*Supporting Information*

# Folate-Targeted Curcumin-Loaded Niosomes for Site-Specific Delivery in Breast Cancer Treatment: In Silico and In Vitro Study

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**Table S1.** Different niosomal formulations for encapsulation of curcumin.

| Formulation | Type of Surfactant | Lipid/Drug (mol ratio) | HLB | Transition temperature , Tc (°C) | Drug concentration(mg/ml) | Sonication time (min) | Surfactant: Cholesterol: DCP (molar ratio) |
|---|---|---|---|---|---|---|---|
| Nio-Cur1 | Span20 | 10 | 8.60 | 16 | 1 | 5 | 2:1:0.05 |
| Nio-Cur 2 | Span60 | 10 | 4.70 | 53 | 1 | 5 | 2:1:0.05 |
| Nio-Cur 3 | Span80 | 10 | 4.30 | -12 | 1 | 5 | 2:1:0.05 |
| Nio-Cur 4 | Span20 | 20 | 8.60 | 16 | 1 | 5 | 2:1:0.05 |
| Nio-Cur 5 | Span60 | 20 | 4.70 | 53 | 1 | 5 | 2:1:0.05 |
| Nio-Cur 6 | Span80 | 20 | 4.30 | -12 | 1 | 5 | 2:1:0.05 |
| Vehicle (Nio) | Span80 | 10 | 4.30 | -12 | - | 5 | 2:1:0.05 |
| PEG-FA@Nio-Cur 3 | Span80 | 20 | 4.30 | -12 | 1 | 5 | 2:1:0.05 |

**Table S2**. Primers and their sequences are used in real-time PCR.

| Gene | Forward Primer | Reverse Primer |
|------|----------------|----------------|
| *Bax* | 5'-CGGCAACTTCAACTGGGG-3' | 5'-TCCAGCCCAACAGCCG-3' |
| *BCL$_2$* | 5-'GGTGCCGGTTCAGGTACTCA-3' | 5'-TTGTGGCCTTCTTTGAGTTCG-3' |
| *p53* | 5'-CATCTACAAGCAGTCACAGCACAT-3' | 5'-CAACCTCAGGCGGCTCATAG-3' |
| *ß-actin* | 5-'TCCTCCTGAGCGCAAGTAC -3' | 5'-CCTGCTTGCTGATCCACATCT-3' |
| *PBGD* | 5'-ATGTCCGGTAACGGCGGC-3' | 5'-CAGCATCGCTACCACAGTGTC-3' |

**Table S3.** Bax, Bcl2, and p53 PDB features

| Target | Resolution | R-Value Free | R-Value Work | R-Value Observed | Total Structure Weight (kDa) | Atom Count | Mutation(s) | Unique protein chains | position | Gene length |
|---|---|---|---|---|---|---|---|---|---|---|
| 6GL8 (Bcl2) | 1.40 Å | 0.194 | 0.178 | 0.179 | 20.95 kDa | 1414 | yes | 1 | 9-206 | 239 |
| 6EB6 (Bax) | 2.02 Å | 0.201 | 0.171 | 0.173 | 21.27 kDa | 1492 | yes | 1 | 1-192 | 192 |
| 6SL6 (p53) | 1.67 Å | 0.180 | 0.163 | 0.164 | 28.18 kDa | 1902 | yes | 1 | 89-311 | 393 |

**Table S4.** Significant Bax, Bcl2, and p53 features in Uniport server.

| Ligand | Subcellular location | | Position of mutagenesis | | |
|---|---|---|---|---|---|
| *Bax* | Isoform Alpha | Mitochondrion | 21 | K → E | Reduces interaction with *Bcl2*L11, homo-oligomerization, and triggering of apoptosis |
| | | Cytoplasm and Cytosol | | | |
| | Isoform Beta | Cytoplasm and Cytosol | 74 | M → D or E | Strongly reduced interaction with MCL1, *Bcl2*, *Bcl2*L1, and *Bcl2*L2. |
| | Isoform Gamma | Cytoplasm and Cytosol | 184 | S → D, E, H, or K | S → D, E, H or K: Constitutive cytoplasmic location. |
| | Isoform Delta | Cytoplasm and Cytosol | 184 | S → V | S → V: Constitutive mitochondrial location. |
| *Bcl2* | 1: Nucleus membrane | | 34 | D → A | Abolishes cleavage by caspase-3 |
| | | | 64 | D → A | No effect on cleavage by caspase-3. |
| | 2: Endoplasmic reticulum membrane | | 138-141 | | Loss of *Bax*-binding and anti-apoptotic activity |
| | | | 144 | W → A | Loss of *Bax*-binding and anti-apoptotic activity |
| | | | 145 | G → A | Loss of *Bax*-binding and anti-apoptotic activity |
| | 3: Mitochondrion Outer membrane | | 145 | G →E | Loss of *Bax*-binding and anti-apoptotic activity |
| | | | 146 | R → A | Loss of *Bax*-binding and anti-apoptotic activity |
| | | | 188 | W → A | Loss of *Bax*-binding and anti-apoptotic activity |
| | | | 190 | Q → L | Partial loss of *Bax*-binding and 50% decrease in anti-apoptotic activity |

