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Strategies of stabilization of zein nanoparticles containing doxorubicin hydrochloride

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Abstract

Hybrid nanoparticles made up of zein and various stabilizers were developed and characterized. In detail, a zein concentration of 2 mg/ml was blended with various amounts of different phospholipids or PEG-derivatives in order to obtain formulations with suitable physico-chemical properties for drug delivery purposes. Doxorubicin hydrochloride (DOX) was used as a model of a hydrophilic compound and its entrapment efficiency, release profile and cytotoxic activity were investigated. Photon correlation spectroscopy showed that the best formulations were obtained using DMPG, DOTAP and DSPE-mPEG2000 as stabilizers of zein nanoparticles, which were characterized by an average diameter of ~100 nm, a narrow size distribution and a significant time- and temperature-dependent stability. The interaction between protein and stabilizers was confirmed through FT-IR analysis, while TEM analysis showed the presence of a shell-like structure around the zein core. The release profiles of the drug from the zein/DSPE-mPEG2000

nanosystems, evaluated at two pHs (5.5 and 7.4), showed a prolonged and constant leakage of the drug. The encapsulation of DOX within zein/DSPE-mPEG2000 nanosystems did not compromise its biological efficacy, demonstrating the potential application of these hybrid nanoparticles as drug carriers.

Keywords: Doxorubicin hydrochloride, nanoparticles, PEG, phospholipids, zein.

1. Introduction

Over the past few years, hybrid nanoparticles have created a growing interest in the field of nanotechnology. Biocompatible hybrid nanosystems are characterized by many advantageous properties such as: i) high physico-chemical stability and structural integrity, ii) low toxicity, iii) opportunity of entrapping hydrophilic and lipophilic molecules iv) customizable surfaces and controlled drug release of entrapped compound(s) [1]. In fact, blending different materials allows the exploitation of the peculiar characteristics of different drug delivery systems [2,3]. Recently, hybrid zein-based nanoparticles have been proposed as stable nanocarriers of active compounds [4]. Zein is a prolamin-rich protein extracted from corn, characterized by a lipophilic nature, insoluble in water and soluble in ethanol/water mixture and other organic solvents [5,6]. The easy production, high availability, great versatility and low costs make this biopolymer a useful material for biomedical, alimentary and pharmaceutical applications [7]. Zein was awarded GRAS status by the U.S. FDA and it has been widely used for the preparation of biodegradable and biocompatible drug delivery systems able to encapsulate hydrophilic and lipophilic compounds [8,9]. Various approaches have been proposed to stabilize zein-based colloidal systems, such as association with surfactants characterized by different physico-chemical properties [10,11], as well as to (phospho)lipids, sugars and other polymers [12-14]. For example, recently, zein-phosphatidylcholine nanoparticles have been developed, which

demonstrate a better stability and retention of various hydrophobic active compounds with respect to the normal zein systems [15]. Moreover, our research team has described the possibility of obtaining biodegradable hybrid drug delivery systems made up of zein and mixtures of phospholipids (phospholipon®) containing all-*trans* retinoic acid (ATRA), demonstrating that the encapsulation of the drug does not compromise its *in vitro* pharmacological activity [16]. In another work, polyethylene glycol (PEG) decorated -zein nanoparticles were used to entrap gallic acid and to protect the compound under simulated gastrointestinal conditions [17]. Among the various approaches widely used to increase the plasma half-time of a colloidal system after parenteral administration, PEGylation remains the best option because the hydrophilic polymer prevents opsonization, even though over the last decade the appearance of PEG-related accelerated blood clearance phenomena have been described [18-21].

Considering the aforementioned findings, in this investigation zein was blended with different kinds of phospholipids or PEG-derivatives in order to evaluate the influence of the lipophilic and hydrophilic residues on the stability of the resulting hybrid nanoparticles. It is worth noting that the systems were prepared without using any electromechanical homogenizer, making the process easier and more ecofriendly. The best resulting colloidal systems were used to evaluate the opportunity of entrapping doxorubicin chloride (Dox), a model of a hydrophilic drug. Dox is an anthracycline able to inhibit topoisomerase II, suppressing cell mitosis, and it is widely used in anticancer therapy for the treatment of many types of tumors [22,23]. Unfortunately, the active compound is characterized by a remarkable cardio- and myelotoxicity and many approaches have been proposed to better exploit its pharmacological efficacy while increasing the safety of healthy tissues [24-28]. Nanoencapsulation within biocompatible

colloidal systems was observed to be a useful strategy that yielded a safer formulation, as was true in the cases of Doxil/Caelyx and Myocet [29,30]. Therefore, blending zein with phospholipids could represent an ecofriendly strategy to obtain biocompatible and biodegradable drug delivery systems to be used for the parenteral administration of various bioactive compounds, while the use PEG-derivatives could offer the possibility to increase their blood residence time and to modulate the architecture of their surface. The development of a novel nanomedicine based on a hybrid system containing Dox could represent an additional therapeutic option for the treatment of tumors.

2. Materials and methods

2.1. Materials

Zein, poly(ethylene glycol)distearate PEG-di-*p*-tosylate, 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide salt (used for MTT testing), phosphate buffered saline (PBS) tablets, dimethyl sulfoxide, and amphotericin B solution (250 μg/ml) were purchased from Sigma-Aldrich (Milan, Italy). Cholesterol-(polyethylene glycol-600), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N [methoxy(polyethylene glycol)-2000] (DSPE-*m*PEG2000), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), dimyristoylphosphatidylglycerol (DMPG), and 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPPC) 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar (Alabaster, Alabama, USA). Doxorubicin hydrochloride (Dox) was acquired from DBA Italia S.r.l. (Segrate, Milan, Italy). Minimum essential medium (DMEM) with glutamine, trypsin, ethylene diamine tetraacetic acid (EDTA) (1×) solution, fetal bovine serum (FBS) and penicillin-streptomycin solution were obtained from Gibco (Life Technologies, Monza, Italy). Human breast cancer cells

(MCF-7) were provided by the IRCCS Azienda Ospedaliera Universitaria San Martino – IST Istituto Nazionale per la Ricerca sul Cancro. All other materials and solvents used in this study were of analytical grade (Carlo Erba, Milan, Italy).

2.2. Preparation of hybrid nanoparticles

Hybrid nanoparticles were prepared by the nanoprecipitation technique.~~,~~ Briefly, various amounts of phospholipids or PEG-derivatives (1-3 mg) were solubilized in 3 ml of a mixture of ethanol/water solution (2:1 v/v) at room temperature; namely, the various stabilizers were firstly solubilized in 2 ml of ethanol; then 1 ml of MilliQ water was added to the organic solvent and the obtained system was mildly stirred for 5 min. Finally, 10 mg of zein were solubilized in the ethanol/water solution. This phase was added to 5 ml of MilliQ water. The resulting suspension was stirred at 600 rpm for 12 h on a magnetic plate in order to promote the evaporation of the organic solvent. The final zein concentration of the sample was 2 mg/ml [14].

The hybrid nanosystems containing Dox were obtained adding the active compound (final concentration of drug 0.2-0.8 mg/ml) to 1 ml of MilliQ water of the organic phase during the sample preparation. The formulations were purified by means of ultracentrifugation at 90k g for 60 min at 4 °C using a Beckman OptimaTL Ultracentrifuge equipped with an F12 fixed-angle rotor or by means of Amicon Ultracentrifugal filters (cut-off 10 kDa, 4000 rpm for 20 min).

2.3. Physico-chemical characterization

The colloidal systems were characterized by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Panalytical Ltd., Spectris plc, England as previously reported [10]. The mean diameter, size distribution and Zeta-potential of the nanoparticles were evaluated

applying a third-order cumulant fitting correlation function A 4.5 mW laser diode operating at 670 nm was used as a light source for size analysis and the back scattered photons were detected at 173°, while the real and imaginary refractive indexes were set at 1.59 and 0.0, respectively. The medium refractive index (1.330), medium viscosity (1.0 mPa s), and dielectric constant (80.4) were set before the experiments. Quartz cuvettes were used for the analysis. Namely, each sample was dispersed in different polar media (see tables 1 and 2) and various pH (1:50 ratio) in order to evaluate the influence of the dispersant on the physico-chemical properties of nanosystems.

The results were reported as the mean of three different experiments (15 determinations for each batch) and indicated as a function of the intensity ± standard deviation.

The kinetic stability was investigated by using a Turbiscan Lab Expert® analyzer (Formulaction, Toulouse, France) and the data were expressed as Turbiscan Stability Index (TSI) as a function of time and temperature [31]. The morphology of the hybrid nanosystems was evaluated through a Gaia 3 (Tescan s.r.o, Brno, Czech Republic) FIB-SEM used for TEM imaging by a bright-field detector as previously reported [16].

2.4. Fourier transform infrared spectroscopy (FT-IR)

The vibrational spectra of zein, Dox, DOTAP and DSPE-PEG2000 and of the freeze-dried hybrid nanoparticles as empty systems or containing Dox were detected by a Nicolet™ iS5 spectrometer coupled with an iD7 Attenuated Total Reflectance accessory (Thermo Fisher Scientific Inc., Waltham, MA, USA). The nanoparticle samples were freeze-dried without using any cryoprotectants, as previously described [32]. The FT-IR spectra were acquired through 64 acquisitions with a resolution of 4 cm^{-1} in a wavenumber range of 500–4000 cm^{-1}. Spectra

analyses were elaborated using OMNIC software, version 9.12.1019. The results reported are a representative analysis of three independent experiments.

2.5. Evaluation of the drug entrapment efficiency and release profiles

The amount of Dox encapsulated within the hybrid nanosystems was evaluated by a spectrophotometric analysis (Perkin Elmer Lambda 35, Waltham, Massachusetts, USA). Firstly, the formulations were purified by means of ultracentrifugation as previously described [33] and the supernatant was analyzed using a spectrophotometer at λ 483 nm.

