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Preservation of Physicochemical Properties in Freeze-Dried Betaine-Glucose Eutectic-Based Curcumin Niosomes using Mannitol as Cryoprotectant

(Pemeliharaan Sifat Fizikokimia dalam Niosom Kurkumin Berasaskan Eutektik Betaina-Glukosa Beku-Kering menggunakan Manitol sebagai Krioprotektan)

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ABSTRACT

This study investigates the preservation of physicochemical properties in freeze-dried eutectic-based curcumin niosomes using mannitol. Three niosomes' formulations, incorporating various natural deep eutectic solvents (NADES) alongside a blank formulation devoid of NADES, were subjected to freeze-drying processes. The particle size (Z-average), polydispersity index (PDI), entrapment efficiency, and zeta potential of the freeze-dried niosomes were assessed upon rehydration. Niosomes were prepared with varying concentrations of the cryoprotectant, mannitol, relative to non-ionic surfactants. Although the pharmaceutical potential of curcumin niosomes is recognized, this research focuses on the specific synergy between the internal BET-GLU NADES core and the external mannitol in maintaining niosome integrity after lyophilization. The incorporation of mannitol markedly improved the physicochemical properties of the freeze-dried eutectic-based niosomes. The use of BET-GLU NADES in the niosome formulation resulted in a reduction in particle size from 870.0 ± 15.52 nm to 418.07 ± 5.95 nm, concomitant with a decrease in PDI from 0.34 ± 0.016 to 0.12 ± 0.04. Moreover, the entrapment efficiency of curcumin in the BG-NADES formulation was 75.75 ± 6.12%, which was higher than the blank formulation (37.52 ± 2.19%). The synergistic addition of 10 g/g of mannitol was identified as the most effective concentration, further increasing entrapment efficiency to 93.24 ± 3.21%. Additionally, the zeta potential slightly increased in the negative direction from -38.87 ± 0.32 mV to -45.33 ± 3.99 mV with the incorporation of mannitol. The *in vitro* drug-release profiles, demonstrating sustained-release kinetics, and the antioxidant assays indicated preserved activity. This study highlights the critical synergistic role of mannitol and NADES in preserving the physicochemical properties of freeze-dried eutectic-based curcumin niosomes, providing a precise framework for stable nano-delivery systems.

Keywords: Cryoprotectant; curcumin; drug delivery; freeze-drying; niosomes

ABSTRAK

Penyelidikan ini mengkaji pemeliharaan sifat fizikokimia dalam niosom kurkumin berasaskan eutektik beku-kering menggunakan manitol. Tiga formulasi niosom, yang menggabungkan pelbagai pelarut eutektik dalam semula jadi (NADES) bersama-sama satu formulasi sifar tanpa NADES, telah melalui proses beku-kering. Saiz zarah (purata-Z), indeks polidispersiti (PDI), kecekapan pemerangkapan dan potensi zeta niosom beku-kering dinilai selepas penghidratan semula. Niosom disediakan dengan kepekatan krioprotektan, manitol yang berbeza-beza berbanding surfaktan bukan ion. Walaupun potensi farmaseutikal niosom kurkumin telah diiktiraf, penyelidikan ini memberi tumpuan kepada sinergi khusus antara teras dalaman NADES BET-GLU dan manitol luaran dalam mengekalkan integriti niosom selepas liofilisasi. Penggabungan manitol telah menambah baik sifat fizikokimia niosom berasaskan eutektik beku-kering dengan ketara. Penggunaan NADES BET-GLU dalam formulasi niosom menghasilkan pengurangan saiz zarah daripada 870.0 ± 15.52 nm kepada 418.07 ± 5.95 nm, disertakan dengan penurunan PDI daripada 0.34 ± 0.016 kepada 0.12 ± 0.04. Selain itu, kecekapan pemerangkapan kurkumin dalam formulasi BG-NADES adalah 75.75 ± 6.12%, iaitu lebih tinggi berbanding formulasi sifar (37.52 ± 2.19%). Penambahan sinergi manitol pada 10 g/g telah dikenal pasti sebagai kepekatan paling berkesan, yang meningkatkan lagi kecekapan pemerangkapan kepada 93.24 ± 3.21%. Tambahan pula, potensi zeta meningkat sedikit ke arah negatif daripada -38.87 ± 0.32 mV kepada -45.33 ± 3.99 mV dengan penggabungan manitol. Profil pembebasan dadah *in vitro* menunjukkan kinetik pembebasan berterusan dan ujian antioksidan menunjukkan aktiviti yang dikekalkan. Kajian ini menyerlahkan peranan sinergi kritikal antara manitol dan NADES dalam memelihara sifat

fizikokimia niosom kurkumin berasaskan eutektik beku-kering, sekali gus menyediakan rangka kerja yang tepat bagi sistem nano-penyampaian yang stabil.

Kata kunci: Krioprotektan; kurkumin; niosom; pengeringan beku; penghantaran dadah

INTRODUCTION

Curcumin has demonstrated remarkable therapeutic potential due to its anti-inflammatory, antioxidant, and anticancer properties. However, the bioavailability of curcumin is often limited by its poor aqueous solubility and rapid metabolism. Researchers have turned to nanotechnology to overcome these challenges, focusing on niosomes as promising carriers for curcumin delivery. Niosomes possess a lipid-based bilayer structure and offer enhanced stability and controlled release. This makes them attractive for encapsulating hydrophobic compounds such as curcumin, thereby shielding them from degradation and facilitating sustained release.

Despite advances, the practical use of niosomes in therapy is hindered by their inherent susceptibility to physical and chemical instability. Challenges in the use of niosomes include drug leakage, vesicle aggregation, fusion, and hydrolysis of encapsulated drugs (Dhanvir & Sandeep 2018). These challenges limit the shelf life of the dispersion, thereby affecting the overall stability of niosomes. Factors such as bilayer defects induced by chemical degradation and external influences, including heating, freezing, and phase transitions, can further contribute to instability (Ingvarsson et al. 2011). A highly effective strategy to mitigate the inherent physical and chemical instabilities of aqueous niosomal dispersions is to apply dehydration techniques, as most degradation processes are accelerated in aqueous environments. Various methodologies, including freeze-drying (lyophilization), spray-drying, and supercritical fluid technology, have been successfully employed to transform liquid niosomes into stable solid-state matrices, thereby extending their shelf-life and preserving their structural integrity.

