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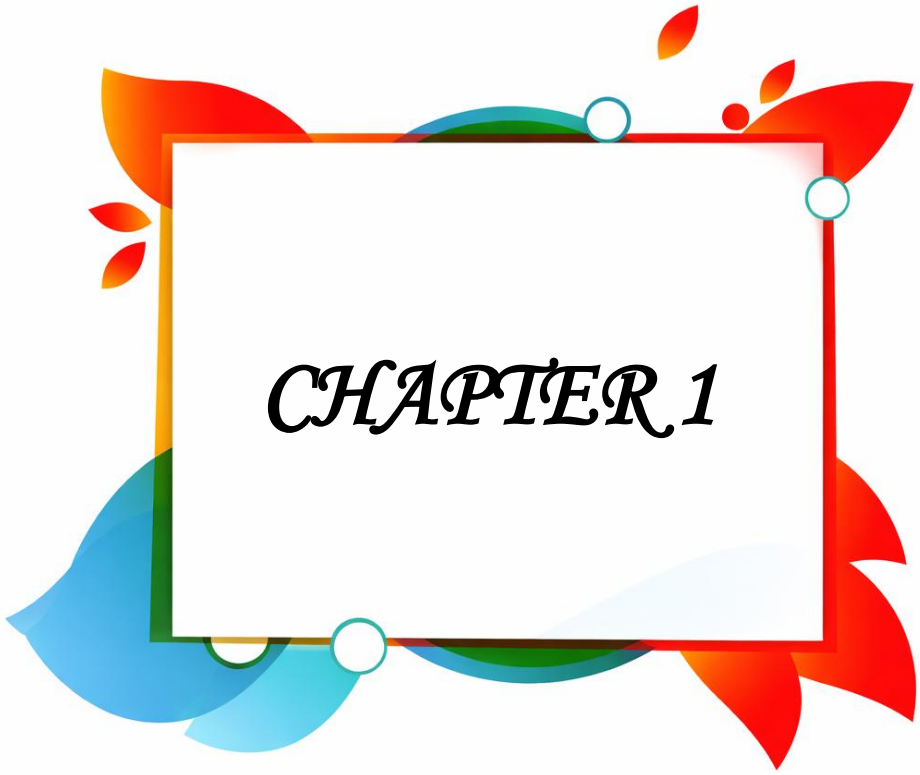
CHAPTER 1

In-Vitro Synergy Testing: Methodological Approaches, Clinical Applications, and Future Perspectives

Meltem Özden[1] & Salih Özden[2]

1- INTRODUCTION

Clinical interest in antimicrobial synergy began to take shape in the 1950s. A well-known early example was reported in patients with enterococcal endocarditis: treatment with penicillin G alone often resulted in high relapse rates, whereas the addition of streptomycin markedly reduced recurrences. Subsequent in vitro and clinical investigations consistently demonstrated the value of antibiotic combinations in managing such infections (Acar, 2000).

The rapid dissemination of multidrug-resistant (MDR) pathogens today poses a serious threat to the effectiveness of existing antibiotics and underscores the urgent need for new therapeutic strategies. At the same time, the development of novel antimicrobial agents has not kept pace with the continual emergence of resistance mechanisms (Doern, 2014; Gaudereto et al., 2020; Laishram et al., 2017).

Combination therapy is regarded as one of the most important strategies in combating antimicrobial resistance. It involves the concurrent use of two or more antimicrobial agents with the aim of achieving a stronger therapeutic effect than single agents alone. The key objectives of such regimens are to broaden the antimicrobial spectrum, reduce dose-dependent toxicity, and suppress the emergence of resistance. Notably, combining drugs with distinct mechanisms of action—for example, cell wall synthesis inhibitors together with aminoglycosides—has been shown to enhance therapeutic success (Acar, 2000; Assefa et al., 2025; Doern, 2014; Jaglal et al., 2025; Laishram et al., 2017).

Using different antibiotic groups in combination, or pairing them with non-antibiotic agents, is regarded as an effective way to slow the development of resistance. By acting on multiple cellular targets at the same time, this strategy

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makes it harder for microorganisms to adapt to several agents simultaneously. In addition, exploiting synergistic interactions can accelerate bacterial clearance and improve overall treatment outcomes (Doern, 2014; Dokumacı, 2024).

In vitro synergy tests aim to evaluate whether the combined efficacy of two or more antimicrobials is greater than the sum of their individual activities. Antimicrobial combinations can exhibit additive, synergistic, or antagonistic interactions. In additive interactions, the total effect of the combination is equal to the sum of the individual effects of the agents, while in synergistic interactions, the antimicrobial activity generated by the combination is higher than the sum of the individual efficacies of the agents. Conversely, in antagonistic interactions, the combination may reduce the effectiveness of the agents (Doern, 2014).

The primary goal of in vitro synergy detected in the laboratory is to achieve the targeted bactericidal effect with minimal risk of toxicity at the site of infection in clinical microbiology. In the presence of resistance genes (e.g., carbapenemases or colistin resistance genes), an antibiotic with a minimum inhibitory concentration (MIC) above clinically significant limits and considered "resistant" can be reactivated thanks to a synergistic co-agent. Synergy tests are of great importance, especially in determining salvage therapy regimens to be applied in patients infected with MDR pathogens. Furthermore, these tests allow for the identification of antagonistic interactions that could potentially fail or harm the patient before in vivo application of combinations (Assefa et al., 2025; Doern, 2014; Dokumacı, 2024; Laishram et al., 2017).

Since evaluating the effectiveness of different combinations in clinical trials is quite challenging from an ethical and practical standpoint, in vitro synergy tests are considered important tools in translational research. These tests provide a scientific basis for identifying antimicrobial combinations that can yield successful clinical results and guide in vivo studies and clinical trials (Assefa et al., 2025; Laishram et al., 2017).

2- FUNDAMENTAL PRINCIPLES AND CLINICAL SIGNIFICANCE OF *IN-VİTRO* SYNERGY TESTING

The effect observed when two antibiotics are used in combination, compared with their individual activities, is defined as synergy, antagonism, or additivity; this distinction is critically important in clinical microbiology laboratories for guiding therapeutic regimens.

- Synergy: The combination of two agents produces antimicrobial activity (i.e. killing) greater than the sum of their individual effects. This is characterized by a net effect that significantly exceeds that of the most active single agent. In the checkerboard method, synergy is defined as a Fractional Inhibitory Concentration Index (FICI) ≤ 0.5, whereas in time-kill assays it corresponds to a reduction of ≥ 2 log10 CFU/mL compared with the most active single agent. Some studies consider a ≥ 3 log10 CFU/mL reduction as the threshold for strong

synergy (Assefa et al., 2025; Doern, 2014; Gómara-Lomero et al., 2023; Laishram et al., 2017; White et al., 1996).

