



# Synthesis of CNP-SAR405 delivery system for improved therapeutics anticancer activity using A549 human lung cancer cell lines

Ruqayyah A. Bashirah<sup>1</sup> · Nur Balqis Zamri<sup>1,3</sup> · Noorjahan Banu Alitheen<sup>1</sup> · Saila Ismail<sup>2</sup> · Mas Jaffri Masarudin<sup>1,3</sup>

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## Abstract

Autophagy, a critical homeostatic process, is increasingly implicated in cancer progression and therapy resistance. SAR405 is a potent inhibitor of the autophagy related PIK3C3/VPS34 complex, offering potential as an anticancer agent. This study reports the synthesis, characterization, and biological evaluation of SAR405-loaded chitosan nanoparticles (CNP-SAR405) designed to improve therapeutic delivery and efficacy in A549 human lung carcinoma cells. CNPs were prepared via ionic gelation using chitosan and sodium tripolyphosphate (TPP), yielding stable monodisperse nanoparticles ~ 77.4 nm, PDI ~ 0.2). Upon SAR405 encapsulation, nanoparticle size increased to ~ 110 nm while maintaining uniform distribution. Encapsulation efficiency reached 80% at 200 nM SAR405, confirmed by UV–Vis spectroscopy. Morphological analyses using FESEM and TEM verified spherical nanoparticle structures, while FTIR confirmed successful SAR405 incorporation. FITC-labelling enabled real-time tracking of intracellular uptake, revealing detectable internalization as early as 12 h post-treatment, with fluorescence intensity peaking at 72 h. In vitro cytotoxicity assays demonstrated enhanced anticancer efficacy of CNP-SAR405 compared to free SAR405, CNP-SAR405 achieved similar cytotoxic effects at 69 nM compared to 100 nM for free SAR405 in A549 cells. Furthermore, co-treatment with the autophagy inducer Torin-2 validated that CNP-SAR405 more effectively inhibited autophagosome formation than SAR405 alone, particularly at the 24-h mark. These findings underscore the potential of chitosan nanoparticle-mediated delivery to increase SAR405 bioavailability and anticancer potency while achieving comparable cytotoxic at a lower dose than free SAR405. The CNP-SAR405 formulation represents a promising nanotechnology-driven approach to targeted lung cancer therapy. All experiments were performed in triplicate biological replicates with technical triplicates, and data were analysed using one-way ANOVA followed by Tukey's multiple comparison post-hoc test ( $p < 0.05$  considered significant).

**Keywords** Anticancer · Autophagy · Chitosan nanoparticles · Nanomedicine · SAR405

## Introduction

Autophagy is a highly conserved catabolic process that plays a pivotal role in maintaining cellular homeostasis by recycling cytoplasmic components such as damaged organelles and misfolded proteins. This lysosome-mediated degradation pathway is critical for cell survival under conditions of nutrient deprivation, oxidative stress, or hypoxia. Beyond its roles in development, immunity, and aging, autophagy is also implicated in neurodegenerative diseases, infections, and cancer. In oncology, autophagy can suppress tumours early by limiting genomic instability, but in advanced stages it often supports cancer cell survival, helping them resist therapy and endure hostile conditions. Studies have shown that autophagy is frequently induced in various cancers following treatment

✉ Mas Jaffri Masarudin  
masjaffri@upm.edu.my

<sup>1</sup> Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

<sup>2</sup> Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

<sup>3</sup> Nanomaterials Synthesis and Characterization Laboratory, Institute of Nanoscience and Nanotechnology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

and contributes to chemoresistance by interacting with signalling pathways such as PI3K/AKT and MAPK (Jalali et al. 2025; Mehta et al. 2024; Ho & Gorski, 2019). Depending on cellular context, autophagy may either promote apoptosis or facilitate survival, highlighting its complex, dualistic nature in tumour biology (Parzych & Klionsky, 2014). Understanding and modulating autophagy in cancer has therefore become a critical focus for therapeutic development.

Traditional cancer treatments have largely focused on eradicating malignant cells by inducing apoptosis or disrupting mitotic processes through DNA damage, radiation, or chemotherapeutics. However, accumulating evidence suggests that targeting cancer cell survival mechanisms, such as autophagy, presents a complementary and potentially more sustainable strategy. Rather than focusing solely on killing tumor cells, this approach seeks to disrupt the intracellular mechanisms that confer treatment resistance, allowing existing therapies to be more effective. Targeting adaptive stress response pathways may result in longer-lasting therapeutic outcomes and decreased likelihood of relapse. Strategies that disrupt cellular adaptation, including nutrient sensing, protein degradation, and stress-induced signalling are emerging as key components of next-generation cancer therapies (Zhang et al., 2022). While autophagy's role in cancer progression and therapy resistance is well documented, few studies have explored the use of nanocarrier systems to improve delivery of selective autophagy inhibitors. This work develops SAR405-loaded chitosan nanoparticles to address solubility and delivery challenges, aiming to improve anticancer efficacy in lung carcinoma cells.

Nanoparticle-mediated drug delivery offers a promising avenue for implementing these non-conventional therapeutic strategies. Previous work in A549 cells has shown that nanoparticle-mediated co-delivery of chemotherapeutics and natural compounds can synergistically enhance anticancer effects (Sachdeva et al. 2023). For example, co-delivery of erlotinib and resveratrol via nanostructured lipid carriers inhibited cell proliferation and induced ROS-mediated apoptosis (Mahoutforoush et al. 2022). These findings support the potential of nanocarrier platforms to improve drug bioavailability and combinatorial therapy outcomes in NSCLC. Chitosan nanoparticles (CNPs) are notable for their biocompatibility, biodegradability, mucoadhesiveness, and capacity to encapsulate both hydrophilic and hydrophobic drugs (Biswas et al. 2025). Chitosan's cationic nature allows for efficient binding to negatively charged cellular membranes, facilitating improved drug internalization and controlled release (Islam et al. 2025). CNPs have been successfully applied in various biomedical fields, including antimicrobial therapy, gene delivery, vaccine development, and notably, cancer treatment. Recent studies show CNPs improve delivery of poorly soluble or unstable phytochemicals and chemotherapeutics, enhancing efficacy while reducing systemic toxicity (Liu et al. 2025; Yoo et al. 2011). Notably,

several studies by Masarudin et al. 2015 have advanced the application of chitosan nanoparticles in drug and gene delivery systems.

In this study, we encapsulated SAR405, a selective autophagy inhibitor targeting the class III PI3K complex, into chitosan nanoparticles using the ionic gelation method. Our primary aim was to improve SAR405's solubility, stability, and intracellular delivery in A549 human lung carcinoma cells, thereby enhancing its therapeutic efficacy.

## Methods

### Materials

Chitosan (CS, low molecular weight), sodium tripolyphosphate (TPP), SAR405 VPS34 inhibitor (Chemical formula:  $C_{19}H_{21}ClF_3N_5O_2$ ), fluorescein 5(6)-isothiocyanate (FITC), thiazolyl blue tetrazolium bromide, 4,6-diamidino-2, glacial acetic acid, sodium hydroxide and hydrochloric acid (analytical grade) were obtained from Sigma-Aldrich, USA. RPMI media 1640, antibiotic-antimycotic (100X), fetal bovine serum and phosphate buffer saline were purchased from Gibco, Life Technologies, USA. Sodium hydroxide pellet, 37% hydrochloric acid, acetic acid glacial, dimethyl sulfoxide and 37% formaldehyde were obtained from Friedmann Schmidt. All reagents, unless stated otherwise, were used without further purification.

### Synthesis of nanoparticles

#### Synthesis of chitosan nanoparticles (CNPs)

CNPs were prepared by ionic gelation method as previously described by Masarudin et al. (2015). Briefly, CS solution and TPP solution were prepared to 1.0 mg/ml concentration by dispersing 25 mg of chitosan and TPP powder in 25 ml of sterile distilled water, exclusively. The resulting CS and TPP solutions were then subsequently diluted to 0.5 mg/ml and 0.7 mg/ml respectively, before being adjusted to pH 5 (CS solution) and pH 2 (TPP solution) using 1 M of NaOH and 1 M of HCl. CNP were subsequently formed by mixing 600  $\mu$ L of CS solution (0.5 mg/ml) with 200  $\mu$ L of TPP solution (0.7 mg/ml). The nanoparticles were then collected by centrifugation at 13,000 rpm for 20 min. Following centrifugation, the top 320  $\mu$ L of the supernatant, containing the monodisperse nanoparticle fraction was carefully collected, while larger aggregates sedimented. This method, as described by Robertson et al. (2016), uses differential centrifugation to enhance size uniformity by selectively isolating the less-aggregated particles, and mixed with 480  $\mu$ L of sterile distilled water (dH<sub>2</sub>O) and used for further experimentation.

### Synthesis of SAR405-encapsulated CNP (CNP-SAR405)

A 3 mM of SAR405 master stock solution (Calbiochem, China) was prepared by dissolving 5 mg SAR405 powder in 4.99 mL of RPMI 1640 media (Gibco, Grand Island, NY, USA) containing 0.03% DMSO. The 3 mM stock was further diluted to 1  $\mu$ M working concentration by diluting the stock prepared in RPMI 1640 media (Gibco, Grand Island, NY, USA). A working concentration of 1  $\mu$ M SAR405 was chosen based on prior  $IC_{50}$  reports for lung cancer cell lines and preliminary cytotoxicity testing in A549 cells, which showed that higher concentrations induced non-specific toxicity. A predetermined volume of 1  $\mu$ M of SAR405 working concentration was prepared and added dropwise to 0.5 mg/ml of 600  $\mu$ L of freshly prepared CS solution. The mixture was left for 5 min to allow the compound to interact with active sites of CS. To form CNP- SAR405, 200  $\mu$ L TPP solution (0.7 mg/ml) was dropwise added into the solution and left for another 5 min at 25°C.

### Synthesis of FITC-CNP and FITC-CNP-SAR405

#### Synthesis of FITC labelled CNPs

FITC labelled CNPs were prepared to track the movement of nanoparticles in vitro. The nanoparticles were fluorescently labelled with FITC via minor modification on the surface of CNPs. Briefly, 0.5 mg/ml of CS solutions were prepared as previously described. Approximately 200  $\mu$ L of 0.25 mg/ml of FITC were added dropwise to the tube containing CS

solutions prior to the addition of 0.7 mg/ml of TPP solutions. Freshly synthesized FITC-CNP and FITC-CNP-SAR405 were used onto A549 cell line for in vitro tracking of nanoparticles. Each batch used for treatment on cells were freshly prepared.

#### Synthesis of FITC labelled CNP-SAR405

For the synthesis of FITC-CNP-SAR405, a solution of FITC (0.25 mg/ml) was dropwise added to the tube containing CS solution (0.5 mg/ml), prior to the addition of 1  $\mu$ M of SAR405 and TPP solution (0.7 mg/ml). Freshly synthesized FITC-CNP-SAR405 was used directly onto A549 cells for in vitro nanoparticles tracking analysis.

### Physico-chemical analysis of synthesized nanoparticles

#### Determination of encapsulation efficiency (%EE) of SAR405 into CNP-SAR405

The %EE for SAR405 in CNP-SAR405 was performed to determine the percentage of SAR405 encapsulated inside CNP-SAR405. Sample preparation was as described in Sect. 3.2.2 and were prepared fresh before the analysis.

