

Formulation, nanoencapsulation and characterisation of mānuka oil and β -triketones for biomedical applications

Ghsaq Alhamdani^{a,*}, Dina Abdelmoneim^{a,*}, Natalie J. Hughes^b, Warwick J. Duncan^a, Chen Chen^a, Niki Hazelton^c, Kai Chun Li^a, Amira Salem^{a,d}, Dawn E. Coates^a

^a Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, Dunedin, New Zealand

^b School of Pharmacy, University of Otago, Dunedin, New Zealand

^c Otago Micro and Nano Imaging Electron Microscopy Suite, University of Otago, Dunedin, New Zealand

^d Oral Health Department, School of Acute and Primary Health, Auckland University of Technology, Auckland, New Zealand

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ABSTRACT

Mānuka oil (MO) is derived from mānuka shrubs in New Zealand (NZ) and presents antimicrobial properties that may help combat antibiotic resistance. However, applications of MO are limited due to volatility, sensitivity to light and heat, and the complexity of controlled administration.

We used ionic gelation to encapsulate MO and its derivative β -triketones (TK) within chitosan nanocarriers. Nanoparticles were characterised for morphology, chemical composition, encapsulation efficiency and release profile. Antimicrobial properties and biocompatibility were evaluated.

Nanoparticles loaded with MO or TK were successfully synthesised with an average size of 331.5 nm (PDI 0.431) and 333.5 nm (PDI 0.503), respectively. Formulations showed positive zeta potentials, stability, high yield (1.5–1.8 g), as well as oil release over 24 h. Bactericidal effect was achieved at concentrations of 3.125–25.000 mg/ml, with no cytotoxic effect. This study has demonstrated the formulation of MO and TK nanoparticles for potential applications in wound dressing, protective coating and targeted drug delivery.

Introduction

Natural phytotherapeutic agents, such as essential oils (EOs), are increasingly used as adjunctive or alternative treatments to synthetic medications. Resistant bacterial and fungal strains continue to rise, increasing the risk of compromised clinical outcomes and the burden on healthcare resources.¹ Previous studies have explored the antimicrobial activity of oils derived from lavender,² thyme,³ peppermint⁴, cinnamon,⁵ clove,⁶ eucalyptus⁷ and tea tree plants.⁸ Mānuka (*Leptospermum scoparium*), a member of the Myrtaceae family, grouped under tea trees, grows widely across NZ and Australia. Traditionally, indigenous populations have used it for centuries as a topical treatment for wounds and skin diseases and as an inhalation remedy for colds and fevers. The antimicrobial properties of MO are attributed to β -triketones: leptospermane, isoletospermane and flavesone, with the highest levels in NZ East Coast chemotypes.⁹

The pharmaceutical applications of EOs are limited by volatility, poor water solubility and cellular penetration, and high sensitivity to ambient conditions (light, oxygen, and heat) due to unsaturated carbon

chains.¹⁰ Allergic reactions have been related to oxidation products of terpenes.¹¹ To address these limitations, nanoencapsulation packages bioactive substances at the nanoscale, preserving their chemical and physical properties and enabling precise targeting.

Ionic gelation, introduced by Pilar Calvo,¹² based on electrostatic interactions between polymeric cations and polyanions (e.g., phosphate ions from sodium tripolyphosphate, TPP) under constant agitation and controlled pH, forming crosslinked spherical particles. The method is efficient, solvent-free, and preserves the chemical structure of oils as no heat or high-pressure homogenization is required.¹³ Successful EOs encapsulation has been reported using ionic gelation, including saffron,¹⁴ neem,¹⁵ palmarosa,¹⁶ lemon,¹⁷ cinnamon and thyme.¹⁸ Chitosan is a natural polymer approved by the Food and Drug Administration (FDA) for food and medical applications. Due to its high solubility, low cytotoxicity, mucoadhesive nature, and inherent antimicrobial properties, it serves as an excellent nanocarrier for EOs.^{19,20}

This study aims to synthesise chitosan nanoparticles loaded with MO and purified TK via ionotropic gelation and evaluate their stability, cytotoxicity, and antimicrobial activity as a potential therapeutic for

* Corresponding authors at: Sir John Walsh Research Institute, Faculty of Dentistry, PO Box 56, University of Otago, Dunedin, 9054, New Zealand.

E-mail addresses: algh812@student.otago.ac.nz (G. Alhamdani), dina.abdelmoneim@otago.ac.nz (D. Abdelmoneim).

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biomedical applications. MO-based biomedical formulations have been reported, including emulsions,⁹ nanoemulsions,²¹ microcapsules,^{22,23} nanocapsules²⁴ and nanotube coatings.²⁵ However, to our knowledge, no delivery system incorporates concentrated TK, as we anticipate a more powerful antimicrobial effect at lower concentrations than MO.

Materials and methods

Synthesis of chitosan nanoparticles

Non-loaded chitosan nanoparticles

Based on the previous work of Ma, Adayi²⁶ and Rampino, Borgogna,²⁷ 0.35% chitosan solution was prepared by dissolving low molecular weight chitosan (Cat. No. 448869-50G, Sigma-Aldrich®, USA) in a 1% glacial acetic acid (Cat. No. 1000632500, Emsure®, Germany), stirred for 48 h and centrifuged at 6000 $\times g$ for 30 min. The supernatant was collected and filtered through a filter (pore size 0.45 μm) to eliminate impurities. Tween-80 (Cat. No. 6224-500 ml, Sigma-Aldrich, USA) was added at a concentration of 1.125% v/v and stirred for 2 h at 50 °C. The solution pH was adjusted to 5.5 using 10 M NaOH.

Sodium triphosphate (TPP) (Cat. No. 238503-25G, Sigma-Aldrich®, St Louis, USA) was dissolved in dH₂O at a concentration of 0.175%. An Eppendorf manual dispenser and automated syringe driver (Graseby MS 16 A, Smith Medical, UK) were used to deliver 20 μl drops of TPP at a chitosan:TPP volume ratio of 2.5:1 under stirring at RT for 30 min (Fig. 1). The mixture was centrifuged at 12000 $\times g$ for 15 min at RT, and the supernatant was collected (approximately 85–90% of the total volume). Cryoprotectant, D-trehalose dihydrate (Cat. No. 90210-250G, Millipore, Germany) was added at a concentration of 5% wt./wt. and vortexed until completely dissolved. The recovered nanoparticles were stored overnight at –80 °C, freeze-dried at 0.1 mbar, –100 °C for 48 h, and the yield was calculated after subtracting the tube weight.

Oil-loaded chitosan nanoparticles

Chitosan solution was prepared as described above. MO (19.9 Tairawhiti pharmaceuticals, NZ) or TK (21.7 Tairawhiti pharmaceuticals, NZ) were added to the chitosan/Tween-80 solution at three concentrations (0.5%, 1% and 1.5% v/v) and stirred at RT for 1 h (Table 1). The pH value was adjusted to 5.5 using 10 M NaOH. Oil-loaded solutions were crosslinked and freeze-dried as previously.

Characterisation of MO and TK

Gas chromatography mass spectrometry (GCMS)

Chemical components of MO and TK were identified using gas chromatography-Flame Ionisation Detector (FID) by Tairawhiti Pharmaceuticals Ltd., NZ. The analyses were performed as described by Porter et al.⁹

Stability of oils

The stability of oils was investigated in an acidic environment. 10 μl of MO/TK was suspended in 10 ml of 1, 5 and 10% AA. Tween-80 was added at a concentration of 1.125%, and samples were incubated at 37 °C. The UV absorbance was measured in triplicate using a plate reader (Clariostar^{plus}, BMG LABTECH, UK) at a wavelength of 280 nm at day 0-day 7. The effect of incubation conditions on MO was also investigated using the same experimental setup at RT, 4 °C, and 37 °C.

Characterisation of chitosan nanoparticles

Size determination of nanoparticles

Dynamic light scattering (DLS) was used to determine the average size, polydispersity index (PDI), ζ -potential of nanoparticle formulations. Supernatants were diluted 1:10 with Milli-Q H₂O (MQ-H₂O), transferred into polystyrene cuvettes (Cat. No. 67.740 Sarstedt, Nümbrecht, Germany) and examined by Malvern Zetasizer Pro Blue Range (ATA Scientific PTY Ltd., NSW, Australia). Freeze-dried nanoparticles were resuspended in MQ-H₂O (5 mg/ml), samples were measured in three runs at 25 °C. Samples of non-crosslinked chitosan solution, MO and TK suspensions in MQ-H₂O (1 μl /10 ml) were used as the backgrounds.

Nanoparticles morphology (Scanning electron microscopy)

Freeze-dried nanoparticles (5 mg) were rehydrated in absolute ethanol for 1 h at RT and vortexed briefly. Ethanol was replaced by reagent-grade hexamethyldisilazane (HMDS; Sigma-Aldrich, 440191), incubated for 15 min at RT, then air-dried. Samples were mounted on aluminium stubs with double-sided carbon tape and coated with ~10 nm of gold/palladium (80:20) using a Quorum Q150V ES PLUS coater (Quorum Technologies, East Sussex, UK) and imaged with a JEOL 6700F field-emission scanning electron microscope (JEOL Ltd., Tokyo, Japan) at 10 kV.

