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Brij-stabilized zein nanoparticles as potential drug carriers

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ABSTRACT

The current study was designed to provide a preliminary physico-chemical characterization of zein nanosystems prepared with various Brij surfactants (for the first time to the best of our knowledge) as a function of various external stimuli such as temperature, pH, serum incubation and the freeze-drying process. The results demonstrate that when Brijs are characterized by unsaturation (C18), considerable stabilization of the colloidal structure is promoted while the length of the polyethylene glycol fraction does not significantly modulate the physico-chemical properties of the nanosystems. Specifically, dynamic light scattering and nanoparticle tracking analysis demonstrated that the use of 0.2 % w/v of Brij O10 promoted the formation of stable zein nanosystems with mean sizes of ~150 nm and a narrow size distribution, preserving their structures at various pHs and temperatures. The use of mannitol as cryoprotectant resulted in a formulation that can easily be re-suspended in water after the freeze-drying process. This nanoformulation demonstrated that it efficiently retained different amounts of both hydrophilic and lipophilic compounds and showed a prolonged release of the entrapped molecules. In addition, the nanosystems showed a favorable degree of *in vitro* safety on various cell lines when a concentration <50 μg/mL of protein was used, demonstrating the potential application of Brij O10-stabilized zein nanoparticles as innovative nanocarriers of several active compounds.

1. Introduction

Over the past decades, the availability of surfactants used to develop stable formulations has significantly increased in pharmaceutical applications as a result of the need to manage and administer new therapeutic compounds characterized by scarce aqueous solubility [1]. Indeed, various strategies involving non-ionic surfactants have been employed to improve the bioavailability as well as the effectiveness of drugs retained by various colloidal systems [2].

Surfactants are amphiphilic, surface-active molecules, generally classified as either ionic or non-ionic compounds based on the charged or uncharged nature of the functional group, respectively [3]. The addition of non-ionic surfactants is a common approach for stabilizing polymeric nanoparticles by reducing the surface tension of the colloids; this promotes interaction between the nanosystems and the medium [4–8].

The most commonly employed non-ionic surfactants belong to the family of polysorbates, poloxamers and poly(vinyl alcohol). These molecules are characterized by a hydrophobic alkyl chain linked to a hydrophilic head with a different number of repeated polyoxyethylene (POE) residues in a range between 10–100 units. For this reason, surfactants of this type are often described as polymeric ethers [2,9,10].

The chain lengths of the various units and the hydrophilic/lipophilic balance (HLB) of the stabilizers can easily be modified by varying the alkyl and PEO portions, thus favoring an employment of the stabilizers in a wide range of fields (e.g., detergents, cosmetics) [11]. Among the ethylene oxide-based surfactants, Brijs have drawn a certain degree of interest from the scientific community because of their potential uses as biodegradation enhancers, micellar catalyzed systems, stabilizers in drug-release systems and components used for developing pH-responsive nanoparticles [12]. Moreover, several papers have described their capacity to sensitize multidrug-resistant (MDR) tumor cells towards chemotherapy through the inhibition of the drug-efflux transporters of the ATP-binding cassette superfamily, such as P-glycoprotein (Pgp), promoting a significant decrease in ATP levels [13]. In addition, the great advantage of Brij derivatives is due to the presence of the

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ether bond in the chemical structure, which is less susceptible to hydrolysis in an aqueous solution as compared to the ester bond of other non-ionic surfactants that may increase the risk of the degradation and instability of the colloidal structures. Indeed, it is well-known that the size of the hydrophilic portion of Brij surfactants can be a crucial factor in their ability to retard the chemical degradation of various compounds in an acidic environment [3,14–17].

Various studies have also shown the ability of polyoxyethylene alkyl ether surfactants to improve the *in vitro* and *in vivo* performance of topically applied ophthalmic drugs [2,18]. In particular, polyoxyethylene oxide-(2)-oleyl ether (Brij O2) and polyoxyethylene oxide-(10)-oleyl ether (Brij O10) are included among the Inactive Ingredients Guide of the Food and Drug administration (FDA).

Considering these features, several Brij derivatives were used in this investigation to stabilize polymeric nanoparticles made up of zein, and this was for the first time to the best of our knowledge (Table 1). Zein is a natural biopolymer, a prolamin-rich storage protein extracted from the endosperm of corn. It has been widely investigated for the development of innovative nanocarriers because it is characterized by peculiar physico-chemical features useful for obtaining biocompatible delivery systems [19]. Indeed, it is widely available and is low cost, cytocompatible and biodegradable and has also been recognized as a safe material (GRAS status) by the FDA [20]. The large amounts of non-polar aminoacids it contains clearly show the predominance of the significant hydrophobic character of the protein which favors the nanoencapsulation of poorly-water soluble compounds [21–23]. Another peculiar characteristic of zein is its inherent antioxidant activity related to the presence of specific aminoacids (i.e. histidine, arginine, leucine, alanine, valine and methionine), as well as the presence of xanthophylls (8–9 %) such as zeaxanthin, β-cryptoxanthin and lutein, which are able to carry on scavenging activity against free radicals and lipid peroxidation, as reported in several works [24–26]. However, various attempts have been made to improve the stabilization of zein nanoparticles by decreasing their hydrophobic attraction and increasing their steric repulsion in order to prevent the adverse physical phenomena that can occur when certain environmental conditions are close to the isoelectric point of the protein (pI 6.2). In addition, several studies have shown that zein nanosystems are generally unstable at high temperatures and in saline solutions [27,28].

A large number of stabilizers have already been tested in the effort to reach this goal, the most important being sodium caseinate [29] lysine [30], dextran sulfate [31], ι-carrageenan [32], chondroitin sulfate [33], poloxamer 407 [34], Tween 80® [35], sodium alginate [36], gum arabic [37], soybean polysaccharide [38] chitosan [39] and D-α-Tocopherol polyethylene glycol 1000 succinate (TPGS) [40]. Recently our research team developed zein nanoparticles decorated with sodium deoxycholate. The addition of 1.25 % w/v of surfactant resulted in nanosystems characterized by considerable long-term stability and a significant retention rate for various active compounds [26,41,42].

The aim of the present study was to evaluate the influence of the various Brij derivatives on the physico-chemical features of zein nanoparticles. In particular, the non-ionic polyoxyethylene alkyl ether surfactants were selected as a function of the length of the alkyl chain, the presence of unsaturation and the number of ethylene oxide units they contain (Table 1). The effects of specific parameters (i.e. temperature, pH, serum incubation, the nature and concentration of cryoprotectants) on the physico-chemical properties of zein nanoparticles as well as on the cytotoxicity, the entrapment efficiency and release profiles of different model compounds were investigated in order to identify the most promising nanoformulations that could be used for oral and parenteral administration.