| | | | |
|---|---|---|---|
| | | 191 | D → A | Partial loss of *Bax*-binding and 50% decrease in anti-apoptotic activity |
| | | 192 | N → A | Partial loss of *Bax*-binding and 50% decrease in anti-apoptotic activity |
| | | 194-197 | Missing | Loss of *Bax*-binding and anti-apoptotic activity |
| | | 200 | E → A | Partial loss of *Bax*-binding and 50% decrease in anti-apoptotic activity |
| | | 15 | S → A | Loss of interaction with PPP2R5C, PPP2CA, and PPP2R1A |
| | | 18 | T → A | No effect on interaction with MDM2 and increase in protein levels after DNA damage |
| | | 20 | S → A | Abolishes phosphorylation site. Abolishes increase in protein levels after DNA damage |
| | | 20 | S → D | Constitutively increased T*p53* protein levels |
| | | 22-23 | LW → QS | Loss of interaction with MDM2, leading to constitutively increased T*p53* protein levels |
| | | 24 | K → R | Abolishes ubiquitination by MUL1 |
| | | 37 | S → D | Abolishes phosphorylation by MAPKAPK5 |
| *p53* | 1: Cytoskeleton | 46 | S → A | Abolishes phosphorylation by DYRK2 and HIPK2 and acetylation of K-382 by CREBBP |
| | 2: Cytoplasm and Cytosol | 46 | Missing | Alters interaction with WWOX |
| | 3: Mitochondrion | 55 | T → A | Blocks phosphorylation by TAF1 |
| | | 183 | S → A | Strongly abolishes phosphorylation |
| | | 183 | S → E | Inhibits slightly its transcriptional activity |
| | 4: Endoplasmic reticulum | 248 | R → A | Does not induce SNAI1 degradation |
| | 5: Nucleus | 269 | S → A | Abolishes phosphorylation |
| | | 269 | S → E | Strongly inhibits its transcriptional activity |
| | | 284 | T → E | Strongly inhibits its transcriptional activity |
| | | 291-292 | KK → RR | Abolishes polyubiquitination by MKRN1 |
| | | 319 | K → A | Loss of nuclear localization, when associated with A-320 and A-321 |
| | | 320 | K → A | Loss of nuclear localization, when associated with A-319 and A-321 |
| | | 321 | K → A | Loss of nuclear localization, when associated with A-319 and A-320 |

| | | |
|---|---|---|
| 333-337 | RGRER→KGKEK | Reduced methylation by PRMT5. Reduced nuclear localization. Decreased binding to promoters of target genes. Reduced transcriptional activity |
| 359 | P → D | Abolishes binding to USP7 |
| 361 | G → E | Abolishes binding to USP7 |
| 362 | S → A | Abolishes binding to USP7 |
| 370 | K → R | Induces a decrease in methylation by SMYD2 |
| 372 | K → R | Induces a decrease in protein stabilization |
| 373 | K → R | Abolishes dimethylation by EHMT1 and EHMT2 |
| 382 | K → A | Abolishes acetylation by CREBBP |
| 382 | K → R | Abolishes monomethylation by KMT5A |
| 383 | L → A | Abolishes S-315 phosphorylation by CDK2/cyclin A |
| 385 | F → A | Reduced SUMO1 conjugation |
| 386 | K → A | Abolishes SUMO1 conjugation, in vitro and in vivo |
| 387 | T → A | No effect SUMO1 conjugation |
| 388 | E → A | Abolishes SUMO1 conjugation |

**Table S5.** Imperative features of all three genes (Bax, Bcl2 and p53 in *Homo sapiens*) from the protparam server.

| gene | Theoretical pI | The estimated half-life | Instability index | Total number of negatively charged residues (Asp + Glu) | Total number of positively charged residues (Arg + Lys) |
|---|---|---|---|---|---|
| *Bax* | 5.08 | 30 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo). | 36.42 (stable) | 23 | 20 |
| *Bcl2* | 6.75 | 30 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo). | 51.63 (unstable) | 22 | 21 |
| *p53* | 8.17 | >20 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). | 70.55 (unstable) | 43 | 45 |

**Table S6.** Validation of three proteins (6GL8(Bcl2), 6EB6(Bax), 6SL6(p53)) by using the SAVES program.

| | Target | ERRAT score | Verify 3D score | z-score | Ramachandran plot | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | | | | Favored region | Additional allowed | Generosity allowed | Disallowed |
| procheck | 6GL8(Bcl2) | 100% | 100% | -6.76 | 96.1% | 3.9% | 0.0% | 0.0% |
| | 6EB6(Bax) | 99.4152% | 98.32% | -7.99 | 94.9% | 5.1% | 0.0% | 0.0% |
| | 6SL6(p53) | 88.1443% | 94.12% | -6.18 | 93.1% | 6.9% | 0.0% | 0.0% |