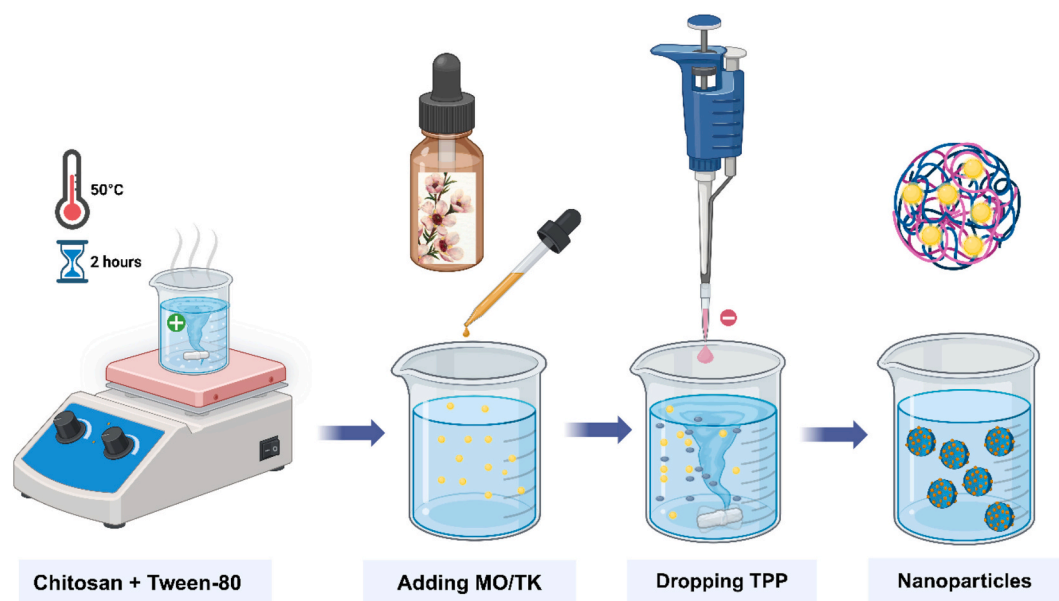


Fig. 1. An illustration of nanoparticle preparation by ionic gelation. Created with BioRender.com.

Table 1Chitosan nanoparticle formulations, bold denotes final formulation adopted (Ch = Chitosan, MO = Mānuka oil, TK = β -triketones, TPP = Sodium Tripolyphosphate).

Sample	Ch wt. (mg/20 ml)	MO/TK conc. (%)	TPP:Ch 8:20 ml (28 ml)	TPP wt. (mg/28 ml)	Ch wt. final volume (mg/28 ml)	MO/TK final conc. (μ l/28 ml)	TPP:Ch mass ratio
Chitosan	70	0	1:2.5	14	70	0	1:5
Chitosan:0.5% MO	70	0.5	1:2.5	14	70	140	1:5
Chitosan:0.5% TK	70	0.5	1:2.5	14	70	140	1:5
Chitosan:1% MO	70	1.0	1:2.5	14	70	280	1:5
Chitosan:1% TK	70	1.0	1:2.5	14	70	280	1:5
Chitosan:1.5% MO	70	1.5	1:2.5	14	70	420	1:5
Chitosan:1.5% TK	70	1.5	1:2.5	14	70	420	1:5

Fourier transform infrared spectrometry (FTIR)

Bruker Alpha II FT-IR Spectrometer (Bruker Optics, USA) interfaced with an ATR (attenuated total reflectance) were used in absorbance mode from 400 cm^{-1} to 4000 cm^{-1} by accumulating 32 scans at a spectral resolution of 4 cm^{-1} . Samples of non-encapsulated MO, TK and oil-loaded nanoparticles were tested after UV radiation at a wavelength of 254 nm for 30 min. All resulting spectra were acquired using OPUS Software (version 5.0 Bruker Optik, Ettlingen, Germany) and the graphs were created using Origin 2025b software.

Oil encapsulation efficiency

The efficiency of MO and TK encapsulation was determined following Subhaswaraj et al.²⁸ The optical density (OD) of recovered supernatant from non-loaded chitosan and oil-loaded nanoparticles was measured using an Ultrospec 3100 Pro spectrophotometer (Amersham Biosciences, UK). Nanoparticle suspensions were diluted with absolute ethanol (1:1 v/v), transferred into an Amicon Ultra-0.5 filter tube (MW:3000 K) (Cat. No. UFC5003, Sigma-Aldrich®, St Louis, USA) and centrifuged at 14000 xg, RT for 30 min. The filtered samples were collected and transferred into an acrylic cuvette (SARSTED, Germany). The absorbance of the non-encapsulated MO and TK was recorded against non-loaded chitosan supernatant (280 nm).

To evaluate the total non-encapsulated oils in the suspension, 0.35% chitosan solution was mixed with 1.125% Tween-80 and MO or TK (0.5, 1 and 1.5%). Non-encapsulated oil formulations were prepared by replacing the TPP with MQ-H₂O. Samples were diluted with absolute ethanol as previously described. Absorbance was measured at 280 nm in triplicate. The encapsulation efficiency (EE) was calculated following the equation.

In vitro oil release profile

25 mg of freeze-dried nanoparticles (non-loaded particles as a control) were resuspended in 1100 μ l of PBS solution and incubated under oscillation (100 rpm) at 37 °C and 4 °C. Samples were centrifuged (13,000 xg) at RT for 5 min. The supernatant was replaced with fresh PBS after 30 min, 1, 4, 24, 48 and 72 h. The absorbance values of the collected supernatant were measured at 280 nm in triplicate using a Clariostar^{plus} spectrophotometer.

Nanoparticle size changes in an acidic environment

The stability of chitosan nanoparticles was evaluated by resuspending chitosan nanoparticles in 1% (pH 2.7) and 10% acetic acid (AA) (pH 2.3). Two sets of samples (5 mg/ml) were prepared: one stored at 4 °C and the other at 37 °C. The size and PDI of the nanoparticles were measured in triplicate via DLS at different time points.

Antimicrobial properties

MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) of chitosan nanoparticles were evaluated against *Staphylococcus aureus* Oxford (*S. aureus* NCTC 6531), *Streptococcus mutans* (*S. mutans* UA159), *Fusobacterium nucleatum* subspecies *Polymorphum* (*F. nucleatum* ATCC 10953) and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans* ATCC 43718). Bacterial

species were initially cultured on Columbia sheep blood agar plates (Cat. No. CBA 1100, Thermo Fisher Scientific- USA), and selected colonies were grown in appropriate culture conditions (Table 2). Fastidious universal medium was prepared following.²⁹

Non-encapsulated MO and TK were tested on *S. mutans* (UA159) and *F. nucleatum* (25586) at concentrations that are roughly equivalent to oil-loaded nanoparticles (17 μ l/ml free oil $\sim$ 100 mg/ml of nanoparticles). Oil concentration was approximated from the initial concentration in nanoparticle formulations loaded with 1% MO or TK, in the presence of 1% v/v of Tween-80. Serial dilution was performed to obtain a range of oil concentrations 17–0.53 μ l/ml.

For clinical application and biological testing of nanoparticle formulations, a final sterilisation technique is required. Autoclaving is not viable for nanoparticles, especially with oil. Hence, we investigated UV and gamma irradiation to preserve chemical and morphological properties of polymeric nanoparticles.^{30,31}

Nanoparticles were sterilised using UV irradiation (254 nm) for 30 min. Treatment solutions were prepared in broth at nanoparticle concentrations of 3.125–100.000 mg/ml in a 96-well flat-bottomed CELL-STAR® 96 W microplate (Cat. No. M0812, Sigma-Aldrich®, St Louis, USA). The bacterial inoculum was diluted to obtain a final bacterial concentration of 5×10^5 CFU/ml (total volume 200 μ l). Nanoparticles were inoculated with the standardised bacterial suspension, and the optical density was measured at 600 nm immediately (time 0), after 24 h of incubation for *S. aureus*, *S. mutans* and *A. actinomycetemcomitans*, and after 48 h for *F. nucleatum*. The MBC was determined by spot-plating of 5 μ l onto agar plates (n = 3) followed by incubation at 37 °C, 5% CO₂ for 24 h, except for *F. nucleatum*, 48 h. The MBC was identified as the lowest nanoparticle concentration that allowed <10 bacterial colonies of the test bacterium to grow on the agar plate.³²

The same experiment was performed on nanoparticles after washing with absolute ethanol (x3) to remove non-incorporated oil. Nanoparticles were resuspended in absolute ethanol (Lab Supply, NZ) and centrifuged at 6000 $\times$ g, RT for 5 min, supernatant removed, and

Table 2

Incubation conditions for tested bacterial species.

Bacteria species	Broth media	Incubation conditions
<i>Streptococcus mutans</i> UA159	Brain Heart Infusion (BHI) (Cat. No. 37500, Becton, Dickinson and Company, Maryland, USA)	Anaerobic, Columbia Blood Agar. 37 °C, 16–18 h
<i>Staphylococcus aureus</i> NCTC6531	Brain Heart Infusion (BHI)	Aerobic, Columbia Blood Agar. 37 °C, 16–18 h
<i>Fusobacterium nucleatum</i> ATCC 10953	Fastidious universal medium supplemented with Sørensen's buffer (67 mmol/L) and glucose (0.3% w/v)- pH 7.20	Anaerobic, Columbia Blood Agar. 37 °C, 48 h
<i>Aggregatibacter actinomycetemcomitans</i> ATCC 43718	Brain Heart Infusion (BHI) + 1 mg/ml Yeast extract powder (Cat. No. YEA03, FORMEDIUM, England).	Anaerobic, Columbia Blood Agar. 37 °C, 24 h

particles placed in a dry incubator at 37 °C overnight.

Therefore, we investigated the effect of gamma irradiation on the antimicrobial properties, as a potential sterilisation technique, was also investigated. Freeze-dried nanoparticles were exposed to 16 kGy (MSD Animal Health, NZ- certificate No. GM029147). Data were analysed using GraphPad Prism (version 10.02).

Biocompatibility of chitosan nanoparticles

Metabolic activity of Human gingival fibroblasts (HGFs). Human gingival fibroblasts (HGFs; NCL2014) were cultured in DMEM with GlutaMAX™ (DMEM; Cat. No. 10569010, Gibco, Life technologies NZ Ltd) supplemented with 10% fetal bovine serum (Cat. No. 10091148, Life technologies, NZ Ltd), 1% antibiotic–antimycotic (100×; Cat. No. 15240–062, Gibco, MT, USA), and 0.5% gentamicin (Cat. No. 15710064, Gibco, Life technologies, NZ). Cells were seeded in triplicate in Transwell® 96-well plates (0.3 µm inserts) (Cat. No. 3385, Corning, New York, USA) at a density of 4,000 cells/well and incubated at 37 °C, 5% CO₂.

