Stewart, 2011). Therefore, our  
603 hyperthermia treatment experiments were performed at two different temperatures, 40 °C and 44  
604 °C, with an exposure period of 30 min. These two temperatures were specifically selected to  
605 evaluate the cytotoxicity effects of mild hyperthermia on A2780/AD ovarian cancer cells at the  
606 accepted lower and upper temperatures. The desired cellular temperatures were achieved by  
607 varying magnetic field amplitude at a constant magnetic field frequency of 393 kHz. Thus,  
608 exposure of A2780/AD cells transfected with IONPs-PEG-LHRH to AMF with field amplitude  
609 of 33.5 kA/m resulted in the maximum temperature of 44 °C, while decreasing field amplitude to  
610 21.2 kA/m lowered cellular temperature to 40 °C. Subsequently, the treated cells were seeded in

611 96 well plates and viability was analyzed 48 hrs after hyperthermia treatment in order to  
612 investigate whether mild hyperthermia promoted apoptosis on drug resistant ovarian cancer cells.  
613 The effects of hyperthermia treatments on the cell viability, at two temperatures (40 °C and 44  
614 °C), can be seen in Figure 7. The results indicated that cell viability decreased by 95% at 44 °C  
615 (Fig.7, bar 4), while mild hyperthermia at a lower temperature (40 °C) was less efficient resulting  
616 in a 72% reduction of cellular viability (Fig.7, bar 5). Experimental controls performed on cell  
617 viability demonstrated that neither IONPs alone (Fig.7, bar 3) nor the applied magnetic field  
618 (Fig.7, bar 2) effected cell viability under the studied experimental conditions. Therefore, heat  
619 generated by the developed delivery system is the main mechanism for the cell death under an  
620 AMF.

### 621 **3.6 Combinatorial treatment**

622 Numerous studies have demonstrated that the combination of mild hyperthermia with a number  
623 of conventional chemotherapeutic agents at lower therapeutic dosages result in additive or  
624 synergistic therapeutic effects (Kulshrestha et al., 2012; Pradhan et al., 2010). Therefore, the  
625 main goal of this work was not to kill the cancer cells with higher concentrations of either  
626 anticancer drug or high temperature, but rather to demonstrate an efficacy of the combinatorial  
627 treatment achieved with the developed delivery system at lower therapeutic doses of mild  
628 hyperthermia and chemotherapeutic agent. Consequently, the concentrations of 1µg/mL of DOX  
629 and 15 µg/mL of IONPs incorporated in the tumor targeted delivery system were employed for  
630 the current study. Our choice was based on the fact that the selected DOX concentration alone  
631 cannot significantly enhance cell apoptosis and leaves over 70% of the cells viable after  
632 treatment (Fig. 5A and Fig. 7, bar 6). Further, the employed concentration of IONPs is not toxic  
633 to A2780 cells (Fig. 15A, Fig. S3B and Fig. 8, bar 3) and results in substantial cellular heat

634 generation required for mild hyperthermia conditions (40-44 °C) under exposure to AMF (Fig.  
635 6B). Because 44 °C is lethal to the cancer cells under the studied conditions (viability of the cells  
636 decreased over 95%), the lowest temperature accepted for mild hyperthermia (40 °C) was chosen  
637 for the combinatorial treatment experiment and was attained by adjusting field amplitude to 21.2  
638 kA/m. Our data demonstrated that the combination of the chemotherapeutic drug and mild  
639 hyperthermia resulted in over 95% cell death (Fig.7, bar 7), while either chemotherapy alone  
640 (Fig.7, bar 6) or nanoparticles-mediated mild hyperthermia (Fig.7, bar 5) decreased cell viability  
641 by 27% and 72%, respectively. It can be concluded that the cytotoxic effect of the combinatorial  
642 treatment achieved with the developed delivery system is superior to each of the two treatments  
643 applied separately, and an evaluation using Valeriote's formula (Pradhan et al., 2010) showed  
644 that the combined effects were synergistic in nature. Obviously, the developed delivery system  
645 can be efficiently used for killing of ovarian cancer cells by only hyperthermia (Fig. 7, bar 4) at  
646 the higher temperature (44 °C). However, to avoid damage to surrounding healthy tissue, it is  
647 highly desirable to keep the local temperature during hyperthermia treatment under 44 °C and  
648 whole body temperatures less than 42 °C, which is the upper limit compatible with life. Thus, an  
649 advantage of the proposed combinatorial treatment approach is based on the concurrent  
650 application of two treatment modalities at their respective safe doses that work by different  
651 mechanisms, thereby increasing tumor cell killing while minimizing overlapping toxicity to  
652 healthy organs.