Lyophilization is a crucial technique for prolonging the stability of niosomes, particularly those containing thermosensitive drugs such as curcumin (Fugita et al. 2012). Despite its advantages, the drying process for niosomes introduces challenges. This complexity arises from the process's intricate nature, as selecting suitable excipients and adjusting process parameters to protect membrane integrity against stresses induced by freezing and dehydration are challenging. Notably, niosomes containing water assemble spontaneously in the presence of water. Consequently, removing water may lead to substantial and, at times, irreversible alterations in their structure (Franzé et al. 2018). The drying method must preserve the structural integrity of niosomes and maintain their physicochemical properties. The objective is for lyophilized niosomes to readily reconstitute into their initial state upon rehydration, exhibiting minimal alterations in their essential properties.

Preparing niosomes using dried formulations while maintaining their physical structure and properties poses a significant challenge. Freezing-induced bilayer shrinkage may damage niosome membranes, dehydration can cause vesicle fusion, and rehydration may trigger a phase transition. To address these issues, a common strategy is to use cryoprotectants to establish stable boundaries between vesicles. Various carbohydrates or polyalcohols, such as mannitol, are considered cryoprotective agents that prevent niosome fusion or degradation during freezing (Stark, Pabst & Prassl 2010). Based on the water replacement hypothesis, cytoprotectants substitute the bound water around the bilayers by interacting with the polar regions of lipid head groups (Chen et al. 2010). Alternatively, cytoprotectants can form a vitreous layer around the bilayer with high viscosity and low mobility that prevents aggregation of niosomes, inhibits phase transitions, and safeguards lipid bilayers from damage caused by ice crystals (Chen et al. 2010). Mannitol offers advantages as a cryoprotectant due to its high glass transition temperature, small microemulsion particle size, and ability to produce crystal-like freeze-dried powders (Wu, Sun & Liu 2019).

In this study, the cryoprotected NADES-based curcumin niosomes are developed for the first time. The use of NADES as a cosolvent is expected to improve the stability and solubility of curcumin. In addition, mannitol is used as a cryoprotectant to protect the curcumin-niosomes formulation from freeze-drying stresses. Besides that, preservation of physicochemical properties in NADES-based curcumin niosomes was explored. The impact of freeze-drying on the niosome suspension was also investigated. Importantly, this paper elucidates the effects of including mannitol in shielding niosomes against fusion and leakage during lyophilization. The protective effects of mannitol were evaluated after rehydration of the freeze-dried niosomes.

MATERIALS AND METHODS

PREPARATION OF NATURAL DEEP EUTECTIC SOLVENT

The following reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA): betaine (≥98% purity), cholesterol (≥92.5% purity), curcumin (>65% purity), D-(-)-fructose (≥99% purity), dihexadecyl phosphate (DCP), sorbitan monostearate (Span 60), and polyoxyethylene sorbitan monostearate (Tween 60). D-(+)-glucose anhydrous (≥99% purity) was obtained from R&M Chemicals (Malaysia), and D-(+)-xylose (≥98% purity) was supplied by Nacalai Tesque (Kyoto, Japan). Chloroform (≥99.8% purity) and

methanol (≥99.8% purity) were purchased from Emsure (Merck, Germany) and LiChrosolv (Merck, Germany), respectively. All chemicals were used as received without further purification.

The synthesis of NADES was accomplished using the heating method. Betaine (BET) serves as the hydrogen bond acceptor (HBA), while glucose (GLU), xylose (XY), and fructose (FRU) act as the hydrogen bond donors (HBD) in the synthesis of NADES. The HBA and HBD were weighed on an analytical balance (OHAUS PX224, Ohaus Corporation, USA) to achieve the target molar ratios (2:1 for BET-GLU NADES; 1:1 for BET-XY and BET-FRU). In a sealed glass vial, the lower-melting-point component was heated until liquefied. The higher-melting-point component was then introduced, and the system was maintained at 70 °C with magnetic stirring (500 rpm) to yield a clear, homogenous liquid. The mixture was then cooled to room temperature. Stability of the resulting NADES was affirmed by the lack of crystal formation over a 24 h observation period.

SYNTHESIS OF EUTECTIC-BASED CURCUMIN NIOSOME

Curcumin-loaded niosomes were formulated using the thin-film hydration technique. Initially, the niosome constituents, a non-ionic surfactant mixture (Span 60 and Tween 60 in a 3:1 molar ratio), cholesterol, and dihexadecyl phosphate (DCP) were combined in a 25:12.5:2.5 molar ratio. These components, along with 25 mg of curcumin, were completely dissolved in a chloroform: methanol (4:1, v/v) solution within a round-bottom flask. Then, NADES (100 mg) was added to form the niosome suspension. The organic solvents were removed via rotary vacuum evaporation (BÜCHI, Switzerland), yielding a thin film on the inner wall of the flask. The film was dried for 24 h. Subsequently, the dried film was hydrated with 10 mL of phosphate-buffered saline (PBS, pH 7.4) for 24 h to form a niosome suspension. Finally, to achieve a smaller niosome size, the suspension was sonicated for 30 min at 60 °C.

PREPARATION OF NIOSOME POWDER USING FREEZE-DRYING METHOD

The niosome suspensions were diluted with an equal volume of PBS to achieve a final lipid concentration of 2.5 mg/mL. This 1:1 dilution was performed to standardize the concentration across all samples and to optimize the surface-area-to-thickness ratio for efficient ice sublimation. To prevent 'melt-back' during the 24 h lyophilization process (-50 °C, 0.5 mbar), the volumes were adjusted so that samples occupied only 10% (5 mL) of the 50 mL tubes' total capacity. Mannitol was dissolved in PBS at concentrations ranging from 1 to 10 g per gram of dry lipids for suspensions containing cryoprotectant. The niosome solution with NADES was diluted with an equivalent amount of PBS in the control group. The niosome suspensions were then stored in a deep freezer for 48 h before they were freeze-dried. The freeze-drying process lasted 24 h at -50 °C in a chamber at a pressure of 0.5 mbar, in accordance with the equipment specifications. The freeze-dried niosomes were reconstituted to their original volume at room temperature in an incubator shaker using PBS. After equilibrating for 1 h, the samples were ready for further testing.

