

Protective Effects of Liposomal Vitamin C on SARS-CoV-2 Target Viral Entry Genes in Renal Cells

Abtin Behmardi¹, Leila Zolghadr^{1,2}, Farzad Rajaei¹,
Saeideh Gholamzadeh Khoei¹, Nematollah Gheibi*¹

Abstract

Background: The kidneys are a potential target for SARS-CoV-2 infection. Ascorbic acid (vitamin C) has been shown to play an important role in reducing the symptoms of SARS-CoV-2. Recently liposomal drug delivery platforms have demonstrated promising results in enhancing the effectiveness of various therapeutics including infectious diseases. In this study, we designed a liposomal delivery system containing vitamin C to evaluate its antiviral efficacy in COVID-19, focusing on its effects on viral entry gene expression in Vero cells.

Methods: Vitamin C was loaded into a liposome made up of hydrogenated soybean phosphatidylcholine, cholesterol, and 1,2-distearoyl-sn-glycero-3 phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000], and their physicochemical properties were assessed. Next, the cytotoxicity of free and liposomal vitamin C on the survival of the Vero cell line was evaluated using the MTT assay. In addition, the expression of viral entry genes, *angiotensin-converting enzyme 2 (ACE2)* and *transmembrane protease serine 2 (TMPRSS2)*, key mediators of SARS-CoV-2 entry into kidney cells, was investigated using RTq-PCR.

Results: Liposomes were successfully loaded with vitamin C, achieving an encapsulation efficiency of 88.03%. The liposomal vitamin C formulation exhibited a brilliant surface morphology as observed by SEM. Both free and liposomal forms of vitamin C showed cytotoxic effects at higher concentrations. Moreover, both forms downregulated the expression of viral entry genes, although the liposomal form showed superior inhibitory performance compared to the free form.

Conclusion: The study suggests liposomal vitamin C as a safe, effective treatment for COVID-19 by targeting viral entry genes in kidney cells, protecting them from viral damage and inflammation.

Keywords: Ascorbic Acid, COVID-19, Liposomes, Renal Cells, Viral Entry.

Introduction

Coronaviruses (CoVs) are a group of positive-strand RNA viruses with an envelope, among which Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has caused the coronavirus disease 2019 (COVID-19) pandemic, resulting in significant global morbidity and mortality (1, 2). Although COVID-19 is primarily a respiratory disease, the kidneys can also be among the target organs

of infection by SARS-CoV-2, leading to acute kidney injury (AKI), particularly in patients with pre-existing kidney disease (3, 4), as previous studies have demonstrated the link between infections and AKI (5).

SARS-CoV-2 consists of four structural proteins: Spike (S), Envelope (E), Nucleocapsid (N), and Membrane (M). The S protein facilitates viral entry into host cells by

1: Cellular and Molecular Research Center, Research Institute for Prevention of Non-Communicable Disease, Qazvin University of Medical Sciences, Qazvin, Iran.

2: Department of Chemistry, Imam Khomeini International University, Qazvin, Iran.

*Corresponding author: Nematollah Gheibi; Tel: +98 2833328213; E-mail: n_gheibi@qums.ac.ir.

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interacting with angiotensin-converting enzyme 2 (ACE2). Transmembrane protease serine 2 (TMPRSS2) cleaves the spike protein, enabling its binding to ACE2 and promoting membrane fusion, a critical step in viral invasion (6). ACE2 is a component of the renin-angiotensin system (RAS), which maintains body homeostasis through two axes: Angiotensin-Converting Enzyme/Angiotensin II/Angiotensin Type 1 Receptor (ACE/Ang II/AT1R) as classical RAS, and Angiotensin-Converting Enzyme 2/Angiotensin 1-7/ Mas Receptor (ACE2/Ang1-7/ MasR) as the alternative RAS (7).

SARS-CoV-2 binding to ACE2 disrupts this balance, leading to inflammation, vascular damage, and multi-organ dysfunction, including acute kidney injury (AKI) (8, 9). Kidney involvement in COVID-19 has gained significant attention, as studies show that *ACE2* and *TMPRSS2* are highly expressed in renal cells, particularly in the proximal tubules, making the kidneys a critical target for SARS-CoV-2 (9). Direct viral invasion, systemic inflammation, and hypoxia all contribute to renal dysfunction in COVID-19 patients (10). Therefore, the modulation of *ACE2* and *TMPRSS2* expression in kidney cells could play a vital role in reducing SARS-CoV-2 infectivity and mitigating renal damage.