#### 653 **4. Conclusions**

654 We have successfully synthesized and tested a tumor-targeting multifunctional delivery system  
655 for combinatorial treatment of ovarian cancer. The developed nanocarriers have a hydrodynamic  
656 diameter less than 100 nm and a zeta potential close to zero, which make them stable in

657 biological media and decreases their elimination by the immune system. The developed delivery  
658 system demonstrated low cytotoxicity and high efficiency in the delivery of DOX, and mild  
659 hyperthermia to drug resistant ovarian cancer cells. The prepared nanocarriers are capable of  
660 drug triggered release in an acidic environment and, therefore, DOX can be predominantly  
661 dissociated from IONPs within a cancer tumor, but not during the systemic circulation after  
662 intravenous administration. Due to superparamagnetic behavior of the developed IONPs, the  
663 delivery system can efficiently heat cancer cells remotely upon exposure to AMF and the desired  
664 temperature can be achieved by varying magnetic field strength. Moreover, lower doses of  
665 chemotherapeutic drug and mild hyperthermia delivered by the developed nanocarrier  
666 synergistically decreased viability of drug resistant ovarian cancer cells. Thus, much higher  
667 therapeutic outcomes with fewer side effects can be reached with combinatorial treatment of  
668 ovarian cancer by employing lower doses of chemotherapeutic drugs and mild hyperthermia  
669 incorporated in the developed delivery system.

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#### 677 **Appendix A. Supplementary data**

678

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811 **Figure Legends**

812 **Figure 1.** Surface-engineered approach for preparation of iron oxide nanoparticle (IONPs)-based  
813 nanocarrier for a targeted co-delivery of anticancer drug and heat. (A) Loading of anticancer  
814 agent (DOX) into the polymer reservoir on IONPs surface. (B) Surface modification of DOX-  
815 loaded IONPs with PEG. (C) Conjugation of LHRH peptide to the distal end of PEG.

816  
817 **Figure 2.** TEM images and size distribution histograms of (A, B) iron oxide nanoparticles  
818 (IONPs) and (C, D) DOX loaded nanoparticles modified with PEG and LHRH peptide (IONPs-  
819 DOX-PEG-LHRH).

820  
821 **Figure 3.** Representative fluorescence microscopy images of A2780/AD cancer cells incubated  
822 for 10 hrs with (A, B) DOX loaded iron oxide nanoparticles (IONPs-DOX), (C, D) IONPs-DOX  
823 modified with PEG (IONPs-DOX-PEG) and (E, F) IONPs-DOX modified with both PEG and  
824 LHRH peptide (IONPs-DOX-PEG-LHRH). A, C and E represent light images of A2780/AD  
825 cells; B, D and F represent fluorescent images of DOX delivered into A2780/AD cells by IONPs  
826 with different modifications. The fluorescent images were taken with the same exposure times to  
827 allow comparisons of DOX internalization efficiency delivered by different systems. The red  
828 arrows indicate strong aggregation of non-modified DOX loaded iron oxide nanoparticles  
829 (IONPs-DOX) in cell culture medium containing 10% of fetal bovine serum.