**Table S7.** Predicted Drug likeness of curcumin according to Lipinski's rule.

| Drug likeness rule | Property (unit) | Rule | Predicted Result curcumin | Predicted Result Ascorbic acid |
|---|---|---|---|---|
| Lipinski's rule | Molecular weight | ≤500 | 368.38gr/mol | 176.12 gr/mol |
| | Lipophilicity (LogP) | ≤5 | 3.03 | 0.39 |
| | Hydrogen bond acceptor | ≤10 | 6 | 6 |
| | Hydrogen bond donors | ≤5 | 2 | 4 |

| | | | Predicted Result | Predicted Result |
|---|---|---|---|---|
| | | | curcumin | Ascorbic acid |
| | Molecular weight | ≤500 | 368.38gr/mol | 176.12 gr/mol |
| | Lipophilicity (LogP) | ≤5 | 3.03 | 0.39 |

**Table S8.** Predicted pharmacokinetic profile of curcumin.

| Category | Property (unit) | Predicted curcumin Result | ADMETLAB standards |
|---|---|---|---|
| Basic physicochemical property | LogP (partition coefficient) (log mol/L) | 3.37 | Favorable: 0< LogP <3<br>LogP <0: high aqueous solubility.<br>LogP >3: poor aqueous solubility. |
| | LogD7.4 (Distribution coefficient D) (log mol/L) | 0.969 | < 1: high solubility, low Permeability, and Metabolism; Permeability possible via paracellular if MW < 200.<br>1 to 3: moderate Solubility, Permeability; and low Metabolism.<br>3 to 5: low solubility; high Permeability; and moderate to high Metabolism.<br>> 5: low Solubility; high Permeability and Metabolism. |
| | Log S(solubility) (Log mol/L) | -4.733 | Favorable: higher than -4 log mol/L<br><10 μg/mL: Low solubility;<br>10–60 μg/mL: Moderate solubility;<br>>60 μg/mL: High solubility |
| Absorption | Papp (Caco-2 permeability) (cm/s) | -5.052 | Favorable: higher than -5.15 Log unit or -4.70 or -4.80 |
| | HIA (Human Intestinal Absorption) (%) | 0.569 | >0.5: HIA positive<br><0.5: HIA negative |
| | Pgp-inhibitor | 0.217 | >0.5: An inhibitor<br><0.5: non-inhibitor |
| | Pgp-substrate | 0.061 | >0.5: A substrate<br><0.5: non-substrate |
| Distribution | PPB (Plasma protein binding) (%) | 87.01 | 90%: highly protein-bound and low therapeutic index. |
| | BBB (Blood brain barrier) (%) | 0.814 | ≥0.1: BBB positive<br><0.1: BBB negative |
| | VD (Volume Distribution) | -0.574 | Favorable: 0.04-20L/kg;<br><0.07L/kg: highly hydrophilic Confined to blood, plasma protein-bound<br>0.07-0.7L/kg: Regularly distributed;<br>>0.7L/kg: highly lipophilic; Bound to tissue components. |

| | | | |
|---|---|---|---|
| | CYP1A2-Inhibitor | 0.833 | >0.5: An inhibitor <br> <0.5: non-inhibitor |
| | CYP1A2-Substrate | 0.504 | >0.5: Substrate <br> <0.5: non-substrate |
| | CYP3A4-Inhibitor | 0.032 | >0.5: An inhibitor <br> <0.5: non-inhibitor |
| | CYP3A4-Substrate | 0.406 | >0.5: Substrate <br> <0.5: non-substrate |
| | CYP2C9-inhibitor | 0.071 | >0.5: An inhibitor <br> <0.5: non-inhibitor |
| | CYP2C9-substrate | -4.733 | >0.5: Substrate <br> <0.5: non-substrate |
| | CYP2C19-inhibitor | 0.437 | >0.5: An inhibitor <br> <0.5: non-inhibitor |
| Metabolism | CYP2C19-substrate | 0.454 | >0.5: Substrate <br> <0.5: non-substrate |
| | CYP2D6-inhibitor | 0.214 | >0.5: An inhibitor <br> <0.5: non-inhibitor |
| | CYP2D6-substrate | 0.858 | >0.5: Substrate <br> <0.5: non-substrate |
| Excretion | Clearance (mL/min/kg) | 1.541 | >15 mL/min/kg: high ; 5mL/min/kg< Cl < 15mL/min/kg: moderate ; <5 mL/min/kg: low |
| | T1/2 (Half-life) (H) | 1.687 | >8h: high; 3h< Cl < 8h: moderate; <3h: low |
| Toxicity | hERG (hERG blockers) | 0.537 | >0.5: A Blocker <br> <0.5: non-blocker |
| | H-HT (Human Hepatotoxicity) | 0.796 | >0.5: HHT positive <br> <0.5: HHT negative |

**Table S9.** Molecular docking of Bax, Bcl2, and p53 genes with curcumin.

| | RMSD lower bond | RMSD upper bond | Binding affinity |
|---|---|---|---|
| Curcumin-Bax | 0 | 0 | -6.3 |
| Curcumin-Bcl2 | 0 | 0 | -5.0 |
| Curcumin-p53 | 0 | 0 | -6.5 |