The UV calibration curve of Dox was:

$y = 0.015599x + 0.068662$

where y is the absorbance and x is Dox concentration, with a correlation coefficient (r^2) value of 0.993. The limit of quantitation was 6.25 μg/mL.

The entrapment efficiency (EE%) was calculated in accordance with the following formula and reported as the mean of three independent experiments:

$$EE\% = \frac{D_E}{D_T} \times 100$$

where D_E is the concentration of Dox retained by the nanosystems and D_T is the theorical amount of the drug initially added.

The release profile of Dox from the hybrid nanoparticles was evaluated at different pHs (5.5 and 7.4) by using the dialysis technique [14]. An isotonic solution of phosphate buffered saline (PBS) 0.01 M was used to obtain the two media, while the pH was adjusted to 5.5 by adding 1 mol/l of hydrochloric acid (pHmeter SevenCompact S210 Mettler Toledo). Briefly, 1 ml of each type of hybrid nanoparticles was placed into dialysis bags, sealed with clips at both ends and transferred to a beaker containing 200 ml of a constantly-stirred PBS solution under

sink conditions. At various incubation times, 1 ml of sample was withdrawn and replaced with the same volume of fresh solution. The collected samples were successively analyzed using the spectrophotometric method previously described.

The percentage of released Dox was calculated as the ratio between the amount of the drug detected in the release solutions (Dox_{REL}) and that contained in the hybrid systems (Dox_{LOAD}) as reported below:

$$\text{Release\%} = \frac{\text{Dox}_{REL}}{\text{Dox}_{TLOAD}} \times 100$$

The results were reported as the mean of three different experiments ± standard deviation.

2.6. *In vitro* toxicity

Human breast cancer cells (MCF-7 and MDA-MB-231) were cultured as previously described [34]. The cytotoxicity of the empty hybrid nanosystems, the Dox-loaded nanoparticles and the free drug was investigated by MTT- testing [10]. The cells were plated in 96-well culture dishes, treated with different concentrations of empty formulations (10, 25, 50 and 100 µg/ml of biomaterial) and incubated for 24, 48 and 72 h. Untreated cells were used as control. Successively, the same experiment was performed testing various concentrations of Dox (0.1, 0.5, 1 and 5 µM) in the free form or entrapped within hybrid nanosystems. Cell viability was evaluated through a microplate spectrophotometer (Multiskan MS 6.0, Labsystems) at a wavelength of 540 nm with a reference at 690 nm.

The cell viability was calculated according to the following equation:

$$\text{Cell viability (\%)} = \frac{\text{AbsT}}{\text{AbsC}} \times 100$$

in which AbsT and AbsC represent the absorbance of the treated and untreated cells, respectively. Each experiment was replicated three times, and cell viability values were the mean of each experiment ± standard deviation.

2.7. Statistical analysis

The statistical analysis of the various experiments was performed by ANOVA and the results were confirmed by a Bonferroni t-test, with a p value of < 0.05 considered statistically significant

3. Results and Discussion

3.1. Physico-chemical characterization of hybrid systems

The concentrations of the different derivatives used and their effects on the mean sizes, surface charges and polydispersity of the hybrid nanoparticles are summarized in Table 1. In detail, the concentration of zein was 2 mg/ml as a consequence of the results previously described concerning the physico-chemical features of the colloidal systems made up of the protein [10]; in fact, higher amounts of the biopolymer promoted the appearance of macroaggregates and sedimentation. Zein-based nanoparticles were stabilized by means of the addition of various derivatives in a range between 0.2-0.6 mg/ml.

Table 1.

Table 1 describes the results obtained by DLS analysis; in particular, DMPG, DSPE-mPEG2000 and DOTAP induced a concentration-dependent stabilization of the colloidal systems. Namely,

0.2 mg/ml of DMPG promoted a decrease in the mean sizes and PdI of the nanosystems with respect to the phospholipid-free formulation, whereas adverse physical phenomena occurred when its concentration was increased. In the case of DOTAP, a concentration of 0.4 mg/ml allowed us to obtain hybrid nanoparticles characterized by a mean diameter of less than 100 nm and a low PdI, while higher amounts of the derivative showed an increase of the polydispersity of the samples. The use of DSPE-mPEG2000 was useful for colloidal stabilization when a concentration of the derivative in the range between 0.4-0.6 mg/ml was used; in fact, lesser amounts of DSPE-mPEG2000 exerted negative effects on the size distribution of the colloidal systems.

The surface charge values of the colloidal systems was significantly influenced by the nature of the phospholipid used. In detail, the systems prepared using DOTAP were characterized by a positive surface charge (~50 mV), while the use of DSPE-mPEG2000 and DMPG promoted a negative zeta potential of the hybrid nanosystems (~ -29 mV and ~-35 mV, respectively) (Figure 1).

Fig. 1.

The surface charge plays a key role in the physical stability of a colloidal system and a Zeta potential close to 0 provokes the occurrence of adverse phenomena such as the appearance of macroaggregates and precipitates [35,36]. This was clear when PEG-tosylate was used as stabilizer of the zein nanosystems; in fact, only the formulation characterized by a positive zeta potential of 20 mV did not show the presence of aggregates or sediments (Table 1).

The stability profiles of the hybrid systems characterized by the best physico-chemical

properties reported in Figure 1 were investigated using the Turbiscan Lab apparatus as a function of time and temperature. In detail, the transmittance and backscattering of the nanoparticles obtained using a concentration of 0.2 mg/ml of DMPG (Z/DMPG 10:1 w/w), 0.4 mg/ml of DOTAP (Z/DOTAP 10:2 w/w) and 0.6 mg/ml of DSPE-mPEG2000 (Z/DSPE-mPEG 10:3 w/w) were evaluated and compared to the stabilizer-free formulation. The selected hybrid nanoparticles were observed to be stable at both 25 °C and 37 °C and no adverse physical phenomena, i.e. sediments, creaming or flocculates appeared (Figure 2).

Fig. 2.

FT-IR analysis provided the evidence of interaction between the stabilizers and the protein. The spectra of the stabilizers, protein, their physical mixtures and hybrid nanoparticles are shown Figure 3. In detail, it is possible to observe that the lipid portions of the surfactants are integrated into the colloidal systems. In fact, the two peaks at 1641 and 1515 cm^{-1} that are the peculiar stretching bands of the amide I and amide II are still visible in the spectra of the hybrid nanoparticles, while the peculiar peaks of the lipid portion of DSPE-mPEG2000 were not observed in the Z/DSPE-mPEG nanoparticles. Specifically, the peaks at 2915 and 2849 cm^{-1} corresponding to the alkyl CH stretching band and at 1734 cm^{-1} corresponding to carbonyl C=O are not observed in the nanoparticles, while the peaks at 1240 and 1105 cm^{-1} corresponding to the C-O-C symmetric/asymmetric stretching, respectively, characteristic of the PEGylated portion of the derivative are visible in the nanosystems. These results suggest a hydrophobic effect and hydrogen-bonding interaction between the derivatives and the protein [12]. This could explain the slight shift of the characteristics peaks of the protein in the case of the hybrid

nanoparticles. Similar results were obtained in the case of the nanosystems made up of zein and DOTAP, suggesting quite similar interactions. In fact, peculiar peaks of the cationic lipid are not observed in the spectrum of the hybrid nanoparticles. In detail, the peaks at 2920, 2851, 1734, 1481, 1465, 1253, 1171 and 747 cm^{-1}, corresponding to the alkyl CH stretching band, carbonyl C=O stretching, to the out- and in- phase-bending of $(N)CH_3$, to the symmetric and asymmetric stretching of the C-O-C of the ester and to the CH=CH out-of-plane deformation, respectively, are not visible in the hybrid nanoparticles, confirming the results previously discussed. The analysis of the Z/DMPG systems evidenced similar results (data not shown).

Fig. 3.

TEM analysis showed the presence of a shell-like structure around the zein core, as already described by our research team when zein was stabilized using various phospholipons, confirming the preferential localization of the stabilizers on the protein surface (Figure S1).

3.2. Influence of doxorubicin hydrochloride on the physico-chemical parameters of hybrid nanosystems

Various amounts of Dox (0.2, 0.4, 0.8 mg/ml) were used during the sample preparation in order to assess the capacity of the best hybrid nanosystems previously described to retain the hydrophilic drug. In detail, the addition of the active compound to the Z/DMPG systems caused a physical destabilization of the colloidal structure, bringing about the formation of macroaggregates and a precipitate. Contrarily, the Z/DOTAP and Z/DSPE-mPEG2000 hybrid systems were able to entrap the drug and a variation of some of the physico-chemical parameters

occurred (Figure 4). The encapsulation of Dox barely influenced the mean sizes of the hybrid nanosystems and their polydispersity index was close to 0.2. Amounts of Dox superior to 0.8 mg/ml were not used because they promoted the appearance of macroaggregates (data not shown). On the other hand, the hydrophilic compound significantly affected the surface charges of the PEGylated colloidal systems. In fact, a proportional increase of the Zeta potential was obtained with respect to the empty formulation when high concentrations of Dox were used, while a value of ~40/50 mV was obtained when the samples were prepared using DOTAP (Figure 4).

Fig. 4.

The physical stability of the hybrid systems was also investigated by multiple light scattering as a function of temperature and incubation time. The TSI profiles demonstrated the absence of adverse phenomena such as creaming, flocculation or sedimentation in the analyzed samples, confirming the data of PCS already discussed (Figure 5). Moreover, a certain stabilization was exerted by an increase in temperature, a phenomenon in agreement with other zein-based systems and related to the rearrangement of the protein (Figure 5) [14,34].

Fig. 5.

3.3. Entrapment efficiency of doxorubicin hydrochloride

The best hybrid nanoformulations previously described were further analyzed in order to evaluate the encapsulation rate of Dox. In detail, the best entrapment efficiency (EE) was

obtained using the Z/DSPE-mPEG system; in detail, ~80% of Dox was encapsulated by the colloidal structure when 0.4 mg/ml of drug were initially used during the sample preparation while higher amounts of the active compound evidenced a significant concentration of unretained molecule (Figure 6).