830  
831 **Figure 4.** Intracellular localization of DOX in A2780/AD cancer cells after incubation with  
832 LHRH targeted drug delivery systems (IONPs-DOX-PEG-LHRH) for (A, B) 3 hrs and (C, D) 24  
833 hrs and free DOX for 3 hrs (E, F) followed by DAPI staining. A, C and E represent light images

834 of A2780/AD cells; B, D and F represent superimposed fluorescence images of DAPI stained  
835 nuclei (blue color) and DOX (red color). Superimposition of fluorescence images allows for  
836 detecting of nuclear localization of DOX resulting in purple color.

837

838 **Figure 5.** (A) *In vitro* cytotoxicity of free DOX, DOX loaded into IONPs-based drug delivery  
839 system (IONPs-DOX-PEG-LHRH) and DOX-free drug delivery system (IONPs-PEG-LHRH)  
840 against A2780/AD human ovarian cancer cells after 24 hrs of incubation. (B) The release  
841 profiles of DOX from IONPs-DOX-PEG-LHRH incubated at 37 °C in 50% of human plasma  
842 and PBS buffer at pH 5.5 and pH 7.4.

843

844 **Figure 6.** (A) Heating profiles of non-modified IONPs and IONPs-DOX-PEG-LHRH of  
845 concentration 0.85 mg/mL subjected to AMF at magnetic field frequency of 393 KHz and  
846 amplitude of 33.5 kA/m. (B) Dynamic temperature profile of A2780/AD cells transfected with  
847 IONPs-PEG-LHRH (Fe concentration- 15 µg/mL) and exposed to AMF (33.5 kA/m and 393  
848 kHz). Cells were allowed to equilibrate to 37 °C in the sample chamber for 7 min before AMF  
849 was turned on. The arrows indicate when AMF was turned on and off, respectively.

850

851 **Figure 7.** Viability of A2780/AD ovarian cancer cells after treatment with the following  
852 formulations (1) control (no treatment), (2) exposure to AMF only (33.5 kA/m and 393 kHz), (3)  
853 IONPs-PEG-LHRH (Fe concentration- 15 µg/mL), (4) 44 °C hyperthermia for 30 min: cells  
854 were incubated with IONPs-PEG-LHRH (Fe concentration- 15 µg/mL) and exposed to AMF  
855 (33.5 kA/m and 398 kHz), (5) 40 °C hyperthermia for 30 min: cells were incubated with IONPs-  
856 PEG-LHRH (Fe concentration- 15 µg/mL) and exposed to AMF (21.2 kA/m and 393 kHz), (6)

857 chemotherapy: cells were treated with IONPS-DOX-PEG-LHRH (Fe concentration- 15  $\mu\text{g}/\text{mL}$ ,  
858 DOX concentration- 1  $\mu\text{g}/\text{mL}$ ) and (7) combinatorial treatment: chemotherapy (DOX  
859 concentration- 1  $\mu\text{g}/\text{mL}$ ) and hyperthermia (40  $^{\circ}\text{C}$  for 30 min). Cells were incubated with  
860 IONPs-DOX-PEG-LHRH and exposed to AMF (21.2 kA/m and 398 kHz).  
861