To determine the encapsulation efficiency of SAR405 encapsulated into CNP, CNP-SAR405 samples were centrifuged at 13,000 rpm for 20 min. The supernatant was collected and was read at A285nm using a UV/VIS spectrometer (NP80, München, Germany). The %EE was calculated using the following formula:

$$\text{Encapsulation Efficiency (\%)}_{\text{Supernatant}} = \frac{A_{285 \text{ of free SAR405}} - A_{285 \text{ of SAR405}}}{A_{285 \text{ of free SAR405}}} \times 100\% \quad (1)$$

### Particle size distribution and polydispersity analysis of nanoparticles

Particle size distribution and polydispersity analysis of nanoparticles were performed to evaluate the size distribution and the polydispersity index (PDI) of synthesized nanoparticles. The size and distribution of synthesized CNPs and CNP-SAR405 were evaluated through DLS using a Malvern Zetasizer Nano S Instrument (Malvern Instruments, Worcestershire, UK). Approximately 500  $\mu$ L of sample was loaded into a disposable cuvette and left to equilibrate for 5 min. An equilibration time of 30 s were needed to ensure sample temperature is equal to cell area temperature and analysis was measured at a measurement angle of 175° at 25°C. Samples were always prepared fresh before each measurement. Each sample was analyzed using three reading cycles and each cycle consists of nine measurements to

ensure samples were stabilized. A total of three batches ( $N=3$ ) were used in this study to obtain average size and PDI.

### Morphological analysis of nanoparticles using electron microscopy

The morphological analyses of nanoparticles using Field-Emission Scanning Electron Microscopy (FESEM) and Transmission Electron Microscopy (TEM) were conducted to determine surface morphology and size estimation of both CNP and CNP-SAR405. The surface morphology and size estimation of CNP and CNP-SAR405 were determined by using field-emission scanning electron microscopy (FESEM). CNPs and CNP-SAR405 were prepared and diluted prior analyses by diluting 10  $\mu$ L samples into 90  $\mu$ L dH<sub>2</sub>O. Approximately 10  $\mu$ L of each sample was added

dropwise onto the surface of an aluminium stub and left to dry for 4 days in a dryer. Dried stubs containing the samples were then coated with gold prior analysed using FEI Nova NanoSEM 230 FESEM (Kensington, Sydney, Australia).

Surface morphology and size estimation analysis of CNP and CNP-SAR405 were also analysed using TEM. This is to analyse morphological images both on the surface as well as the inner structure of samples. CNP and CNP-SAR405 were freshly prepared before placed on the surface of copper grid. Samples were left overnight under direct heat source to let dry prior placed and analysed under TECNAI G2 F20 (Pleasanton, CA, USA) transmission electron microscope utilizing voltages from 20 to 200 kV and standard magnification from 22× to 930 k×. Images were acquired using an SC1000 ORIUS CCD (Pleasanton, CA, USA) camera.

#### **Determination of free functional groups of samples using fourier transform infrared analysis (FTIR)**

FTIR analysis was used to determine the presence of free functional groups and the functional groups presence in CS, TPP, CNP, CNP-SAR405 samples. Prior to FTIR analysis, samples were freeze dried using a COOL safe 95–15 PRO freeze drier (SCANVAC, UK) for approximately 48 h. Samples in powder form were then analysed using a Spectrum 100 (Perkin Elmer, USA) using attenuated total reflectance (ATR) method at an infrared frequency range of 200–4000  $\text{cm}^{-1}$ . Following acquisition of IR spectra, the results were subsequently recorded in table form.

#### **Statistical Analysis**

All experiments were performed in triplicate ( $n=3$  independent replicates). Data are expressed as mean  $\pm$  standard deviation (SD). Statistical significance was assessed using one-way ANOVA followed by Tukey's post-hoc test. A  $p$ -value  $< 0.05$  was considered statistically significant.

#### **In vitro study of A549 cell line treatment using CNPs and CNP-SAR405**

##### **Establishment and maintenance of A549 human lung cancer cell line**

Human lung cancer cell (A549 cell line) was cultured in T75 flasks and were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% antibiotics/antimycotics solution (Gibco, Grand Island, NY, USA). Cultured cells were placed in a humidified incubator at 37°C with 5%  $\text{CO}_2$ . *In-vitro* study was conducted for 72 h due to limitations in terms of nutrient supply within media and cell-plate used throughout this study.

#### **Determination of in vitro cellular cytotoxic effects using mtt assay**

MTT assay was performed to evaluate toxicity of the synthesized compounds toward the A549 cells. Prior to each treatment, cells were seeded into 96-well plate at a density of  $8 \times 10^3$  cells per well and allowed to grow for 24 h. Cells were treated with CNP, CNP-SAR405, Torin, Rapamycin and SAR405 for treatments at specific timeframes, including 0 h, 24 h, 48 h, and 72 h. Sample were prepared and diluted via serial dilution using autoclaved distilled water from the highest concentration of 0.5 mg/ml for CNP and CNP-SAR405, 50 nM for Torin, 2  $\mu\text{M}$  for Rapamycin and 500 nM for SAR405. A stock of 5 mg/ml MTT were prepared prior to the assay in Phosphate Buffer Saline (PBS) (Gibco, Grand Island, NY, USA) and were further diluted into 0.5 mg/ml using RPMI 1640 media (Gibco, Grand Island, NY, USA). After 72 h post treatment, media containing samples were discarded and was washed twice with PBS (Gibco, Grand Island, NY, USA). A 100  $\mu\text{l}$  of 0.5 mg/ml thiazolyl blue tetrazolium bromide solution was added into each well, the plate was covered with aluminum foil and were incubated for 4 h at 37 °C. Following incubation, the thiazolyl blue tetrazolium bromide solutions were removed and a 100  $\mu\text{l}$  of DMSO were added into each well. The thiazolyl blue tetrazolium bromide absorbance then was read at 570 nM using Bio-Rad iMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA).

The second MTT assay was conducted to evaluate toxicity level of co-treatment of different concentration of Torin-2 with CNP-SAR405 towards A549 cells. Prior to each treatment, cells were seeded into 96-well plate at a density of  $8 \times 10^3$  cells per well and allowed to grow for 24 h. Cells were treated with Torin-2 and CNP-SAR405 at specific timeframes, including 0 h 24 h, 48 h, and 72 h. Torin-2 was prepared and diluted to 200 nM and 20 nM respectively from a stock of 2  $\mu\text{M}$ , while CNP-SAR405 was prepared and diluted via serial dilution using autoclaved distilled water from the highest concentration of 0.5 mg/ml. A stock of 5 mg/ml thiazolyl blue tetrazolium bromide were prepared prior to the assay in PBS (Gibco, Grand Island, NY, USA) and were further diluted into 0.5 mg/ml using RPMI 1640 media (Gibco, Grand Island, NY, USA). After 72 h post treatment, media containing samples were discarded and was washed twice with PBS (Gibco, Grand Island, NY, USA). A 100  $\mu\text{l}$  of 0.5 mg/ml thiazolyl blue tetrazolium bromide solution was added into each well, the plate was covered with aluminium foil and were incubated for 4 h at 37°C. Following incubation, the thiazolyl blue tetrazolium bromide solutions were removed and a 100  $\mu\text{l}$  of DMSO were added into each well. The thiazolyl blue tetrazolium bromide absorbance then was read at 570 nM using Bio-Rad iMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA). All assays

were performed in triplicate (three biological replicates), with each experiment repeated independently at least three times. After incubation with MTT solution, formazan crystals were dissolved in DMSO and absorbance was measured at 570 nm using a microplate reader. Background absorbance at 630 nm was subtracted prior to analysis. Cell viability was calculated as a percentage relative to untreated control cells. Data are presented as mean  $\pm$  standard deviation (SD). Statistical significance was assessed using one-way ANOVA followed by Tukey's post-hoc test (GraphPad Prism v9.0), with  $p < 0.05$  considered significant. For all assays, free SAR405 was tested at concentrations equivalent to those encapsulated in CNP-SAR405, and blank CNPs (without drug) were included as nanoparticle controls. Untreated cells served as negative controls.

#### Determination of intracellular localization of FITC-CNP and FITC-CNP-SAR405

The accumulation of FITC-CNP and FITC-CNP-SAR405 was qualitatively determined using IX3P2F/Olympus fluorescence inverted microscope (Olympus, Germany). Briefly, A549 cells were cultured in T75 flasks and were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% antibiotics/antimycotics solution (Gibco, Grand Island, NY, USA). Cultured cells were placed in humidified incubator at 37°C with 5% CO<sub>2</sub>. In vitro accumulation analysis was performed to evaluate accumulation of both FITC- CNPs and FITC-CNP-SAR405 using IX3P2F/Olympus fluorescence inverted microscope (Olympus, Germany).

Cells were seeded into 12-well plate at a density of  $5 \times 10^4$  cells per well and were allowed to grow for 24 h prior treatment. Cells were treated with FITC-CNP and FITC-CNP-SAR405 for treatments. Each sample was prepared and diluted to the optimum concentration of 0.5 mg/ml for CNP and FITC-CNP, 0.25 mg/ml for FITC, 100 nM for SAR405, CNP-SAR405 and FITC-CNP-SAR405. After 72 h post treatment, media containing samples were discarded and washed with PBS (Gibco, Grand Island, NY, USA) to ensure no traces of samples were left. Prior to visualization, approximately 2 ml of formaldehyde (4% v/v) were added into each well and were incubated for 10 min. Following incubation, neutral buffered formalin was removed and washed with PBS (Gibco, Grand Island, NY, USA) twice. In each well, approximately 2 drops of NucBlue™ Fixed Cell ReadyProbes™ Reagent (DAPI) (Life Technologies, Grand Island, NY) and ActinRed™ 555 ReadyProbes™ Reagent (Rhodamine phalloidin) (Life Technologies, Grand Island, NY) was used in each well and were incubated for 30 min at room temperature prior imaging. After incubation, DAPI

and actin red were washed using  $1 \times$  autoclaved PBS (Gibco, Grand Island, NY, USA) twice. The accumulation of FITC-CNPs and FITC-CNP-SAR405 were observed under fluorescence microscope using DAPI fluorescence filter at 360 nm excitation wavelength and mCherry fluorescence filter at 540 nm excitation wavelength respectively.

#### Detection of autophagosomes using flow cytometric analysis autophagy detection using flow cytometry analysis

A cyto-ID autophagy detection kit was used to detect autophagy process with presence of autophagosomes. A549 cell line were cultured in T75 flasks and were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% FBS (Gibco, Grand Island, NY, USA) and 1% antibiotics/antimycotics solutions (Gibco, Grand Island, NY, USA). Cultured cells were placed in humidified incubator at 37°C with 5% CO<sub>2</sub>.

Cells were seeded into 12-well plate at a density of  $5 \times 10^4$  cells per well and were allowed to grow for 24 h prior treatment. Cells were co-treated with SAR405 and Torin-2 and CNP-SAR405 and Torin-2. Each sample was prepared and diluted to the concentration of 0.5 mg/ml for CNP, 20 nM for Torin and 200 nM for SAR405. After 72 h post treatment, media containing samples were discarded and was washed twice using  $1 \times$  autoclaved PBS (Gibco, Grand Island, NY, USA). A 500  $\mu$ l of Trypsin was added into each well, followed by incubation at 37°C for 5 min. Cells were then transferred into new tubes and centrifuged at 1000 rpm for 5 min. Supernatant from each tube were removed and CYTO- ID® Autophagy Detection Kit (ENZ-51031) (Enzo Life Sciences) was added into each tube for 30 min incubation in the dark. All samples were analyzed using NovoCyte Flow Cytometry Instrument (Acea Biosciences Inc., San Diego, USA).

