Research Article

Nano Biomed Eng

Multivitamin–Cisplatin Encapsulated Chitosan Nanoparticles Modulate DDX3X Expression in Cancer Cell Lines

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Received: Jun. 15, 2022; **Revised:** Jan. 23, 2022; **Accepted:** Mar. 23, 2023

Citation: R. Othayoth, K. Khatri, R. Gadicherla, et al. Multivitamin–cisplatin encapsulated chitosan nanoparticles modulate DDX3X expression in cancer cell lines. *Nano Biomedicine and Engineering*, 2023.

http://doi.org/10.26599/NBE.2023.9290008

Abstract

Vitamin supplementation during chemotherapy has often been associated with lower recurrence and mortality rates in cancer patients. We had previously demonstrated that the multivitamin (C, D3, and B12)–cisplatin nanoparticle complex—NanoCisVital (NCV)— could alleviate chemotherapy-induced cancer fatigue. Chitosan is frequently used in functional nanomaterials to encapsulate drugs, because it is biodegradable, biocompatible, and non-toxic. The chitosan-based NCVs were prepared, and their physicochemical properties, size, and stability were evaluated before assessing their effect on cancer cell lines. The multivitamin mixture is packed in the core, and cisplatin is loaded at the periphery of the nanoparticle. This encapsulation facilitates the slow and sequential release of peripheral cisplatin and the core multivitamin combination. By increasing the amounts of vitamin and drug-encapsulated nanoparticles in breast and cervical cancer cell lines, the viable cell percentage was calculated. DDX3X promotes cancer cell proliferation, invasion, and metastasis, while Ki-67 promotes active cell proliferation in all cell types. DDX3X is elevated in several cancer types, and breast cancer cells express it abnormally. The Ki-67 protein is a biomarker of cell proliferation that is present throughout all active stages of the cell cycle but undetectable in the resting state. The expression of the *DDX3X* and *Ki-67* genes is altered in NCV-treated cells. This study uses *DDX3X* and *Ki-67* gene expression as a comparative measuring tool for the anti-cancer and cell proliferation effects of cisplatin and vitamins, respectively.

Keywords: NanoCisVital (NCV); Ki-67; DDX3X; cancer cell lines; cancer-related fatigue; chemoprevention; nano-formulation; transmission electron microscope; atomic force microscope; cytotoxicity

Introduction

Multimodality therapies are often used in advanced hospitals to treat cancer. Despite the improved efficacy and higher survival rates, cancer patients often suffer from a series of side effects; fatigue is a typical and distressing side effect of cancer treatment [1]. Patients may experience fatigue before the onset of treatment, and it may worsen throughout the treatments, including radiation [2], chemotherapy [3], hormonal, and/or

biological treatments [4]. The prevalence of fatigue during a course of treatment is estimated to range from 25% to 99% based on the kind of treatment received and the assessment method [5]. In the majority of studies, fatigue is moderate in 30%–60% of patients, which may lead to treatment discontinuation in some cases [6, 7]. Long-term cancer survivorship studies report persistent fatigue in approximately 25%–30% of patients up to 10 years after cancer diagnosis [8, 9]. Fatigue may also account for nutritional insufficiency, especially vitamin deficiencies, which may manifest in metabolic dysregulation and accelerated catabolism. Vitamin deficiency triggers an inflammatory response that stimulates tumor growth by activating the surrounding stroma, particularly neovascularization [10]. Conversely, two randomised, large-scaled, and controlled studies have shown a reduced incidence of cancer in males taking a daily multivitamin dosage compared to placebo [11].