2. Materials and methods

2.1. Materials

Zein, bromophenol blue, rhodamine B, were all purchased from Sigma-Aldrich (Milan, Italy). Ethanol was purchased from Carlo Erba SpA (Rodano, Italy). Brij L4 (polyoxyethylene oxide (4)-lauryl ether), Brij C10 (polyoxyethylene oxide-(10)-cetyl ether), Brij S20 (polyoxyethylene oxide (20)-stearyl ether), Brij O2 (polyoxyethylene oxide (2)-oleyl ether) and Brij O10 (polyoxyethylene oxide (10)-oleyl ether) were purchased from Croda (Snaith, United Kingdom). Cellulose membrane for the release studies (MW 50 kDa) was obtained from Spectrum Laboratories Inc. (Eindhoven, Netherlands).

2.2. Preparation of Brij-stabilized zein nanoparticles

The nanoprecipitation technique was used to obtain zein nanoparticles as previously reported [41]. Specifically, 3.3 mg/mL of zein was dissolved in 3 mL of an ethanol/water solution (2:1 v/v) at room temperature and added to 5 mL of MilliQ water containing different amounts of surfactants. The resulting suspensions were then homogenized by means of an Ultraturrax (model T25, IKA® Werke GmbH and Co, Staufen, Germany) at 24,000 rpm for 1 min.

Zein nanoparticles containing rhodamine B and bromophenol blue were obtained by adding different amounts of the compounds to the aqueous and organic phases, respectively, according to their physico-chemical features. Successively, the samples were placed on a magnetic stirrer (600 rpm, 12 h) until the evaporation of the organic solvent was complete. The nanoparticles were then purified using the dialysis technique (cut-off 50 kDa) which removed the unreacted compounds before *in vitro* experimentation.

The morphology of the nanoparticles was investigated by means of Transmission Electron Microscopy (TEM) as already described (Fig. S1) [43].

2.3. Physico-chemical characterization of Brij-stabilized zein-nanosystems

The average diameter, size distribution and surface charges of the zein nanoparticles were investigated using a Zetasizer Nano ZS (Malvern Panalytical Ltd., Spectris plc, England) applying the third order cumulant fitting correlation function [44,45]. The results are the mean of three different measurements performed in triplicate on three different samples (10 determinations for each sample) ± standard deviation and expressed as a function of the intensity parameter [46].

The particle concentration and particle size distribution of the zein nanoparticles were also evaluated through nanoparticle tracking analysis (NTA), using a Nanosight NS300 with a 488 nm laser at 25 °C (Malvern Panalytical Ltd., Spectris plc, England) [47].

Table 1
Chemical properties of Brij derivatives used.

Trade name	Chemical name	Molecular formula	HLB	MW (g/mol)	CmC (mM)	Density (g/mL)
Brij L4	Polyoxyethylene oxide-(4)-lauryl ether	$C_{12}H_{25}(OCH_2CH_2)_4OH$	9.7	362	6.51×10^{-5}	0.95
Brij C10	Polyoxyethylene oxide-(10)-cetyl ether	$C_{16}H_{33}(OCH_2CH_2)_{10}OH$	12.9	683	3.5×10^{-3}	0.977
Brij S20	Polyoxyethylene oxide-(20)-stearyl ether	$C_{18}H_{37}(OCH_2CH_2)_{20}OH$	15	1152	0.006	0.893
Brij O2	Polyoxyethylene oxide-(2)-oleyl ether	$C_{18}H_{35}(OCH_2CH_2)_2OH$	5	356.58	–	0.912
Brij O10	Polyoxyethylene oxide-(10)-oleyl ether	$C_{18}H_{35}(OCH_2CH_2)_{10}OH$	12.4	709	0.029	1

The stability profiles of the various zein formulations were evaluated by means of a Turbiscan Lab Expert® (Formulaction, Toulouse, France), as a function of incubation time and temperature and the resulting data were expressed as the Turbiscan Stability Index (TSI) [48].

2.4. Influence of serum proteins and pH on the stability of zein nanosystems

The influence of serum proteins on the stability of zein nanosystems was performed by incubating the samples in 70 % FBS and their sizes were monitored over time. Briefly, 1 mL of FBS solution was added to 200 μL of formulation and the resulting suspension was incubated at 37 °C for 48 h while being stirred at 600 rpm. The mean sizes of the samples were analyzed at various incubation times (0.5, 1, 2, 3, 4, 6 and 24 h) as previously described [49,50].

The influence of the pH on the physico-chemical features of the various zein nanosystems was also investigated by incubating the samples in deionized water at different pH values (4.0, 7.0, 10.0), using 1 mol/l of NaOH or HCl [42].

2.5. Cytotoxicity of zein nanoparticles

In order to assess the cytotoxicity of the zein nanoparticles, C-28 (human chondrocytes) Nthy-ori 3–1 (human primary thyroid follicular epithelial cells), A549 (human lung cancer cells) and BCPAP (human papillary thyroid carcinoma cell lines) were incubated in plastic culture dishes (100 mm × 20 mm) in a water-jacketed CO_2 incubator at 37 °C (5% CO_2) using D-MEM or RPMI1640 with glutamine, supplemented with penicillin (100 UI/mL), streptomycin (100 μg/mL), amphotericin B (250 μg/mL) and FBS (10 %, v/v) [43]. The cell viability of surfactant-free zein nanosystems and Brij O10-stabilized zein nanocarriers was evaluated by MTT assay as previously reported [41]. In detail, the cells were plated in 96-well culture dishes (7×10^3 cells/0.2 mL) and treated with increasing concentrations of nanoparticles (1, 10, 50, 100 μg/mL of zein) at different incubation times (24, 48 and 72 h). Successively, 20 μl of tetrazolium salt solubilized in phosphate-buffered solution (5 mg/mL) were added to each well and the plates were incubated again for 3 h. They were then analyzed using a microplate spectrophotometer (xMARK™ BIORAD) at a wavelength of 540 nm with reference at 690 nm. Untreated cells were used as a control. Cell viability, expressed as percentage, was evaluated as the mean of 5 different experiments ± standard deviation and was obtained using the following equation:

$$\text{Cell viability (\%)} = \text{AbsT/AbsC} \times 100 \quad (1)$$

in which AbsT is the absorbance of the treated cells and AbsC is the absorbance of the untreated cells (control).