- Additivity: The effect of the combination is equal to the sum of the activities of the individual agents. This is generally accepted when the FICI value falls between 0.5 and 1 ($0.5 < FICI \leq 1$) (Doern, 2014; Özseven, Sesli Çetin, & Özseven, 2012).
- Indifference: The combined use of agents does not produce a meaningful difference compared with their individual effects. In the checkerboard method, this corresponds to $1 < FICI \leq 4$, or in a time-kill assay, to a change of <2 log10 CFU/mL (Brennan-Krohn & Kirby, 2019a).
- Antagonism: The combination yields less activity than the individual agents, meaning one drug inhibits or reduces the effect of the other. In the checkerboard method, antagonism is defined as FICI> 4, while in the time-kill assay, it is indicated by an increase of >2 log10 CFU/mL in bacterial counts compared with the most active single agent (Acar, 2000).

Clinical Significance in Microbiology

The primary purpose of detecting in vitro synergy in clinical microbiology is to achieve bactericidal activity at the site of infection while minimizing toxicity. In the presence of resistance genes (such as carbapenemases or colistin resistance determinants), an antibiotic classified as "resistant" due to MIC values above clinical breakpoints may regain effectiveness when combined with a synergistic partner. Synergy testing is therefore crucial in designing salvage regimens for patients infected with multidrug-resistant (MDR) pathogens. Importantly, these assays also allow clinicians to exclude potentially antagonistic interactions before in vivo application, thereby reducing the risk of therapeutic failure or patient harm (Clinical and Laboratory Standards Institute [CLSI], 1999; Gaudereto et al., 2020; Özseven, Sesli Çetin, & Özseven, 2012; Jaglal et al., 2025).

3- CLASSICAL *IN-VİTRO* SYNERGY TESTS

The principal methods used to evaluate synergy in vitro include the checkerboard assay, the multiple-combination bactericidal test (MCBT), the E-test, and time-kill curve analysis (Doern, 2014).

Checkerboard Assay

One of the most commonly used methods in in vitro synergy testing is the checkerboard (CB) arrangement method, a modification of the minimum inhibitory concentration (MIC) analysis, in which the inhibitory effect of two different antibiotics on a bacterial isolate is tested with various concentration combinations (Doern, 2014; Özseven, Sesli Çetin, & Özseven, 2012). If the two drugs, when used together, show a stronger activity than the additive effect, this

combination is considered synergistic. When interpreting the obtained results, the MIC values of specific concentration combinations of each antibiotic are calculated using the following formulas, and the interpretation is based on Table 1 (Laishram et al., 2017; Çali & Çelik, 2024).

$$\text{FIC A} = \frac{\text{MIC of agent A in combination}}{\text{MIC of agent A alone}}$$

$$\text{FIC E} = \frac{\text{MIC of agent E in combination}}{\text{MIC of agent E alone}}$$

$$\Sigma\text{FICI} = \text{FIC A} + \text{FIC E}$$

Table 1. Interpretation of ΣFICI results

∑FICI Değeri	**Result**
∑FICI< 0.5	Synergistic Effect
0.5<∑FICI<1	Additive Effect
1<∑FICI<4	Indifferent Effect
∑FICI≥4	Antagonistic Effect

The manual setup of the checkerboard assay involves calculations, serial dilutions, and pipetting steps, making it a labor-intensive process prone to human error. These limitations have restricted the use of synergy testing primarily to retrospective evaluations of a limited number of antibiotic combinations and bacterial isolates, often resulting in inconsistent findings across studies. Moreover, the complexity of synergy assays has hindered their routine application in clinical microbiology laboratories. It has led to a near absence of in vitro synergy data in clinical trials of combination therapies. To improve the efficiency and throughput of the checkerboard method, automated systems have been developed that dispense antibiotic stock solutions into microplate wells with precision and consistency. This automated checkerboard approach enables rapid, high-capacity synergy testing. Nevertheless, the method has inherent limitations: as a modification of the MIC assay, it provides data only on growth inhibition. It does not yield information on bactericidal activity or time-dependent effects of antibiotics. Therefore, to validate checkerboard synergy results and to determine whether identified synergistic combinations are bactericidal, time-kill analyses on a limited number of isolates are recommended (Brennan-Krohn & Kirby, 2019b; Doern, 2014).

Time-Kill Assay (TKA)

Time-kill assay (TKA) are derived from minimum bactericidal concentration (MBC) testing. The MBC test determines the concentration of an antimicrobial required to kill 99.9% of the inoculum after 24 hours of exposure. This principle also forms the basis of the MCBT (Minimum Bactericidal Concentration-Based Testing) method. However, its use is limited because it relies on a definition of

complete killing (99.9%) tested at a single fixed time point and considered somewhat arbitrary. Unlike the checkerboard method, which evaluates the killing effect at a fixed time point and determines the optimal concentration, TKAs determine the killing rate. This can be a more meaningful measure in predicting patient outcomes. In 1999, the Clinical Laboratory Standards Committee (CLSI) published a guideline for bactericidal assays. This guideline presented a standard protocol for time-kill assays. According to this protocol, TKAs are analyzed in large volumes (≥10 ml); glass tubes are used; bacterial inoculum is added to a culture medium containing the desired concentration of antimicrobial. The inoculum is incubated for a total of 48 hours, and 0.5 ml samples are taken at specific intervals (usually at 4, 8, 10, 12, and 24 hours) and cultured for colony counting. The obtained time-colony count data are shown graphically as a function of time. In time-kill analyses, synergy is defined as a difference of ≥2 $\log_{10}$ between the result of the antimicrobial combination and the result of the most effective component (Clinical and Laboratory Standards Institute [CLSI], 1999; Doern, 2014; Norden, Wentzel, & Keleti, 1979).

Multiple-Combination Bactericidal Antimicrobial Test (MCBT)

The multiple-combination bactericidal test is performed in 96-well microplates. Each well is inoculated with a standardized bacterial suspension together with different antimicrobial combinations and incubated for 48 hours. After incubation, all wells without visible turbidity are subcultured onto drug-free medium to verify whether ≥99.9% killing has been achieved. Antagonism is defined as the addition of a second antibiotic to an agent that is bactericidal alone, resulting in bacterial growth. Although this method can detect bactericidal activity, interpretation of the results is not always straightforward. For example, an increase in bactericidal activity of a drug that is not bactericidal on its own can only be considered synergistic. The use of this test has been largely limited to identifying antagonistic combinations among agents employed in the treatment of respiratory infections in patients with cystic fibrosis, rather than to systematically detect synergy (Laishram et al., 2017).

E-test Based MIC: MIC Ratio Methods

E-test strips, which contain a gradient of antimicrobial concentrations, have been employed to identify synergistic combinations. Several approaches using the E-test have been described, including the E-test cross method, fixed-ratio E-tests, the E-test agar method, and the E-test MIC: MIC method (Laishram et al., 2017).

In the E-test MIC: MIC method, which may be performed using agar gradient diffusion (E-test strips) or microdilution, two drugs are combined at fixed ratios based on their individual MICs (e.g., 1:1, 1:2, or 1:4). Synergy indices are then calculated according to these ratios. Although this method offers a less labor-intensive alternative to the checkerboard assay in clinical microbiology laboratories, its concordance with the time-kill assay—the gold standard—has been reported to vary, particularly for Gram-negative pathogens (Assefa et al., 2025; White et al., 1996).