There is a growing evidence to support the assumption that supplementing chemotherapy medications with vitamins may lower cancer incidence [12]. Vitamins, one of the most researched nutritional ingredients, have displayed the inhibitory action of free radicals by preventing carcinogen formation [13]. Higher doses of antioxidant vitamins such as vitamin A, vitamin C, and vitamin E were found to reduce cancer risks by preventing DNA damage from reactive oxygen species [14]. Though vitamin D is not an antioxidant, a combination of vitamin D3 (cholecalciferol) at 1 100 IU/day and calcium at 1 450 mg/day has been shown to reduce the frequency of invasive cancer by 60% [15]. Also, by increasing the expression of E-cadherins (the principal epithelial intercellular adherence proteins), the incidence of cancer is reduced [16, 17]. A sufficient intake of B-complex vitamins and vitamin B12 (cobalamin) has also been shown to reduce the risk of cervical cancer and assist in the production of amino acids, blood cells, and physically active nerves [18]. The use of nutritional supplements could pose serious health risks and have a negative impact on the chances of curing or controlling cancer when compared to conventional cancer treatment. Combinatorial regimens, when compared to a single therapy, enhance anti-cancer properties while maintaining tissue homeostasis [19–21].

The vitamins protect the homeostasis of tissue from cancer's long latency. Vital issues such as probable toxicity, efficacy, and dose-dependent effects have been observed in vitamin chemoprevention. Therefore, the administration of vitamins and chemotherapeutics by nano-delivery methods can reduce vitamin-induced toxicity and side effects [22–26]. Vitamin-rich nanoparticles and chemotherapeutic drugs can reduce the resistance commonly observed in cancer cells and tumors. This combination is known as nanomedicine, and it has numerous therapeutic applications. The long-term treatment of cancer drugs in combination with vitamins is unknown, so this study aims to define the chemo-preventive properties involved in cancer progressions.

In this study, a multivitamin nano–cisplatin formulation called NanoCisVital (NCV) was developed using chitosan biopolymers. Chitosan is selected in our study, due to its biodegradability, biocompatibility, non-toxicity, and antibacterial properties [27]. The chitosan nanoparticles have demonstrated good absorption properties, biocompatibility, enhanced permeability retention (EPR) effects, and increased blood circulation times that can improve drug delivery to tumor sites [28]. The physicochemical properties of chitosan-based NCV were characterized, including its chemical state, oxidation state, growth kinetics, ligand binding arrangement and density, and surface composition. We evaluated the effects of NCV on cell lines derived from breast cancer (MDA-MB-231 and MCF-7) and cervical cancer (HeLa and SiHa). The cell lines were treated with a combination of drugs and vitamins. DDX3X promotes the proliferation, invasion, and metastasis of cancer cells, whereas Ki-67 promotes cell proliferation. Overexpression of DX3X is observed in breast cancer cells. The Ki-67 protein is present during all active phases of the cell cycle but is difficult to detect during the quiescent phase. The expression patterns of the well-known proliferative marker gene *Ki-67* [29], and the cisplatin-sensitive gene *DDX3X* [30] were investigated to determine whether the treatment altered their expression. Our findings could lead to a new therapeutic strategy for preventing chemotherapy-induced fatigue.

Experimental

Materials

The following analytical-grade reagents were purchased from Hi-Media (Mumbai, India): extra-

pure chitosan powder (low-molecular-weight), sodium tri-poly phosphate (TPP), vitamin B12 (cobalamin), vitamin C (ascorbic acid), vitamin D3 (cholecalciferol), fetal bovine serum (FBS), Dulbecco's Modified Eagle medium (DMEM), and other common reagents. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) and dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich Chemicals Private Limited (Bangalore, India). Cisplatin was purchased from Hetero Healthcare Limited (Himachal Pradesh, India). The Qiagen (India) supplied the total RNA isolation kit, Takara (Mumbai, India) supplied the real-time polymerase chain reaction (PCR) kit and complementary DNA synthesis kit.