Fig. 6.

But the EE drastically decreased when the initial drug concentration used was increased in the case of the Z/DOTAP 10:2 systems (from ~74% to ~34% using 0.2 and 0.8 mg/ml of the active compound, respectively), demonstrating a lower interaction rate between this formulation and the hydrophilic molecule with respect to the PEGylated nanocarriers (Figure 6). These results are noteworthy if compared to the encapsulation rate of the surfactant-free systems; in fact, a significantly lower amount of Dox was retained by the zein nanoparticles (40% when 0.2 mg/ml of drug were used) confirming once again the fundamental role exerted by the phospholipids in the stabilization of the colloidal formulation (Table S1).

In addition, FT-IR analysis demonstrated a noteworthy interaction of Dox with the zein/DSPE-mPEG2000 hybrid nanoparticles. In fact, as shown in Figure 7, the characteristic peaks of the zein/DSPE-mPEG2000 were not influenced by the encapsulation of Dox. On the contrary, the peculiar peaks of the chemotherapeutic drug are not observed after its encapsulation within the colloidal systems (Figure 7). Specifically, those at 1729, 760 and 687 cm^{-1} corresponding to the C=O stretching, the CH=CH out-of-plane deformation and to the C=C ring bend respectively, are not visible in the nanoparticles containing Dox, while other peculiar peaks at 3314 and 1578 cm^{-1} related to aminic groups overlap with those of the empty nanoparticles.

Fig. 7.

3.4. Effect of pH and dispersion medium

The influence of the pH and of the dispersion medium on the physico-chemical properties of the hybrid nanoparticles was also evaluated. After *in vivo* administration, in fact, the nanoparticles were able to reach various body compartments characterized by different pHs; for this reason it is important to assess and predict the behavior of the nanosystems under different physio-pathological conditions.

Table 2 shows the physico-chemical parameters of the various formulations as a function of the medium and pH. Only Z/DSPE-mPEG 10:3 w/w nanoparticles preserved their physico-chemical properties in the various conditions; in fact the use of 0.6 mg/ml of the PEGylated phospholipid avoided the aggregation of zein nanoparticles, demonstrating that both PDI and mean sizes were suitable for parenteral administration (Table 2). The same investigation was performed after the encapsulation of Dox and similar physico-chemical parameters of the formulations with respect to empty systems were obtained.

Table 2.

3.5. Release profiles

The *in vitro* release profiles of Z/DSPE-mPEG 10:3 containing various amounts of Dox were evaluated in two solutions. It is well known that the tumor microenvironment is characterized by an acid pH [7,37,38]. Therefore, the drug leakage from hybrid systems was

evaluated in PBS at pHs of 5.5 and pH 7.4 mimicking the tumor and plasmatic conditions, respectively. The results demonstrated that the release profiles of Dox were similar in all samples. In fact, after an initial burst release probably due to the desorption of the hydrophilic active compound from the surface of the nanosystems, the leakage of Dox was constant and prolonged. This biphasic release behavior is in agreement with that of other vegetal protein-based nanosystems containing Dox [39,40] or other hydrophilic compounds [11]. As shown in Figure 8, a slightly higher percentage of Dox is released by the systems in acidic conditions during the first hours.

This release trend could help to avoid the premature plasmatic drug leakage of Dox, preventing toxicity against healthy tissues and favoring the accumulation of the bioactive within the tumor compartment.

Fig. 8.

3.6 Evaluation of cytotoxicity

Considering these results, the hybrid nanosystems made up of Z/DSPE-mPEG 10:3 w/w prepared with 0.4 mg/ml of Dox were selected in order to evaluate their cytotoxic profiles on human breast cancer cells.

The empty colloidal systems evidenced a significant degree of toxicity after 72 h incubation at a biomaterial concentration of 100 µg/ml that was never reached during subsequent tests, data in agreement with that obtained when using zein/phospholipid hybrid nanoparticles [10].

The encapsulation of Dox within zein/DSPE-mPEG2000 nanosystems did not compromise its biological efficacy and a similar cytotoxic profile with respect to the free form of the drug was

obtained (Figure 9). In detail, a very good degree of antitumor efficacy was evident at concentrations of Dox higher than 0.5μM which was directly related to the incubation time on both the cell lines. Even better cytotoxicity was obtained on MDA-MB-231 cells probably as a consequence of their greater responsiveness to the drug [41]. These results are in agreement with those described in other experimental investigations focused on the encapsulation of antitumor compounds within zein-based formulations and to their cell uptake [34,35,42,43].

Fig. 9.

4. Conclusions

Stable hybrid zein-based nanoparticles able to retain Dox have been herein proposed as a novel potential nanomedicine. The use of a PEGylated derivative increased the stability of the zein nanosystems in different media and pHs and enhanced the amount of encapsulated drug. Nowadays, the PEGylation of colloidal drug delivery systems is still being disputed. PEG is widely employed in various pharmaceutics for different purposes [44-47]; PEG decreases the opsonization of nanoparticles and their uptake by the macrophages, increasing the accumulation of the entrapped drug(s) in several types of solid tumors through a passive phenomenon [36,48]. On the other hand, multiple administrations of PEGylated formulations induces the formation of anti-PEG antibodies, promoting the accelerated blood clearance phenomenon and increasing the risk of hypersensitivity [49,50]. These features are controversial and several investigations are in progress in order to understand the *in vivo* fate of PEGylated nanosystems, and this polymer still remains the best option to confer long circulation properties to a colloidal carrier [19,51]. DSPE-mPEG2000 was shown to be a useful candidate for the stabilization of zein nanoparticles, promoting the formation of hybrid systems able to retain Dox while preserving its *in vitro*

pharmacological efficacy. The *in vivo* activity, together with the pharmacokinetic and biodistribution profiles of the formulation need to be evaluated in order to assess the suitability of the nanohybrids for anticancer applications and to understand the potential impact of the previously discussed issues on the translation of this nanomedicine into clinical trials.

Author Contributions

N.A. and D.C. designed the experiments, analyzed data and prepared the manuscript. N.A. and A.G. prepared the nanosystems and evaluated their physico-chemical characteristics. N.A. and S.V. performed the experiments of cell viability and contributed to the analysis of the results. M.C.S. performed the TEM analyses. N.A., A.G, M.F. and D.C. acquired data and provided a critical revision. M.F. and D.C. contributed reagents and materials. All authors discussed the results and approved the final version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest in this work.

References

[1] T. Date, V. Nimbalkar, J. Kamat, A. Mittal, R.I. Mahato, D. Chitkara, Lipid-polymer hybrid nanocarriers for delivering cancer therapeutics, J. Control. Release. 271 (2018) 60–73. https://doi.org/10.1016/j.jconrel.2017.12.016.

[2] A. Mukherjee, A.K. Waters, P. Kalyan, A.S. Achrol, S. Kesari, V.M. Yenugonda, Lipid–

polymer hybrid nanoparticles as a next-generation drug delivery platform: State of the art, emerging technologies, and perspectives, Int. J. Nanomedicine. 14 (2019) 1937. https://doi.org/10.2147/IJN.S198353.

[3] R.J.C. Bose, R. Ravikumar, V. Karuppagounder, D. Bennet, S. Rangasamy, R.A. Thandavarayan, Lipid–polymer hybrid nanoparticle-mediated therapeutics delivery: advances and challenges, Drug Discov. Today. 22 (2017) 1258–1265. https://doi.org/10.1016/j.drudis.2017.05.015

[4] S.A.A. Radwan, W.H. El-Maadawy, C. Yousry, A.N. ElMeshad, R.A. Shoukri, Zein/phospholipid composite nanoparticles for successful delivery of gallic acid into ahscs: Influence of size, surface charge, and vitamin a coupling, Int. J. Nanomedicine. 15 (2020) 7995. https://doi.org/10.2147/IJN.S270242.

[5] A.L. Martínez-López, C. Pangua, C. Reboredo, R. Campión, J. Morales-Gracia, J.M. Irache, Protein-based nanoparticles for drug delivery purposes, Int. J. Pharm. 581 (2020) 119289. https://doi.org/10.1016/j.ijpharm.2020.119289.

[6] P.H.L. Tran, W. Duan, B.J. Lee, T.T.D. Tran, The use of zein in the controlled release of poorly water-soluble drugs, Int. J. Pharm. 566 (2019) 557–564. https://doi.org/10.1016/j.ijpharm.2019.06.018.

[7] A. Gagliardi, E. Giuliano, E. Venkateswararao, M. Fresta, S. Bulotta, V. Awasthi, D. Cosco, Biodegradable polymeric nanoparticles for drug delivery to solid tumors, Front. Pharmacol. 12 (2021) 601626. https://doi.org/10.3389/fphar.2021.601626.

[8] R. Paliwal, S. Palakurthi, Zein in controlled drug delivery and tissue engineering, J. Control. Release. 189 (2014) 108–122. https://doi.org/10.1016/j.jconrel.2014.06.036.

[9] G. Labib, Overview on zein protein: A promising pharmaceutical excipient in drug

delivery systems and tissue engineering, Expert Opin. Drug Deliv. 15 (2018) 65–75. https://doi.org/10.1080/17425247.2017.1349752.

[10] A. Gagliardi, D. Paolino, M. Iannone, E. Palma, M. Fresta, D. Cosco, Sodium deoxycholate-decorated zein nanoparticles for a stable colloidal drug delivery system, Int. J. Nanomedicine. 13 (2018) 601. https://doi.org/10.2147/IJN.S156930.

[11] A. Gagliardi, S. Voci, M.C. Salvatici, M. Fresta, D. Cosco, Brij-stabilized zein nanoparticles as potential drug carriers, Colloids Surfaces B Biointerfaces. 201 (2021) 111647. https://doi.org/10.1016/j.colsurfb.2021.111647.