Freeze-dried nanoparticles were pre-weighed and UV-sterilised for 30 min. Suspensions were prepared in DMEM (3.125–100 mg/ml) and gently oscillated for 5–10 min to ensure dispersion. After 24 h, the culture medium was replaced with 230 µL fresh DMEM in the lower chamber and 75 µL nanoparticle suspension in the insert, followed by incubation at 37 °C and 5% CO₂. After 20 h, PrestoBlue® (10% v/v) (Cat. No. A13261, Life Technologies, Oregon, USA) was added, and cells were incubated for 4 h. Fluorescence was measured using a CLARIOstar^{Plus} plate reader (BMG LABTECH, Germany) at 24 and 48 h (Ex 560 nm/Em 590 nm). Untreated cells served as controls, and DMEM was used for background correction. Data are presented as fluorescence intensity and percentage of control using GraphPad Prism v10.0.2.

Fluorescent staining. Phalloidin cell-labelling reagent (Alexa Fluor™ Phalloidin 647, Cat. No. A22287, Thermo Fisher Scientific, USA) was used to stain HGFs in combination with 4',6-diamidino-2-phenylindole (DAPI) (Cat. No. D3571, Thermo Fisher Scientific, USA). After 72 h of incubation with nanoparticles, cells were washed with PBS, fixed with 10% buffered neutral formalin solution (Cat. No. BSP10C, LabServ,

Table 3
Average size and polydispersity index (PDI) for chitosan nanoparticle formulations.

Sample	Average size before FD (nm)	PDI before FD	Average size after FD (nm)	PDI after FD	Zeta potential value	Average yield (g)
A. Control (Background)						
Chitosan solution	1143.00 ± 43.62	0.5615 ± 0.0200	–	–	–	–
MO solution in H ₂ O	10.20 ± 0.10	0.1186 ± 0.0300	–	–	–	–
TK solution in H ₂ O	10.20 ± 0.03	0.1207 ± 0.0100	–	–	–	–
Chitosan:1% MO	24.30 ± 1.58	0.2936 ± 0.0400	–	–	–	–
Chitosan:1% TK	16.90 ± 0.32	0.2978 ± 0.0300	–	–	–	–
B. Chitosan solution filtration						
Chitosan-filtered	300.90 ± 5.44	0.2517 ± 0.0100	–	–	–	–
Chitosan-non-filtered	527.50 ± 14.10	0.4071 ± 0.0600	–	–	–	–
C. Nanoparticles prepared by syringe driver (supernatant, TPP dropped by syringe driver)						
Chitosan	409.40 ± 5.95	0.3293 ± 0.0100	474.40 ± 20.55	0.6887 ± 0.0600	+30.29	1.36
Chitosan:1% MO	605.80 ± 182.02	0.4782 ± 0.0900	704.30 ± 342.14	0.6900 ± 0.1400	+5.793	1.33
Chitosan:1% TK	294.80 ± 7.38	0.4474 ± 0.0400	580.60 ± 177.93	0.6706 ± 0.0800	+30.73	1.48
Nanoparticles prepared by the Eppendorf dispenser						
D. Nanoparticles collected from the bottom fraction (with trehalose)						
Chitosan	663.10 ± 19.11	0.5133 ± 0.0400	4771.00 ± 1583.00	0.7298 ± 0.2000	–	–
Chitosan:1% MO	572.30 ± 20.88	0.5643 ± 0.1000	4150.00 ± 1080.03	0.8601 ± 0.2400	–	–
Chitosan:1% TK	1008.00 ± 168.10	0.6458 ± 0.1000	859.60 ± 74.88	0.7165 ± 0.1400	–	–
E. Nanoparticles collected from supernatant (no trehalose)						
Chitosan	317.80 ± 4.97	0.4900 ± 0.0200	5018.00 ± 1123.01	0.9917 ± 0.1700	–	–
Chitosan:1% MO	323.80 ± 52.20	0.4981 ± 0.1100	2678.00 ± 183.44	0.6239 ± 0.2500	–	–
Chitosan:1% TK	317.30 ± 8.53	0.5222 ± 0.0100	873.60 ± 207.38	0.6018 ± 0.0300	–	–
F. Nanoparticles collected from supernatant (with trehalose)						
Chitosan	319.70 ± 5.60	0.3237 ± 0.0080	333.80 ± 16.3	0.5499 ± 0.0030	+23.37	1.52
Chitosan:0.5% MO	220.20 ± 4.87	0.5051 ± 0.0100	352.70 ± 2.70	0.6089 ± 0.0400	+26.09	1.75
Chitosan:0.5% TK	264.00 ± 4.26	0.3051 ± 0.0200	487.10 ± 87.23	0.6464 ± 0.0500	+28.82	1.65
Chitosan:1% MO	257.20 ± 18.9	0.4682 ± 0.0900	331.50 ± 15.11	0.4306 ± 0.0200	+27.04	1.59
Chitosan:1% TK	274.60 ± 0.83	0.3228 ± 0.0100	333.50 ± 2.95	0.5036 ± 0.0300	+28.04	1.83
Chitosan:1.5% MO	214.70 ± 8.80	0.5472 ± 0.0200	315.30 ± 41.01	0.7274 ± 0.0800	+24.92	2.34
Chitosan:1.5% TK	267.30 ± 7.86	0.3019 ± 0.0130	302.30 ± 8.36	0.7189 ± 0.0600	+25.39	1.64
G. Chitosan-MO very top layer (Non-encapsulated MO)						
Chitosan:1% MO (syringe driver)	1112.00 ± 187.38	0.5925 ± 0.0400				
Chitosan:1% MO (Eppendorf dispenser)	1664.00 ± 481.29	0.9307 ± 0.1500				
H. Nanoparticles prepared with 2% Tween-80 (supernatant no trehalose)						
Chitosan:1% MO	231.7 ± 41.57	0.5966 ± 0.1900	851.00 ± 138.27	1.0000 ± 0.0100	–	–

Thermo Fisher Scientific, NZ) and incubated at RT for 15 min. Samples were washed with PBS ($\times 3$) and incubated with 1% TritonTM X-100 detergent solution (Cat. No. T-8787, Sigma-Aldrich®, USA) at RT for 15 min. Cells were washed with PBS and stained by adding 200 μ l of phalloidin labelling reagent (Alexa FluorTM 647) and incubated at RT for 60 min. Cells were then washed with PBS, stained with 300 nM DAPI, and incubated at 37 °C for 5 min. After a final PBS wash, cells were visualised using an EVOSTM M5000 Imaging System at 650/668 nm (excitation/emission) for phalloidin and 357/447 nm for DAPI. Cell counts were performed on DAPI images in triplicate using ImageJ software (v2.14).

Statistical analysis

Statistical analysis was performed with one-way or two-way ANOVA, using GraphPad Prism (version 10.0.2). All results were reported as mean with standard deviation ($\pm$ SD) and considered statistically significant when the P value < 0.05 .

Results

Chitosan nanoparticles preparation and size measurement

Different formulations of chitosan nanoparticles, as summarised in Table 3, were successfully prepared on the bench to encapsulate MO or β -triketones at concentrations of 0.5%, 1% and 1.5%.

Non-loaded chitosan nanoparticles

The chitosan:TPP volume ratio (2.5:1) and solution pH of 5.5 were applied for all formulations based on our previous study optimisation.³³ After crosslinking with TPP, the chitosan solution formed a turbid suspension attributed to nanoparticle formation. The DLS of chitosan solution reveals a noticeable reduction in the average size of chitosan particles from 1143 nm to 320 nm and PDI 0.5 to 0.3 after crosslinking, indicating nanoparticle formation (Fig. 2A and D). High-speed

centrifugation (12,000 $\times g$) introduced phase separation into a pellet at the bottom and an opaque supernatant. This process was essential to separate the unreacted components and the large-sized particles. Despite the addition of the surfactant (Tween-80) and the cryoprotectant (trehalose), nanoparticles collected from the bottom fraction were large (600–1000 nm) and showed a high tendency to aggregate, with size increased up to 4000 nm (PDI 0.7–0.8) after freeze-drying (Suppl. Fig. 2). While smaller, more uniformly distributed nanoparticles were collected from the supernatant regardless of the method used to deliver the TPP drops (Suppl. Fig. 3). Chitosan nanoparticles without trehalose exhibited a clumpy, undesirable appearance after drying (PDI 0.99) (Table 3E). Generally, nanoparticles prepared via a syringe driver were slightly larger (400 nm) than those from a manual dispenser (300 nm), but they maintained uniformity with PDIs around 0.3. Therefore, nanoparticles collected from supernatant prepared with an Eppendorf dispenser were selected for further investigation.

Oil-loaded chitosan nanoparticles

Considering the acidic nature of MO and TK, there was a slight decline in the pH of chitosan solutions after oil incorporation (pH 5.3–5.4); hence, pH was re-adjusted to 5.5. Before crosslinking, DLS of MO and TK presented a single sharp peak around 10 nm (Fig. 2B and C), which slightly shifted to 24.3 and 16.9 nm when resuspended (PDI 0.29) in chitosan solution (Suppl. Fig. 4). As the TPP was introduced, suspension turbidity was noted, and phase separation was obtained after centrifugation. A thin white oily layer was observed floating on the top of the MO suspension regardless of the oil concentration or TPP incorporation technique. This layer was composed of non-encapsulated oil particles sized 1660 and PDI 0.93 (Suppl. Fig. 5). The TK suspension tended to have a yellow aggregation at the bottom after separation.