Methods

Preparation of NCV

The ionic gelation method was used to make the chitosan nanoparticles [31]. To make the chitosan solution, low-molecular-weight chitosan was dissolved in a 1% (volume fraction) acetic acid solution. After adding the sodium tri-polyphosphate (TPP) solution, a cross-linking agent, to the chitosan solution and stirring gently for 1 h at room temperature, chitosan nanoparticles spontaneously formed. The 2:1 volume ratio of nanoparticles in opaque suspension was obtained by taking a 1 g/L chitosan solution and a 0.4 g/L TPP solution. The minimum amount of vitamins required for the cancer patients is identified as 1, 75, and 1 mg per day for vitamin B12, vitamin C, and vitamin D3, respectively [32–39]. 5 mg/mL cisplatin is given to the breast cancer patient in one chemo cycle [40, 41]. Based on the standard vitamin and cisplatin regimes in our previous studies we prepared various concentrations of cisplatin (0.5, 1, 2, 3, 4, and 5 mg/mL)–chitosan nanoparticles. Among all, 2 mg/mL cisplatin encapsulated chitosan nanoparticles possessed properties of better encapsulation efficiency (62%), anti-inflammatory, anti-proliferative, and anti-angiogenesis [42]. Therefore, in this study, we used the same concentrations to monitor cellular cytotoxicity and gene expression analysis using breast and cervical cancer cell lines. The vitamin B12 (1 mg/mL), vitamin C (75 mg/mL), and cisplatin (2 mg/mL) were dissolved in water containing 2 g/L TPP. Vitamin D3 (1 mg/mL) was dissolved in ethanol and further added to a 2 g/L TPP solution. Cisplatin–vitamin-loaded nanoparticles were created by dissolving cisplatin and vitamins in an aqueous chitosan solution and further homogenising them using a magnetic stirrer at 500 r/min for 6 h at the same time. In a 20 μL of glycerol bed, the nanoparticles were centrifuged for 25 min at 15 000 r/min (Eppendorf, Germany). The obtained pellet was again suspended in a sucrose solution (5%) and freeze-dried using a freeze dryer (Operon, Korea). The nanoparticles were kept at 4 °C for further use.

Characterization of nanoparticles

The transmission electron microscopy (TEM) of the JEOL JEM 2100 PLUS was used to assess the morphology of the blank nanoparticles (BNPs), vitamin B12, vitamin C, and vitamin D3-loaded nanoparticles (VIT NPs), and NCV. TEM analysis was carried out by placing a drop containing a suspension of nanoparticles on a copper network covered by carbon and enabling the dissipation of water in a vacuum-enabled dryer. The grid with nanoparticles was scanned for images of TEM. They were further recorded by atomic force microscopy (AFM) using a Bruker Multimode-8 scanning probe microscope system. The roughness of these nanoparticles was measured using AFM. The thermal behavior of the nanoparticles was analysed by differential scanning calorimetry (DSC 4000, Perkin Elmer). A sample of 4–8 mg was scanned within a temperature range of 0–250 °C at a heating rate of 15 °C per minute per cycle in an inert atmosphere maintained by nitrogen purging at a rate of 360 cm^3/min. The phase changes and thermal stability were analysed using a thermogravimetric analyser (TGA) from TA Instruments Inc., SDT Q600 V20.9 Build 20 Universal V4.5A. For TGA runs, samples of 4–8 mg were weighed in the platinum pan and scanned from 0 to 400 °C at a rate of 10 °C per minute per cycle. The weight loss and decomposition were measured during the heating range. Purging nitrogen at a rate of 360 cm^3/min maintained an inert atmosphere. An aluminum, the empty pan was taken as a reference. The X-ray diffraction (XRD) patterns of BNP, VIT NP, and NCV were obtained with a Bruker D8 Focus diffractometer, operating at 40 kV and 35 mA with Cu Kα radiation (λ = 1.54060 Å). The XRD patterns were obtained in fixed-time mode with a scan rate of 50 s^{-1} and 2θ ranging from

10° to 80° at room temperature.

