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Drug Delivery to Oral Cancer Treatment

RESEARCH ARTICLE

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# Enhanced Antibacterial, Anti-Biofilm, and Anticancer Activities of Liposome-Encapsulated Selenium Nanoparticles: A Novel Therapeutic Approach

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## Abstract

**Background:** This study investigates the green synthesis of selenium nanoparticles (SeNPs) and their encapsulation in liposomes as a novel drug delivery system to enhance the antibacterial and anticancer properties of SeNPs. Liposomes are well-known for their ability to improve the biological activity of encapsulated drugs, making them a promising candidate for targeted therapies, particularly in oral cancer treatment. **Methods:** Biosynthesised SeNPs were incorporated into liposomes via the thin-film hydration technique. Particle size and zeta potential were quantified by dynamic light scattering (DLS), whereas encapsulation efficiency (EE) was determined spectrophotometrically (UV–Vis). **Results:** The physicochemical properties of the liposome-loaded SeNPs were characterized, revealing an average size of 270 nm, spherical morphology, and an encapsulation efficiency of 50.5%. The release profile of SeNPs from the liposomes demonstrated a controlled release of 61% over 64 hours, while free SeNPs released 100% of their content during the same period. The antibacterial and anti-biofilm activities of both free and liposome-loaded SeNPs were tested against standard pathogenic bacterial strains, with the liposome formulation showing enhanced efficacy. The cytotoxicity assay revealed that liposome-loaded SeNPs exhibited significantly higher cytotoxic effects on oral cells compared to free SeNPs, indicating improved therapeutic potential. **Conclusion:** The study demonstrates that liposome-loaded SeNPs are an effective and biocompatible drug delivery system with notable antibacterial, anti-biofilm, and anticancer properties, making them a promising candidate for targeted drug delivery in oral cancer therapy.

**Keywords:** Antibacterial activity- anticancer activity- oral cancer therapy- liposomes

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## Introduction

Various diseases have threatened human life, continually engaging the human mind in the search for therapeutic solutions [1-7]. Over the past few decades, sweeping advances in technology from cutting-edge materials and nanotech to artificial intelligence, modern imaging tools, and data science have empowered researchers and clinicians across the health and life sciences to create new ways to prevent, detect, and treat illness. By uniting insights from chemistry, biology, and clinical practice, experts have produced smarter therapies, safer materials, and more tailored care plans, helping people live longer and enjoy better overall well-being [8-21]. Cancer remains one of the most formidable health challenges worldwide, claiming millions of lives each year. Its complex nature and diverse manifestations drive relentless research efforts to improve prevention, early diagnosis, and targeted therapies [22-36]. There are various methods for treating cancer, but one of the newest approaches is drug delivery [37-45]. Selenium nanoparticles (SeNPs) have emerged as an innovative and potent therapeutic agent in the fields of antibacterial, anti-biofilm, and anticancer therapies. Selenium nanoparticles have shown significant antimicrobial activity, including combating biofilm formation by pathogens like Staphylococcus

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aureus [46]. Moreover, SeNPs are effective against various oral pathogens responsible for biofilms, such as *C. albicans* and *S. mutans* [47]. Selenium, a trace element with recognized antioxidant properties, plays a vital role in cellular processes. The generation of reactive oxygen species (ROS) by SeNPs is a primary mechanism inducing oxidative stress and apoptosis in cancer cells [48]. Additionally, SeNPs are effective at inducing oxidative stress and cell cycle arrest through pro-oxidant activity, contributing to their chemotherapeutic potential [49]. The growing interest in SeNPs is attributed to their superior biological properties, including their significant antimicrobial activity. SeNPs exhibit broad-spectrum antimicrobial activity, targeting bacteria, fungi, and viruses with mechanisms involving ROS and biofilm disruption [50]. Furthermore, these nanoparticles effectively prevent bacterial adhesion and quorum sensing, limiting biofilm formation [51]. Their small size, high surface-to-volume ratio, and ease of modification make SeNPs a highly versatile platform for drug delivery systems. These properties enable SeNPs to enhance drug delivery by improving the targeting of cancer cells and increasing therapeutic indices [52]. Functionalized SeNPs further improve drug delivery efficiency by targeting specific cancer cells and reducing off-target effects [53]. Recent advances in nanomedicine have led to the exploration of various nanocarriers to improve the efficacy and bioavailability of SeNPs. Among these carriers, liposomes have gained considerable attention due to their ability to encapsulate both hydrophobic and hydrophilic substances, providing controlled drug release, improved stability, and enhanced delivery to specific target sites [54]. Liposomes are lipid-based vesicles that offer a biocompatible and non-toxic medium for the encapsulation of drugs, ensuring their protection from degradation in the body and facilitating their targeted release [55]. Liposome-encapsulated nanoparticles can also enhance cellular uptake, reduce systemic side effects, and improve therapeutic outcomes. These carriers are effective in increasing drug retention and bioavailability while minimizing toxic side effects by targeting the release of therapeutic agents to specific cells or tissues [56]. Additionally, their versatility in encapsulating various drugs has led to advancements in cancer therapy, with liposomes achieving enhanced tumor targeting and controlled drug release [57]. The combination of selenium nanoparticles (SeNPs) with liposomes is particularly promising in the context of bacterial infections and cancer treatment. In bacterial infections, SeNPs have been shown to disrupt bacterial cell membranes and inhibit biofilm formation, a critical feature of pathogenic bacteria's resistance mechanisms [51, 58]. When encapsulated in liposomes, SeNPs demonstrate enhanced antibacterial and anti-biofilm activities, potentially overcoming the limitations of traditional antibiotics [59-60]. Moreover, the anticancer potential of SeNPs is increasingly recognized, particularly in the treatment of oral cancer. Studies have demonstrated that SeNPs can induce apoptosis in cancer cells by activating various signaling pathways, including the upregulation of apoptotic genes such as CASP3 and CASP9 [48-49, 61]. Encapsulation of SeNPs in liposomes significantly enhances their anticancer efficacy through prolonged release, increased intracellular accumulation, and targeted delivery to cancer cells [48, 62]. The physicochemical properties of selenium nanoparticles (SeNPs), such as their particle size, surface charge, and morphology, are critical factors influencing their biological activity and therapeutic potential. These parameters significantly determine their interactions with cells and their overall therapeutic effectiveness [51]. The encapsulation of SeNPs in liposomes improves their solubility, stability, and bioavailability, enabling more effective and sustained drug release. Liposomal formulations provide a more stable environment for SeNPs, reducing premature degradation and enhancing bioavailability [52, 63]. Liposome formulations are also capable of modulating the pharmacokinetics of SeNPs, reducing their toxicity while maximizing their therapeutic effects. This is achieved by optimizing the delivery profile, ensuring prolonged circulation time, and minimizing off-target effects [49]. The controlled release of SeNPs from liposomes can be carefully tailored to match the needs of specific treatments, enhancing the selectivity of the drug and minimizing off-target effects. This targeted approach not only improves the therapeutic index but also reduces potential side effects associated with non-specific drug distribution [48, 63]. In this study, we aim to explore the green synthesis of SeNPs, their encapsulation in liposomes, and the subsequent evaluation of their antibacterial, anti-biofilm, and anticancer properties. We will characterize the physicochemical properties of the liposome-loaded SeNPs, including their size, morphology, and encapsulation efficiency. The release profile of SeNPs from the liposomes will also be assessed to determine the controlled release kinetics. Finally, we will investigate the biological activities of the liposome-loaded SeNPs, including their antibacterial effects against standard pathogenic bacterial strains, anti-biofilm activity, and anticancer efficacy on CAL-27 oral cancer cells.