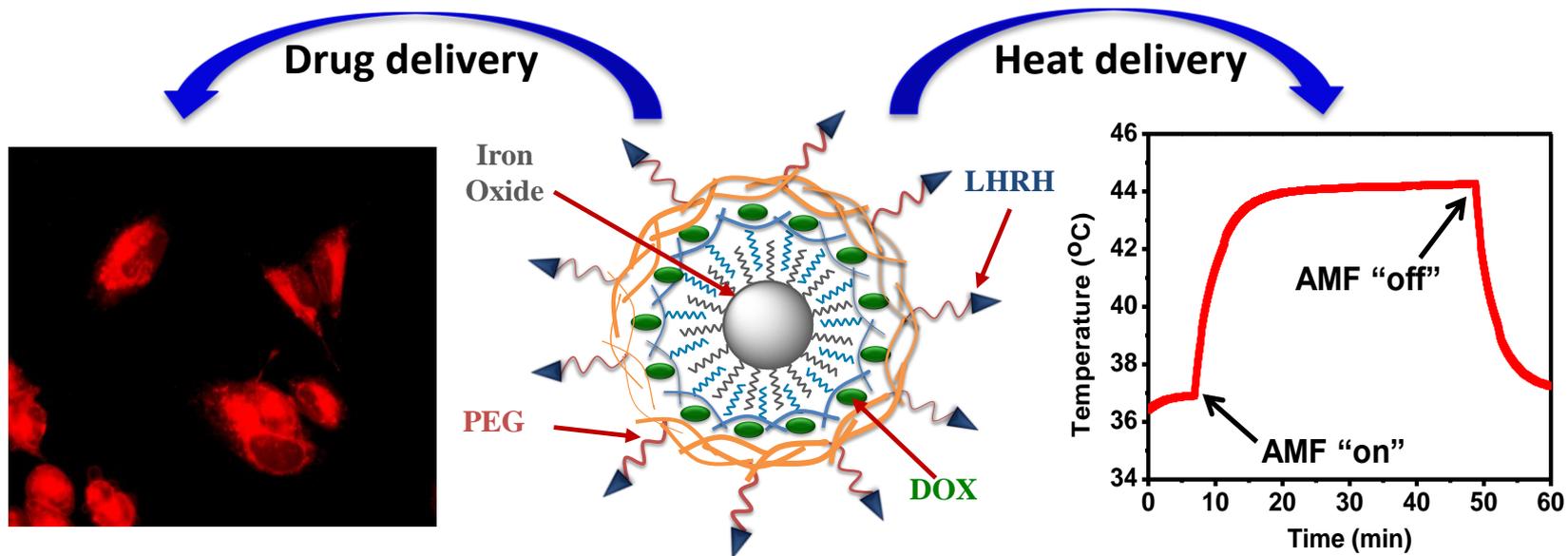
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Formulations	Size (nm) <sup>a</sup>	Size (nm) <sup>b</sup>	PDI <sup>b</sup>	Zeta potential (mV)
IONPs	34.2 ± 2.1	70.8 ± 0.2	0.141 ± 0.010	+38.0 ± 1.8
IONPs-DOX	37.5 ± 2.1	86.4 ± 0.7	0.129 ± 0.012	+42.6 ± 0.7
IONPs-DOX-PEG-LHRH	43.5 ± 2.8	97.1 ± 1.0	0.218 ± 0.037	+4.9 ± 0.9

862 **Table 1.** Physicochemical characterization of iron oxide nanoparticle-based formulations863 <sup>a</sup> TEM measurements<sup>b</sup> DLS measurements