In vitro cytotoxicity study (MTT assay)

To determine the cytotoxicity of nanoparticles, 5 × 10^3 MCF-7, MDA-MB-231, HeLa, and SiHa cells per well were seeded and cultured. Cells were treated with an increasing concentration (0, 50, 100, 150, 200, and 250 mmol/L) of various nanoparticles in DMSO for 24 h. Following drug treatment, the cells were washed with 1× phosphate buffered saline (PBS), and 100 µL of fresh culture medium was added to the cells. The 20 µL of MTT solution (5 mg/mL) was added to the cells, and the plate was incubated for 3–4 h in a CO_2 incubator at 37 ºC. The purple color obtained by dissolving the formazan crystals in 100 µL of DMSO was estimated by measuring the absorbance at 540 nm in a PerkinElmer plate reader. The percentage inhibition of nanoparticles, drug, and vitamins was calculated as

$$\frac{\overline{OD}_{BNPTC} - \overline{OD}_{DVLNPTC}}{\overline{OD}_{BNPTC}} \times 100 \quad (1)$$

where $\overline{OD}_{BNPTC}$ represents the mean optical density ($\overline{OD}$) of blank nanoparticle-treated cells, and $\overline{OD}_{DVLNPTC}$ represents the $\overline{OD}$ of drug and vitamin-loaded nanoparticle-treated cells.

The half-maximal inhibitory concentration (IC50) values listed in Table 1 were calculated using the graph pad prism software (Graph Pad Software Inc., CA, USA).

Gene expression study

To identify changes at the molecular level, MCF7, MDA-MB-231, HeLa, and SiHa cell lines were treated for 24 h with different concentrations of cisplatin, vitamins, and NCV. Following incubation, total RNA was extracted from the treated cell lines using the RNeasy Mini Kit (Qiagen), followed by a reverse transcription reaction using a PrimeScript cDNA synthesis kit (Takara) to generate first-strand cDNAs to be used in a quantitative real-time polymerase chain reaction (qRT-PCR). Expression levels of *DDX3X* and *Ki-67* were quantified in a CFX96 real-time PCR system (Bio-Rad) using TB green premix Ex Taq II (Takara). The primers were as follows: *GAPDH*, 5 ′-TGACATCAAGAAGGTGGTGA-3 ′ (forward) and 5 ′-TCCACCACCCTGTTGCTGTA-3′ (reverse); *DDX3X*, 5′-GGAGGAAGTACAGCCAGCAAAG-3 ′ (forward) and 5 ′-CTGCCAATGCCATCGTAATCACTC-3 ′ (reverse); and *Ki-67*, 5 ′-TCCTTTGGTGGGCACCTAAGACCTG-3 ′(forward) and 5 ′- TGATGGTTGAGGTCGTTCCTTGATG-3 ′ (reverse). 5 ′-TGACATCAAGAAGGTGGTGA-3 ′ (forward) and 5 ′-TCCACCACCCTGTTGCTGTA-3 ′ (reverse); *DDX3X*, 5 ′-GGAGGAAGTACAGCCAGCAAAG-3 ′ (forward) and 5 ′-CTGCCAATGCCATCGTAATCACTC-3′ (reverse); and *Ki-67*, 5′-TCCTTTGGTGGGCACCTAAGACCTG-3 ′(forward) and 5 ′-TGATGGTTGAGGTCGTTCCTTGATG-3 ′ (reverse). The conditions for qRT-PCR were as described in the qRT-PCR kit, with an annealing temperature of 56 °C for 30 s, and data were normalized to GAPDH before being quantified using a relative relationship method.

Statistical analysis

All experiments were repeated three times, and the results are presented as the ± mean standard deviation (SD). To determine statistical significance, we performed a one-way analysis of variance at $P < 0.05$.

