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RESEARCH ARTICLE

Development and *In Vitro* Assessment of Butyrylcholinesterase-Loaded Micelles for Protection Against Central Neurotoxicity Induced by Organophosphate Exposure

Gizem Ruya TOPAL[*], Ozgur ESIM[**°]

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SUMMARY

Organophosphate (OP) compounds are highly toxic chemicals widely used as pesticides and nerve agents, causing severe peripheral and central nervous system toxicity through disruption of cholinergic signaling. The therapeutic efficacy of butyrylcholinesterase (BChE), a promising bioscavenger against OP poisoning, is limited by poor penetration across the blood-brain barrier (BBB). In this study, biomimetic rhamnolipid-based micelles were developed as a nanocarrier system to enhance BChE delivery and improve protection against OP-induced central neurotoxicity. BChE-loaded micelles were prepared using an ultrasonication-assisted homogenization method and optimized through a 3^2 factorial design using lecithin and rhamnolipid as formulation variables. Particle size (PS), polydispersity index (PDI), and zeta potential (ZP) were evaluated as critical quality attributes. The optimized formulation exhibited a particle size of 171.2 ± 5.79 nm, PDI of 0.45 ± 0.03, and zeta potential of −16.6 ± 3.6 mV. Encapsulation efficiency was high, indicating successful incorporation of BChE into the micellar structure. In vitro release studies demonstrated a sustained release profile compared with free BChE. FT-IR and TEM analyses confirmed compatibility of formulation components and nearly spherical morphology of the micelles. Cytotoxicity studies on CaCo-2 and SH-SY5Y cell lines showed that the developed micelles were well tolerated at therapeutically relevant concentrations (0.1–100 μM), while also reducing the cytotoxicity associated with free BChE exposure. These findings suggest that rhamnolipid-based BChE-loaded micelles represent a promising strategy for central nervous system-targeted detoxification and may offer an effective therapeutic platform for the management of OP-induced neurotoxicity.

Keywords: *Butyrylcholinesterase, micelles, organophosphate toxicity, SH-SY5Y, Caco-2.*

Organofosfat Maruziyetinin Neden Olduğu Santral Nörotoksisiteye Karşı Koruma İçin Butirilkolinesteraz Yüklü Misellerin Geliştirilmesi ve İn Vitro Değerlendirilmesi

ÖZ

Organofosfat (OP) bileşikleri, pestisit ve kimyasal savaş ajanı olarak yaygın şekilde kullanılan, kolinerjik sinyal iletimini bozarak ciddi periferik ve merkezi sinir sistemi toksisitesine neden olan yüksek derecede toksik kimyasallardır. OP zehirlenmesine karşı umut verici bir biyolojik detoksifikasyon enzimi olan bütirilkolinesterazın (BChE) terapötik etkinliği, kan-beyin bariyerini (BBB) sınırlı düzeyde geçebilmesi nedeniyle kısıtlanmaktadır. Bu çalışmada, BChE taşınımını artırmak ve OP kaynaklı santral nörotoksisiteye karşı korumayı geliştirmek amacıyla biyomimetik ramnolipit bazlı miseller nanotaşıyıcı sistem olarak geliştirilmiştir. BChE yüklü miseller, ultrasonik destekli homojenizasyon yöntemi ile hazırlanmış ve lesitin ile ramnolipidin formülasyon değişkeni olarak kullanıldığı 3^2 faktöriyel deney tasarımı ile optimize edilmiştir. Partikül boyutu (PS), polidispersite indeksi (PDI) ve zeta potansiyeli (ZP) kritik kalite parametreleri olarak değerlendirilmiştir. Optimize edilen formülasyonun partikül boyutu 171,2 ± 5,79 nm, PDI değeri 0,45 ± 0,03 ve zeta potansiyeli −16,6 ± 3,6 mV olarak belirlenmiştir. Enkapsülasyon etkinliğinin yüksek bulunması, BChE'nin misel yapısına başarılı şekilde yüklendiğini göstermiştir. İn vitro salım çalışmaları, serbest BChE'ye kıyasla kontrollü salımın sağlandığı bir profil ortaya koymuştur. FT-IR ve TEM analizleri, formülasyon bileşenleri arasında uyumluluk bulunduğunu ve misellerin yaklaşık küresel morfolojiye sahip olduğunu doğrulamıştır. CaCo-2 ve SH-SY5Y hücre hatlarında gerçekleştirilen sitotoksisite çalışmaları, geliştirilen misellerin terapötik olarak anlamlı konsantrasyonlarda (0,1–100 μM) iyi tolere edildiğini ve serbest BChE uygulamasına bağlı sitotoksisiteyi azalttığını göstermiştir. Bu bulgular, ramnolipit bazlı BChE yüklü misellerin merkezi sinir sistemine hedefli detoksifikasyon için umut verici bir yaklaşım olduğunu ve OP kaynaklı nörotoksisitenin tedavisinde etkili bir terapötik platform sunabileceğini göstermektedir.

Anahtar Kelimeler: *Butirilkolinesteraz, miseller, organofosfat toksisitesi, SH-SY5Y, Caco-2.*

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INTRODUCTION

Organophosphate (OP) compounds are a group of chemicals with high toxic potential, widely used as pesticides and developed as nerve agents. Exposure to OPs can occur through inhalation, ingestion, and dermal absorption. OP poisonings resulting from accidental or intentional exposure are among the most common poisonings worldwide. OP compounds cause approximately three million poisoning cases annually and hundreds of thousands of deaths each year in developing countries (Robb, Regina, & Baker, 2023). OP poisoning disrupts neurotransmitter signaling and physiologically results in a cholinergic crisis. Along with significant peripheral effects, OP exposure may also lead to neuropathological injury and neurobehavioral impairments. It is known that organophosphate compounds exert their effects by binding to butyrylcholinesterase (BChE) (Bennion et al., 2013). However, effective treatment is limited by the blood-brain barrier (BBB) and other central nervous system (CNS) barriers.