**Table S10.** The residues in the hydrogen bond between Bax, Bcl2, p53 and curcumin.

| Proteins | p53 | Bax | bcl2 |
|----------|-----|-----|------|
| Residues | ARG110 – PHE 113 – LEU111 – ASN268 – GLN144 – GLN104 – HIS115 – TYR126 – ASP228 – TRP146 – PHE113 – SER90 | LYS119 – LYS123 – LEU122 – LYS128 – ALA35 – THR127 – ASP84 – ILE80 – ARG34 – MET38 | TYR108 – SER205 – LEU201 – ARG107 – TYR202 – ARG129 – ARG146 – ASP103 – SER205 – ARG107 |

**Table S11.** Vesicle size, PDI, and EE % of various Cur-Nio and PEG-FA@Nio-Cur. Data are represented as mean ± SD, n = 3.

| Formulation | Vesicle Size (nm) | Polydispersity index (PDI) | EE (%) |
|---|---|---|---|
| Nio-Cur1 | 333.20±21.07 | 0.250±0.021 | 97.8778±0.2962 |
| Nio-Cur 2 | 237.97±4.56 | 0.390±0.005 | 96.7111±0.3469 |
| Nio-Cur 3 | 218.30±12.41 | 0.200±0.010 | 92.7111±0.4194 |
| Nio-Cur 4 | 357.03±41.03 | 0.290±0.013 | 95.7667±1.3017 |
| Nio-Cur 5 | 272.40±18.98 | 0.280±0.025 | 94.8222±0.0962 |
| Nio-Cur 6 | 221.50±13.31 | 0.290±0.022 | 91.9944±0.0096 |
| Vehicle (Nio) | 164.80±5.42 | 0.180±0.013 | - |
| PEG-FA@Nio-Cur 3 | 187.13±7.55 | 0.160±0.033 | 98.2517±0.7851 |

**Table S12.** The release kinetic models and the parameters seen for niosomal formulations.

| Release Model | Zero-Order | Korsmeyer-Peppas | | First-Order | Higuchi |
|---|---|---|---|---|---|
| | $R^2$ | $R^2$ | n | $R^2$ | $R^2$ |
| Free Curcumin (pH 7.4-37 °C) | 0.4048 | 0.7030 | 0.3084 | 0.8669 | 0.6418 |
| Cur-Nio (pH 7.4-37 °C) | 0.8185 | 0.9089 | 0.5598 | 0.8771 | 0.9396 |
| Cur-Nio (pH 5.4-37 °C) | 0.8134 | 0.9326 | 0.4446 | 0.9113 | 0.9343 |
| PEG-FA@Nio-Cur (pH 7.4-37 °C) | 0.7915 | 0.9103 | 0.5053 | 0.8715 | 0.9209 |
| PEG-FA@Nio-Cur (pH 5.4-37 °C) | 0.7346 | 0.9164 | 0.3620 | 0.8764 | 0.8820 |
| Free Curcumin (pH 7.4-25 °C) | 0.5709 | 0.7732 | 0.5844 | 0.9303 | 0.7425 |
| Cur-Nio (pH 7.4-25 °C) | 0.8304 | 0.9110 | 0.6245 | 0.8720 | 0.9473 |
| Cur-Nio (pH 5.4-25 °C) | 0.7924 | 0.9128 | 0.4518 | 0.8632 | 0.9227 |
| PEG-FA@Nio-Cur (pH 7.4-25 °C) | 0.7689 | 0.9024 | 0.5207 | 0.8229 | 0.9059 |
| PEG-FA@Nio-Cur (pH 5.4-25 °C) | 0.7520 | 0.9035 | 0.3986 | 0.8590 | 0.8927 |

**(A)**

| | Mean (mV) | Area (%) | Width (mV) |
|---|---|---|---|
| Zeta Potential (mV): -28.7 | Peak 1: -28.7 | 100.0 | 8.12 |
| Zeta Deviation (mV): 8.12 | Peak 2: 0.00 | 0.0 | 0.00 |
| Conductivity (mS/cm): 0.162 | Peak 3: 0.00 | 0.0 | 0.00 |
| Result quality Good | | | |

**(B)**

| | Mean (mV) | Area (%) | Width (mV) |
|---|---|---|---|
| Zeta Potential (mV): -22.6 | Peak 1: -22.6 | 100.0 | 4.01 |
| Zeta Deviation (mV): 4.01 | Peak 2: 0.00 | 0.0 | 0.00 |
| Conductivity (mS/cm): 1.30 | Peak 3: 0.00 | 0.0 | 0.00 |
| Result quality Good | | | |

**(C)**

| | Mean (mV) | Area (%) | Width (mV) |
|---|---|---|---|
| Zeta Potential (mV): -8.1 | Peak 1: -8.1 | 100.0 | 3.65 |
| Zeta Deviation (mV): 3.65 | Peak 2: 0.00 | 0.0 | 0.00 |
| Conductivity (mS/cm): 0.669 | Peak 3: 0.00 | 0.0 | 0.00 |
| Result quality Good | | | |