## Materials and Methods

*Materials*

Lecithin, cholesterol, polyethylene glycol (PEG) 3350, along with ethanol, isopropanol, and dimethyl sulfoxide (DMSO), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Additionally, culture medium RPMI 1640, Dulbecco's Modified Eagle Medium (DMEM), phosphate-buffered saline (PBS), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Penicillin/Streptomycin (100X solution) were obtained from Gibco (Thermo Fisher Scientific, USA). The CAL-27 oral squamous cell carcinoma (OSCC) cell line was acquired from the National Cell Bank of Iran (NCBI), Pasteur Institute of Iran, ensuring authentication and adherence to quality control standards.

*Green Synthesis of Selenium Nanoparticles (SeNPs)*

The aerial parts of Trifolium cherleri were procured from the Iran Biological Reserves Center (Plant Bank) under the designated herbarium accession number 1368 and were

taxonomically validated by an expert botanist. The plant material was subjected to controlled desiccation, ensuring minimal photodegradation by maintaining light-shielded and aerated conditions. Following complete dehydration, the plant was mechanically pulverized using an industrial-grade electric milling system, yielding a homogeneous fine powder. For the preparation of the aqueous botanical extract, precisely 20 g of the processed plant powder was immersed in 100 mL of distilled water, wherein phytochemical extraction was conducted via the maceration method, ensuring optimal solubilization of bioactive compounds. Upon completion of the extraction period, the resultant crude extract was subjected to sequential filtration through high-purity Whatman filter paper (Germany) to eliminate residual particulates and obtain a clarified extract. Subsequently, for the green synthesis of selenium nanoparticles (SeNPs), an alkaline sodium selenite ($Na_2SeO_3$) solution (1 mM, 300 mL) was freshly prepared under sterile conditions. A meticulously measured 15 mL aliquot of the T. cherleri aqueous extract was gradually introduced into the selenite solution under ambient reaction conditions, wherein the reduction of selenium ions was initiated, manifesting a characteristic colorimetric transition, indicative of nanoparticle formation. The synthesized SeNPs were then subjected to multiple purification cycles, incorporating repeated centrifugation and washing with ultrapure distilled water, effectively removing unreacted precursors and organic residues. Finally, the purified SeNPs were lyophilized and stored under desiccated ambient conditions for subsequent physicochemical characterization and biomedical assessments.

*Preparation of liposome loaded SeNPs*

To fabricate liposome-encapsulated selenium nanoparticles (SeNPs), precise quantities of cholesterol (75 mg), lecithin (130 mg), and polyethylene glycol (PEG) 3,350 (14 mg) were dissolved in an organic solvent system comprising 10 mL of chloroform. The resulting lipidic solution underwent continuous agitation to ensure complete solubilization and homogeneity of the constituents. Subsequently, the lipid-solvent mixture was transferred into a specialized rotary evaporation flask and subjected to vacuum-assisted thin-film formation under controlled thermodynamic conditions (60°C, 150 rpm), facilitating the gradual and complete evaporation of the organic solvent, thereby generating a uniform dry lipid film along the inner surface of the flask. During the hydration phase, a pre-formulated phosphate-buffered suspension of SeNPs (1 mg/mL, 10 mL) was introduced onto the lipid film under elevated thermal conditions (60°C). The system was continuously agitated using a rotary evaporator set at 120 rpm for 30 minutes, allowing optimal hydration and self-assembly of the lipid bilayer around the selenium nanoparticles. To achieve uniform nano-scale dispersion and size reduction, the resulting liposomal SeNP suspension was subjected to ultrasonic treatment (probe sonication) for 5 minutes, effectively reducing the particle size by disrupting and homogenizing the vesicle structures, yielding a monodisperse liposomal formulation optimized for enhanced bioavailability, and therapeutic efficacy.