The BBB is primarily a membranous barrier that separates the brain from the systemic circulation. It consists of a specialized semipermeable endothelial cell membrane system that maintains homeostasis in the brain by protecting it from harmful substances and toxins circulating in the blood while allowing the passage of essential nutrients (Zlokovic, 2008). OPs exert their toxic effects by binding to BChE; however, peptide-based agents with poor barrier permeability demonstrate limited therapeutic efficacy because of the BBB effect (Bennion et al., 2013). These challenges emphasize the importance of nanotechnology in modifying or controlling cellular-level processes to obtain the desired properties. Owing to their nanoscale size, nanoparticles can cross the BBB and represent an effective alternative to conventional drug delivery methods and other therapeutic approaches (Mittal et al., 2022).

Nanoparticles offer an advantageous approach for enhancing CNS delivery. Nanotechnology offers significant potential to enhance therapeutic and diagnostic strategies for CNS disorders while improving efficient drug delivery. Through nanoengineering, drugs can be designed to cross the BBB, modulate signaling pathways, target specific cells, enable effective gene delivery, and support the regeneration and protection of nerve cells (Ali & Chen, 2015). Numerous carrier systems, including liposomes, SLNs, nanoemulsions, dendrimers, and micelles, have been applied in CNS disorders, and successful outcomes have been achieved in overcoming the BBB (Mittal et al., 2022).

Glycolipid biosurfactants are structures composed of mono- and disaccharides linked by glycosidic bonds to hydroxylated carboxylic acids. They are commercially available and, more importantly, are considered toxicologically safe while exhibiting strong surface activity. Rhamnolipids, glycolipid surfactants with reported antioxidant and anticancer properties, have recently gained attention for biomedical applications. Studies have demonstrated that rhamnolipids, glycolipid-derived biosurfactants, can enhance both paracellular and transcellular transport across Caco-2 monolayers, an *in vitro* model of the human small intestinal epithelium, while also suppressing P-gp activity (Ghazaei, 2021). Several studies have already employed rhamnolipids as surfactants for the formation or stabilization of nanosystems (Azevedo et al., 2021). Micelles are nanometer-sized colloidal aggregates that spontaneously form with a lipophilic core and a hydrophilic exterior layer. They have been extensively utilized to increase the aqueous solubility of poorly soluble drugs and offer promising features as carriers for drug delivery applications (Torchilin, 2005). Especially, lipid-core micelles can incorporate a wide range of poorly soluble drugs and exhibit high stability, both in maintaining their structural integrity and in retaining the encapsulated compounds. Moreover, numerous studies have reported that their application in CNS delivery provides advantages by facilitating the passage of active compounds across

biological barriers (Micheli, Bova, Magini, Polidoro, & Emiliani, 2012).

Although BChE has previously been incorporated into liposomal and polymersomal systems for organophosphate detoxification, the development of biomimetic rhamnolipid-based micelles for BChE delivery has not been reported to date. Therefore, this study aimed to develop rhamnolipid-based micelles as a novel nanocarrier platform to enhance BChE delivery for the treatment of organophosphate-induced central neurotoxicity. In addition to serving as biocompatible carrier materials, rhamnolipids possess membrane-interacting properties that may facilitate transport across biological barriers. Following formulation optimization and physicochemical characterization, the biological performance of the optimized micelles was evaluated using CaCo-2 and SH-SY5Y cell models.

MATERIALS AND METHODS

Materials

Butyrylcholinesterase, lecithin, Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and DMSO were purchased from Sigma (Steinheim, Germany). Rhamnolipid was obtained from AGAE Technologies, Corvallis, USA. Butyrylcholinesterase ELISA Kit was purchased from BT-Lab (Shanghai, China). Dulbecco's Modified Eagles Medium with high glucose, L-glutamine, sodium pyruvate (DMEM), trypsin-EDTA (0.25%), penicillin-streptomycin, and sterile PBS solution were provided from Vivacell (Denzlingen, Germany). All other chemicals used were of analytical grade.

Preparation and optimization of micelles

Rhamnolipid-based micelles were prepared using an ultrasonication-assisted homogenization method. Different ratios of phospholipid and rhamnolipid were dissolved in 20 mL of phosphate-buffered saline at pH 7.0. The solution was heated in a water bath at 35 °C for 15 minutes and then homogenized at room temperature (25 °C) using a high-speed homogenizer (Ultra-Turrax, IKA T-25, Staufen im Breisgau, Germany) at 13,500 rpm for 5 minutes. For the preparation of BChE-loaded micelles (BChE-micelles), 2 mg of BChE was first dissolved in PBS before the addition of the phospholipid and surfactant mixture, followed by stirring for 30 minutes (Niaz, Shabbir, Noor, & Imran, 2019).

Before the experimental design, preliminary formulation studies were conducted to identify the critical formulation variables affecting micelle formation. Several formulation, and processing parameters, including lecithin concentration, rhamnolipid concentration and homogenization conditions, were initially evaluated. Based on these screening studies, lecithin and rhamnolipid concentrations were identified as the most influential formulation variables, whereas the remaining process parameters were fixed at their optimized levels. Therefore, a 3^2 full factorial design was selected to optimize the formulation.

The 3^2 full factorial design was considered appropriate because only two independent variables, lecithin (X_1: 25, 37.5, and 50 mg) and rhamnolipid (X_2: 0, 12.5, and 25 mg), were investigated at three levels. This design enables simultaneous evaluation of the main effects, interaction effects, and potential curvature of the selected variables while requiring a relatively small number of experimental runs.

Particle size (PS), polydispersity index (PDI), and zeta potential (ZP) were selected as the response variables because they represent the critical quality attributes (CQAs) of nanocarrier systems. Particle size strongly influences cellular uptake, biodistribution, and transport across biological barriers, particularly in central nervous system-targeted delivery. The PDI reflects the homogeneity of the nanoparticle population and serves as an indicator of formulation quality and reproducibility. Zeta potential provides information on surface charge and colloidal stability by influencing electrostatic interactions between particles. Collectively, these physicochemical characteristics provide a comprehensive assessment of formulation

quality and were therefore selected as the primary responses for optimization.