**Figure S1.** Zeta potential of Nio (A), Nio-Cur (B), and PEF-FA@Nio-Cur (C).
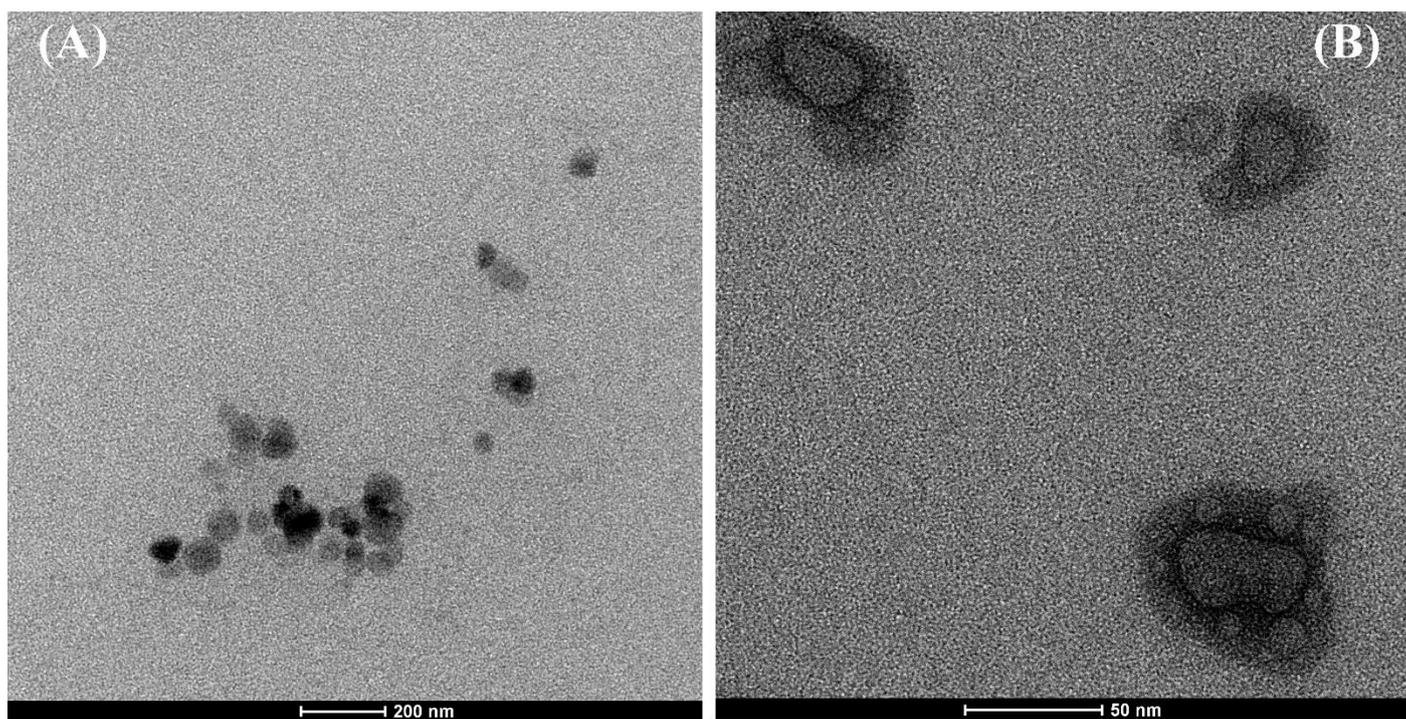
**Figure S2.** TEM image of the prepared optimized; Nio-Cur (A) and PEG-FA@Nio-Cur (B).
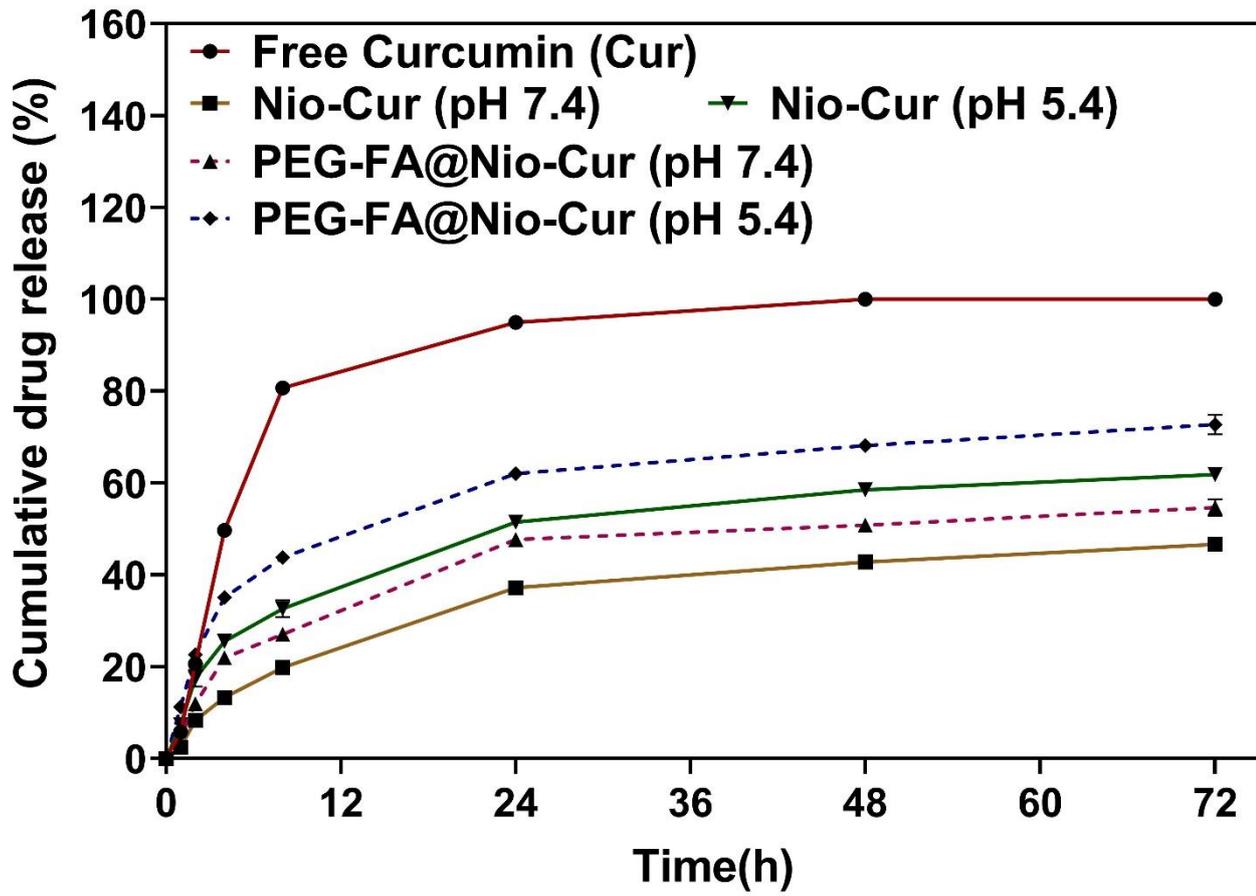
**Figure S3.** In vitro release of Cur from Nio-Cur3 and PEG-FA@Nio-Cur3 at pH 7.4 and pH 5.4 in 25 °C
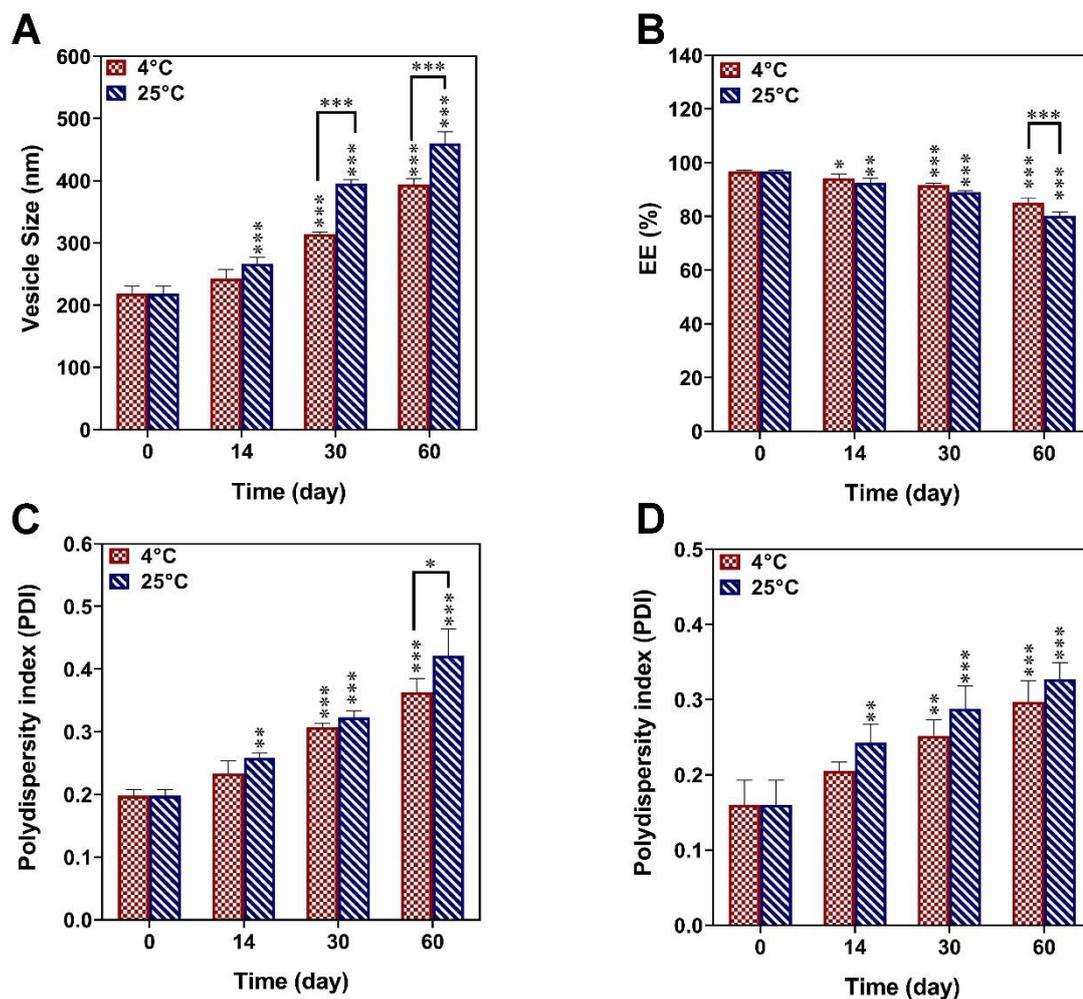