*Nanoparticle Characterization*

The particle size and zeta potential of the synthesized nanoparticles were characterized using the Dynamic Light Scattering (DLS) technique, utilizing a Zetasizer (Nano ZS3600, Malvern Instruments, UK). Additionally, a detailed morphological analysis of the nanoparticles was conducted to evaluate their surface structure and distribution. To prepare the samples for this analysis, the nanoparticle suspension was lyophilized, following the addition of 3% mannitol as a cryoprotectant, ensuring the preservation of the nanoparticle integrity during the freezing and drying process. The resulting nanoparticle powder was then examined using an advanced electron microscopy technique: Scanning Electron Microscopy (SEM) (S-4160 Scanning Electron Microscope, Hitachi, Japan), which provided high-resolution images of the surface morphology, offering detailed insights into the internal structure of the SeNPs at the nanoscale. The encapsulation efficiency (EE%) of the liposomal formulation was determined to assess the proportion of SeNPs successfully encapsulated within the liposomes relative to the total amount of SeNPs used in the preparation. The liposome-loaded SeNPs were subjected to centrifugation at 4°C and 14,000g for 60 minutes, which facilitated the separation of encapsulated SeNPs from the free, unencapsulated particles. Following centrifugation, the supernatant containing the free SeNPs was carefully removed, and the absorbance was measured at 265 nm using a spectrophotometer to quantify the amount of free SeNPs. The amount of encapsulated SeNPs was determined by subtracting the amount of free SeNPs from the initial total amount of SeNPs used in the formulation. The encapsulation efficiency was then calculated using the following formula:

Encapsulation Efficiency (EE%)= (Amount of free Drug−Amount of primary Drug/Amount of primary Drug) ×100

*Drug release study*

The drug release profile of the SeNPs formulations was assessed using a dynamic release system. In this procedure, 3 mL of the liposome-loaded SeNPs and an equivalent volume of free SeNPs were each placed in separate dialysis bags with a molecular weight cutoff of 10 kDa. These dialysis bags were then immersed in a beaker containing 40 mL of phosphate-buffered saline (PBS), maintained at a constant temperature of 37°C to simulate physiological conditions. The beakers were placed on a magnetic stirrer to ensure continuous mixing, promoting the diffusion of the SeNPs through the dialysis membrane. At specific time intervals, 1 mL of the PBS solution containing the diffused SeNPs from the dialysis bag was withdrawn, and an equal volume of fresh PBS (also pre-warmed to 37°C) was added to maintain the volume and ensure the continuous release of the drug. Sampling was conducted at various time points to monitor the release dynamics, specifically at 4, 8, 12, 24, 36, 54, and 64 hours. After each sampling, the optical

absorption of the collected solution was measured using a UV spectrophotometer at a wavelength of 265 nm, which corresponds to the absorbance peak of the SeNPs. The data collected from these time points were used to plot a cumulative release curve, which shows the percentage of SeNPs released over the 64-hour period. This graph provides valuable insights into the release kinetics of both the liposome-loaded SeNPs and free SeNPs, allowing for a direct comparison of their release profiles under similar experimental conditions.

*Antimicrobial activity*

To evaluate the antimicrobial effects of both liposome-loaded SeNPs and free SeNPs, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) methods were employed. These tests were performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines using a serial dilution technique, with triplicate testing to ensure reproducibility and accuracy. The tests were carried out against pathogenic microbial strains including Staphylococcus aureus ATCC 25923 and Enterococcus faecalis ATCC 29212. In the procedure, a 5 µL aliquot of microbial culture, standardized to a 0.5 McFarland turbidity, was added to each well of a 96-well microplate, followed by the addition of 95 µL of Mueller Hinton Broth (MHB) to complete the inoculum preparation. Subsequently, a range of concentrations of both liposome-loaded SeNPs and free SeNPs, varying from 4.125 µg/mL to 66 µg/mL, was introduced to the wells. The MIC was determined as the lowest concentration of the tested formulation at which no visible bacterial growth occurred. To determine the MBC, the contents of the wells where bacterial growth was inhibited were subcultured onto fresh Mueller Hinton Agar plates, and the lowest concentration where no bacterial colony growth was observed was considered the MBC, representing the concentration required to kill the bacteria. As controls, a well containing only Mueller Hinton Broth medium without bacteria served as the negative control, and a well containing only the standard bacterial culture without any treatment was used as the positive control, ensuring proper validation of the experiment.

*Time kill assay*

The antibacterial effect of liposome-loaded SeNPs, free SeNPs, and free liposomes against specific pathogenic bacteria was evaluated over 64 hours using the time-kill assay method in a 96-well plate. In short, 100 µL of liposome-loaded SeNPs and free SeNPs at sub-MIC concentrations were added to wells containing 100 µL of a $10^5$ CFU/mL bacterial suspension. After incubation at 37°C, the optical absorbance of the samples at OD 600 nm was measured at 4, 8, 12, 24, 50, and 64 hours using a microplate reader.

*Anti bioflm activity*

To assess the anti-biofilm activity of liposome-loaded selenium nanoparticles (SeNPs), free SeNPs, and free liposomes, a crystal violet (CV)-based microtiter plate assay was employed. Standard bacterial strains were cultured in 96-well plates at 37°C for 24 hours. Following incubation, the wells were washed three times with phosphate-buffered saline (PBS) to remove any unadhered bacterial cells. Subsequently, the bacterial cultures were exposed to sub-minimum inhibitory concentration (sub-MIC) levels of liposome-loaded SeNPs, free SeNPs, or free liposomes for another 24 hours at 37°C. After treatment, the wells were rinsed three times with PBS and fixed with methanol for 15 minutes. The plates were air-dried for 30 minutes, and 100 µL of a 0.1% crystal violet solution was added to each well for 20 minutes at room temperature. Following incubation, the wells were washed with distilled water, and 100 µL of 33% acetic acid was applied to each well to solubilize the dye. The optical density (OD) of each well was measured at 570 nm. The absorbance values obtained were averaged and compared with those of the control group, which consisted of untreated bacterial strains.