864



**Nanocarrier for concurrent delivery of chemotherapeutic drug and heat**

Figure 1

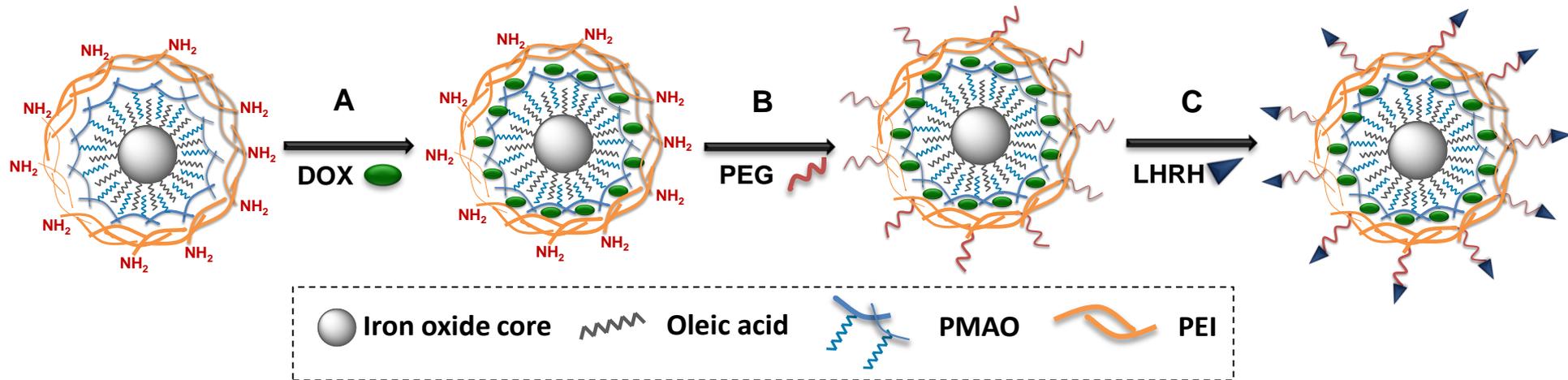


Figure 2

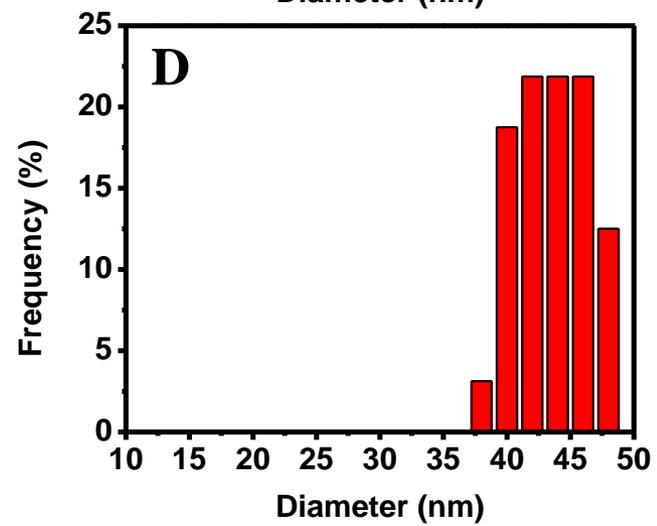
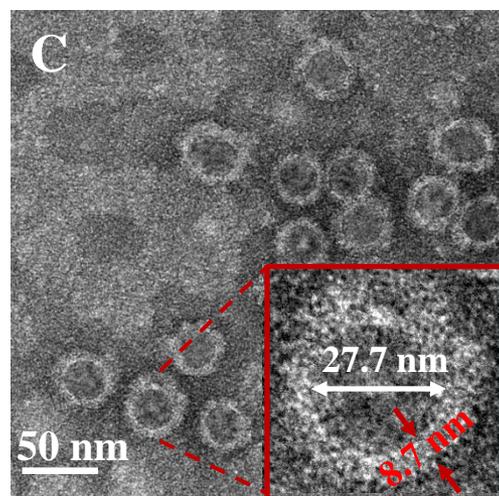
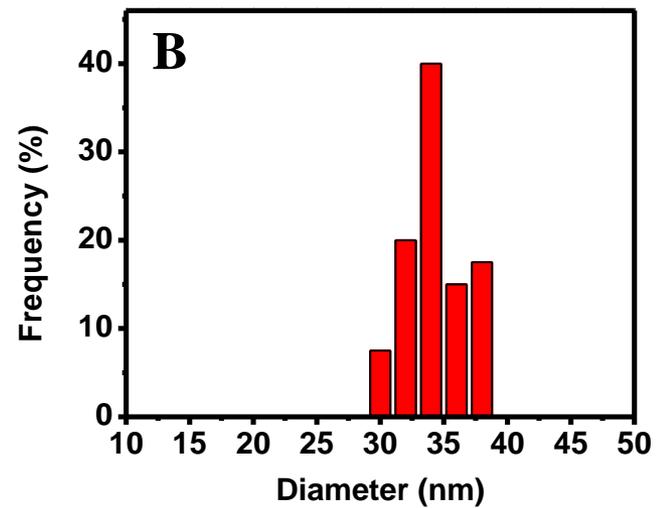
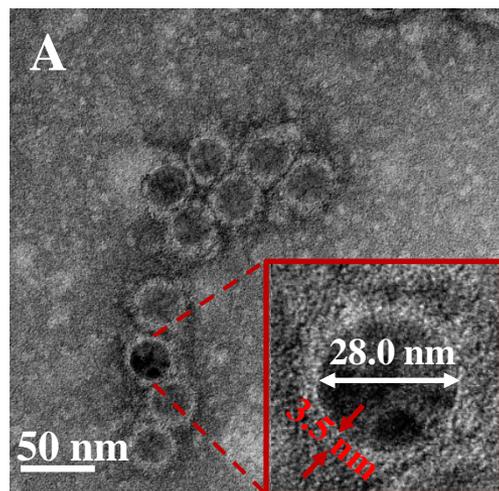