2.6. Freeze-drying of Brij-stabilized zein nanosystems

Zein nanoparticles were lyophilized using a VirTis SP Scientific Sentry 2.0 instrument equipped with a vacuum pump (model B14, Carpanelli S.p.A. Bologna, Italy). In detail, 500 μL of each formulation were enriched with 5 and 10 % w/v of different cryoprotectants (glucose, mannose, sucrose, trehalose and mannitol) and placed in pyrex glass vials which were then immersed in liquid nitrogen for 2 min. Subsequently, the samples were placed in the freeze-drying chamber and freeze-dried for 24 h. The temperature and pressure of the condenser were about −55 °C and 30–50 mT, respectively. At the end of the process, the resulting powder was rehydrated with the same volume of sublimated water and subjected to photon correlation spectroscopy analysis [43].

2.7. Entrapment efficiency of model compounds

The entrapment efficiency of rhodamine B (a model of the hydrophilic compound) and bromophenol blue (a model of the lipophilic molecule) within zein nanoparticles was assessed by means of spectrophotometric analysis. The colloidal formulations, prepared with different amounts of fluorescent compounds, were centrifuged at 90k rpm for 1 h using an ultracentrifuge Beckman Optima TL (Fullerton, CA). The resulting pellet was incubated for 48 h either in water or ethanol, the choice being based on the chemical features of the entrapped probe. The amount of model compounds contained in the solution was then analyzed spectrophotometrically (Perkin Elmer Lambda 35, Waltham, Massachusetts, USA) at λ_{max} 544 and 350 nm for rhodamine B and bromophenol blue, respectively. No interference was observed for the empty zein formulations. The amount of the encapsulated molecule (EE%) was calculated as the percentage of the amount retained by the polymeric (De) structure with respect to that initially added during the preparation of the sample (Da).

$$\text{EE\%} = \text{De/Da} \times 100 \quad (2)$$

The amount of Brij O10 integrated into the zein nanoparticles was quantified through a colorimetric assay [51]. The loading capacity (LC) was expressed as the ratio between the amount of entrapped compound with respect to the total weight of the nanoparticles according to the following equation:

$$\text{LC \%} = \text{Amount of entrapped compound/total weight of nanoparticles} \times 100 \quad (3)$$

2.8. Evaluation of release profiles of model compounds from Brij - stabilized zein nanoparticles

The release profiles of the model compounds retained by the zein nanoparticles were investigated using the dialysis method and implementing cellulose acetate tubes (Spectra/Por with molecular cutoff 12k–14k by Spectrum Laboratories Inc.) [52]. A PBS solution (pH 7.4, 0.1 M) constantly stirred and warmed to 37 ± 0.1 °C was used as the release fluid for the compounds in order to operate under sink conditions. The percentage of released probe was calculated using the following equation:

$$\text{Release (\%)} = \text{probe}_{rel}/\text{probe}_{load} \times 100 \quad (4)$$

where probe_{rel} is the amount of released compound at the time t and probe_{load} is the amount of molecule entrapped within the nanoparticles.

2.9. 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 zein nanoparticles

The physico-chemical and technological features of nanosystems need to be fully explored during the phases of pre-clinical characterization [53]. In this context, the European Nanomedicine Characterisation Laboratory (EUNCL) and the US National Cancer Institute Nanotechnology Characterization Laboratory (NCI-NCL) have established that the preclinical characterization of nanoparticle-based formulations requires critical quality attributes such as the mean size and the polydispersity index. These can be assessed by the combination of multiple high-resolution measurements i.e. dynamic light scattering (DLS) and Nanoparticle tracking analysis (NTA) in order to simplify the

translation of successful nanomaterials from the laboratories to the clinic [54,55].

Recently our research team described the physico-chemical properties of zein systems evidencing a direct correlation between the protein concentration and the formation of well-defined nanoparticles. In particular, it was interesting to observe the variation of the surface charges of surfactant-free nanocarriers as a function of the protein concentration, which showed negative values at low amounts of biopolymer and positive values when the zein concentration was increased [41]. These systems were shown to be influenced by both physical and chemical stress and the use of sodium deoxycholate greatly stabilized the polymeric structure, favoring the development of drug delivery systems able to retain various active compounds [41,42]. Based on these results, the first step of this investigation was to evaluate the influence of the non-ionic Brij surfactants on the formation of zein nanoparticles (Table 2).

The addition of 0.002 % w/v of the lauryl ether derivative Brij L4 (alkyl chain with 12 C atoms as a lipophilic residue) to the water phase during sample preparation, favored only a slight stabilization of the resulting nanoparticles at room temperature, while larger amounts of surfactant induced the formation of polydisperse nanoparticles and macroaggregates. The use of Brij C10 (alkyl chain with 16 C atoms), allowed the formation of colloidal systems with a mean diameter of about 100–150 nm, but promoted no significant improvement in the polydispersity index with respect to the surfactant-free formulation (Table 2). In addition, the increase of the temperature up to 37 °C confirmed this trend and favored an increase in the mean diameter of the systems (Fig. 1).

Good results were obtained when small amounts of Brij S20 were used. This is a compound characterized by a stearyl-based alkyl chain, which promoted the development of stable and monodisperse structures, suitable for intravenous administration (Table 2). Unfortunately, also in this case, the heating process provoked significant destabilization of the colloidal systems, suggesting that the rise in temperature probably caused the modulation of the protein conformation and the collapse of the systems, which is in agreement with the data previously reported [41]. Moreover, zein nanoparticles decorated with Brij O2 (oleyl-2-based derivative) showed a direct correlation between the surfactant concentration and the stability of the nanocarriers. In fact, the addition of small amounts of Brij O2 (0.002–0.020% w/v) resulted in nanosystems of mean sizes ranging between 100 and 130 nm, while a significant increase of this parameter was obtained at greater concentrations of the compound and higher temperatures (Fig. 1).

Table 2
Composition and physico-chemical properties of zein-based nanoparticles prepared using 2 mg/mL of protein and various amounts of Brij surfactants.

Stabilizer	Concentration (% w/v)	Mean Sizes (nm)	Polydispersity Index	Zeta Potential (mV)
–	–	106 ± 1	0.20 ± 0.01	20 ± 0.8
Brij L4	0.002	117 ± 1	0.19 ± 0.01	9.1 ± 1.6**
	0.020	310 ± 10**	0.27 ± 0.03	10.8 ± 0.6**
	0.100	741 ± 117**	0.70 ± 0.04**	8.2 ± 1.7**
	0.200	913 ± 23**	0.32 ± 0.01*	12.3 ± 3.4**
Brij C10	0.002	119 ± 1	0.22 ± 0.01	9.9 ± 0.5**
	0.020	152 ± 2**	0.16 ± 0.03	6.1 ± 1.5**
	0.100	151 ± 1**	0.20 ± 0.01	8.2 ± 0.4**
	0.200	129 ± 3*	0.28 ± 0.04*	7.3 ± 2.3**
Brij S20	0.002	106 ± 1	0.16 ± 0.01	8.1 ± 0.4**
	0.020	157 ± 2**	0.15 ± 0.02	4.8 ± 0.5**
	0.100	148 ± 1**	0.25 ± 0.03	7.1 ± 1.5**
	0.200	146 ± 1**	0.22 ± 0.01	8.3 ± 0.5**
Brij O2	0.002	101 ± 1	0.18 ± 0.02	10.8 ± 0.6**
	0.020	130 ± 1*	0.12 ± 0.01	10.2 ± 1.9**
	0.100	269 ± 1**	0.21 ± 0.02	9.3 ± 1.6**
	0.200	368 ± 5**	0.17 ± 0.03	11.1 ± 2.7**
Brij O10	0.002	112 ± 1	0.17 ± 0.01	9.3 ± 0.8**
	0.020	133 ± 7*	0.17 ± 0.03	7.2 ± 0.5**
	0.100	134 ± 1*	0.18 ± 0.02	8.4 ± 0.9**
	0.200	176 ± 1**	0.17 ± 0.02	10.2 ± 0.6**

