

Transmucosal Delivery of miR-30c-5p by Chitosan Nanoparticles for Oral Squamous Cell Carcinoma

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Background: Oral squamous cell carcinoma (OSCC) remains difficult to treat with current modalities. miR-30c-5p, a tumor-suppressive microRNA frequently downregulated in OSCC, inhibits cancer cell proliferation and migration. However, its clinical application is limited by poor stability and inefficient uptake. To address these issues, miR-30c-5p was encapsulated into chitosan nanoparticles (CS-NPs) using ionic gelation to enhance delivery and protect against degradation.

Methods: miR-30c-5p-loaded CS-NPs were characterized for particle size, zeta potential, morphology, and encapsulation efficiency. HSC-3 and OEC-M1 cells were treated with free miRNA, CS-NPs, or CS-miRNA-NPs at final concentrations of 5%, 10%, 25%, and 50% (v/v) in culture medium. Cellular uptake was assessed by confocal microscopy. Ex vivo porcine buccal membrane studies evaluated mucosal penetration. Cytotoxicity was determined using MTT assays, while gene regulation was analyzed via quantitative polymerase chain reaction and Western blotting.

Results: The prepared CS-NPs had particle sizes ranging from 434 to 452 nm and encapsulation efficiencies between 79% and 87%. Confocal imaging revealed significantly greater cytoplasmic uptake of CS-miRNA-FAM NPs versus free miRNA. Ex vivo studies showed that CS-miR-30c-5p-FAM NPs penetrated mucosa up to 80–160 μ m with a 5.42-fold higher fluorescence intensity than free miR-30c-5p-FAM. Cytotoxicity testing showed high cell viability (>93%) for all treatments at concentrations $\leq$ 25% (v/v). At 50% (v/v) nanoparticle suspension, viability significantly decreased in OEC-M1 cells (84.41% for naked miRNA, 54.52% for CS-NPs, 61.10% for CS-miRNA-NPs; $P < 0.001$). After 48 h, greater reductions were observed at 50% (v/v), with cell viability in HSC-3 cells decreasing to 85.55% (free miRNA), 42.72% (CS-NPs), and 51.82% (CS-miRNA-NPs), and in OEC-M1 cells to 73.98%, 33.00%, and 39.89%, respectively. Functional assays showed vimentin mRNA reductions of 85% in HSC-3 and 30% in OEC-M1, with protein decreases confirmed by Western blot.

Conclusion: CS-NPs enhance miRNA delivery and gene-silencing efficacy in OSCC cells. These findings support CS-based systems for miRNA therapeutics in oral cancer.

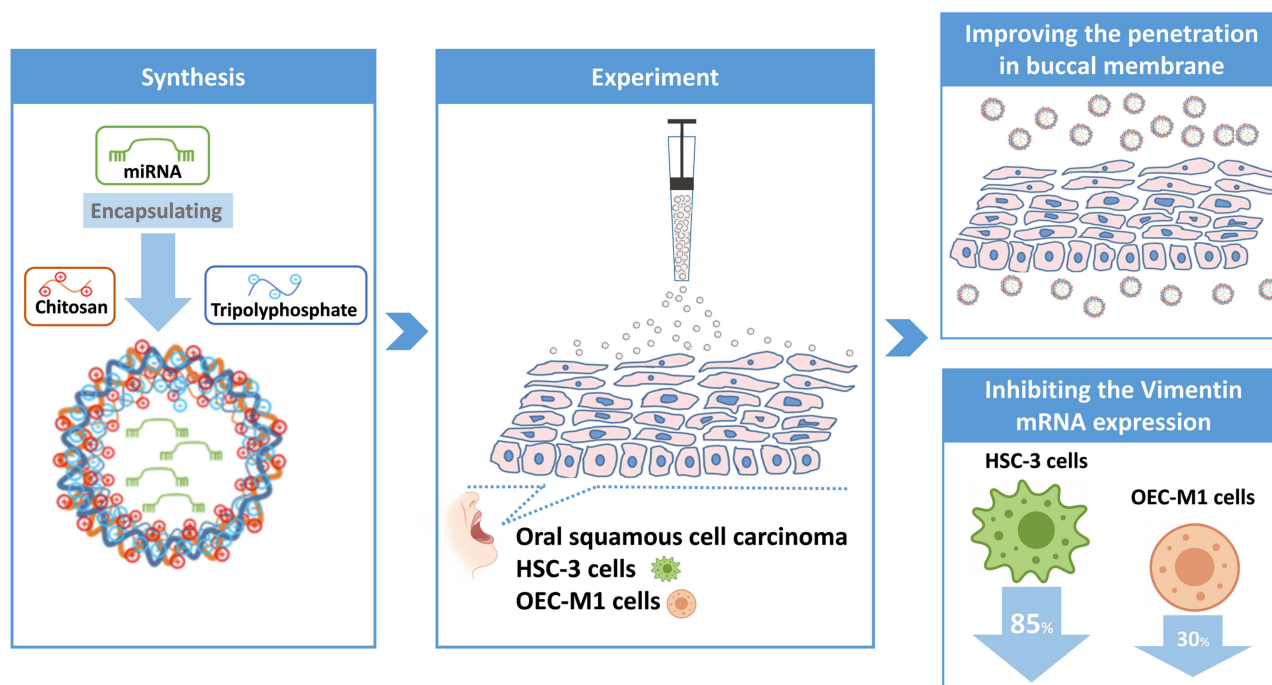
Keywords: oral squamous cell carcinoma, microRNA, transmucosal, buccal delivery, chitosan nanoparticles

Introduction

MicroRNAs (miRNAs) play a critical role in medicine because of their ability to regulate gene expression at the post-transcriptional level. In recent years, miRNAs have emerged as promising biomarkers for cancer diagnosis¹ and therapeutic targets,² enabling personalized medicine by tailoring treatments to individual genetic profiles.

miR-30c-5p (miR-30c), a member of the miR-30 family, has been implicated in various tumors. In gastric cancer, its downregulation correlates with tumor-node-metastasis (TNM) stage and lymphatic metastasis, and it inhibits migration and invasion by targeting metastasis-associated protein 1 (MTA1).³ In ovarian cancer, miR-30c-5p reduces cisplatin resistance and inhibits epithelial-mesenchymal transition (EMT) by targeting DNA methyltransferase-1 (DNMT1).⁴ In

Graphical Abstract



glioma, its upregulation suppresses proliferation and invasion while promoting apoptosis via Bcl-2 downregulation and caspase activation.⁵ Han et al identified miR-30c as a tumor suppressor in invasive micropapillary breast carcinoma, inhibiting proliferation and metastasis by targeting metadherin.⁶ Additionally, circulating miR-30c is a promising breast cancer biomarker with greater sensitivity and specificity than traditional markers such as CA 15-3 and CEA.⁷ Similarly, in non-small cell lung cancer patients treated with tyrosine kinase inhibitors (TKIs), higher miR-30b/c levels were linked to improved prognosis, supporting their potential as biomarkers for TKI response.⁸

Multiple lines of evidence further support the tumor-suppressive role of miR-30c-5p specifically in oral squamous cell carcinoma (OSCC). In patient saliva samples, miR-30c-5p levels were significantly reduced, with strong diagnostic performance (AUC = 0.82, sensitivity = 86%, specificity = 74%).⁹ Bioinformatic analyses revealed that its predicted targets are enriched in oncogenic pathways such as p53 and Wnt, and higher expression of these targets is associated with poorer overall survival in OSCC patients. In addition, miR-30c-5p was consistently downregulated in OSCC tissues, Head and neck squamous cell carcinoma (HNSCC) cell lines, and tobacco-induced tongue cancer models.¹⁰ Functional studies further demonstrated that re-expression of miR-30-5p family members in head and neck cancer cells led to significant reductions in oncogenes involved in proliferation (eg, EGFR, MET, IGF1R) and migration (eg, ITGA6, SERPINE1), suggesting that miR-30c-5p regulates multiple malignant phenotypes in OSCC.¹⁰ Given its involvement in multiple cancers, miR-30c-5p plays a critical regulatory role in multiple cancers with significant potential both as a diagnostic biomarker and as a therapeutic target, offering opportunities for early detection and personalized treatment in various malignancies, including OSCC.