**Figure S4. A**) Size stability evaluation of Nio-Cur3, **B**) EE (%) stability evaluation of Nio-Cur3, **C**) PDI stability evaluation of Nio-Cur3, **D**) PDI stability evaluation of PEG-FA@Nio-Cur3, after two months of storage at $4 \pm 2°C$ and $25 \pm 2 °C$. Data are represented as mean $\pm$ SD and n=3; $P<.001***$, $P<0.01 **$, $P<0.05 *$.
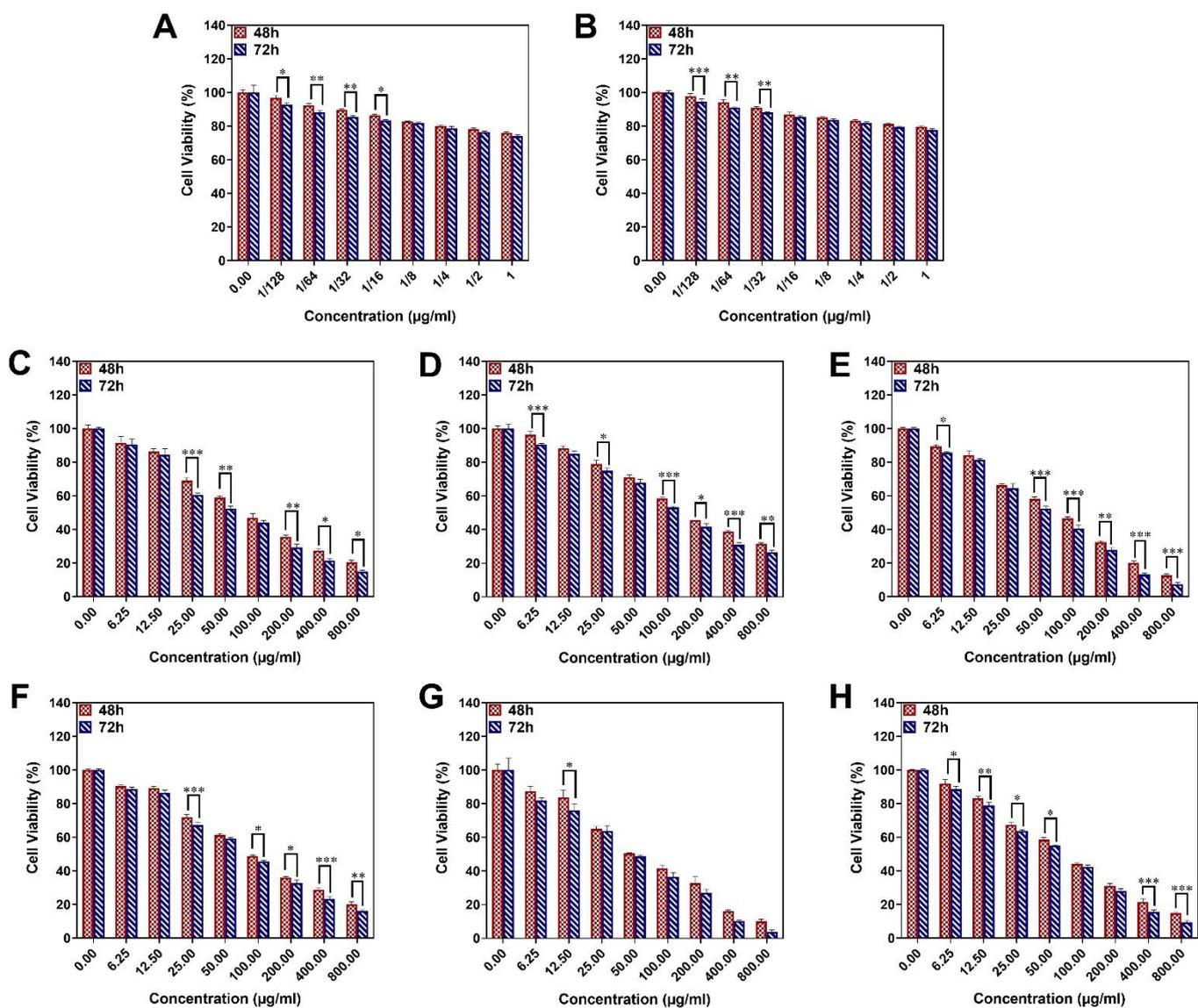