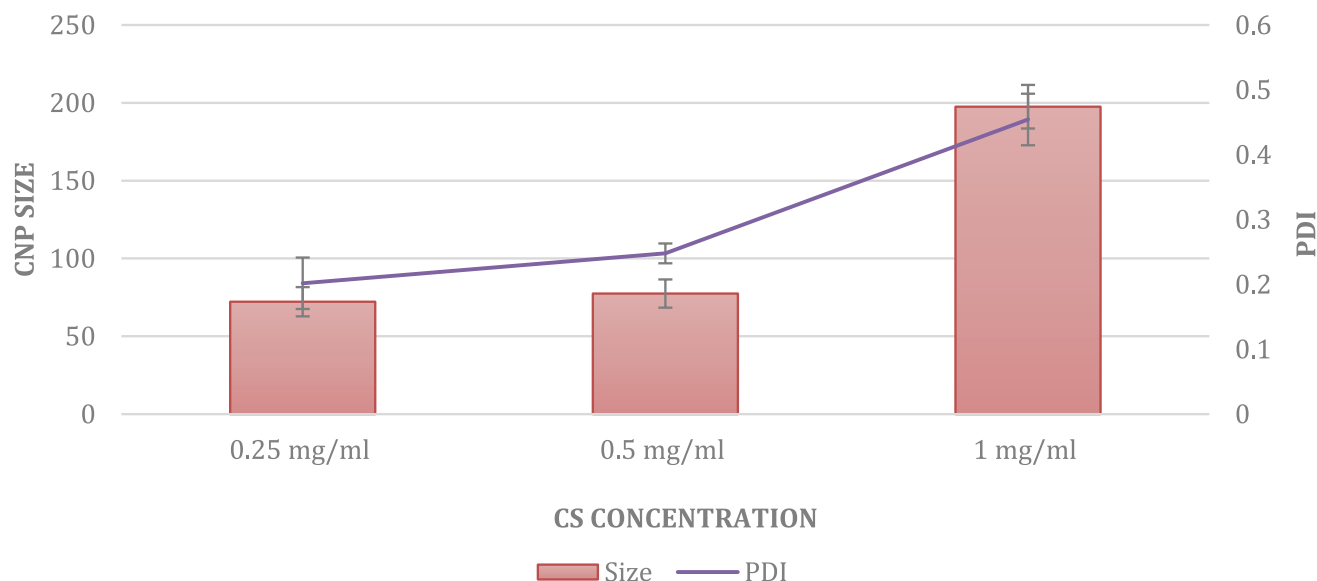
## Results and Discussion

### Synthesis of CNPs and CNP-SAR405

#### Size and polydispersity index of synthesized nanoparticles

The formation of chitosan nanoparticles (CNPs) occurred through crosslinking between chitosan (CS) with sodium tripolyphosphate (TPP), a process known as ionic gelation. Figure 1 presents the effect of three different chitosan (CS) concentrations on the formation of CNPs with a target size of  $< 100$  nm and low polydispersity index (PDI). Figure 1 shows the size of synthesized CNPs using three different concentrations of CS. This was done to determine the optimal CS concentration and formulation to produce

## Synthesis of CNP



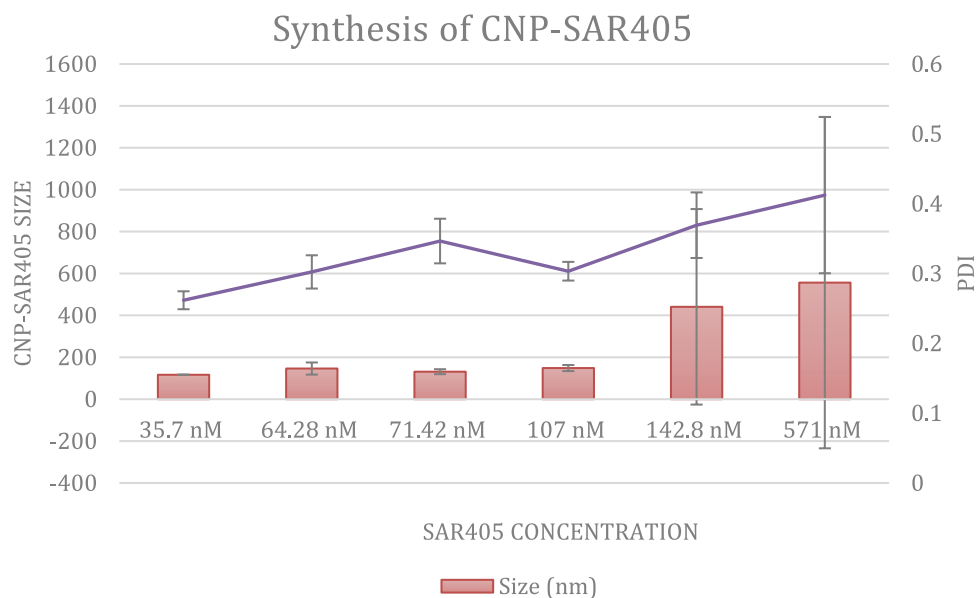
**Fig. 1** Effect of chitosan concentration on CNP particle size and polydispersity index (PDI). Formulations with 0.25 mg/mL and 0.5 mg/mL CS yielded particles < 100 nm with low PDI (0.20–0.25),

indicating stability and uniformity, while 1 mg/mL CS produced larger, less uniform particles (> 100 nm, PDI 0.40). Data represent mean  $\pm$  SD,  $n=3$ ,  $p<0.05$

nanoparticles < 100 nm with a low value of PDI. The parameter CNP-F1 (0.25 mg/ml of CS with 0.7 mg/ml of TPP) produced CNPs resulting in 72.2 nm and a PDI of 0.2. The parameter CNP-F2 (0.5 mg/ml of CS with 0.7 mg/ml of TPP) produced CNPs of 77.4 nm in particle size, and a PDI of 0.2, while parameter CNP-F3 (1 mg/ml of CS with 0.7 mg/ml of TPP) produced nanoparticles with a diameter size of 197.5 nm with a PDI of 0.4. The smaller particle

sizes observed at 0.25 mg/mL and 0.5 mg/mL CS are attributed to the lower amount of free CS polymer available for interaction, compared to the 1 mg/mL concentration. The excess CS in the 1 mg/mL formulation likely contributed to uncontrolled cross-linking with TPP, producing larger and less uniform particles. The low PDI values of 0.20 and 0.25 at 0.25 mg/mL and 0.5 mg/mL CS, respectively, indicate high stability and a narrow particle size distribution. In

**Fig. 2** Effect of SAR405 concentration on the size and PDI of CNP-SAR405 formulations. Increasing SAR405 concentration from 35.7 nM to 571 nM progressively enlarged particle size (116 nm to 556 nm) and reduced uniformity. Optimal stability was observed between 35.7–107 nM. Data represent mean  $\pm$  SD,  $n=3$ ,  $p<0.05$



contrast, the higher PDI value of 0.40 at 1 mg/mL suggests reduced uniformity and stability, possibly due to increased polymer entanglement and random cross-linking.

Based on both size and homogeneity, the 0.5 mg/mL CS concentration was selected for subsequent experiments. Figure 2 illustrates the impact of six different SAR405 concentrations on the synthesis of CNP-SAR405 formulations. At the lowest concentration of 35.7 nM SAR405, the resulting CNP-SAR405 had a size of 116.5 nm and a PDI of 0.26. Increasing the SAR405 to 64.28 nM yielded particles of 146.3 nm with a PDI of 0.34. At 71.42 nM, the CNP-SAR405 size was 131.2 nm with a PDI of 0.34, while 107 nM SAR405 produced particles of 148.6 nm and a PDI of 0.30.

At higher SAR405 concentrations, a dramatic increase in particle size was observed. Encapsulation of 142.8 nM SAR405 resulted in CNPs measuring 440.8 nm with a PDI of 0.36. The highest concentration tested, 571 nM, produced CNP-SAR405 with a size of 556.4 nm and a PDI of 0.41. The progressive increase in particle size with rising SAR405 concentration is attributed to greater interaction between SAR405 and the free amine groups of the CS polymer. It is proposed that SAR405, being negatively charged, binds to the positively charged amine groups of CS prior to TPP cross-linking, thus altering nanoparticle formation.

The marked increase in particle size at 142.8 nM and 571.0 nM SAR405, compared to 148.6 nm at 107 nM suggests an excessive cross-linking activity between SAR405, TPP, and CS. The inclusion of additional anions from both

SAR405 and TPP likely reduced the number of free amine groups available on CS for consistent cross-linking, resulting in larger and more polydisperse particles. Therefore, SAR405 concentrations ranging from 35.7 nM to 107 nM were selected for subsequent experiments due to their more favourable resulting CNP particle sizes and PDI values, indicating greater stability and uniformity.

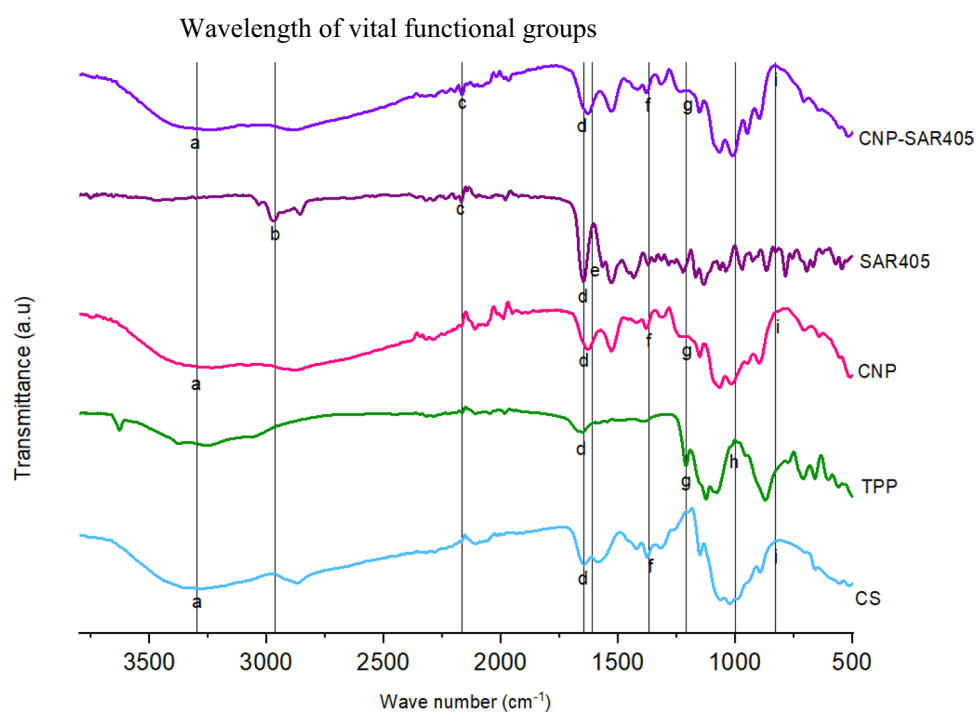
### Determination of vital functional group in nanoparticle samples via FTIR

Fourier-transform infrared spectroscopy (FTIR) analysis was conducted in this study to identify the key functional groups present in the nanoparticle samples. The presence or absence of specific functional groups was monitored with the addition of each component during the formation of chitosan nanoparticles (CNPs) and the subsequent formulation of SAR405-loaded CNPs (CNP-SAR405).