Particle size (PS), polydispersity index (PDI), zeta potential (ZP) measurements

PS, PDI, and ZP of the formulations were characterized. PS and PDI were measured by photon correlation spectroscopy (PCS) using a Nicomp Z3000 (PSS, New York, USA). ZP was determined by laser Doppler velocimetry. All analyses were performed in triplicate.

Encapsulation efficiency (EE%)

The amount of BChE encapsulated in the obtained micelles was determined using an ELISA kit. The method is based on the formation of thiocholine from the BChE substrate in the presence of BChE, followed by the reaction of the generated thiocholine with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), producing a yellow chromophore measured at 412 nm. This method enables measurements at low concentrations.

EE% was determined after removing the non-encapsulated BChE by centrifugation. Briefly, freshly prepared BChE-loaded micelles were centrifuged at 13,000 rpm for 20 min at 4 °C, and the supernatant containing the free (non-encapsulated) BChE was carefully collected. The micellar pellet was washed with PBS and centrifuged again under the same conditions to ensure complete removal of residual free BChE. The purified micelles were then disrupted in 5 mL ethanol, and the encapsulated BChE content was quantified using an ELISA kit. EE% percentage was calculated using the following equation (Sezigen, Esim, Sarper, & Savaser, 2020):

EE% = [Final amount of active material/Initial amount of active material] × 100 (1)

***In vitro* release studies**

The *in vitro* release behavior of BChE from the micelles was investigated using the dialysis bag diffusion technique under sink conditions. Formulation samples were transferred into dialysis membranes (12–14 kDa molecular weight cut-off, Spectrum Labs, California, USA) and immersed in 30 mL of PBS maintained at 37 ± 1 °C under continuous shaking at 50 rpm. At predetermined time intervals, aliquots were withdrawn from the release medium and immediately replaced with an equal volume of fresh PBS to maintain constant volume and sink conditions throughout the experiment. The amount of released BChE was quantified using an ELISA kit.

Fourier-transform infrared (FT-IR) spectroscopy studies

FT-IR was performed to investigate the compatibility between BChE and the formulation components and to confirm the successful encapsulation of BChE within the micelles. FT-IR spectra of the individual components and the optimized formulation were recorded using a Spectrum 400 spectrometer (PerkinElmer, USA) over the range of 600-4000 cm^{-1}.

TEM analysis

TEM imaging was performed to evaluate the morphology and particle size of the micelles. For this procedure, a carbon-coated copper grid was placed on a drop of micellar aqueous dispersion (100 μg/mL) for 10 min, and excess fluid was removed with filter paper. The specimen was then negatively stained by contacting the grid with a 2% w/v aqueous phosphotungstic acid solution (pH 7.0) for 10 s, followed by blotting to remove remaining liquid. The grid was subsequently air-dried and examined under the electron microscope using TEM.

Cytotoxicity studies

Cytotoxicity studies were carried out using a model barrier cell line (CaCo-2) and a model efficacy cell line (SH-SY5Y). Cells were seeded into 96-well plates at a density of 5×10^4 cells/well and allowed to attach overnight under standard culture conditions (37 °C, 5% CO_2). The experiments were initiated after adequate surface attachment had been achieved. The cells were then treated with free BChE and BChE-loaded micelles at concentrations ranging from 0.1 to 100 μM for 24, 48, and 72 h. After the incubation period,

the treatment medium was removed, and MTT solution (0.5 mg/mL) was added to each well, followed by incubation for 4 h. The resulting formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm using a microplate reader. Cell viability was calculated relative to untreated control cells (Fotakis & Timbrell, 2006).

RESULTS AND DISCUSSION

Preparation and *in vitro* characterization studies

Micelles were successfully prepared, and the formulation compositions together with the obtained results were summarized in Table 1.

Table 1. Optimization of lecithin-rhamnolipid micelles

Code	Lecithin (mg)	Rhamnolipid (mg)	PS (nm±SD)	PDI (mean±SD)	ZP (mv±SD)
F1	50	0	692±21.0	0.80±0.40	-31.3±5.1
F2	25	0	498.6±33.0	0.73±0.08	-23.1±1.4
F3	37.5	12.5	226.6±0.3	0.33±0.01	-23±1.3
F4	25	25	169.1±5.4	0.41±0.02	-11±2.4
F5	50	12.5	298.2±1.9	0.46±0.02	-16±1.7
F6	25	12.5	240.6±6.1	0.50±0.03	-43±0.9
F7	37.5	25	223.7±1.5	0.37±0.01	-15±2.1
F8	37.5	0	819.1±12.2	0.90±0.09	-29±4.9
F9	50	25	227.4±5.7	0.35±0.03	-17±0.5

For PS, the quadratic model was found to be significant (Table 2.). The response surface plot illustrating the effect of formulation variables on PS is also presented in Figure 1. Rhamnolipid ratio was the only factor influencing PS, exhibiting an inverse relationship, where increasing rhamnolipid content resulted in smaller particle sizes. In the absence of rhamnolipid, particle size varied between 498.6 nm and 819.1 nm depending on the lecithin concentration. With increasing rhamnolipid concentration, particle size ranged from 226.6 to 298.2 nm at 12.5 mg and from 169.1 to 227.4 nm at 25 mg, respectively. Rhamnolipid is a surfactant characterized by high surface activity and a low critical micelle concentration (0.01/0.2 g/L) (Arkhipov, Arkhipov, Petrova, & Filippov, 2023). In lecithin, this value is approximately 2.5 g/L (Maher, Geoghegan, & Brayden, 2023). The fact that only rhamnolipid influenced particle size may be attributed to its micelle-forming efficiency at the selected concentrations.