Generally, the length of miRNA is approximately 19–25 nucleotides, and it is presented as single-stranded RNA. Due to their nucleotide composition and multiple phosphate groups, miRNAs exhibit high polarity and hydrophilicity. Nevertheless, these characteristics limit their passive diffusion across cellular membranes.¹¹ Moreover, the delivery of miRNAs has several limitations. miRNAs are highly susceptible to degradation under ambient conditions, particularly due to RNase activity. As a result of the negative charges, miRNAs are poorly captured by the cell membrane by passive

diffusion.¹² Moreover, off-target effects, short half-life in the blood circulation, and poor stability in blood due to nucleases action interfere with the efficacy of miRNA.¹³

Previous studies have attempted different various delivery strategies to overcome these problems, including viral systems or nonviral systems. Several viral vectors have been used to deliver miRNA, including using adenovirus (Ad),¹⁴ adeno-associated virus (AAV),¹⁵ helper-dependent adenovirus (HD Ad),¹⁶ self-complementary adeno-associated virus (scAAV),¹⁷ and human immunodeficiency virus (HIV).¹⁸ These viral vectors exhibit high gene transduction efficiency and targeted delivery; however, concerns regarding immunogenicity and biosafety limit their clinical application. In contrast, nonviral delivery systems each face some limitations, including lipid-based carriers have low transfection efficiency and are hard to scale up;¹⁹ polymeric nanoparticles pose biodegradability and toxicity concerns;²⁰ inorganic vectors offer rapid uptake but poor biocompatibility,²¹ and exosomes, though tissue-specific, are costly to produce.¹³ These drawbacks limit the clinical translation of miRNA therapies.

The negatively charged nature and large molecular size of miR-30c-5p (23 base pairs), combined with its susceptibility to rapid degradation, present significant challenges for its delivery to the buccal system. To address these issues, we selected chitosan (CS) as the material for nanoparticle formulation.²² CS is a natural polysaccharide composed of repeated glucosamine and N-acetyl-glucosamine units. Its cationic nature allows for efficient and rapid complex formation with negatively charged miRNAs,²³ enhancing the incorporation efficiency of the miR-30c-5p payload. Additionally, the hydroxyl and amino groups on CS enable chemical conjugation with specific ligands, making it suitable for targeted therapy.²⁴ Furthermore, CS material has the ability to open intercellular tight junctions, thereby enhancing the intracellular transport of miRNAs and other therapeutic payloads.²⁵ In addition, its mucoadhesive properties make CS an attractive material for the formulation of various nanoparticles, particularly for oral drug delivery applications.²⁶ Previous studies have successfully utilized trimethyl CS and chitosan–hyaluronic acid nanoparticles as carriers for delivering miR-126 and miR-21 in cardiovascular and osteogenesis applications.^{27,28} Santos-Carballal et al also utilized chitosan nanoparticles (CS-NPs) to deliver miR-145 for breast cancer (MCF-7) treatment.²⁹

This study aimed to develop self-assembled CS-NPs as a delivery system to localize and target miR-30c-5p to oral cavity cancers. To assess the efficacy of intracellular delivery, *in vitro* cellular uptake and permeation studies were conducted to elucidate the behavior of nanoparticle delivery.

Materials and Methods

Materials

CS (620 kDa, degree of deacetylation 90%, viscosity 115 cps) and sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium phosphate dibasic (Na₂HPO₄) and citric acid were obtained from Merck (Darmstadt, Germany). All other chemicals were analytical grade and were obtained from Mallinckrodt (Staines-upon-Thames, UK).

Quantification of miR-30c-5p

All synthetic miRNAs used in this study, including the miR-30c-5p mimic and the fluorescently labeled control miRNA (miRNA-FAM), were custom-synthesized and HPLC-purified by GenePharma Co., Ltd. (Shanghai, China). According to the manufacturer, the 6-carboxyfluorescein (FAM) fluorophore was pre-attached to the 5' position of a modified nucleic acid monomer, which was then used during the synthesis of the miRNA sequence. Quantification of the FAM-labeled miRNAs was conducted using a VICTOR™ X2 multilabel microplate reader (PerkinElmer, USA) with excitation at 494 nm and emission at 519 nm. The calibration curve exhibited good linearity ($r=0.97$). Both the nanoparticle-encapsulated group and the free miRNA-FAM control group were prepared using the same FAM-labeled miRNA to ensure consistent fluorescence detection and reliable comparative analysis.

Preparation of miRNA-Loaded CS-NPs

Briefly, 0.2% CS was dissolved in 0.4% acetic acid; and 10 N NaOH was used to adjust to the pH 5. For the preparation of the 0.2% TPP solution stock solution, 0.1 N HCl was used to adjust to the pH 9.2. The TPP solution was added to the CS solution using a syringe pump at a controlled flow rate of 1 mL/min under continuous stirring at 850 rpm for 15 min at

room temperature to form a colloidal CS-NPs suspension. Subsequently, miR-30c-5p was added to the freshly prepared CS-NPs and gently stirred at 850 rpm for an additional 15 min to facilitate complex formation. All miRNA-loaded CS-NPs (CS-miRNA NPs), including those loaded with FAM-labeled miRNA (CS-miRNA-FAM NPs), were freshly prepared prior to each analysis.

Size Distribution and Zeta Potential of miRNA-FAM Loaded CS-NPs

The particle size (d), polydispersity index (PDI), and zeta potential of CS-NPs were obtained using dynamic light scattering (DLS) (ELSZ-2000, Otsuka Electronic, Hirakata, Japan). All nanoparticles were measured directly rather than by dilution.

Encapsulation Efficiency of miRNA-FAM Loaded CS-NPs

The encapsulation efficiency was achieved using an ultracentrifugation method. miR-30c-5p-FAM-loaded CS-NPs were separated by centrifugation at 48,000×g for 1 h at 4°C (CS150 GXL, Hitachi, Japan). The supernatant and pellet were used to determine the concentration of miRNA in unencapsulated and encapsulation fractions, respectively. The FAM-labeled miRNA in the supernatant and pellet fractions was quantified using a VICTOR™ X2 multimode microplate reader (PerkinElmer, USA). The encapsulation efficiency of miRNA-FAM-loaded CS-NPs was calculated using the following equation:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Concentration of miRNA}_{(\text{pellet})}}{\text{Concentration of miRNA}_{(\text{total})}}$$

Gel Electrophoresis Retardation Assay

We used the gel electrophoresis retarding assay to evaluate interactions with miRNA and CS. A total of 33 ng of miR-30c-5p-FAM-loaded CS-NPs (CS-miR-30c-5p-FAM NPs) mixed with 5 µL Healthview™ Nucleic Acid Stain were loaded in each well, which contained 1.2% agarose gel in Tris-acetate-EDTA (TAE) buffer. The gel was electrophoresed at a voltage of 80 V for 15 min using TAE as the running buffer. The same amount of free miR-30c-5p-FAM solution was loaded as a control group. The results were visualized under a UV illuminator (Bio-Rad Laboratories, CA, USA).

Morphology Analysis Using Transmission Electron Microscopy (TEM)

The morphology and structural characteristics of the CS-miR-30c-5p NPs were analyzed using TEM, a high-resolution imaging technique that utilizes electron beams to visualize nanoscale structures operated at an accelerating voltage of 120 kV. A drop of sample was applied to a carbon film-covered copper grid to form a thin film specimen, which was stained with 1% phosphotungstic acid. Negative stain was used for CS-NPs to increase the resolution of the morphology. The sample was then vacuumed for 24 h and photographed by TEM (JEM-2000EXII, TEM JEOL, Japan).

Cell Culture

The human OSCC cell lines HSC-3 and OEC-M1 were used in this study. The HSC-3 (Product Code: SCC193) and OEC-M1 (Product Code: SCC180) cell lines were both obtained from Sigma-Aldrich (Merck KGaA, Germany). HSC-3 cells were cultured in MEM (Gibco, USA), and OEC-M1 cells were cultured in RPMI-1640 medium (Gibco, USA). The medium was supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 1 mM sodium pyruvate (Thermo Fisher Scientific, USA), and 1X penicillin-streptomycin-amphotericin B solution (PSA; Merck Millipore, USA). Cultures were maintained in an atmosphere of 95% air and 5% CO₂ at 37°C.