[12] L. Dai, R. Li, Y. Wei, C. Sun, L. Mao, Y. Gao, Fabrication of zein and rhamnolipid complex nanoparticles to enhance the stability and in vitro release of curcumin, Food Hydrocoll. 77 (2018) 617–628. https://doi.org/10.1016/j.foodhyd.2017.11.003.

[13] Y. Yuan, H. Li, C. Liu, J. Zhu, Y. Xu, S. Zhang, M. Fan, D. Zhang, Y. Zhang, Z. Zhang, Fabrication of stable zein nanoparticles by chondroitin sulfate deposition based on antisolvent precipitation method, Int. J. Biol. Macromol. 139 (2019) 30–39. https://doi.org/10.1016/j.ijbiomac.2019.07.090.

[14] A. Gagliardi, N. Ambrosio, S. Voci, M.C. Salvatici, M. Fresta, D. Cosco, Easy preparation, characterization and cytotoxic investigation of 5-Fluorouracil-loaded zein/sericin nanoblends, J. Mol. Liq. 366 (2022) 120344. https://doi.org/10.1016/j.molliq.2022.120344.

[15] S.S. Hong, R.K. Thapa, J.H. Kim, S.Y. Kim, J.O. Kim, J.K. Kim, H.G. Choi, S.J. Lim, Role of zein incorporation on hydrophobic drug-loading capacity and colloidal stability of phospholipid nanoparticles, Colloids Surfaces B Biointerfaces. 171 (2018) 514–521. https://doi.org/10.1016/j.colsurfb.2018.07.068.

[16] A. Gagliardi, S. Voci, E. Giuliano, M.C. Salvatici, M. Celano, M. Fresta, D. Cosco, Phospholipid/zein hybrid nanoparticles as promising carriers for the protection and delivery of all-trans retinoic acid, Mater. Sci. Eng. C. 128 (2021) 112331. https://doi.org/10.1016/j.msec.2021.112331.

[17] H.A. Wiggers, M.T. Fin, N.M. Khalil, R.M. Mainardes, Polyethylene Glycol-Stabilized Zein Nanoparticles Containing Gallic Acid, Food Technol. Biotechnol. 60 (2022) 145–154. https://doi.org/10.17113/ftb.60.02.22.6981.

[18] P. Laverman, M.G. Carstens, O.C. Boerman, E.T.M. Dams, W.J.G. Oyen, N. van Rooijen, F.H.M. Corstens, G. Storm, Factors affecting the accelerated blood clearance of polyethylene glycol-liposomes upon repeated injection, J. Pharmacol. Exp. Ther. 298 (2001) 607–612.

[19] D. Shi, D. Beasock, A. Fessler, J. Szebeni, J.Y. Ljubimova, K.A. Afonin, M.A. Dobrovolskaia, To PEGylate or not to PEGylate: immunological properties of nanomedicine's most popular component, poly (ethylene) glycol and its alternatives, Adv. Drug Deliv. Rev. (2021) 114079. https://doi.org/10.1016/j.addr.2021.114079.

[20] A.S.A. Lila, H. Kiwada, T. Ishida, The accelerated blood clearance (ABC) phenomenon: clinical challenge and approaches to manage, J. Control. Release. 172 (2013) 38–47. https://doi.org/10.1016/j.jconrel.2013.07.026.

[21] G. Pasut, F.M. Veronese, State of the art in PEGylation: the great versatility achieved after forty years of research, J. Control. Release. 161 (2012) 461–472. https://doi.org/10.1016/j.jconrel.2011.10.037.

[22] S. Rivankar, An overview of doxorubicin formulations in cancer therapy, J. Cancer Res. Ther. 10 (2014) 853–858. https://doi.org/10.4103/0973-1482.139267.

https://doi.org/10.4103/0973-1482.139267.

[23] R. Ratan, S.R. Patel, Chemotherapy for soft tissue sarcoma, Cancer. 122 (2016) 2952–2960. https://doi.org/10.1002/cncr.30191.

[24] O. Tacar, P. Sriamornsak, C.R. Dass, Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems, J. Pharm. Pharmacol. 65 (2013) 157–170. https://doi.org/10.1111/j.2042-7158.2012.01567.x.

[25] A. Shafei, W. El-Bakly, A. Sobhy, O. Wagdy, A. Reda, O. Aboelenin, A. Marzouk, K. El Habak, R. Mostafa, M.A. Ali, A review on the efficacy and toxicity of different doxorubicin nanoparticles for targeted therapy in metastatic breast cancer, Biomed. Pharmacother. 95 (2017) 1209–1218. https://doi.org/10.1016/j.biopha.2017.09.059.

[26] M. Fojtu, J. Gumulec, T. Stracina, M. Raudenska, A. Skotakova, M. Vaculovicova, V. Adam, P. Babula, M. Novakova, M. Masarik, Reduction of doxorubicin-induced cardiotoxicity using nanocarriers: a review, Curr. Drug Metab. 18 (2017) 237–263. https://doi.org/10.2174/1389200218666170105165444.

[27] M.E.R. O'Brien, N. Wigler, M. Inbar, R. Rosso, E. Grischke, A. Santoro, R. Catane, D.G. Kieback, P. Tomczak, S.F. Ackland, Reduced cardiotoxicity and comparable efficacy in a phase III trial of pegylated liposomal doxorubicin HCl (CAELYX^{TM}/Doxil®) versus conventional doxorubicin forfirst-line treatment of metastatic breast cancer, Ann. Oncol. 15 (2004) 440–449. https://doi.org/10.1093/annonc/mdh097.

[28] N. Ambrosio, S. Voci, A. Gagliardi, E. Palma, M. Fresta, D. Cosco, Application of Biocompatible Drug Delivery Nanosystems for the Treatment of Naturally Occurring Cancer in Dogs, J. Funct. Biomater. 13 (2022) 116. https://doi.org/10.3390/jfb13030116.

[29] Y.C. Barenholz, Doxil®—The first FDA-approved nano-drug: Lessons learned, J.

Control. Release. 160 (2012) 117–134. https://doi.org/10.1016/j.jconrel.2012.03.020.

[30] U. Kanwal, N. Irfan Bukhari, M. Ovais, N. Abass, K. Hussain, A. Raza, Advances in nano-delivery systems for doxorubicin: an updated insight, J. Drug Target. 26 (2018) 296–310. https://doi.org/10.1080/1061186X.2017.1380655.

[31] D. Cosco, D. Paolino, F. De Angelis, F. Cilurzo, C. Celia, L. Di Marzio, D. Russo, N. Tsapis, E. Fattal, M. Fresta, Aqueous-core PEG-coated PLA nanocapsules for an efficient entrapment of water soluble anticancer drugs and a smart therapeutic response, Eur. J. Pharm. Biopharm. 89 (2015) 30–39. https://doi.org/10.1016/j.ejpb.2014.11.012.

[32] S. Voci, A. Gagliardi, M.C. Salvatici, M. Fresta, D. Cosco, Influence of the dispersion medium and cryoprotectants on the physico-chemical features of gliadin-and zein-based nanoparticles, Pharmaceutics. 14 (2022) 332. https://doi.org/10.3390/pharmaceutics14020332

[33] A. Gagliardi, D. Paolino, N. Costa, M. Fresta, D. Cosco, Zein-vs PLGA-based nanoparticles containing rutin: A comparative investigation, Mater. Sci. Eng. C. 118 (2021) 111538. https://doi.org/10.1016/j.msec.2020.111538.

[34] A. Gagliardi, S. Voci, S. Bonacci, G. Iriti, A. Procopio, M. Fresta, D. Cosco, SCLAREIN (SCLAREol contained in zeIN) nanoparticles: Development and characterization of an innovative natural nanoformulation, Int. J. Biol. Macromol. 193 (2021) 713–720. https://doi.org/10.1016/j.ijbiomac.2021.10.184.

[35] H. Liang, Q. Huang, B. Zhou, L. He, L. Lin, Y. An, Y. Li, S. Liu, Y. Chen, B. Li, Self-assembled zein–sodium carboxymethyl cellulose nanoparticles as an effective drug carrier and transporter, J. Mater. Chem. B. 3 (2015) 3242–3253. https://doi.org/10.1039/c4tb01920b.

[36] M. Ibrahim, E. Ramadan, N.E. Elsadek, S.E. Emam, T. Shimizu, H. Ando, Y. Ishima, O.H. Elgarhy, H.A. Sarhan, A.K. Hussein, Polyethylene glycol (PEG): The nature, immunogenicity, and role in the hypersensitivity of PEGylated products, J. Control. Release. 351 (2022) 215–230. https://doi.org/10.1016/j.jconrel.2022.09.031.

[37] E. Boedtkjer, S.F. Pedersen, The acidic tumor microenvironment as a driver of cancer, Annu. Rev. Physiol. 82 (2020) 103–126. https://doi.org/10.1146/annurev-physiol-021119-034627

[38] V. Huber, C. Camisaschi, A. Berzi, S. Ferro, L. Lugini, T. Triulzi, A. Tuccitto, E. Tagliabue, C. Castelli, L. Rivoltini, Cancer acidity: An ultimate frontier of tumor immune escape and a novel target of immunomodulation, Semin. Cancer Biol., (2017).74–89. https://doi.org/10.1016/j.semcancer.2017.03.001.