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

Another surfactant used in the preparation phase of the nanosystems was Brij O10, which has a chemical structure comparable to that of Brij O2, but having a greater number of polyoxyethylene residues. The addition of this molecule promoted the formation of nanocarriers with an average diameter of less than 150 nm and a narrow size distribution. It was interesting to observe that the use of greater concentrations of Brij O10 (0.2 % w/v) slightly increased the mean sizes of the nanoparticles (180 nm), but left them with dimensions that were still compatible with potential systemic administration. In addition, it can be observed in Fig. 1 that Brij O10 significantly improved the stability of the colloidal systems against the destabilizing phenomena induced by the heating process.

The surfactant-free zein nanoparticles showed a positive surface charge while the addition of Brij derivatives promoted a slight decrease of this parameter. In fact, the ~20 mV zeta-potential of the formulations with no surfactant decreased to ~10 mV when Brij molecules were used. A possible explanation of this result could be related to the adsorption of the surfactant onto the particle surface, leading to a modulation of the shearing plane as a consequence of the interaction between the Brij-residues and the protein. Similar results have been obtained with zein nanoparticles prepared with other non-ionic surfactants such as Tween 80 as previously reported [41,56]. However, the surface charge could be modulated as a function of the proposed application, administration route (i.e. oral, parenteral) and the required therapeutic outcome. For example, the positively charged systems had several advantages over the anionic or neutral nanoparticles. In particular, they demonstrated a huge potential as gene delivery systems due to their strong ability to interact with negatively-charged genetic material as well as to interact with biological barriers [57,58]. Moreover, Fig. 1A shows that zein nanoparticles prepared with Brij C10, Brij S20 and Brij O10 preserved their diameters up to 37 °C with respect to the surfactant-free formulation, suggesting the capacity of the non-ionic surfactant to improve the thermal stability of the colloidal systems (Fig. 1A). The graphic representation of the size distribution of the samples prepared with 0.2 % w/v of the various surfactants confirms the aforementioned trend of the protective features exerted by the stabilizers towards the zein nanoparticles (Fig. 1B). In particular, the carriers prepared with Brij O10 gave the best results on the polydispersity index, confirming the crucial role this compound plays in the stabilization of the nanosystems. This may be related to the presence of the ideal oleyl ether and polyoxyethylene residues present in the surfactant structure, which could be arranged between the lipophilic and hydrophilic portions of the protein, respectively, contributing significantly to the stabilization of the particle structure.

The thermal stability of the various Brij-stabilized zein-based nanoparticles was also investigated at 50 °C and 60 °C (Table S1), except for Brij derivatives L4 and Brij O2 which evidenced the formation of aggregates as well as large and heterogeneous population at 37 °C. For this reason, their physico-chemical features were not evaluated at higher temperatures.

The nanoparticles containing Brij S20 and C10 at a concentration of 0.2 % w/v evidenced an increase of the mean sizes and polydispersity index when the temperature increased up to 60 °C (Table S1). On the contrary, the sample prepared using Brij O10 as stabilizer showed the lowest increase of the mean sizes with respect to the other Brij derivatives, confirming the efficacy of this surfactant to protect zein nanoparticles from the destabilizing phenomena promoted by the heating process.

The size of the nanoparticles was confirmed by NTA analysis which evidenced no significant differences compared to the results obtained by dynamic light scattering (Fig. S1). Indeed, the zein nanoparticles