MTT Assay

HSC-3 and OEC-M1 cells were seeded at 5000 cells per well in 96-well plates and cultured for 24 h. The cells were then treated with free miRNA, CS-NPs, or CS-miRNA NPs at final concentrations of 5%, 10%, 25%, and 50% (v/v), prepared by mixing the appropriate volume of each test sample with culture medium. For the CS-miRNA NPs group, the estimated final concentrations of encapsulated miRNA delivered to the cells were approximately 10.84, 21.68, 54.2, and 108.4 nM,

corresponding to 0.0823, 0.1646, 0.4115, and 0.8230 $\mu\text{g/mL}$ at 5%, 10%, 25%, and 50% (v/v), respectively. These values were calculated based on an initial miRNA input of 500 nM and an encapsulation efficiency of 86.68%. After 24 or 48 h of incubation at 37°C, MTT solution (0.5 mg/mL) was added and incubated for 3 h. The solution was removed and the formazan crystals were dissolved in 100 μL of DMSO per well. The absorbance at 590 nm was measured using a Benchmark Plus microplate reader (BIO-RAD, USA). Cell viability was calculated relative to the 0% (v/v) control group, which received no treatment. All experiments were performed in triplicate with four replicate wells for each sample and control.

Assessment of the Internalization of CS-Based Nanoparticles in OSCC Cell Monolayers by Confocal Laser Scanning Microscopy

HSC-3 and OEC-M1 cells were seeded into 8-well cell culture slides at a density of 1×10^4 cells per well. Cells were treated with 20% CS-NPs, either unloaded (CS-NPs) or loaded with control miRNA labeled with FAM (CS-miRNA-FAM NPs). This concentration was selected based on cytotoxicity data, which indicated that 25% CS-NPs slightly reduced the viability of OEC-M1 cells after 48 hours. Following a 48-h treatment period, the cells were subjected to confocal laser scanning microscopy (CLSM) and imaged using a Zeiss LSM700 confocal microscope (Carl Zeiss, Germany). Four groups were analyzed: (1) negative control (no miRNA or nanoparticles), (2) CS-miRNA-FAM NPs, (3) free miRNA-FAM, and (4) CS-NPs. To visualize the cytoplasm, GAPDH was immunostained using a primary antibody (Cell Signaling Technology, USA) and an Alexa Fluor 594-conjugated secondary antibody (Jackson ImmunoResearch, USA), producing red fluorescence. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole) (blue). Colocalization of the green FAM signal with red GAPDH staining was used to confirm that the delivered miRNA localized within the cytoplasm rather than remaining on the cell surface. All samples were imaged under identical acquisition settings within the same session, including laser power, detector gain, exposure time, and z-stack parameters, to ensure consistency across treatment groups. Semi-quantitative analysis of intracellular fluorescence intensity was performed using ImageJ software (version 1.53t, National Institutes of Health, USA).

Establishment of an in vitro Buccal Epithelium Model

TR146 cells, a human buccal carcinoma cell line, were utilized to create a multilayer structure that represented the buccal epithelium. The cells were seeded on permeable polyethylene terephthalate (PET) inserts with a pore size of 0.45 μm and an area of 0.33 cm^2 , at a specified density of 26,400 cells/ cm^2 (JET BIOFIL, China). The cells were then cultured in DMEM (Gibco, USA) supplemented with 200 μM glutamine, 1x PSA, 10% FBS, 1x non-essential amino acids (NEAA; Gibco, USA), and 1 mM sodium pyruvate. The cell culture medium was replenished every three days. The integrity of the TR146 cell layers was assessed by measuring transepithelial electrical resistance (TEER). Electrical resistance (Ω) across the cell layers was measured every three days using the Millicell[®] ERS-2 voltohmmeter with chopstick electrodes (Merck KGaA, Germany). TEER values ($\Omega \cdot \text{cm}^2$) were calculated using the following formula:

$$\text{TEER} = (R_{(\text{insert with TR146})} - R_{(\text{insert without TR146})}) \times \text{area}$$

Where $R_{(\text{insert with TR146})}$ is the resistance measured across the insert containing TR146 cells and $R_{(\text{insert without TR146})}$ is the resistance of cell-free inserts incubated for 3 days in culture medium. This TEER measurement was used to assess the integrity of the cell layers, with higher values indicating a more intact barrier. TR146 cells were cultured for 25 days based on TEER monitoring, which showed peak barrier integrity at day 22 and stabilization by day 25. This duration aligns with previous studies,^{30,31} where TR146 cells are typically cultured for around 3 to 4 weeks to establish a functional epithelial barrier. Slight variations in timing are expected due to differences in experimental conditions.

Permeation of CS-miRNA-FAM NPs Across TR146 Cell Layers

TR146 cells were seeded on permeable supports and cultured in DMEM. On day 25, CS-NPs, loaded with or without miRNA-FAM, were applied to the top of each permeable support, alongside TR146 cells or in their absence. After 16, 20,

24, and 48 h of culture, the medium from the underside of the permeable supports was collected, and fluorescence signals were recorded. The penetration ratio was calculated using the formula:

$$\text{Penetration ratio} = \frac{S_{+TR146+CS-miRNA-FAM\ NPs} - S_{medium} - S_{CS-NPs}}{S_{-TR146+CS-miRNA-FAM\ NPs} - S_{medium} - S_{CS-NPs}}$$

This calculation involved subtracting signals from samples containing only the culture medium or CS-NPs. The control groups consisted of signals from the bottom of permeable supports lacking cells but containing CS-miRNA-FAM NPs. Subsequently, all signals were normalized to that of the control groups. By normalizing to the control group, the results indicated the relative efficiency of the CS-NPs in crossing the TR146 cell barrier.

Transmucosal ex vivo Permeations

The miR-30c-5p-FAM signals were scanned at different depths of the buccal membrane using the CLSM technique. The excised porcine buccal membrane was placed on the microscope slide, oriented with the surface epithelium side facing the cover glass. The Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) was used with an argon laser beam at an excitation wavelength of 488 nm and an emission wavelength of 543 nm. CLSM was used to slice the buccal membrane into sections 20 µm thick along the z-axis. Using CLSM with Leica Confocal Software version 2.61 (Leica Microsystems, Wetzlar, Germany), the intensity of miR-30c-5p-FAM versus the permeable depths were measured. All samples were imaged under identical acquisition settings within the same session across all treatment groups, ensuring consistent fluorescence intensity and depth measurements. The intensity of the CLSM images was also quantified with Leica Confocal Software. The data are shown as arbitrary units.

RNA Extraction and Quantitative Real-Time PCR (QPCR)

Total RNA was extracted from each sample using the NucleoSpin RNA kit (MACHEREY-NAGEL, Germany) and reverse transcribed to first-strand cDNA according to the manufacturer's instructions (Applied Biosystems, USA). For QPCR, 20 ng of cDNA was combined with KAPA SYBR Fast QPCR Master Mix (Sigma-Aldrich, USA) and primers specific to Vimentin and GAPDH. Primer sequences were designed using the Universal ProbeLibrary Assay Design Center (Roche Applied Science, Germany). The thermal cycling protocol included initial denaturation at 95°C for 3 min, followed by 50 cycles of denaturation at 95°C for 3 s and annealing/extension at 60°C for 30s.

Western Blot Analysis

OSCC cells were treated with 20% CS-miR-30c-5p NPs or CS-mimic NC NPs (CS-NPs loaded with non-targeting negative control miRNA mimic) and subsequently lysed using RIPA buffer (Cell Signaling Technology, MA, USA) with 1% protease inhibitor. Lysates were placed on ice for 5 min and sonicated for 20s. Equal amounts of proteins were separated by SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% BSA, the membranes were incubated overnight at 4°C with primary antibodies against vimentin or β-actin (Cell Signaling Technology, USA), followed by a 1-h incubation at room temperature with HRP-conjugated secondary antibodies. Protein bands were visualized using the ECL Detection System (GE Healthcare, UK), with β-actin as the loading control.