Results and Discussion

Morphology and characterization of NCV

Cancer therapeutic procedures necessitate the development of novel strategies that are cost-effective and have fewer and less severe side effects to be more acceptable to patients. Nanoparticles, novel anticancer compounds, have displayed toxicity at low levels [43]. Several investigations have been carried out on conjugating cytotoxic medicines to nano molecules to improve their anti-cancer activities while lowering their systemic side effects [44]. Among the hydrophilic polymers, chitosan has been extensively studied. The NCVs in this work were

Table 1 IC50 values of cisplatin, cisplatin NP, vitamins-loaded NP, NCV, vitamin B12 NP, vitamin C NP, vitamin D3 NP, and blank NP on MCF7, HeLa, MDA-MB-231, and SiHa cells in micromole per liter (µmol/L)

Serial No.	Cell lines	Cisplatin	Cis NP	B12+C+D3 NP	NCV	B12 NP	C NP	D3 NP	Blank NP
1	MCF7	108	84.158	814	490	827	245.8	544.9	420.18
2	HeLa	89.93	118.6	1652	536.8	1986	111.66	13219	604.26
3	MDAMB231	121.96	111.092	841.7	206.4	275.2	211	256.35	353.94
4	SiHa	113.43	254.99	4636	827.36	7227	7897	804.43	680.06

created to target anticancer activity with minimal or no side effects using chitosan and were characterised using several biophysical approaches.

The surface morphological characterization and size confirmation of BNP, VIT-NPs, and NCV were determined using HRTEM. According to HRTEM images, NCV were spherical, with a particle size distribution in the 200 nm range (Fig. 1). The size of nanoparticles affects their pharmacokinetics significantly. Larger particles are normally eliminated by macrophages, while nanoparticles smaller than than 300 nm in diameter can activate the complement system, resulting in blood clearance. These nanoparticles have high cellular uptake and good suspensible properties. Hence, it can easily penetrate the arterial walls. Furthermore, the smaller particles' surface area to volume ratio is larger, which can result in a slow drug diffusion rate and a high drug loading capacity [45]. The morphology of NCV is spherical, and no aggregation was observed. All the nanoparticles were separated and uniformly synthesized. The maximum roughness peak (Rmax) height was observed at 59 nm (Fig. 2). The high average roughness enhances the binding capacity for cisplatin, vitamins, and nanoparticles; therefore, drug delivery is more effective [46].

DSC studies confirm the physical status of the encapsulation of drugs inside the nanoparticles. BNPs showed two endothermic peaks at 67.94 and 223.78 °C (Fig. 3(a)); VIT-NPs showed one common endothermic peak similar to blank chitosan nanoparticles at 67.94 °C and two new endothermic peaks at 194.39 and 233.19 °C (Fig. 3(b)). The presence of exothermic peaks in VIT-NPs is due to the acquisition of thermal stability due to the encapsulated vitamins in the chitosan nanoparticles. The NCV showed multiple endothermic and exothermic peaks. The highest expanded endothermic

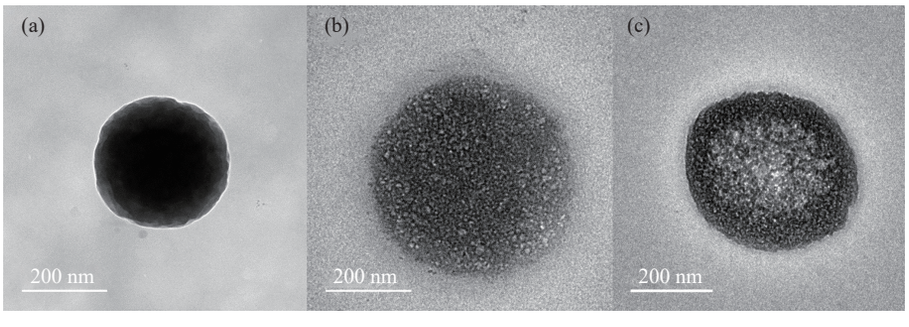

Fig. 1 Characterization of NCV. Note: The TEM images show the surface morphological characterization of **(a)** BNP, **(b)** VIT-NP, and **(c)** NCV.