DETERMINATION OF ENTRAPMENT EFFICIENCY

The drug entrapment efficiency (EE%) was determined indirectly by quantifying the unentrapped curcumin in the supernatant, following the methods of Bashkeran et al. (2024) and Nadzir et al. (2017). A 10 mL sample was centrifuged at 4,500 rpm and 25 °C for 30 min. The resulting pellet was discarded, and the supernatant underwent centrifugation under identical conditions for 30 min. Following centrifugation, the supernatant was combined with water to achieve a total volume of 10 mL. This mixture was then centrifuged under the same conditions for 60 min. The resulting supernatant was withdrawn and preserved for analysis. The total amount of unentrapped curcumin, representing curcumin in the final supernatant, was quantified using UV-Vis spectroscopy. The EE% is determined by Equation (1).

$$\textit{Entrapment Efficiency \%} = \frac{\textit{Total amount of curcumin} - \textit{unentrapped curcumin}}{\textit{Total amount of curcumin}} \times 100 \quad (1)$$

Equation (2) was used to determine the drug leakage percentage.

$$\textit{Drug Leakage \%} = \frac{\textit{Drug entrapped (before freeze drying} - \textit{after freeze drying)}}{\textit{Drug entrapped before freeze drying}} \times 100 \quad (2)$$

CURCUMIN NIOSOMES' SIZE ANALYSIS

Dynamic light scattering was used to determine the size of the niosomes. Prior to measurement, the hydrated freeze-dried niosomes were diluted by adding 50 µL of the suspension to 1 mL of distilled water. The diluted sample was then loaded into a disposable folded capillary cell (DTS1060). Measurements were conducted at a constant temperature of 25 °C for 120 s. The Z-average (mean hydrodynamic diameter) and Polydispersity Index (PDI) were determined through the cumulative analysis of the correlation function provided by the Zetasizer software.

DETERMINATION OF THE ELECTROPHORETIC MOBILITY OF NIOSOMES

Using the Zetasizer Nano ZS, the electrophoretic mobility of the curcumin niosome was determined. Prior to

measurement, the sample was diluted with distilled water and then put into the electrophoretic cells of the Zetasizer. The experiments were conducted at 25 °C and a field strength of 20 V cm^{-1}. The information on the curcumin niosome charges was determined by observing the particles' electrophoretic mobility. From the collected data, the mean values of zeta potential and the corresponding standard deviation were computed directly.

PHOTOCHEMICAL STABILITY

The photochemical stability of the encapsulated curcumin was evaluated against a control of non-encapsulated (free) curcumin. A niosome suspension and a free curcumin solution, each containing an equivalent amount of curcumin (25 mg), were placed in separate sealed glass Petri dishes. These samples were exposed to sunlight for 12 h per day over 28 days. Aliquots were withdrawn for analysis at intervals of 7, 14, 21, and 28 days. To quantify the remaining curcumin, each 0.1 mL sample aliquot was added to 10 mL of methanol. The mixture was agitated at 160 rpm for 10 min, followed by a 4 h incubation at room temperature to ensure complete niosome disruption and curcumin extraction. The mixture was then centrifuged at 4,000 rpm for 10 min and filtered. UV-Vis spectroscopy was used to quantify the curcumin content in the resulting supernatant. The degradation kinetics were analysed by plotting the percentage of curcumin retained over time. The apparent degradation rate constant (k) was determined from the slope of the graph. The half-life ($t_{1/2}$), which is the time taken for the curcumin concentration to reduce by 50%, was calculated using Equation (3), where A_0 is the initial concentration.

$$t_{1/2} = \frac{A_0}{2k} \tag{3}$$

DETERMINATION OF ANTIOXIDANT ACTIVITY

The antioxidant capacity of the formulations was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. A stock solution of 0.1 mM DPPH was prepared by dissolving 4 mg of DPPH in 100 mL of methanol. For the analysis, the niosome suspensions were serially diluted in methanol to achieve a final concentration range of 1 to 25 mg/mL. The assay was performed by mixing 1 mL of each diluted niosome sample with 3 mL of the 0.1 mM DPPH solution. A control was established by combining 1 mL of methanol with 3 mL of the 0.1 mM DPPH solution. These solutions were thoroughly shaken and left in the dark at room temperature for 30 min. Following the incubation period, the absorbance of each mixture was measured at a wavelength of 517 nm. The percentage of DPPH radical scavenging activity was determined using Equation (4):

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \tag{4}$$

In this equation, A_0 is the absorbance of the control sample (DPPH solution mixed with methanol), and A_1 is the absorbance of the test sample (DPPH solution mixed with the niosome formulation). All measurements were performed in triplicate.

In vitro DRUG RELEASE STUDIES

The *in vitro* release profile of curcumin from the niosomes was investigated using a dialysis bag diffusion technique. A 2 mL aliquot of the niosome suspension was loaded into a pre-swelled dialysis membrane (12,000 Da MWCO, 25 mm flat width), which was then securely sealed. The sealed bag was submerged in 100 mL of phosphate-buffered saline (PBS, pH 7.4), which served as the release medium. The entire apparatus was maintained at 37 ± 0.5 °C with continuous agitation at 150 rpm. At predetermined time intervals, 1 mL aliquots of the release medium were withdrawn for analysis. To maintain sink conditions, the sampling volume was immediately replaced with an equal volume of fresh, pre-warmed PBS. The concentration of released curcumin in each aliquot was quantified by UV-visible spectrophotometry at a wavelength of 424 nm. All release studies were performed in triplicate, and the percentage of curcumin released was determined according to Equation (5).

$$\textit{Drug release \%} = \frac{\textit{Released amount of curcumin}}{\textit{Total amount of curcumin}} \times 100 \tag{5}$$

STATISTICAL ANALYSIS

Data are expressed as mean ± Standard Deviation (SD) for three independent replicates (n=3). Statistical significance was evaluated using a paired t-test in Microsoft Excel to compare the observed experimental measurements of eutectic-based niosomes against the reference (blank) niosome dataset. A p-value less than 0.05 (p<0.05) was established as the threshold for statistical significance. In all figures, statistical significance is indicated by the absence of overlap between the SD error bars, which represents a mathematically distinct difference between the test formulations and the control.

RESULTS AND DISCUSSION

FREEZE-DRIED NIOSOMES WITHOUT CRYOPROTECTANT

Figure 1 presents the entrapment efficiency and drug leakage values for the four formulations: blank curcumin niosome, BG (BET-GLU NADES niosome), BF (BET-FRU NADES niosome), and BX (BET-XY NADES-based niosome).

The baseline formulation, which did not include a specific NADES, exhibited a moderate entrapment efficiency of 37.52 ± 2.19%. This value serves as a reference point for evaluating the improvements achieved by incorporating specific NADES into subsequent formulations. The observed trend highlights the formulation-dependent influence of NADES on entrapment efficiency. Among these, the BG niosome exhibited a notable advantage, with a relatively higher entrapment efficiency of 75.75 ± 6.12% compared with the other formulations.