Statistical Analysis

All experimental data were repeated at least three times and presented as mean±standard deviation (SD). Statistical analyses of control and treatment data were performed using Student's *t*-test and analysis of variance (ANOVA). All tests were 2-tailed and differences were statistically significant at *P*<0.05.

Results

Characterization of miR-30c-5p-FAM Loaded CS-NPs Delivery System

Schematic representation of the synthesis process for miRNA-loaded CS-NPs by ionic gelation method (Figure 1A). The resulting nanoparticles provide protection against degradation and enhance cellular uptake, facilitating effective delivery for therapeutic applications. Table 1 shows the physicochemical properties of empty CS-NPs and miR-30c-5p-FAM-

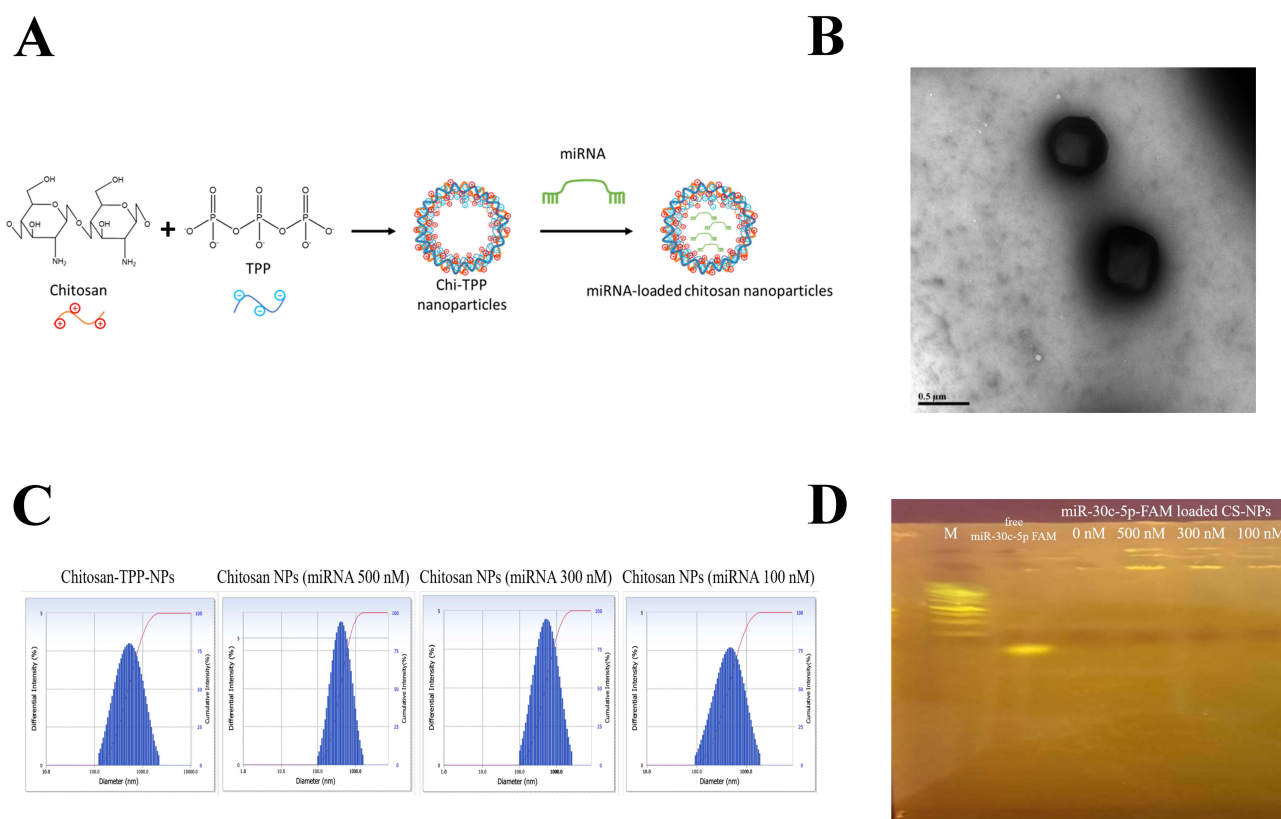


Figure 1 Preparation and characterization of miR-30c-5p-loaded CS-NPs. **(A)** Schematic representation of the encapsulation process of miR-30c-5p within CS-NPs. **(B)** Representative TEM image showing the morphology of the miR-30c-5p-loaded CS-NPs. **(C)** Particle size distribution of CS-NPs encapsulating various concentrations of miRNA. **(D)** Gel electrophoresis retardation assay of miR-30c-5p-FAM encapsulated in CS-NPs. Agarose gel electrophoresis demonstrating the retardation effect of CS-NPs on miR-30c-5p-FAM at various concentrations. Lanes are as follows: M (size marker), free miR-30c-5p-FAM (unencapsulated control), and CS-NPs encapsulating 0 nM, 100 nM, 300 nM, and 500 nM of miR-30c-5p-FAM, showing the efficiency of miRNA encapsulation.

loaded CS-NPs. The schematic diagram of miR-30c-5p-FAM-loaded CS-NPs shows the particle size, PDI, zeta potential, and entrapment efficiency. Empty CS-NPs exhibited a particle size of ~536 nm, a strong positive charge (+34.58 mV), and a well-defined PDI (0.28). Loading miR-30c-5p-FAM significantly decreased the size to ~450 nm ($P < 0.05$). The zeta potential was not significantly different with or without miR-30c-5p-FAM ($P > 0.05$). The entrapment efficiency ranged from 79% to 87% at various loading concentrations. TEM imaging of 500 nM miR-30c-5p-FAM-loaded CS-NPs showed predominantly spherical morphology with a size of ~500 nm (Figure 1B). The size distribution of CS-miR-30c-5p-FAM

Table 1 Physicochemical Characteristics of miRNA-Loaded CS-NPs

miRNA Concentration (nM)	Average Size (nm)	PDI	Zeta Potential (mV)	Entrapment Efficiency (%)
Empty NPs	536.43±13.11*	0.28±0.01	34.58±0.33	—
500	434.67±10.07	0.25±0.02	30.36±0.69	86.68±2.56
300	452.80±24.13	0.26±0.01	32.57±0.58	80.30±1.45
100	448.07±16.50	0.26±0.02	32.76±0.25	79.02±1.18

Notes: All values are presented as mean±SD, (n=3). * $P < 0.05$.

Abbreviation: PDI, Polydispersity index.

NPs at different concentrations was well represented (Figure 1C). A gel retardation assay confirmed the encapsulation of miR-30c-5p-FAM in lanes 4, 5, and 6 (Figure 1D).

Cytotoxicity of Free miRNA and CS-Based Nanoparticles in Oral Squamous Cancer Cells

The cytotoxic effects of free miRNA, CS-NPs, and CS-miRNA NPs were evaluated in HSC-3 and OEC-M1 oral cancer cells following treatment with 5%, 10%, 25%, and 50% (v/v) for 24 and 48 hours (Figure 2A). In HSC-3 cells, all treatment conditions exhibited minimal cytotoxicity at concentrations up to 25% (v/v), with cell viability consistently exceeding 93% at both time points ($P > 0.05$). However, at 50% (v/v) for 48 hours, a significant reduction in cell viability was observed for both CS-NPs ($42.72 \pm 4.47\%$, $P < 0.001$) and CS-miRNA NPs ($51.82 \pm 0.55\%$, $P < 0.001$), whereas free miRNA caused only a moderate decrease ($85.55 \pm 1.90\%$, $P < 0.001$). At 48 hours in OEC-M1 cells, cytotoxicity was also evident at 25% (v/v), with viability reduced to $93.32 \pm 1.22\%$ for free miRNA ($P < 0.001$), $87.56 \pm 4.22\%$ for CS-NPs ($P = 0.0176$), and $89.50 \pm 1.15\%$ for CS-miRNA NPs ($P < 0.001$). A more substantial decline was observed at 50% (v/v), with cell viability dropping to $73.98 \pm 2.55\%$, $33.00 \pm 0.97\%$, and $39.89 \pm 1.00\%$ for free miRNA, CS-NPs, and CS-miRNA NPs, respectively ($P < 0.001$ for all).