Dropping TPP with a syringe driver resulted in larger nanoparticles, especially for MO-loaded particles, as freeze-dried particles sized 700 nm (Table 3C and Suppl. Fig. 6). This can be attributed to variability in the size and the speed of the TPP drops. Oil-loaded nanoparticles crosslinked with Eppendorf-controlled TPP-drops and collected from the

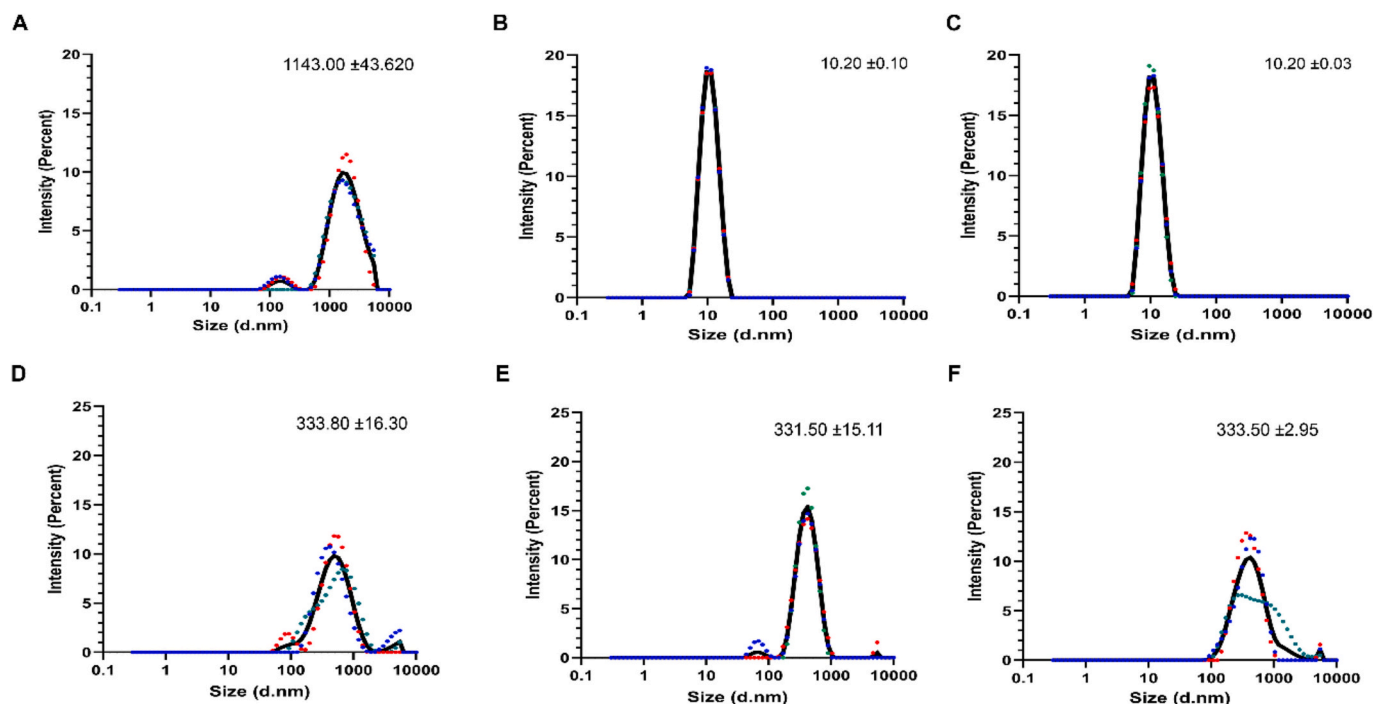


Fig. 2. Dynamic light scattering (DLS). (A) Chitosan solution. (B) MO in Milli-Q H₂O. (C) TKs in Milli-Q H₂O. (D) Non-loaded chitosan nanoparticles (Eppendorf dispenser), collected from supernatant after freeze-drying. (E) Chitosan-1% MO nanoparticles (Eppendorf dispenser) collected from the supernatant after freeze-drying. (F) Chitosan-1% TK nanoparticles (Eppendorf dispenser), collected from the supernatant after freeze-drying. TPP = sodium tripolyphosphate. N = 3 (dotted lines), the black line represents the average of three runs.

supernatant were relatively smaller (200–500 nm) with PDIs 0.3–0.5, indicating uniformity, regardless of the oil concentration. Nanoparticles loaded with 0.5% and 1.5% of MO or TK demonstrated higher PDI values after freeze-drying, which indicated clumping (Suppl. Fig. 7 and Suppl. Fig. 8). In contrast, formulations with 1% MO or TK exhibited more consistent average size and dispersity indices before and after freeze-drying (PDI: 0.3–0.5) (Fig. 2E and F). In addition, a higher concentration of Tween-80 (2% v/v) did not enhance nanoparticles' PDIs after freeze-drying (Suppl. Fig. 9).

Zeta potential results indicate favourable cationic charges and moderate stability of particles with the highest values for TK-loaded nanoparticles compared to non-loaded nanoparticles. Low Zeta potential values with MO nanoparticles prepared with the syringe driver, signify unstable formulations and clumping, aligning with high PDI.

Characterisation of actives

Gas chromatography mass spectrometry (GCMS)

GCMS analysis identified 31 components in MO with the greatest concentrations for leptospermone (17%) and trans-calamenene (13%), followed by flavesone, isoleptospermone and cadinene (Table 4). Several unidentified compounds were recorded (sum of peak areas = 17.65%) at retention times between 10.68 and 45.07. The purified β -triketone extract was 99.74% pure, with approximately 3 times more β -triketone concentration than MO (28.97%). The GC–MS profiles of MO and TK are shown in Suppl. Fig. 10 and Suppl. Fig. 11.

Stability of the MO and TK

The stability of non-encapsulated MO and TK was evaluated at 37 °C and low pH to study the effect of a simulated body environment on the oil present on the nanoparticle surface or as-released oil. Absorbance of MO and TK samples was measured at 280 nm to evaluate the effect of an acidic environment anticipated during future applications. Control (50% ethanol) showed a gradual increase in absorbance values over time. The OD of TK remained constant throughout the experiment (3.3–3.4). In contrast, all MO samples exhibited a marked increase in absorbance values during the first 24 h of incubation, but subsequently, maintained a stable reading around 3.2 (Fig. 3A).

Further investigation to clarify the MO behaviour was conducted in

the most acidic pH (10% AA, pH 2.29) compared to MQ-H₂O, incubated at 4 °C, RT or 37 °C for 24 h. As expected, samples prepared in AA had a lower pH (2.3) compared to the MO suspension in H₂O (pH 4.2) with no change over time. A slight increase in the absorbance was recorded in the MO/AA suspension. The absorbance of MO in H₂O was consistent at about 3.2 after 24 h (Fig. 3B and C).

Chitosan nanoparticle characterisation

Morphology

SEM images confirmed the morphological features of chitosan nanoparticles, as individual nanoparticles embedded within a trehalose matrix and identified with a scale bar that is consistent with the average size suggested by DLS (Fig. 4).

Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of chitosan solution, MO, TK, and crosslinked nanoparticles are shown in Fig. 5 and Suppl. Table 1. The chitosan-TPP interaction was confirmed in the FTIR spectrum as the amide band shifted to 1408 and 1569 cm⁻¹, attenuation of CH bending at 2930 cm⁻¹, and the appearance of new phosphate-related peaks at 1155, 1244, and 1043 cm⁻¹ (Fig. 5Ai). After crosslinking, chitosan nanoparticles retained characteristic peaks at 1408 and 1569 cm⁻¹, confirming nanoparticle formation. Oil-loaded nanoparticles displayed merged bands at ~1730, 1645, 1248, and 1088 cm⁻¹, alongside chitosan peaks, confirming oil incorporation (Fig. 5Aii). The FTIR spectrum of non-encapsulated MO and TK, as shown in Suppl. Fig. 12A, exhibited a similar spectrum after UV irradiation (254 nm for 30 min), as both MO and TK maintained their major peaks of conjugated ketones (1672 cm⁻¹, 1725 cm⁻¹), terpenoid C–O at 1046 cm⁻¹, and cyclic alkene C=C at 1550 cm⁻¹.

The spectra of oil-loaded nanoparticles were also investigated after UV irradiation (254 nm for 30 min). Results in Suppl. Fig. 12B also indicate no significant variations, as major peaks were maintained. Chitosan-TPP interaction was identified at 1408 cm⁻¹ and 1567 cm⁻¹. The CH bending of chitosan appeared at 2932 cm⁻¹. Peaks related to oil incorporation were presented at 1737 cm⁻¹, 1645 cm⁻¹ and 1088 cm⁻¹.

Table 4

Gas chromatography mass spectrometry analysis of MO and TKs, bold denotes the triketones component in MO.

GC peak	Retention time (minutes)	Compound name	Peak area %	GC peak	Retention time (minutes)	Compound name	Peak area %
A. Gas chromatography analysis of MO (MO; 19.9 Tairawhiti Pharmaceuticals, NZ)							
1	7.20	α -Pinene	1.81	17	30.30	aromadendrene	1.42
2	8.58	β -Pinene	0.17	18	30.90	α -humulene	0.29
3	9.20	myrcene	0.24	19	31.21	α -amorphene	0.17
4	10.29	p-cymene	0.09	20	32.36	β -selinene	3.51
5	10.58	1,8-cineol	0.20	21	32.85	α -selinene	4.16
6	11.93	β -ocimenes	0.11	22	33.12	α -muurolene	0.80
7	14.44	linalool	0.15	23	33.61	γ -cadinene	0.66
9	26.36	α -cubebene	3.22	24	33.91	trans-calamenene	13.85
10	27.30	α -ylangene	0.19	25	34.17	δ -cadinene	5.21
11	27.56	α -copaene	4.84	26	34.52	Cadina-1,4-diene	0.28
12	28.21	β -elemene	1.36	27	34.66	flavesone	5.67
13	29.08	α -gurjunene	0.81	28	36.17	caryophyllene oxide	5.41
14	29.36	β -caryophyllene	2.19	29	38.01	isoleptospermone	5.41
15	39.06	α -eudesmol	1.30	30	38.43	leptospermone	17.39
16	39.30	β -eudesmol	0.41	31	45.28	grandiflorone	0.50
Total β -triketones: 28.97%							
B. Gas chromatography analysis of β -triketones (TK; 21.7 Tairawhiti Pharmaceuticals, NZ)							
1	32.81	unidentified	0.11	4	37.64	isoleptopermone	17.15
2	34.30	flavesone	25.13	5	38.09	leptospermone	55.99
3	36.22	unidentified	0.15	6	45.19	grandiflorone	1.47
Total β -triketones: 99.74%							