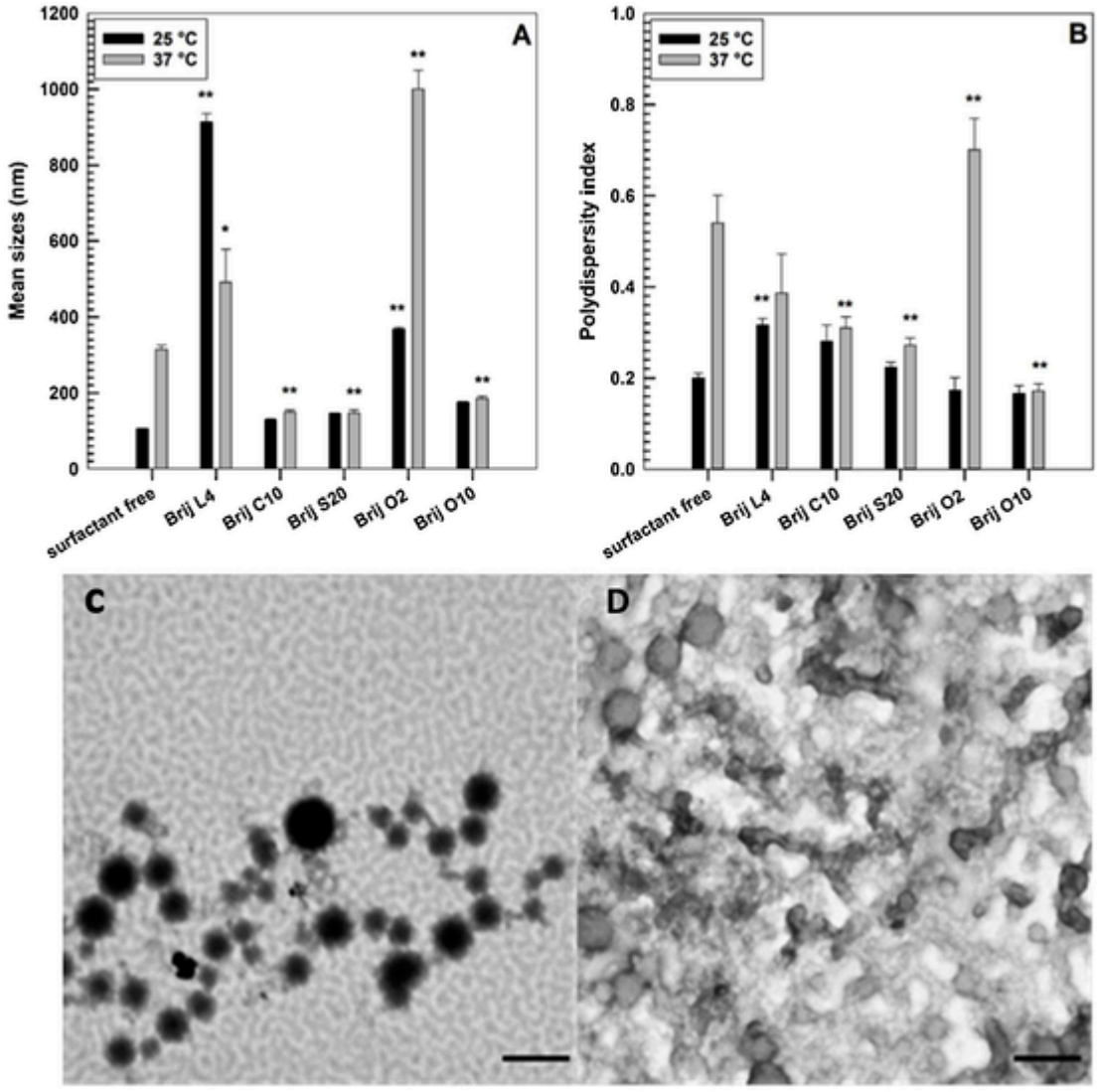

Fig. 1. Evaluation of the mean sizes (A) and polydispersity index (B) of zein nanoparticles (2 mg/mL of protein) stabilized by using various Brij derivatives (0.2 % *w/v* of surfactant) as a function of the temperature. *p < 0.05; **p < 0.001, as compared to the surfactant-free formulation. TEM micrographs of zein nanoparticles prepared with 2 mg/mL of zein and 0.2 % w/v of Brij O10 (C) and 0.2 % w/v of Brij L4 (D). Scale bar =200 nm.

prepared with Brij O10 at 0.2 % w/v showed an average diameter of about 150 nm with a homogeneous dimensional distribution of the population, confirming the capacity of this surfactant to interact positively with the protein. In this regard, the combination of multiple characterization methods could be an effective strategy for removing the uncertainties that each technique used individually may cause, thus providing a more accurate representation of the dimensional distribution of the samples [59,61].

In addition, zein nanoparticles prepared with Brij O10 were characterized by a smooth spherical shape while the formulation prepared with Brij L4 showed the presence of large aggregates and a heterogenous population, confirming the photon correlation spectroscopy data (Fig. 1C and D).

3.2. Stability evaluation of zein nanoparticles

The stability of the formulations prepared with the different Brij molecules was also investigated using the Turbiscan Lab® Expert apparatus and expressed as TSI [62,63]. Specifically, as can be seen in Fig. S2, the presence of small amounts of Brij L4 (0.002 % w/v) gave the characteristic TSI profiles of unstable formulations, suggesting a time-dependent variability of the particle distribution, while the use of Brij C10 and Brij S20 as surfactants evidenced a considerable decrease in the TSI values, indicating significant stabilization of the nanosystems. However, as shown in Fig. S2 the concentration of the surfactant affects the kinetic profiles of the nanoparticles; indeed, high concentrations of both molecules promoted an increase of the TSI slopes evidencing the presence of some adverse phenomena.

A different trend was observed with the formulations prepared with Brij O2 and Brij O10 (Fig. S2). Indeed, the zein nanoparticles stabilized with different amounts of both surfactants showed no significant variation of the TSI profiles, confirming the positive influence of the compounds on the colloidal structure. This finding is probably related to the presence of an unsaturated oleyl-based alkyl chain which contributes to an effective stabilization of the colloidal structure. This suggests that the lipophilic moiety of both stabilizers seems to have a predominant role in this stabilization with respect to the length of the polyethylene glycol chain. Considering the data previously discussed, the nanoparticles prepared with 0.2 % w/v of Brij O10 provided the best results and were the most suitable systems to be proposed for the delivery of compounds.

3.3. Impact of pH on the physico-chemical features of zein nanoparticles

In order to investigate the influence of pH on the behavior of the various zein samples, the mean sizes and zeta potential values were evaluated as a function of different values of this parameter (Fig. 2). As reported in our previous work, surfactant-free nanoparticles had an average diameter >1000 nm at pH values above 6.0 due to destabilizing events that come about which are related to the isoelectric point of protein (pI 6.2), while at pH 4.0 they maintained their normal diameters (<300 nm) [41].

The lack of electrical repulsion between zein nanoparticles in alkaline environments promoted the decrease of their surface charges, data in agreement with other experimental works [64], while in an acidic environment, the Z-potential shifted toward positive values, due to the protonation of the residues of the biopolymer (Fig. 2B) [65]. The presence of Brij derivatives (S20, O2 and O10) minimized the negative effect induced by the pH variation on the nanosystems, preventing the formation of macroaggregates. In particular, even though the addition of the investigated Brij derivatives showed similar zeta potential values at all pH values, the use of Brij 010 evidenced no significant variation in the mean sizes.

This is probably related to the peculiar physico-chemical characteristics of the unsaturated O10 derivative which promoted the formation of favorable protein-surfactant interactions, confirming the capacity of this surfactant to preserve the colloidal structure of the zein nanoparticles at all the pH ranges.

3.4. Serum stability

Zein nanoparticles were incubated in 70 % FBS at 37 °C and their size was investigated for up to 24 h. The surfactant-free nanoparticles showed a constant increase in their average size, probably due to the progressive interaction with the serum proteins that came about (Fig. 2C). Similar results were obtained with the samples prepared with Brij