As shown in Fig. 3, chitosan (CS), CNP, and CNP-SAR405 exhibited similar sets of characteristic peaks. Peak “a” appeared at a wavenumber of  $3300\text{ cm}^{-1}$  and corresponds to both the primary amine group (N-H2 stretching) and the hydroxyl group (O-H stretching) associated with alcohols. Peak “b,” located at  $2964\text{ cm}^{-1}$ , is attributed to the carbon hydrogen stretching vibration present in SAR405. Peak “c,” observed at  $2167\text{ cm}^{-1}$ , represents multiple groups including alkynes (C triple bond C), allenes, ketenes, isocyanates, and isothiocyanates.

Peak “d,” at  $1645\text{ cm}^{-1}$ , corresponds to the carbonyl group in amides (C=O) and the amine group (N-H

**Fig. 3** Fourier-transform infrared (FTIR) spectra of CS, TPP, SAR405, CNPs, and CNP-SAR405. Characteristic peaks confirm successful crosslinking and SAR405 encapsulation, including amine ( $3300\text{ cm}^{-1}$ ), carbonyl ( $1645\text{ cm}^{-1}$ ), and phosphate groups ( $1208\text{ cm}^{-1}$ ,  $1000\text{ cm}^{-1}$ )



stretching), which are present in CS, TPP, CNP, SAR405, and CNP-SAR405. Peak “e,” at  $1612\text{ cm}^{-1}$ , is associated with carbon double bonds (C equal C) and primary amine groups (N H stretching). Peak “f,” observed at  $1369\text{ cm}^{-1}$ , represents the hydroxyl group from alcohols and is evident in CS, CNP, and CNP-SAR405.

Peak “g” at  $1208\text{ cm}^{-1}$  corresponds to the phosphorus oxygen double bond (P equals O) and is characteristic of inorganic phosphate groups present in TPP, CNP, and CNP-SAR405. Peak “h,” located at  $1000\text{ cm}^{-1}$ , signifies the phosphorus oxygen single bond (P O) in phosphate esters and is unique to TPP. Lastly, peak “i,” at  $826\text{ cm}^{-1}$ , is attributed to the carbon hydrogen stretching vibration in aromatic rings, which appears in CS, CNP, and CNP-SAR405 Table 1.

Peak a to i showed the functional groups that are presence in CS, TPP, SAR405, and CNP-SAR405.

### Percentage of encapsulation efficiency of SAR405 into CNPs

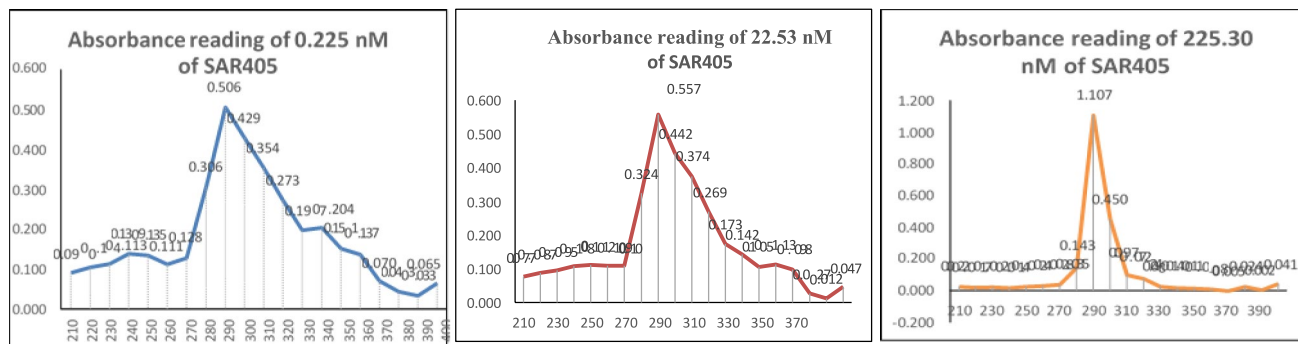
Encapsulation efficiency refers to the percentage of SAR405 successfully being encapsulated within chitosan

nanoparticles to form CNP-SAR405. The wavelength of maximum absorbance of SAR405 was determined by using series of different wavelengths to determine its lambda max. Figure 4 (A) showed the absorbance reading of  $0.1\text{ }\mu\text{g/ml}$  and the wavelength with the highest absorbance reading of 0.506 was 290 nm; Fig. 4 (B) showed the absorbance reading of  $10\text{ }\mu\text{g/ml}$  of SAR405 and the wavelength with the highest absorbance reading of 0.557 was 290 nm; (C) showed the absorbance reading of  $100\text{ }\mu\text{g/ml}$  of SAR405 and the wavelength with the highest absorbance reading of 1.107 was 290 nm. In a previous study conducted by Alamassi et al., 2023, it was stated that Aurone derivatives can inhibit autophagy process in human cells via either starvation or mTOR inhibitor. The selection of Aurone derivatives through a series of structure-based virtual screening (Alamassi et al., 2023) and the lambda max for aurone derivatives are between 225 to 254 nm (Kontoghiorghes 2022). These findings are similar with findings for SAR405 in this study.

Figure 5 shows the percentage encapsulation efficiency of SAR405 into CNPs. Three different concentrations of SAR405 used to be encapsulated into CNPs were 50 nM of

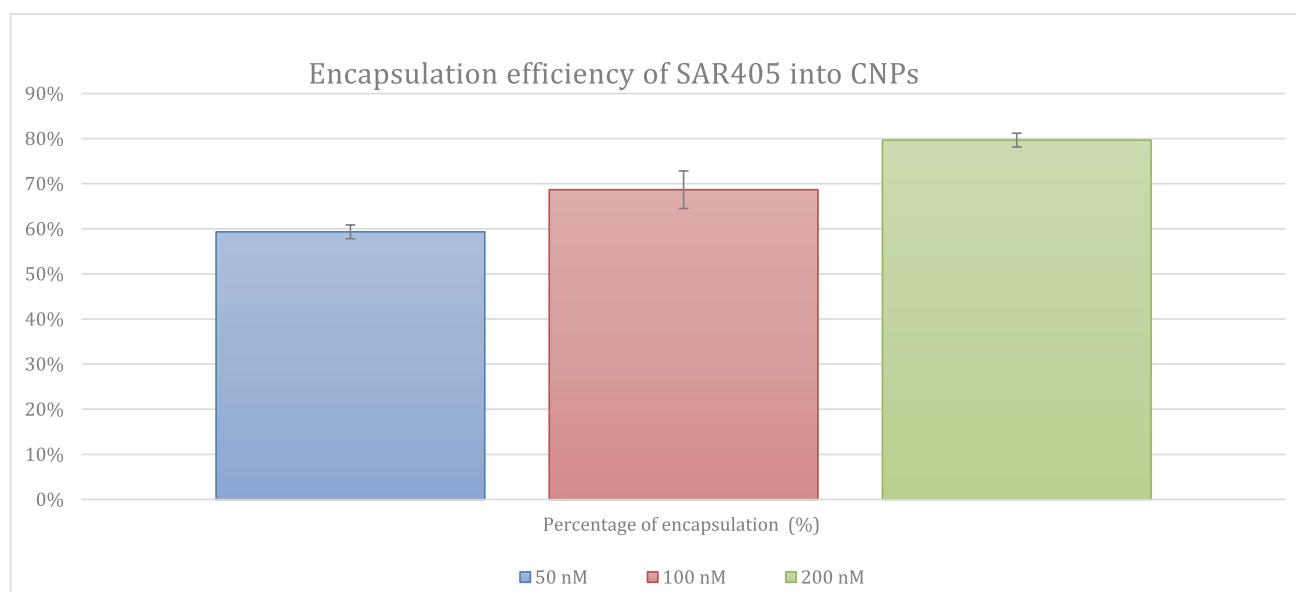
**Table 1** List of functional groups found in CS, TPP, CNP, SAR405 and CNP-SAR405

| Peak | Functional groups                                       | Wave-number ( $\text{cm}^{-1}$ ) | CS | TPP | CNPs | SAR405 | CNP-SAR405 |
|------|---------------------------------------------------------|----------------------------------|----|-----|------|--------|------------|
| a    | N-H2 I, O–H stretch                                     | 3300                             | ✓  | -   | ✓    | -      | ✓          |
| b    | CH stretch                                              | 2964                             | -  | -   | -    | ✓      | -          |
| c    | $\text{C}\equiv\text{C}$ , $\text{X}=\text{C}=\text{Y}$ | 2167                             | -  | -   | -    | ✓      | ✓          |
| d    | C=O amide, N–H stretch                                  | 1645                             | ✓  | ✓   | ✓    | ✓      | ✓          |
| e    | C=C alkene                                              | 1612                             | -  | -   | -    | ✓      | -          |
| f    | N–H stretch                                             | 1369                             | ✓  | -   | ✓    | -      | ✓          |
| g    | C-O alcohols                                            | 1208                             | -  | ✓   | ✓    | -      | -          |
| h    | P=O inorganic phosphate group                           | 1000                             | -  | ✓   | -    | -      | ✓          |
| i    | P-O Phosphate ester, C-H aromatic stretch               | 826                              | ✓  | -   | ✓    | -      | ✓          |



**Fig. 4** The absorbance reading of different concentrations of SAR405 to determine the wavelength of maximum absorbance for SAR405. The SAR405 compound was determined to possess lambda max of 285 nm and it is concentration dependent. The absorbance reading of

SAR405 at  $0.1\text{ }\mu\text{g/mL}$ ,  $100\text{ }\mu\text{g/mL}$ , and  $100\text{ }\mu\text{g/mL}$  possess lambda max of 285 nm.  $P$  values  $< 0.05$ . Each experiment was replicated 3 times



**Fig. 5** Encapsulation efficiency of SAR405 into CNPs at 50, 100, and 200 nM. Encapsulation increased proportionally with concentration, reaching ~80% at 200 nM. Data represent mean  $\pm$  SD,  $n = 3$ ,  $p < 0.05$

SAR405, 100 nM of SAR405 and 200 nM of SAR405. The percentage of 50 nM of SAR405 encapsulated into CNPs were 59%, 100 nM of SAR405 were 69% and 100 nM of SAR405 were 80%. Based on these data, the percentage of SAR405 encapsulated into CNPs was found to increase with the concentration of SAR405 used as well. This was expected because the negatively charged SAR405 interacts with positively charged chitosan nanoparticles prior the addition of TPP. It was also found that the lower the concentration of SAR405 used, the lower the interaction occurred with chitosan before formation of CNP-SAR405, resulting in lower encapsulation efficiency using 50 nM SAR405. The higher the concentration of SAR405 used for encapsulation, the higher the interaction with chitosan before formation, resulting in higher encapsulation efficiencies using 200 nM of SAR405. The addition of the negatively charged compound of SAR405 was thought to interact with protonated amine groups from CS.

As the concentration of SAR405 increases, more anions were supplied to interact with protonated amine groups from CS prior the addition of TPP resulting in increasing percentage of SAR405 being encapsulated into CNPs. Previous study conducted by Masarudin et al., 2015 showed that the encapsulation of 5-Fluorouracil (5-FU) into CNPs increases as the mass ratio between 5-FU and CNPs increases. The 5-FU used in this study was negatively charged, to which is similar with SAR405, and its interaction with CS to form 5-FU encapsulated within chitosan nanoparticles is similar to interactions between SAR405 with CS before the formation of CNP-SAR405 nanoparticles. Based on findings in this analysis and previous

study conducted, it can be postulated that the percentage of encapsulation efficiency increases as the concentration of compounds increases.

