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Green Nanoemulsion Water/Ethanol/Transcutol/LabM-Based Treatment of Pharmaceutical Antibiotic Erythromycin-Contaminated Aqueous Bulk Solution

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Cite This: *ACS Omega* 2022, 7, 48100−48112

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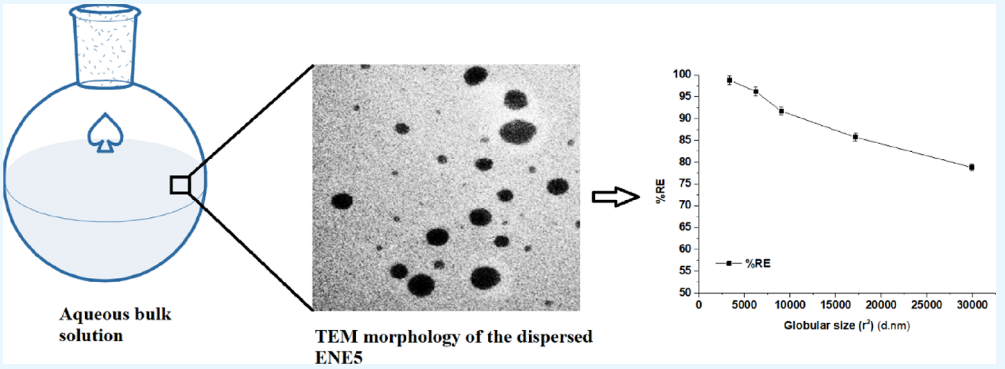

ABSTRACT: Contaminated wastewater released from hospital, domestic, and industrial sources is a major challenge to aquatic animals and human health. In this study, we addressed removal of erythromycin (ERN) from contaminated water employing water/ethanol/Transcutol/Labrafil M 1944 CS (LabM) green nanoemulsions as a nanocarrier system. ERN is a major antibiotic contaminant harming aquatic and human lives. Green nanoemulsions were prepared and evaluated for size, size distribution (measuring polydispersity index), stability, zeta potential, refractive index, and viscosity. Transmission electron microscopy (TEM) was used to visualize morphological behavior. The treated-water was analyzed for ERN by the spectroscopy, scanning electron microscopy−energy-dispersive X-ray analysis mode (SEM−EDX), and inductively coupled plasma−optical emission spectroscopy (ICP−OES) techniques. We studied factors (composition, size, viscosity, and time of exposure) affecting removal efficiency (%RE). The obtained green nanoemulsions (ENE1−ENE5) were stable and clear (<180 nm). ENE5 had the smallest size (58 nm), a low polydispersity index value (0.19), optimal viscosity (∼121.7 cP), and a high negative zeta potential value (−25.4 mV). A high %RE value (98.8%) was achieved with a reduced size, a high water amount, a low Capryol 90 content, and optimal viscosity as evidenced by the obtained results. Moreover, contact time had insignificant effect on %RE. UV−vis spectroscopy, SEM−EDX, and ICP−OES confirmed the absence of ERN from the treated water. Conclusively, ERN can easily be removed from polluted water employing green nanoemulsions prepared from the optimized excipients, and evaluated characteristics.

INTRODUCTION

Macrolide antibiotics (erythromycin, azithromycin, and clarithromycin) are potential broad-spectrum drugs possessing efficient therapeutic efficacy against several bacterial (Gram-positive and Gram-negative) infections. A regular therapeutic application in healthcare systems and large-scale production in industrial bodies led to prominent aqueous pharmaceutical contaminants emerging through effluents. Eventually, these macrolides were disposed slowly in drinking water, surface water, groundwater, and aquatic ecosystems.[1] Notably, prolonged presence of (at a trace level) these drugs in the aquatic system (environment) has drawn the attention of various scientists due to their adverse health consequences on aquatic lives and threat to public health. These serious adverse effects are associated with chemical instability, slow accumulation to toxic concentrations, and high biological activity.[1,2] Al-Maadheed et al. investigated erythromycin (ERN) as a major pharmaceutical contaminant (∼5.2 μg/L) among several

Received: September 21, 2022
Accepted: November 18, 2022
Published: December 12, 2022

antibiotics available in hospital and domestic wastewater influents.[3] In several countries (China, India, Germany, Spain, Taiwan, and the United Kingdom), the effluent wastewater was identified with a high content (2−2.05 μg/L) of ERN as a pharmaceutical contaminant with a low removal efficiency (80%).[4] Moreover, the occurrence values of ERN were found to be in the range of 470−810 ng/L and 510−850 ng/L in the influent and the effluent of wastewater treated from sewage water, respectively. These findings were obtained by collecting water samples from Hong Kong and Shenzhen Province of China.[5] The European Union (EU) released a "watch list" in 2015, wherein macrolides were the least studied contaminants (15.6%) among antibiotics (CECs 2015).[6]

Various conventional methods (physical, chemical, and microbial) have been exploited to remove macrolide contaminants from wastewater obtained from different water resources.[7,8] These conventional methods are the least efficient (unable to remove contaminants completely from water), hectic, and costly; have a high probability of microbial growth; and are difficult to scale up for bulk cleaning.[7] Moreover, photocatalytic degradation, Fenton reaction, UV light application, ultrasound (low frequency ultrasound ∼40 kHz), and adsorption (activated carbon) based methods are commonly used techniques for remove trace amounts of macrolides present in aquatic systems despite several limitations.[7−9] Recently, we reported green nanoemulsions as nanocarriers for removing few macrolides and anti-tubercular pharmaceuticals (clarithromycin, azithromycin, and rifampicin) contaminating aqueous solutions.[10−12] These nanoemulsions are isotropic, and thermodynamically stable, composed of water, lipid, surfactant and co-surfactant. ERN is a poorly water-soluble (0.15 mg/mL) drug as evidenced by its log *P* value (2.6−3.06). The drug is a potential contaminant in aquatic systems that needs to be removed using the established economic and efficient method.[13] There are several factors affecting the removal efficiency of the drug from contaminated water such as (a) the components (water and oil phases) of the nanoemulsion, (b) the physicochemical properties (viscosity, globular size, ferrous ions, hydrogen peroxide, and refractive index) of the nanoemulsion, (c) the degree of polluted water, (d) method efficiency, and (e) other factors (pH, UV frequency, reducing agent, microbial inhibition by antibiotics).[7−9] From the literature, it was concluded that the percent removal efficiency (%RE) of macrolides was comparatively lower than that of fluoroquinolones and the %RE values of ERN were 74 and 79% in wastewater treatment plants in Beijing and the United Kingdom (and Australia), respectively.[14,15] The drug can be estimated using previously reported methods based on the physicochemical nature of the drug.[16,17] The selection of excipients was based on various factors such as (a) the solubility of the drug, (b) the hydrophilic−lipophilic balance (HLB) value of the excipient, (c) medium-chain triglycerides capable of self-emulsification after dispersion into water, (d) capability of forming an emulsion through self-emulsification into water and subsequent adsorption of the lipophilic drug, (e) safety and biocompatibility, and (f) cost-effectiveness and ease of access. Labrafil M 1944 CS (LabM) consists chemically of mono-, di-, and triglycerides and PEG-6 (MW 300) mono- and diesters of oleic acid (C18:1) (source: Gattefossé leaflet). It has the capability to self-emulsify into water to load poorly water-soluble drugs due to lipophilic−lipophilic (HLB of LabM = 9) interactions and other cohesive interactive forces (polarity energy, hydrogen bonding energy, and dispersion energy) working in tandem. Transcutol HP is chemically a diethylene glycol monoethyl ether and extensively exploited to remove diclofenac as a contaminant from water with a high removal efficiency (94.5%).[18] The combination of Transcutol HP (THP) and ethanol significantly reduced surface tension due to their combined effect. Thus, these excipients can be suitable for removing ERN from aqueous systems.

Notably, there was no report published so far on removal of the potential toxic contaminant "erythromycin" employing green nanoemulsions. Components of nanoemulsions were scrutinized based on solubility profiles, environmental safety concerns, and ternary phase diagrams. Pseudo-ternary phase diagrams (PTDs) indicated the most robust values of surfactant-to-co-surfactant ratios (S_{mix}). The prepared nanoemulsions were characterized in terms of thermodynamic stability, globular size, polydispersity index (PI), shape, viscosity, refractive index (RI), and zeta potential (ZP). Finally, we investigated the effect of oil and water phases on % RE followed by a complete confirmation test using UV−vis spectroscopy and high-performance liquid chromatography (HPLC)-based analysis. SEM−EDX and ICP−OES confirmed the absence of the drug in the treated water.