Collectively, these findings indicate that CS-NPs and CS-miRNA NPs are biocompatible at concentrations up to 25% (v/v), but higher concentrations and prolonged exposure can induce marked cytotoxicity, particularly in the more sensitive OEC-M1 cell line. In comparison, free miRNA alone exhibited limited cytotoxicity, likely due to its poor cellular uptake and rapid degradation in the absence of nanoparticle protection.

Comparative Cellular Uptake of CS-miRNA-FAM NPs and Controls Observed by CLSM

To assess intracellular uptake efficiency, CLSM imaging combined with ImageJ-based semi-quantitative colocalization analysis was performed (Figure 2B). The negative control group (cells stained with DAPI and GAPDH only) showed minimal colocalized green and red fluorescence, with mean pixel counts (FAM–GAPDH colocalization) of 4.67 ± 4.51 in HSC-3 cells and 1.00 ± 1.73 in OEC-M1 cells, and was used as the background reference for signal quantification. In HSC-3 cells, the free miRNA-FAM group showed similarly low colocalized signal (1.33 ± 1.53), indicating poor cytoplasmic uptake of naked miRNA. The CS-NPs group, which lacked FAM-labeled miRNA, exhibited a slightly higher signal (31.00 ± 20.66), likely attributable to the intrinsic autofluorescence of chitosan. Although the weak green fluorescence likely resulted from chitosan autofluorescence rather than FAM labeling, its cytoplasmic localization suggests limited nanoparticle uptake. In contrast, the CS-miRNA-FAM NPs group demonstrated a dramatic increase in colocalized fluorescence ($28,816.67 \pm 18,941.00$), significantly higher than the negative control ($P = 0.0289$), free miRNA-FAM ($P = 0.0289$), and CS-NPs ($P = 0.0290$), confirming effective cytoplasmic delivery.

In OEC-M1 cells, a similar trend was observed. The negative control, free miRNA-FAM, and CS-NPs groups showed minimal colocalization signals (1.00 ± 1.73 , 5.67 ± 3.79 , and 4.00 ± 6.08 , respectively), whereas the CS-miRNA-FAM NPs group exhibited a marked increase ($30,606.33 \pm 15,075.04$), with statistically significant differences compared to each of the other groups ($P = 0.0123$ vs all).

Penetration of CS-miRNA-FAM NPs Through the TR146 Oral Mucosal Barrier Model

Filter-grown TR146 cells were incubated in specified media, and TEER values were monitored over 25 days. Figure 3A shows TEER values rising initially before plateauing, indicating the formation of a stable barrier. The permeation dynamics of CS-miRNA-FAM NPs across TR146 layers were further analyzed. An increased penetration ratio at earlier time points suggested efficient permeation between 16 and 24 h (Figure 3B). At 48 h, the penetration ratio plateaued, indicating a saturation threshold for CS-miRNA-FAM NPs translocation or an adaptive cellular response. Figure 3C shows the distribution of CS-miRNA-FAM NPs through the TR146 model. Magnified views of each layer in the bottom row reveal a nanoparticle gradient from the fifth to the first layer, highlighting the functional barrier properties of the

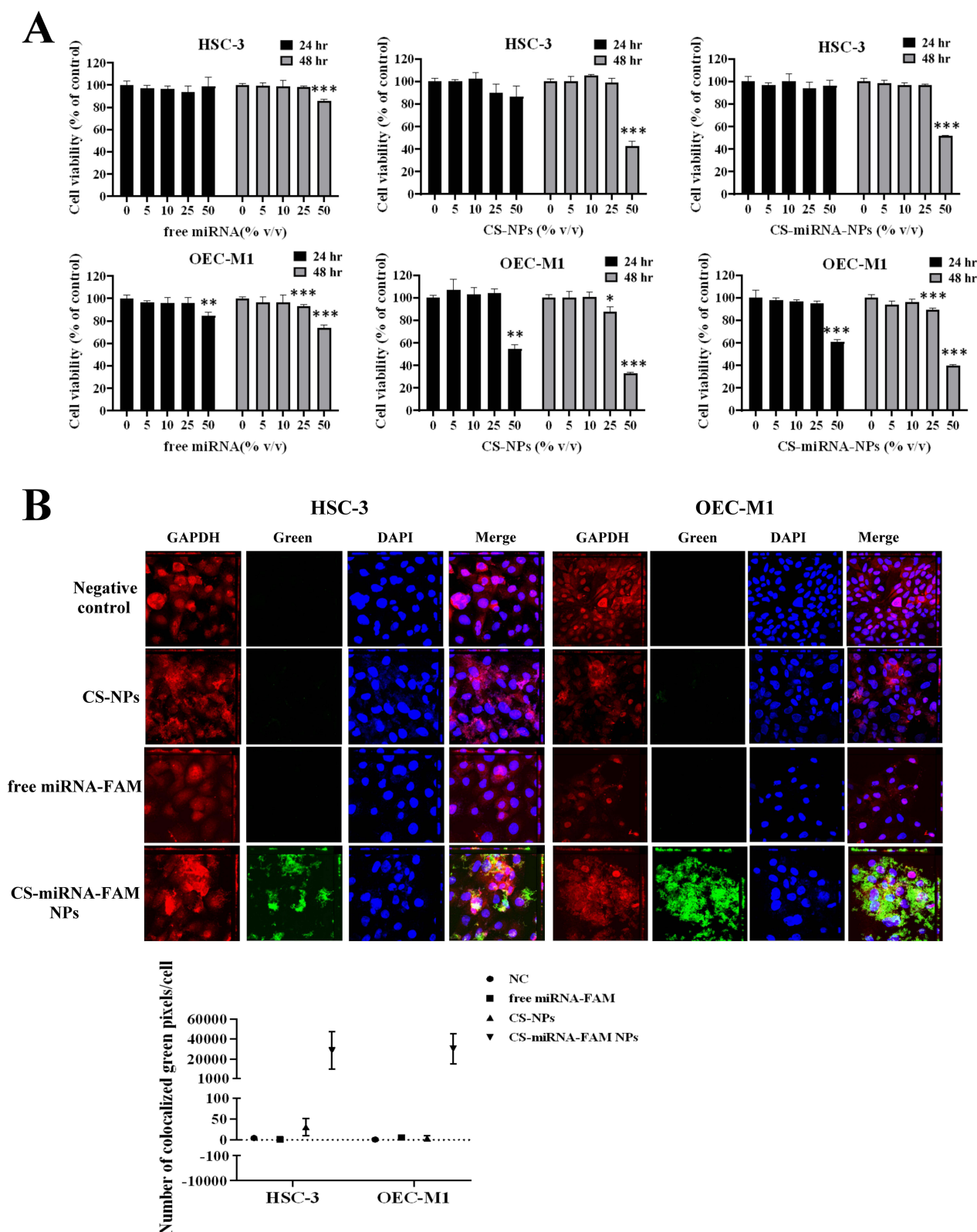


Figure 2 Cytotoxicity and cellular uptake of chitosan-based miRNA NPs in oral squamous carcinoma cells. **(A)** HSC-3 and OEC-M1 cells were treated with free miRNA, CS-NPs, or CS-miRNA-NPs at final concentrations of 0% (control), 5%, 10%, 25%, and 50% (v/v) for 24 or 48 hours. The y-axis represents the percentage of cell viability relative to the untreated control group. The error bars indicate the standard deviation of four replicate wells from three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **(B)** CLSM images showing the intracellular uptake of free miRNA-FAM, CS-NPs, and CS-miRNA-FAM NPs in HSC-3 and OEC-M1 cells after 48 hours of treatment. The negative control group consisted of untreated cells. Cell nuclei were stained with DAPI (blue), and the cytoplasmic region was labeled using GAPDH immunofluorescence (red). Green fluorescence indicates FAM-labeled miRNA. Quantification was performed using ImageJ to count the number of green pixels colocalized with red fluorescence per cell. Bar graphs represent the mean $\pm$ SD from three representative CLSM images per treatment group. $P < 0.05$.