[39] F. Dong, X. Dong, L. Zhou, H. Xiao, P.-Y. Ho, M.-S. Wong, Y. Wang, Doxorubicin-loaded biodegradable self-assembly zein nanoparticle and its anti-cancer effect: Preparation, in vitro evaluation, and cellular uptake, Colloids Surfaces B Biointerfaces. 140 (2016) 324–331. https://doi.org/10.1016/j.colsurfb.2015.12.048

[40] L. Zha, B. Wang, J. Qian, B. Fletcher, C. Zhang, Q. Dong, W. Chen, L. Hong, Preparation, characterization and preliminary pharmacokinetic study of pH-sensitive Hydroxyapatite/Zein nano-drug delivery system for doxorubicin hydrochloride, J. Pharm. Pharmacol. 72 (2020) 496–506. https://doi.org/10.1016/j.colsurfb.2015.12.048

[41] A. Gagliardi, D. Cosco, B.P. Udongo, L. Dini, G. Viglietto, D. Paolino, Design and characterization of glyceryl monooleate-nanostructures containing doxorubicin hydrochloride, Pharmaceutics. 12 (2020) 1017. https://doi.org/10.3390/pharmaceutics12111017

[42] A. Jain, G. Sharma, V. Kushwah, G. Ghoshal, A. Jain, B. Singh, U.S. Shivhare, S. Jain, O.P. Katare, Beta carotene-loaded zein nanoparticles to improve the biopharmaceutical attributes and to abolish the toxicity of methotrexate: A preclinical study for breast cancer, Artif. Cells, Nanomedicine, Biotechnol. 46 (2018) 402–412. https://doi.org/10.1080/21691401.2018.1428811

[43] S.A. Sabra, A.O. Elzoghby, S.A. Sheweita, M. Haroun, M.W. Helmy, M.A. Eldemellawy, Y. Xia, D. Goodale, A.L. Allan, S. Rohani, Self-assembled amphiphilic zein-lactoferrin micelles for tumor targeted co-delivery of rapamycin and wogonin to breast cancer, Eur. J. Pharm. Biopharm. 128 (2018) 156–169. https://doi.org/10.1016/j.ejpb.2018.04.023.

[44] A.A. D'souza, R. Shegokar, Polyethylene glycol (PEG): a versatile polymer for pharmaceutical applications, Expert Opin. Drug Deliv. 13 (2016) 1257–1275. https://doi.org/10.1080/17425247.2016.1182485.

[45] N. Shah, M. Hussain, T. Rehan, A. Khan, Z.U. Khan, Overview of polyethylene glycol-based materials with a special focus on core-shell particles for drug delivery application, Curr. Pharm. Des. 28 (2022) 352–367. https://doi.org/10.2174/1381612827666210910104333.

[46] Z.A. Bachir, Y. Huang, M. He, L. Huang, X. Hou, R. Chen, F. Gao, Effects of PEG surface density and chain length on the pharmacokinetics and biodistribution of methotrexate-loaded chitosan nanoparticles, Int. J. Nanomedicine. 13 (2018) 5657. https://doi.org/10.2147/IJN.S167443.

[47] A. Kolate, D. Baradia, S. Patil, I. Vhora, G. Kore, A. Misra, PEG—a versatile conjugating ligand for drugs and drug delivery systems, J. Control. Release. 192 (2014) 67–81. https://doi.org/10.1016/j.jconrel.2014.06.046.

[48] R.M. Bukowski, C. Tendler, D. Cutler, E. Rose, M.M. Laughlin, P. Statkevich, Treating cancer with PEG Intron: pharmacokinetic profile and dosing guidelines for an improved interferon-alpha-2b formulation, Cancer Interdiscip. Int. J. Am. Cancer Soc. 95 (2002) 389–396. https://doi.org/10.1002/cncr.10663

[49] G.T. Kozma, T. Shimizu, T. Ishida, J. Szebeni, Anti-PEG antibodies: Properties, formation, testing and role in adverse immune reactions to PEGylated nano-biopharmaceuticals, Adv. Drug Deliv. Rev. 154 (2020) 163–175. https://doi.org/10.1016/j.addr.2020.07.024.

[50] W.-A. Chen, D.-Y. Chang, B.-M. Chen, Y.-C. Lin, K. Barenholz, S.R. Roffler, Antibodies against Poly (ethylene glycol) Activate Innate Immune Cells and Induce Hypersensitivity Reactions to PEGylated Nanomedicines, ACS Nano. (2023). https://doi.org/10.1021/acsnano.2c12193.

[51] J.L. Markman, A. Rekechenetskiy, E. Holler, J.Y. Ljubimova, Nanomedicine therapeutic approaches to overcome cancer drug resistance, Adv. Drug Deliv. Rev. 65 (2013) 1866–1879. https://doi.org/10.1016/j.addr.2013.09.019.

Table 1. Physico-chemical properties of hybrid nanosystems made up of zein (2 mg/ml) and various derivatives used as stabilizers.

Derivatives	***Concentration (mg/ml)***	***Mean sizes (nm)***	***PDI***	***Zeta potential (mV)***	***Adverse physical phenomena***
-	-	154 ± 10	0.259 ± 0.009	8 ± 2	

DMPG	0.2	84** ± 1	0.121** ± 0.020	-36** ± 6	
DMPG	0.4	97** ± 2	0.252 ± 0.008	-35** ± 1	
DMPG	0.6	194** ± 3	0.273 ± 0.005	-44** ± 1	Macroaggregates
DOTAP	0.2	97** ±1	0.195* ± 0.018	28** ± 2	
DOTAP	0.4	99** ± 1	0.162** ± 0.014	47** ± 3	
DOTAP	0.6	102** ± 1	0.213* ± 0.007	53** ± 3	
DPPC	0.2	2[illegible] ± 3	0.373** ± 0.017	-8** ± 1	
DPPC	0.4	>1000**	0.850** ± 0.2	-6** ± 1	Macroaggregates
DPPC	0.6	>1000**	1.00**	-12** ± 4	Macroaggregates
LPPC	0.2	163 ± 1	0.233* ± 0.005	11 ± 1	
LPPC	0.4	173* ± 1	0.332* ± 0.014	15* ± 1	
LPPC	0.6	194** ± 8	0.356** ± 0.006	12* ± 1	

PEG-distearate	0.2	168* ± 1	0.159** ± 0.007	5 ± 1	
PEG-distearate	0.4	168* ± 1	0.190* ± 0.044	10 ± 1	Sediments
PEG-distearate	0.6	138** ± 1	0.290* ± 0.006	2* ± 1	Macroaggregates
PEG-Tosylate	0.2	120** ± 17	0.113** ± 0.006	2* ± 1	Macroaggregates
PEG-Tosylate	0.4	95** ± 1	0.150** ± 0.020	20* ± 2	
PEG-Tosylate	0.6	152 ± 5	0.[illegible]** ± 0.024	-4** ± 1	Sediments
CHOL-PEG 600	0.2	507* ± [illegible]	0.234* ± 0.066	-13** ± 1	
CHOL-PEG 600	0.4	184** ± 1	0.178* ± 0.021	-19** ± 1	Sediments
CHOL-PEG 600	0.6	267** ± 13	0.337 ** ± 0.001	-14** ± 1	Sediments
DSPE-mPEG 2000	0.2	132** ± 2	0.320* ± 0.011	-9** ± 1	
DSPE-mPEG 2000	0.4	110** ± 2	0.222* ± 0.008	-22** ± 1	
DSPE-mPEG	0.6	74** ± 1	0.177* ±	-29** ± 2	

2000			0.005		

DMPG: dimyristoylphosphatidylglycerol; DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane; DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; LPPC: 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine; PEG-distearate: poly(ethylene glycol)distearate; PEG-Tosylate: poly(ethylene glycol)-di-p-tosylate; CHOL-PEG 600: Cholesterol-(polyethylene glycol-600); DSPE-mPEG2000: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000].

The analysis was performed in NaCl 1 mM with a dilution 1:50.

*p < 0.05, **p < 0.001. with respect to the stabilizer-free formulation.

Table 2. Physico-chemical properties of stabilizer-free zein nanoparticles (2 mg/ml) and hybrid nanosystems as a function of the dispersant medium and pH.

	stabilizer-free		*Z/DSPE-mPEG 10:3 w/w*		*Z/DOTAP 10:2 w/w*	
	Mean sizes (nm)	PDI	Mean sizes (nm)	PDI	Mean sizes (nm)	PDI
pH 4	291** ± 15	0.301** ± 0.051	71 ± 2	0.198 ± 0.013	796** ± 71	0.364** ± 0.043
pH 7	>1000**	0.451** ± 0.065	74 ±1	0.207 ± 0.038	>1000**	0.317** ± 0.033
pH 10	>1000**	0.381** ± 0.051	153** ± 13	0.119* ± 0.013	>1000**	0.467** ± 0.019
Glucose 5%	711** ± 142	0.243 ±	79* ± 2	0.210 ±	110* ± 1	0.221* ±

w/v		0.012		0.07		0.006
PBS	853** ± 100	0.209 ± 0.064	80* ± 1	0.190 ± 0.02	>1000**	0.324** ± 0.034
NaCl 0.9% w/v	852** ± 65	0.284 ± 0.084	68* ± 2	0.199 ± 0.009	>1000**	0.232* ± 0.041

*p < 0.05, **p < 0.001. with respect to the formulation analyzed in NaCl 1 mM.

Figure captions

Fig. 1. Mean sizes, polydispersity index A) and zeta potential B) of zein nanoparticles (2 mg/ml) as stabilizer-free formulation or as hybrid systems prepared with DMPG (0.2 mg/ml), DOTAP (0.4 mg/ml) and DSPE-mPEG2000 (0.6 mg/ml). *p < 0.05, **p < 0.001. with respect to the stabilizer-free formulation.

Fig. 2. Turbiscan stability index profiles of hybrid nanosystems made up of stabilizer-free zein (2 mg/ml) and DMPG (0.2 mg/ml); DOTAP (0.4 mg/ml) and DSPE-mPEG2000 (0.6 mg/ml) as a function of time and temperature

Fig. 3. FT-IR spectra of zein, stabilizers, their physical mixture and hybrid nanoparticles.

Fig. 4. Mean sizes, polydispersity index and Zeta potential of Z/DSPE-mPEG 10:3 w/w (panels A and C) and Z/DOTAP 10:2 w/w (panels B and D) hybrid nanoparticles prepared with various amounts of doxorubicin hydrochloride (Dox). *p < 0.05, **p < 0.001. with respect to the empty formulation.