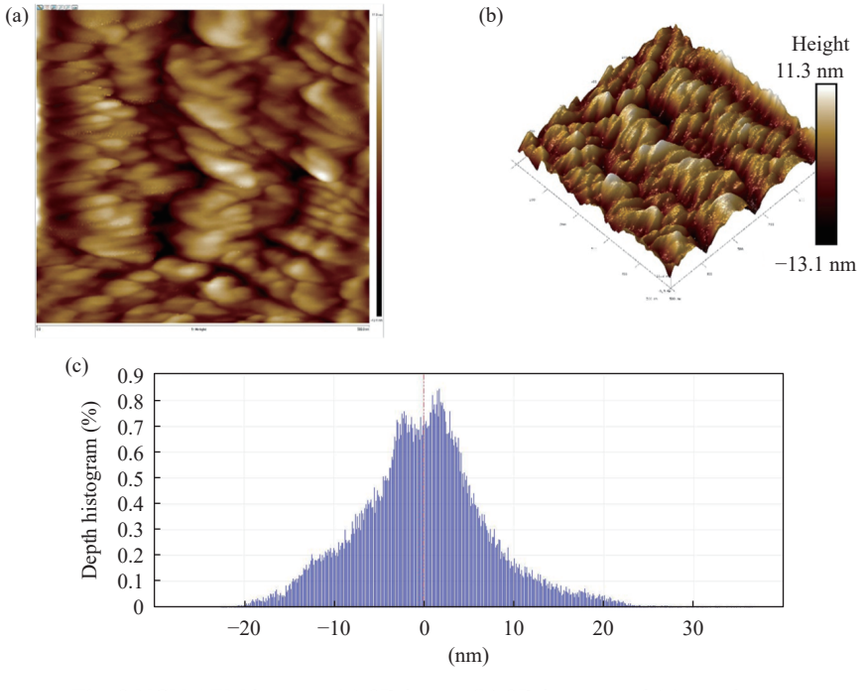

Fig. 2 NCV AFM images: **(a)** 2D image **(b)** 3D image, and **(c)** roughness.

peak was observed from 36.45 to 156.70 °C. Further endothermic and exothermic peaks were shown from 204.73 to 244.45 °C, suggesting the incorporation of cisplatin in the core of the vitamin nanoparticle matrix (Fig. 3(c)). The successful integration of the drug and vitamins inside the nanoparticles was assessed by DSC. The common endothermic peak is due to the decomposition of chitosan in the nanoparticles, and the other two different endothermic peaks are due to the stronger interactions between vitamins and chitosan nanoparticles. Multiple endothermic and exothermic peaks at higher temperatures are due to the hydrophilic nature of vitamins and cisplatin. In this study, the expanded peak in the chitosan nanoparticles encapsulated with vitamins and cisplatin (NCV) showed the partial or complete encapsulation of drug and vitamins inside the chitosan nanoparticles.

The thermogravimetric analysis (TGA) is performed to determine the degradation temperature (T_d) of the active component included in the encapsulated material at which the maximum weight loss occurs. BNPs (Fig. 4(a), VIT-NPs (Fig. 4(b), and NCV (Fig. 4(c)) all had three levels of weight loss. The different temperature ranges and concentrations of cisplatin loaded into chitosan nanoparticles will result in different slopes. The highest T_d values were found between 250 and 350 °C, where major weight loss was recorded. The weight of cisplatin and vitamin-loaded chitosan nanoparticles decreased with increasing temperature levels, ranging from 0 to 800 °C, and it was observed that cisplatin and vitamin-loaded nanoparticles had new T_d at 200 and 250 °C, respectively. The weight losses indicate the degradation of drugs and vitamins encapsulated inside the nanoparticles. The TGA results suggest that cisplatin and vitamins were successfully loaded into the chitosan nanoparticles.