Table 2. Effects of model parameters on the particle size of lecithin-rhamnolipid micelles

ANOVA Analysis of Variance table for the quadratic model						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	**418876.2**	**5**	**83775.24**	**9.265393**	**0.0482**	**significant**
A-lesitin	15944.42	1	15944.42	1.763424	0.2762	
B-ramnolipit	**321785**	**1**	**321785**	**35.58886**	**0.0094**	
AB	4563.003	1	4563.003	0.50466	0.5287	
A^2	9471.467	1	9471.467	1.047527	0.3814	
B^2	67112.27	1	67112.27	7.422498	0.0723	
Residual	27125.21	3	9041.736			
Cor Total	446001.4	8				

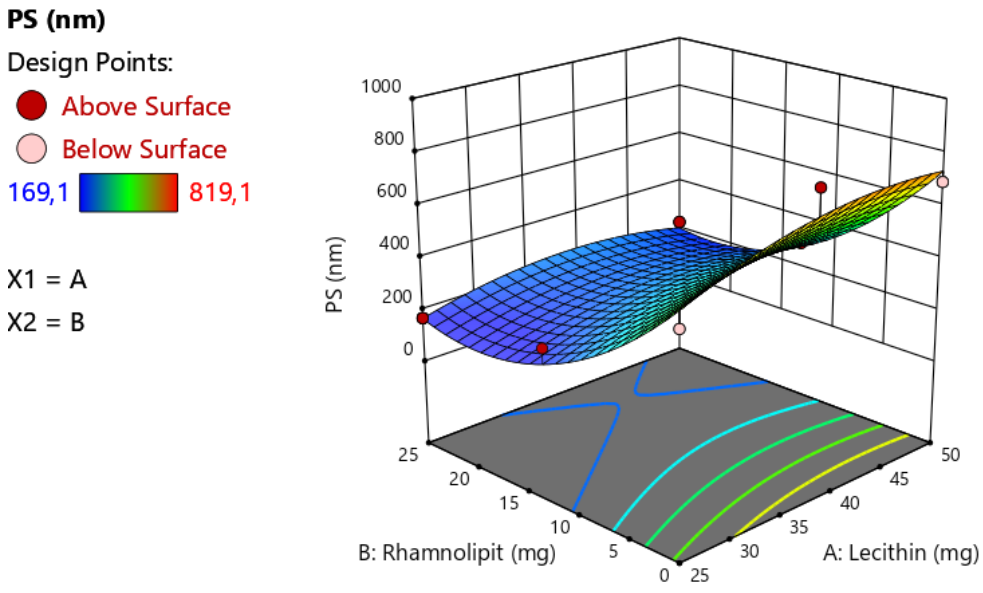


Figure 1. Response surface plot showing the influence of formulation variables on PS

For the PDI, a significant linear model was obtained, with rhamnolipid concentration again identified as the only significant variable. The significance table corresponding to the obtained results is presented in Table 3. The response surface plot showing the influence of formulation variables on PS is presented in Figure 2. It was determined that the only parameter affecting the PDI values of the prepared micelles was the rhamnolipid ratio. This may again be attributed to the low critical micelle concentration of rhamnolipid.

Table 3. Effects of model parameters on the polydispersity index of lecithin-rhamnolipid micelles

ANOVA Analysis of Variance table for the linear model						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	**0.268426**	**2**	**0.134213**	**8.974457**	**0.0157**	**significant**
A-lesitin	3.27E-05	1	3.27E-05	0.002184	0.9642	
B-ramnolipit	**0.268394**	**1**	**0.268394**	**17.94673**	**0.0055**	
Residual	0.08973	6	0.014955			
Cor Total	0.358156	8				

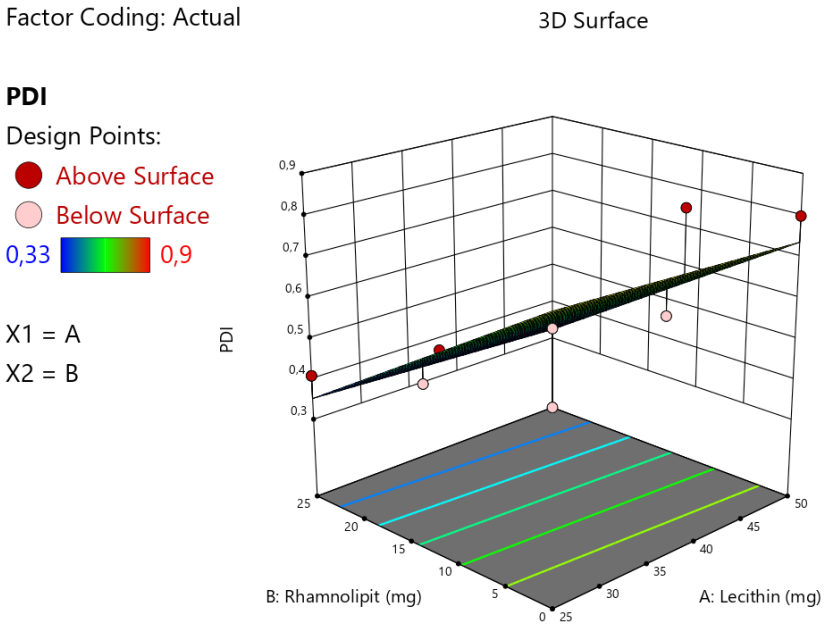


Figure 2. Response surface plot showing the influence of formulation variables on PDI

When the results were evaluated in terms of zeta potential, none of the models yielded significant results. The significance table corresponding to the obtained findings is presented in Table 4. This may be attributed to the fact that zeta potential is influenced simultaneously by multiple factors. Since the effect was limited only to the two polymers used in the study, no statistically significant results were obtained.

Table 4. Model-related results of the zeta potential measurements of lecithin-rhamnolipid micelles

Sequential Model Sum of Squares [Type I]						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Mean vs Total	4807.111	1	4807.111			Suggested
Linear vs Mean	294.8333	2	147.4167	1.775906	**0.2479**	Suggested
2FI vs Linear	1	1	1	0.010059	**0.9240**	
Quadratic vs 2FI	82.94444	2	41.47222	0.300443	**0.7604**	

According to the desirability function analysis of the experimental design, the optimum levels of the independent variables were determined to be 25 mg of lecithin and 24.6 mg of rhamnolipid. The optimization goals were established as minimizing PS, maintaining the PDI within the range of 0.3-0.5, and targeting a ZP between -15 and -45 mV, resulting in the highest overall desirability value (D = 0.867). The predicted responses together with the experimentally obtained values are presented in the corresponding tables. Using these optimized conditions, the model-directed formulation generated micelles with an average particle size of 198.5 nm, a PDI of 0.32, and a zeta potential of -21.9 mV.