These findings support the potential of chitosan nanoparticles as effective carriers for SAR405, improving intracellular delivery and enhancing inhibition of autophagy in A549 cells. This aligns with recent reports demonstrating that nanoparticle-based strategies can modulate autophagy and apoptosis pathways to enhance therapeutic efficacy in cancer cells, including A549 (Mahoutforoush et al., 2023).

### Morphological studies of nanoparticles samples

The morphological characteristics of the individual components, chitosan (CS) polymer, sodium tripolyphosphate (TPP), and SAR405 were evaluated using field-emission scanning electron microscopy (FESEM) to assess surface morphology, particle size, and homogeneity. Figure 6 showed the morphology of CS, TPP, and SAR405, respectively. Both CS and TPP appeared irregular in shape and relatively large prior to nanoparticle synthesis. In contrast, SAR405 exhibited small, circular structures that tended to agglomerate with nearby SAR405 particles.

According to a previous study by Liu et al., 2025 synthesized CNPs typically exhibit a spherical morphology with sizes below 100 nm. As shown in Fig. 6, the synthesized CNPs in this study displayed a spherical shape with particle sizes ranging from 48.5 nm to 80.0 nm before drug encapsulation. Following SAR405 encapsulation, Fig. 7 illustrates an increase in particle size to values greater than 100 nm, accompanied by slight irregularities in shape.

The observed increase in CNP size after SAR405 encapsulation was initially detected via dynamic light scattering (DLS), and this trend was further confirmed through FESEM imaging. The size expansion and minor deviations in particle shape are likely due to the incorporation of SAR405 into the nanoparticle matrix. A similar phenomenon was reported by Yoo et al., 2011 who observed significant size increases in CNPs post-encapsulation with varying concentrations of L-ascorbic acid (LAA). Likewise, Hassan et al., 2018 reported notable morphological differences in CNPs post-encapsulation with glutamic acid (GA). The DLS results from this study are consistent with these findings and are further validated by the FESEM observations.

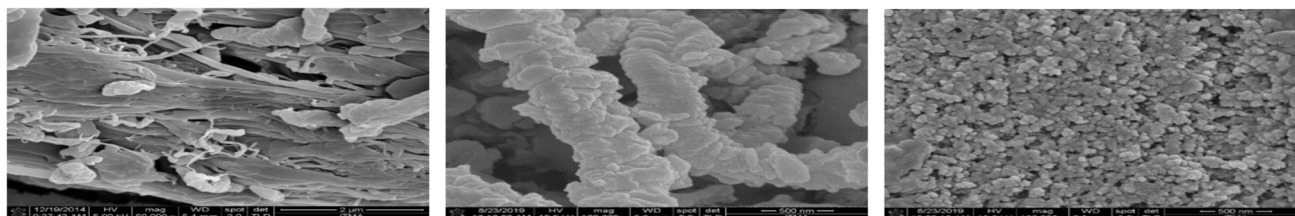
Further morphological analysis was conducted using transmission electron microscopy (TEM) to gain higher-resolution insights. Figure 8 show TEM images of synthesized CNPs and CNP-SAR405, respectively. The synthesized CNPs maintained a spherical shape with sizes ranging from 24.2 nm to 40.8 nm. For CNP-SAR405, particle sizes ranged from 59.4 nm to 80.3 nm. The spherical

morphology observed in TEM was consistent with that seen in FESEM images; however, particle sizes appeared smaller under TEM. This discrepancy is attributed to the higher resolution of TEM and the differences in detection mechanisms, resulting in variation in perceived particle size between both microscopy methods.

## Inhibition of autophagy in autophagy-induced A549 cell line

### The cytotoxic level of CNPs, CNP-SAR405, SAR405 and torin-2

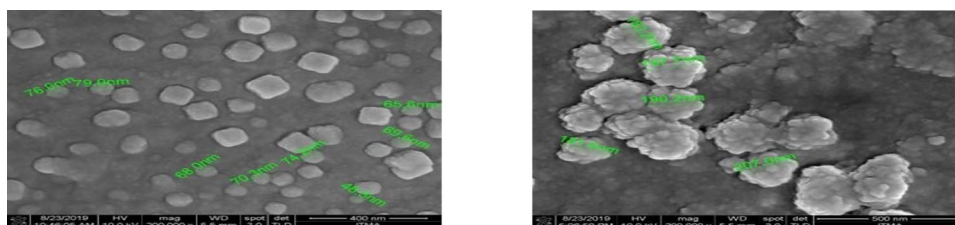
Autophagy plays an important role in maintaining homeostasis in cellular level, development, and disease. However, autophagy has been known to contribute to the advances of malignant cells, by allowing cancer cells to adapt and thriving through various biological stressors. SAR405 is an autophagy inhibitor compound that has been studied to inhibit autophagy process in cells. Through inhibiting basal autophagy, it also inhibits cancer progression that are



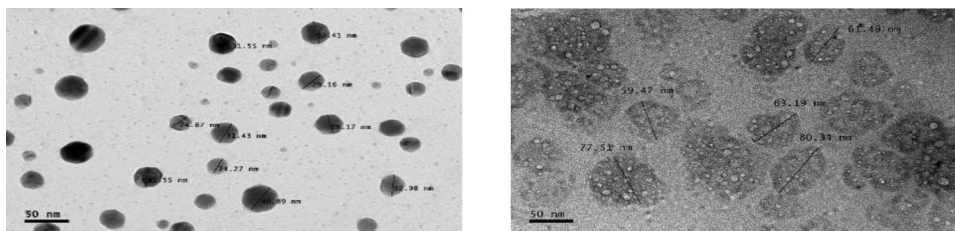
**Fig. 6** The morphological study of (A) CS, (B) TPP and (C) SAR405 Were Conducted Using Field-Emission Scanning Electron Microscopy (FESEM) to Analyse Its Surface Morphology, Size and Homogeneity. Difference in shape and sizes was observed for CS, TPP and SAR405 indicating that the nanoparticle structures were formed post

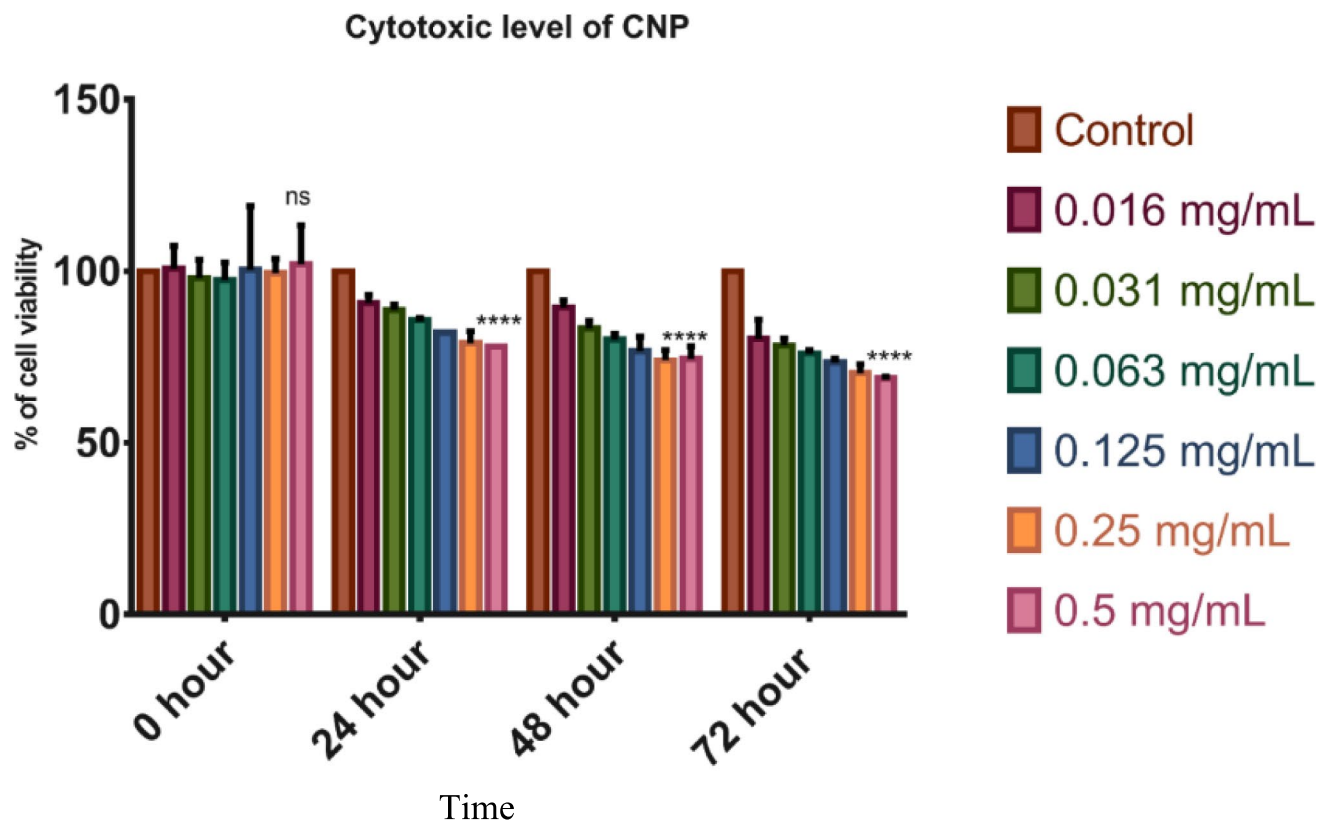
cross-linking between CS and TPP. Scale magnification for CS (A) was at 2  $\mu$ M, and scale magnification for TPP (B) and SAR405 (C) was at 500 nm. Scale bar for CS is at 2  $\mu$ m. A larger scale was used to visualize the broader chitosan structure, as imaging at nanometer resolution does not adequately display the overall morphology

**Fig. 7** FESEM images of CNPs (A) and CNP-SAR405 (B). Blank CNPs exhibited particle sizes < 100 nm, while SAR405 encapsulation increased average particle size to > 100 nm, confirming successful drug loading



**Fig. 8** TEM images of CNPs (A) and CNP-SAR405 (B). CNPs showed spherical morphology (24–40 nm), while encapsulation increased particle size to 59–80 nm. TEM analysis confirmed uniform distribution and structural integrity





**Fig. 9** MTT assay results showing cell viability of A549 cells treated with different concentrations of CNPs (0.0156–0.5 mg/ml) at 0, 24, 48, and 72 h. Data are presented as mean  $\pm$  SD ( $n=3$ ). All values were normalized to untreated controls (set as 100%). Statistical analysis was performed using one-way ANOVA with post-hoc Tukey's test; comparisons versus untreated control were considered significant

at  $p < 0.05$ , with exact  $p$ -values reported in Supplementary Table. The MTT assay reflects cell viability/proliferation only and does not measure autophagosome formation or flux. Accordingly, these results are interpreted solely in terms of viability outcomes, while autophagy-related effects are evaluated separately using CYTO-ID staining and qualitative microscopy

autophagy-dependent" when treated with anti-cancer compounds, to which activates cells death pathway.