Ascorbic acid (vitamin C) has emerged as a promising candidate for COVID-19 therapy due to its potent antioxidant, anti-inflammatory, and immunomodulatory properties (11). Numerous studies have shown that vitamin C plays an important role in preventing and reducing the severity of many types of viral and bacterial infections (12). For example, giving vitamin C to septic mice with acute respiratory distress syndrome (ARDS) strengthened the epithelial barrier and improved alveolar fluid clearance by reducing the expression of inflammation-related genes (13, 14). Furthermore, vitamin C deficiency is directly associated with increased influenza A-induced lung pathology in mice (15). Arvinte et al. reported low vitamin C levels among critically ill COVID-19 patients admitted to intensive care units (ICUs) in the US (16). Another study also reported that low

levels of vitamin C were associated with acute respiratory distress syndrome in COVID-19 patients (17). Additionally, vitamin C has been shown to reduce interleukin-7 (IL-7)-induced ACE2 expression in endothelial cells, suggesting a potential mechanism for limiting SARS-CoV-2 entry (18).

Despite its potential, the bioavailability of vitamin C is limited due to rapid metabolism and degradation in the digestive system (19). Liposomal systems enhance vitamin C stability, absorption, and controlled release, addressing its bioavailability limitations (20). Liposomes, composed of lipid bilayers, are particularly effective at delivering hydrophilic compounds like vitamin C, protecting them from degradation and improving therapeutic efficacy (21). Studies have demonstrated the advantages of liposomal vitamin C, including increased plasma concentrations without gastrointestinal side effects, which enables its use at higher therapeutic doses (22, 23).

This study aims to evaluate the therapeutic potential of free and liposomal vitamin C in targeting kidney cells, focusing on the modulation of *ACE2* and *TMPRSS2* to mitigate SARS-CoV-2-induced kidney damage.

Materials and Methods

Reagents

The Vero cell line (the epithelial kidney tissue normal cells, ATCC CCL-81™) was purchased from the Iranian Biological Resource Center (IBRC). MTT and vitamin C were obtained from Sigma-Aldrich (USA) and additional consumables including chloroform were prepared from Merck (Germany). Trypsin-EDTA, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from BioIdea (Iran), and DSPE-PEG2000, HSPC, cholesterol, and a dialysis membrane were purchased from Avanti Polar Lipids (USA). RNX-Plus Kit and PCR primers, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), *ACE2*, and *TMPRSS2* were purchased from Cinnagen (Iran) and SinaColon (Iran) respectively. cDNA synthesis kit and qRT-PCR SYBR green

master mix were purchased from Yekta Tajhiz Arma (Iran) and Amplicon (Denmark) respectively.

Liposome Preparation

The synthesis of liposomes was carried out using a modified version of the standard thin film hydration technique (24). Initially, a lipid mixture comprising hydrogenated soy phosphatidylcholine (HSPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG2000) was dissolved in ethanol and chloroform at a 3:7 (v/v) ratio. The molar ratios of the lipids were set at 70:25:5, respectively. A total of 4.5 mg of vitamin C was added to this lipid solution in a round-bottom flask. The solvent was evaporated at 49 °C to achieve a thin lipid film. The resulting lipid film was thoroughly dried and rehydrated with 3 ml of phosphate-buffered saline (PBS) at pH 7.4, then manually shaken to ensure the film detached from the flask wall and dissolved uniformly. The rehydrated liposomal suspension was then homogenized at 3000 rpm for 5 minutes at 25 °C to reduce the particle size. Following homogenization, the liposomes underwent probe sonication at 75% amplitude in an ice compartment for 10 minutes to further refine their size and uniformity. To ensure sterility and achieve the desired size distribution, using sterile mixed cellulose ester (MCE) filters (0.22 µm), the liposomal suspension was filtered seven times (Sigma-Aldrich, USA). Finally, the liposomes were subjected to freeze-drying in a lyophilizer for 48 hours to obtain stable liposomal powders.