The results obtained from the formulation re-prepared using the specified amounts are presented in the table 5 below. Subsequent studies were carried out based on this formulation. Following optimization of the blank micelle formulation based on PS, PDI, and ZP, BChE was incorporated into the optimized formulation. Therefore, EE was determined only for the optimized BChE-loaded micelles used in the subsequent characterization and biological studies.

Table 5. Results of the suggested formulation by Design-Expert

Code	Lecithin (mg)	Rhamnolipit (mg)	PS (nm±SD)	PDI (mean±SD)	ZP (mv±SD)	EE% (mean±SD)
Optimum formulation	25	24.6	171.2±5.79	0.45±0.03	-16.6±3.6	21.1±3.2

Based on the desirability function, the optimized formulation (25 mg lecithin and 24.6 mg rhamnolipid) demonstrated a PS of 171.2 ± 5.79 nm, a PDI of 0.45 ± 0.03, and a ZP of −16.6 ± 3.6 mV.

The optimized formulation exhibited a PS of 171.2 nm, which is considered suitable for intranasal and central nervous system (CNS)-targeted delivery. Nanocarriers with particle sizes below 200 nm are generally associated with improved mucosal permeation, enhanced cellular uptake, and more efficient transport across biological barriers, including the BBB (Muntimadugu et al., 2016; Saraiva et al., 2016). Therefore, the obtained PS is expected to facilitate effective nose-to-brain delivery while minimizing particle aggregation.

The optimized formulation exhibited a PDI value of 0.45, indicating an acceptable and relatively homogeneous particle size distribution. Although PDI values below 0.30 represent highly monodisperse systems, values below 0.50 are generally considered acceptable for lipid-based nanocarriers and reflect satisfactory formulation quality and reproducibility (Danaei et al., 2018). The obtained PDI suggests that the developed micelles possess adequate uniformity for pharmaceutical applications.

Although no statistically significant relationship was found between the formulation variables and zeta potential, all formulations exhibited negative surface charges ranging from −11 to −43 mV. The optimized formulation showed a zeta potential of −16.6 ± 3.6 mV. In lipid-based nanocarrier systems, surface charge contributes to colloidal stability by reducing particle aggregation through electrostatic repulsion. Although absolute zeta potential values greater than ±30 mV are generally considered indicative of highly electrostatically stabilized systems, steric stabilization provided by phospholipids and rhamnolipids can also contribute substantially to the physical stability of micellar formulations (Azevedo et al., 2021; Honary & Zahir, 2013). Therefore, the obtained zeta potential together with the relatively low PDI suggests that the optimized formulation possesses acceptable colloidal stability.

EE of the optimized BChE-loaded micelles was determined to be 21.1 ± 3.2%. Although this value is lower than those commonly reported for hydrophobic small-molecule drugs, it is considered acceptable for a hydrophilic macromolecule such as BChE. The relatively moderate EE may be attributed to the hydrophilic nature and large molecular size of BChE, which limit its efficient association with the micellar matrix during the self-assembly process. Nevertheless, the obtained EE was sufficient to enable subsequent physicochemical characterization and biological evaluation of the optimized formulation. Furthermore, the sustained release profile and enhanced biocompatibility observed for the BChE-loaded micelles suggest that the encapsulated enzyme remained successfully associated with the micellar system and retained its functionality. These findings demonstrate that the developed rhamnolipid-based micelles are capable of incorporating therapeutically relevant amounts of BChE and represent a promising platform for enzyme delivery (Simon et al., 2025; Torchilin, 2006).

Collectively, these physicochemical characteristics indicate that the optimized micelles possess suitable size, acceptable homogeneity, and adequate colloidal stability for subsequent biological evaluations. Therefore, this formulation was selected for further *in vitro* studies.

Results of *in vitro* release studies

The release study was conducted over a period of 24 hours, and the release profiles of the active sub-

stance and the loaded particles are presented in Figure 3. Free BChE exhibited a relatively rapid release, reaching approximately 95% cumulative release after 24 h. In contrast, BChE-loaded micelles displayed a slower and more sustained release pattern throughout the study, with approximately 65% cumulative release at 24 h. The delayed release from the micellar formulation indicates successful encapsulation of BChE within the carrier system and suggests that diffusion of the enzyme from the micellar matrix governed the release process.

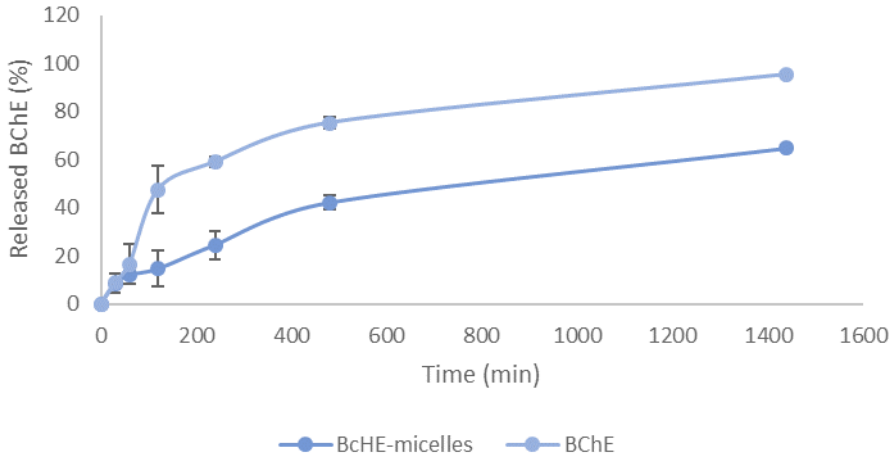


Figure 3. Release profiles of BChE and BChE-micelles for 24 h

Unlike poorly water-soluble small-molecule drugs, BChE is a highly water-soluble enzyme. Therefore, the sustained release observed from the micellar formulation cannot be attributed to dissolution-limited release but rather to the encapsulation of BChE within the micellar structure, which acts as a diffusion barrier and prolongs enzyme release.