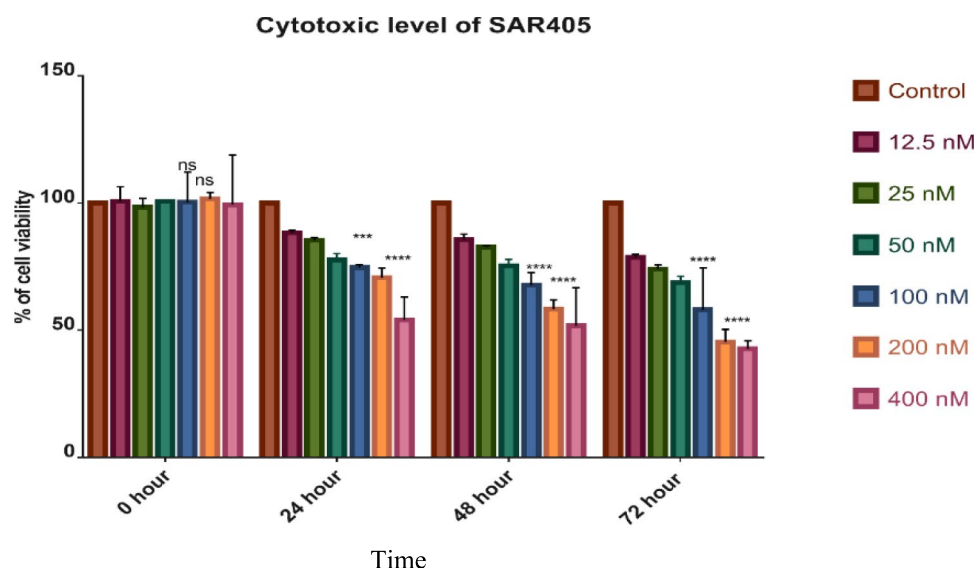
The cytotoxic levels for CNPs, SAR405, CNP-SAR405 and Torin-2 on A549 cells were observed to ensure that the concentrations used were not toxic to the cells. This is crucial because any observed toxicity would affect cellular autophagic processes Poillet-Perez et al., 2019. Figure 9 shows the cytotoxic analysis of CNPs on A549 cell line through MTT assay. Viability of A549 cells 72-h post treatment with 0.5 mg/ml blank CNPs were higher than 50% post treatment and was considered to not have any significant cytotoxic effects as no  $IC_{50}$  value was recorded. This observation was congruent with a study by Masarudin et al., 2015, which showed that CNPs was not cytotoxic below 0.74 mg/ml in A549 cell line (Masarudin et al., 2015).

MTT analysis was done to study the cytotoxic level that are derived from viable cells of SAR405 on A549 cell line at different timepoints. High toxicity of autophagy inhibitors such as SAR405 often limits its adoption in subsequent clinical stages Alvarez-Meythaler et al. 2020. Figure 10 showed

the cell viability of A549 cells upon SAR405 treatment. At concentrations of 200 nM SAR405 the percentage of viability of cells was 45.3% at 72 h post treatment, while viability increased at lower concentrations of 100 nM SAR405 of 58% at the same timepoint. For SAR405 to be considered not toxic, percentage of cell viability should be more than 50% at 72 h post treatment.

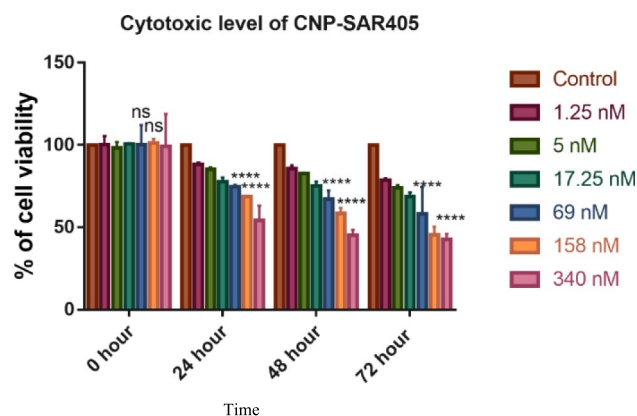
Similar studies conducted by Pasquier 2015 showed that starvation-induced autophagy activities were inhibited by SAR405 at an  $IC_{50}$  of 419 nM. Another similar study conducted using another autophagy inhibitor known as Chloroquine, CQ, showed an  $IC_{50}$  of 53.01  $\mu$ mol/L (Jia et al., 2018). Based on previous studies conducted as well as findings in this study showed that autophagy inhibitors have high toxicity level on cells but able to inhibit starvation-induced autophagy activity in cells in a dose-dependent manner.

Encapsulation of SAR405 into a nano-sized vehicle such as CNPs was found to increase its efficiency for autophagy activity inhibition in A549 cell line with minimal toxicity effect on cells. As the CNPs that used was determined to be



**Fig. 10** Cytotoxicity of SAR405 on A549 cells assessed by MTT assay. Cell viability (%) measured at 0, 24, 48, and 72 h. All values were normalized to untreated controls (set as 100%). MTT assay of A549 cells treated with SAR405 at concentrations ranging from 15.6 to 500 nM for up to 72 h. Cell viability is expressed as mean  $\pm$  SD

of three independent replicates ( $n=3$ ). One-way ANOVA with Tukey's post-hoc test showed no significant differences compared with untreated controls ( $p>0.05$ ). These results indicate that SAR405 treatment maintains general cell viability, supporting that its inhibitory effects on autophagy are not attributable to cytotoxicity



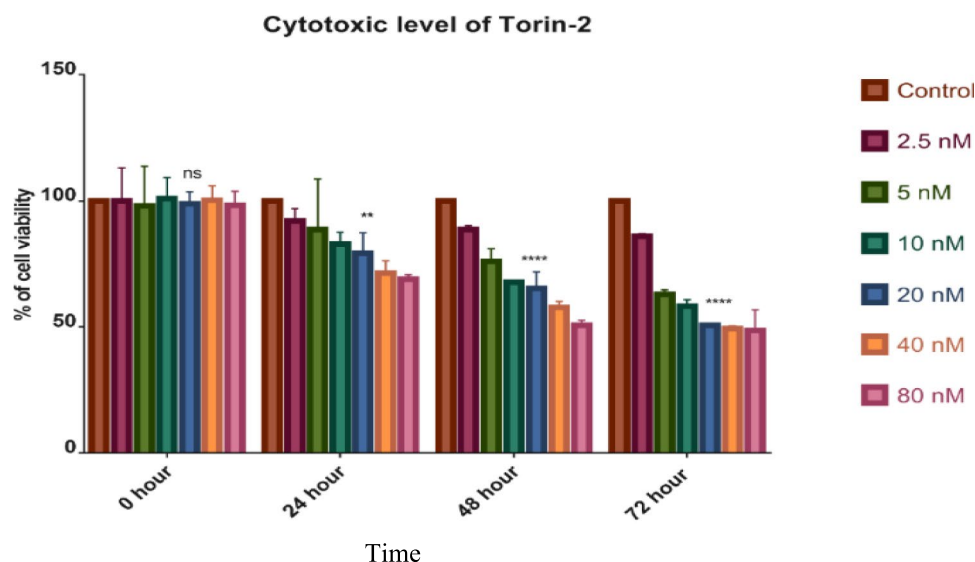
**Fig. 11** MTT assay of A549 cells treated with CNP-SAR405 demonstrated a marked reduction in viability compared with blank CNPs. All values were normalized to untreated controls (set as 100%). At 0.5 mg/ml for 72 h, cell viability decreased to  $24.0\% \pm 1.2$  versus  $91.2\% \pm 2.1$  in blank CNPs ( $n=3$ ). One-way ANOVA with Tukey's post-hoc test confirmed statistical significance ( $p<0.001$ ). These findings indicate that CNP-mediated delivery of SAR405 reduces cell viability in a dose- and time-dependent manner, while complementary assays are required to confirm the link with autophagy inhibition

non-toxic to A549 cell line, any cytotoxic effect observed using CNP-SAR405 is attributed to the SAR405 encapsulated. Figure 11 showed the percentage of cell viability after a series of treatments with CNP-SAR405 at different timepoints. Percentage of cells viability of A549 cells after treatment with 158 nM of CNP-SAR405 falls to 45.3% at 72-h

post treatment, while percentage of cell viability when treated with 69 nM of CNP-SAR405 is 58%. The mortality rate of cells when treated with 158 nM of SAR405 is 54.7%. The same mortality rate was achieved when cells were treated with 200 nM of SAR405 at the same timepoint as showed earlier. When cells were treated with 69 nM of SAR405, the mortality rate is only 42% while the same mortality rate was achieved when cells were treated at a much higher concentration of 100 nM of SAR405 as showed in previous MTT analysis. Based on these findings it can be postulated that when treated onto cells, CNP-SAR405 requires 45% less the concentration of SAR405 to achieve the same percentage of cell viability. As mentioned previously, CNPs has the ability to enter and accumulate before releasing its cargo inside cells. With the encapsulation of SAR405 into CNPs, the entrance and accumulation within cells increases its efficiency which resulted in requiring half the concentration of SAR405 to achieve the same percentage of viability.

As shown in Fig. 12, an autophagy inducer, Torin-2 was used in this study to induce the autophagy process in A549 cell line. Torin-2 was treated onto cells to assess its cytotoxic level in a dose-dependent manner. Figure 12 showed the percentage of cell viability when Torin-2 was treated onto cells at 4 different timepoints. At concentration of 20 nM of Torin-2, the percentage of cell viability is at 50.6% at 72-h timepoint. For Torin-2 to be considered not toxic, the percentage of cell viability must be at least 50% at 72-h timepoint, at which Torin-2 is able to induce autophagy activity in cells but at the same time does not result in cell death. Previous

**Fig. 12** Cytotoxicity of Torin-2 on A549 cells assessed by MTT assay. Viability was measured at 0, 24, 48, and 72 h across a concentration range of 1.56–50 nM. All values were normalized to untreated controls (set as 100%). Torin-2 treatment produced a time-dependent decrease in viability, with reductions becoming most pronounced at 48 and 72 h. At 72 h, viability decreased to 64% (50 nM), 54% (25 nM), and 43–47% (1.56 nM) compared with untreated controls ( $n=3$ ). One-way ANOVA with Tukey's post-hoc test indicated significance ( $p<0.0001$ )



study conducted by Pasquier, 2015 stated that Torin-2 is toxic to ACHN and 786-o cell lines at higher concentration. Another study conducted stated that Torin-2 has a high anti-proliferation activity when treated onto cells and has an  $IC_{50}$  of nanomolar range (Gremke et al., 2020). Findings in both previous studies are similar to findings in MTT analysis of Torin-2 represented in Fig. 12. Based on these findings, it can be postulated that Torin-2 is highly toxic and can cause cell death when treated at high concentration. Thus, in this study 20 nM concentration of Torin-2 was used as co-treatment to induce autophagy onto A549 cell line at which the concentration does not cause cell viability to drop below 50%.