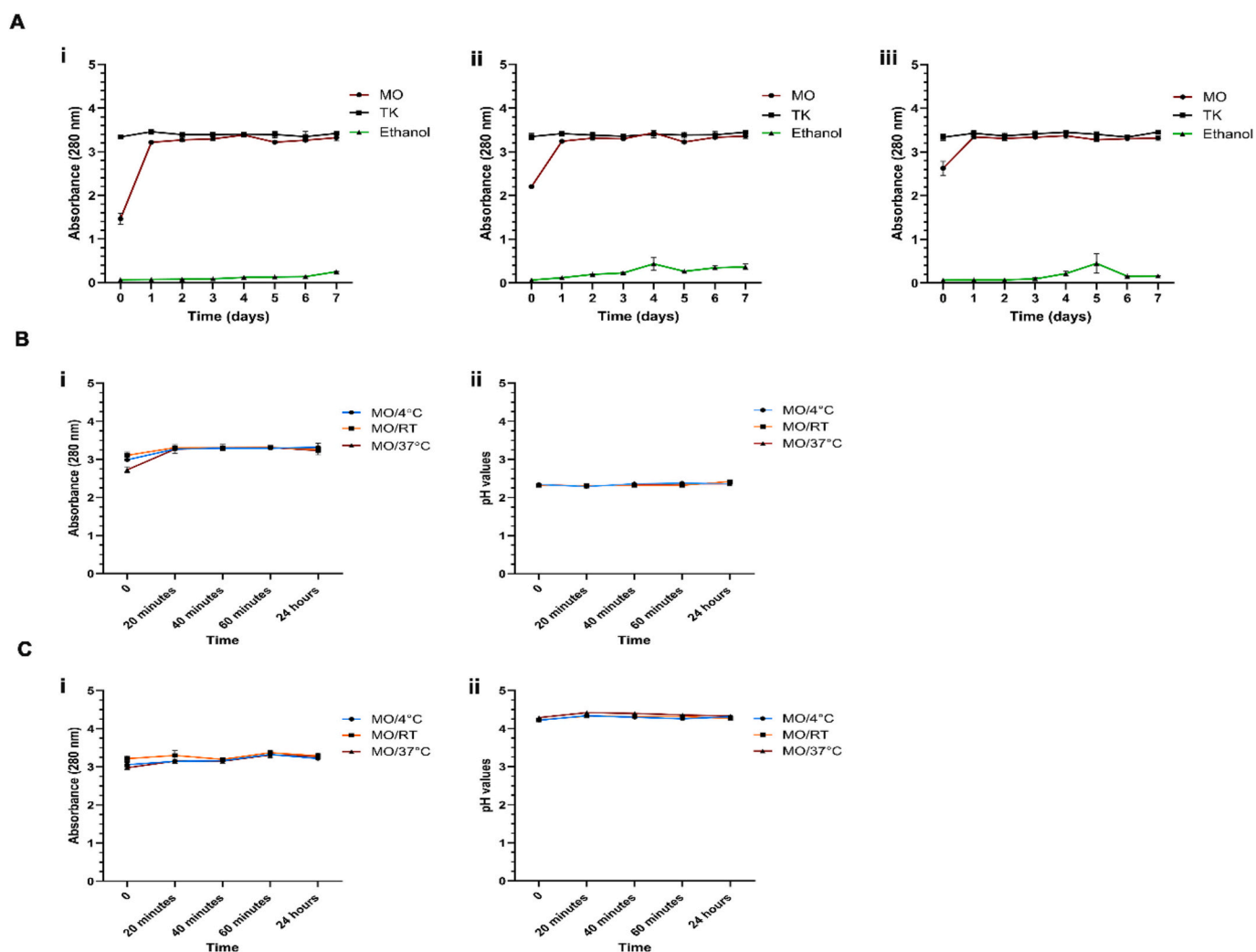


Fig. 3. (A) Stability of MO and TKs at 1 ml/ml in AA at 37 °C: (i) 1% (pH 2.91), (ii) 5% (pH 2.49), (iii) 10% (pH 2.29), compared with 50% ethanol. (B). Stability of MO at 1 µl/ml in 10% AA in different incubation conditions: (i) Absorbance of MO. (ii) pH of MO. (C) Stability of MO at 1 µl/ml in MQ-H₂O in different incubation conditions: (i) Absorbance of MO. (ii) pH of MO. Samples tested in triplicate. Mean $\pm$ SD.

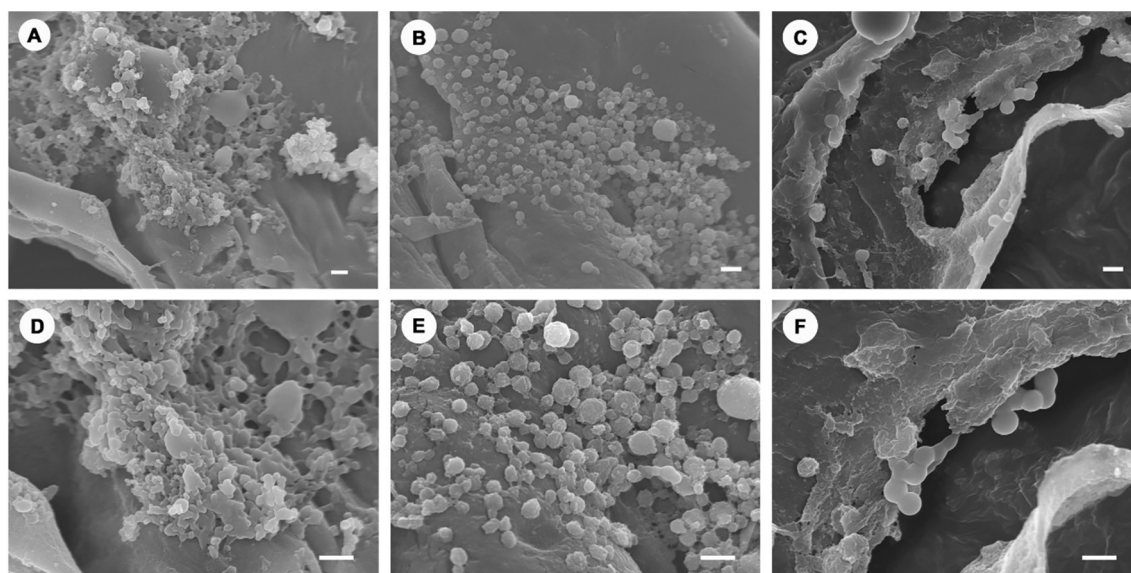


Fig. 4. SEM images showing the morphology of: (A) and (D) Non-loaded chitosan nanoparticles. (B) and (E) Chitosan-1% MO nanoparticles. (C) and (F) Chitosan-1% TK nanoparticles. Scale bar = 1 µm.

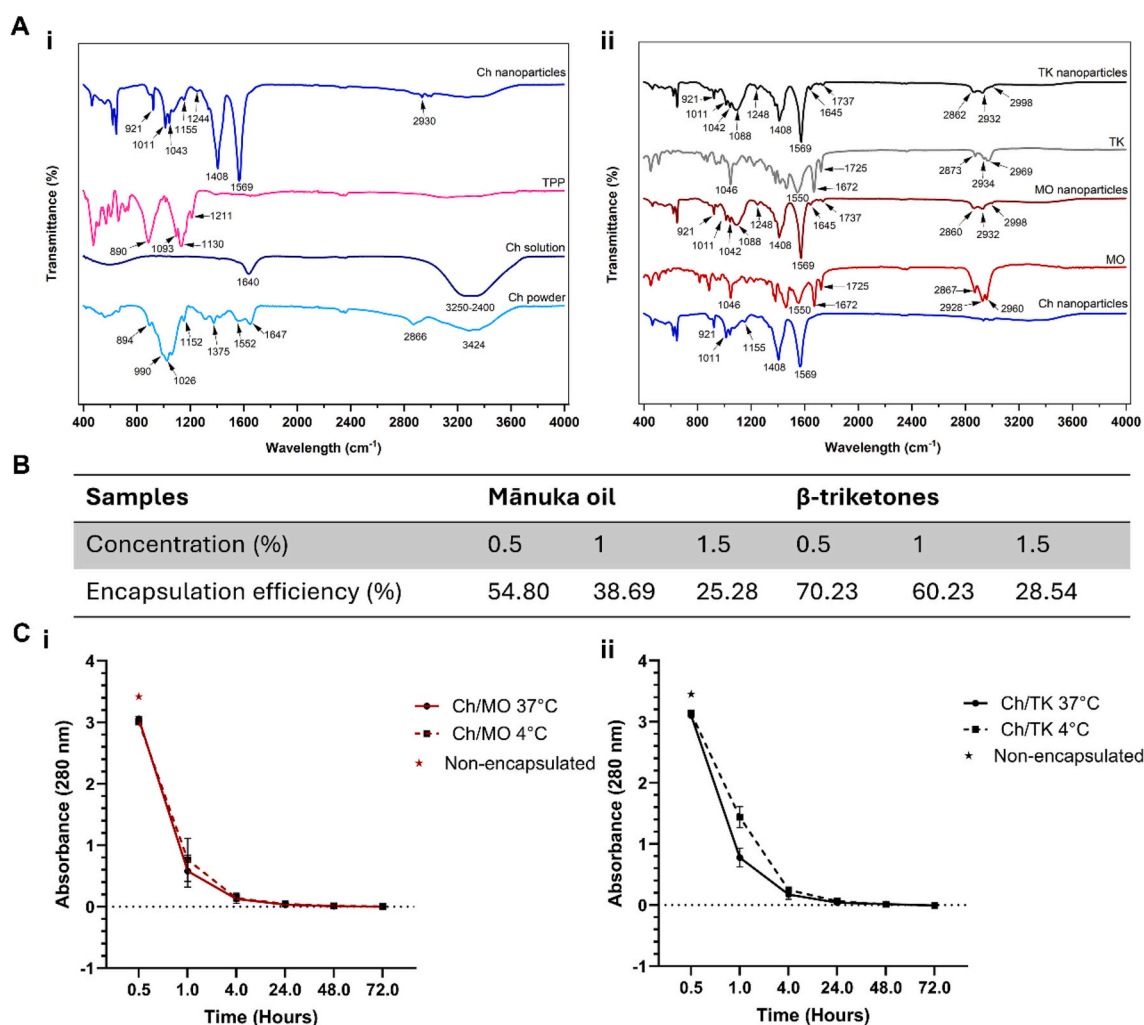


Fig. 5. (A). FTIR spectra of (i) Chitosan powder (Ch powder), 0.35% chitosan solution (Ch solution), sodium tripolyphosphate powder (TPP) and freeze-dried non-loaded chitosan nanoparticles (Ch nanoparticles). (ii) MO and TK; freeze-dried chitosan nanoparticles, chitosan-1% MO nanoparticles (MO nanoparticles) and chitosan-1% TK nanoparticles (TK nanoparticles). Wavelength range 4000–400 cm⁻¹. (B) Oil encapsulation efficiency of chitosan nanoparticles. (C) Oil release profile in PBS: (i) Chitosan-1% MO nanoparticles. (ii) Chitosan-1% TK nanoparticles. N = 3, mean ± SD.