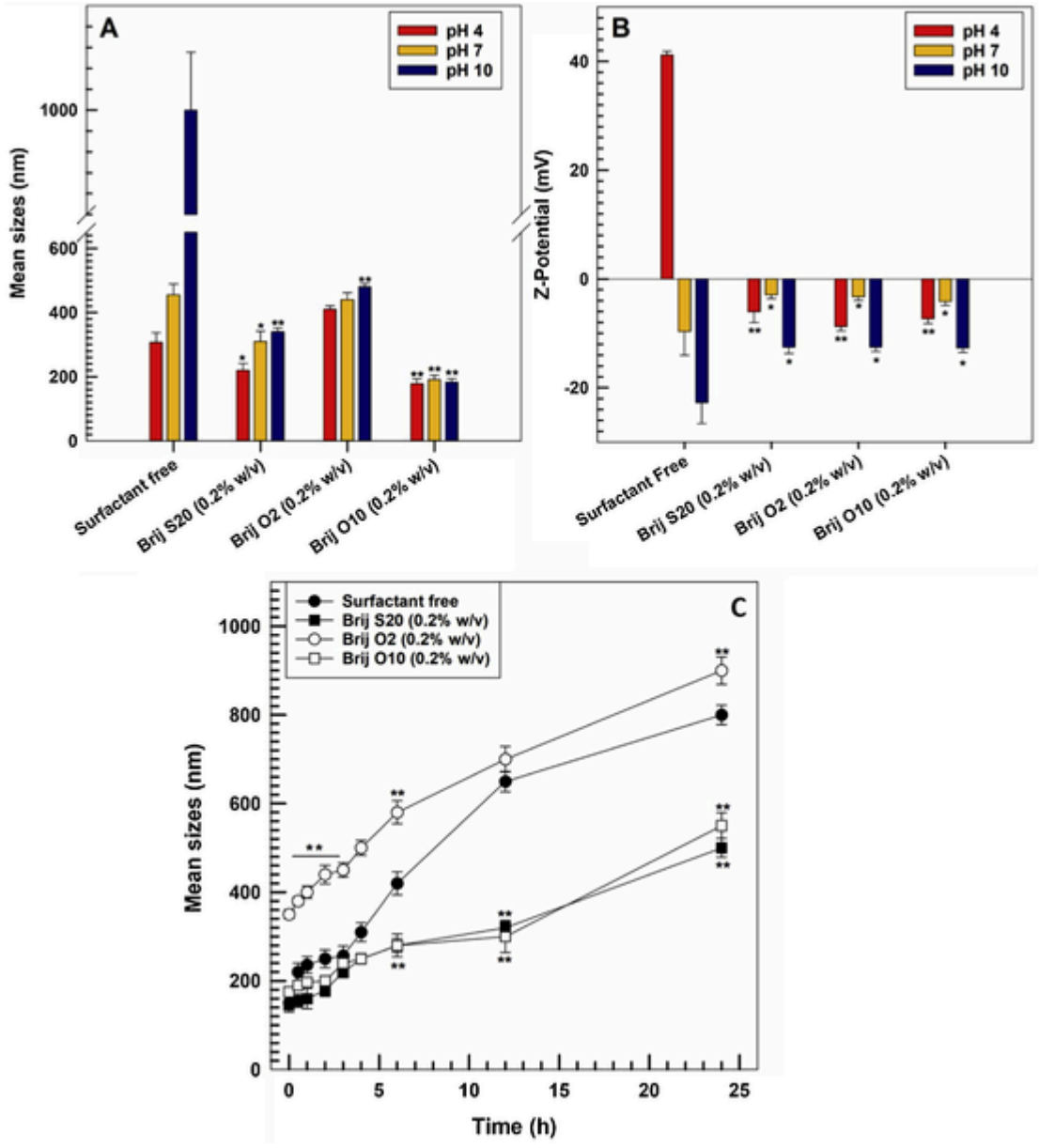

Fig. 2. Influence of pH on the average size (A) and zeta potential values (B) of zein nanoparticles (2 mg/mL of protein) prepared with different Brij derivatives at a concentration of 0.2 % w/v. Influence of serum (C) on the stability of zein nanoparticles prepared using a protein concentration of 2 mg/mL as a function of incubation time. *p < 0.05; **p < 0.001, as compared to the surfactant-free formulation.

O2, although the initial average diameter of these systems was greater as compared to the mean size of the surfactant-free nanoparticles when incubated in serum (Table 2, Fig. S4). On the other hand, the zein nanoparticles stabilized with Brij S20 or Brij O10 provided suitable serum stability; the average size was <200 nm in the first hours of incubation and only a slight increase of their diameter with respect to the other formulations was observed over time.

However, the results require additional studies concerning the nature of the plasmatic proteins adsorbed onto the colloidal surface after incubation in serum in order to fully understand the *in vivo* fate of the nanosystems at systemic, tissue, and cellular levels [66].

3.5. Freeze-drying of zein nanoparticles

A mandatory step in providing a conceivable method for promoting the long-term storage stability of nanoparticles is the evaluation of the physico-chemical characteristics of a colloidal system after the freeze-drying process. As reported above, Brij O10 was proposed as a promising stabilizer for use in obtaining stable zein nanoparticles. For this reason, lyophilization studies were performed on the zein nanosystems prepared with Brij O10 (0.2 %w/v), as a function of various cryoprotectants. As shown in Table 3, the formulation prepared with 5% w/v of mannitol gave the best results because the mean sizes of the nanosystems were preserved after the freeze-drying procedure, results that are in agreement with previous studies [67,68]. In fact, these nanosystems showed an average diameter of about 150 nm and a homogeneous and monodisperse size distribution. This finding could be related to the physico-chemical features of mannitol (a non-reducing sugar) which can prevent potential Maillard reactions between the excipient and the protein, a phenomenon that often occurs in protein-based nanoformulations following the lyophilization procedure [69].

The addition of all the other cryoprotectants caused a noticeable increase in the aforementioned parameters, and the presence of macroaggregates was observed upon rehydration. The failure of these cryoprotectants to prevent the aggregation of the zein nanoparticles may be due to their chemical structure and/or to a decreased interaction with the nanosystems following the freeze-drying process [70]. Moreover, the nanoformulations were characterized by a positive surface charge that was, however, close to neutrality, suggesting a certain difficulty in obtaining stable freeze-dried nanoparticles These results point the way towards effectively obtaining the long-term stability of Brij O10-stabilized zein nanoparticles by rehydrating a powder-based formulation that could be used for pharmaceutical purposes.

3.6. Cytotoxicity of surfactant-free and Brij O10-stabilized zein nanoparticles

During the phases of characterization of a novel colloidal system, cytotoxicity evaluation is another fundamental aspect to be investigated because the biocompatibility of the new formulation can affect its real clinical application [71,72]. For this reason, different amounts of surfactant-free and Brij O10-stabilized zein nanoparticles were tested as a function of both polymer concentration and incubation time on the C-28, Nthy-ori 3–1, A549 and BCPAP used as model of normal and cancer human cell lines. The fact that Brij-stabilized nanosystems are cationic in nature could promote the efficacious delivery of a drug to the lungs so A549 cells were chosen as a model of non-small cell lung cancer, while the BCPAP cells were selected as a model of poorly-differentiated thyroid carcinoma [73]. Surfactant-free zein nanoparticles promoted a decrease in cell viability by 25 %–30 % only after 72 h incubation at high protein concentrations (100 μg/mL). This was true for all cell lines, confirming the low toxicity of this biopolymer as described in our previous investigations (Fig. 3) [26,41,42]. On the other hand, the formulation decorated with Brij O10 showed greater cytotoxicity at a polymer concentration of ≥25 μg/mL after only 24 h incubation on all the cell lines investigated, demonstrating that this is the maximum formulation concentration that can be used for *in vitro* experiments and confirming the results obtained using negatively-charged sodium deoxycholate-stabilized nanosystems [41].