MATERIALS AND METHODS

Materials. ERN (>99%), methanol, ethanol, acetonitrile, Tween 80, propylene glycol (PG), ethylene glycol (EG), and poly(ethylene glycol 400) (PEG400) were procured from Sigma-Aldrich (Mumbai, India). LabM and diethylene glycol monoethyl ether (Transcutol as THP) were gifted by Gattefossé (France). Capmul MCM C8 (CMC8) (mixture primarily of caprylic and capric acids) and Capryol 90 (CAP90) were generously provided by Abitec (Janesville, WI). Olive and clove oils were obtained from a local in-house chemical shop.

Methods. *Analytical Method.* The drug was quantified using a reversed-phase HPLC method using a Waters-based system (Waters, Empower 2.0, 34 Maple Street, Milford, MA 01757, United States). Analysis was performed using a C18 column (150 × 4.6 mm, 5 μm packing particle size) operating at 40 °C. The mobile phase (pH = 9.0) comprised acetonitrile (ACN) and a phosphate buffer solution (0.02 M K_2HPO_4) in a 40:60 ratio. The buffer solution contained 0.1% formic acid. The injection volume, run time, and flow rate were set as 30 μL, 15 min, and 1 mL/min, respectively. The wavelength of the drug for analysis was 205 nm. Analysis, data acquisition, and reporting were performed using the Waters Empower 2.0 chromatography data software. The limit of detection and the limit of quantification were 0.01 and 0.5 μg/L, respectively.[16,17] The established regression coefficient (r^2) value was obtained as 0.999 for the working standard calibration curve. Analysis was replicated for statistical calculation.

Thermal Analysis of ERN Using Differential Scanning Calorimetry. The drug is a white solid and crystalline powder. To determine its melting point (fusion temperature) and enthalpy of fusion, the differential scanning calorimetry (DSC) technique (DSC-50, Shimadzu, Japan) was used for thermal analysis. Analysis was carried out by purging N_2 gas (50 cm^3/min) as a coolant.[18] A precisely weighed content of ERN (4.0 mg) was hermetically crimped within an aluminum pan. The same was transferred to a furnace with the help of a sample holder. Exposure of the drug to humid conditions was avoided,

https://doi.org/10.1021/acsomega.2c06095
ACS Omega 2022, 7, 48100−48112

and the crimped sample was immediately kept in the furnace. Thermal analysis of the sample was performed by heating at a rate of 10 °C/min up to 200 °C, and the sample was subsequently cooled to room temperature before the next cycle.

Assessment of Solubility in Various Excipients. To scrutinize the best components of green nanoemulsions (GNEs), it was a prerequisite to investigate the solubility of ERN in oils (LabM, CMC8, clove and olive oils), surfactants (THP, and Tween 80), and co-surfactants (ethanol, PEG400, PG, and EG). The study was conducted at 40 ± 1 °C for 48 h in each excipient until saturation. Therefore, a weighed amount of the drug was constantly added up to saturated solubility using a shaker water bath (Remi-Equipment, Mumbai, India). Each sample mixture was contained in a clear glass vial. At the end, the sample mixture was centrifuged at 12,000 rpm for 20 min (Centurion Scientific Lab, Church Farm, Stoughton, Chichester, West Sussex PO18 9JL, United Kingdom) and the supernatant was dissolved in methanol. The drug solution was filtered using a membrane filter and analyzed using the HPLC technique. The drug content was assayed at absorption λ_{max} of 205 nm.[19] The analysis was replicated for mean and standard deviation (n = 3, mean ± SD).

Pseudo-Ternary Phase Diagrams. Generally, water-in-oil (w/o) types of GNEs were prepared using oil, water, surfactants, and co-surfactants. A solubility study suggested that LabM, THP, ethanol, and water can serve as oil, surfactant, co-surfactant, and aqueous component, respectively. The ratio of the surfactant to the co-surfactant is termed "S_{mix}", and this is an important mixture ratio for preparation of a stable nanoemulsion. Therefore, several S_{mix} ratios (1:1, 1:2, 2:1, and 1:3) were tried to identify a stable and maximally delineated nanoemulsion in PTDs. A series of trial GNEs was prepared using a slow emulsification and titration method.[10] The mixed blend of S_{mix} and oil phase was slowly titrated against the aqueous phase at various ratios (1:9 to 9:1). The prepared nanoemulsion was physically inspected for benchtop stability overnight. The appearance of any signs of instability (turbidity, phase separation, and creaming) was recorded if they developed. The unstable nanoemulsions were dropped from further study. The total percent content of all three components was always 100% in each formulation. Several phase diagrams were constructed using the titration data, and the nanoemulsion was selected based on the maximally delineated zone in the PTD followed by physical benchtop stability at room temperature. Transparent and isotropic nanoemulsions were subjected to further studies.

Cycles of Freeze−Thaw and Centrifugation Study. GNEs are considered kinetically stable when subjected to thermal (high and subsequent low temperatures) and physical stress (centrifugation). Therefore, ENE1−ENE5 were passed through subsequent cycles of low (−21 °C), intermediate (4 °C), and high (45 °C) temperatures followed by centrifugation (Beckman Coulter, Indianapolis, IN 46268, United States) at 36,000 × g for 10 min.[20] Briefly, a small amount (5 mL) of the sample was packed into glass vials and stored at the pre-set temperature of an incubator. Initially (freeze−thaw cycles as step 1), ENE1−ENE5 were stored at the lowest temperature (−21 °C) for 24 h and then transferred to room temperature (25 °C) to attain their former stable state (clear, isotropic, and transparent) within 5 min. The same step was repeated for intermediate and high temperatures. Thus, cycles of thermal exposure (from low temperature to high and vice versa) of the samples were repeated thrice and the samples were inspected carefully at room temperature. Second, the stable nanoemulsions from ENE1 to ENE5 were selected and subjected to ultracentrifugation (repeated cycles). In this step (step 2), 2 mL of the sample was transferred to a centrifugation tube and centrifugation was performed for 10 min. Each sample was properly inspected for any sign of instability. Finally, each sample was exposed to repeated cycles of heating and cooling steps (by storing at 4.0 and 40.0 °C) after step 2 for 48 h.[22] Now, nanoemulsion passing the previous steps and step 3 were eligible for further characterizations and %RE and other studies.

Evaluation Parameters of ENE1−ENE5. Nanoemulsions were characterized for globular size (diameter), size distribution (PI), shape, viscosity, RI, and ZP. These characteristic features helped obtain conclusive findings in terms of evaluation parameters.

Size and Size Distribution as PI. The globular size and PI values of nanoemulsions (ENE1−ENE5) were estimated using a Malvern Zetasizer (Nano ZS90 Zetasizer, Worcestershire, United Kingdom). The samples were completely diluted using water so that they can be easily analyzed without interference. The diluted sample was transferred to a cuvette for analysis. The working principle of the Zetasizer is based on "dynamic light scattering (DLS) by particles" moving in Brownian motion within an aqueous dispersive medium. The technique measures the intensity of the light scattered by the dispersed nanoscale globules within the dispersing medium. Analysis was performed at a scattering angle of 90° and 25 °C in triplicate to get the mean value. The average size (Dz) was estimated by eq 1:

$$Z, \text{ average globular size (Dz)} = \left[\frac{\sum (I)}{\sum \left(\frac{I}{d}\right)}\right] \quad (1)$$

where the terms "d" and "I" represent the size of globules and the scattered light intensity, respectively. In practice, the sizes (diameters) obtained from electron microscopy (transmission electron microscopy (TEM)) and DLS differ due to differences in the working principles and the sample processing. This may be considered an instrumental error due to preferentially adsorbed small droplets by the grid of TEM. The DLS signal detects the scattered light counts. The fold error (FE) is estimated using eq 2.[7,21]

$$\text{FE} = 10^{1/n \times \sum \log(\text{size,Zetasizer}/\text{size,TEM})} \quad (2)$$

where "n" represents repetitions of the study.

Undiluted sample was used to measure zeta potential using a Malvern Zetasizer. The sample holder was properly cleaned using running water and syringe. The sample holder was filled with the sample, and both openings were completely closed using plastic caps. The adhered sample or flown sample on the surface of the sample holder was properly removed using cotton or tissue paper. The sample was analyzed by placing in the analysis chamber followed by closing the lid. The analysis was carried out at room temperature.