E-test methods have some limitations. These include the inability to determine the interaction between more than two antimicrobial agents and the limited gradient of antimicrobial concentrations on the strip. Calculating the FIC index for microorganisms with MIC values higher than the highest concentration on the strip can be difficult and lead to misinterpretations. Furthermore, the detection of antagonistic combinations will be limited for such isolates. When using the E-test cross-method, mild antagonisms may not be detected due to overlapping strips (Doern, 2014; Laishram et al., 2017).

There are certain interpretation criteria in synergy analysis using e-tests. According to these criteria, a decrease of 3 or more dilutions from the MIC value is considered synergy, a decrease of 2 or 3 dilutions from the MIC value is considered additive effect, a decrease of 2 dilutions from the MIC value is considered independent effect, and an increase of 3 or more dilutions from the MIC value is considered antagonistic effect (Lewis et al., 2002).

Disk Diffusion (Disk Approximation) Method

For routine laboratories, this method offers a practical and cost-effective alternative. A bacterial suspension is spread onto a standard Mueller–Hinton agar plate, and two antibiotic disks are positioned at a defined distance. After incubation, the merging of inhibition zones between the disks, producing a "bridging" effect, is interpreted as synergy. In contrast, truncation or flattening of the zones is taken as evidence of antagonism. Although straightforward, the main drawback of this approach is its inability to generate quantitative data and its weak correlation with time-kill assay outcomes (low kappa agreement) (Clinical and Laboratory Standards Institute [CLSI], 1999; Özseven, Sesli Çetin, & Özseven, 2012).

4- CONTEMPORARY AND ALTERNATIVE METHODS

To overcome the limitations of classical approaches, reduce workload, and facilitate the discovery of novel combinations, high-throughput agar screening (sHTSS) and repurposing of FDA-approved non-antibiotic drugs have been developed.

High-Throughput Synergy Screens (HTSS)

Semi-quantitative high-throughput screening techniques have revolutionized microbial synergy research by enabling the simultaneous evaluation of thousands of compound combinations using a single agar tray (OmniTray) or multi-well systems. In this method, a sub-inhibitory concentration (1/8×–1/2× MIC) of a primary antibiotic is incorporated into the bottom agar layer, while the target bacterium is inoculated into the top agar layer. Test compounds from the library are then applied, and radial measurements of inhibition zones allow rapid identification of "hit" compounds that potentiate the activity of the primary antibiotic (Gómara-Lomero et al., 2023).

Synergy Screening via Repurposing of FDA-Approved Drugs

To circumvent the high cost and time required for de novo antimicrobial development, libraries of FDA-approved drugs—already characterized in terms of pharmacokinetics/pharmacodynamics (PK/PD) and toxicity profiles—are subjected to synergy screening. This approach has revealed that compounds originally indicated for non-antibacterial purposes, such as antivirals, antineoplastics, or anti-inflammatory agents, can exhibit strong synergy with last-resort antibiotics, thereby restoring susceptibility in MDR pathogens (Gómara-Lomero et al., 2023).

5- LIMITATIONS OF *IN-VITRO* SYNERGY TESTING

Synergy assays offer considerable promise, yet several obstacles limit their routine use in clinical laboratories. The absence of a universally accepted gold standard, the weak correlation between in vitro results and in vivo dynamics, and the time- and cost-intensive nature of these methods remain major barriers to widespread implementation.

Lack of Standardization

A key obstacle in clinical application is the lack of fully standardized protocols endorsed by international reference bodies such as EUCAST or CLSI. Experimental variables—including the choice of agar versus broth media (MHB vs. agar), inoculum size, and incubation time—can markedly influence FICI values or log-based killing outcomes. For example, synergy observed in agar-based sHTSS may be confirmed in liquid checkerboard assays at rates as low as 15.09% (Clinical and Laboratory Standards Institute [CLSI], 1999; Gómara-Lomero et al., 2023; Özseven, Sesli Çetin, & Özseven, 2012).

Risk of Limited Clinical Translation

In vitro assays cannot fully reproduce the dynamic pharmacokinetic and pharmacodynamic (PK/PD) processes of the human body. Factors such as immune responses, local drug concentrations at infection sites (for example, epithelial fluids), pharmacokinetic variability, and protein binding are not adequately reflected under static laboratory conditions. As a result, combinations that appear synergistic in vitro may not necessarily improve survival or clinical outcomes in patients. Even highly promising interactions can fail therapeutically if effective serum concentrations cannot be achieved in vivo (Assefa et al., 2025; Özseven, Sesli Çetin, & Özseven, 2012; Laishram et al., 2017; Brennan-Krohn & Kirby, 2019b).

Time and Cost Constraints

Integrating gold standard methods, such as time-kill assay, into routine laboratory procedures is impractical due to the intensive labor involved, sequential dilutions, and sampling spread over 48 hours. Using dozens of plates or tubes in time-kill or checkerboard assays leads to both material (cost) loss and a delay of at least 24-48 hours (workload) in obtaining results (White et al., 1996, Laishram et al., 2017, Gaudereto et al., 2020).

6- FUTURE PERSPECTİVES

The future of synergy research lies in rapid flow cytometry analyses, hollow fiber infection models that replicate drug dynamics, and molecularly guided personalized therapies.

AI- and Automation-Based Synergy Screening

Future directions in microbiology emphasize the integration of robotic automation and machine learning into laboratory workflows. Large compound libraries can now be screened *in silico* with AI-based models, which estimate potential synergy or antagonism from physicochemical features before experimental validation. At the same time, automation platforms are being adopted in clinical laboratories to ease workload and reduce human error. Flow cytometry instruments, such as the Sysmex UF-5000, allow rapid assessment of viable and non-viable bacteria through fluorochrome staining, delivering results within a few hours rather than days. Moreover, advanced bioreactor systems like the Hollow Fiber Infection Model reproduce dynamic antibiotic concentrations, enabling in vitro data to be aligned with clinical PK/PD parameters. Collectively, these innovations have proven valuable for optimizing dosing strategies and assessing drug interactions in combination therapy (Gómara-Lomero et al., 2023; Laishram et al., 2017; Sanz-García et al., 2026).

The incorporation of AI-driven analytical platforms, novel automated synergy testing devices, flow cytometry, and advanced genotypic methods into clinical practice is expected to transform the management of resistant infections. Rather than relying on empirical broad-spectrum antibiotics, clinicians will be able to identify patient-specific combination therapies within hours. This strategy enables the tailoring of optimized regimens by considering not only the pathogen's resistance profile but also individual clinical parameters, including renal function (Brennan-Krohn & Kirby, 2019b; Jaglal et al., 2025).