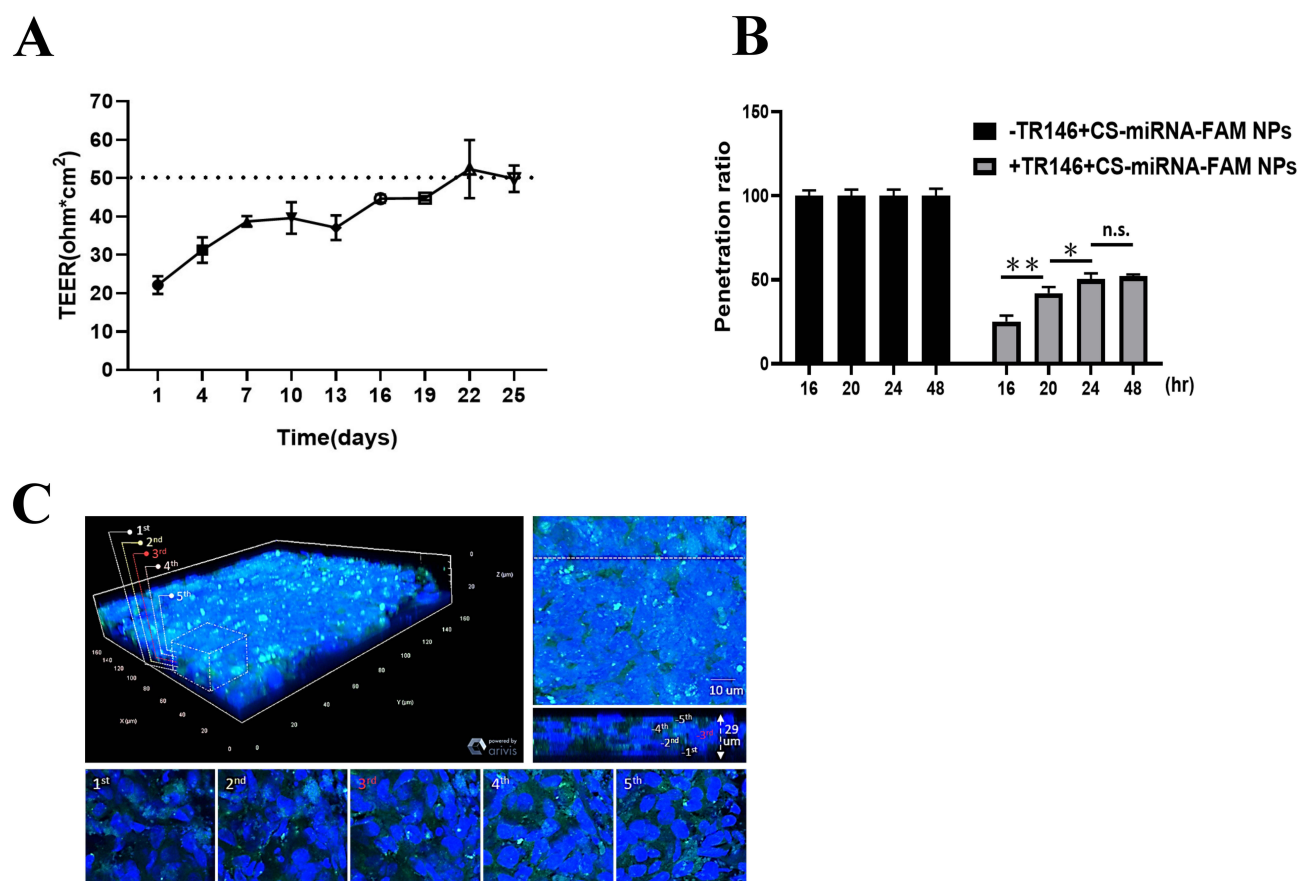


Figure 3 Characterization of TR146 cell multilayers as a model for buccal epithelium and the penetration of CS-miRNA NPs. **(A)** TEER values of TR146 cell multilayers over a period of 25 days. **(B)** The penetration ratio of fluorescently labeled CS-miRNA NPs into and through TR146 cell multilayers over time, with and without the presence of TR146 cells. * $P < 0.05$; ** $P < 0.01$; n.s., no significance. **(C)** Visualization of the penetration of CS-miRNA-FAM NPs into TR146 cells and across the multilayer barrier. On day 25 of culture, the cell layers were exposed to CS-miRNA-FAM NPs for 48 h and then analyzed by CLSM. The top left image showed a three-dimensional reconstruction of the multilayered cell model with CS-miRNA-FAM NPs distributed across different layers (1 to 5). The adjacent images displayed corresponding two-dimensional sections at increasing depths, with the green fluorescence indicating the presence of CS-miRNA-FAM NPs, and blue fluorescence (DAPI) highlighting cell nuclei.

multilayered structure. These findings show that CS-miRNA-FAM NPs traverse the TR146 barrier rather than adhering, underscoring their potential to deliver therapeutic cargo in nanoparticle-mediated systems.

Ex vivo Penetration Behaviors

To investigate the penetration behavior of miR-30c-5p-FAM, fluorescence microscopy and CLSM were used to assess penetration depth. miR-30c-5p-FAM localized within the porcine buccal membrane after delivery via the CS-NPs system (Figure 4A and B). To determine precise localization, xyz optical scanning analyzed the relationship between fluorescence intensity and penetration depth determined using CLSM (Figure 5). Free miR-30c-5p-FAM (as a negative control) also showed negligible fluorescence, due to rapid degradation or poor penetration, indicating limited stability and permeability. In contrast, CS-miR-30c-5p-FAM NPs showed strong fluorescence, confirming significant penetration and effective delivery into the submucosal layers.

Figure 5A shows the optical scans obtained at 20 μm increments across ten sections of the porcine buccal membrane, generating a cumulative xyz image. Figure 5B profiles depth versus fluorescence intensity. CS-miR-30c-5p-FAM NPs increased localization 5.42-fold compared with the control, with maximum penetration depths reaching 80 μm and extending up to 160 μm .