Determination of Viscosity. Rheological assessment of the developed nanoemulsions (ENE1−ENE5) was required to ensure their consistency and flow behavior. Initially, the developed nanoemulsions (w/o type) were relatively more viscous than the respective dispersed nanoemulsion resulting in an oil-in-water (o/w) type. The viscosity was measured

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ACS Omega 2022, 7, 48100−48112

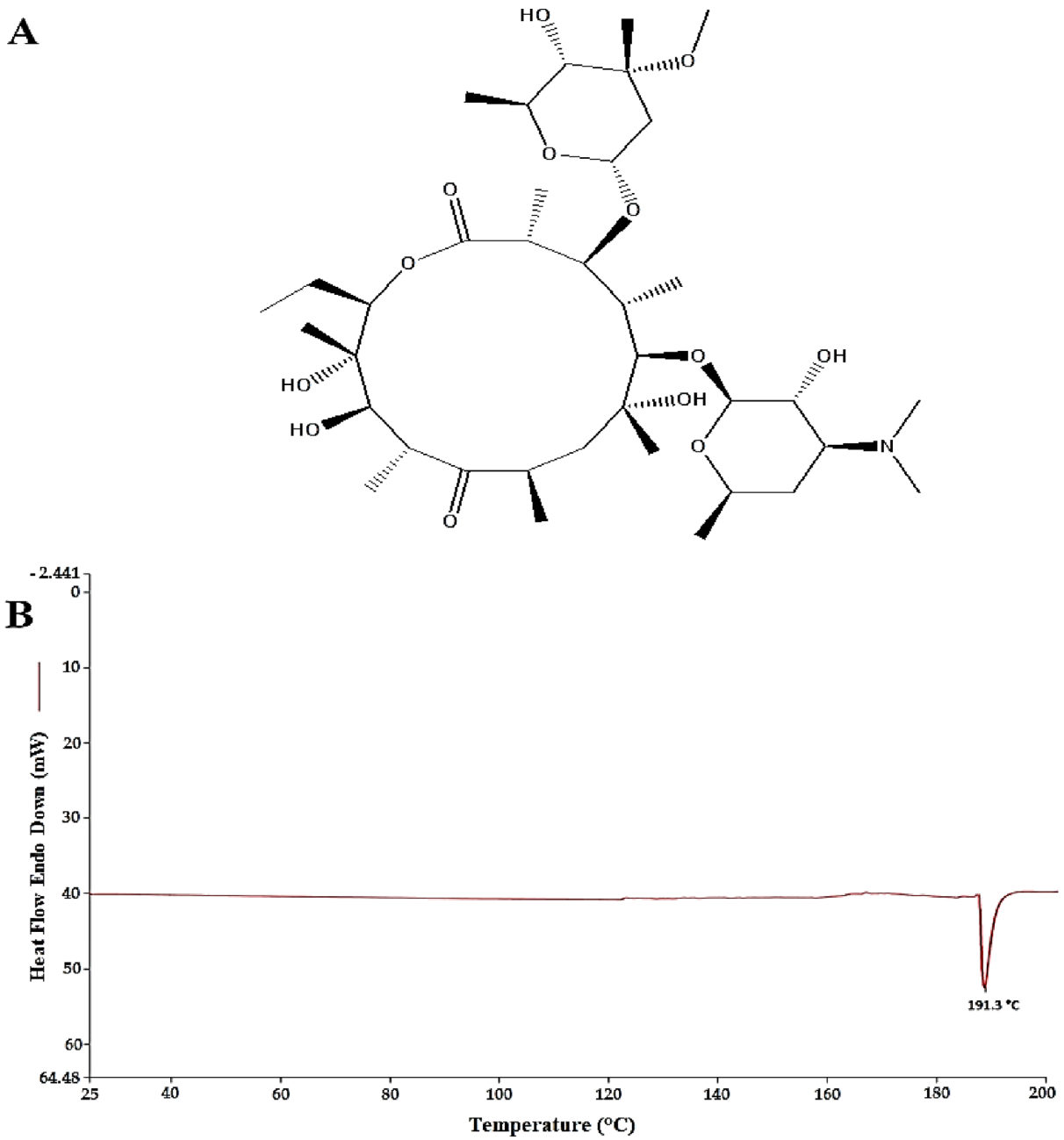

Figure 1. (A) Chemical structure of erythromycin (ERN) and (B) a DSC thermogram of neat ERN.

using a Bohlin viscometer (Bohlin Visco 88, Malvern, United Kingdom) at 25 °C and a shearing rate of 0−100 s^{-1}.[21] The viscometer was assembled with two prime parts (cone and plate) that were coaxially fixed in a vertical position. The cone along with the shaft was fixed to the roof of the solid plate form (upper section) and allowed to slowly touch the plate of the bottom. There was a slight minute gap for sample processing between them. The placed sample was allowed to be processed between the cone and plate for a given time point. In general, o/w ENEs were comparatively less viscous than the corresponding dispersed (w/o) ENEs.[10]

Measurement of RI. The prepared ENE1−ENE5 were transparent and stable. The optical behaviors of ENE1−ENE5 were assessed by measuring their RI values and comparing these against those of neat water (Milli-Q water) and oil (LabM). The RI value can be correlated with the isotropic nature of ENE1−ENE5. The value of RI was measured using an Abbe-type refractometer (Bausch and Lomb Optical Company, Rochester, NY, United States) by placing a drop of the sample on a clean glass slide at room temperature.[22] According to the "effective medium theory", the RI difference (X) is proportional to the surfactant content and mathematically defined as follows:

$$X = \frac{(n_o - n)}{n_o} \tag{3}$$

where "n" and "n_o" represent the RI values of pure water and ENE, respectively. This optical behavior is dependent upon several factors such as drop volume, size, and viscosity.[23]

Preparation of the Stock Solution. The aqueous solubility of ERN is limited at 25 °C (0.15 mg/mL).[13] The drug was reported to be soluble in dimethyl sulfoxide (DMSO) and

https://doi.org/10.1021/acsomega.2c06095
ACS Omega 2022, 7, 48100−48112

ethanol (6−30 mg/mL) at room temperature (25 °C). Therefore, a stock solution of ERN was prepared in water containing a trace concentration of DMSO (1% v/v) and the final concentration was 100 ppm. Several dilutions were made to achieve a concentration range of 0.1−100.0 ppm. A quantitative analysis was carried out using a validated HPLC method.

Percent Removal Efficiency (%RE). ERN is a hydrophobic pharmaceutical contaminant with poor aqueous solubility as described before. In order to investigate the %RE of the drug from an aqueous solution (model solution at lab scale), a known content (1 g) of ENE was dispersed in 10.0 mL of a freshly prepared solution (100 ppm). Both mixtures (stock solution and ENE) were forcibly vortexed for 5−30 min and kept aside for 20 min (benchtop standing) at room temperature (25 °C). Each ENE was individually dispersed with the aqueous solution for 5, 15, and 30 min. Now, the mixed drug−nanoemulsion system was de-stabilized by storing at freezing temperature (−21 °C) for 30.0 min and subsequently heating at 60.0 °C for 120 min. The procedure resulted in two separate phases. Thus, the unstable system was centrifuged (10,000 × g for 20 min) to obtain a supernatant without the drug.[12] The drug was quantified for different time samples and ENE1−ENE5 nanoemulsions. The total drug content (X_t) at time point "t" was quantified and expressed in "ppm/g". The hydrophobic ERN was adsorbed onto the globular surface of each nanoemulsion and assayed at explored time points (5, 15, and 30 min) using eq 4:

$$Q = [(X_O - X_t)/w] \times V \quad (4)$$

Thus, %RE was calculated using eq 5:

$$\%RE = [(X_O - X_t)/X_O] \times 100 \quad (5)$$

"X_O" and "X_t" indicate the contents (ppm) of ERN at "0" and "t" time points, respectively. Moreover, "V", "w", and "m" are the volume dispersed (mL), the total weight (g), and the mass of GNE (g), respectively.[24]

Morphological Assessment. Morphological assessment of ENE5 was conducted using TEM (Philips, Tecnai, Eindhoven, Netherlands). The optimized ENE5 was dispersed in the drug stock solution and stirred for 5.0 min in a glass beaker. The sample from the beaker was used for morphological visualization (shape) and size assessment of the globular structure. This tool visualized the ENE5 nanoemulsion after dispersion in the stock solution. The sample to be processed was placed on a copper grid and stained using a negative staining agent (0.1% phosphotungstic acid). The sample was air-dried overnight before scanning by TEM at varied voltages and resolutions. The globular size was also determined to calculate the FE value (eq 2).

Assessment of the Treated Water Using Spectroscopy, SEM−EDX, and ICP−OES Methods. The water sample treated by ENE5 was individually analyzed using UV−vis spectroscopy and inductively coupled plasma−optical emission spectroscopy (ICP−OES). In spectrophotometer, the treated water sample and the aqueous drug bulk solution were subjected to absorbance at 205 nm, respectively. Moreover, both samples were scanned with a UV−vis spectrophotometer for the characteristic peaks of the drug.