Determination of Encapsulation Efficiency (EE%)

Liposomes were separated from non-entrapped vitamin C through ultracentrifugation. The concentration of free vitamin C in the supernatant was determined using a spectrophotometer at a wavelength of 295 nm. A standard curve was used to quantify the vitamin C present in the supernatant. The

calibration curve exhibited a high coefficient of determination ($R^2 = 0.999$).

Encapsulation efficiency was then calculated using Equation provided below.

$$\%EE = \frac{\text{Amount of vitamin C used} - \text{amount of free vitamin C}}{\text{Amount of vitamin C used}} \times 100$$

Characterization of Liposomes

Fourier-Transform Infrared Spectroscopy (FTIR)

A FTIR spectrometer (FTIR, Bruker Tensor, USA) was employed to analyze the functional groups of free vitamin C, empty liposomes, and vitamin C-loaded liposomes. The FTIR spectra were recorded for both vitamin C and liposomal formulations and across the wavenumber range of 400 to 4000 cm^{-1} .

Morphology Characterization

The morphology of the liposomes was analyzed through scanning electron microscopy (SEM). For sample preparation, the liposomes were freeze-dried to obtain a powdered form. The lyophilized powders were subsequently examined using an SEM instrument (SU660, Hitachi, Japan).

Kinetics of Vitamin C Release

Various kinetic models are commonly employed to describe the drug release process. In this study, various models, including zero-order, first-order, Higuchi, Hixson-Crowell, and Peppas, were employed to analyze the release kinetics of vitamin C from liposomes.

In zero-order systems, the drug is released at a constant rate, regardless of the concentration. The first-order model describes release in which the rate depends on the drug concentration. Higuchi's model explains release via Fickian diffusion, showing a linear relationship with the square root of time. Hixson-Crowell's model accounts for changes in particle size and surface area. The Peppas model categorizes release into four types: Fickian, case II, non-Fickian, and super case II, based on the release exponent. The percentage of drugs released versus log time follows a linear pattern in the Peppas model (25, 26).

Cell Culture

The Vero cell line was used in this study due to its high expression of *ACE2* and *TMPRSS2* genes. In addition, many studies have shown that Vero cells have a high viral titer (27). The Vero cell line was grown in plastic flasks (75cm² and 25cm²) using DMEM high glucose supplemented with 10% FBS and 1% antibiotic (penicillin-streptomycin) and incubated at 37 °C, with 95% humidity and 5% CO₂.

Cell Viability Assay

After reaching 80-90% confluence, the cells were trypsinized and seeded into a 96-well microplate. After 24 h, untreated cell samples were used as the positive control. Cell treatment was performed at diverse concentrations of 100, 250, 500, 1000, and 10000 µM of free and liposomal forms of vitamin C for 24 h. MTT test was performed to evaluate the toxicity and find the best dose of free and liposomal forms of vitamin C on Vero cells. After replacing the culture medium of the treated cells with 100 µl of MTT solution, it was incubated (4h, 37 °C). Then, after removing the MTT solution, the formed formazan crystals were dissolved in 200 µl of dimethyl sulfoxide (DMSO). Eventually, absorbance was determined at 570 nm by an Elx800 ELISA microplate reader (USA).

Real time quantitative PCR (RTq-PCR)

A 12-well plate (25×10⁴ cells/well) was used to seed Vero cells and incubate at 37 °C. After 24 h, according to the results of the MTT test, the concentration of 1000 µM was utilized for cell treatment. The RNA was extracted using the Total RNA Extraction Mini Kit after 48 h. The RNA yield was determined using a NanoDrop spectrophotometer (Epoch, BioTek, USA). Then, reverse transcription was carried out on the extracted RNA using the cDNA synthesis kit. For real-time PCR, RTq-PCR SYBR green master mix and Real-Time PCR Machine (Rotor-Gene 6000, Qiagen, USA) were utilized. GAPDH was used as an internal control (Table 1). Amplification was done based on the following temperature

program: First step: 95 °C for 3min, then 45 cycles and 40 cycles including 15 s at 95 °C, 15 s at 54 °C and 15 s at 72 °C for *ACE2* and *TMPRSS2*. The process was done in three stages and the relative expression of genes was calculated by the 2^{-ΔΔCT} method.