This superior entrapment is governed by the Water Replacement Theory, where the polyols in the BET-GLU NADES core act as internal stabilizers. As water is removed, the hydroxyl (-OH) groups of the glucose and betaine form a robust hydrogen-bonding network with the curcumin and the niosomal polar headgroups, effectively substituting for the hydration shell and locking the curcumin within the niosomal structure (Crowe, Carpenter & Crowe 1998; Nystedt, Grønlien & Tønnesen 2021). Additionally, curcumin exhibits higher solubility in NADES with glucose as the HBD. The BF niosome demonstrated a favourable entrapment efficiency of 67.23 ± 4.18%, indicating that BET-FRU NADES also contributes positively to the encapsulation of curcumin. While slightly lower than BG, this value suggests that variations in the NADES composition influence the encapsulation process. The entrapment efficiency of the BX niosome, although still substantial at 60.76 ± 7.13%, is lower than that of the BG and BF formulations. This indicates that BET-XY NADES may have a less pronounced impact on enhancing curcumin encapsulation than BET-GLU and BET-FRU NADES. Efficient entrapment of curcumin is vital for achieving a higher payload and improved therapeutic outcomes.

The drug leakage values represent the amount of drug released from each formulation and serve as a critical indicator of bilayer integrity post-lyophilization. Among the formulations, BG exhibited the lowest leakage (22.71 ± 8.18%), which was significantly lower than that of the blank formulation (49.31 ± 5.12%). The magnitude of this improvement is substantial, as the incorporation of BET-GLU NADES reduced drug leakage by more than half compared to the standard aqueous-core carrier. This suggests that the NADES core acts as a secondary internal cryoprotectant.

Among the eutectic-based niosomes, BG remained the most effective, followed by BF (29.34 ± 4.91%) and BX (35.35 ± 4.01%). This can be attributed to the hydroxyl groups of the glucose and betaine within the NADES core, which form a robust hydrogen-bonding network with the niosomal polar headgroups. This interaction effectively substitutes for the hydration shell lost during dehydration, locking the curcumin within the niosomal structure and preventing the membrane rupture typically observed in unprotected formulations (Crowe, Carpenter & Crowe 1998). Consequently, formulation BG demonstrates the highest efficiency in retaining the drug, while the blank formulation, lacking these internal interactions, shows the least stability.

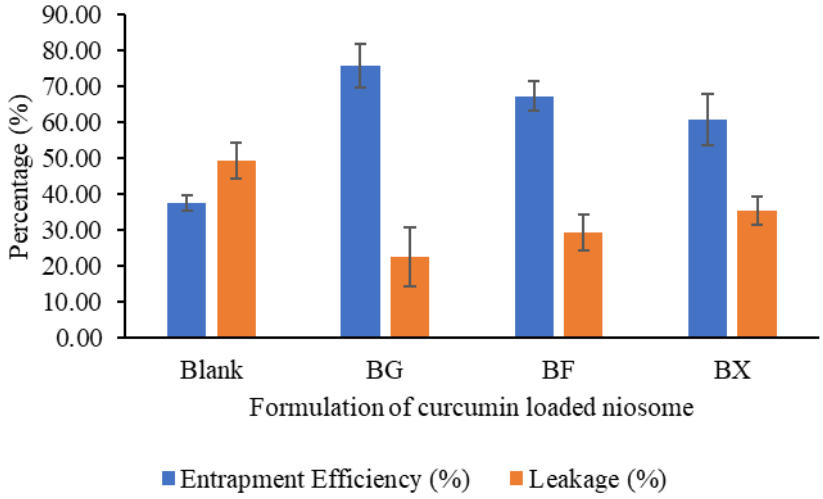
Freeze-drying without a cryoprotectant significantly affects the niosome particle size (Z-average), as shown in Table 1. While the removal of water content typically leads to a dry, porous matrix that induces particle aggregation or fusion (Stark, Pabst & Prassl 2010), the incorporation of NADES clearly mitigated these effects. The magnitude of improvement is most evident in the BG niosomes, which exhibited the smallest particle size (870.0 nm), a nearly 40% reduction compared to the baseline blank formulation (1442.7 nm). This suggests that the BET-GLU NADES core functions as a secondary internal cryoprotectant.

According to the Vitrification Theory, the high-viscosity nature of the NADES core facilitates the formation of an amorphous matrix that restricts the molecular mobility of the lipid bilayers. This reduces the frequency of vesicle collision and subsequent fusion during the ice crystal growth phase (Arakawa et al. 2001). The trend in size, BG (870.0 nm) < BF (954.2 nm) < BX (1014.3 nm), correlates with the different hydrogen-bonding densities of the sugar-based HBDs, with glucose providing the most effective stabilization. This reduction in particle size is crucial for enhancing curcumin's bioavailability, as smaller vesicles provide a larger surface area for cellular uptake and for improved interactions with target cells (Nowroozi et al. 2018).

The Polydispersity Index values provide valuable insights into the uniformity of the particle size distribution post-lyophilization. BG niosomes exhibit a remarkably low PDI of 0.243, which represents a statistically significant (p<0.05) improvement in homogeneity compared to the blank niosome formulation, which exhibited the highest PDI at 0.743. This narrow and uniform distribution suggests that the incorporation of BET-GLU NADES ensures a more stable formulation during the stresses of freeze-drying. Mechanistically, this is attributable to the sugar-based NADES core (betaine and glucose), which facilitates the formation of a rigid, amorphous glassy matrix. This high-viscosity matrix immobilizes the niosome vesicles, preventing the random, non-homogeneous aggregation that typically leads to high PDI values in unprotected formulations (Arakawa et al. 2001).

In contrast, BF (PDI 0.278) and BX (PDI 0.350) niosomes displayed slightly broader size distributions, indicating that the specific HBA-HBD combination in the glucose-based NADES provides superior homogenizing effects. The correlation between low PDI values and smaller particle sizes highlights the stabilizing role of BET-GLU NADES in achieving uniform niosome assemblies. This uniformity is vital for consistent drug delivery and long-term stability, as it significantly reduces the risks of localized aggregation over time.

The zeta potential values show significant distinctions in surface charge characteristics. These differences have



Results are presented as mean ± SD (n=3). The entrapment efficiency was determined by an indirect method quantifying unentrapped curcumin in the supernatant. Statistical significance (p<0.05) is indicated by the absence of overlap between the standard deviation error bars of the eutectic-based formulations and the blank control

FIGURE 1. Entrapment efficiency (EE%) and drug leakage (%) of freeze-dried curcumin niosomes

TABLE 1. Physicochemical properties of various niosome formulations post-lyophilization

Formulation	Z-average size (nm)	PDI	Zeta potential (mV)
Blank	1442.7 ± 117.41	0.743 ± 0.10	-16.22 ± 3.37
BG	870.0 ± 15.52[a]	0.243 ± 0.10	-38.87 ± 0.32[a]
BF	954.2 ± 43.22[a]	0.278 ± 0.06	-34.37 ± 3.36[a]
BX	1014.3 ± 127.82[a]	0.350 ± 0.19	-33.87 ± 1.00[a]

