**Supplementary**

**1. Experimental section**

**1.1. Physiochemical characterization of drug-loaded zein NS**

**1.1.1. Particle size, zeta potential analysis**

Photon correlation spectroscopy (PCS) was used for determination of particle size (PS) and polydispersity index (PDI) of NPs via a NanoZS/ZEN3600 Zetasizer. The PS was measured with the non-invasive backscattering technology at a detection angle of 173° after dilution with purified water to an appropriate concentration. All of the DLS measurements were carried out at 25°C for three repeated measurements. The zeta potential of the NPs was estimated following the same method previously used by our group [[1](#_ENREF_1)].

**1.1.2. Drug loading and encapsulation efficiency**

To determine % EE, the centrifuged NS sediment (20 mg) was treated with 10 mL ethanol (95% v/v) under sonication, for 5 min, to dissolve the encapsulated drug(s). The solution was then filtered through a 0.22 µm membrane filter, diluted with ethanol (95% v/v) and analyzed using HPLC developed and validated method [[2](#_ENREF_2)].

An Agilent 1260 Infinity HPLC system equipped with a quaternary pump, an autosampler, Diode Array Detector DAD, and an Agilent Chemstation data processing system were used for the analysis. A gradient elution composed of the mobile phase; methanol: water (60:40 for 4 minutes then ramped linearly to 90:10, over 12 minutes) pumped at 1.5 mL/min for 4 min then turned to 1 mL/min till the end of run. EXM, LUT and Flutamide (Internal standard) were measured at 246 nm, 350 nm and 300 nm, respectively. The injection volume was 50 𝜇L. The chromatographic separation of the mixture was accomplished using an Inertsil ODS-3 C18 reversed-phase column (250 X 4.6 mm, 5µm, GL Sciences, Torrance, USA) and maintained at 25±2 °C.

The encapsulation efficiency for each formula was calculated using the following equations:

**1.1.3. *In vitro* drug release**

The dialysis membrane method was used to investigate the *in-vitro* release of drugs from the single or dual drug-loaded zein NS as compared to *in-vitro* release of free drug solution. Different drug-loaded zein NS formulae (equivalent to 2 mg EXM) were placed in a cellulose ester dialysis bags (12–14 kDa MWCO VISKING dialysis tubing, SERVA, Germany). The bags were then suspended in a beaker and dialyzed against 200 mL phosphate buffer (pH 7.4) containing 0.5% w/v Sodium lauryl sulfate in accordance with the FDA-approved dissolution medium for EXM. The release process remained in sink condition at 37±0.5°C for 24 hr in a shaking water bath at 100 rpm. At designated time intervals, samples of 2 mL of the dialyzing medium were withdrawn and replaced with the same volume of release medium. All samples were filtered through a 0.22 µm membrane filter, and the released EXM and LUT were quantitively analyzed by HPLC.

For analyzing the kinetics modelling of EXM release from the developed optimized NS, the obtained release profiles were fitted into zero-order, first order and Higuchi equations. The model with the highest calculated correlation coefficients was considered as the best fitting model. To understand the release mechanism, the release data were subjected to the kinetic analysis using Korsmeyer–Peppas model correlating drug release to time by the simple exponential equation for the fraction of drug release [[3](#_ENREF_3)].

*Mt /M∞= Ktn (Equation S2)*

Where Mt /M∞ is the proportion of drug released at time t, k is the kinetic constant and the release exponent n has been proposed as indicative of the drug release mechanism. All measurements were carried out in triplicates and values expressed as mean ± S.D.

**1.2. Morphological analysis (TEM)**

One drop of dilute sample was placed on a copper grid and subsequently stained with uranyl acetate solution (1%w/v) for 30 seconds. The excess solution was drawn off with a filter paper and then sample was air-dried. The surface morphology of the NS was inspected and photographed using transmission electron microscopy (Instrument JFC-1100E JEOL, Japan).

**1.3. Physical Stability**

The physical stability, of the three optimized formulae (M2, L3 and P2), was monitored according to terms of time and temperature of storage. Therefore, aliquots of non-diluted dual drug-loaded zein NS were stored in sealed tubes in a refrigerator at 4±1 °C. PS, PDI and ZP of the three formulae were monitored at different time points for a period of 3 months.