Statistical Analysis

The difference between the treatment groups was determined using one-way ANOVA. Duncan's multiple range test was employed to measure significant differences between data groups using SPSS v20 and GraphPad Prism 9 (P< 0.05).

Results**Characterization of Liposomes Physically and Chemically**

SEM imaging was employed to observe the morphology and structure of liposomes, including both empty and vitamin C-loaded variants. The SEM images revealed that both types of liposomes had a generally spherical shape and a consistent appearance (Fig. 1). Additionally, the liposomes loaded with vitamin C displayed greater brightness in comparison to the empty liposomes.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was used to examine the interactions between components by observing changes in vibration frequencies. The FTIR spectrum of empty liposomes (Figure 2) showed aromatic rings at 800-1000 cm⁻¹, C=C stretching at 1600 cm⁻¹, C-H groups between 2500-2900 cm⁻¹, and OH groups between 3300-3700 cm⁻¹. The spectrum of pure L-Ascorbic Acid revealed peaks at 1674 cm⁻¹ (C=C) and 1322 cm⁻¹ (enol-hydroxyl). After encapsulation, new peaks appeared at 3311.88 cm⁻¹, 1635.01 cm⁻¹, 1567.45 cm⁻¹, and 1377.59 cm⁻¹. These peaks correspond to the hydroxyl, band due to the scissor bending vibration of molecular water, acidic asymmetric stretch and C-H deformations of –CH₂ or –CH₃ groups (lignin) in aliphatic respectively. Following the encapsulation of vitamin C, a shift towards higher wavenumbers (3405 cm⁻¹) was observed in the

position of this peak. This shift indicated the involvement of hydrogen bonding, particularly in the hydroxyl groups. This suggests

hydrogen bonding plays a key role in vitamin C encapsulation into liposomes.

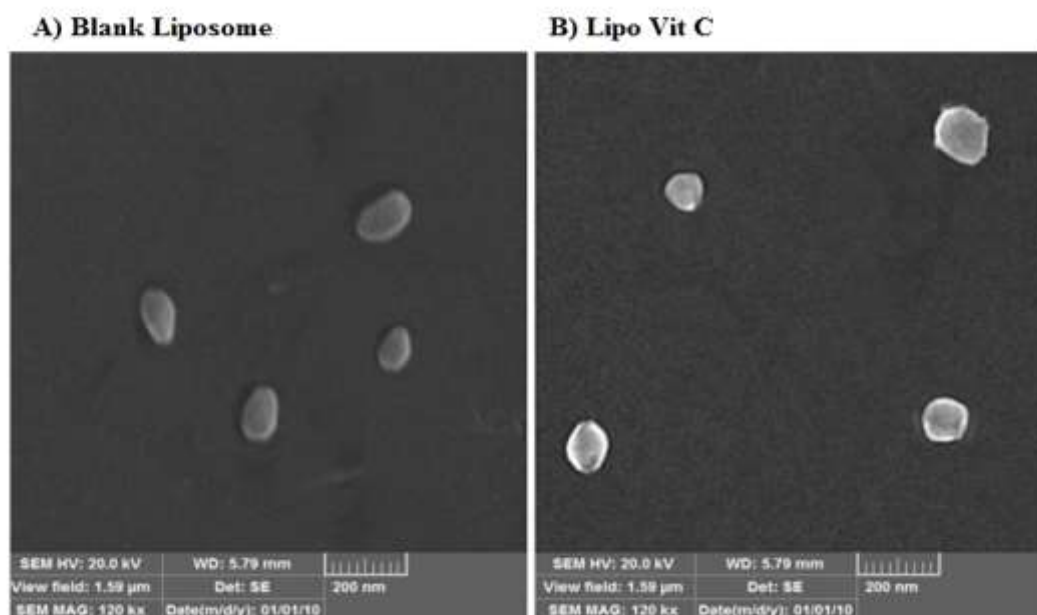


Fig. 1. The SEM images at magnifications of 1.59 μm. (A) Blank liposomes and (B) Vitamin C-loaded liposomes. The Vitamin C-loaded liposomes appear brighter than the blank ones, which indicate successful encapsulation and differences in surface characteristics due to the presence of Vitamin C.