Data are expressed as mean ± SD (n=3). Statistical significance was evaluated using a paired t-test against the blank control (p<0.05). Superscript [a] denotes values that are statistically distinct from the reference baseline

crucial implications for the stability and susceptibility to aggregation of the formulations. BG niosomes exhibit an elevated negative zeta potential of -38.87 ± 0.32 mV, signalling a robust surface charge that fosters enhanced electrostatic repulsion between particles. This suggests heightened stability and a diminished probability of aggregation. Similarly, BF niosomes showcase a substantial negative zeta potential of -34.37 ± 3.36 mV, indicative of strong electrostatic repulsion and favourable stability. Although marginally less negative than BG, the absolute value remains high, affirming a stable surface charge. In contrast, BX niosomes display a negative zeta potential of -33.87 ± 1.00 mV, representing a stable surface charge, albeit slightly less negative than BG and BF. The distinct zeta potential trends may be attributed to the unique interactions introduced by BET-XY NADES on the niosome surface. Conversely, lacking specific NADES, the blank formulation exhibits a lower negative zeta potential of -16.22 ± 3.37 mV. While still negative, this reduced value implies diminished electrostatic repulsion, potentially rendering the blank niosomes more prone to aggregation. The comparative analysis underscores the impact of different NADES compositions on surface charge, emphasizing the need for tailored formulations to achieve specific stability objectives. Higher zeta potential values, indicative of greater stability and reduced aggregation, are critical for safeguarding the integrity of niosome formulations during production and drug delivery. Based on the comprehensive analysis of entrapment efficiency, particle size, PDI, and zeta potential, the BG niosome was chosen for further investigation. Its superior performance across these key parameters makes it a promising candidate for curcumin delivery.

FREEZE-DRIED NIOSOMES WITH CRYOPROTECTANT

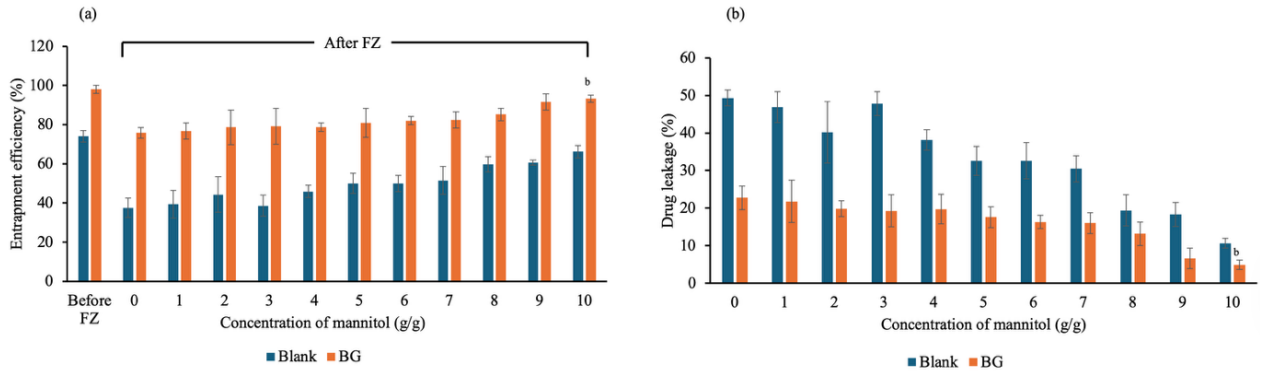
Figure 2 presents the entrapment efficiency and drug leakage from the blank niosome and the BG niosome. It is evident that the inclusion of mannitol generally correlates with an increase in the entrapment efficiency and a reduction in drug leakage for both the blank and BG niosomes.

The leakage percentages gradually decrease with a marginal increase in entrapment efficiency across the concentration range, indicating that mannitol is effective as a cryoprotectant in reducing drug leakage during freeze-drying and enhancing entrapment efficiency. A dose-dependent relationship is also observed between mannitol concentration and its cryoprotective effect on niosome leakage and entrapment. At a mannitol concentration of 1 g/g, the blank niosome leaks 49.31% and entraps 37.52% of curcumin, whereas the BG niosome leaks 22.71% and has an entrapment efficiency of 76.66%. As the mannitol concentration rises to 10 g/g, curcumin leakage drops significantly to 10.60% for the blank niosome and 4.86% for the BG niosome. As for the entrapment of curcumin, curcumin's entrapment efficiency increased 66.18% for blank niosome and 93.24% for BG niosomes. When comparing the two formulations, BG consistently demonstrates lower drug leakage and higher entrapment percentages than the blank niosome at all mannitol concentrations. This indicates that the BG formulation, which includes BET-GLU NADES, provides greater protection for curcumin encapsulation and against drug leakage during freeze-drying than the blank niosome.

It is crucial to maintain the physical integrity of niosomes throughout the freeze-drying process, which can be achieved by incorporating cryoprotectants into the finished formulations prior to freeze-drying. As shown in Figure 3, niosomes lacking cryoprotectant in their composition experience a substantial increase in size and PDI following the freeze-drying process. An insufficient polyalcohol concentration may result in an incomplete coating of the glassy matrix surrounding nanovesicles, thereby fostering aggregation (Date, Samad & Devarajan 2010). Optimal outcomes, characterized by smaller particle sizes and a uniform suspension, are achieved at higher mannitol concentrations. Notably, a mannitol ratio of 10:1 yields the smallest particle size of 400 nm and the lowest PDI of 0.2. Although higher molar ratios of mannitol were tested to reduce size and PDI and match the original niosomal size, elevated mannitol concentrations resulted in niosomal size and PDI similar to those achieved with the 10:1 mass ratio.