**1.4. Solid state characterization**

**1.4.1. FTIR Spectroscopy**

The FTIR spectra of pure drugs and drug-loaded nanocarriers were obtained via FTIR spectrometer (spectrum RXI, Perkin Elmer, USA). Samples were finely ground with infra-red grade dry potassium bromide then pressed into pellets. The spectra were recorded in the transmission range of 4000 to 450 cm-1 at room temperature[[4](#_ENREF_4)].

**1.4.2. DSC Thermograms**

DSC thermograms were recorded for free drugs and drug-loaded nanocarriers using a DSC 6 differential scanning calorimeter (Perkin Elmer, USA). Each sample (5 mg) was weighed precisely, placed onto flat-bottomed aluminum pan and scanned between 50-400°C with a constant heating rate of 10°C/min in presence of nitrogen atmosphere (flow rate 20 ml/min)[[5](#_ENREF_5)].

**1.5. *In-vitro* hemolysis**

Hemolytic toxicity of EXM/LUT-loaded zein NS (M2), Lf-coated EXM/LUT-loaded NS (L3) and PEGylated EXM/LUT-loaded NS (P1 and P2) were investigated [[6](#_ENREF_6)]. Rat blood samples were collected from retro-orbital plexus into test tubes containing EDTA, centrifuged, washed twice with normal saline (0.9% w/v) and then diluted with normal saline to obtain 10 mL of a final concentration of 2 % v/v RBCs suspension. 2 mL of each formula were incubated with an equal volume of RBCs suspension at 37±0.5 ºC for 2 hr, in the shaking water bath at 50 rpm. Then, samples were centrifuged at 3000 rpm for 5 min and hemoglobin content was quantitatively determined, in the supernatant, by spectrophotometric analysis at λmax 540 nm. A negative control was prepared by mixing 2 mL of the RBC suspension with 2 mL of saline, while a positive control (100% hemolysis) was induced by treating the RBCs with 1% w/v Triton X100. The hemolytic rates of the samples were calculated as the following equation:

Where, *At* represents absorbance value of test sample, *Anc* and *Apc* stand for absorption value of negative and positive controls, respectively.

**1.6. *In-vitro* serum stability**

To assess the physical stability of zein NS in an appropriate biological medium, the stability of the three optimized formulae; M2, L3 and P2 in 10% w/v fetal bovine serum (FBS) solution (pH 7.4) was determined. Each formula was incubated in a shaking water bath at 37±0.5°C under mild stirring with an equal volume of 10% w/v FBS for 6 hr. At each time interval (0, 1, 2, 4 and 6 hr), 50 µL of the mixture was withdrawn then diluted with distilled water (1:50 v/v) to measure the PS and PDI.

**1.7. Scaling-up**

A 5-fold scaled up of combined EXM/LUT-loaded NS formula was developed following the formerly employed procedure of preparation of M2 with exception in quantities of the materials used. To the produced NS suspension, an aqueous mannitol solution (2.5% w/v) was added, under magnetic stirring for 10 min. This preparation was fed into a B-290 mini-spray dryer (Büchi, Flawil, Switzerland) with inlet temperature of 105 °C, outlet temperature of 55 °C, aspiration air of 100%, feed flow of 2.5 mL/min, spraying pressure of 5.0–5.8 mbar and air flow rate of 600 L/hr [[7](#_ENREF_7)]. The spray-drying yield was calculated by dividing the weights of the spray-dried powders collected by the total initial mass of solids in the preparation submitted to drying. PS, PDI and ZP of the reconstituted spray-dried NS were evaluated.

Moreover, the percentage drug loading (% LE) and % EE were calculated. To calculate % LE, an aliquot of accurately weighed 200 mg of the spray-dried NS was treated with 50 mL 95% ethanol under ultrasonication. This solution was then filtered through a 0.22 µm membrane filter and injected into the HPLC. % LE was calculated using the following equation:

**1.8. *In vivo* studies**

**1.8.1. Animals**

Female BALB/C mice (7-8 weeks of age, 25 ± 5 g) were housed in a pathogen-free environment at a 4–5 mice/cage under standard conditions of light illumination, relative humidity, and temperature, and they had free access to standard laboratory food and water throughout the study. All procedures were performed according to a protocol approved by the Animal Care and Use Committee of the Faculty of Pharmacy, Alexandria University, Alexandria, Egypt, and in accordance with regulations of the National Research Council’s guide for the care and use of laboratory animals.