*Evaluation of bioflm gene expression level*

The bacterial strains used in the study were exposed to sub-inhibitory concentrations (Sub-MIC) of liposome-loaded selenium nanoparticles (SeNPs), free SeNPs, and free liposomes. Following treatment, RNA was extracted from the bacterial strains using the Qiagen RNA extraction kit (USA), strictly adhering to the manufacturer's protocol. cDNA synthesis was then carried out using the QuantiTect Reverse Transcription kit from Fermentas (Lithuania), following standard procedures for reverse transcription. To assess the expression of biofilm-associated genes, quantitative Real-Time PCR (qRT-PCR) was employed, focusing on genes such as icaD, which plays a key role in biofilm formation, and Ace, which is involved in collagen adhesion in Enterococcus faecalis. Additionally, the 16S rRNA gene was used as a housekeeping gene to normalize the results, serving as an internal control. Finally, the relative expression levels of the biofilm-related genes were quantified using the ΔΔCt method. A detailed list of the primer sequences utilized for the study is provided in Table 1.

*Cell toxicity test*

The cytotoxicity of SeNPs loaded into liposomes, free SeNPs, and free liposomes was evaluated using a colorimetric MTT assay on oral cancer cell line,

Table 1. The Primer Sequences of Bioflm-Related Genes

| Gene | Primer sequence (5′–3′) | Ref |
|---|---|---|
| *icaD* | F ATGGTCAAGCCCAGACAGAG RAGTATTTTCAATGTTTAAAGCAA | [64] |
| *Ace* | F GGAGAGTCAAATCAAGTACGTTGGTT RTGTTGACCACTTCCTTGTCGAT | [65] |
| *16SrRNA* | F TATGGAGGAACACCAGTGGCGAAG RTCATCGTTTACGGCGTGGACTACC | [66] |

including CAL 27. These cell lines were sourced from the Pasteur Institute Iran Cell Bank, located in Tehran, Iran. Initially, the cells were seeded into a 96-well plate at a density of 10,000 cells per well and allowed to incubate for 24 hours. Subsequently, the cells were treated with varying concentrations of liposome-loaded SeNPs and free SeNPs, ranging from 4.125 to 66 µg/mL. After a 48-hour incubation period, MTT dye solution (5 mg/mL in PBS) was introduced to the wells and maintained in the incubator for an additional 3 hours. Following this, the supernatant was carefully removed, and 100 µL of dimethyl sulfoxide (DMSO) solution was added to each well to dissolve the formazan crystals. The absorbance of the wells was measured at 570 nm using a microplate reader. The percentage of cell survival was then calculated using the following formula:

Cell survival % = (optical absorbance of treated cells / optical absorbance of control) × 100

*Statistical analysis*

Statistical analysis was performed by repeating all tests three times. The results were evaluated using GraphPad Prism software (version 8), applying a one-way analysis of variance (ANOVA), with a significance level set at $p<0.05$.

## Results

*Green Synthesis and Characterization of Selenium Nanoparticles (SeNPs) and Liposome-Encapsulated SeNPs*

The powdered form of T. cherleri was immersed in a solvent for a 24-hour period to facilitate extraction. Following this, the resultant extract was filtered using Whatman filter paper. The reduction of sodium selenite solution was initiated at ambient temperature upon the addition of the *T. cherleri* extract, leading to a noticeable transition in color from yellow to a reddish hue, signifying the formation of selenium nanoparticles (SeNPs). The occurrence of SeNPs was validated through scanning electron microscopy, revealing that the nanoparticles are predominantly spherical in shape as depicted in Figure 1A. As illustrated in Figure 1B, the maximum absorption peak of the nanoparticles was recorded at 265 nm, affirming the biosynthetic production of selenium nanoparticles. Further characterization demonstrated that the average size and zeta potential of these nanoparticles were 270.3 ± 19.6 nm, as shown in Figure 1C, and -21.9 ± 1.7 mV, respectively. Moreover, the encapsulation and loading efficiency of the drug within these nanoparticles were quantitatively assessed to be 50.5 ± 3.7%.

*Drug release study*

Figure 2 illustrates the cumulative release profiles

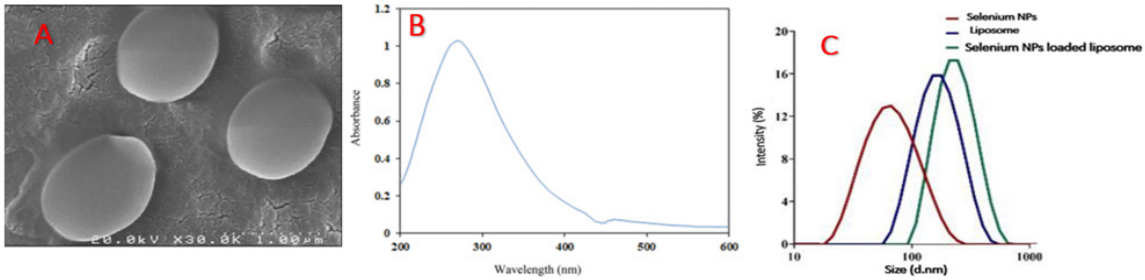


Figure 1. Characterization of the Synthesized Selenium Nanoparticles (SeNPs) and Liposome-Encapsulated SeNPs includes: (A) Scanning Electron Microscopy (SEM) of the green-synthesized SeNPs, (B) Ultraviolet-Visible (UV-Vis) Spectroscopy Analysis of the biosynthesized SeNPs, and (C) Dynamic Light Scattering (DLS) Analysis of the Liposome-Loaded SeNPs.