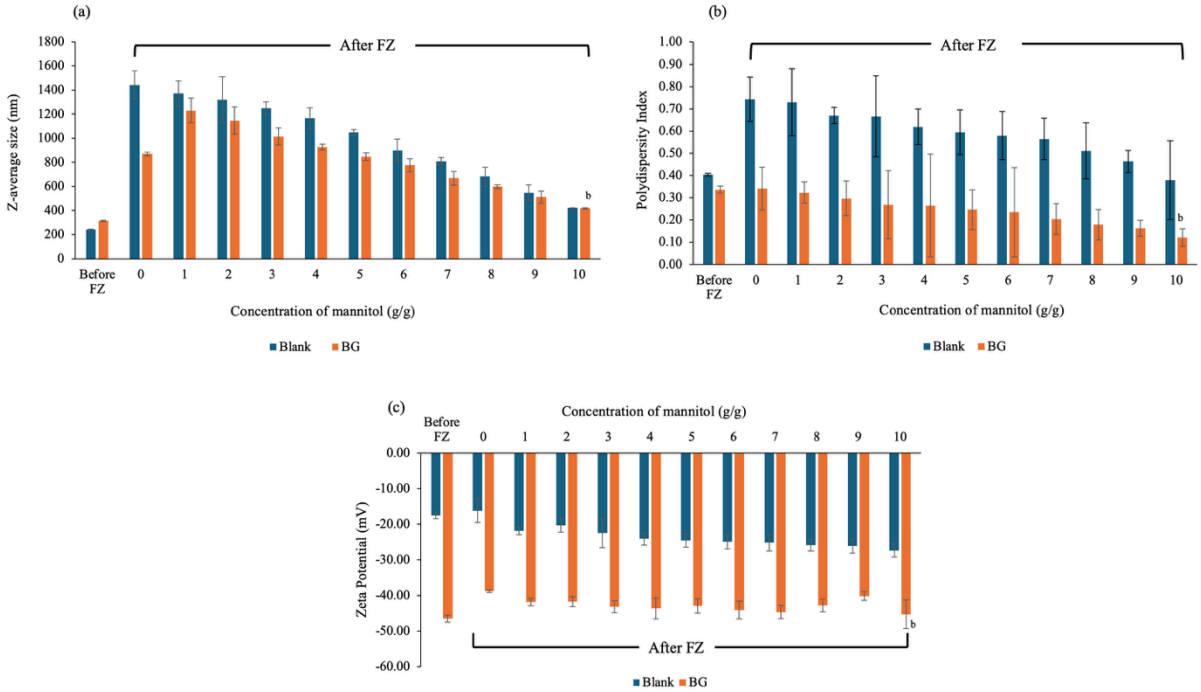
The effectiveness of cryoprotectants in stabilizing niosomes can be explained by several theories. The Water Replacement Theory posits that protectors stabilize by displacing bound water from the bilayers. Particularly at low hydrations, this replacement happens through interactions with the polar area of the lipid head group. According to the Vitrification Theory, the mobility of niosomes is limited throughout the freeze-drying process because the cryoprotectant forms a highly viscous matrix surrounding the vesicle (Chen et al. 2010; Sun et al. 1996). Another, less common theory, known as the kosmotropic effect, suggests that structural disruptions are caused by cryoprotectants' interaction with water. This disruption plays a preventive role during freeze-drying by reducing water content at the membrane interface (Ingvarsson et al. 2011).

The zeta potential results for niosome dispersions in deionized water (0.01 g/100 mL) are presented in Figure 3(c). The addition of cryoprotectant has a notable impact on the zeta potential of the niosomes, as the zeta potential values generally increase with higher concentrations of mannitol, indicating an improvement in the stability and surface charge characteristics of the niosome formulations. This phenomenon suggests that mannitol plays a crucial role in enhancing electrostatic repulsion between particles, thereby preventing aggregation and maintaining the niosomes' integrity during freeze-drying. The positive correlation between mannitol concentration and zeta potential values underscores the importance of optimizing cryoprotectant concentrations to achieve desired stability and surface charge properties in niosome formulations. This finding is consistent with the notion that cryoprotectants



Data are expressed as mean ± SD (n=3). Superscript [b] indicates a statistically significant improvement (p<0.05) compared to the non-cryoprotected BG formulation, demonstrating the protective role of mannitol in maintaining vesicle integrity

FIGURE 2. (a) Entrapment efficiency and (b) drug leakage of BG-based niosomes as a function of mannitol concentration



Values represent the mean ± SD (n=3). Statistical significance was determined via a paired t-test. Superscript [b] denotes a statistically significant improvement (p<0.05) in the 10 g/g mannitol formulation compared to the non-cryoprotected BG control (0 g/g). The absence of overlap between error bars for the 10 g/g sample confirms its superior stabilization effect

FIGURE 3. (a) Z-average size, (b) PDI, and (c) zeta potential of freeze-dried BG niosomes as a function of mannitol concentration

form a protective matrix around the niosomes, reducing mobility and thereby facilitating retention of their physicochemical properties.

The maximum enhancement in zeta potential was observed for the BG niosome formulation containing 10 g/g mannitol. This increased surface charge corresponds with the improved dispersibility noted for both blank and eutectic-based niosomes after the incorporation of a cryoprotectant. These observations can be attributed to modifications of the vesicle's surface chemistry. The components of the DESs, for example, may alter the prevalence of oxygen-containing functional groups on the niosome surface, consistent with the reduction effects reported by Al-Risheq et al. (2021). The ionization of these surface groups generates the electronegativity that determines the negative zeta potential values. Generally, a zeta potential value with a magnitude greater than ± 30 mV indicates sufficient electrostatic repulsion for a dispersion to be physically stable. Formulations with values exceeding ± 40 mV are considered to possess excellent long-term stability (Hayyan et al. 2015). The higher dispersibility of eutectic-based niosomes relative to the blank can be attributed to the simultaneous functionalization and reduction effects of the respective DESs. The deterioration of dispersibility in freeze-dried samples, in the absence of cryoprotectants, could be ascribed to the removal of some hydrophilic functional groups during freeze-drying, which leads to dehydration of the niosomes and diminishes the electrostatic repulsion between particles, consequently lowering the zeta potential (Zhang et al. 2020). Secondly, in the absence of cryoprotectants, niosomes are more prone to aggregation during freeze-drying, thereby reducing their effective surface charge. Thirdly, freeze-drying can damage the lipid bilayers of niosomes, disrupting their surface properties and altering charge distribution, thereby reducing the zeta potential. Lastly, the formation of ice crystals during freezing can physically disrupt the niosome structure, resulting in alterations in the surface charge distribution and a lower zeta potential. Collectively, the absence of cryoprotectants during freeze-drying renders niosomes vulnerable to various detrimental effects, ultimately impacting their zeta potential.