**1.8.2. Development of tumor model**

Female BALB/C mice (7-8 weeks of age) were housed in a pathogen-free environment at 7 mice/cage. They were provided with autoclaved and non-fluorescent mouse chow and water. Ehrlich ascites tumor (EAT) cells, supplied from National Institute of Cancer, Egypt, were collected from the ascitic fluid of BALB/C mice harboring 8–10 days old ascitic tumor. Approximately, 107 of EAT cells suspended in PBS were injected into the left side of the mammary fat pad of BALB/C female mice. Tumor growth was assessed daily until its volume reached 100 mm3. Tumor volume was calculated by measuring both perpendicular diameters of the tumor using a micrometer based on the following equation [[8](#_ENREF_8)]:

where W is tumor width, L is tumor length.

**1.8.3. Tumor growth biomarkers**

Excised tumors were homogenized using cold PBS to make a final 40% tissue homogenate. Rat Aromatase (ARO) BioAssay™ ELISA Kit (Rat) Cat No. 023576 was purchased from US Biological Life Sciences, USA. Cyclin-D1 (CD1) ELISA Kit (EIAab® Catalog No: E0585r) was purchased from Cedarlane Laboratories USA Inc., USA. Angiogenesis was determined by measurement of the level of the angiogenic factor; vascular endothelial growth factor (VEGF-1) using "VEGF-1 ELISA Kit" (RayBio Tech Inc., USA). Apoptosis induction was detected by measurement of tissue caspase-3 level using "Caspase-3 (Casp-3) ELISA Kit” (WKEA Med Supplies Corp., USA). The markers were quantified according to the manufacturer’s protocol.

**1.8.4. Histopathological analysis**

10% neutral formalin was used for fixation of the tumor samples for 24 h at room temperature. A 5 μm thick section were brought down to distilled water, stained with H & E for 5 min and 2 min, respectively, dehydrated in alcohol and mounted in Canada balsam, then examined microscopically.

The necrosis in the excised mammary tumor was assessed semi-quantitatively by examining 10 random sections (×40) from each excised tumor and scoring on a scale from 1 to 4 for the following criterion: Score 4; section in poorly differentiated tumor showing >50% necrosis, Score 3; section in poorly differentiated tumor showing about 35% necrosis, Score 2; section in poorly differentiated tumor showing about 25% necrosis, Score 1; section in poorly differentiated tumor showing about 10% necrosis. The mean value of all 10 scores was computed for each excised tumor and expressed as Mean of necrosis scale ± S.E

The mean value of all 10 scores was computed for each excised tumor and expressed as Mean of necrosis scale ± S.E.

**1.9. Statistical analysis**

All measurements were carried out in triplicate and values are presented as the mean ± S.D. For all *in-vitro* characterization, analysis of Variance test (ANOVA) and Tukey’s Multiple Comparison test were used to compare mean values between groups. Statistical analysis of *in-vivo* pharmacokinetic and anti-tumor efficacy were performed using the software package Prism® 5.0 (GraphPad Software, Inc., CA and USA). The difference was considered significant when *p*-values < 0.05.

**2. Results**

**Figures**

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**Figure S1**. *In-vitro* release profiles showing; the influence of (A) stabilizer type, (B) EXM concentrations, and (C) zein concentrations with a fixed ratio of zein: EXM (3:1) on the *in-vitro* release of EXM from zein NS. (D) *in-vitro* release of LUT from LUT-loaded zein NS in PBS pH 7.4; 0.5% (w/v) SLS at 100 rpm and 37±0.5 ºC.