Elucidation of Molecular Mechanisms

Investigating the molecular pathways behind synergistic interactions at the transcriptomic and metabolomic levels is of great importance. Fully elucidating the gene expression repressive roles of drugs on bacterial outer membrane protein channels (porins), efflux pumps, and DNA repair mechanisms will pave the way for targeted, rational combination designs. Direct links are beginning to be established between synergy levels and molecular resistance mechanisms in the targeted bacteria (e.g., specific KPC, NDM, or OXA-type carbapenemases). It has been found that the combination of colistin and meropenem shows specific synergy only in strains harboring certain carbapenemases (such as NDM), and synergy prediction is predicted through molecular diagnostics. For example, some studies have shown that the combination of colistin and meropenem exhibits the highest synergy (68.8%), particularly in strains producing blaNDM (Doern, 2014; Gaudereto et al., 2020; Jaglal et al., 2025; Laishram et al., 2017).

Personalized Combination Therapies

With the introduction of next-generation sequencing (NGS) technologies into clinical microbiology laboratories, it has become possible to identify specific resistance genes (e.g., blaKPC, blaNDM, mcr-1) in bacteria isolated from patients within hours. In the future, the goal is to develop “personalized” or “tailored” combination therapies specific to each patient's individual isolate by combining this genetic data with automated analysis results obtained from in vitro synergy tests (Assefa et al., 2025; Gaudereto et al., 2020).

7- Conclusion

In vitro synergy testing, powered by innovative automation devices and drug repositioning strategies, provides critical guidance in the management of antimicrobial combinations against resistant bacteria.

Summary of Current Advances

In vitro synergy tests are the strongest scientific basis for clinicians and researchers in breaking the antimicrobial resistance spiral. Since increasing resistance rates in bacterial infections make treatment with single drugs nearly impossible, combinations validated by in vitro synergy tests (time-kill, E-test, checkerboard) (e.g., ceftazidim/avibactam-aztreonam, meropenem-tigecycline, or non-FDA drugs/phytochemicals) are providing new avenues for the medical field. Valuable data from classic time-kill and checkerboard methods are now supplemented by innovative approaches such as high-throughput screening (sHTSS), repositioning of FDA-approved drugs, and phytochemical adjuvant approaches. Time and standardization constraints are being addressed with automated flow cytometry devices and hollow-fiber models (Assefa et al., 2025; Doern, 2014; Gómara-Lomero et al., 2023; Jaglal et al., 2025; Laishram et al., 2017; Sanz-García et al., 2026).

Contributions to Clinical and Research Fields

In vitro synergy testing provides a translational foundation for clinical decision-making in complex, life-threatening infections, particularly in pediatric and extensively drug-resistant (XDR) cases where standard algorithms fail. Rationally generated data in vitro are valuable when systematically transferred to preclinical animal models and subsequently to controlled human trials. A correctly identified synergistic combination not only overcomes resistant pathogens but also reduces drug dosages, enhances patient safety, lowers treatment costs, and most importantly, prolongs the clinical lifespan of existing antibiotics. Full integration of artificial intelligence and genomic technologies into this process is expected to define rational success criteria in the global fight against resistant infections. As testing methods become standardized and supported by in vivo models, synergy-guided antimicrobial therapies are anticipated to further improve patient survival outcomes (Assefa et al., 2025; Jaglal et al., 2025).

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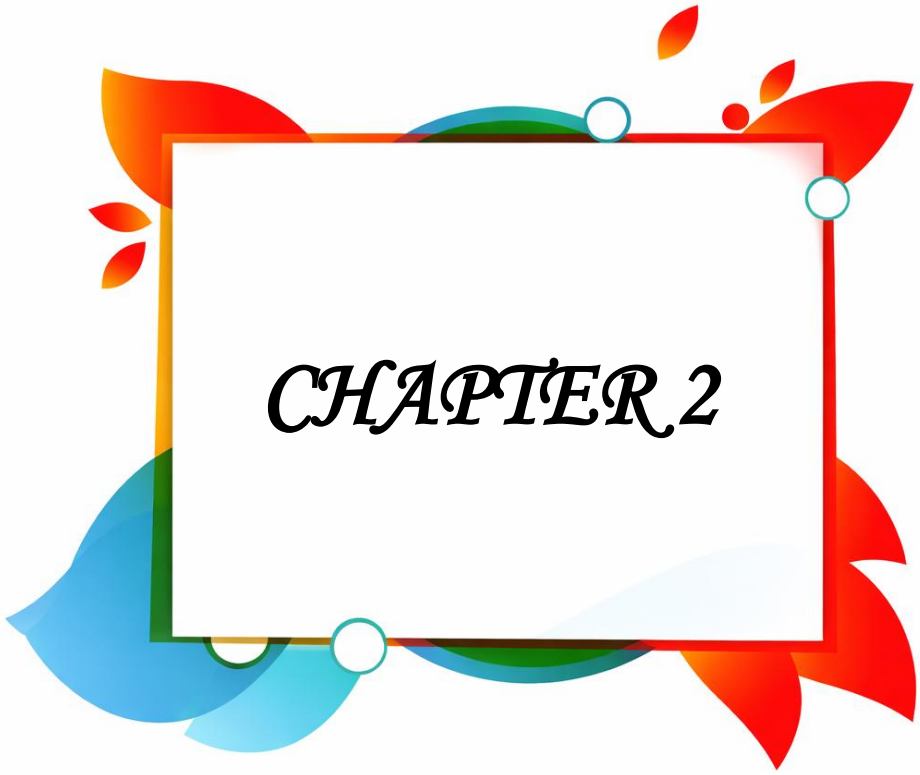
CHAPTER 2

Liposomes as Advanced Carrıers for Essential Oils: From Structural Design to Antimicrobial Functionality

Mehzat Altun[1] & İlke Karakaş[2]

Introduction

Bacterial infections are still among the leading causes of treatment-related complications and continue to create a significant global healthcare burden. Over recent decades, the widespread emergence of multidrug-resistant microorganisms has made infection control increasingly difficult. In addition, the ability of bacteria to form biofilms further decreases antibiotic susceptibility and weakens the effectiveness of host immune responses (Mandal et al., 2014). Because of these limitations, there has been growing interest in identifying alternative antimicrobial strategies and discovering new bioactive substances. Natural products remain one of the most valuable resources in this field, particularly since a considerable proportion of Food and Drug Administration-approved small-molecule drugs are either directly obtained from natural sources or developed based on naturally occurring compounds (Praditya et al., 2019).

Essential oils (EOs) are aromatic and volatile plant-derived mixtures composed mainly of monoterpenes and sesquiterpenes. Besides these major constituents, they may also contain alcohols, aldehydes, ketones, esters, ethers, and various terpenoid derivatives, depending on the botanical source. Such chemically diverse mixtures can be isolated from a wide range of aromatic plants belonging to more than sixty different plant families (Raut et al., 2014). Owing to their biological and pharmacological properties, EOs have attracted considerable attention in complementary and alternative medicine applications over the past years (Millezi et al., 2019).

Despite their promising antimicrobial potential, the practical application of EOs remains limited because of several inherent physicochemical disadvantages. Their hydrophobic character causes low solubility in aqueous systems, which may reduce their bioavailability and restrict formulation possibilities.

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Furthermore, many EO components are chemically unstable and can easily undergo oxidation or hydrolytic degradation under environmental conditions (Hosseini et al., 2013; Trinetta et al., 2017). High volatility also represents another important drawback, particularly during storage and processing. These issues have encouraged researchers to investigate suitable carrier systems capable of improving EO stability while providing more controlled and efficient delivery (Vivas et al., 2019).