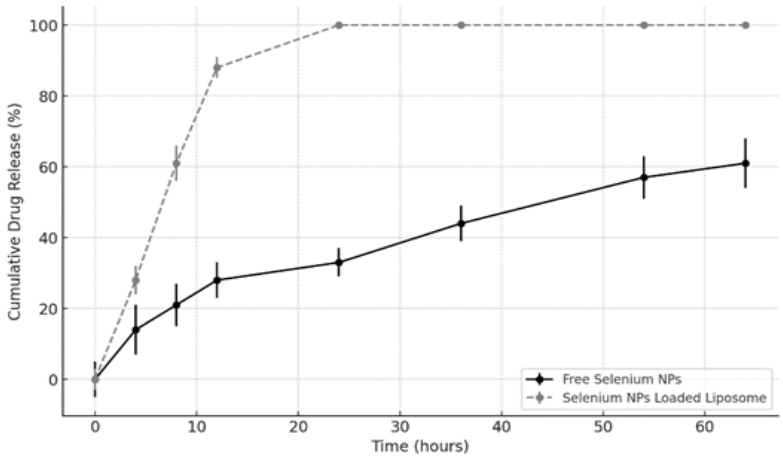


Figure 2. The Release Profile of SeNPs Encapsulated in Liposomes at 64 hours. Data are expressed as mean ± SD from three independent experiments.

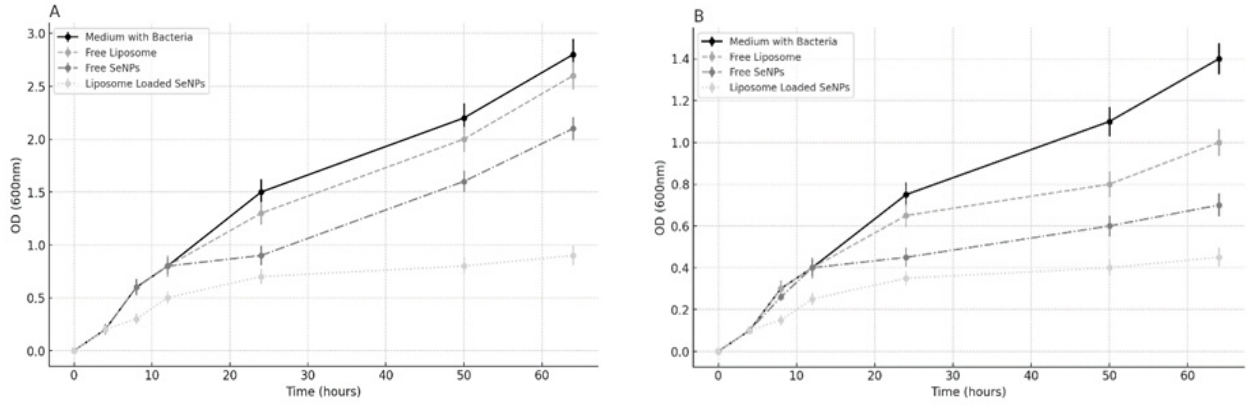


Figure 3. Antibacterial Efficacy of Free Selenium Nanoparticles and Liposome-Encapsulated Selenium Nanoparticles Against Selected Pathogenic Bacteria Including Staphylococcus aureus (A), and Enterococcus faecalis (B).

Table 2. Antibacterial Effects of Free Selenium Nanoparticles and Liposome-Encapsulated Selenium Nanoparticles Against Selected Pathogenic Bacteria.

| Bacteria | MIC/MBC of free SeNPs (µg/ml) | MIC/MBC of liposome-loaded SeNPs (µg/ml) | SubMIC value of free SeNPs/ liposome-loaded SeNPs (µg/ml) |
|---|---|---|---|
| Staphylococcus aureus | 16.5/33 | 4.125/8.25 | 8.25/>4.125 |
| Enterococcus fecalis | 33/66 | 4.125/8.25 | 16.5/>4.125 |

of both free and liposome-encapsulated selenium nanoparticles (SeNPs) in a PBS release medium over a period of 64 hours. To mimic the ex vivo release conditions more closely to those of in vivo, a PBS medium with a pH of 7.2 was employed. The data indicate that the release of SeNPs from the liposomal carriers (61%) is lower than that of the free SeNPs (100%) over the same period. Specifically, 88% of the free SeNPs were released within the first 12 hours, compared to only 28% from the liposome-encapsulated SeNPs. The release kinetics from the liposomes are characterized by two distinct phases: an initial rapid release phase from 0–12 hours where SeNPs are quickly discharged into the medium, followed by a prolonged slow-release phase extending up to 64 hours. Overall, the pattern of drug release from liposomes is initially rapid and then tapers off over time.

*Antibacterial Activity of Selenium Nanoparticles*

In this study, the antimicrobial activity of free selenium nanoparticles (SeNPs) and liposome-loaded SeNPs was quantitatively assessed against two pathogenic bacteria, Staphylococcus aureus and Enterococcus faecalis. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for both formulations of selenium nanoparticles. For Staphylococcus aureus, the MIC and MBC values for the liposome-loaded SeNPs, which were 4.125 and 8.25 µg/ml respectively, represented a fourfold reduction compared to the free SeNPs with values of 16.5 and 33 µg/ml. Similarly, for Enterococcus faecalis, the liposome-loaded SeNPs decreased the MIC and MBC eightfold, with values of 4.125 and 8.25 µg/ml compared to 33 and 66 µg/ml for the free SeNPs. Additionally, the sub-MIC values, which indicate a sustained inhibitory effect of the nanoparticles at concentrations below the MIC, were recorded for both formulations against both bacteria. These results demonstrate a significant enhancement in the antimicrobial activity of the liposome-loaded SeNPs, which not only