Figure 3

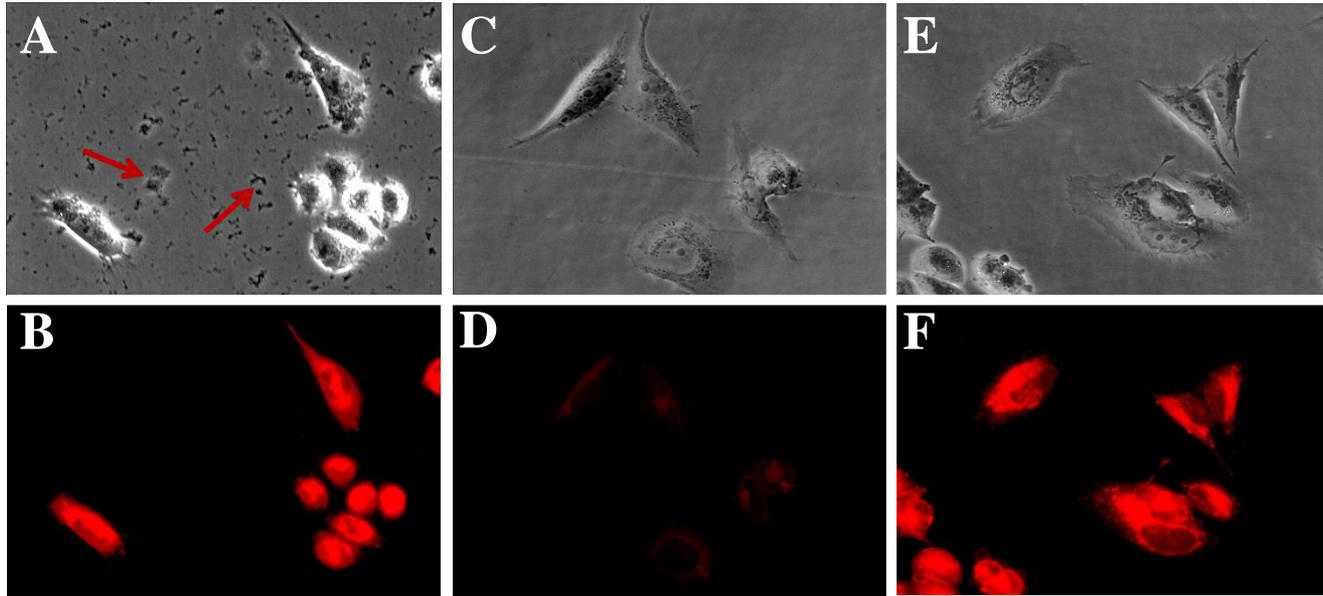


Figure 4

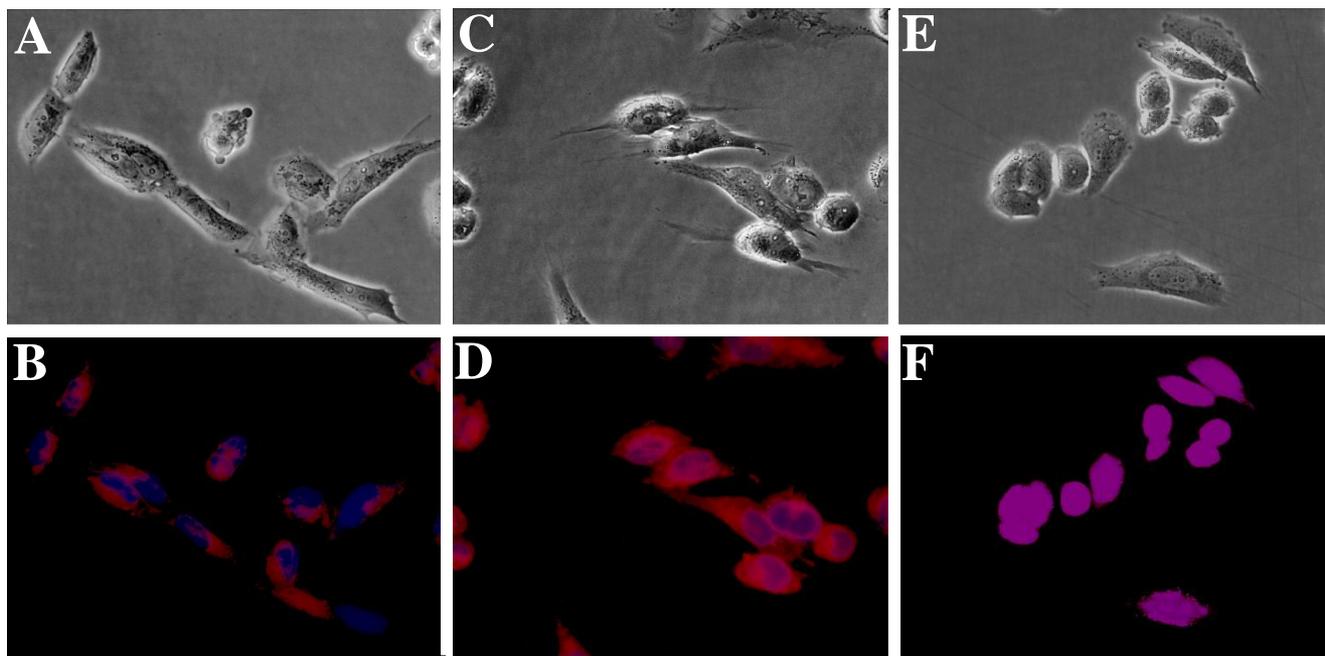