Among the available nanoscale delivery approaches, liposomes have emerged as particularly attractive systems for encapsulating bioactive compounds. Their structural characteristics enable modulation of drug release, enhancement of skin penetration, and facilitation of transdermal transport. Liposomal systems may also improve the stability and safety profile of antimicrobial agents, thereby increasing their therapeutic effectiveness (Colzi et al., 2015; Moghimipour et al., 2012; Santo et al., 2014). Encapsulation strategies involving nanoparticles and liposomes have therefore been proposed as useful tools for overcoming challenges associated with antimicrobial resistance (Colzi et al., 2015). In the case of EOs, liposomal incorporation can help minimise instability-related problems without significantly altering the original chemical composition of the oils. Previous studies have further demonstrated that EO-loaded nanoliposomes may exhibit antibiofilm (Cui et al., 2016), quorum-sensing inhibitory (Nazareth et al., 2021), and antibacterial activities (Zabihi et al., 2017). against different pathogenic microorganisms.

Consequently, the incorporation of EOs into liposomal nanocarriers is increasingly regarded as a promising strategy for enhancing physicochemical stability, improving delivery efficiency, and strengthening antimicrobial performance. This chapter discusses recent progress in liposome-based EO delivery systems, with particular emphasis on preparation methods, stability-associated properties, physicochemical characterisation, and antibacterial activities reported in the literature.

Structural Features of Liposomes

Liposomes are extensively applied nanocarriers owing to their excellent biocompatibility, biodegradability, and low immunogenicity, which enable the efficient delivery of both hydrophilic and hydrophobic molecules. They can enhance drug solubility and allow for controlled, targeted, and prolonged release through surface modification. Several liposome-based formulations have been clinically approved, particularly for cancer therapy and infectious diseases, while many others are under clinical investigation (Nsairat et al., 2022). Structurally, liposomes are spherical vesicles formed by the self-assembly of natural or synthetic lipids in aqueous media. They consist of one or more concentric phospholipid bilayers surrounding an aqueous core and typically range in size from 50 to 500 nm (Jha et al., 2016; Sun et al., 2014).

Liposomes were first reported in the 1960s by Alec D. Bangham and colleagues (Bangham and Horne, 1964). Since their discovery, liposomes have been extensively studied as colloidal vesicular structures arising from the self-assembly of amphipathic lipids, especially phospholipids, in aqueous environments (Sebaaly et al., 2016). Liposomes are structurally organised as single or multiple lipid bilayer membranes enclosing an aqueous core, where the polar head groups are oriented toward both aqueous interfaces, contributing to bilayer stability (Nisini et al., 2018). The development of liposomal formulations depends on several critical parameters, including the type of phospholipids used, head group characteristics, fatty acyl chain length, and the molar ratios of the components. These factors collectively influence the safety, stability, and overall therapeutic performance of liposomal systems (Kapoor et al., 2017). Glycerophospholipids represent the primary structural components of liposomes; these amphipathic molecules consist of a glycerol backbone linked to a phosphate group and fatty acyl chains, which can be saturated or unsaturated (Pinot et al., 2014).

Natural phospholipids can be classified into several major groups based on their organic head groups, including phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidylserine (PS) (Tsuji et al., 2019). Alongside these naturally derived lipids, synthetic phospholipids can be generated through targeted chemical modifications of both the polar head groups and hydrophobic tails. Such modifications allow the development of well-defined and systematically tailored phospholipid derivatives (Van Hoogevest and Wendel, 2014). Owing to their amphiphilic nature, phospholipids readily self-organize in aqueous media to form structures that closely resemble biological membranes. This biomimetic behavior facilitates favorable interactions with mammalian cell membranes, thereby enhancing cellular internalization and improving intracellular uptake (He et al., 2019).

Alongside phospholipids, various auxiliary components are often incorporated into liposomal formulations to improve their stability and performance. Common examples include cholesterol, glycols (e.g., propylene glycol and polyethylene glycol), and polymers such as chitosan. These additives can influence not only the physicochemical properties of liposomes but also their biological interactions, including potential effects on healthy tissues and modulation of the immune system, either by stimulating or suppressing immune responses (Inglut et al., 2020). Among these components, cholesterol plays a key role in regulating membrane properties. Its incorporation into the lipid bilayer modulates membrane fluidity and rigidity, reduces permeability, and enhances stability by promoting tighter phospholipid packing and improved bilayer organization (Bozzuto and Molinari, 2015; Lee et al., 2005; Sharifi et al., 2019). Another widely used approach involves grafting glycols, particularly PEG, onto

the liposomal surface. PEGylation has been reported to significantly prolong the circulation half-life of liposomes, increasing it from a few minutes in conventional formulations, to several hours (Beltrán-Gracia et al., 2019). Chitosan are also utilized for surface modification, where they form a protective coating around the vesicles. This modification improves liposome stability and broadens their potential use in oral drug delivery applications. (Caddeo et al., 2017).

Surfactants play a pivotal role in liposomal systems by reducing interfacial tension and modulating both drug loading and release profiles (Cipolla et al., 2014). As amphiphilic single-chain molecules, they can increase bilayer fluidity and vesicle deformability by partially destabilising the lipid membrane. Commonly used surfactants include sodium cholate, Span 60/80, and Tween 60/80, which are widely applied in liposomal formulations to adjust membrane properties and improve performance (Lee et al., 2005).

Liposomes are categorised based on their structural characteristics, particularly the number of lipid bilayers (lamellarity) and vesicle size. Accordingly, they are classified as unilamellar vesicles (ULVs; all size ranges), multilamellar vesicles (MLVs; typically >500 nm), and multivesicular vesicles (MVVs; typically >1000 nm) (Akbarzadeh et al., 2013; Emami et al., 2016). ULVs, characterised by a single lipid bilayer, are grouped on the basis of size into SUVs (20–100 nm), LUVs (>100 nm), and GUVs (>1000 nm). Due to their single bilayer structure, ULVs are generally more suitable for encapsulating hydrophilic compounds. In contrast, MLVs are defined by an onion-like structure composed of multiple concentric lipid bilayers. MVVs, on the other hand, consist of multiple non-concentric small vesicles enclosed within a larger lipid structure, making them particularly advantageous for encapsulating high volumes of hydrophilic substances (Emami et al., 2016; Maherani et al., 2011). In addition, an advanced vesicular system known as multicompartment liposomes (MCLs) has been developed. These structures comprise heterogeneous vesicular systems connected through a shared bilayer interface, enabling the simultaneous delivery of multiple bioactive molecules within a single carrier platform (Al-Jamal and Kostarelos, 2007; Catalan-Latorre et al., 2016).