Encapsulation efficiency

Fig. 5B shows distinct patterns for MO-loaded nanoparticles compared with those containing TK. Loading higher concentrations of MO and TK decreased encapsulation efficiency, reaching 25.28% at 1.5% MO and 28.54% at 1.5% TK. This can be attributed to suspension saturation with floating oil molecules interfering with the chitosan's ability to crosslink with TPP.

Oil release profile

The oils released from chitosan nanoparticles were detected using UV and plotted against the absorbance values of non-encapsulated oils (Fig. 5C). The graph demonstrates a high average absorbance (3.3) after 30 min, with a noticeable decline subsequently, without a clear effect of the incubation temperature. After 48 h of incubation, no MO or TK was detected.

Nanoparticle size changes in an acidic environment

Non-loaded nanoparticles swelled after 24 h in AA, while those in PBS remained unchanged (Fig. 6A). Oil-loaded nanoparticles showed formulation-dependent behaviour. Chitosan-0.5% MO nanoparticles exhibited a slight size increase after 3–5 days in both media. Nanoparticles containing 1% MO showed no significant size changes in AA (240–265 nm) but displayed early variability in PBS ($P = 0.0046$).

Increasing MO concentration to 1.5% resulted in a progressive size increase in AA, with particle size nearly doubling from 358.8 nm to 785.4 nm ($P = 0.0002$) (Fig. 6Biii). All TK-loaded formulations remained stable in acidic conditions, whereas size fluctuations were observed in PBS (Fig. 6C). Overall, nanoparticles loaded with 1% MO or TK exhibited minimal size variation in both media, and this formulation was therefore selected for extended incubation studies at body temperature.

At 37 °C, non-loaded particles showed minimal size changes in 1% and 10% AA solutions over time. However, MO nanoparticles responded differently to a 10% AA solution with an approximately 4-fold increase in size from a mean of 265 nm to 1087 nm. TK-loaded nanoparticles showed some small but significant size changes over time of incubation (Fig. 7). Detailed data of nanoparticle size change based on DLS measurements in different environments are provided in Suppl. Table 2 and Suppl. Table 3.

Antimicrobial activity of chitosan nanoparticles

Nanoparticles used for this study were sterilised either with UV (254 nm, 30 min) or 16 KGy gamma irradiation. All tested bacteria were inhibited or killed within 24–48 h of incubation with nanoparticle formulations at concentrations between 3.125 mg/ml and 100 mg/ml (Table 5A, Suppl. Fig. 14). Non-loaded chitosan nanoparticles showed

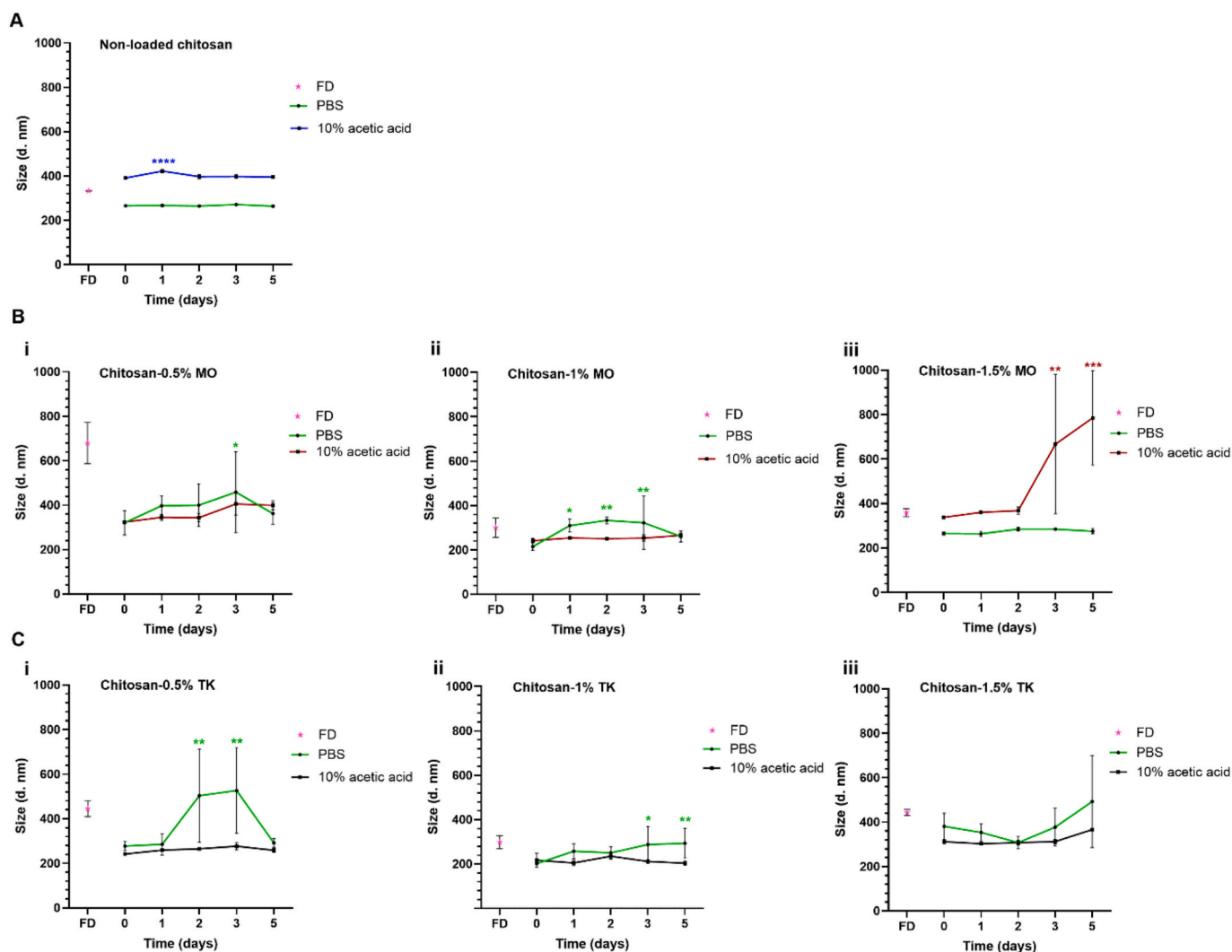


Fig. 6. Chitosan nanoparticle size change after incubation in PBS (pH 7.4) or 10% AA (pH 2.29) at 4 °C for 5 days, as measured by DLS. (A) Non-loaded chitosan nanoparticle. (B) Chitosan nanoparticles loaded with MO. (i) Chitosan-0.5% MO nanoparticles. (ii) Chitosan-1% MO nanoparticles. (iii) Chitosan-1.5% MO nanoparticles. (C) Chitosan nanospheres loaded with TK. (i) Chitosan-0.5% TK nanoparticles. (ii) Chitosan-1% TK nanoparticles. (iii) Chitosan-1.5% TK nanoparticles. Statistical analysis with two-way ANOVA (Uncorrected Fisher LSD), reporting the difference from time 0 (10 min of incubation). d. nm = diameter of nanoparticle in nm. FD = average particle size of freeze-dried nanoparticles in MQ-H₂O. N = 3, mean ± SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

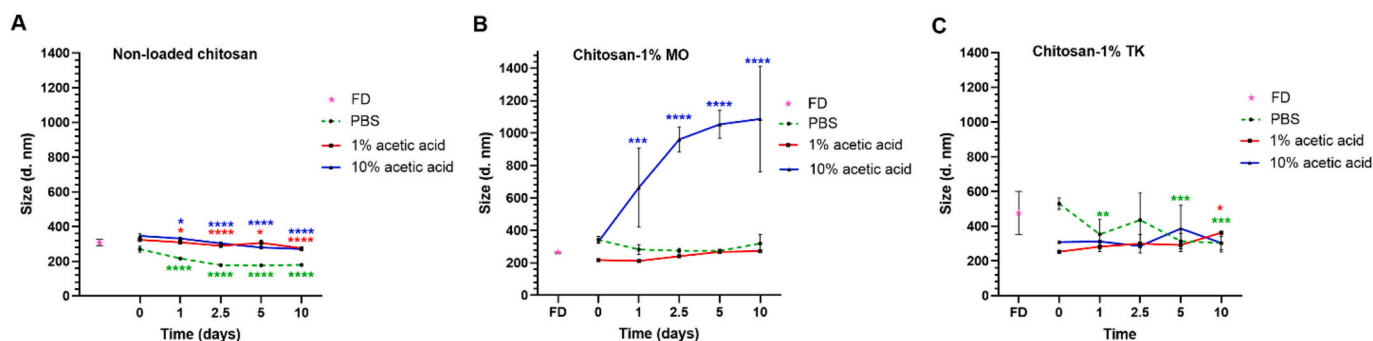


Fig. 7. Chitosan nanoparticles size change after incubation in PBS (pH 7.4), 1% (pH 2.91) and 10% AA (pH 2.29) at 37 °C, for 10 days, as measured by DLS. (A) Non-loaded chitosan nanoparticles. (B) Chitosan-1% MO nanoparticles. (C) Chitosan-1%TK nanoparticles. Statistical analysis with two-way ANOVA (Uncorrected Fisher LSD), reporting the difference from timepoint 0 (10 min of incubation). d. nm = diameter of nanoparticle in nm. FD = average particle size of freeze-dried nanoparticles in MQ-H₂O. Three readings were recorded for each sample. Mean ± SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

an inhibiting effect at higher concentrations compared to particles loaded with MO and TK. Generally, Gram-negative species were more sensitive to chitosan-1% MO nanoparticles than Gram-positive species,

with inhibition of *A. actinomycetemcomitans* and *F. nucleatum* achieved at as little as 3.125 mg/ml. It is noteworthy that *S. aureus* showed the highest level of resistance among the tested species, with 25 mg/ml of

Table 5

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of chitosan nanoparticles against bacterial species.