PHOTOCHEMICAL STABILITY STUDIES

The curcumin retention percentage provides valuable insights into the photochemical stability of freeze-dried curcumin niosomes, both in their blank formulation and when formulated with 100 mg of BET-GLU NADES, as shown in Figure 4. The curcumin retention reflects the amount of curcumin effectively encapsulated within the niosome structure while being exposed to sunlight for 28 days. A higher curcumin retention suggests better protection of curcumin from external factors, such as

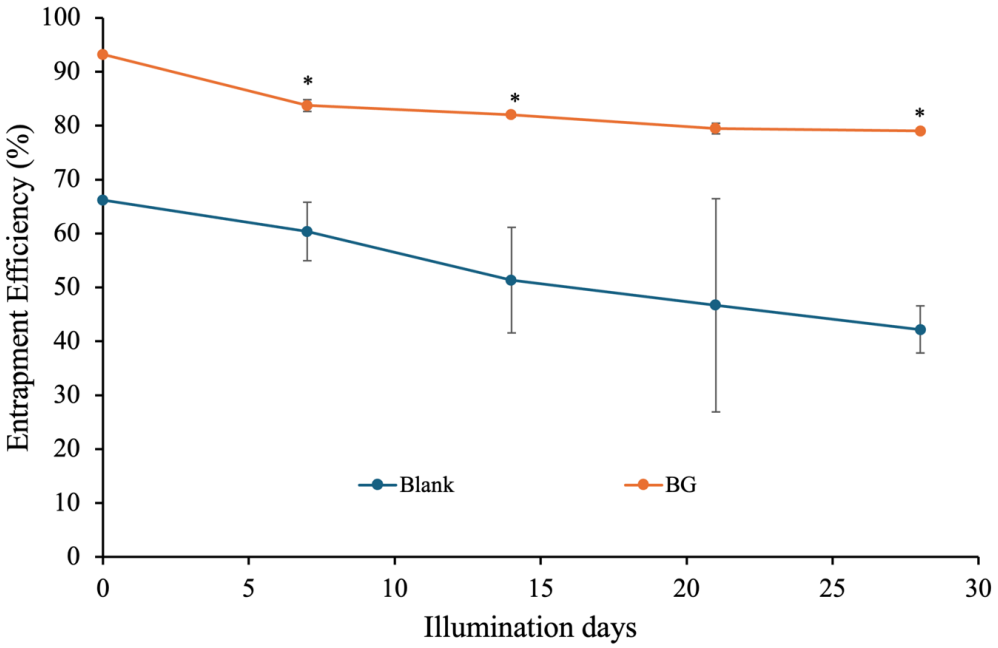
sunlight, which can induce photochemical degradation. The data show that both the blank and BG niosomes exhibit a decline in curcumin retention over the 28-day period. However, the BG niosomes consistently maintained higher retention values than the blank niosomes throughout the study. This indicates that the presence of BET-GLU NADES enhances the ability of the niosomes to retain curcumin within their structures, thereby potentially improving photochemical stability.

The photodegradation half-life of curcumin was determined using zero-order kinetics. In the BG niosome, at a curcumin entrapment efficiency of 93.24%, the half-life was calculated to be 85.90 ± 1.77 days, with a high correlation ($r^2 > 0.80$). Conversely, the blank niosome with a lower curcumin entrapment of 66.18% degraded rapidly, achieving a photodegradation half-life of 17.81 ± 2.31 days, with a strong correlation ($r^2 > 0.98$). NADES play a pivotal role in enhancing the photostability of curcumin, as indicated by the observed half-lives. By stabilizing curcumin's molecular structure, NADES effectively shields it from breakdown when exposed to light. This stabilization is further reinforced by NADES' ability to lock curcumin in a stable conformation, particularly its colourless diketo form, thus, reducing the likelihood of photodegradation. Additionally, NADES facilitate the rapid formation of supersaturated curcumin solutions in aqueous media, reducing the risk of precipitation and subsequent degradation. Most notably, beyond mere protection, NADES actively boost curcumin's phototoxic effect (Wikene, Bruzell & Tønnesen 2015).

ANTIOXIDANT STUDIES

The antioxidant properties of the blank and BG niosomes were assessed using the DPPH radical scavenging assay to evaluate the impact of the lyophilization process on bioactive stability. Before freeze-drying, both formulations exhibited distinct antioxidant activity, with the blank niosomes demonstrating an IC_{50} of 22.09 mg/mL, while the curcumin-loaded BG niosomes exhibited a significantly lower IC_{50} of 8.94 mg/mL (Figure 5). Statistical analysis confirmed that the enhancement of radical scavenging activity in the BG formulation was statistically significant ($p<0.05$). This indicates that the incorporation of the BET-GLU NADES core enhances the antioxidant capacity of the niosome system relative to the control.

Following the freeze-drying process, the IC_{50} value of the blank niosomes shifted from 22.09 to 19.08 mg/mL, while the IC_{50} of the BG niosomes increased from 8.94 to 15.08 mg/mL. The minor increase in the IC_{50} of the eutectic-based niosomes suggests a slight reduction in antioxidant activity post-lyophilization. Mechanistically, this change may arise from the mechanical stress of ice crystal growth during freezing or decreased accessibility of reactive groups due to steric interactions within the bilayer membrane (de Azambuja et al. 2015).



Values represent the mean ± SD (n=3). The retention percentage was determined relative to the initial curcumin content. Statistical significance was evaluated by comparing the BG formulation against the blank control at each time point using a paired t-test. Asterisks (*) denote a statistically significant difference ($p<0.05$) on that specific day

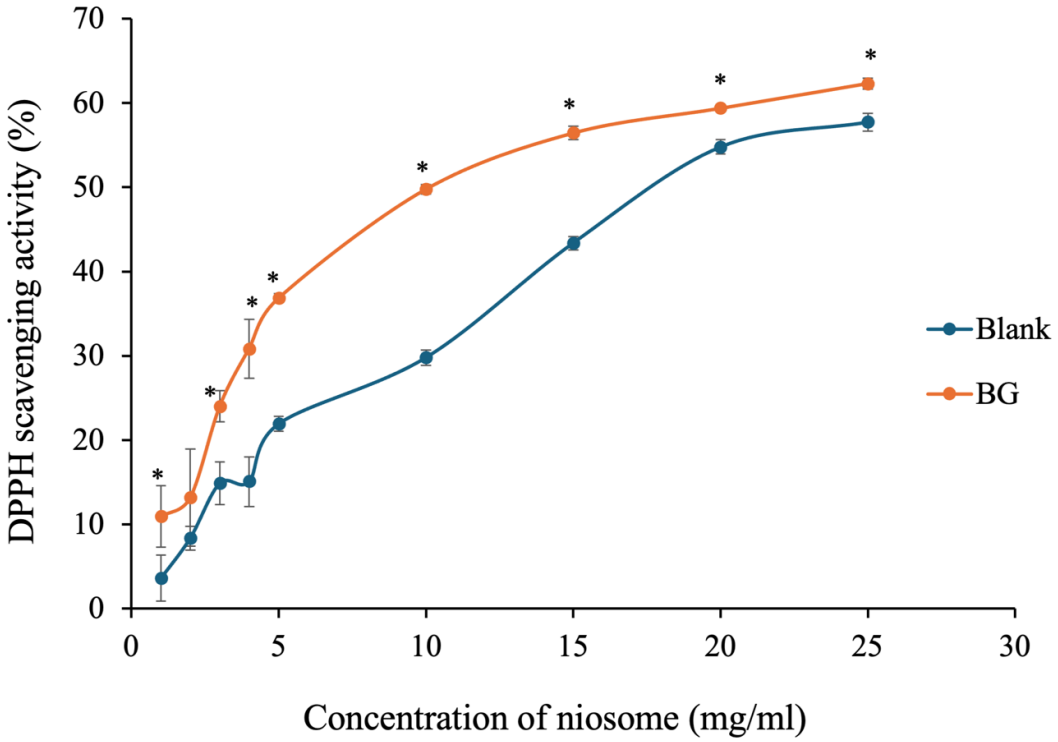
FIGURE 4. Photochemical stability of curcumin-loaded niosomes over a 28-day illumination period