FT-IR analysis

The FT-IR spectra of BChE, lecithin, rhamnolipid, cholesterol, and the optimized BChE-loaded micelles are presented in Figure 4. The spectrum of the loaded micelles predominantly exhibited the characteristic absorption bands of the lipid components, whereas the characteristic bands of BChE became markedly less pronounced. This masking effect is attributed to the successful incorporation of BChE within the micellar matrix. Furthermore, no additional absorption bands or significant shifts in the characteristic peaks were observed after micelle preparation, indicating that no chemical interaction or incompatibility occurred between BChE and the excipients (Kannan, 2013). These findings suggest that BChE was physically encapsulated within the micelles while preserving the chemical integrity of both the enzyme and the formulation components.

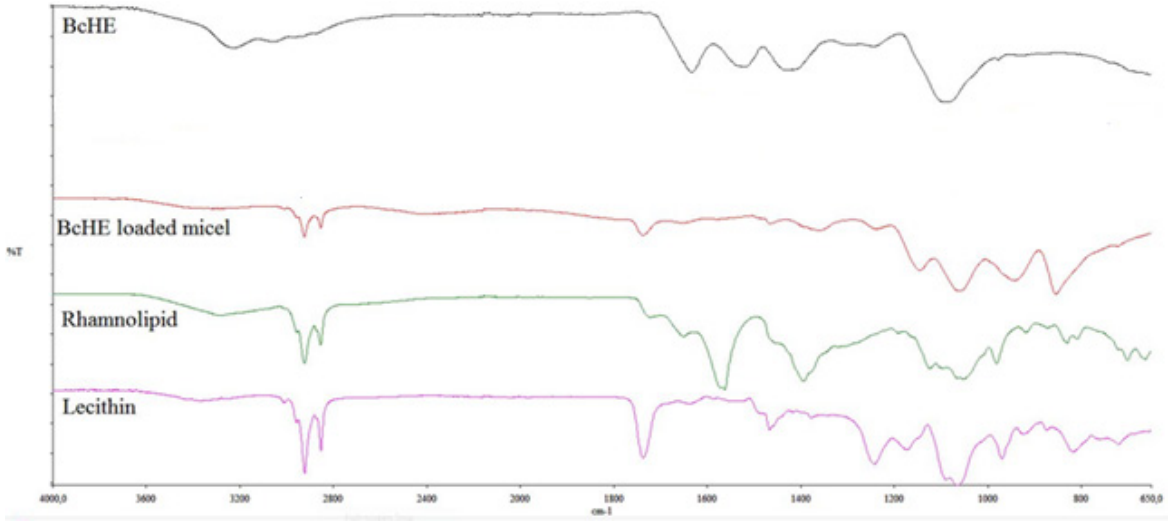


Figure 4. FT-IR Spectra of BChE, rhamnolipid, lecithin and loaded micelles

TEM analysis

TEM images (Figure 5.) revealed nearly spherical particles, in agreement with the measured particle size values.

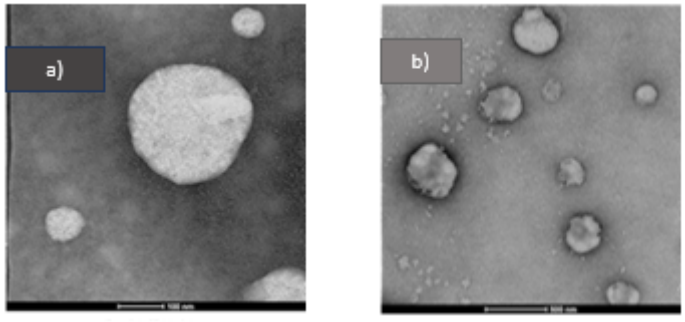


Figure 5. TEM Images of BcHE-micelles

Cell culture studies

Results of MTT assay

As shown in Figure 6, none of the formulations exhibited cytotoxic effects on the CaCo-2 cell line after 24 and 48 hours of exposure. The IC50 value is defined as the concentration corresponding to 50% cell viability on the graph, indicating 50% cell death (He et al., 2016). In the present study, since no formulation caused 50% or greater cell death at 24 or 48 hours, it was demonstrated that BChE-micelles could be safely applied within the dose range of 0.1-100 μM.

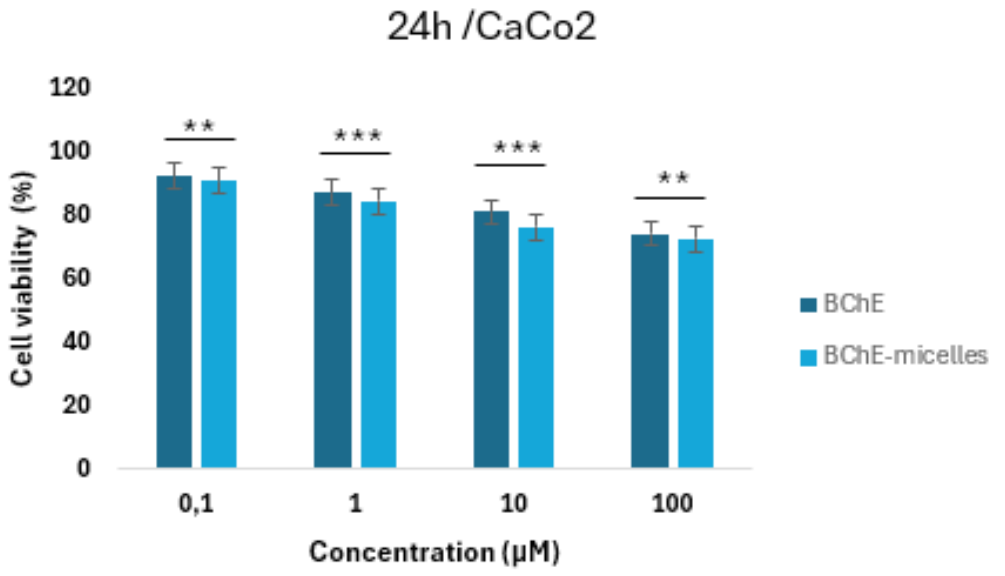
Statistical analysis demonstrated that BChE-loaded micelles significantly improved cell viability compared with free BChE at all tested concentrations and incubation periods (24, 48, and 72 h; $p < 0.05$).