Bacteria species	Classification	MIC (mg/ml)			MBC (mg/ml)		
		Ch	Ch/MO	Ch/TK	Ch	Ch/MO	Ch/TK
A. MIC and MBC before washing and Gamma radiation (UV irradiated)							
<i>Streptococcus mutans</i> UA159	Gram +ve, facultative anaerobe	50.000	12.500	6.250	50.000	6.250	6.250
<i>Staphylococcus aureus</i> NCTC6531	Gram +ve, facultative anaerobe	100.000	12.500	6.250	100.000	25.000	12.5000
<i>Fusobacterium nucleatum</i> ATCC10953	Gram -ve, anaerobic	3.125	3.125	3.125	12.500	3.125	3.125
<i>Aggregatibacter actinomycetemcomitans</i> ATCC 43718	Gram -ve, facultative anaerobe	6.250	3.125	3.125	25.000	12.500	6.250
B. MIC and MBC after washing and gamma radiation.							
1. Washed particles							
<i>S. mutans</i> UA159	Gram +ve, facultative anaerobe	25.000	12.500	12.500	50.000	25.000	25.000
2. Gamma-radiated particles (non-washed)							
<i>S. aureus</i> NCTC6531	Gram +ve, facultative anaerobe	50.000	3.125	3.125	100.000	12.500	6.250
C. MIC and MBC of non-encapsulated MO and TK							
<i>Streptococcus mutans</i> UA159	Gram +ve, facultative anaerobe		3.125 (0.53 µl/ml)	3.125 (0.53 µl/ml)			
<i>Fusobacterium nucleatum</i> 25586	Gram -ve, anaerobic		6.25 (1.06 µl/ml)	3.125 (0.53 µl/ml)			

nanoparticles required to achieve a bactericidal effect. *F. nucleatum* showed significant sensitivity to the lowest concentration of 3.125 mg/ml ($P < 0.0001$). The same concentration of TK nanoparticles inhibited the growth of *A. actinomycetemcomitans* ($P < 0.0001$). However, a two-fold increase was required to obtain a bactericidal effect. Chitosan-1% TK nanoparticles displayed greater antimicrobial efficacy with lower MICs and MBCs for all tested species. Nanoparticle formulations were also tested against *S. mutans* after being washed with ethanol to eliminate the effect of the non-encapsulated oils. The MIC of MO and TK-loaded nanoparticles was constant or slightly increased (Table 5B-1). These results confirm that much of the bactericidal effect after washing was due to encapsulated oils. As a potential sterilisation method for future applications, we evaluated the impact of gamma irradiation on the antimicrobial activity of nanoparticles against *S. aureus* (Table 5B-2). Irradiation of 16 kGy reduced the bacteriostatic effect to 3.125 mg/ml and the bactericidal doses for MO and TK-loaded nanoparticles to 12.5 and 6.25 mg/ml, respectively. Suppl. Fig. 15 shows the MIC graphs of the non-encapsulated MO and TK. A highly potent inhibitory effect was observed with free oil forms at a concentration of 0.53 µl/ml (equivalent to 3.125 mg/ml of nanoparticles). However, the primary goal of our study is encapsulation to provide protection, controlled release of the active ingredients, and enhance biocompatibility.

Metabolic activity of human gingival fibroblasts (HGFs)

Cells incubated with up to 50 mg/ml of non-loaded chitosan and chitosan-1% TK nanoparticles exhibited similar or higher metabolic activity compared to controls (0 mg/ml nanoparticles) after 48 h of incubation. Moreover, results show a pattern of substantially reduced metabolic activity with 100 mg/ml of nanoparticles for all groups. However, the effects of MO or TK were not different from those of unloaded nanoparticles. Viable cells were positively stained with DAPI and phalloidin to observe cell morphology and allow counting (Fig. 8B). When examining cell number, the lowest values were for the 50 and 100 mg/ml groups, with a dose response evident (Fig. 8A,iv and Fig. 8B). While at lower concentrations of nanoparticles (3.125 and 6.25 mg/ml), we observed a noticeable enhancement in cell count and morphology (Suppl. Fig. 16).

Discussion

In this study, we successfully encapsulated MO and the purified TK

into spherical chitosan nanoparticles (average 300–500 nm diameter) using the ionic gelation technique. The oil-loaded nanoparticles demonstrated good biocompatibility and antimicrobial activity, highlighting their potential as a drug-delivery system.

Nanoparticles can be precisely controlled in size, shape, and surface charge, which influence treatment outcomes. Particle size is inversely related to drug loading capacity, with smaller particles providing greater encapsulation efficiency.³⁴ A particle size of around 100 nm is considered optimal for cellular interaction.³⁵ Conversely, larger particles may enhance drug delivery efficiency. Huang et al. reported increased vancomycin loading in PLGA/PLGA-PE nanoparticles sized 350–500 nm.³⁶ Similarly, higher encapsulation efficiency was observed as PLGA particle size increased from 160 to 230 nm.³⁷ Nanoscale delivery systems are predominantly spherical, as this geometry enhances stability, lowers surface free energy, improves drug adsorption and encapsulation, and facilitates mobility in biological systems.³⁸

Zeta potential is an important indicator of nanoparticle stability and affects initial adsorption and cell entry.³⁹ Positively charged nanoscale particles can overcome systemic and cellular barriers and serve as effective vehicles for active agents. Kure et al. reported MO nanocapsules within the same range as our nanoparticles,²⁴ attributing the negative charge of MO nanoentities (−12 to −10 mV) to anionic ions from sodium alginate. In contrast, all our formulations were positively charged (+5 to +30 mV), which can be attributed to the cationic nature of the chitosan wall. Similar results were reported by Yilmaz et al.⁴⁰ for chitosan nanoparticles loaded with *Origanum vulgare* essential oil (+25 to +47 mV). Overall, high zeta potential values are generally required for nanosystem stability.⁴¹

Ionic gelation is based on the reaction between chitosan NH_3^+ and TPP phosphoric ions in an acidic environment, forming an opalescent suspension.⁴² The cationic nature of chitosan drives interaction with anions (e.g., phosphate, sulphate), initiating gelation with TPP. This method was used to synthesise MO- and TK-loaded chitosan nanoparticles. Although simple, control of particle size and uniformity remains challenging. Maintaining pH < 6.5 enhances solubility and amine protonation, contributing to mucoadhesive and antimicrobial properties. However, pH < 5.5 reduces particle size (~100 nm) and increases PDI.⁴³ In this experiment, adding TPP at pH > 5.5 produced no turbidity. Thus, chitosan-MO/TK suspensions were crosslinked at pH 5.5 to achieve consistent size and dispersity. Alkaline TPP (pH 9) resulted in larger particles, higher yield and enhanced encapsulation efficiency.