In the case of scanning electron microscopy−energy-dispersive X-ray analysis mode (SEM−EDX) (Jeol, Tokyo, Japan) and the ICP−OES technique (Thermo Fisher Scientific, Bremen, Germany), the samples were analyzed by elemental analysis. The prime detection element for the drug is the "N" atom. The presence of N atoms in water may confirm ERN in the treated water. Therefore, other heavy metals may be present in trace amounts or even below the detection limit. In practice, water contains various dissolved salts (sodium chloride, bicarbonates and carbonates of calcium, magnesium, and sodium) responsible for temporary and permanent hardness. To avoid detection interference, we used Milli-Q water free from any heavy salts (such as sulfates of Mg and Ca) responsible for permanent hardness. The procedure was adopted based on our previous experience with slight modifications.[25]

RESULTS AND DISCUSSION

ERN and Thermal Analysis. ERN is a well-established macrolide broad-spectrum antibiotic and frequently employed to treat several bacteria caused infections (bactericidal and bacteriostatic). Chemically, the drug is a conjugate base of an ERN A (1+) and derived from erythronolide A (cyclic ketone) (Figure 1A). The drug is associated with poor aqueous solubility (0.15 mg/mL) and has a log P of 2.6−3.06 and a pK_a value of 8.8.[13,25] The drug is considered one of the most common pharmaceutical contaminants present in aquatic systems through effluents disposed from hospital, sewage, and industrial wastes in several countries. Thermal analysis result showed 191.3 °C as the melting point of ERN, which is in agreement with the reported value (191−193 °C).[19] A sharp endothermic peak was obtained without drug degradation over the explored temperature range as shown in the thermogram in Figure 1B. Thus, there was no apparent shift of the characteristic endothermic peak, suggesting the purity of the drug and chemical stability over the temperature range.

Solubility Studies in Excipients. ERN is a poorly water-soluble drug (0.15 mg/mL) at physiological pH.[13] Its poor aqueous solubility may be due to its long cyclic hydrocarbon chain, high molecular weight, and lipophilic nature (log P = 2.3) (Figure 1A). It was required to select suitable components for nanoemulsion preparation. Therefore, solubility studies of ERN in various excipients were carried out, and the results are shown in Figure 2. The findings suggested that hydrophobic ERN was maximally soluble in LabM, THP, and ethanol.

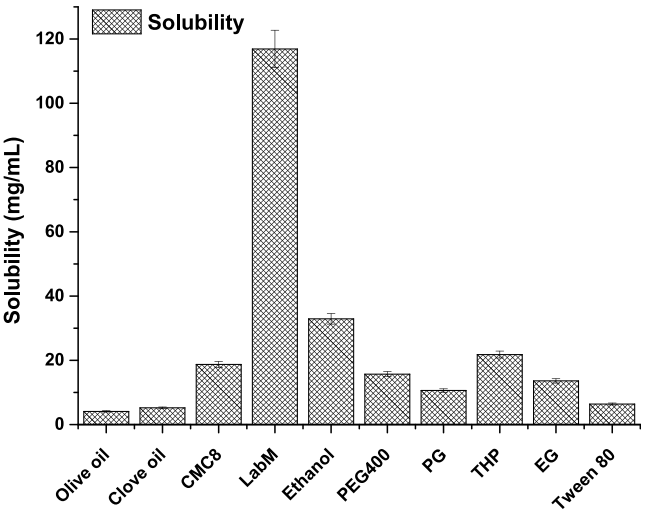

Figure 2. Solubility study of ERN in various solvents, surfactants, co-surfactants, and lipids (n = 3, mean ± SD).

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ACS Omega 2022, 7, 48100−48112

Among oils, the solubility values of ERN in olive oil, clove oil, CMC8, and LabM were found as 4.1 ± 0.22, 5.2 ± 0.2, 18.7 ± 0.94, and 116.9 ± 5.8 mg/mL, respectively. Among surfactants, the solubility values were observed as 21.8 ± 0.89 and 6.4 ± 0.31 mg/mL in THP and Tween 80, respectively. Among co-surfactants, the values of solubility in ethanol, PG, EG, and PEG400 were found to be 32.9 ± 1.6, 10.6 ± 0.52, 13.6 ± 0.5, and 15.7 ± 0.67 mg/mL, respectively. The solubility values of ERN in ethanol and LabM were 30.0 and 1120 mg/mL, respectively, as published before.[26] Ethanol and THP showed good solubility of ERN, which may be correlated to hydrogen bonding-based interaction (14 H-bond acceptor counts and 5 H-bond donor counts) and lipophilic nature of the drug.[25] Moreover, THP is chemically a 2-(2-ethoxyethoxy)ethane possessing one free hydroxyl group (OH−) and ERN possesses four hydroxyl groups responsible for hydrogen bonding solubility interaction as shown in Figure 1A.[27,28] The improved solubility of ERN in ethanol and THP may be attributed to the hydrogen bonds formed between the free OH− groups of ERN and excipients.[28] Thus, THP, LabM, and ethanol were selected as suitable components for preparation of GNEs.

PTDs and Prepared Nanoemulsions. A series of "water/ethanol/THP/LabM" nanoemulsions were prepared employing the screened lipid (LabM), surfactant (THP), and co-surfactant (ethanol) and aqueous phase (water). The ratio of the surfactant to the co-surfactant (S_{mix}) was varied to determine the most stable and most robust GNE. Components were screened considering the solubility at a fixed temperature. Therefore, it was a prerequisite to construct PTDs to identify the suitable S_{mix} ratio (1:1, 2:1, 1:2). Several nanoemulsions based on these ratios were synthesized, and the right ratio was indicated by the maximally delineated area in the PTD.[12] Data obtained from titration for constructing the PTD suggested that Figure 3A exhibited maximum delineated zone of GNE possessing an S_{mix} ratio of 1:2 among them. Furthermore, this value of S_{mix} was taken as the most robust ratio for a stable, isotropic, and transparent GNE at the optimum concentration. A summary of the compositions of various GNEs is presented in Table 1. It was noticed that the equal ratio of surfactant to co-surfactant gave limited delineated zone of nanoemulsion. However, increasing the concentration of the co-surfactant over that of the surfactant was found to have relatively important impact on the delineated zone. This can be observed in Figure 3B (S_{mix}) where the delineated zone was found to be maximum as compared to others (S_{mix} = 1:1 and S_{mix} = 2:1). The delineated zone was found to be slightly decreased on increasing the concentration of the surfactant (S_{mix} = 2:1) as compared to that of the co-surfactant (Figure 3C). The result of the PTD revealed that 65.0% w/w water was solubilized using 22.0% w/w S_{mix} (1:2) as shown in Figure 3B (Figure 3B exhibits the maximized delineated area of the GNE). Moreover, an S_{mix} ratio of 2:1 (22.5% w/w) solubilized ∼48.2% w/w water as observed in the delineated zone in Figure 3C. Thus, Figure 3B shows that the maximum solubilization of water (∼65.0% w/w) by 22.0% w/w S_{mix} (2:1) emulsifies 27% w/w LabM. In conclusion, THP served as a potential surfactant for emulsifying water in an organic phase and delineated maximum zone in PTD. Both LabM (medium-chain triglycerides) and THP have already been explored as established green excipients to tailor nanoemulsions and used to eliminate the trace content of a lipophilic drug as a contaminant (diclofenac sodium) in an aquatic system.[29]

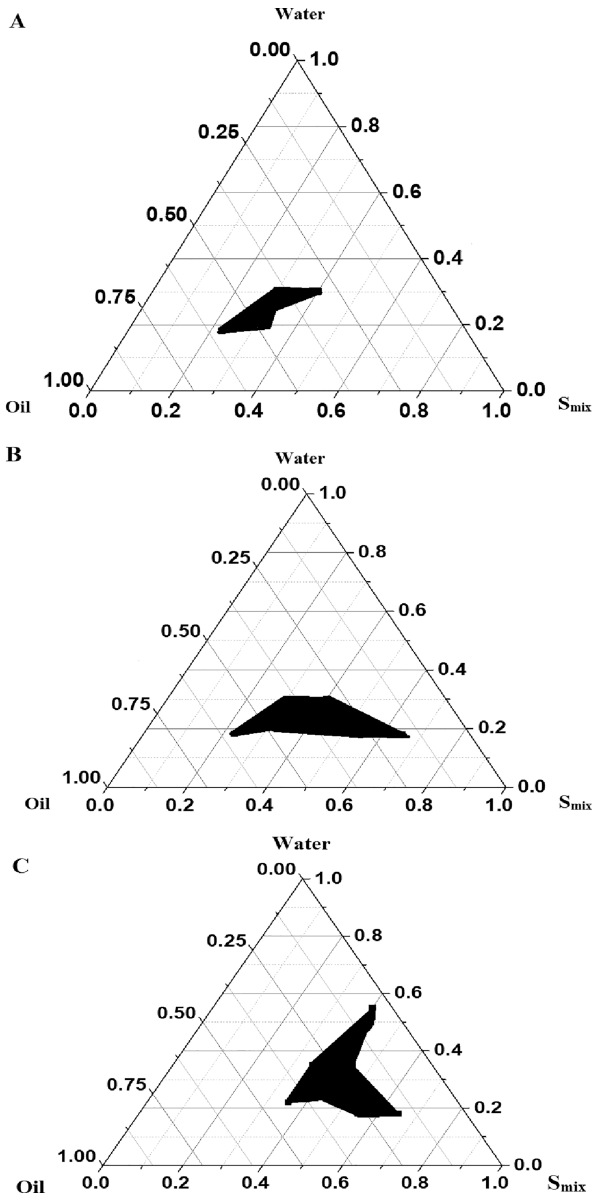

Figure 3. Pseudo-ternary phase diagrams of ERN-loaded nanoemulsions with varied S_{mix} ratios: (A) 1:1, (B) 2:1, and (C) 1:2.