Closer examination of the figure revealed that all treatments exhibited dose-dependent cytotoxicity, and some applications caused marked cytotoxicity at 100 μM after 72 hours. Studies investigating the cytotoxicity of BChE are limited, and none of the available studies were conducted using the same cell lines employed in the present work. Our findings indicated that treatment with BChE, particularly at high con-

centrations, resulted in more than 50% cell death at 72 hours.

When comparable studies were reviewed, no clear evidence of cytotoxic properties of BChE was reported in either case. However, in the study conducted by Yu et al. (2021), the highest concentration used in cytotoxicity assays was 180 µg/mL (500 nM). In the present study, substantially higher doses were tested, while lower doses of BChE demonstrated low cytotoxicity. The study by Mirzaei et al. (2021) was less explicit regarding experimental details. The concentrations used in cytotoxicity assays were not clearly specified; however, based on the presented graphs, even 200 µM BChE was reported to show no toxic effect. It should be noted that this study was performed using the PC12 cell line (Mirzaei et al., 2021).

MTT assay results demonstrated dose-dependent cytotoxicity in both cell lines, with significant effects observed for some treatments at 100 µM after 72 hours of exposure. All tested compounds and formulations showed cytotoxicity risk only at 100 µM after 72 hours, while remaining safe at lower concentrations and shorter incubation periods. Evaluation of the prepared formulations showed that none of them reached the IC50 value at any tested concentration or time interval. In addition, BcHE-micelles application was found to reduce the cytotoxicity of these active substances within the concentration range of 0.1-100 µM. Therefore, the prepared formulations were considered more suitable than direct administration of the active substance for investigating efficacy without causing damage to the BBB.



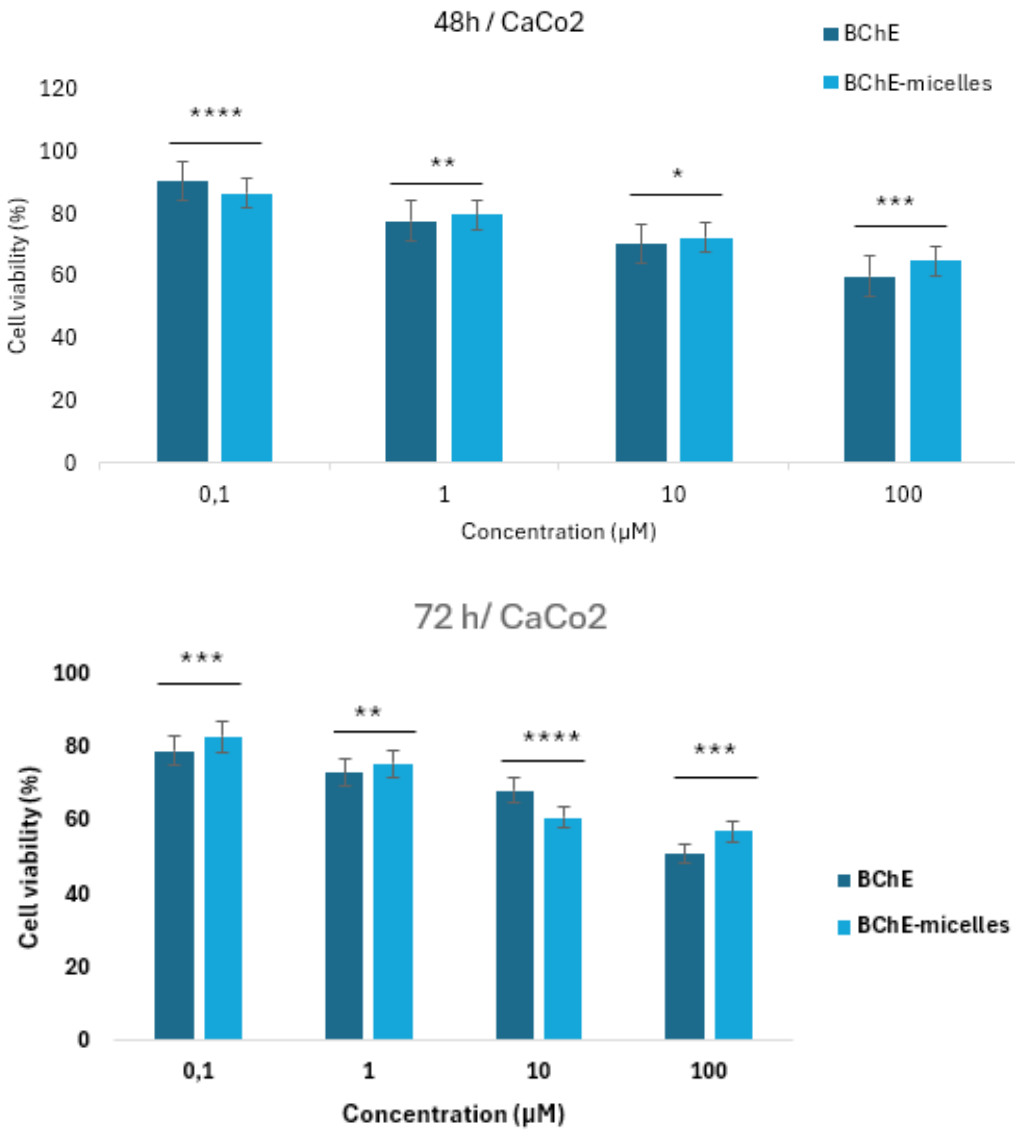


Figure 6. MTT results of BcHE and BcHE-micelles at 24 h, 48 h, and 72 h on the CaCo-2 cell line. Data are presented as mean ± SD (n = 3). Statistical significance between free BChE and BChE-loaded micelles at the corresponding concentration was analyzed using Student's t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

To evaluate the efficacy of the formulations on neuronal cells, where the principal therapeutic effect is expected, cytotoxicity analyses were performed using the SH-SY5Y neuroblastoma cell line. As shown in Figure 7, the obtained results were found to be similar to the cytotoxicity profile observed in the CaCo-2 cell line presented in Figure 6. Statistical analysis revealed that BChE-loaded micelles significantly improved cell viability compared with free BChE at all tested concentrations in SH-SY5Y cells throughout the incubation period ($p < 0.001$).