3.7. Entrapment efficiency and release profiles of model compounds

The ability of the polymeric matrix to effectively retain active compounds characterized by different physico-chemical features is another aspect to be evaluated during the pre-formulation studies, so rhodamine B and bromophenol blue were chosen as hydrophilic and lipophilic model drugs, respectively. Fig. 4 shows the retention of both molecules in the colloidal structure as a function of the amount of drug initially added. It is interesting to observe that there is significant retention of the lipophilic molecule even when a great amount of the compound is used to prepare the colloidal systems, as in this case. Specifically, the addition of 0.6 and 0.8 mg/mL of bromophenol blue to the organic phase favored an encapsulation of the probe in the polymeric matrix of about ~80 % and ~77 %, respectively. This was mainly due to the hydrophobic interactions occurring between the dibromophenyl residue and the apolar groups of zein, as well as the hydrogen bonds between the hydroxyl portions of the molecule and the carbonyl units of the biomaterial. This confirmed the previous results when zein gels containing bromophenol blue were used [74,75]. Moreover, the significant encapsulation of the hydrophobic compound within zein nanosystems is in agreement with several other investigations already published,

Table 3
Physicochemical features of zein nanoparticles (2 mg/mL of protein) prepared with Brij O10 (0.2 % w/v) after the freeze-drying process with respect to the amount of cryoprotectant used.

Cryoprotectant	Concentration (% w/v)	Mean sizes (nm)	Polydispersity index	Zeta potential (mV)
before		176 ± 1	0.166 ± 0.017	10.2 ± 0.6
–	–	≥1000**	0.911 ± 0.104**	5.1 ± 1.5*
Glucose	5	≥1000**	0.940 ± 0.063**	2.3 ± 2.6**
	10	≥1000**	0.961 ± 0.068**	5.1 ± 1.7*
Mannitol	5	149 ± 3	0.182 ± 0.012	9.2 ± 0.9
	10	≥1000**	0.791 ± 0.218**	4.3 ± 4.5**
Mannose	5	≥1000**	0.794 ± 0.181**	4.1 ± 0.8**
	10	867 ± 9**	0.532 ± 0.041**	6.2 ± 1.7*
Sucrose	5	496 ± 18**	0.476 ± 0.014**	8.8 ± 1.5
	10	≥1000**	0.616 ± 0.153**	6.9 ± 1.8
Trehalose	5	≥1000**	0.908 ± 0.082**	6.1 ± 1.6*
	10	≥1000**	0.834 ± 0.144**	3.4 ± 0.6**

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

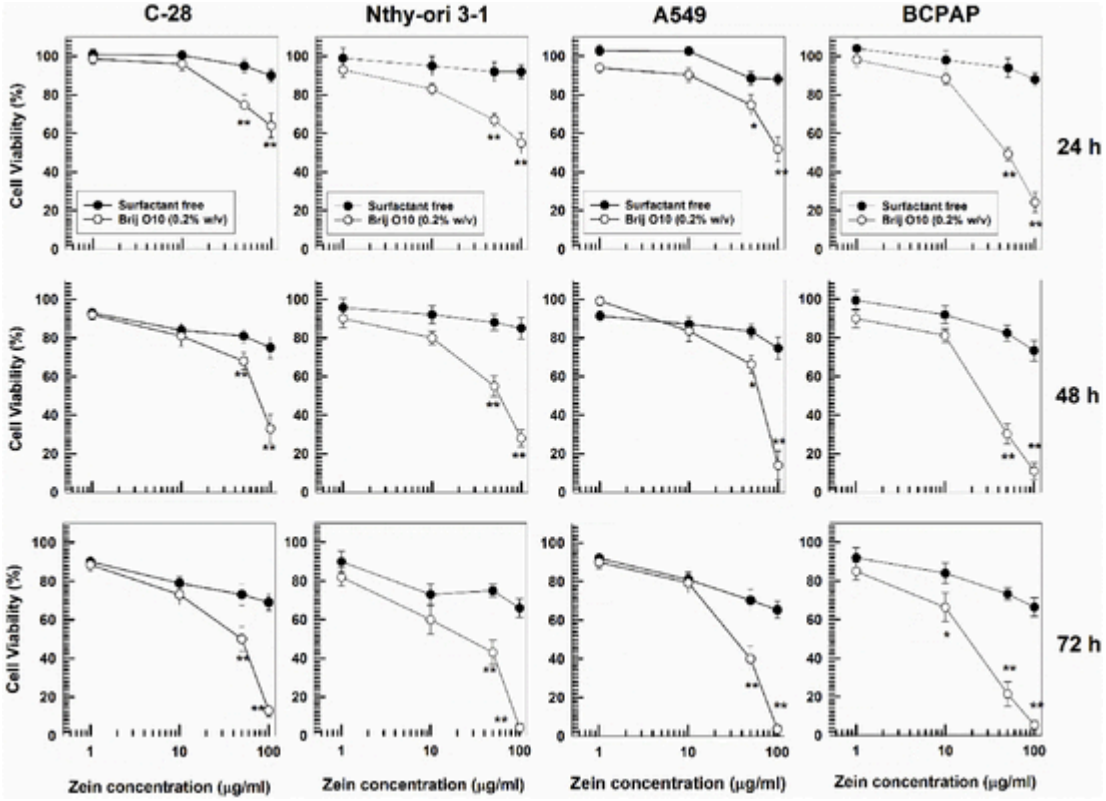

Fig. 3. *In vitro* cytotoxicity of zein nanoparticles on C-28, Nthy-ori 3-1, A549 and BCPAP cell lines as a function of protein concentration and incubation time. The data are expressed as a percentage of cell viability assessed by MTT testing. The results were obtained from the average of four different experiments ± standard deviation. * < 0.05; ** < 0.001 with respect to the surfactant-free formulation.