Fig. 5. Turbiscan stability index (TSI) profiles of hybrid nanosystems made up of zein-based hybrid nanosystems as a function of the Dox concentration, time and temperature.

Fig. 6. Entrapment efficiency (%) of Dox within hybrid zein nanosystems as a function of the

amount of drug used during the preparation of the samples.

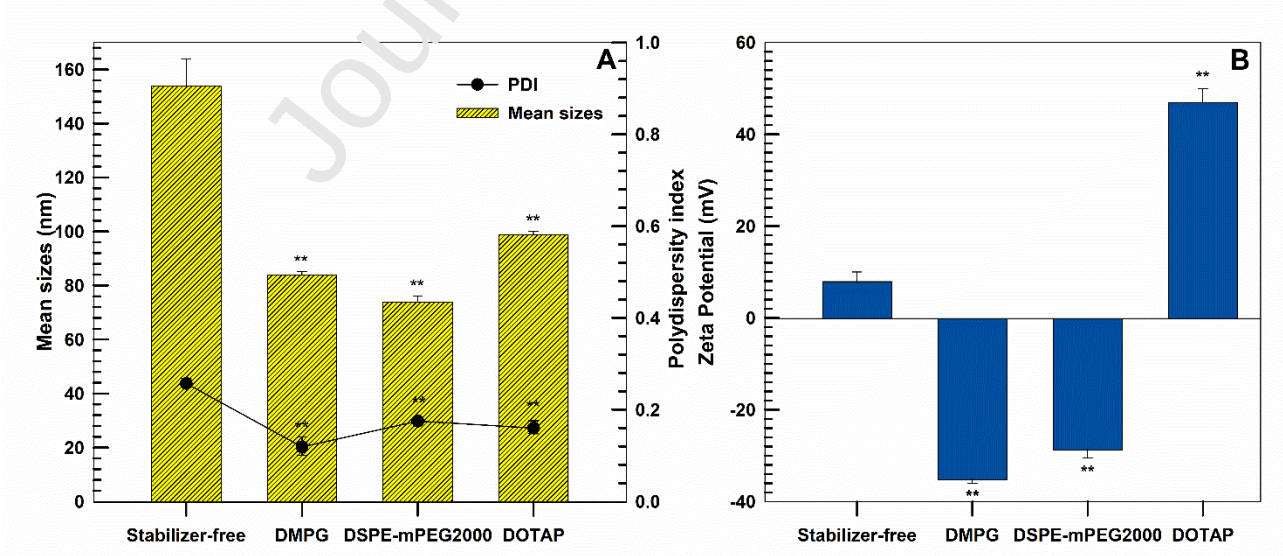
Fig. 7. FT-IR spectra of Dox, zein/DSPE-mPEG2000/Dox physical mixture, zein/DSPE-mPEG2000 (10:3 w/w) hybrid systems and Dox-loaded zein/DSPE-mPEG2000 (10:3 w/w) hybrid systems.

Fig. 8. Release profile of Dox from Z/DSPE-mPEG (10:3 w/w) hybrid nanosystems at pH 5.5 (A) and 7.4 (B).

Fig. 9. *In vitro* toxicity of Dox and Dox-loaded zein/DSPE-mPEG2000 (10:3 w/w) nanoparticles (nanoDox) on MCF-7 and MDA-MB-231 cells as function of the drug concentration and incubation time.

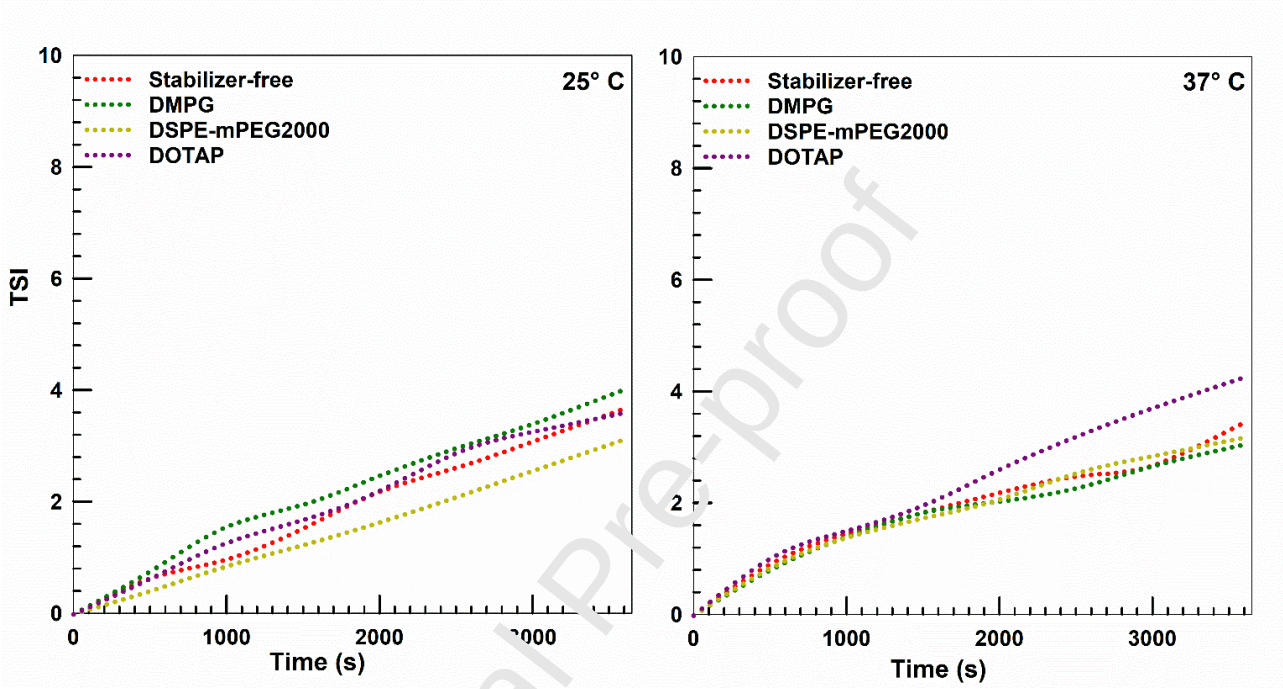
Stabilization strategies for zein nanoparticles containing doxorubicin hydrochloride

Fig. 1.



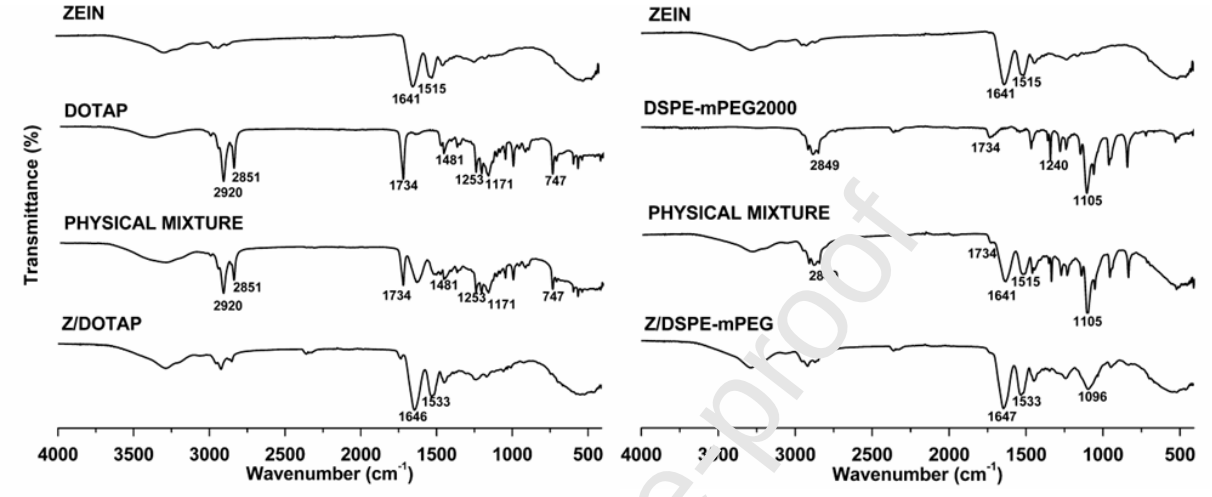
Stabilization strategies for zein nanoparticles containing doxorubicin hydrochloride

Fig. 2.