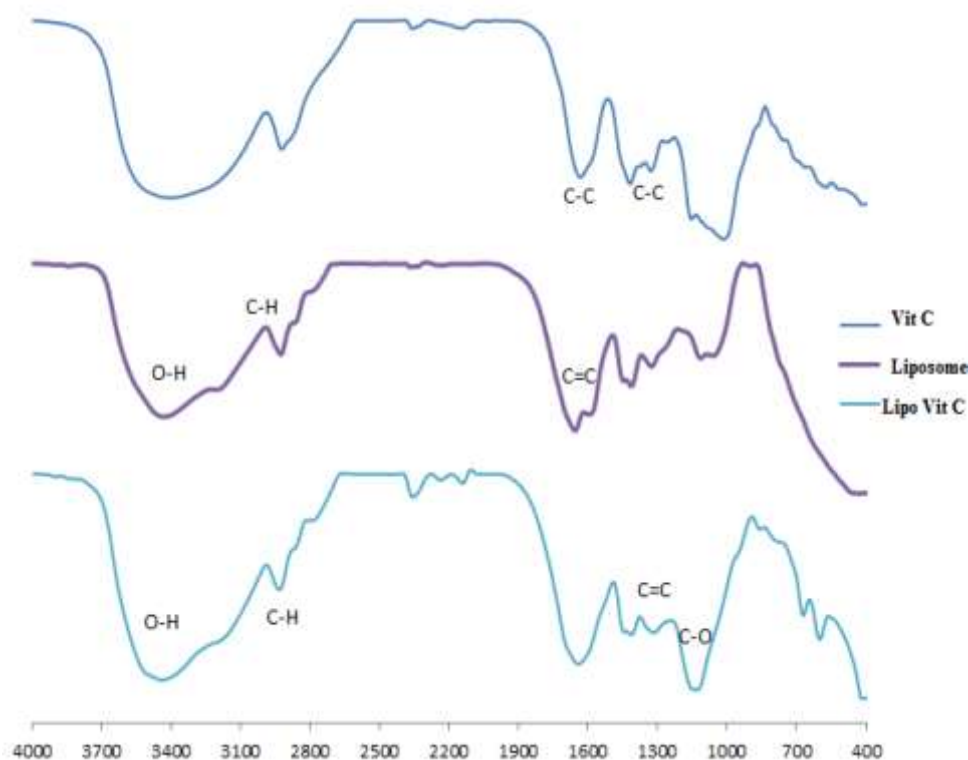


Fig. 2. Comparative FTIR spectrum of Vitamin C, Liposome, and Liposomal Vitamin C. The results show changes in vibrational peaks after encapsulation, indicating interactions between components and the role of hydrogen bonding in the encapsulation process.

Vitamin C Release from Liposomes and Modeling

At pH 7.4 and over various time points (0.5 to 24 h), cumulative vitamin C release from liposomes was measured. Using the standard curve from encapsulation efficiency, the

release was monitored. The release increased gradually during the first 8 h and then plateaued. The maximum release was approximately 93% (Fig. 3). Kinetic studies identified the Peppas model as the best fit for the release process at pH 7.4 (Table 2).

Table 1. The sequences of RTq-PCR primers.

Genes	Primer sequence	Refseq mRNA Accession
<i>ACE2</i>	F:5'- GCCTCCTCTCCTACTTTG -3' R:5'- CTCAGCCCATCTTCTTCC -3'	NM_021804
<i>TMPRSS2</i>	F:5'- TGGGAAGTTTCAAATCAGC -3' R:5'- GCATTCTTGGACGAGGG -3'	NM_005656
<i>GAPDH</i>	F:5'-CAATGACCCCTTCATTGACC-3' R:5'-TGGAAGATGGTGTGATGGGATT-3'	NM_002046.7