Figure 5

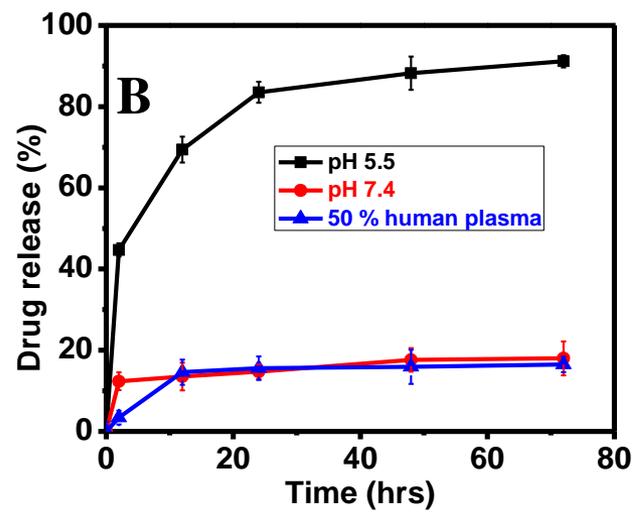
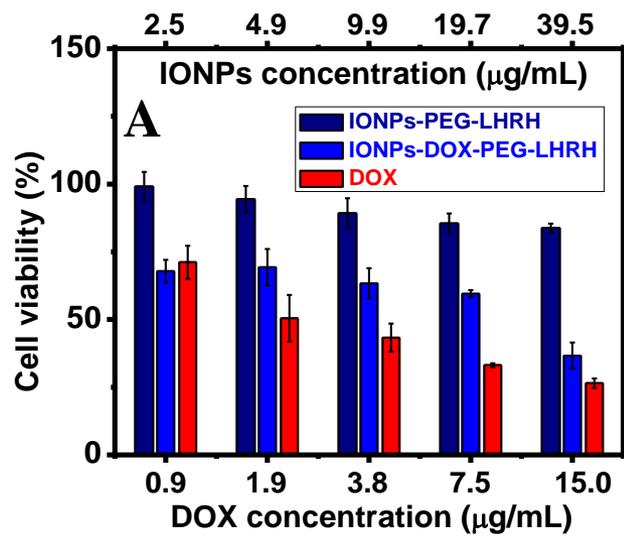