Table 1. Summary of the Compositions of Green Nanoemulsions[a]

	composition (% w/w)				
code	THP	water	ethyl alcohol	LabM	S_{mix}
ENE1	18	5.5	36	40.5	1:2
ENE2	18	8.5	36	37.5	1:2
ENE3	18	11.5	36	34.5	1:2
ENE4	18	14.5	36	31.5	1:2
ENE5	18	17.5	36	28.5	1:2

[a]Note: LabM, Labrafil M; THP; Transcutol HP.

Benchtop stability was assessed at room temperature to inspect any signs of physical instability. ENE1−ENE5 were synthesized as per Figure 3B and subjected to further evaluations.

Freeze−Thaw Cycles and Centrifugation: A Thermodynamic Study. In general, GNEs are considered to be kinetically stable systems. Physically, these are isotropic, transparent, and thermodynamically stable systems. A nano-

https://doi.org/10.1021/acsomega.2c06095
ACS Omega 2022, 7, 48100−48112

carrier possessing a size of 100 nm or smaller is considered stable under thermal stress (~−21 to 40 °C) and physical stress (centrifugation). Pharmaceutically, liquid nanoemulsions, microemulsions, and emulsions may probably exhibit physical instability (phase separation or creaming or precipitation) during transportation and storage due to temperature variation and mechanical stress. This was a reason for carrying out a thermodynamic stability study of the developed nanoemulsions to ensure physical stability under explored temperatures and centrifugation forces. The results showed that ENE1−ENE5 were found to be stable after exposure to series of low and high temperatures as shown in Table 2. These isotropic mixtures revealed no signs of physical instability. This may be due to the substantially firm mono or multilayer of S_{mix} at the interfaces responsible for reducing surface tension and preventing globules from coalescing.[30]

Table 2. Various Cyclic Steps of Thermodynamic Stability Assessment of ENE1−ENE5[a]

	thermodynamic stability steps				
code	centrifugation	cooling (4 °C)	freezing (−21 °C)	thawing (45 °C)	S_{mix}
ENE1	√	√	√	√	1:2
ENE2	√	√	√	√	1:2
ENE3	√	√	√	√	1:2
ENE4	√	√	√	√	1:2
ENE5	√	√	√	√	1:2

[a]Note: the √ mark indicates a passed step.

Evaluation Parameters of GNEs. In Table 3, the results of size, PI, ZP, RI, and viscosity are summarized. The value of the globular size ranged as 58−173 nm for ENE1−ENE5, respectively. The values of PI and ZP were found to be in the ranges of 0.19−0.42 and 25.4−30.2 (−mV), respectively, for ENE1−ENE5. The ZP values were found to be negative due to the LabM content of the organic phase composed of oleoyl polyoxyl-6 glycerides.[31] LabM contains seven lipids (palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, arachidic acid, and eicosenoic acid), and oleic acid is the major component (58−80%) among them.[31] These lipids contributed a maximum negative ZP (~25 to 30 mV) as the surface charge on globules to keep dispersed nanoscale globules distant (electrostatic repulsion bearing similar charges) from each other. ENE1 and ENE5 showed the highest (173 ± 9 nm) and the lowest values (58 ± 6 nm) of globular sizes among them. This decreased value of the globular size in ENE5 may be correlated with its relatively low content of oil as compared to that of ENE1.[12,26] A similar pattern was observed in PI values where ENE5 exhibited a good globular size distribution as evidenced with its low value (0.19). The values of the globular size and PI of ENE5 can be further visualized using TEM for estimating FE later. The values of viscosity ranged as 121.7−209.6 cP for ENE1−ENE5, respectively. ENE5 revealed the lowest value of viscosity (121.7 cP) as compared to ENE1, which may be due to it having the lowest value of the oil content and the highest content of the aqueous phase. The viscosity of a nanoemulsion indicates flow and efficient dispersion in an aqueous system if it is dispersed. In general, a viscous liquid or nanoemulsion (w/o) takes a longer time to get dispersed in an aqueous system as compared to a less viscous nanoemulsion (o/w) due to the relative content of the oil phase.

In Table 3, the values of RI ranged as 1.3286−1.3589 (ENE1−ENE5). The values of RI were slightly found to be decreased with a decrease in oil content. RI is an optical property of an isotropic system and signifies (physicochemical) interaction among the drug and components.[32] In the present investigation, the RI values of all developed nanoemulsions are close to the RI value of water (1.33−1.42), suggesting a transparent, stable, and isotropic nature.[33,34] The observed values of RI for blank water and LabM were found to be 1.345 and 1.458, respectively, which are in close agreement with the values from the source label leaflet (1.465−1.475) (Gattefossé leaflet). Thus, there were no significant differences ($p > 0.05$) in RI values for ENE1−ENE5, which signified fundamentally thermodynamically stable nanoemulsions.

Impact of Water and LabM Contents on the Globular Size of Nanoemulsions. A nanoemulsion consists of at least two distinct phases of opposite polarity (water as the hydrophilic phase and oil as the lipophilic phase) and stabilized (maintained in a metastable form) using a surfactant(s) by dissipating the excess energy at the newly developed interface between the two phases.[34] The globular size values were found to be reduced with an increased concentration of water (from 5.5 to 17.5%), whereas these values were observed to be increased with an increased content of LabM (from 28.5 to 40.5%) (Table 3, Figure 4A). Aside from these two phases, many factors that have profound effect on the globular size of GNEs such as phase volume fraction, type and content of the surfactant, interfacial properties (HLB), chemical properties, viscosity, and the compositions of the two phases. However, little is known about the impact of these factors on the globular size and stability of nanoemulsions and, thus, how these influence the removal efficiency of water contaminants.[34] Objectively, the removal efficiency of a drug from a contaminated aqueous system depends upon the stability of the nanoemulsion, the globular size, and encapsulation efficiency (lipophilic−lipophilic interaction) after dispersion of the w/o emulsion in an aqueous system. Therefore, the globular size and the compositions of the two phases have crucial impact on the removal efficiency. At this level of

Table 3. Various Characterization Parameters of ENE1−ENE5[a]

	result (mean ± standard deviation)				
code	size (nm)	PI	η (cP)	refractive index	ζ (mV)
ENE1	173 ± 9	0.42	209.6 ± 6.1	1.3589 ± 0.003	−30.2 ± 4.7
ENE2	131 ± 7	0.39	191.4 ± 5.9	1.3504 ± 0.002	−28.7 ± 4.2
ENE3	95 ± 8	0.33	165.0 ± 4.2	1.3488 ± 0.003	−27.5 ± 3.8
ENE4	79 ± 6	0.29	142.9 ± 4.0	1.3323 ± 0.003	−27.1 ± 3.2
ENE5	58 ± 6	0.19	121.7 ± 3.2	1.3286 ± 0.004	−25.4 ± 3.0

[a]Note: PI, polydispersity index; ζ, zeta potential; and η, viscosity.