Vesicle size affects liposome circulation time and drug loading capacity. Liposomes of 50–150 nm are generally preferred for drug delivery. Cellular uptake occurs via endocytosis (Kelly et al., 2011), fusion (Caracciolo et al., 2005), phagocytosis (Storm et al., 1995), and membrane adsorption (Bozzuto et al., 2015). Larger liposome size favours higher encapsulation efficiency of hydrophilic compounds, while increasing the number of lipid bilayers leads to a decrease (Ong et al., 2016). Besides vesicle size, the degree of lamellarity influences the encapsulation capacity of liposomes (Akbarzadeh et al., 2013; Olusanya et al., 2018). Beyond structural features, liposome–cell interactions are

influenced by several additional parameters, including lipid composition (Düzgünes and Nir, 1999), vesicle size, surface charge (Lee et al., 1993), targeting ligands displayed on the liposomal surface, and the surrounding biological context (Kono et al., 1999).

Encapsulation Methods for Liposomes

Different techniques are used in the production of liposomes, including formulation and size reduction approaches, which play a crucial role in defining key characteristics such as particle size, lamellarity, and encapsulation efficiency (Pattni et al., 2015).

Conventional techniques

A variety of conventional approaches have been developed for liposome preparation; however, the most commonly employed approaches include thin-film hydration, reverse-phase evaporation, solvent injection, and detergent removal techniques (Karn et al., 2013; Meure et al., 2008). Despite methodological differences, these techniques generally follow a series of fundamental steps: (i) dissolution of lipid components in suitable organic solvents, (ii) removal of the organic phase, (iii) isolation and purification of the resulting liposomal vesicles, and (iv) physicochemical characterisation of the prepared liposomes (Akbarzadeh et al., 2013).

Mozafari (Heating) Method

The heating method, also known as the Mozafari method, was first introduced in 2005. Due to its avoidance of organic solvents and detergents, it is considered a highly advantageous approach for liposome preparation (Mozafari and Mortazavi, 2005). In this technique, lipids are hydrated with agents such as glycerin or propylene glycol at temperatures above the phospholipid phase transition temperature (40–120 °C). The required temperature applied depends on the formulation components, cholesterol content, and the nature of the encapsulated compound (Mozafari et al., 2008).

The Mozafari method is widely regarded as a rapid, simple, scalable, and non-toxic method that can achieve high encapsulation efficiency. Moreover, liposomes produced at elevated temperatures may often be used directly without the need for additional sterilisation steps (Nkanga et al., 2019). Although this technique is commonly applied for the encapsulation of plant oils, there are currently no reports of its use for EOs, likely due to their thermal sensitivity and volatile nature (Lammari et al., 2021).

Thin-film hydration method

Thin-film hydration, commonly termed the Bangham method, is the earliest described method for liposome production (Bangham et al., 1967). Within this technique, lipids are first dissolved in organic solvents (e.g. chloroform, ether, or

methanol), followed by solvent evaporation using a rotary evaporator to obtain a thin lipid film, which is subsequently hydrated with an aqueous phase. Resulting in liposome formation. The structural characteristics of the vesicles depend on hydration conditions: vigorous agitation yields heterogeneous multilamellar vesicles, whereas mild hydration can produce giant unilamellar vesicles (Isalomboto Nkanga et al., 2019; Monteiro et al., 2014).

Despite its simplicity, the method has several limitations, including the formation of large and heterogeneous vesicles, low encapsulation efficiency, difficulty in complete solvent removal, and challenges in scale-up (Meure et al., 2008). Therefore, additional processes such as sonication and extrusion are often required to obtain smaller and more uniform vesicles, which increases processing time. Moreover, the need for organic solvents and the possible presence of residual solvents in the final product may lead to toxicity concerns and reduced stability of liposomal systems (Lammari et al., 2021).

Reverse-phase evaporation

This is an alternative technique for liposome preparation. Phospholipids are first dissolved in an organic solvent and re-dispersed in a mixture such as diethyl ether and/or isopropyl ether. An aqueous phase is subsequently introduced to generate a water-in-oil emulsion (Pattni et al., 2015), which is then subjected to sonication, leading to the formation of reverse micelles and a homogeneous system. Removal of the organic solvent under reduced pressure subsequently produces a viscous gel, which transforms into a liposomal suspension (Akbarzadeh et al., 2013; Maherani et al., 2011). This technique is particularly advantageous due to its relatively high encapsulation efficiency (Monteiro et al., 2014; Wagner and Vorauer-Uhl, 2011). However, its main limitations include the exposure of bioactive compounds to organic solvents and sonication, which may impact their stability (Antimisiaris et al., 2007).

Solvent injection techniques

Liposomes can also be prepared via solvent injection methods, in which lipids are first dissolved in an organic solvent and rapidly injected into an aqueous phase, leading to spontaneous vesicle formation (William et al., 2020). Among these, the ethanol injection method is widely preferred due to its simplicity, reproducibility, rapid application, and ease of scale-up, while minimising lipid degradation and oxidative changes (Justo and Moraes, 2010).

This technique is commanly used for the encapsulation of plant oils, and a key advantage of this method is the formation of submicron liposomes (<100 nm) with a narrow size distribution without the need for sonication or extrusion (Stano et al., 2004). Sebaaly et al. (2015) showed that clove EO encapsulated in soybean phospholipid liposomes via ethanol injection was protected from UV-induced degradation without reducing its DPPH antioxidant activity. In addition, ethanol

is considered a suitable solvent for in vivo drug delivery applications when used at low concentrations, as indicated in the European Pharmacopoeia (Marasini et al., 2017).

However, this technique has certain limitations, such as the limited solubility of certain lipids in ethanol, the potential formation of heterogeneous vesicles under inadequate mixing conditions, poor encapsulation efficiency for hydrophilic compounds, and challenges in achieving complete solvent removal (Çağdaş et al., 2014; Maherani et al., 2011).

Detergent removal method

In this approach, phospholipids are first solubilized in the presence of detergents above their critical micelle concentration, forming mixed micells (Isalomboto Nkanga et al., 2019). Liposome formation is then induced by the gradual removal of detergent, commonly via dialysis or column chromatography, allowing phospholipids to self-assemble into vesicular systems in an aqueous medium (Akbarzadeh et al., 2013; Pattni et al., 2015).

The lipid-to-detergent ratio largely governs the final vesicle size and uniformity and the kinetics of detergent removal (Maherani et al., 2011; Wagner and Vorauer-Uhl, 2011). Despite its effectiveness, the method is limited by potential residual detergent contamination, possible interactions with encapsulated agents, and relatively long processing times (Meure et al., 2008; Schubert, 2003).

Novel approaches in liposome preparation

Recent developments in liposome technology primarily aim to improve scalability, broaden compatibility with various lipids and therapeutic agents, and enhance structural and functional performance, particularly for industrial applications (Pattni et al., 2015). Many of these novel strategies are derived from modifications of conventional techniques. For instance, cross-flow injection (Wagner technique) (Wagner et al., 2002) and membrane contactor systems are improved versions of ethanol injection, designed to provide better process control and reproducibility (Patil and Jadhav, 2014).

Liposome functionality can also be tailored through surface engineering. Charged liposomes, commonly formulated using lipids such as oleic acid (anionic lipid) or trimethylammonium chlorid (DOTAP; cationic lipid), exhibit improved colloidal stability due to electrostatic repulsion, while cationic systems are particularly effective for nucleic acid delivery in gene therapy applications (San et al., 1993).