The X-ray diffraction patterns were taken for blank chitosan nanoparticles. The BNP exhibited a characteristic crystalline peak at 2θ = 19.47°, which was shifted slightly to a higher diffraction angle (Fig. 5(a)). This indicates that the chitosan in the nanoparticles has a better crystalline nature. The chitosan only contained one broad diffraction peak,

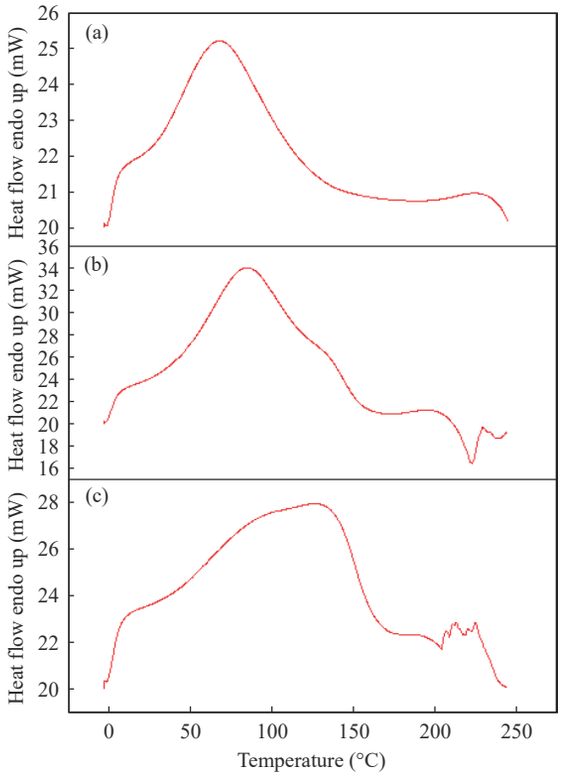

Fig. 3 DSC thermographs of **(a)** BNPs, **(b)** VIT-NPs, and **(c)** NCV.

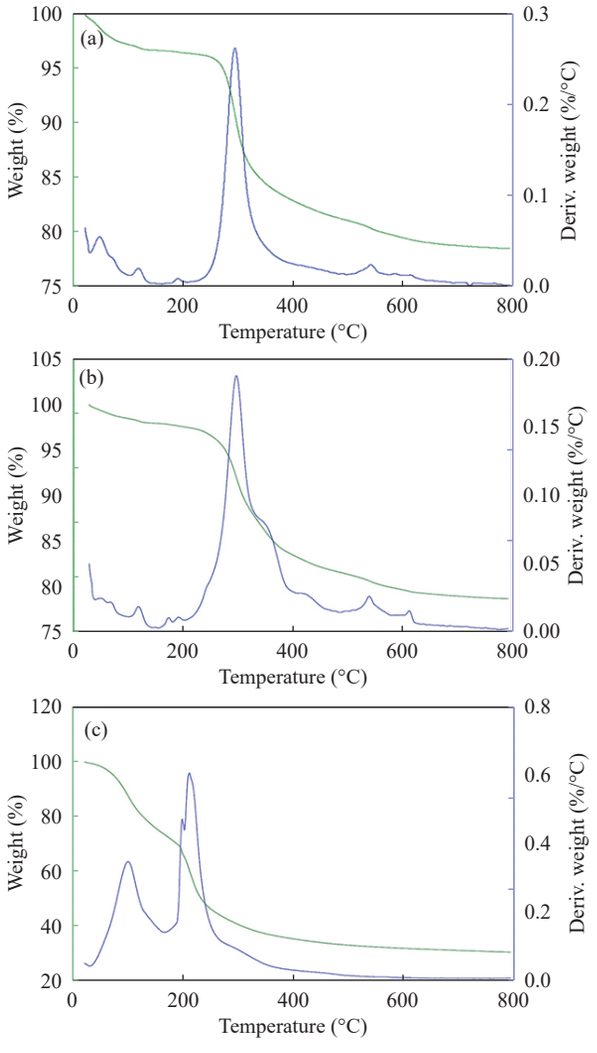

Fig. 4 TGA curve of of **(a)** BNP, **(b)** VIT-NP, and **(c)** NCV.