**Table S1**: Composition and physicochemical properties of EXM-loaded zein NS.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Code | Zein  % (w/v) | Stabilizer concentration  (% w/v) | EXM | | Particle  Size  (nm) | PDI | Zeta Potential  (mV) |
| Theoretical  (mg) | % EE |
| F0 | 1.00 | L-S75 (0.45%)  PLX (0.9%) | - | - | 210.5±3.6 | 0.21±0.04 | -36.6 |
| F1 | 1.00 | HPMC (1%) | 10 | 73.5 | 492.3±10.7 | 0.54±0.07 | -15.0 |
| F2 | 1.00 | SC (1%) | 10 | 88.3 | 500.9±2.5 | 0.25±0.06 | -35.7 |
| F3 | 1.00 | L-S75 (0.45%)  PLX (0.9%) | 10 | 68.7 | 237.2±3.3 | 0.11±0.02 | -37.9 |
| F4 | 1.00 | L-S75 (0.45%)  PLX (0.9%) | 6 | 61.8 | 229.5±3.8 | 0.14±0.03 | -37.9 |
| F5 | 1.00 | L-S75 (0.45%)  PLX (0.9%) | 20 | 83.5 | 338.2±2.4 | 0.48±0.02 | -37.9 |
| F6 | 1.67 | L-S75 (0.45%)  PLX (0.9%) | 16 | 64.3 | 300.3±6.4 | 0.33±0.01 | -38.3 |
| F7 | 2.33 | L-S75 (0.45%)  PLX (0.9%) | 23 | 80.2 | 373.4±5.3 | 0.20±0.03 | -46.2 |

**Table S2**: Physicochemical properties of combined EXM/LUT-loaded zein NS.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Code | % EE | | Particle Size  (nm) | PDI | Zeta Potential  (mV) |
| EXM | LUT |
| T1 | - | 63.0 | 315.4±3.2 | 0.24±0.04 | -31.6 |
| E1 | 67.3 | - | 344.5±2.7 | 0.27±0.03 | -37.4 |

**Table S3**: Release kinetic parameters for EXM from optimized EXM/LUT-loaded zein NS formulae in PBS pH 7.4; 0.5% (w/v) SLS at 100 rpm and 37±0.5 ºC.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Nanospheres code** | **Zero-order model** | | **First-order model** | | **Higuchi model** | | **Korsmeyer-Peppas model** | | |
| K0\*  (% h-1) | R2 | K1\*  (% h-1/2) | R2 | KH\*  (% h-1) | R2 | KKP\*  (% h-n) | n\* | R2 |
| **M2** | 4.730 | 0.2283 | 0.154 | 0.9079 | 20.722 | 0.9408 | 27.934 | 0.374 | 0.9957 |
| **L3** | 3.356 | 0.3120 | 0.083 | 0.3792 | 15.242 | 0.7756 | 25.142 | 0.285 | 0.9862 |
| **P2** | 3.583 | 0.6976 | 0.075 | 0.9456 | 14.847 | 0.9344 | 17.136 | 0.430 | 0.9496 |

K0, K1, KH and KKP are the release rate constant for zero-order, first-order, Higuchi and Korsmeyer-Peppas models, respectively. n is the release exponent for Korsmeyer-Peppas model.

**Table S4**: Release kinetic parameters for LUT from optimized EXM/LUT-loaded zein NS formulae in PBS pH 7.4; 0.5% (w/v) SLS at 100 rpm and 37±0.5 ºC.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Nanospheres code** | **Zero-order model** | | **First-order model** | | **Higuchi model** | | **Korsmeyer-Peppas model** | | |
| K0\*  (% h-1) | R2 | K1\*  (% h-1/2) | R2 | KH\*  (% h-1) | R2 | KKP\*  (% h-n) | n\* | R2 |
| **M2** | 3.466 | 0.7063 | 0.109 | 0.0343 | 16.344 | 0.4797 | 31.971 | 0.208 | 0.9808 |
| **L3** | 2.839 | 0.8545 | 0.053 | 0.3945 | 12.653 | 0.8623 | 19.281 | 0.320 | 0.9924 |
| **P2** | 3.255 | 0.6698 | 0.098 | 0.0313 | 15.466 | 0.4956 | 29.579 | 0.220 | 0.9306 |

K0, K1, KH and KKP are the release rate constant for zero-order, first-order, Higuchi and Korsmeyer-Peppas models, respectively. n is the release exponent for Korsmeyer-Peppas model.

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