**Figure S5.** Cytotoxicity effect analysis of MCF7 **(**A**:** vehicle **(**Nio**)**, C**:** Cur, E**:** Nio-Cur, and G**:** and PEG-FA@Nio-Cur**)** and 4T1 **(**B**:** vehicle **(**Nio**)**, D**:** Cur, F**:** Nio-Cur, and H**:** and PEG-FA@Nio-Cur**)** cell lines against different fabricated niosomal and non-niosomal formulation treatment in 48hr 72hr **(**B and E**)** by various concentration**.**
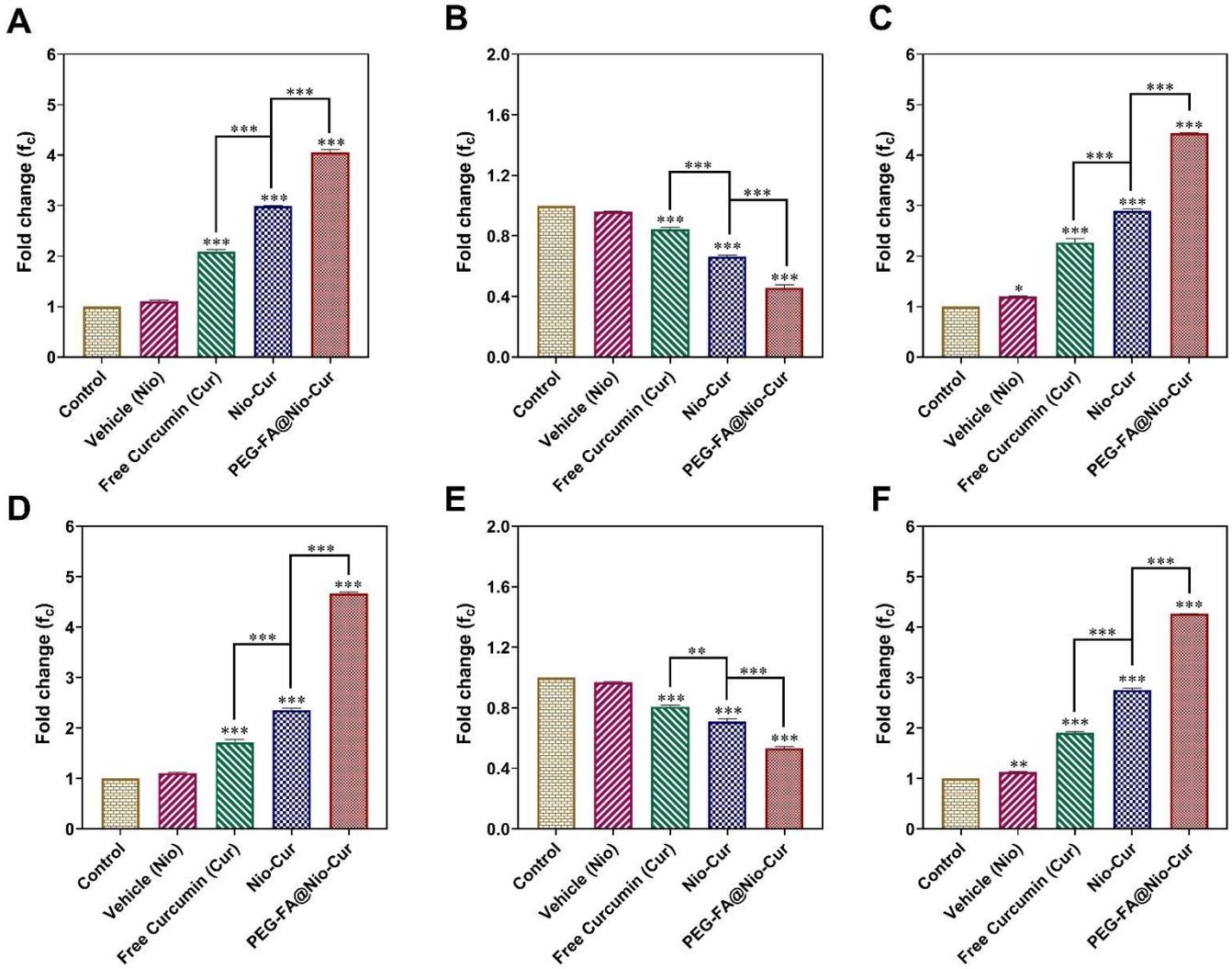
**Figure S6**. A. Expression levels of Bax, B. Bcl2, and C. p53 genes in MCF7 cells and D. Ex-pression levels of Bax, E. Bcl2, and F. p53 genes in 4T1 cells after those breast cancer cells were exposed to Vehicle (Nio), Free Curcumin, Nio-Cur, and PEG-FA@Nio-Cur (PBGD as housekeeping gene). Data represent means ± standard deviations (n=3).    For all charts, ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$.