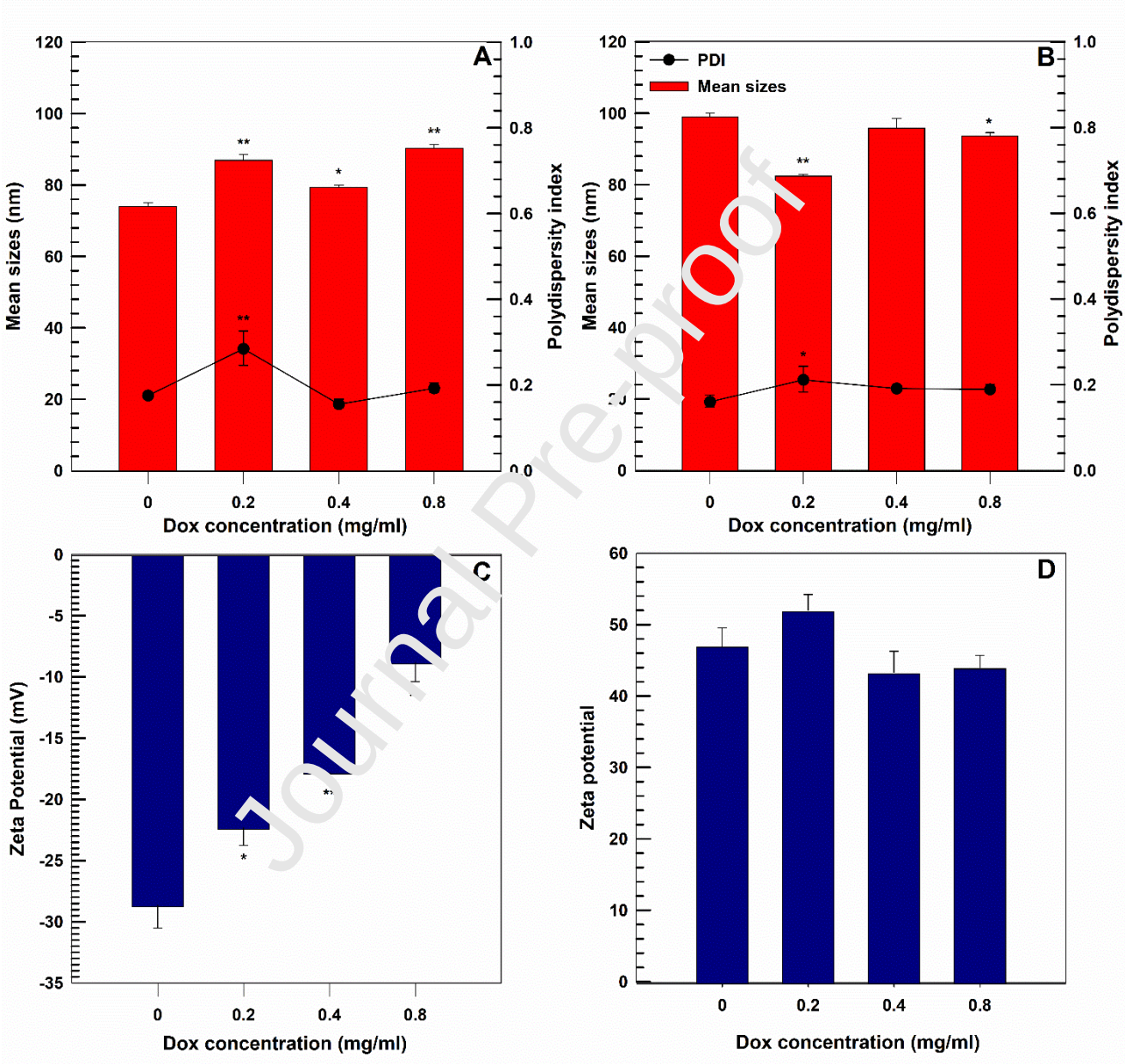
Stabilization strategies for zein nanoparticles containing doxorubicin hydrochloride

Fig. 3.



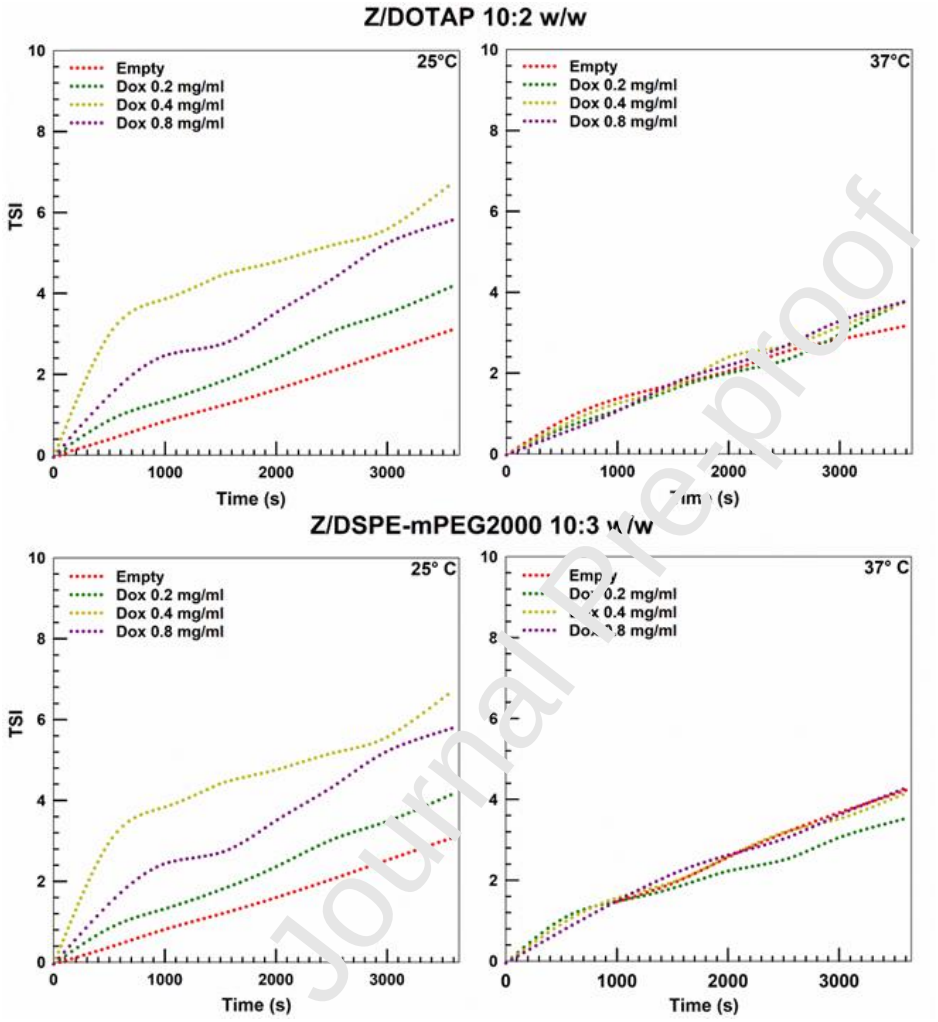
Stabilization strategies for zein nanoparticles containing doxorubicin hydrochloride

Fig. 4.



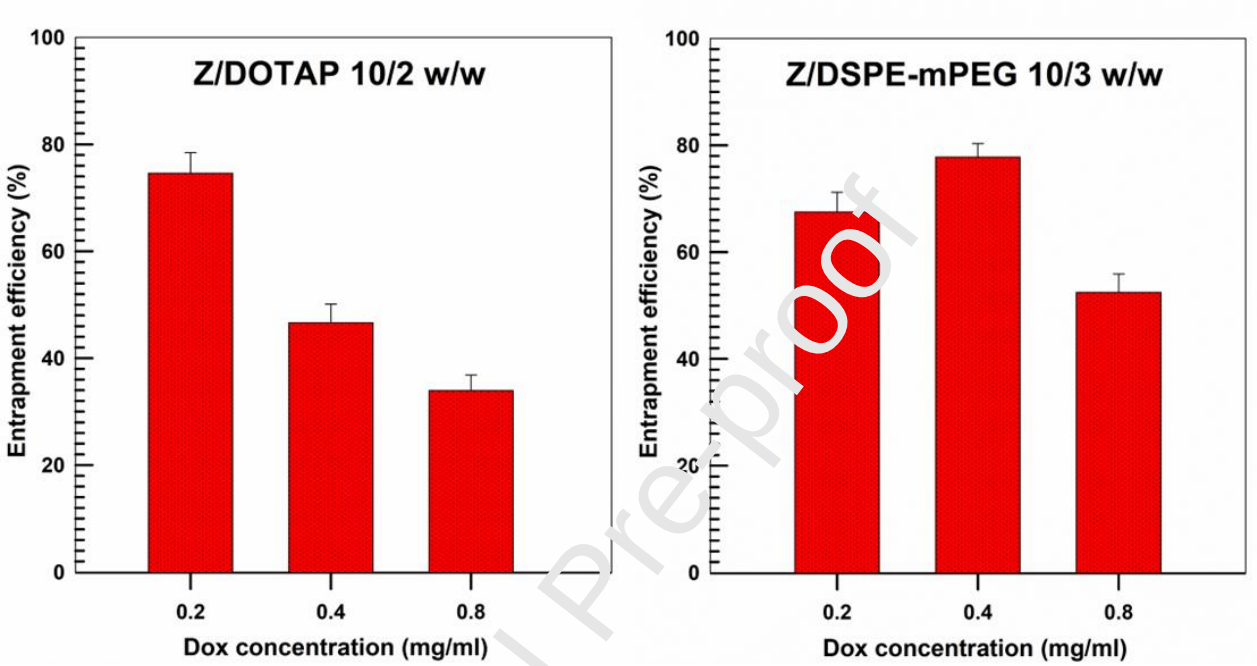
Stabilization strategies for zein nanoparticles containing doxorubicin hydrochloride

Fig. 5.