A further advancement involves “stealth” or sterically stabilised liposomes, typically coated with polymers or hydrophilic molecules such as PEG, PVA, or hyaluronic acid (Sunamoto et al., 1986). PEGylated liposomes, also known as

stealth liposomes, prolong systemic circulation. They also enhance tumour accumulation (Allen, 1994; Paolino et al., 2010; Vaage et al., 1993). Doxil® represents the first clinically approved PEGylated liposomal formulation (He et al., 2018). In addition to PEGylation-based stealth systems, alternative polymer-coated liposomal strategies have also been developed. Lin et al. (2018) demonstrated a new approach for the encapsulation of thyme EO within ε-polylysine-coated solid liposomes. Solid liposomes are regarded as next-generation nanosystems, offering enhanced stability and prolonged storage life compared with conventional aqueous liposomal formulations (Guan et al., 2015).

Third-generation systems include actively targeted liposomes, which are functionalized with ligands that enable receptor-mediated uptake (Allen and Cullis, 2013; Leserman et al., 1980), and improving selective delivery to diseased cells, which increases therapeutic efficacy and reduces systemic toxicity. However, direct drug–ligand conjugation may compromise receptor recognition (Kaneda, 2000), whereas nanocarrier-mediated targeting preserves ligand functionality and enhances site-specific drug accumulation (Allen and Cullis, 2013), allowing dose reduction and minimising adverse effects (Bertrand and Leroux, 2012).

Microfluidic systems enable rapid and controlled liposome formation under laminar flow. They produce uniform vesicles with tunable size and polydispersity by adjusting flow parameters. This improves reproducibility and scalability while reducing cost and reagent consumption (Zhang and Sun, 2021).

In addition, stimulus-responsive (smart) liposomes have been engineered to release their payload in response to environmental stimuli such as pH, temperature, redox state, enzymatic activity, ultrasound, or electromagnetic fields. These systems enable controlled and triggered drug release, and recent studies have also explored liposomal encapsulation of bioactive gases and ultrasound-triggered delivery to enhance therapeutic outcomes (Lee and Thompson, 2017).

In recent years, liposomes have been employed for the encapsulation of bioactive gases and/or therapeutic agents to facilitate ultrasound-triggered drug release and enhanced drug delivery (Sun, 2017); gas-containing liposomes (bubble liposomes) offer promising applications in gene and drug delivery systems (Katsuji et al., 2008).

Overall, liposome preparation techniques can be broadly classified according to the loading strategy. Passive loading methods involve mechanical strategies such as film hydration, sonication, extrusion, freeze–thaw cycles, and French press. They also comprise solvent-based methods including ethanol or ether injection, reverse-phase evaporation, and supercritical fluid processes (Akbarzadeh et al., 2013; Monteiro et al., 2014).

Physicochemical properties of liposomes

The therapeutic effectiveness and biological behaviour of liposomal systems are primarily determined by the physicochemical characteristics of their lipid bilayer structures. Parameters such as membrane stiffness, bilayer thickness, fluidity, surface electrostatic charge, and curvature play a collective role in regulating drug encapsulation efficiency, structural integrity, release profiles, and the interaction involving of liposomes with cells and biological environments (Briuglia et al., 2015; Jokerst et al., 2011; Róg et al., 2009).

A key challenge in nanoliposomal formulation is controlling the physicochemical properties that determine in vivo performance and accurately characterizing these systems. Parameters such as composition, phase transition temperature, loading efficiency, vesicle size, morphology, lamellar structure, surface charge, and release profile are critical for stability and reliable drug delivery (Danaei et al., 2018). These parameters are measured using complementary separation, light scattering, microscopic, and spectroscopic methods. Characterisation of nanoliposomes is performed using complementary physical, chemical, and microscopic techniques, high-performance liquid chromatography (HPLC), ultraviolet–visible spectroscopy (UV–Vis), Fourier-transform infrared spectroscopy (FT-IR), differential scanning calorimetry (DSC), and fluorescence spectroscopy. In addition, biocompatibility is assessed through cytotoxicity assays such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Cell Counting Kit-8 (CCK-8); however, since each method has its own limitations, the combined use of multiple techniques is necessary for a reliable and comprehensive evaluation (Zhang et al., 2026).

Encapsulation efficiency and drug loading, measured using UV–Vis spectrophotometry, are key indicators of nanoliposomal performance. High encapsulation means better drug incorporation with less loss, while proper drug loading helps maintain effective therapeutic levels (Liu et al.,2024). Microscopic techniques are important for characterizing nanoliposomes because they show detailed structural and morphological features. TEM allows for direct visualization of the internal structure and overall shape, while SEM provides clear information about the surface. CLSM, on the other hand, is useful for tracking how liposomes are distributed in cells or tissues using fluorescence labelling. (Liu et al., 2024). These methods allow direct visualization of individual particles, which makes it possible to evaluate both their size and shape. In particular, TEM and cryogenic TEM are commonly used for detailed imaging of liposomal systems (Isalomboto Nkanga et al., 2019).

Particle size and distribution are usually measured by laser particle size analysis or dynamic light scattering (DLS), along with the polydispersity index (PDI) and which indicates whether the formulation is monodisperse or

polydisperse. A PDI value of 0.3 or lower is generally regarded as acceptable, and suggests a fairly uniform liposome population (Danaei et al., 2018). Liposome size is an important parameter, particularly for inhalation and parenteral applications (Laouini et al., 2012), since smaller liposomes are able to remain in systemic circulation for longer periods (Sercombe et al., 2015). For drug delivery applications, the optimal liposome size generally falls within the range of 50–200 nm (William et al., 2020).

Zeta potential is commonly used to estimate liposome stability. Low or near-neutral values lead to easier aggregation due to weak repulsion, while high positive or negative values improve stability by preventing particle aggregation (Laouini et al., 2012). Fluorescence spectroscopy and DSC are commonly used to evaluate the membrane structure, fluidity, and thermal stability of nanoliposomes. These techniques can also reveal changes in phase transition temperature and membrane organization caused by interactions between active compounds and the lipid bilayer, which helps confirm successful encapsulation and overall structural stability (Pan et al., 2018).

Antimicrobial Effects of Liposome-Encapsulated Essential Oils

The control of microbial infections represents a major focus of pharmacological research; however, the increasing prevalence of antibiotic resistance has intensified the need for alternative antimicrobial strategies. In this context, natural compounds such as EOs have gained growing scientific attention. (Cowan,1999).

The hydrophobic nature, high volatility, chemical instability, and potential toxicity of EOs significantly limit their applications. These drawbacks can be effectively mitigated through encapsulation within delivery systems, which enhances bioavailability and physicochemical stability while simultaneously reducing volatility and toxicity (Cimino et al., 2021).