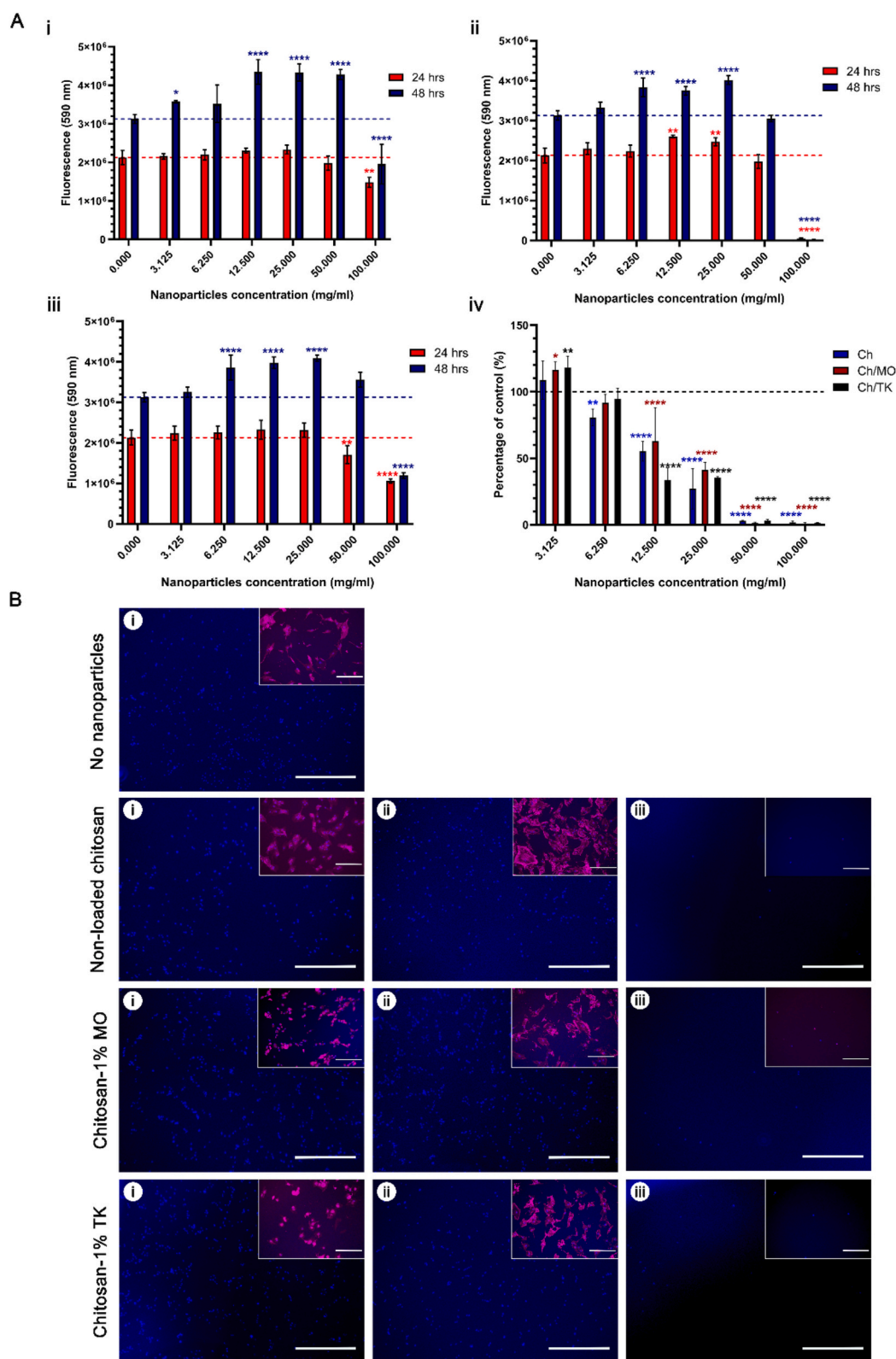


Fig. 8. Cytotoxicity of chitosan nanoparticles on human gingival fibroblasts (HGFs). (A) Fluorescence values of PrestoBlue® after 48 h incubation. (i) Non-loaded chitosan nanoparticles. (ii) Chitosan-1% MO nanoparticle. (iii) Chitosan-1% TK nanoparticles. (iv) Percentage of control based on the cell count from DAPI images after 72 h incubation. Statistical analysis using two-way ANOVA with Uncorrected Fisher LSD test, mean $\pm$ SD; * $P < 0.05$, ** $P < 0.01$, **** $P \leq 0.0001$. Ch = non-loaded chitosan nanoparticles, MO = chitosan-1% MO nanoparticles, TK = chitosan-1% TK. The dotted line represents control (non-treated cells growing on tissue culture plastic). (B) HGFs stained with phalloidin and DAPI after 72 h incubation. Images labelled (i) represent cells treated with nanoparticles at a concentration of 12 mg/ml, images labelled (ii) represent cells treated with nanoparticles at a concentration of 25 mg/ml and images labelled (iii) represent cells treated with 50 mg/ml. Scale bar for DAPI images is 750 μ m. Scale bar for inset Phalloidin images is 300 μ m.

MO/TK-loaded nanoparticles showed an initial burst release in the first hour of incubation in PBS, followed by a slower, sustained release over 24 h. This can be explained by desorption of oil molecules from the particle wall during the early stage, compared to later release driven by oil diffusion. Similar findings were reported for chitosan nanoparticles loaded with BSA.⁴⁴ The final mass ratio had a limited effect on particle size, PDI and aggregation behaviour. At pH 5.5, a chitosan: TPP ratio of 2.5:1 v/v (5:1 mass ratio) produced particles with improved size distribution compared to acidic solutions (pH 3–4).⁴⁴

Freeze-drying improves the long-term stability of nanoparticle formulations. However, freezing- and dehydration-induced stresses can compromise nanoparticle stability, promote aggregation, and reduce storage life. Trehalose, a disaccharide cryoprotectant, outperforms other sugars in preserving nanoparticle structure after lyophilisation due to its low hygroscopicity, flexible hydrogen-bonding, and low chemical reactivity.⁴⁵ A concentration of 5% v/v provided effective protection for our formulations. DLS results (Table 3) show minimal changes in particle size and PDI in non-loaded and 1% oil-loaded chitosan particles, consistent with previous studies.^{45–47} Trehalose was more critical than the surfactant, as higher particle size and PDI were observed in its absence in the absence, despite increasing the Tween-80 from 1.25% to 2% (Table 3H).

Our results suggest a minimal effect from altering the concentration of loaded MO and TK on the average particle size. However, we observed an inverse relationship between oil concentration and their encapsulation efficiency for both MO and TK nanoparticles. The efficiency of encapsulation decreased to half as the oil concentration increased from 0.5 to 1.5% due to encapsulation limitations. Similar findings were reported by Nguyen¹⁶ and Turan and Aydin⁴⁸ as they studied factors that can affect the entrapment efficiency of essential oils. They indicated that at low concentrations, the amount of oil is very low compared to the polymer. As the oil loading increases, the encapsulation efficiency can increase up to the saturation status; if exceeded, the encapsulation efficiency will decrease.

Considering the sensitivity of MO and TK to ambient conditions, including light, temperature and acidity, chitosan encapsulation protects and stabilises the active components in the biological environment. To expand clinical applications, we assessed nanoparticle stability and size changes at low pH 2–3 (simulating stomach acid)⁴⁹ or infected wounds (pH 5–5.5).⁵⁰

Results indicated nanoparticle stability during the time required for complete oil release. Previous studies discussed possible adverse effects of low pH on EOs, causing hydrolysis, isomerisation (hydrocarbon structure alteration) and accelerating oil oxidation.⁵¹

There is growing evidence supporting the strong antimicrobial activity of MO against a wide range of pathogens, primarily through bacterial cell wall disruption, lysis, and morphological alterations.⁵² MO inhibits the growth and adhesion of oral plaque species, *P. gingivalis* and *S. mutans*,⁵³ as well as *Streptococcus sanguinis*, *Staphylococcus epidermidis*, and *E. coli*,²⁵ *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Proteus mirabilis*.⁵⁴ Compared with tea tree, eucalyptus, lavender, and rosemary oils, lower concentrations of MO are required to achieve inhibitory and bactericidal effects against oral pathogens.¹⁹

The antimicrobial effect of non-encapsulated oil was discussed in limited previous studies, with an MBC concentration of 0.25 µg/ml against *S. mutans* JC2⁵⁵, an MIC of 0.513% v/v against *S. aureus*⁵⁶ and 480 µg/ml against *S. sobrinus*.⁵⁷ Antimicrobial efficiency was obtained against Gram-negative species as well, such as *P. gingivalis* (0.06–0.03%), *F. nucleatum* ATCC 25586 (µg 0.03) and *A. actinomycetecomitans* (0.13%),⁵³ and a wide range of bacterial strains.⁵⁸ Our team has previously reported the bactericidal activities of mānuka oil via the microbroth dilution method, as it showed an inhibitory effect against dental plaque species at concentrations between 0.1 and 0.01% w/w.⁹ For the current formulations, we roughly estimated the oil concentration based on the initial incorporation. The closest estimate as about 17 µl/ml (equivalent to 100 mg/ml of freeze-dried

nanoparticles) and we performed serial dilution to obtain the same range of nanoparticle concentrations presented in Table 5 and Suppl. Fig. 14. The MIC results for non-encapsulated oil are presented in Suppl. Fig. 15.

The chemical composition of the MO in this study is consistent with reports for *Leptospermum scoparium*, with East Cape-harvested plants exhibiting the highest TK content (~29%).⁹

The Gas chromatography analysis results indicated 3-times higher levels of TKs in the purified form (99.74%) compared to MO. This can explain the results from the antimicrobial assay, as half the concentration of TK-loaded nanoparticles (6.25 mg/ml) could inhibit the growth of *S. aureus* and *S. mutans* compared to MO-loaded nanoparticles (12.5 mg/ml). The tested Gram-negative species exhibited higher sensitivity to MO- and TK-loaded nanoparticles, in contrast with the findings of Christoph, Kaulfers.⁵⁹ The antimicrobial activity of chitosan alone was described against a wide variety of fungi and bacteria.⁶⁰ Our results confirm the previous findings and highlight the synergistic antimicrobial activity achieved by using chitosan as a nanocarrier system for MO and TK. In alignment with previous studies, much lower concentrations were required to obtain a bactericidal effect from free, non-encapsulated oil (MBC = 0.53 µl/ml) of MO and TK. However, nanoencapsulation with a biocompatible polymer like chitosan provides sustained oil release to inhibit bacterial growth and reduce the potential cytotoxic effect.

Another important aspect is biocompatibility. Without encapsulation, MO (≥ 0.1% v/v) was reported to be cytotoxic to HGFs, causing a 50% reduction in cell viability within 48 h.⁹ Another study reported a dose-dependent reduction in viability and proliferation of human fibroblasts (CUA-4) and fibrosarcoma cells (HT-1080) following exposure to MO, along with significant cleavage of poly (ADP-ribose) polymerase (PARP) in both cell lines, indicating apoptosis.⁶¹ Thus, incorporating MO and TK into biocompatible materials such as chitosan may reduce cytotoxicity while enabling delivery of higher therapeutic concentrations. Oil-loaded nanoparticles at concentrations up to 25 mg/ml enhanced metabolic activity compared to control, whereas morphological changes and a significant reduction in cell count were observed at 50 and 100 mg/ml Fig. 8A,vi and Fig. 8B.

This study is limited to in vitro findings; further work is required to demonstrate that nanoencapsulated oil can be effectively delivered from a suitable carrier in an in vivo wound model while maintaining biocompatibility, antimicrobial efficacy and healing potential.

To conclude, MO and TK nanoparticles showed favourable stability, biocompatibility and antimicrobial activity. These nanoparticles can be incorporated into a wide variety of biomedical devices, including tissue regeneration membranes or scaffolds, as a drug delivery system to improve treatment outcomes.

CRedit authorship contribution statement

Ghsaq Alhamdani: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dina Abdelmoneim:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Conceptualization. **Natalie J. Hughes:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Warwick J. Duncan:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Conceptualization. **Chen Chen:** Writing – review & editing, Visualization, Methodology, Investigation. **Niki Hazelton:** Writing – review & editing, Visualization, Methodology, Data curation. **Kai Chun Li:** Writing – review & editing, Supervision, Software, Methodology, Investigation, Conceptualization. **Amira Salem:** Writing – review & editing, Validation, Methodology. **Dawn E. Coates:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors occasionally used Grammarly and Microsoft 365 Copilot for proofreading and grammar corrections. The authors reviewed and edited the output as needed and take full responsibility for the content of the published article.

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Declaration of competing interest

Ghsaq Alhamdani, Dina Abdelmoneim, Natalie J. Hughes, Warwick J. Duncan, Chen Chen, Amira Salem and Dawn E. Coates are inventors on the “Novel antimicrobial compositions” patent that is related to the nanoparticles investigated in this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nano.2026.103013>.

Data availability

The authors confirm that the data for this study are available within the article and the supplementary information.

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