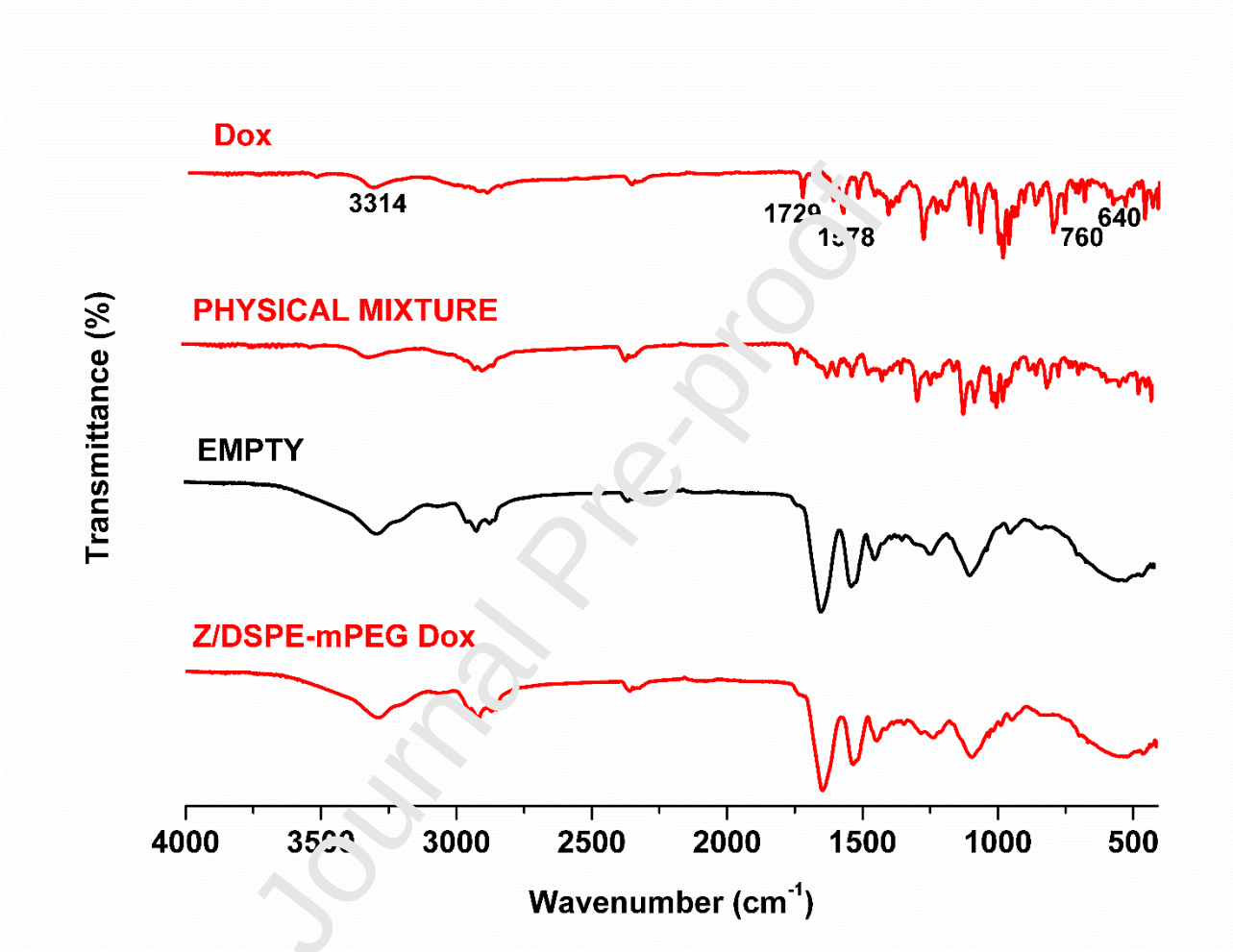
Stabilization strategies for zein nanoparticles containing doxorubicin hydrochloride

Fig. 6.



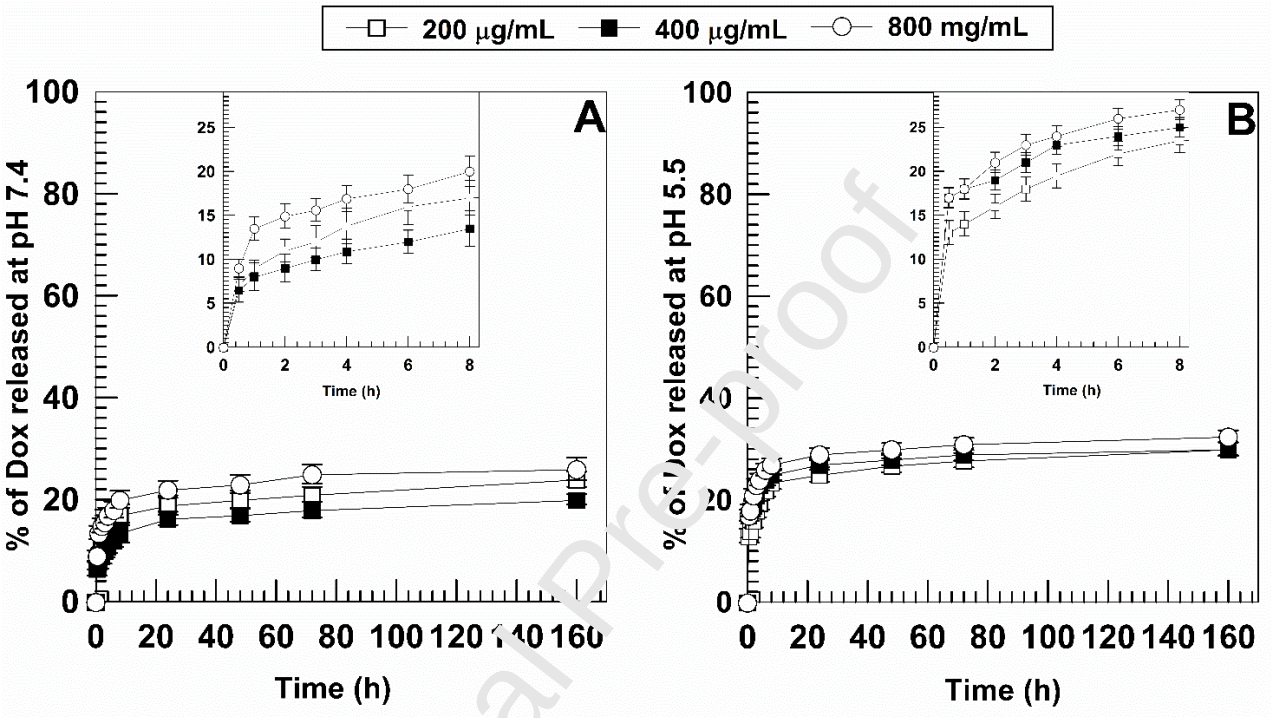
Stabilization strategies for zein nanoparticles containing doxorubicin hydrochloride

Fig. 7.



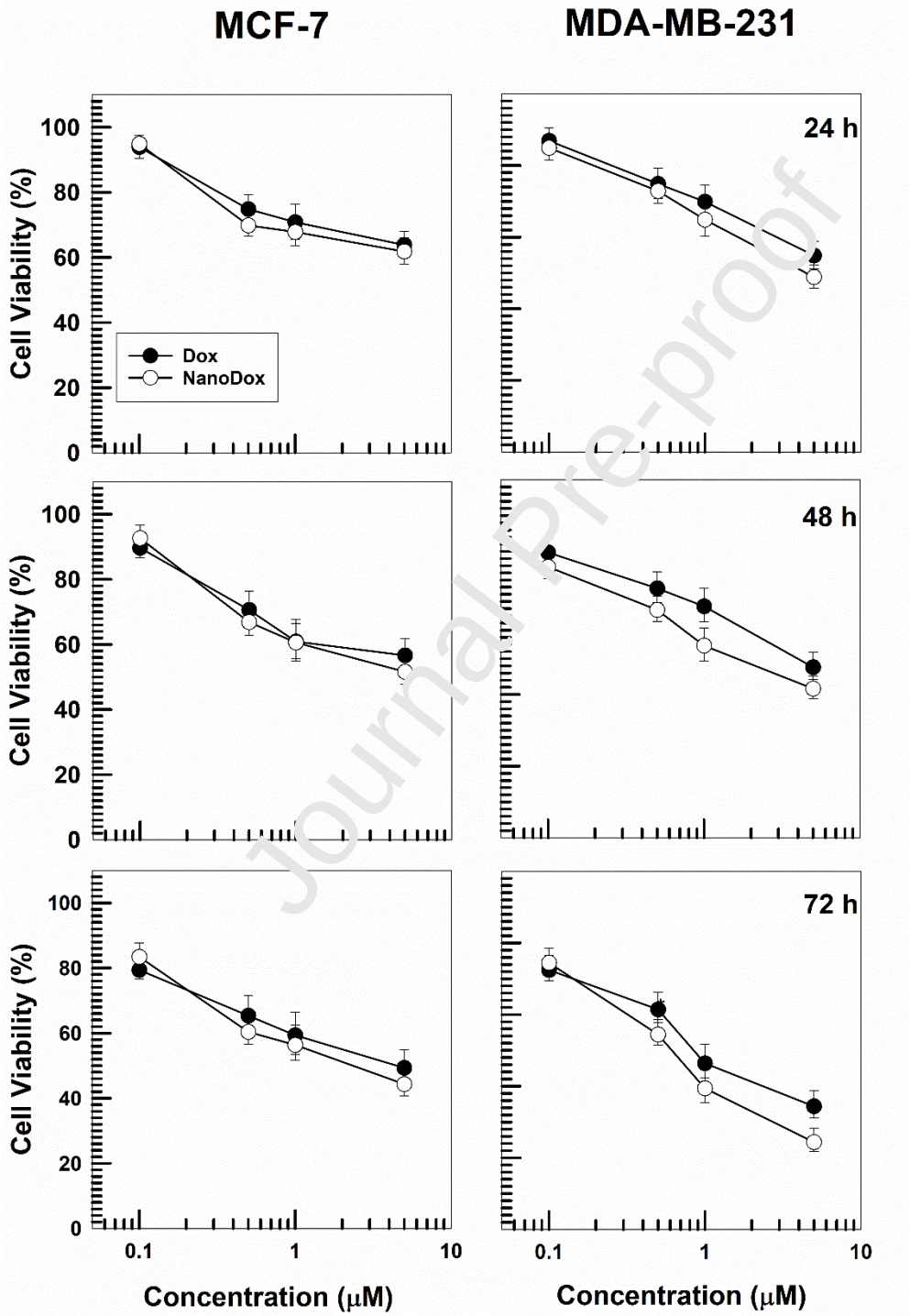
Stabilization strategies for zein nanoparticles containing doxorubicin hydrochloride

Fig. 8.



Stabilization strategies for zein nanoparticles containing doxorubicin hydrochloride

Fig. 9.



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

A stable nanoformulation made up of zein and phospholipids was developed

The characteristics of (phospho)lipid derivatives influenced the sizes of nanoblends

DSPE-mPEG2000 efficiently stabilized the zein nanoparticles

The nanoblends retained doxorubicin hydrochloride and a slow release was obtained

The encapsulation of the antitumor compound did not compromise its *in vitro* activity