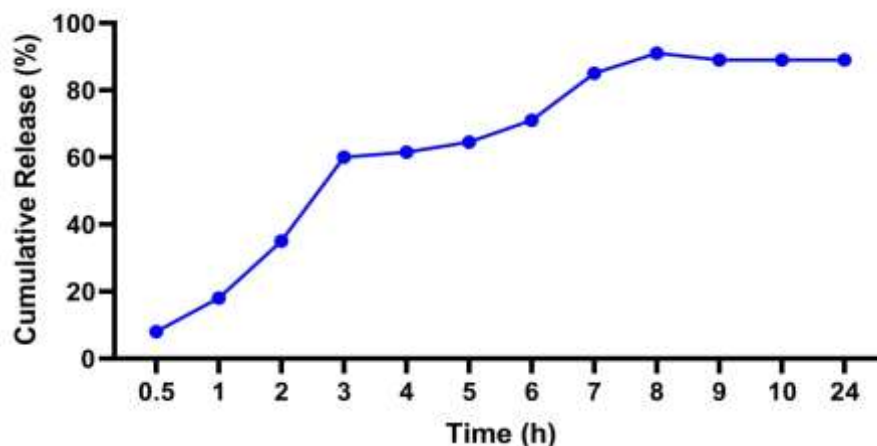


Fig. 3. Vitamin C release pattern from liposomal form, for 24 h and pH 7.4. The release gradually increased within the first 8 h and then plateaued. The maximum release reached approximately 93%, with the Peppas model providing the best kinetic fit.

Table 2. Kinetic Analysis of Vitamin C Release from Liposomes at pH 7.4.

pH=7/4	factor	Kinetic Models
8.33	K0	Zero order
0.743	R ²	
28246.3	Ss	
-0.083	K ₁	One order
0.883	R ²	
46.03	Ss	Higuchi
31.47	K _H	
0.956	R ²	
4392.5	Ss	Peppas
1/24	K _p	
0/967	R ²	
2.51	Ss	Hixone Corel
0.607	N	
-0.138	K _C	
0.952	R ²	
50.11	Ss	

Cell Viability Assay

Vero cells were treated with various concentrations of 100, 250, 500, 1000, and 10000 μM of free and liposomal forms of vitamin C. Our findings showed that the cytotoxic effects of vitamin C (free and liposomal) at high concentrations (in 1000 to 10000 μM) increased within 24 h (Fig. 4). In addition, the IC₅₀ of the free and liposomal forms of vitamin C were calculated at 3477 μM and 2700 μM , respectively.

ACE2 and TMPRSS2 Gene Expression

The RTq-PCR method was used to investigate the expression changes of ACE2 and TMPRSS2. The concentration of 1000 μM of the free and liposomal forms of vitamin C was chosen for RTq-PCR analyses. Free and liposomal forms of vitamin C decreased the expression of ACE2 and TMPRSS2 at the mRNA levels liposomal form of vitamin C was more successful (Fig. 5).