Liposomes are widely recognised as key nanocarriers in biomedical and biotechnological fields, with applications spanning drug delivery, gene transfer, biosensing, and vaccine development. Their adjustable size, compositional flexibility, and surface properties enable efficient encapsulation of both hydrophilic and hydrophobic molecules. From a structural perspective, liposomes are nanoscale, spherical vesicular systems consisting of phospholipid bilayers, with typical diameters ranging between 50 and 200 nm. This dual encapsulation capacity enables efficient loading of diverse therapeutic molecules, positioning liposomes as versatile nanocarriers for therapeutic, diagnostic, and immunisation purposes. The clinical relevance of liposomal systems is well demonstrated by FDA-approved formulations such as Doxil, AmBisome, and Onivyde (Khodadadi et al., 2025).

Liposomes are widely utilised in the food and healthcare applications, particularly for the delivery of bioactive constituents such as EOs (Li et al., 2022). Encapsulation of an active compound within liposomes protects it from degradation while also enhancing its solubility (Lee, 2020). Liposomes are nanoscale spherical vesicles formed by phospholipid bilayers and are considered highly effective carriers for EOs in antimicrobial applications. Their bilayer structure closely resembles that of natural biological membranes, enabling interactions such as fusion or adhesion with both bacterial and mammalian cell membranes, which in turn enhances the transport and intracellular uptake of bioactive molecules (Bedoya-Agudelo et al., 2024; Nasr et al., 2021). This biomimetic characteristic offers a distinct advantage in addressing bacterial resistance, as it facilitates more efficient delivery of active compounds, allowing them to evade or interfere with bacterial defence mechanisms (Lohith Kumar et al., 2018; Sherry et al., 2013).

Several studies have highlighted the potential of liposomes as delivery systems for EOs. Valenti et al. (2001) demonstrated that the *Santolina insularis* EO exhibited notable in vitro antiherpetic activity against herpes simplex virus type 1, and its incorporation into liposomes improved the stability of the formulation. Multilamellar and small unilamellar vesicle formulations were also developed to increase the antiviral effectiveness of *Artemisia arborescens* L. EO (Sinico et al., 2005). Similarly, an increase in antifungal activity has been observed following the liposomal encapsulation of EO extracted from eucalyptus leaves (Moghimipour et al., 2012).

Nano-liposomal EO formulations were prepared from a mixture of peppermint, eucalyptus, tea tree, and clove EOs diluted with coconut oil, and incorporated into a topical cream. These formulations exhibited antimicrobial activity against *E. coli* and *B. subtilis*, with efficacy comparable to kanamycin. Liposomes were prepared using soybean lecithin, phytosterol, and α-tocopherol, and subsequently converted into nanoliposomes via sonication, homogenisation, and extrusion (Al-Ogaidi et al., 2022).

Liposomal encapsulation improved the aqueous solubility of EOs and significantly enhanced their antimicrobial activity, as evidenced by reduced minimum inhibitory concentration (MIC) values. Liposomes containing *Cymbopogon citratus* EO showed the strongest inhibition against *S. aureus,* while *C. winterianus* liposomes showed the highest activity against *Enterococcus faecalis*. Notably, *C. winterianus* liposomes maintained particle stability for 72 h without phase separation. Overall, soy lecithin-based liposomal formulations markedly enhanced the antibacterial efficacy of Cymbopogon EOs, supporting their potential as a sustainable strategy against antimicrobial resistance (Elmi et al., 2025).

Cinnamon EO nanoliposomes exhibited strong antibacterial and antibiofilm activity against *S. aureus*, while liposomal formulation enhanced its stability and prolonged its release profile (Ellboudy et al., 2023). In addition, *Salvia officinalis* EO–loaded liposomes were prepared via thin-film dispersion followed by ultrasonic hydration to enhance oil stability and enable controlled release. The formulation also exhibited notable antioxidant capacity and pronounced antibacterial activity against *Pseudomonas* spp (Wang et al., 2025). Furthermore, Liolios et al. (2009) reported that liposomal encapsulation of *Origanum dictamnus* L. EO and its major constituents increased vesicle stability and enhanced antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and *Listeria monocytogenes* compared with the free oil. Similarly, Najafi et al. (2020) reported the antibacterial activity of lecithin/cholesterol-based liposomes loaded with *Ferula gummosa* (barije) EO against *E.coli*.

Palmas et al. (2020) recently developed liposomal formulations of *Citrus limon* var. *pompia* EO as a potential gargle for oropharyngeal disorders. Citral-loaded liposomes were biocompatible, protected cells from oxidative stress–induced damage, promoted wound healing by accelerating closure of keratinocyte monolayer lesions, and inhibited *Streptococcus mutans* proliferation. Hammoud et al. (2020) demonstrated that encapsulating EO constituents (estragole, eucalyptol, isoeugenol, pulegone, terpineol, and thymol) in a drug-in-cyclodextrin-in-liposome system enhances their stability, shelf life, and bioactivity.

Liposomal encapsulation of curcumin and *Lippia origanoides* EO represents a promising delivery strategy that enhances physicochemical stability, and antimicrobial activity while maintaining biocompatibility, as the formulations inhibited *S. aureus* ATCC 25923 and showed no cytotoxicity, even increasing metabolic activity in VERO cells (Bedoya-Agudelo et al., 2024). Similarly, liposomes loaded with *Salvia triloba* and *Rosmarinus officinalis* EOs remained stable for one month at 4°C, and showed significant antibacterial activity against *Klebsiella pneumoniae* compared with the free EOs, indicating reduced volatility and enhanced biological performance (Risaliti et al., 2019).

Liposomal formulations of *Zataria multiflora* Boiss EO demonstrated lower MIC values against *E. coli* O157:H7 in both broth media and minced meat systems, supporting their potential as natural preservatives in the food industry (Khosravi-Darani et al., 2016). Furthermore, De Alteriis et al. (2021) showed that *Lavandula angustifolia* EO, in both free and liposomal forms, effectively eradicated primary and persister *Candida auris* biofilms. The EO induced intracellular ROS production, resulting in oxidative stress–mediated cell damage and biofilm disruption in a dose-dependent manner, associated with altered expression of key genes including ALS5, ERG11, CDR1, and HOG1.

Notably, Dai et al. (2026) demonstrated that surface charge plays a crucial role in the interaction of *Litsea cubeba* EO-loaded ionic liposomes with *L. monocytogenes* biofilms. Anionic liposomes showed enhanced penetration and superior removal of mature biofilms, whereas cationic liposomes exhibited stronger surface adhesion via electrostatic interactions and were more effective in controlling surface contamination, highlighting charge-engineered systems as a promising antibiofilm strategy for food safety applications.

Conclusion

In conclusion, incorporating EOs into delivery systems, particularly liposomes, represents an effective approach to overcome limitations such as volatility, instability, and poor water solubility while preserving their biological activity. Liposomal formulations can enhance bioavailability and biocompatibility, improve the solubility of poorly water-soluble compounds, and provide controlled as well as sustained release, thereby increasing their therapeutic and antimicrobial potential for pharmaceutical, food, and cosmetic applications.

Future research should focus on the development of scalable and patentable formulations suitable for industrial production. In addition, the antimicrobial mechanisms and toxicological profiles of EO-loaded liposomes should be comprehensively evaluated through in vitro and in vivo studies to support their safe and effective clinical use.

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