Figure 6

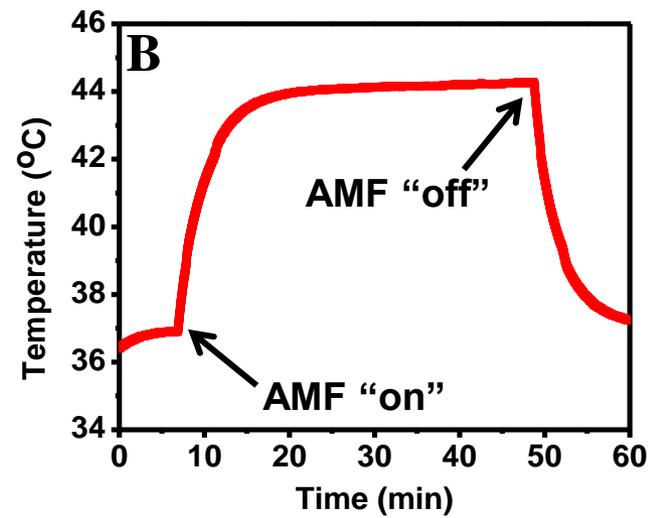
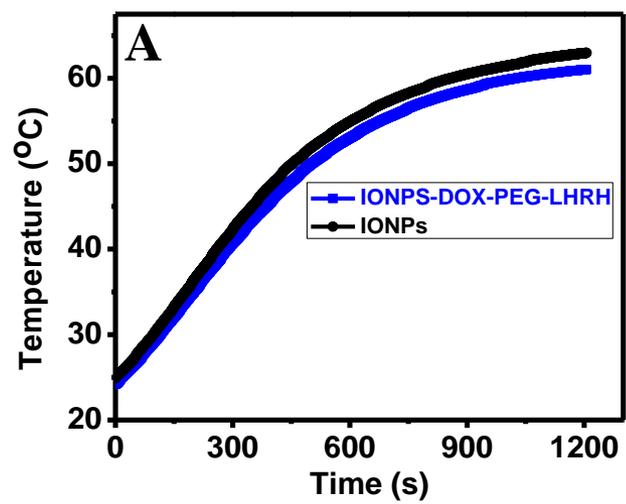
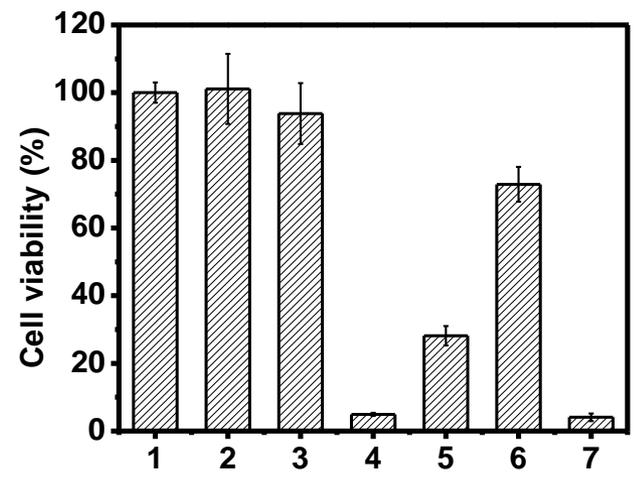


Figure 7



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