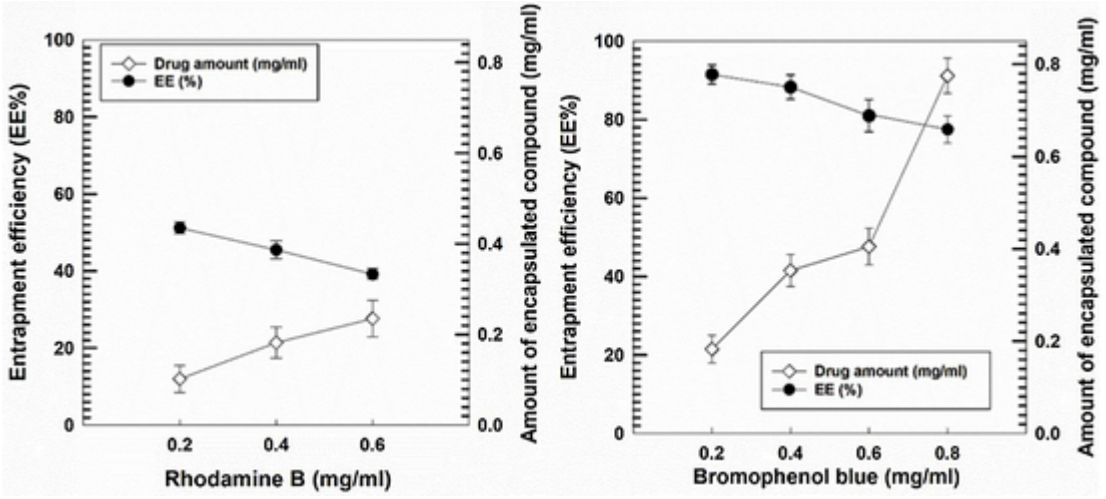

Fig. 4. Evaluation of the entrapment efficiency of rhodamine B and bromophenol blue in zein nanosystems (2 mg/mL of protein) prepared with Brij O10 (0.2 % w/v). The data represent the average of three experiments ± standard deviation.

which evidence the great ability of the biopolymer to efficiently entrap poorly water-soluble compounds [76,77].

Conversely, it is possible to observe that the addition of different amounts of rhodamine B promoted a substantial decrease of its entrapment efficiency within the zein nanoparticles. Specifically, ~40 % of rhodamine B was retained by the polymeric matrix when 0.6 mg/mL of probe was initially used, while increasing amounts of the compound favored the formation of macroaggregates and sediments, suggesting a conspicuous destabilization of the colloidal structure (Fig. 4). This phenomenon is related to a significant decrease in drug retention probably as a consequence of the saturation of the physical compartments able to interact with the compound. In addition, the zein nanoparticles containing both model compounds showed a considerable drug loading capacity suggesting the noteworthy ability of the protein matrix to efficiently retain several drugs, as previously reported (Table S2) [26,42].

The influence of the physico-chemical properties of the model drugs on the colloidal systems was also confirmed through investigation of the release profiles of the entrapped compounds. As shown in Fig. 5, the amphiphilicity of zein protein showed a constant and prolonged release of both probes over time, corroborating the data previously reported [75].

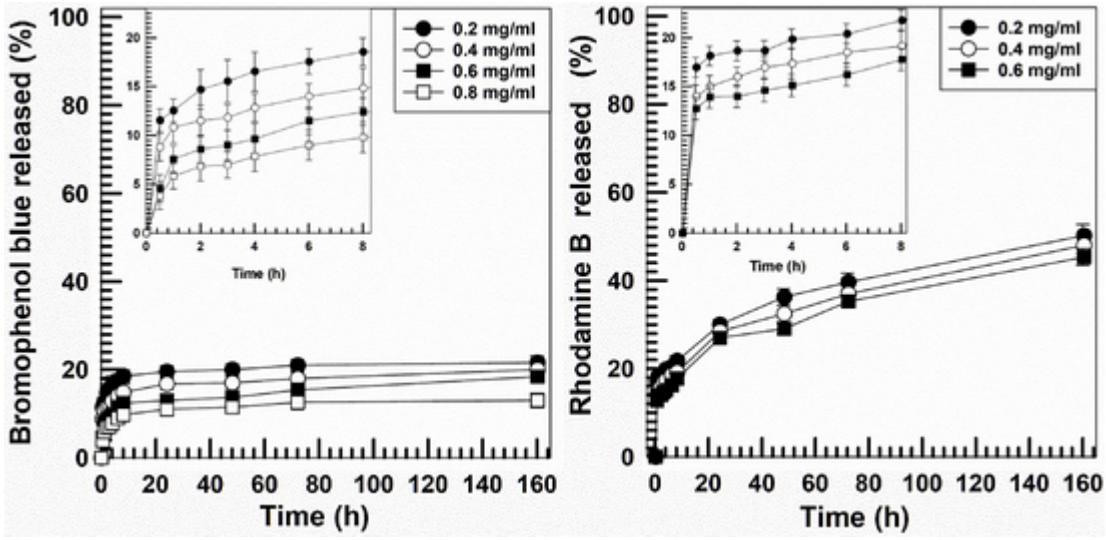

Fig. 5. Release profiles of bromophenol blue and rhodamine B from zein nanoparticles (2 mg/mL of protein) prepared with Brij O10 (0.2 % w/v). Values represent the mean of three different experiments ± standard deviation.

Namely, the release profile of rhodamine B from the zein nanoparticles was modulated by the amount of the encapsulated probe, evidencing significant interaction between the carboxyl and amine groups of the molecule and also the polar moieties of the biopolymer [74,78]. In particular, the formulation containing the higher concentration of the hydrophilic dye was characterized by a drug release of ~40 % after 7 days of analysis (Fig. 5). However, the slower release profile of bromophenol blue suggests the greater ability of the protein-based system to retain the compound as a consequence of the lipophilic nature of the model drug. These findings demonstrate the potential application of Brij O10-stabilized zein nanosystems as suitable carriers for the controlled release of both hydrophilic and lipophilic drugs.

4. Conclusions

The peculiar features of zein used as a natural polymer to develop nanoparticles has been drawing increasing attention due to its ability to encapsulate a broad range of bioactive compounds for food, pharmaceutical and biomedical purposes [79]. In this work, yellow zein was the raw material employed with the aim of developing an innovative low-cost nanoformulation useful for various applications. The physico-chemical features as well as the time- and temperature-stability of the resulting nanosystems have shown that they are influenced by the addition of different types of Brij surfactants. In this regard, among the emulsifiers that were analyzed, Brij O10 gave the best results because it affected neither the average diameter nor the size distribution of the nanoparticles, but rather improved the physical stability of the colloids.

The use of mannitol as cryoprotectant resulted in a powder formulation that is easily rehydrated in water, thus evidencing the formation of a system with good storage features. Zein nanoparticles have also been shown to efficiently retain different amounts of both hydrophilic and lipophilic compounds and to confer a prolonged release of the entrapped molecules. Brij O10 stabilized the zein nanosystems, giving a favorable *in vitro* safety profile on various cell lines up to a concentration of <50 μg/mL of protein. The presence of Brij O10 in the colloidal structure may furnish the formulation with an intrinsic antitumor property, as a consequence of the inhibitory activity exerted by the surfactant on the active efflux pumps that are over-expressed in many tumors. Additional investigation is in progress in order to evaluate this aspect.

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Uncited reference

[60].

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2021.111647.

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