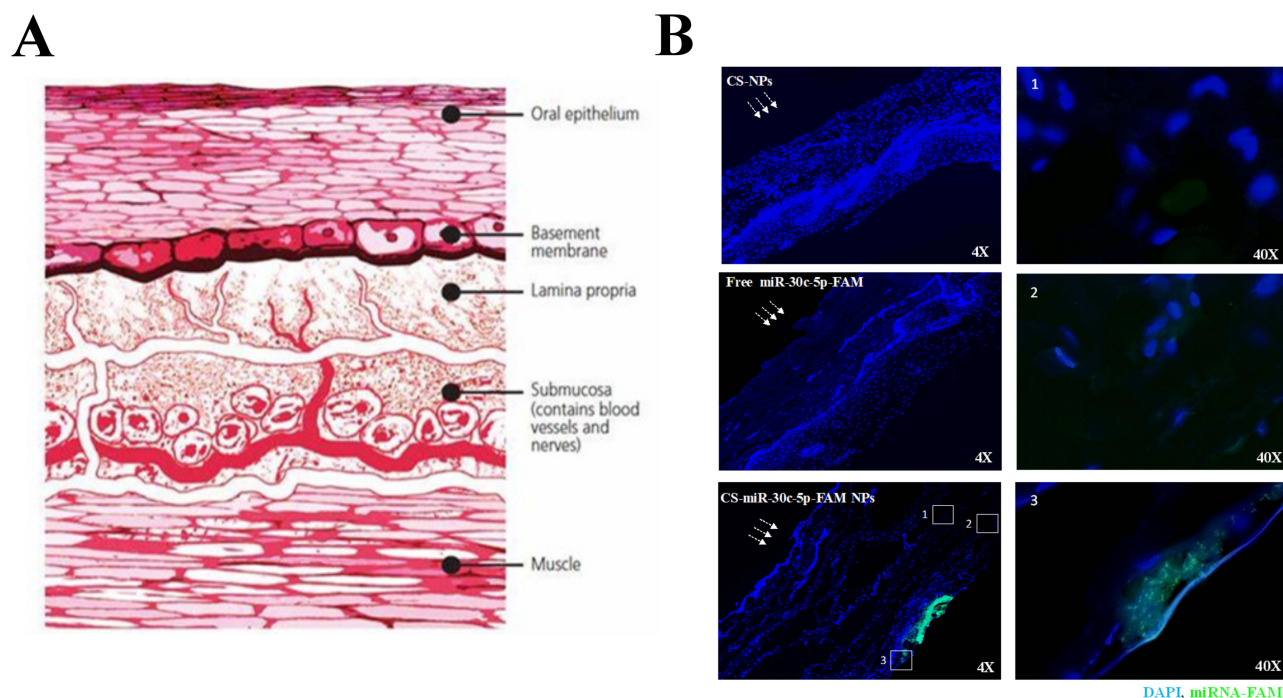


Figure 4 Histological and fluorescence analysis of miRNA-FAM nanoparticle penetration in porcine buccal membrane. **(A)** Schematic illustration of a histological cross-section of the porcine buccal mucosa stained to show the structural layers: oral epithelium, basement membrane, lamina propria, submucosa (containing blood vessels and nerves), and muscle layer. **(B)** Fluorescence microscopy images at 4x magnification showing the distribution of CS-miR-30c-5p-FAM NPs within the buccal membrane. The upper image in the left panel shows the CS-NPs treatment. The middle image indicates tissue treated with free miR-30c-5p-FAM only. The lower image demonstrates tissue treated with miR-30c-5p-FAM loaded nanoparticles. Highlighted regions (1, 2, 3) on the right panel provide close-up views, showing detailed localization of CS-miR-30c-5p-FAM NPs within the cells. Arrows in all images point to areas of CS-miR-30c-5p-FAM NPs delivery.

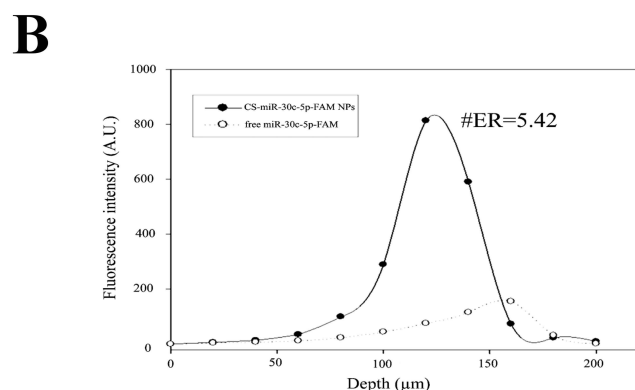
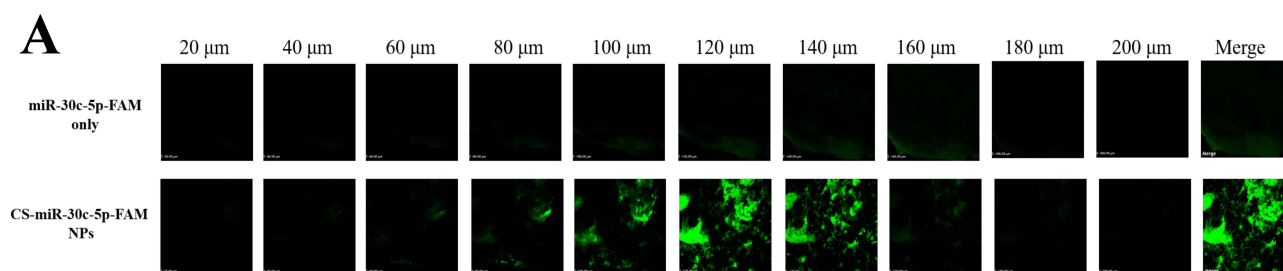


Figure 5 CLSM images of miR-30c-5p-FAM alone and CS-miR-30c-5p-FAM NPs in porcine buccal membrane after 24 h in situ buccal delivery. **(A)** Sequential xyz plane scans of porcine buccal membrane treated with aqueous miR-30c-5p-FAM solution and CS-miR-30c-5p-FAM NPs, with the full membrane thickness divided into 10 sections from the top surface (left to right). **(B)** Cumulative xyz images of all optical sections were merged across a 0–200 μm xyz scan. Fluorescence intensity profiles relative to buccal membrane depth were quantified from CLSM micrographs for both the free miR-30c-5p-FAM and CS-miR-30c-5p-FAM NPs groups. The enhancement ratio (#ER) was determined as the ratio of the integrated fluorescence intensity of CS-miR-30c-5p-FAM NPs to that of miR-30c-5p-FAM alone.

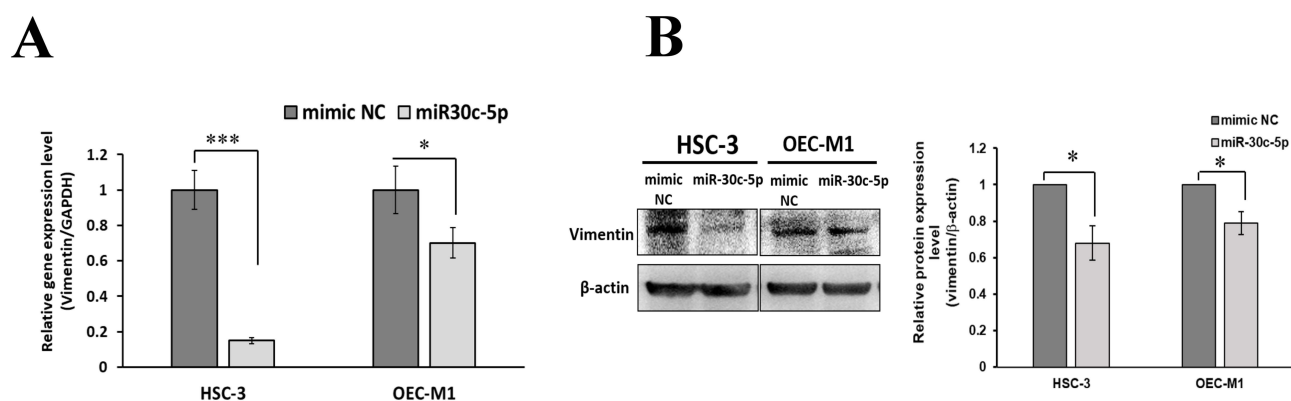


Figure 6 Impact of CS-miR30c-5p NPs-mediated transfection on vimentin expression. **(A)** The bar graph illustrates the relative mRNA expression of vimentin in HSC-3 and OEC-M1 cell lines following transfection with CS-NPs encapsulating either mimic NC or miR-30c-5p. **(B)** Western blot analysis showing vimentin protein levels with β -actin as the loading control. Both mRNA and protein levels are normalized to the mimic NC group, set at 1. Data are presented as mean $\pm$ SD from three independent biological replicates. * P <0.05; *** P <0.001.

Functional Release of miR30c-5p from CS-NPs Downregulated Vimentin Expression

This experiment aimed to validate that CS-NPs encapsulated miR-30c-5p and released it into cells after uptake, to allow its biological functions. Vimentin was selected as a downstream target based on miRDB prediction analysis (<https://mirdb.org/>), which identified it as a putative target gene of miR-30c-5p with a high confidence score. Additionally, as a central marker of EMT, vimentin plays a critical role in tumor invasion and progression in OSCC.³² Quantitative analysis showed that transfection with CS-miR-30c-5p NPs significantly reduced vimentin mRNA levels in HSC-3 cells by 85% (P <0.001) compared to the CS-mimic negative control (mimic NC, Figure 6A). In OEC-M1 cells, vimentin expression decreased by approximately 30% (P <0.05). Western blot analysis confirmed this downregulation at the protein level, with densitometry supporting reduced Vimentin expression after CS-miR-30c-5p NPs treatment (Figure 6B). These results demonstrate that CS-NPs effectively deliver and release miR-30c-5p into cells, enabling it to regulate target gene expression.