However, the high retention of bioactivity (>85%) in the BG formulation is supported by water replacement and vitrification. As water is removed during sublimation, the glucose component of the NADES core acts as a chemical substitute for water. The hydroxyl (-OH) groups of the polyol form hydrogen bonds with the curcumin and the niosome headgroups, effectively replacing the hydration shell and maintaining the native, bioactive conformation of the molecules (Arakawa et al. 2001; Crowe, Carpenter & Crowe 1998). Furthermore, the addition of mannitol facilitates the formation of a rigid, amorphous sugar-glass matrix. This high-viscosity matrix immobilizes the niosomal vesicles, preventing the molecular mobility that typically leads to membrane rupture and oxidative degradation of phenolic compounds (Arakawa et al. 2001; Borges et al. 2023; Crowe, Carpenter & Crowe 1998).

Despite the slight shift in IC_{50}, the change is considered biologically insignificant, as the BG niosomes retained considerable antioxidant activity. This demonstrates that the combination of a eutectic core and a mannitol cryoprotectant provides an effective barrier against prolonged freezing and dehydration, supporting the potential of these niosomes as robust antioxidant delivery systems.

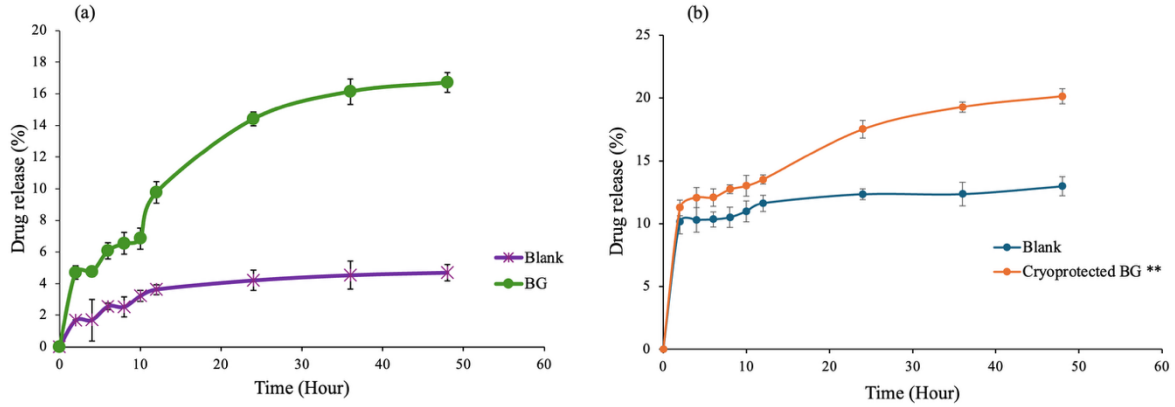
In vitro DRUG RELEASE

The drug release profiles of the blank and BG niosomes under freeze-dried conditions without and with 10 g/g of mannitol were evaluated at various time points and shown in Figure 6. The trends observed in the drug release were analysed based on percentages (%) over time. At the initial time points (0 to 4 h), both the blank and BG niosomes exhibited a gradual increase in drug release percentages. This phase often involves releasing loosely bound or surface-bound drug molecules from the niosome vesicles. From 6 to 12 h, the drug release profiles entered a sustained release phase, characterized by a slower but consistent increase in drug release percentages. This phase reflects the gradual diffusion of encapsulated drug molecules through the niosome lipid bilayers or pores, contributing to sustained drug release. Comparing the drug-release profiles of freeze-dried formulations with and without a cryoprotectant, it is evident that both mannitol-containing formulations exhibited relatively consistent release patterns and were slightly higher over the study duration. This stability ensures predictable, controlled drug-release kinetics, particularly in pharmaceutical applications. Notable differences were observed in the drug release profiles between the blank and BG niosomes, particularly



Data are presented as the mean ± SD (n=3). The antioxidant capacity of the BG formulation was compared against the blank niosome control using a paired t-test. Asterisks (*) denote concentrations where the BG formulation exhibited a statistically significant enhancement in scavenging activity (p<0.05) compared to the control

FIGURE 5. DPPH radical scavenging activity (%) of curcumin-loaded niosomes at various concentrations (1-25 mg/mL)



Values represent mean ± SD (n=3). Double asterisks (**) in the legend indicate that the cryoprotected formulation exhibited a highly significant difference (p<0.01) compared to the non-cryoprotected control across all time points

FIGURE 6. Comparative *in vitro* release profiles of non-cryoprotected (0 g/g mannitol) and cryoprotected (10 g/g mannitol) BG niosomes

at later time points (24 to 48 h). BG niosomes exhibited slightly higher drug release percentages compared to the blank niosomes under freeze-dried conditions with and without the presence of mannitol. This could be attributed to the influence of BET-GLU NADES on the membrane properties of the niosomes, affecting drug diffusion rates.

CONCLUSION

This study was undertaken to evaluate the impact of freeze-drying and cryoprotectants on the physicochemical integrity and bioactive stability of eutectic-based curcumin niosomes. The key finding demonstrates that the synergistic use of BET-GLU NADES and 10 g/g mannitol optimally preserved niosome properties post-lyophilization. The incorporation of BET-GLU NADES resulted in a significant reduction in particle size to 418.07 ± 5.95 nm and a decrease in PDI to 0.12 ± 0.04. Furthermore, entrapment efficiency was maximized at 93.24 ± 3.21%. These results confirm that the NADES core and mannitol provide a dual-shielding effect that maintains the structural and antioxidant properties of the carrier during dehydration stresses. Overall, this work provides a precise framework for using green solvents to enhance the shelf life of nanostructures, highlighting their potential as stable platforms for delivering thermosensitive bioactive compounds. Despite these promising results, several limitations should be noted. This study relied exclusively on *in vitro* release and stability assays; therefore, further *in vivo* investigations are required to confirm the biological performance and safety of the NADES-based system. Additionally, while mannitol was identified as highly effective, this research focused on a single cryoprotectant and a single HBA:HBD ratio for the NADES core. Future studies should explore various cryoprotectant types and varying NADES molar ratios to further optimize the formulation for specific pharmaceutical applications.

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