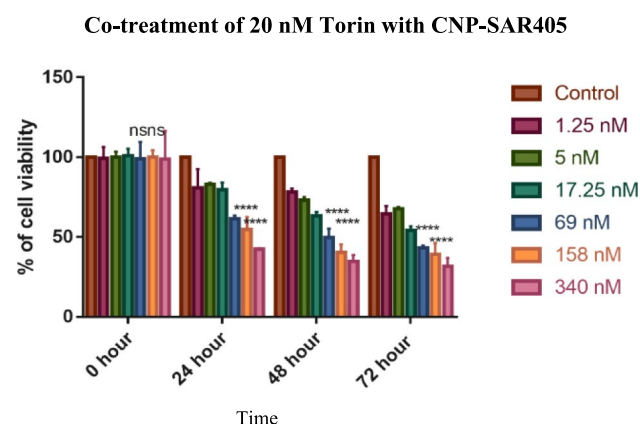
Encapsulation of SAR405 into a nano-sized vehicle such as CNPs was found to increase its efficiency for autophagy activity inhibition in A549 cell line with minimal toxicity effect on cells. As the CNPs that used was determined to be non-toxic to A549 cell line, any cytotoxic effect observed using CNP-SAR405 is attributed to the SAR405 encapsulated. Figure 11 showed the percentage of cell viability after a series of treatments with CNP-SAR405 at different timepoints. Percentage of cells viability of A549 cells after treatment with 158 nM of CNP-SAR405 falls to 45.3% at 72-h post treatment, while percentage of cell viability when treated with 69 nM of CNP-SAR405 is 58%. The mortality rate of cells when treated with 158 nM of SAR405 is 54.7%. The same mortality rate was achieved when cells were treated with 200 nM of SAR405 at the same timepoint as showed earlier. When cells were treated with 69 nM of SAR405, the mortality rate is only 42% while the same mortality rate was achieved when cells were treated at a much higher concentration of 100 nM of SAR405 as showed in previous MTT analysis. Based on these findings it can be postulated that when treated onto cells, CNP-SAR405 requires 45% less the concentration of SAR405 to achieve the same percentage of cell viability. As mentioned previously,

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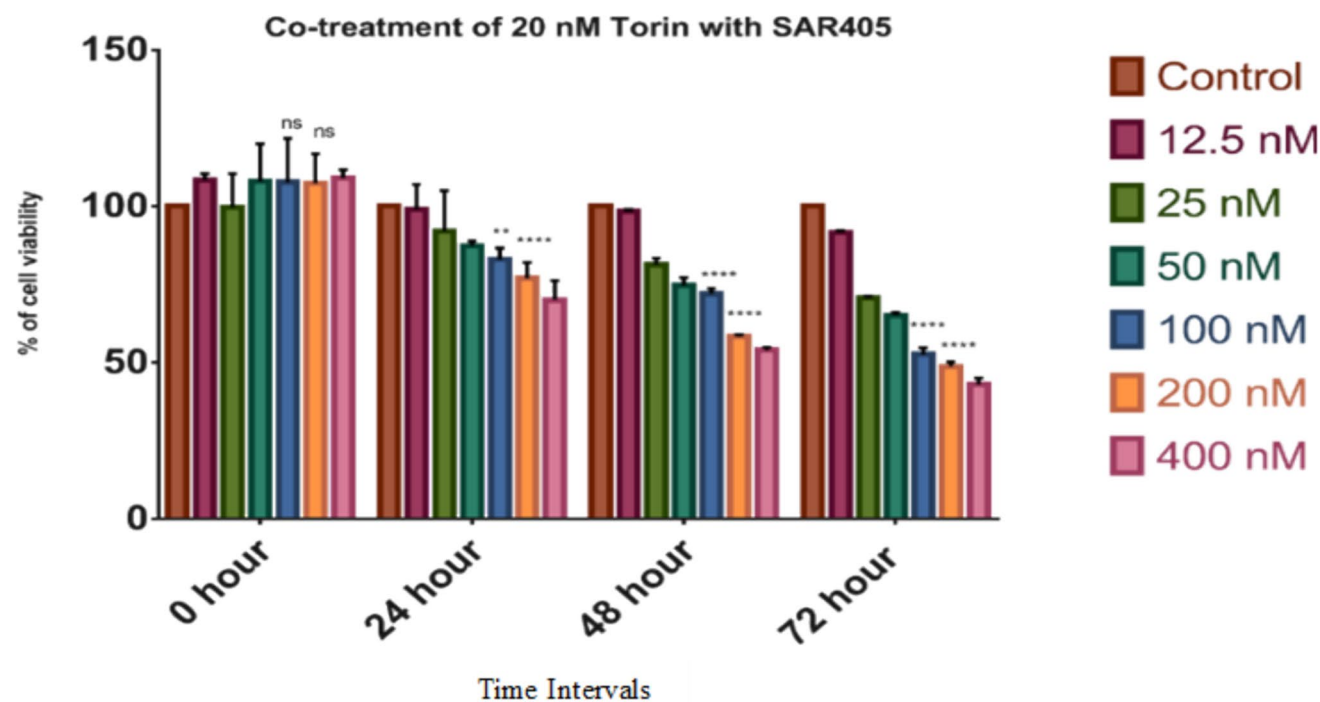
#### The cytotoxic level of co-treatment of torin-2 with CNP-SAR405

MTT analysis of co-treatment between CNP-SAR405 with Torin-2 at different concentrations was conducted to



**Fig. 13** Cytotoxicity of Torin-2 in combination with CNP-SAR405 on A549 cells assessed by MTT assay. Viability was measured at 0, 24, 48, and 72 h across a concentration range of 12.5–400 nM. All values were normalized to untreated controls (set as 100%). Combination treatment produced a time-dependent reduction in viability, with the greatest decreases observed at 48 and 72 h. At 72 h, viability decreased to 73% (340 nM), 72% (158 nM), and 61–63% (69 nM–1.25 nM) compared with untreated controls ( $n=3$ ). One-way ANOVA indicated a significant time-dependent effect ( $p<0.0001$ ), although concentration-dependent differences were not statistically significant

assessed the cytotoxic level of both compounds when it is co-treated on A549 cell line. Although cytotoxic level of CNP-SAR405 and Torin-2 on A549 cell line has been assessed in earlier analysis, co-treatment study using both compounds may alter its cytotoxic level when treated onto cells at the same time. Figure 13 showed the cytotoxic level of 20 nM Torin-2 co-treated with CNP-SAR405 on A549 cell line in a dose dependent manner at 0-h, 24-h, 48-h and 72-h. When A549 cell line was co-treated with 20 nM of Torin-2 and 158 nM of CNP-SAR405, the percentage of cell viability is at 39%. The percentage of cell viability is at 43% when co-treated with 20 nM of Torin-2 and 69 nM of CNP-SAR405. The percentage of cell viability drop below 50% shows high cytotoxic level of compounds treated onto cells. The 20 nM of Torin-2 used in this analysis may be the main contributor to high cytotoxic level which resulted in high cells mortality rate. The co-treatment analysis was repeated using 20 nM of Torin-2 with CNP-SAR405. Figure 14 showed cytotoxic level of 20 nM of Torin-2 co-treated with SAR405 onto A549 cell line at 0-h, 24-h, 48-h and 72-h. The percentage of cell viability when treated with 20 nM of Torin-2 with 158 nM of SAR405 is at 39.3% and the percentage of cell viability when treated with 20 nM of Torin-2 when treated with 69 nM of Torin-2 is at 50%. The concentration of Torin-2 at 20 nM with 69 nM



**Fig. 14** Cytotoxicity of 20 nM of Torin with SAR405 on A549 cells assessed by MTT assay. Cell viability was measured at 0, 24, 48, and 72 h across concentrations of 12.5–400 nM. SAR405 treatment produced variable effects on viability across doses, with modest reductions at 48 h (62–79%) but recovery or maintenance of viability at

72 h (77–102%), suggesting limited sustained cytotoxicity. All values were normalized to untreated controls (set at 100%). One-way ANOVA with Tukey's post-hoc test indicated no statistically significant differences compared with untreated controls ( $p>0.05$ ,  $n=3$ )

concentration of CNP-SAR405 are used in this study based on the data obtained.

### Fluorescent trafficking of FITC-CNP-SAR405 in A549 Cell Line

Chitosan nanoparticles possess the inherent ability to enter and accumulate within cells. Due to their nanoscale dimensions and biocompatible, non-toxic nature, chitosan nanoparticles are widely regarded as effective vehicles for the delivery of therapeutic agents and proteins (Jaferník et al., 2023). However, in their native form, chitosan nanoparticles are colorless and do not emit fluorescence under fluorescence microscopy. To enable their visualization, a surface modification was performed using a fluorophore known as fluorescein isothiocyanate (FITC). This modification introduces a green fluorescence signal that becomes visible when the particles are being analyzed using a fluorescence microscope. The tagging occurs through the interaction between the primary amine groups on the chitosan backbone and the isothiocyanate groups on FITC, resulting in covalent bonding and functionalization of the nanoparticle surface (Ray et al., 2025).

FITC-labelled CNPs loaded with SAR405 (FITC-CNP-SAR405) were applied to A549 human lung carcinoma cells at the following time intervals post-treatment: (A) 0 h, (B) 6 h, (C) 12 h, (D) 24 h, (E) 48 h, and (F) 72 h. As shown in Fig. 15, no green fluorescence was detected at the 0-h and 6-h time points. Fluorescence became first

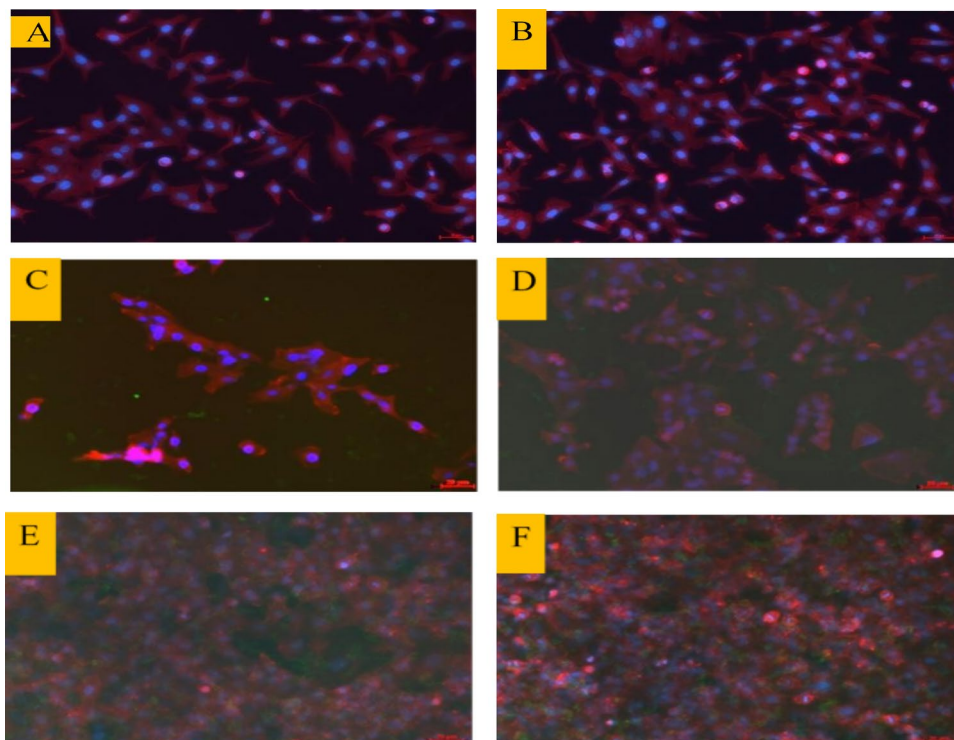
detectable at 12 h post-treatment and continued to accumulate in A549 cells through to the 72-h time point.

The purpose of this tracking study was to evaluate the cellular uptake and retention of FITC-CNP-SAR405 without inducing cytotoxic effects. In addition, understanding the dynamics of nanoparticle accumulation is critical in determining appropriate dosages for intracellular delivery of therapeutic compounds. In this study, SAR405 was delivered into A549 cells via chitosan nanoparticles that had undergone a minor fluorescent modification to facilitate visualization under the microscope.