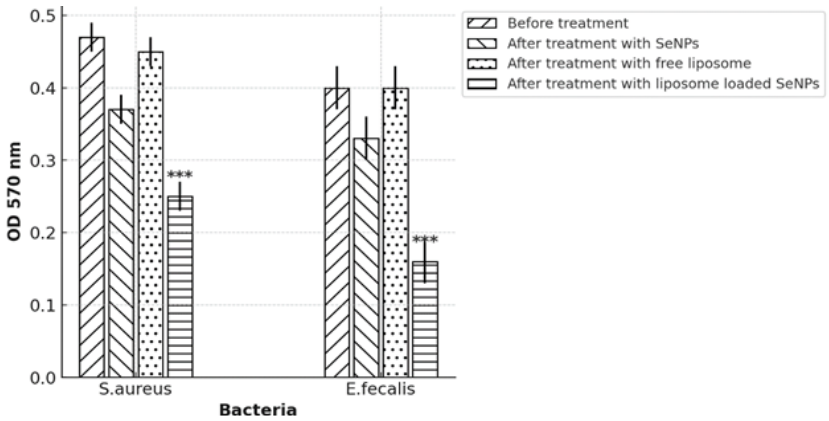


Figure 4. Anti-Biofilm Activity of free SeNPs, Free Liposomes, and Liposome-Loaded SeNPs is Shown. Values are expressed as mean ± SD for three replicates. Error bars denote standard deviations. ***p < 0.001.

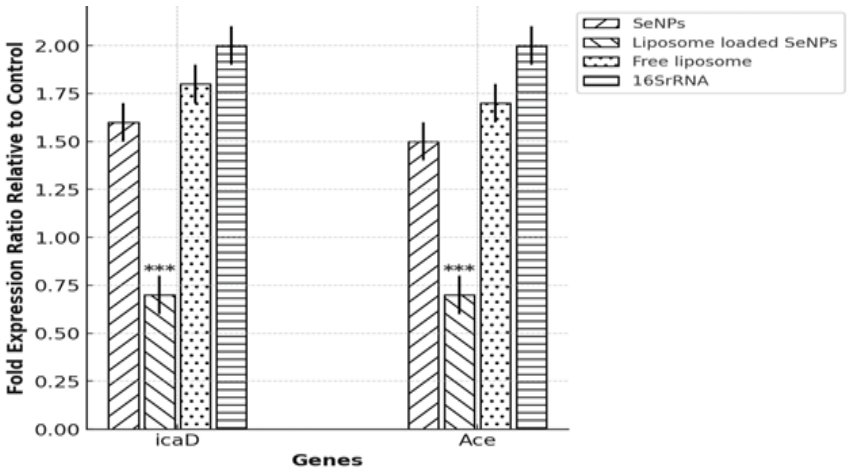


Figure 5. Analysis of Biofilm-Related Gene Expression Following Treatment with free SeNPs, Free Liposomes, and Liposome-Loaded SeNPs. Data represent n=3, ***p<0.0001.

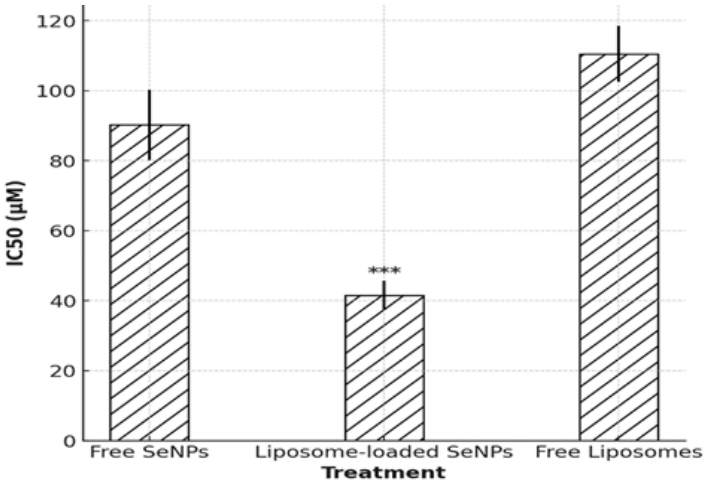


Figure 6. *In vitro* Cytotoxicity of Free SeNPs, Liposome Loaded SeNPs, and Free Liposome on CAL-27cell line. n=3, *** p<0.0001.

exhibit stronger antimicrobial effects compared to the free nanoparticles but also, considering their gradual release and sustained activity, may be more suitable for therapeutic applications. Also, the results indicated that the free liposomes exhibited no antimicrobial effects (Table 2).

*Time kill assay*

To investigate the bactericidal profile of liposome-loaded SeNPs and free SeNPs, we employed the time-kill assay technique against strains of S. aureus and E. faecalis. For this assay, sub-MIC concentrations of both liposome-loaded and free SeNPs were utilized over a period of 64 hours. After 64 hours, it was evident that the antibacterial effects of the liposome-loaded SeNPs were significantly greater than those of the free SeNPs (Figure 3).

*Anti bioflm activity*

The anti-biofilm properties of liposome-loaded SeNPs, free liposomes, and free SeNPs were assessed using the crystal violet staining technique. According to Figure 4, liposome-loaded SeNPs demonstrated significantly enhanced anti-biofilm effects compared to free SeNPs. Moreover, liposome-loaded SeNPs were shown to reduce biofilm formation by two to three times relative to free SeNPs. In contrast, free liposomes, serving as the control, exhibited no anti-biofilm activity.

*Bioflm gene expression analysis*

To evaluate the impact of liposome-loaded SeNPs, free SeNPs, and free liposomes on the expression levels of biofilm-associated genes, the Real-Time PCR technique was employed. The strains were first treated with their respective sub-MIC concentrations, followed by RNA extraction and cDNA synthesis. The expression levels of the biofilm-related genes icaD and Ace were then analyzed. The findings revealed a significant reduction in the expression of biofilm-forming genes in strains treated with liposome-loaded SeNPs compared to those treated with free SeNPs and free liposomes (Figure 5).