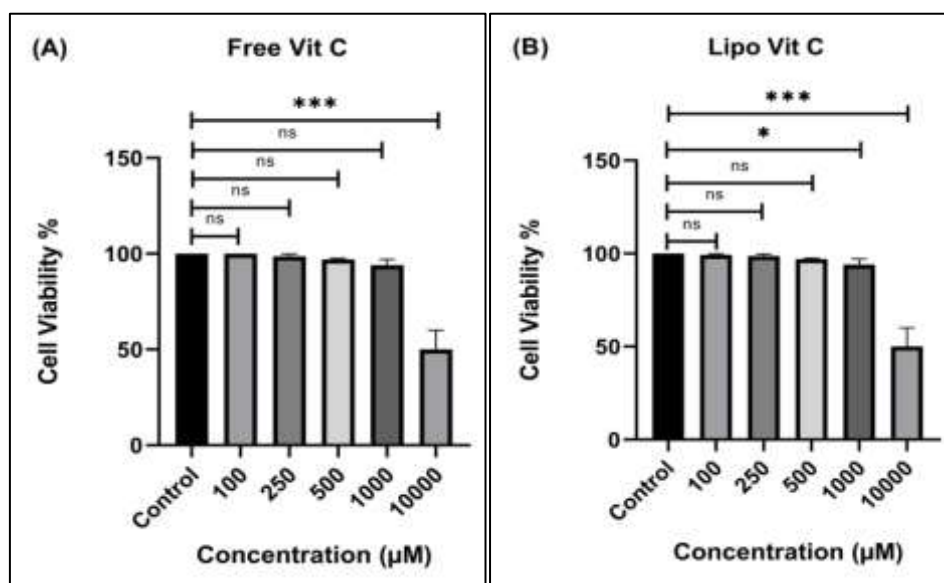


Fig. 4. The results of MTT assay in treatment with free form and liposomal (A) Free vitamin C, and (B) Liposomal vitamin C, for 24h. High concentrations (1000 to 10000 μM) of both forms exhibited cytotoxicity. A dose-dependent cytotoxicity was observed, with a lower IC₅₀ in the liposomal form, P-value<0.05*/ P-value<0.001***.

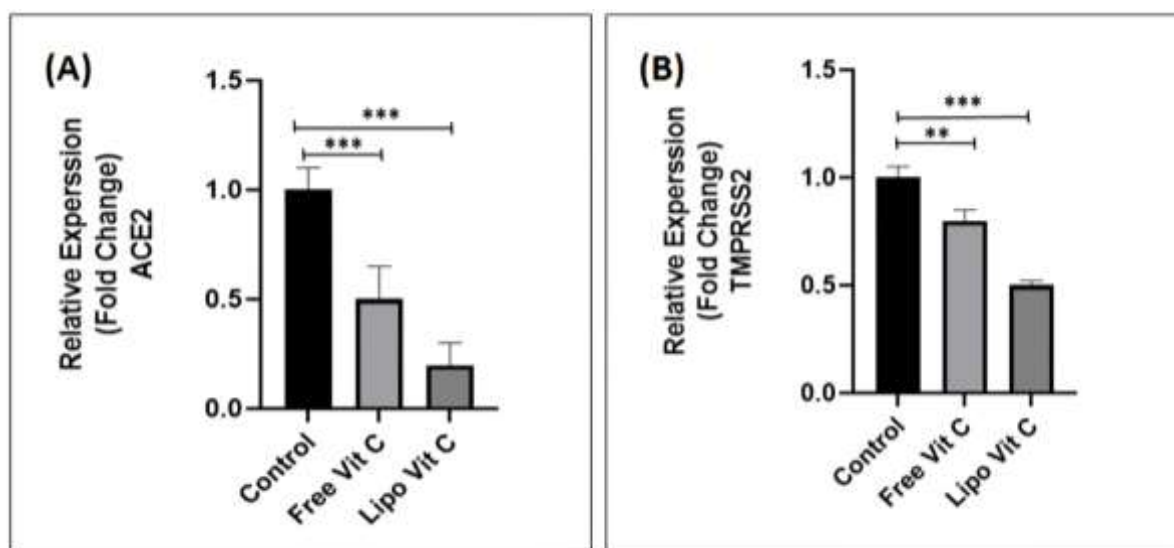


Fig. 5. The effect of vitamin C on the mRNA expression of A) ACE2, and B) TMPRSS2 genes on Vero cell line in 1000 μM of concentration. Both free and liposomal forms of vitamin C reduced the expression of ACE2 and TMPRSS2, with the liposomal form showing a more significant effect. (P-value<0.01**/ P-value<0.001***).

Discussion

This study explored the potential of liposomal vitamin C as a therapeutic agent to mitigate SARS-CoV-2 infection, particularly by modulating the expression of *ACE2* and *TMPRSS2* genes in kidney Vero cells. These two genes play a crucial role in viral entry into host cells, and their downregulation may reduce the susceptibility of cells to viral invasion (28). The results from this study not only support previous findings but also provide new insights into the effectiveness of liposomal vitamin C in targeting the kidney as a desirable site for SARS-CoV-2 infection.