At the 0-h time point, as illustrated in Fig. 15 (A), no green fluorescence was observed. This early time point is likely too short for the uptake process to begin and for any accumulation to occur within the cells. A previous study by Pawaskar et al., 2024 reported that FITC-labelled chitosan nanoparticles can become observable under fluorescence microscopy as early as 6 h post-treatment. However, in this case, at 6 h (Fig. 15 (B)), the internalized amount of FITC-CNP-SAR405 was likely below the detection threshold of fluorescence microscopy, resulting in no observable signal. This supports the hypothesis that a minimum concentration threshold must be reached for successful fluorescence detection.

At 12 h post-treatment (Fig. 15 (C)), green fluorescence was clearly visible, indicating the onset of nanoparticle uptake by the A549 cells. As the time point progressed from 12 to 24 h (D), 48 h (E), and 72 h (F), fluorescence intensity increased correspondingly. This trend suggests a progressive

**Fig. 15** Fluorescence tracking of FITC-CNP-SAR405 uptake in A549 cells at 0, 6, 12, 24, 48, and 72 h. Green fluorescence indicates intracellular accumulation, detectable from 12 h and increasing through 72 h



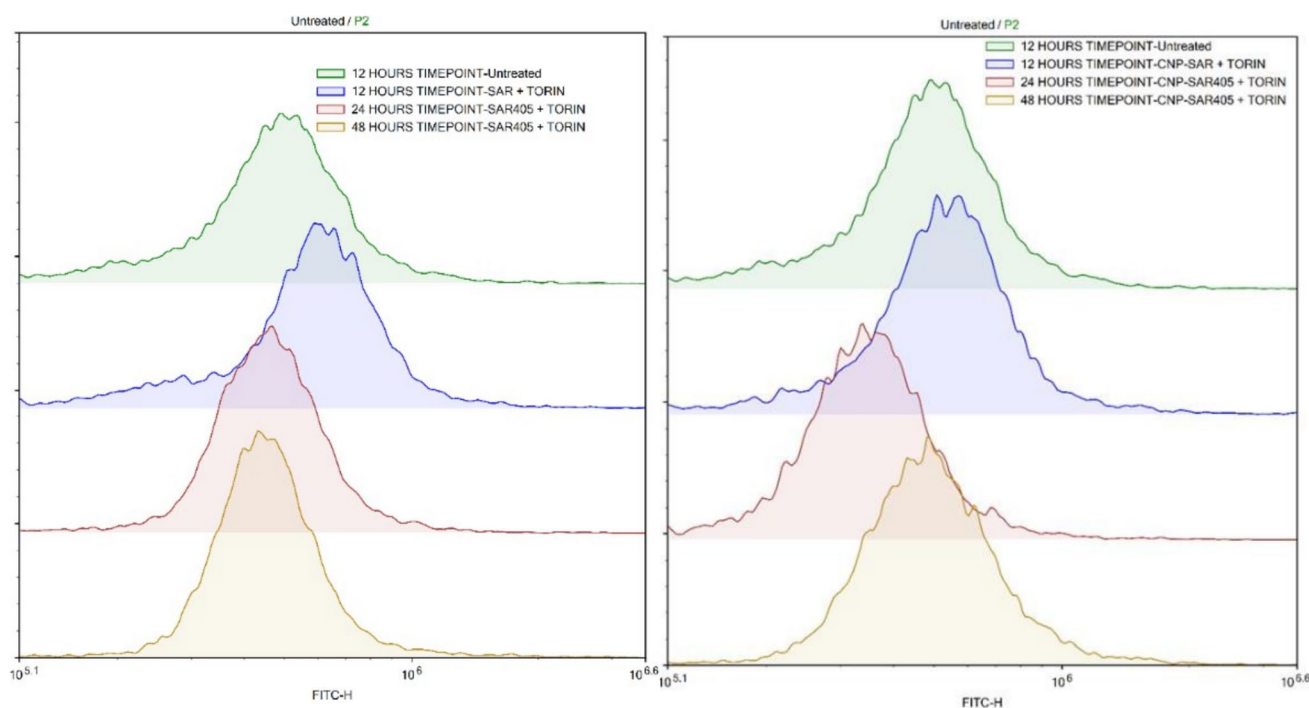
and time-dependent accumulation of FITC-CNP-SAR405 within the cells. The increase in green fluorescence intensity over time indicates not only enhanced uptake but also prolonged retention of nanoparticles within the intracellular environment. While fluorescence microscopy provides valuable qualitative insights, its relatively lower sensitivity may fail to detect early or minimal internalization, as observed in the 6-h time point. Therefore, fluorescence microscopy should be complemented by more sensitive analytical techniques such as flow cytometry.

#### Autophagosomes detection in A549 cell line using Cyto-ID

Autophagy is a highly conserved cellular pathway that enables the degradation and recycling of proteins, lipids, and damaged organelles via the lysosomal system. It is particularly important under stress conditions, allowing cells to maintain homeostasis, preserve metabolic balance, and promote survival (Parzych & Klionsky, 2014). A key morphological hallmark of this process is the autophagosome, a transient double-membrane vesicle that encapsulates cytoplasmic material and delivers it to lysosomes for degradation (Khandia et al., 2019). Because the number and turnover of autophagosomes are directly linked to autophagy activity, their detection has become a standard readout in autophagy research.

Several methods exist to assess autophagy, including immunoblotting for LC3-II accumulation, degradation of p62/SQSTM1, and fluorescence-based microscopy of LC3 puncta (Yoshii and Mizushima, 2017; Poillet-Perez et al., 2019). However, these methods can be technically demanding and may require fixation, antibody staining, or genetic modification of cells. As an alternative, functional dye-based assays provide a convenient approach to quantify autophagosome content in live cells. In this study, we used the Cyto-ID Autophagy Detection Kit, which employs a cationic amphiphilic tracer (CAT) dye that selectively labels autophagic vesicles. Fluorescence intensity detected by flow cytometry is proportional to the abundance of autophagosomes, enabling comparative assessment of autophagy levels across different treatments (Mohammed et al., 2017).

Figure 16 shows the flow cytometry analysis of the formation of autophagosomes in A549 cell line. The goal of this experiment was to evaluate whether nanoparticle-encapsulation of SAR405 enhances its ability to inhibit autophagy in human lung carcinoma (A549) cells under conditions of pharmacological induction. SAR405 is a selective inhibitor of VPS34, a class III PI3K required for autophagosome nucleation. Free SAR405 is known to limit autophagy but suffers from issues of solubility and delivery efficiency. To overcome these limitations,



**Fig. 16** Flow cytometry analysis of autophagosome formation in A549 cells. Untreated cells represent basal autophagy. In the presence of Torin-2 plus free SAR405, autophagosome accumulation remained elevated at 12 h but returned to basal levels by 24 h. In contrast, Torin-2 plus CNP-SAR405 maintained basal-like autophagosome lev-

els from 12 h onward, indicating earlier and stronger suppression. By 48 h, both treatments converged to basal levels, consistent with the transient nature of Torin-2 induction. Data represent qualitative fluorescence profiles from three biological replicates

we encapsulated SAR405 in chitosan nanoparticles (CNP-SAR405) and compared its inhibitory activity with the free drug. In all conditions, autophagy was induced by Torin-2, a potent mTOR inhibitor that activates autophagosome formation. Thus, the experimental design tested whether SAR405, in free or nanoparticle form, could suppress autophagy in the presence of a strong upstream inducer. Untreated cells, which lacked both Torin-2 and inhibitors, served as the basal control for physiological autophagy.

To establish a baseline, untreated A549 cells were analysed to determine the level of autophagosomes present under normal culture conditions. This “untreated” group served as a measure of basal autophagy, reflecting the steady-state turnover of cellular components in the absence of external inducers or inhibitors. As expected, the basal signal was relatively stable and provided a reference point against which Torin-2-induced changes could be assessed.

At the 12-h time point, Cyto-ID analysis revealed clear differences between formulations. Cells treated with Torin-2 plus free SAR405 showed fluorescence levels shifted slightly to the right of the untreated basal profile, indicating that autophagosome accumulation remained above basal levels despite the presence of SAR405. This suggests that, in the early phase of induction, SAR405 alone was not sufficient to fully suppress Torin-2-mediated autophagy. In contrast, cells treated with Torin-2 plus CNP-SAR405 displayed fluorescence intensities comparable to untreated controls. This finding implies that nanoparticle-encapsulation enhanced SAR405 bioavailability, enabling earlier suppression of autophagosome accumulation. The difference at 12 h highlights the importance of delivery systems in overcoming cellular uptake and drug stability limitations.

By 24 h, the inhibitory effect of free SAR405 became more apparent. Cells treated with Torin-2 plus free SAR405 showed a leftward shift relative to the 12-h profile, with Cyto-ID fluorescence approaching basal levels. This indicates that SAR405 was eventually able to counteract Torin-2-induced autophagy, albeit with a delayed onset. In the nanoparticle-treated group, fluorescence intensity remained at basal levels, consistent with the effect already observed at 12 h. Thus, while both formulations ultimately suppressed autophagy at 24 h, CNP-SAR405 achieved the effect earlier and sustained it more consistently.

At the 48-h time point, both treatment groups (Torin-2 + SAR405 and Torin-2 + CNP-SAR405) showed Cyto-ID profiles indistinguishable from untreated basal controls. This suggests that the Torin-2-driven induction was transient and largely resolved by this later stage, regardless of treatment formulation. Although the convergence of effects at 48 h might indicate a natural decline of autophagy induction, the key distinction lies in the kinetics of suppression: nanoparticle delivery provided an immediate inhibitory

effect at 12 h, while free SAR405 required up to 24 h to achieve comparable outcomes.

Taken together, these results demonstrate that CNP-SAR405 exerts a more rapid and pronounced inhibitory effect on autophagosome accumulation in A549 cells than free SAR405 under Torin-2-induced conditions. The accelerated inhibition observed with the nanoparticle formulation likely reflects improved cellular uptake and sustained intracellular release of SAR405, which together enhance its functional impact. While the Cyto-ID assay provides strong qualitative evidence of differential kinetics, it does not directly measure autophagic flux. Therefore, complementary validation using LC3-II turnover assays or p62 degradation would be necessary to confirm the full extent of inhibition. Nonetheless, within the scope of this study, the Cyto-ID data support the conclusion that nanoparticle delivery significantly improves SAR405's pharmacological profile and effectiveness as an autophagy inhibitor.

## Conclusion

The present study successfully demonstrated the encapsulation of SAR405 into chitosan nanoparticles (CNPs). Encapsulation efficiency and particle size increased with higher SAR405 loading, confirming the robustness and adaptability of the formulation. Fluorescent labelling with FITC enabled direct visualization, verifying efficient uptake and accumulation of CNP-SAR405 in A549 cells. Cytotoxicity testing confirmed that blank CNPs were non-toxic, supporting their suitability as a nanosized drug delivery vehicle.

Functionally, CNP-SAR405 exhibited greater inhibitory effects on autophagosome accumulation in A549 cells compared with free SAR405, with the most pronounced activity observed within the 12–24 h window under Torin-2-induced autophagy. These findings indicate that nanoparticle-mediated delivery can enhance the intracellular performance of SAR405, leading to earlier and more consistent inhibition of autophagy.

Collectively, this work highlights CNP-SAR405 as a promising nanocarrier system for autophagy modulation. While our findings provide strong *in vitro* evidence of improved delivery and activity, further mechanistic studies and *in vivo* validation will be essential to determine its therapeutic potential and applicability in cancer therapy.

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**Data availability** All source data for this work (or generated in this study) are available upon reasonable request.

## Declarations

**Ethics approval** Not applicable – the study used established human cell lines and does not involve animals or patient material.

**Competing interests** The authors declare no competing interests.

The authors declare no competing interests.

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