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ACS Omega 2022, 7, 48100−48112

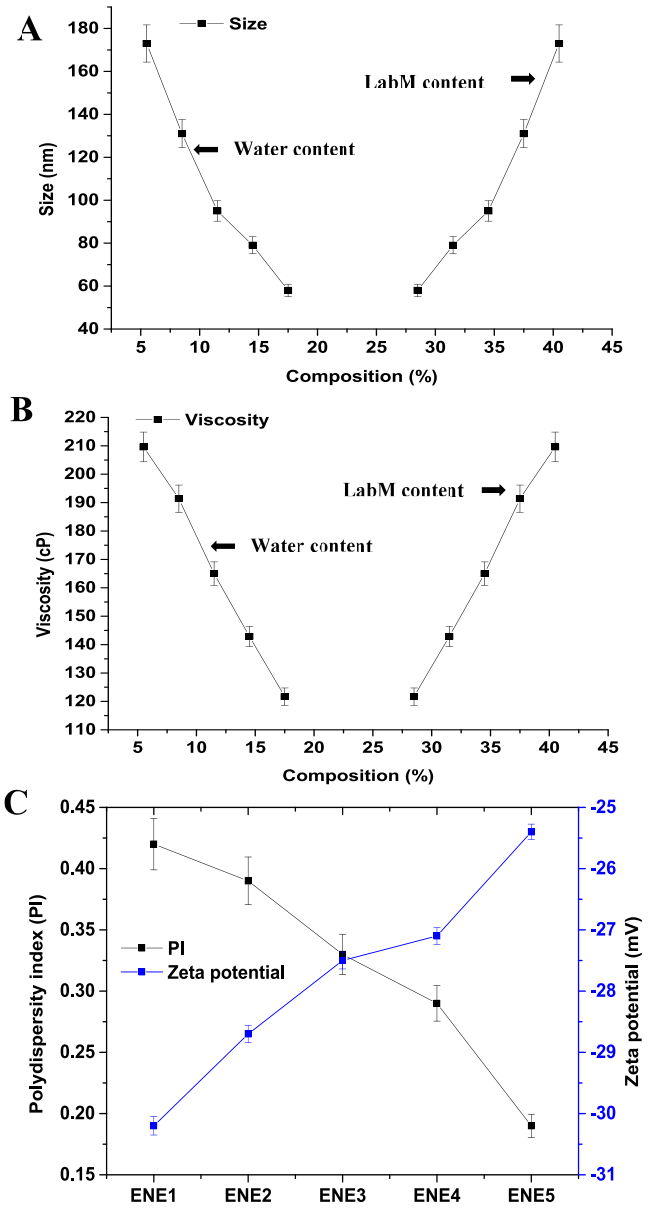

Figure 4. (A) Impact of composition of ENE1−ENE5. (a) Impact of composition (water and lipid content) on globular size, (b) viscosity (cP), and (c) PI and zeta potential.

discussion, it is essential to describe that an oil phase of optimum viscosity and low polarity could be suitable for a stabilized GNE for maximized removal efficiency for a w/o type of GNE.[35] Thus, these properties can, at least in part, contribute to obtaining a stabilized w/o GNE (ENE1−ENE5) with inner water globules against shear−induced stress during transport, manufacturing, and other mechanical stresses.[35] In Figure 4A, ENE5 illustrates the lowest size, which may be due to the low amount of LabM and maximum water content. A similar trend was observed with our previous findings where a water/Transcutol/Capryol-based GNE was used to remove clarithromycin (macrolide antibiotic) from a contaminated aqueous system.[12]

Impact of Water and LabM Contents on the Viscosity of Nanoemulsions. The viscosity and HLB values of LabM are reported to be in the range of 75−95 cP at 20 °C and 3−4, respectively (Labrafil, Gattefossé, 2022). The results of ENE1−ENE5 are compiled in Table 3, wherein ENE5 and ENE1 exhibited the minimum (121 cP) and the maximum (209 cP) values of viscosity, respectively. It is quite clear from the obtained results that the viscosities of nanoemulsions are relatively higher than that of neat oil LabM. This can be correlated to get an improved stable nanoemulsion with optimum viscosity to impart stability of aqueous globules in the dispersed phase against shear-induced stress. Moreover, ENE5 may be considered desirable among them due to its low viscosity for rapid emulsification after dispersion in an aqueous phase and good flow behavior of the water/ethanol/Transcutol/LabM nanoemulsion. The results showed that viscosity values constantly decreased with an increased content of water (from ENE1 to ENE5), and this can be correlated with the decreased oil phase fraction volume in the nanoemulsion. In contrast, the viscosities of nanoemulsions from ENE5 to ENE1 were found to be increased due to the increased content of LabM at a fixed temperature (Figure 4B). The measured values of "viscosity" can also be correlated with globule size. In general, the viscosity of a nanoemulsion depends upon several other factors not encountered here. These factors may be shape, globular size, volume fraction of phases, temperature variation, forces, conformational changes under stressed conditions, and composition polarity.[12] In this study, ENE1−ENE5 are a w/o type of nanoemulsion, which is converted to an o/w type of GNE after dispersion in an aqueous bulk solution (containing the drug as a contaminant). The viscosity values of the former nanoemulsion are always greater than those of the latter one due to the oil phase as the continuous phase (the former ENE1−ENE5). The results showed that viscosity values decreased with an increased content of water (from 5.5 to 17.5%), whereas these (∼121 to 210 cP) linearly increased with an increased content of LabM (28.5 to 40.5%) (Figure 4B). In the case of the dispersed nanoemulsions of ENE1−ENE5 to the respective o/w type of ENE1−ENE5 nanoemulsions, the values of viscosity are expected to be reduced owing to preferential solvation of oil globules in the aqueous phase.[36] Thus, the viscosity of a GNE is a critical factor for selection, design, and carrying out of wastewater treatment by the treatment plant (pumping, mixing, processing, storing, and transportation).[36] In conclusion, the influence of water and oil content on the viscosity of nanoemulsions is portrayed in Figure 4B and the obtained result is in accordance with our recently published report on clarithromycin.[12,37]

Impact of Water and LabM Contents on the PI and ZP of Nanoemulsions. The results of PI and ZP are summarized in Table 3, wherein ENE1 exhibited the highest value of PI (0.42), which may be due to poor emulsification attributed to low-HLB LabM.[38] The PI values of water/ethanol/Transcutol/LabM nanoemulsions (ENE1−ENE5) ranged as 0.42−0.121, which signify a uniform globular distribution (Table 3). The relationship of these with PI is demonstrated in Figure 4C. It is obvious from Figure 4C that the PI values decreased from ENE1 to ENE5, which may be correlated with the decreased concentration of LabM and increased water content. The lowest value of PI of ENE5 is quite suitable for GNEs in terms of consistency, abundant available surface area, and probably high % RE of ERN. ENE1−ENE5 are a w/o type of nanoemulsion that exhibited a negative ZP due to LabM as the continuous oil phase. Chemically, LabM is a mixture of several fatty acids (seven) and oleic acid as major components (up to 80%). Therefore, the negativity of the ZP can be attributed to fatty acids present

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ACS Omega 2022, 7, 48100−48112

in LabM of nanoemulsions. These values were found to be decreased (from ∼−30 to −25 mV) with a decreasing content of LabM from ENE1 to ENE5 as shown in Figure 4C. Notably, the nanoemulsion (o/w) obtained after dispersion of the respective w/o type of nanoemulsion (ENE1−ENE5) in an aqueous bulk solution still had a negative ZP with no significant difference ($p > 0.05$) due to lipid content (data not presented). Thus, ZP was mainly influenced by the content of LabM (possessing fatty acids). Thus, these high values (± 25−30 mV) of ZP supported the kinetically stable ENE1−ENE5 under mechanical and thermal stresses subjected to thermodynamic stability testing as evidenced in various studies.[12,30,36]

Adsorption Study: Percent Removal Efficiency (%RE). ERN is a macrolide antibiotics with poor aqueous solubility and limited dissolution. Short half-life (1−1.5 h), instability in acidic gastric juice, unpleasant taste, and low oral bioavailability (∼30%) of the drug resulted in topical application and subsequently led to high effluent to the environment in original form.[19] Several conventional methods have been implemented to remove ERN from aquatic systems (domestic and hospital effluents, wastewater, industrial and municipal effluents). However, these methods faced various challenges such as (a) inability to remove the contaminant completely, (b) expensive process, (c) complex and time-consuming process, and (d) critical task to decontaminate trace pollutant. Moreover, several factors (nature of the drug, adsorbent, process parameters, and level of contamination in water) have great impacts on the removal efficiency of the trace contaminant found in aquatic systems.[37] Considering these, we investigated the %RE of water/ethanol/Transcutol/LabM nanoemulsions (ENE1−ENE5) from an aqueous system containing "ERN" at varied time points. The results are illustrated in Figures 5 and 6.

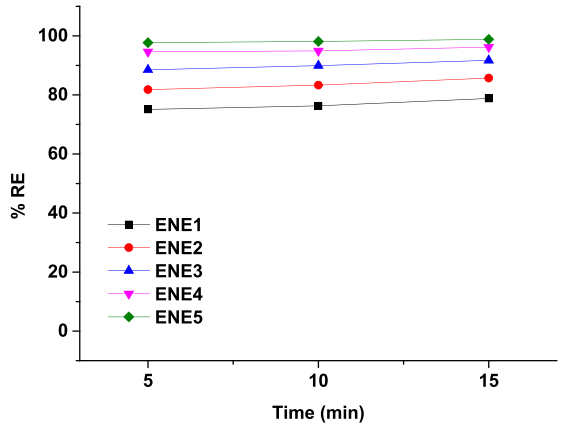

Figure 5. Impact of contact time (min) on %RE by ENE1−ENE5.

In this study, we explored the impact of exposure time, globular size of the nanoemulsion, and composition (water and LabM contents) on %RE. The impact of the exposure time (5, 10, and 15 min) of ENE1−ENE5 (water/ethanol/Transcutol/LabM) on the %RE of ERN after dispersion with an aqueous bulk solution was studied, and the results are shown in Table 4.