We initially developed and characterized a liposomal structure containing vitamin C. The SEM and FTIR results confirmed the morphology of the liposomes and significant hydrogen bonding interactions, indicating successful encapsulation of vitamin C. Moreover, the sustained release profile of vitamin C from liposomes, achieving 93% release after 24 h, at pH 7.4, suggests that liposomes can provide a controlled release of vitamin C, a crucial factor for maximizing its therapeutic effects while minimizing side effects. These findings align with previous studies demonstrating that liposomal formulations can enhance the stability and bioavailability of encapsulated compounds such as vitamin C (19, 29). The controlled release of vitamin C is particularly important in the context of SARS-CoV-2 treatment, as vitamin C has been shown to possess antiviral, anti-inflammatory, and immune-modulatory properties (30). It appears that the liposomal system can prolong the presence of vitamin C in the bloodstream, thereby enhancing its antiviral efficacy and immune-modulating effects.

In the following, Cytotoxicity assays revealed that free and liposomal vitamin C reduced cell viability at high concentrations, with the liposomal formulation exhibiting a slightly lower IC₅₀ (2700 μ M compared to 3477 μ M for the free form). This suggests that liposomal vitamin C can achieve similar therapeutic effects at lower concentrations,

minimizing the risk of toxicity and gastrointestinal side effects. It has been reported that high doses of vitamin C are associated with gastrointestinal side effects (31). Our findings are consistent with the study showing that liposomal formulations reduce the toxicity of high-dose compounds by improving their solubility and bioavailability, thus reducing side effects and systemic toxicity (32).

While high doses of vitamin C have shown promise in preventing and treating viral infections, they are often associated with gastrointestinal disturbances and contraindications in individuals with kidney impairments or hemochromatosis (33, 34). With its enhanced bioavailability, liposomal vitamin C offers a potential solution by delivering therapeutic doses at lower concentrations, minimizing these side effects and providing a safer alternative for patients with underlying conditions.

Next, we examined the expression of *ACE2* and *TMPRSS2* genes using the RTq-PCR method and the most compelling result of this study was the downregulation of *ACE2* and *TMPRSS2* gene expression following treatment with both free and liposomal vitamin C. *ACE2* serves as the primary receptor for SARS-CoV-2, while *TMPRSS2* is crucial for the cleavage of the viral spike protein, a step necessary for viral entry (35). Given that *ACE2* receptors are abundantly expressed on renal tubular cells, the kidneys have been increasingly recognized as major targets of SARS-CoV-2. COVID-19-induced AKI is a common complication, and studies have shown that *ACE2* and *TMPRSS2* are implicated in the pathogenesis of renal damage during SARS-CoV-2 infection (36, 37).

By downregulating *ACE2* expression, liposomal vitamin C may limit the virus's ability to enter and replicate within kidney cells, potentially reducing the incidence of COVID-19-associated AKI. Our findings align with studies showing that vitamin C can modulate *ACE2* expression, thus providing protection against viral entry (38). Furthermore, vitamin C has been shown to

promote *ACE2* degradation, thereby reducing viral load and mitigating the severity of infection (39).

Interestingly, liposomal formulations have been shown to enhance the intracellular delivery of therapeutic agents, resulting in more pronounced biological effects compared to free drug formulations (40, 41). This is in agreement with our study, where liposomal vitamin C exhibited a more significant downregulation of *ACE2* and *TMPRSS2* compared to the free form in kidney cells.

In conclusion, our findings support the use of liposomal vitamin C as a potentially effective treatment for reducing SARS-CoV-2 infection by modulating *ACE2* and *TMPRSS2* expression. The enhanced bioavailability, controlled release, and lower toxicity profile of liposomal vitamin C make it a promising therapeutic option for COVID-19, particularly for patients with renal dysfunction.

While this study provides promising *in vitro* evidence of the potential benefits of liposomal vitamin C in reducing SARS-CoV-2 infection in kidney cells, further *in vivo* studies are needed to validate these findings in animal models and human trials. Given the critical role of *ACE2* in SARS-CoV-2 entry and the

high expression of *ACE2* in kidney cells, liposomal vitamin C could offer a novel therapeutic approach for preventing or treating COVID-19-associated renal complications. Additionally, clinical trials are necessary to determine the optimal dosing and safety profiles of liposomal vitamin C in COVID-19 patients, particularly those with pre-existing kidney dysfunction.

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Conflict of Interest

All the authors have declared no conflicts of interest.

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