*In vitro cytotoxicity*

The cytotoxicity assessment of free SeNPs, free liposomes, and liposome-loaded SeNPs on oral cancer cell line over 48 hours revealed that liposome-loaded SeNPs exhibited the highest cytotoxic effect compared to free

SeNPs, as the IC50 value for CAL-27 cells treated with liposome-loaded SeNPs was 41.52±4.1 µM, significantly lower than that of free SeNPs, which was 90.2±7.2 µM. This highlights the notable cytotoxic efficacy of the liposome-loaded formulation (Figure 6).

## Discussion

The integration of nanotechnology into biomedical applications has led to significant advancements in antimicrobial and anticancer therapies. Among various nanomaterials, selenium nanoparticles (SeNPs) have gained attention due to their unique biological properties, including their ability to combat bacterial infections, disrupt biofilm formation, and induce selective cytotoxicity in cancer cells [67]. However, the efficacy of free SeNPs is often limited by factors such as instability, rapid aggregation, and uncontrolled release, which can reduce their therapeutic potential [68]. Liposomal encapsulation offers a strategic solution to these challenges by enhancing the bioavailability and controlled delivery of SeNPs [69]. The lipid bilayer of liposomes provides a protective environment that prevents nanoparticle degradation while ensuring sustained and targeted release [70]. This improved formulation not only enhances bacterial membrane penetration, making SeNPs more effective against resistant bacterial strains and biofilms, but also increases cellular uptake in cancer treatment, reducing systemic toxicity [49, 71]. The combination of nanotechnology with drug delivery systems like liposomes represents a promising approach for developing next-generation antimicrobial and anticancer agents with improved efficacy and safety [72]. In this research, the use of T. cherleri extract as a reducing agent in the green synthesis of selenium nanoparticles (SeNPs) represents an environmentally friendly approach. This method has distinct advantages over traditional chemical synthesis methods, which often involve toxic solvents and reagents [73]. The color change from yellow to red, resulting from the reduction of sodium selenite and the formation of SeNPs, serves as a visual indicator of nanoparticle synthesis and can be useful for monitoring the reaction without complex equipment [74]. SEM images confirm that the nanoparticles are predominantly spherical. This shape and uniformity can impact the biological activity and stability of the nanoparticles as spherical nanoparticles are often preferred due to better cellular uptake [75]. The average size of the nanoparticles, which is 270.3 nm, and a zeta potential of -21.9 mV indicate moderate stability of these nanoparticles in suspension. The importance of zeta potential in predicting the colloidal stability of SeNPs in suspension is crucial, as negative values indicate repulsion between particles, reducing their tendency to aggregate [76]. An encapsulation efficiency of 50.5% is notable for potential therapeutic applications, where higher encapsulation efficiency often correlates with better therapeutic efficacy and reduced toxicity. Factors that could influence the encapsulation efficiency include the lipid composition of the liposomes, the method of encapsulation, and the interaction between the SeNPs and the liposomal membrane [77-78]. The drug release study demonstrated that liposome-encapsulated selenium nanoparticles (SeNPs) exhibited a controlled and sustained release profile compared to free SeNPs. Over 64 hours, only 61% of the encapsulated SeNPs were released, while free SeNPs were fully discharged, with 88% released within the first 12 hours. This controlled release behavior aligns with previous findings on nanoparticle encapsulation, where liposomal carriers provide a protective barrier that slows drug diffusion and enhances stability [79]. The biphasic release pattern observed an initial rapid release followed by a prolonged slow-release phase suggests that encapsulation effectively modulates the release kinetics. This is likely due to the initial burst of surface-adsorbed nanoparticles, followed by gradual diffusion through the liposomal bilayer [80]. Such a controlled release minimizes burst effects, prolongs nanoparticle availability, and enhances therapeutic potential by reducing toxicity risks [81]. The presence of sub-MIC values in both bacterial strains further supports the sustained antimicrobial activity of liposomal SeNPs, suggesting a prolonged inhibitory effect even at concentrations below the MIC. This controlled release mechanism prevents rapid degradation or aggregation of SeNPs, maintaining their antibacterial action over time. Similar findings have been reported in previous studies, where selenium nanoparticles exhibited prolonged antimicrobial efficacy due to their controlled release and enhanced stability [82]. Additionally, selenium nanoparticles in chitosan solutions have demonstrated strong antimicrobial properties against Streptococcus mutans, Lactobacillus acidophilus, and Candida albicans, reinforcing the idea that nanoparticle formulations can sustain antibacterial effects for extended periods [83]. The enhanced antibacterial potential of selenium nanoparticles against multidrug-resistant bacteria has also been highlighted, suggesting their role as potential alternatives to conventional antibiotics [84]. Furthermore, studies have confirmed that selenium nanoparticles exhibit significant antibacterial effects due to their oxidative stress-inducing properties and ability to disrupt bacterial cell membranes [85]. The absence of antimicrobial activity in free liposomes in our study further confirms that the observed effects were solely due to the SeNPs, rather than the liposomal carrier itself. These findings align with previous studies that have demonstrated the improved antimicrobial potency of nanocarrier systems by increasing nanoparticle stability and bioavailability [81]. Liposomal encapsulation not only enhances the controlled release of SeNPs but also minimizes potential cytotoxicity, making it a promising approach for antimicrobial applications [86]. The time-kill assay results confirm the superior bactericidal activity of liposome-loaded selenium nanoparticles (SeNPs) over free SeNPs, highlighting the benefits of nanocarrier-based drug delivery. Their sustained antibacterial effects over 64 hours suggest a controlled release mechanism that enhances bacterial inhibition. This improved efficacy is likely due to increased stability, bioavailability, and gradual release from the liposomal carrier, preventing nanoparticle aggregation and degradation [87]. Additionally, encapsulated nanoparticles exhibit prolonged retention and enhanced bacterial penetration, further increasing their