The results showed insignificant impact of contact time on %RE for ENE1−ENE5 nanoemulsions after dispersion with a bulk aqueous solution as observed in Table 4. ENE1 exhibited %RE values of about 75, 79, and 83% at 5, 10, and 15 min of exposure, respectively. Moreover, ENE5 exhibited %RE values

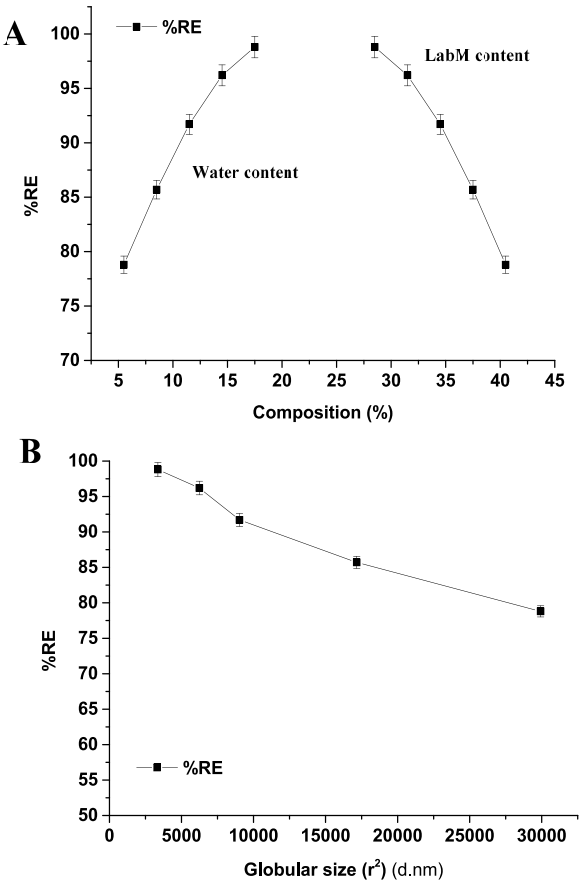

Figure 6. Impact of various factors on %RE by ENE1−ENE5: (A) impact of composition on %RE and (B) impact of square of globular size (r^2) on %RE.

Table 4. %RE of ENE1−ENE5 at Varied Time Points[a]

	percent removal efficiency (%RE)		
NE code	5.0 min	10.0 min	15.0 min
ENE5	97.7 ± 5.1	98.1 ± 5.5	98.8 ± 6.1
ENE4	94.6 ± 4.4	94.9 ± 4.7	96.2 ± 5.3
ENE3	88.5 ± 4.1	89.9 ± 4.4	91.7 ± 3.9
ENE2	81.8 ± 3.3	83.3 ± 4.1	85.7 ± 4.5
ENE1	75.1 ± 3.8	76.3 ± 4.0	78.8 ± 3.9

[a]Note: %RE, percent removal efficiency.

of about 97.7, 98.1, and 99.8% at 5, 10, and 15 min of exposure, respectively. Considering the studied factors, ENE5 can be the most robust and efficient GNE among the developed nanoemulsions, which may be correlated with the composition, low viscosity, small size, and nature of the drug. The highest %RE associated with ENE5 can be related to several combined factors playing in tandem. However, the potential factors are surface area available for adsorption, viscosity, polarity, lipophilicity of ERN, oil, and optimized processes.[12] Moreover, the contact time of GNE with the bulk aqueous solution was not significant, which may be due to efficient emulsification of the w/o GNE (ENE1−ENE5) in the aqueous system (Figure 5).

Second, LabM served as the prime adsorbent in nanoemulsions due to lipophilic−lipophilic interactions. Water is the internal phase of ENE1−ENE5 nanoemulsions (w/o), which are converted to their respective o/w type of

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ACS Omega 2022, 7, 48100−48112

nanoemulsion with the aqueous phase as the external phase (in the new system). Thus, ERN, being a lipophilic drug candidate, exhibits preferential solubilization with an organic phase (LabM) due to lipophilic−lipophilic interaction and adsorption. Therefore, water and LabM contents have significant impact on the values of %RE (Figure 6). The %RE values were found to increase with an increase in water content, whereas these values were observed to decrease with an increase in LabM content. From ENE1 to ENE5, there was a regular reduction in LabM, which resulted in efficient emulsification in the aqueous bulk solution. As a result of this, ENE5 provided maximum adsorptive surface area for the drug.[12,36,38] ERN, being a lipophilic drug, was substantially adsorbed to the organic phase of the nanoemulsion. In contrast, ENE1 was composed of the maximum LabM content and possessed a relatively greater globular size. This resulted in limited available surface area for adsorption of ERN. Figure 7 illustrates the

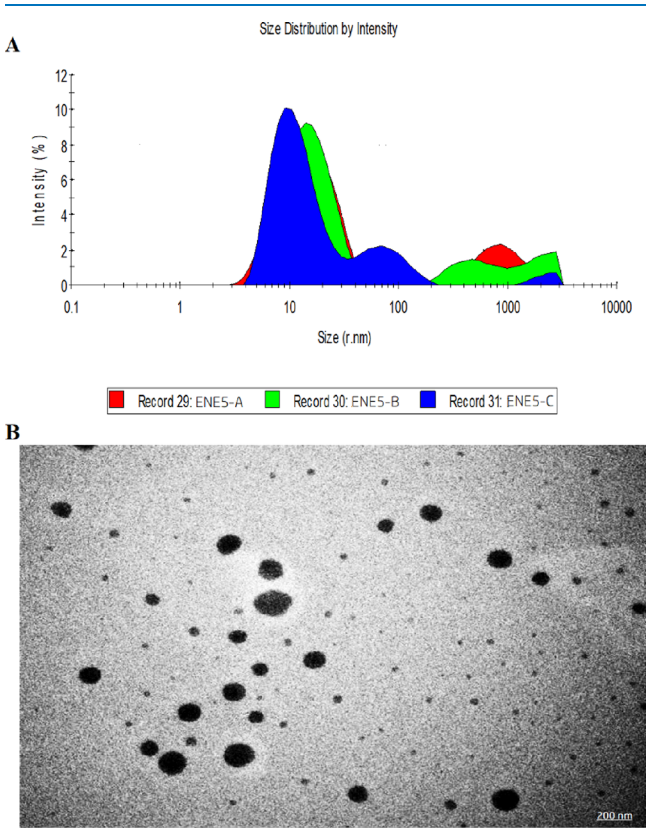

Figure 7. (A) Globular size distribution intensity of ENE5 (n = 3) and (B) morphological assessment of ENE5 using transmission electron microscopy (TEM) visualized at a magnification of 80,000× and a voltage of 80 kV.

impact of globular size on %RE. A similar trend was observed when lipophilic pharmaceutical contaminants (rifampicin, clarithromycin, and azithromycin) were successfully decontaminated at laboratory scale using GNEs.[10−12] Thus, removal efficiency is achieved by the combined impact of composition (water, oil, and surfactant), globular size, viscosity, polarity, and process variables.[37,38] It is noticeable that increasing the content of LabM (from 28.5 to 40.5%) and decreasing the content of water (from 17.5 to 5.5%) resulted in a progressive increase in %RE value (Figure 6). The %RE of ERN is based on the adsorption ability onto the globular surface of the organic surface (LabM). Thus, globular size, surface area of the nanoemulsion after dispersion, viscosity, and lipophilic nature of LabM worked together for efficient removal of ERN. Conclusively, ENE5, having a low size (58 nm), the lowest viscosity (∼122 cP), and uniformly distributed (PI) nanoglobules (0.19), was an optimized green nanoemulsion for the purpose.

Morphological Assessment of ENE5 Dispersed in an Aqueous Bulk Solution. ENE5 was finally selected for morphology assessment. In order to confirm the globular size of dispersed ENE5 in the aqueous bulk solution and its (ENE5) inversion into an o/w type of new ENE5, Zetasizer and TEM studies were performed. The results are exhibited in Figure 7A,B. It is apparently visible that ENE5 showed majority of globular size distribution falling down below 100 nm with slight variations in triplicate study. This further supported the maximum available surface area for adsorption of ERN after dispersion in an o/w type of GNE. Moreover, the TEM report confirmed that the spherical nanoscale globules of ENE5 (o/w) were clearly dispersed and stable when visualized after 15 min of dispersion. The globular size assessed using TEM also confirmed a size dimension below 100 nm with slight variations. Therefore, both values were used to calculate "FE" and the calculated value of FE was found to be 1.7 (below 2.0) within an acceptance range.[11]

Confirmation Test for the Cleaned Water. Among the explored ENE1−ENE5, ENE5 was considered the most suitable, efficient, and robust GNE. Therefore, ENE5-treated water was processed for UV scanning and absorbance. The bulk aqueous drug solution (100 times diluted to get 0.001 μg/mL) and the treated water were scanned in photometry mode. The result showed that there was a prominent absorbance peak for the drug present in the bulk aqueous solution, suggesting the presence of the drug before treatment. In the case of the treated water, there was no any apparent peak, indicating the absence of the drug or being due to the detection limit. Moreover, both of the samples were analyzed against water as the control (reference sample). The aqueous drug solution showed 0.0629 as the absorbance value at 205 nm, whereas the treated water sample could not show any absorbance value, suggesting the absence of the drug or being due to the detection limit. To support the UV finding, the samples were processed using the HPLC method. There was a prominent peak obtained at 11.5 min (retention time) for the drug solution (0.1 ng/mL), whereas the treated water did not reveal any peak (chromatogram data not reported here).