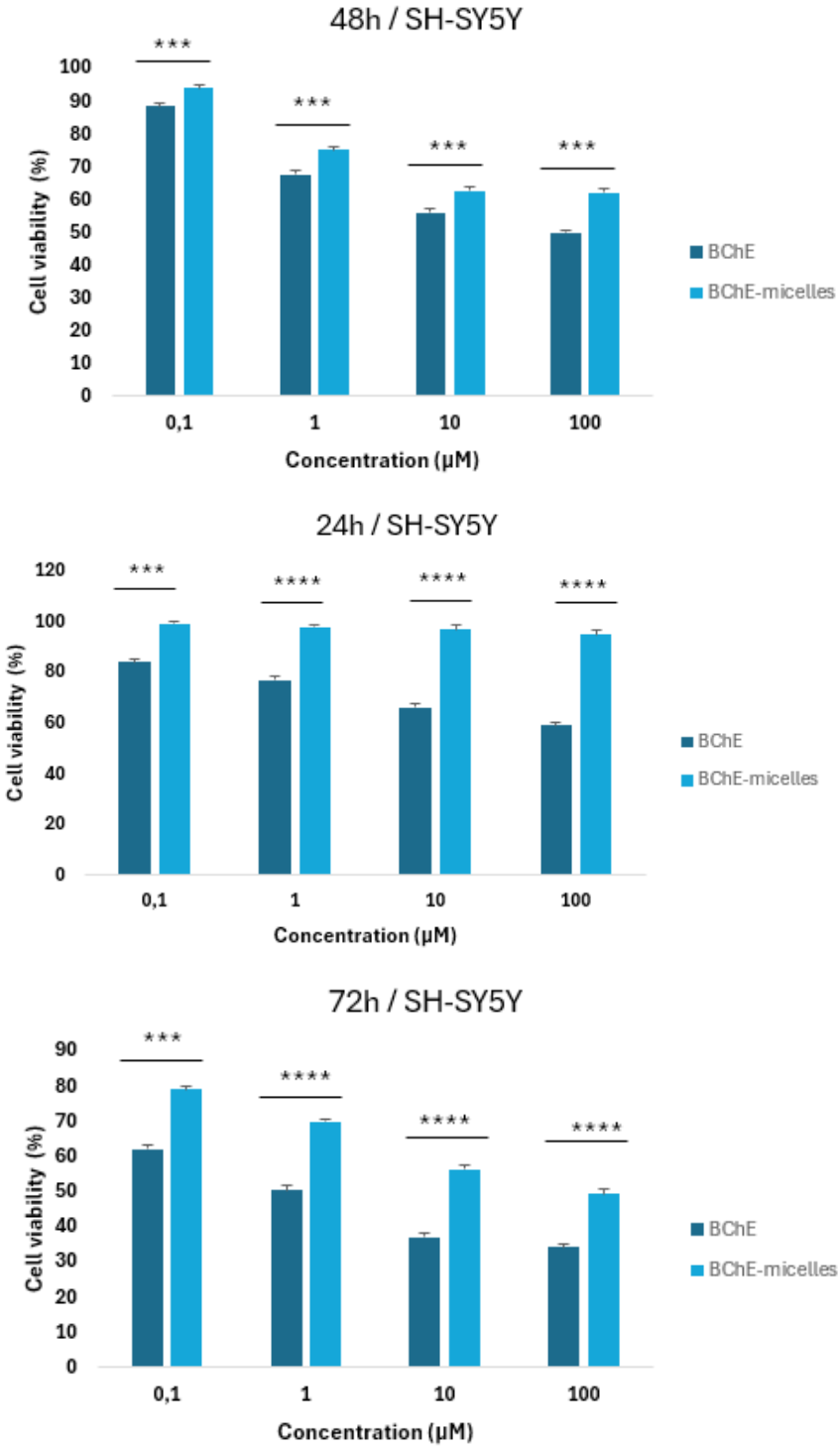


Figure 7. MTT results of BcHE and BChE-micelles at 24 h, 48 h and 72 h on the SH-SY5Y cell line. Data are presented as mean ± SD (n = 3). Statistical significance between free BChE and BChE-loaded micelles at the corresponding concentration was analyzed using Student's t-test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

CONCLUSION

In this study, rhamnolipid-based micelles loaded with BChE were successfully developed and characterized for intranasal administration. The prepared micelles exhibited favorable PS, PDI, ZP, and EE%. A controlled *in vitro* release profile was achieved, characterized and CNS permeability was enhanced compared with the free enzyme.

Cytotoxicity studies conducted on CaCo-2 and SH-SY5Y cell lines confirmed that the formulations were safe at therapeutic concentrations. This study differs from previous studies by incorporating rhamnolipid-based biomimetic carriers for BChE delivery, taking advantage of their inherent neuroprotective properties. Overall, this work presents a novel strategy for CNS-targeted detoxification of organophosphates and provides valuable insights into the design of safe and effective enzyme delivery platforms. These findings may serve as a useful basis for the development of more efficient therapeutic approaches against organophosphate poisoning.

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AUTHOR CONTRIBUTION STATEMENT

Conceptualization, methodology, formal analysis, investigation, visualization, writing-original draft (GRT), Conceptualization, methodology, formal analysis, resources, data curation, writing – review & editing, project administration (ÖE).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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