Discussion

The present study rigorously evaluated the potential of CS-NPs as an effective delivery system for miR-30c-5p, aimed at the therapeutic management of oral cancer. CS, derived from chitin, served as the biopolymer foundation for these nanoparticles. The ionic gelation method was employed for the synthesis of CS-NPs, utilizing CS and TPP to induce complexation.²² This method is pivotal as it directly influences the nanoparticle's physicochemical characteristics such as particle size, stability, and surface charge.³³

The preparation of drug-loaded CS-NPs derivatives is generally achieved by two main techniques: nanoencapsulation and chemical modification.²² Nanoencapsulation relates to the formation of a nanostructure that contains absorbed drug at the surface or within the nanoparticle. We hypothesized that miRNA was electrostatically encapsulated within the CS-NPs matrix via ionic gelation, forming a stable complex during nanoparticle formation, as confirmed by gel retardation assay. The phenomenon is similar to that observed in several studies.²³

The pH of the CS solution critically influences the formation and size of the nanoparticles. When the pH of the CS solution was 5, this generated CS-NPs with a smaller diameter. Protonation refers to the addition of protons (H^+) to a molecule, which can influence its overall charge and behavior.²⁴ The addition of TPP for cross-linking with CS leads to the formation of denser nanoparticles, as evidenced by TEM morphology. However, increased protonation of amine groups due to the addition of TPP can disrupt the ionic bonds between CS and TPP, potentially causing agglomeration or clustering of nanoparticles.³⁴ Therefore, controlling the pH of the CS solution and the amount of TPP added is crucial during nanoparticle preparation to ensure stability and uniformity of the particles.

The therapeutic application of miRNAs offers unique benefits compared to traditional gene therapy methods that utilize larger molecules such as mRNAs or DNA. First, miRNAs target a wider range of biological processes because a single miRNA can regulate numerous mRNAs simultaneously. Additionally, therapeutic strategies using miRNA

inhibitors and miRNA mimics can both enhance and suppress the activity of different genes.^{35,36} This contrasts with siRNAs and mRNAs, which are typically limited to either suppressing or promoting the activity of a single gene, respectively.

Moreover, the small size of miRNAs facilitates their efficient encapsulation into nanoparticles, enhances their cellular delivery, and supports their function at the cytoplasmic level.¹³ Indeed, our findings underscore that CS-NPs can be adeptly loaded with miR-30c-5p, achieving high entrapment efficiencies ranging from 79% to 87%. Notably, this loading significantly reduces the nanoparticle size to approximately 450 nm, which is a critical factor in enhancing cellular uptake, as evidenced by the pronounced internalization and localization of miR-30c-5p observed in CLSM images.

It is generally accepted that nanoparticles smaller than 200 nm are predominantly internalized through receptor-mediated endocytosis pathways, such as clathrin-mediated endocytosis (CME) and caveolae-mediated endocytosis (CvME).³⁷ These pathways are widely active in epithelial cells and involve membrane-coated vesicles typically 50–100 nm in diameter. CME is well-characterized and facilitates uptake of particles around 100 nm or less, while CvME accommodates smaller vesicles (~50–80 nm) associated with lipid rafts. Due to these vesicle size limitations, nanoparticles under 200 nm are often considered optimal for efficient and regulated uptake.

Nonetheless, multiple studies have demonstrated that particles ≥ 200 nm can also be effectively internalized in oral epithelial tissues. For instance, 200 nm polystyrene nanoparticles penetrated more deeply than 25 or 50 nm particles in porcine buccal mucosa,³⁸ and 210 nm nanoparticles delivered via mucoadhesive films reached the spinous and basal layers of human buccal mucosa.³⁹ Roblegg et al reported that 200 nm cationic particles reached submucosal tissue in porcine mucosa, while smaller 20 nm anionic particles remained superficial.⁴⁰ Similarly, in Caco-2 intestinal epithelial cells, CS-NPs exhibited enhanced uptake within the 200–600 nm range, with reduced internalization at 800 nm.⁴¹ Collectively, these findings suggest that our CS-NPs (~450 nm, positively charged) fall within a size range suitable for endocytic uptake and mucosal transport. Since effective intracellular delivery also requires successful miRNA loading, a gel retardation assay was conducted. The results showed that miR-30c-5p-FAM was fully retained by the nanoparticles, indicating strong electrostatic binding between chitosan and the miRNA for stable delivery.

The buccal barrier presents unique characteristics that significantly influence the permeability of substances through the buccal mucosa. The TR146 cell line, derived from human buccal carcinoma, is especially useful for the *in vitro* testing of nanoparticles in drug delivery applications.^{30,42} Its human origin makes it relevant for clinical applications, as it effectively mimics the structure and function of the oral mucosal barrier. TR146 cells are capable of forming tight junctions, enabling evaluation of mucosal permeability and nanoparticle transport. Although their carcinoma origin may cause variability in tight junction protein expression compared to normal mucosa, prior studies have demonstrated the expression of key markers such as claudin-1, claudin-4, and ZO-1, supporting their use in barrier integrity assessments.^{42,43} In this study, barrier formation was evaluated by TEER, a standard and non-invasive method for assessing monolayer integrity. The gradual increase and plateau in TEER values indicated the development of a cohesive epithelial barrier. However, as TEER values may not fully reflect the structural organization of tight junctions in TR146 cells,⁴⁴ complementary methods such as immunofluorescence staining or paracellular flux assays should be considered in future studies for more comprehensive evaluation.^{31,45} Despite these considerations, the TR146 model still offers consistent and reproducible conditions for experimental studies, which are essential for investigating different nanoparticle designs and their biological effects.

In addition to the TR146 cell line, we utilized porcine buccal mucosa for our *ex vivo* barrier model, chosen specifically because, unlike most rodent models, it does not possess a keratinized layer,^{30,46} which makes it more analogous to human buccal mucosa and aligns better with our study's objectives. Our findings also revealed that the zeta potential of CS-NPs remained stable irrespective of miRNA encapsulation, indicating a robust structural integrity crucial for navigating through biological barriers. This stability proved vital in our penetration studies using both the TR146 and the porcine buccal mucosa models, where the nanoparticles demonstrated effective permeation capabilities. These results highlight their potential utility in clinical settings for delivering therapeutic agents across dense tissue barriers.

In this study, consistent with previous research,⁴⁷ CS-NPs exhibited minimal cytotoxic effects at lower concentrations across various cell lines, emphasizing their biocompatibility and potential for medical and pharmaceutical applications. However, at higher concentrations (50%), a significant reduction in cell viability was observed after 24 and 48 h of

exposure. This decrease in viability was not solely due to the intrinsic properties of CS-NPs but was also influenced by environmental factors in the cell culture. Specifically, at a 50% concentration, the substantial dilution of the culture medium may have reduced the availability of essential nutrients, thereby affecting cell survival. Additionally, the intrinsic acidity of CS-NPs may have led to significant pH fluctuations, further contributing to the observed cytotoxic effects. These findings highlight the importance of optimizing nanoparticle concentrations to achieve a balance between therapeutic efficacy and potential cytotoxicity.

More importantly, the marked reduction in vimentin expression at both mRNA and protein levels following treatment with CS-miR-30c-5p NPs demonstrates the efficiency of CS-NPs in delivering encapsulated miRNA and facilitating its intracellular release. This result confirms that CS-NPs not only enable cellular uptake but also successfully release miR-30c-5p, allowing it to exert its gene-regulatory function. The observed downregulation of vimentin further supports the successful intracellular activity of the delivered miRNA, validating the efficiency of CS-NPs as an miRNA delivery system. Although our study focused on vimentin as the primary target of miR-30c-5p, it is known that miRNAs can regulate multiple genes through partial sequence complementarity. Future transcriptome-wide analyses will help clarify potential off-target interactions and provide a more comprehensive understanding of miR-30c-5p function.

A limitation of this study is that, while TEER was used to assess barrier formation in the TR146 model, we did not directly evaluate the expression or localization of specific tight junction proteins such as ZO-1, occludin, or claudins. Given that TEER reflects overall ionic resistance rather than protein-level structure, it may not fully capture tight junction integrity, particularly in carcinoma-derived cells like TR146. Future studies incorporating complementary methods—such as immunofluorescence staining or paracellular flux assays—are recommended to further validate epithelial barrier properties.

Conclusion

This study highlights the strong potential of CS-NPs as a stable and effective delivery system for miR-30c-5p in oral cancer therapy. Based on physicochemical characterization, cellular uptake analysis, mucosal penetration studies, and gene-silencing validation, our data demonstrate that CS-NPs not only enhance miRNA stability and protect it from degradation, but also facilitate efficient intracellular delivery and biological activity in target cells. These findings support the use of CS-NPs for localized miRNA delivery and provide a promising foundation for future translational applications in nucleic acid therapeutics.

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Disclosure

The authors report no conflicts of interest in this work.

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