SEM−EDX and ICP−OES results are presented in Figure 8, wherein SEM−EDX and ICP−OES techniques assessed the trace content of metals, non-metals, and N atoms (present in the drug). It is obvious from results that the treated water contains mainly alkali and alkali earth metals such as potassium (K), sodium (Na), and calcium (Ca) due to the slight hardness of water and dissolved trace water. In the case of heavy metals such as Cu (copper) and Ti (titanium), they are present at 0.05 ± 0.003 and 0.026 ± 0.0012 ppm, respectively. However, Zn, As, and Fe were found to be below the detection limit in ICP−OES. Notably, the assessment of the "N" atom to ensure the presence of ERN confirmed. Thus, the SEM−EDX and ICP−OES techniques ensured the absence of the drug in the water treated by ENE5.

Mechanistic Perspective. The developed isotropic and thermodynamically stable nanoemulsions were efficient and stable after dispersion in an aqueous solution. ERN is a lipophilic contaminant (in trace concentration) and insoluble

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ACS Omega 2022, 7, 48100−48112

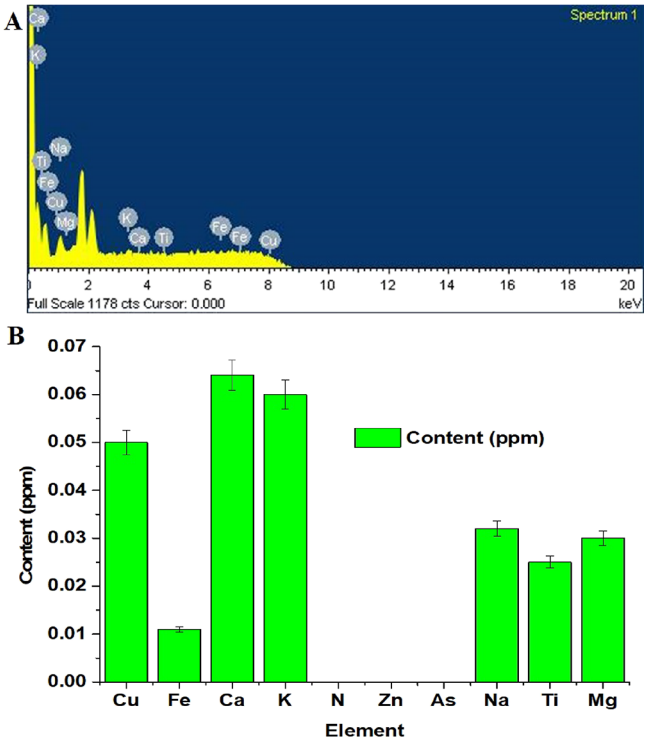

Figure 8. (A) SEM−EDX and (B) ICP−OES results of the treated water revealing elements present in the water (ppm). The absence of the "N" atom indicates the absence of ERN (the N atom is present in the structure of ERN).

in water. Various publications hypothesized that a lipophilic drug results in precipitation in an aqueous medium when the dispersed nanoemulsion is exposed to a high temperature (60 °C). In brief, the exposure of the mixture to ∼60 °C for 3 - 4 h) cracked the emulsion into aqueous and oil phases.[12] This is a forced instability caused by thermal stress (Figure 9). Both phases are separated out, and ERN is precipitated at the bottom and adsorbed as well (due to lipophilic−lipophilic interaction and high solubility in LabM) to the organic phases separated out from nanoemulsion.[12,36] The drug is preferentially adsorbed onto the organic phase of ENE1−ENE5 (Figure 9). Eventually, the obtained aqueous phase was found to be clear and free of the contaminant. This was further confirmed by taking the UV absorbance and scanning using a spectrophotometer. There was no observed absorbance and apparent characteristic peak using the obtained treated water (data not included).

CONCLUSIONS

Conventional methods are challenged with several limitations such as inefficient removal of trace contents of pharmaceuticals, expensive method, lack of specificity, complex process, and being time consuming. Therefore, GNE-based removal of trace contents of antibiotics such as ERN is an efficient and simple approach to removing ERN from contaminated water resources such as wastewater disposed from industrial waste, domestic water, and hospital, healthcare section, and municipal wastewater. An effluent introduced to an aquatic system results in negative health impact on the flora and fauna of the aquatic ecosystem and human health. The developed simple water/ethanol/Transcutol/LabM GNE was found to be stable under thermal and mechanical stresses. The %RE was influenced by several factors such as the content of water, LabM, viscosity, and globular size. Fundamentally, a reduced globular size resulted in a profound increase in the available surface area for adsorption of ERN to the organic phase (LabM). Reduced viscosity had great impact on prompt emulsification of ENE5 for adsorptive removal of ERN at ambient temperature. Reduced content of LabM and maximized content of water were the most optimizing parameters for efficient removal of ERN. Therefore, reduced size, minimum viscosity, high content of water, and low value of LabM dictated the selection of the most robust GNE (ENE5). Spectroscopy, SEM−EDX, and ICP−OES techniques ensured the absence of ERN. Conclusively, ENE5 may be the most simple, economic, eco-

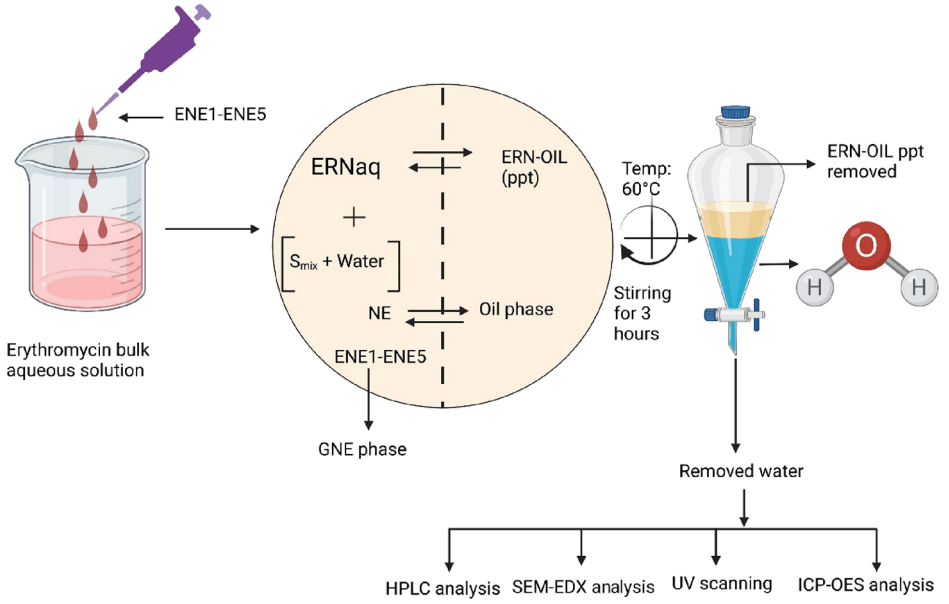

Figure 9. Proposed mechanistic illustration of ERN removal using ENE1−ENE5 from the aqueous drug solution and further analysis of the treated water using various techniques.

https://doi.org/10.1021/acsomega.2c06095
ACS Omega 2022, 7, 48100−48112

friendly, and highly efficient system from decontaminating ERN from aquatic systems.

■ ASSOCIATED CONTENT

Data Availability Statement

Availability of data and materials is not applicable.

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A.H. contributed to conceptualization, funding acquisition, drafting, and writing. M.A.A. contributed to data curation and analysis. S.S.I. contributed to software, data curation, and formal review. M.S.A. contributed to review, resources, and visualization. O.A.A. contributed to software and analysis and methodology.

Funding

The authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number IFKSURG-2-053.

Notes

The authors declare no competing financial interest.

The study does not involve any animal or animal-related experiments.

The article does not involve any human or animal studies.

All authors agreed to publish the article.

■ ACKNOWLEDGMENTS

The authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number IFKSURG-2-053.

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https://doi.org/10.1021/acsomega.2c06095
ACS Omega 2022, 7, 48100−48112