antimicrobial potential [88]. These findings align with previous studies indicating that nanocarrier-based drug delivery systems significantly enhance the pharmacokinetics and efficacy of antimicrobial agents [70]. The findings from the crystal violet staining assay highlight the superior anti-biofilm properties of liposome-loaded selenium nanoparticles (SeNPs) compared to free SeNPs, reinforcing the advantages of nanocarrier-based drug delivery systems for combating biofilm-associated infections. Biofilms pose a significant challenge in antimicrobial therapy due to their ability to protect bacterial cells from conventional treatments, making the development of effective anti-biofilm agents crucial [89]. The results demonstrate that liposome-loaded SeNPs reduced biofilm formation by two to three times compared to free SeNPs. This enhanced efficacy can be attributed to the improved penetration of liposome-encapsulated SeNPs into biofilms, where the lipid-based carriers facilitate deeper diffusion through the biofilm matrix [90]. Additionally, liposomal encapsulation provides a sustained release profile, prolonging the antimicrobial activity of SeNPs within the biofilm environment [70]. The complete lack of anti-biofilm activity observed in free liposomes confirms that the biofilm inhibition effect is specifically due to the SeNPs rather than the liposomal carrier itself. This finding further supports the role of selenium nanoparticles as potent antimicrobial and anti-biofilm agents, with liposomal formulations enhancing their therapeutic efficacy [91]. The gene expression analysis using Real-Time PCR revealed that liposome-loaded selenium nanoparticles (SeNPs) significantly downregulated biofilm-associated genes icaD and Ace, which are critical for biofilm formation in Staphylococcus aureus and Enterococcus faecalis. This stronger suppression compared to free SeNPs suggests that liposomal encapsulation enhances nanoparticle stability, prolongs retention, and improves bacterial penetration, ensuring sustained inhibition of biofilm formation [89]. The controlled release mechanism of liposomal SeNPs allows continuous gene suppression, reducing bacterial adhesion and biofilm stability [92]. The ability of SeNPs to inhibit biofilm formation at the genetic level aligns with previous studies that have shown selenium's effectiveness in disrupting quorum sensing-regulated processes, which are critical for bacterial communication and biofilm development [93]. Moreover, selenium's ability to interfere with *E. coli* O157:H7 biofilms by reducing exopolysaccharide synthesis and biofilm gene expression further supports its potential as an effective antimicrobial agent [94]. Additionally, green-synthesized SeNPs have been shown to significantly reduce biofilm formation in multidrug-resistant Klebsiella pneumoniae, highlighting their broad-spectrum anti-biofilm properties [71].The cytotoxicity assessment of liposome-loaded selenium nanoparticles (SeNPs) against oral cancer cells (CAL-27) demonstrates their superior anticancer potential compared to free SeNPs. The significantly lower IC50 value (41.52±4.1 μM) for liposome-loaded SeNPs compared to free SeNPs (90.2±7.2 μM) suggests that liposomal encapsulation enhances the bioavailability and cellular uptake of SeNPs, leading to more effective cytotoxicity. This increased potency can be attributed to the ability of liposomal carriers to facilitate nanoparticle stability, prolong systemic circulation, and improve intracellular delivery [86].

This study highlights the potential of liposome-loaded selenium nanoparticles (SeNPs) as an advanced and biocompatible drug delivery system with significant antibacterial, anti-biofilm, and anticancer properties. The encapsulation of SeNPs within liposomes enhances their stability, bioavailability, and controlled release, ensuring prolonged therapeutic effects and reduced toxicity. The antibacterial efficacy of liposomal SeNPs, demonstrated by their superior bactericidal and biofilm-inhibiting activity, suggests their potential for combating multidrug-resistant bacterial infections. Additionally, the anti-biofilm properties of liposome-loaded SeNPs, supported by their ability to downregulate key biofilm-associated genes, indicate their effectiveness in preventing and disrupting biofilm formation, which is a major challenge in chronic infections. In the context of oral cancer therapy, liposome-loaded SeNPs exhibited significantly enhanced cytotoxicity against cancer cells compared to free SeNPs, as evidenced by their lower IC50 values. The improved intracellular uptake and sustained release from liposomal carriers contribute to enhanced apoptosis induction, likely through oxidative stress mechanisms and mitochondrial dysfunction. This targeted and prolonged anticancer effect minimizes off-target toxicity, making liposomal SeNPs a safer and more effective alternative to conventional chemotherapeutics. Overall, the results of this study suggest that liposome-loaded SeNPs hold great promise as a multifunctional nanoplatform for targeted drug delivery in oral cancer therapy, while simultaneously offering potent antimicrobial and biofilm-disrupting capabilities. Future research should focus on optimizing their formulation, investigating their pharmacokinetics, and conducting in vivo studies to further validate their clinical applicability.

*Future Research*

By leveraging cutting-edge, integrated technologies alongside advancements in chemistry and biology, substantial progress has been achieved in tackling a wide range of chronic diseases that afflict modern society. Among these innovations, nanotechnology has emerged as a pivotal tool, significantly contributing to the treatment of various conditions, including cancer, as well as driving advancements in other industrial fields. Continued evolution in these technologies holds great promise for further breakthroughs in both healthcare and industry [45, 95-103, 104-106].

## Author Contribution Statement

All authors contributed equally in this study.

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*Ethics approval*

This study did not involve any original data collection or human subjects, and therefore, ethical approval was

not required.

*Conflict of interests*

The authors have no competing interests to declare that are relevant to the content of this article.

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