which is centered at 2θ = 19.47°. And similar studies also showed different diffraction peaks at 2θ = 19.47°, revealing a typical property of semi-crystalline chitosan. Vitamin-loaded chitosan nanoparticles showed a similar chitosan peak at 2θ = 19.37° and vitamin peaks at 2θ = 33.23°, 34.09°, and 35.56° (Fig. 5(b)), which represents the incorporation of vitamins into nanoparticles. The cisplatin and vitamin-loaded nanoparticles exhibited similar chitosan and vitamin peaks and showed a cisplatin peak at 2θ = 23.56°. This indicates the crystalline nature of chitosan with vitamins and cisplatin. The XRD analysis showed the crystalline nature of different nanoparticles, and at high intensities, prominent peaks appeared. The less intense peaks represent their amorphous nature, and the broad, high-intensity peaks represent the diffuse scattering property of the nanoparticles. After reacting with TPP, vitamins, and cisplatin, the conversion of amorphous chitosan into a crystalline form increased the intensity of the NCV diffraction peaks (Fig. 5(c)). The diffraction peaks of BNP, VIT-NP and NCV show the reaction of chitosan with the TPP and crystallised structures of BNP, VIT-NP, and NCV, along with vitamins and cisplatin. The XRD pattern's broad, low-intensity peaks may be attributable to the small particle size and imperfect internal structure. This indicates that a high percentage of these particles

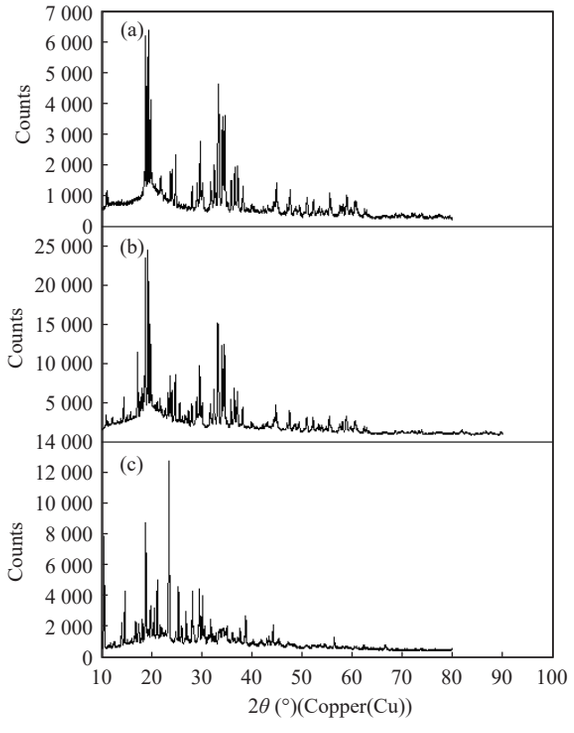

Fig. 5 XRD analysis of **(a)** BNPs, **(b)** VIT-NPs, and **(c)** NCV.

are amorphous. Hence, XRD results confirmed that the synthesised NCV is amorphous in nature. Very low intensity and less noticeable peaks in NCV diffraction graphs show a complex network structure of interpenetrating polymer chains of chitosan cross-linked by the TPP counterion. This low-peak diffraction graph showed the formation of chitosan nanoparticles with a strong interaction between the chitosan and TPP counterions. In the case of NCV, after cross-linking with tripolyphosphate, the crystallised chitosan is converted to an amorphous form, resulting in a slower rate of diffraction peaks.

Effect of nanoparticles on the growth and viability of cancer cells

Cisplatin is a well-known anticancer drug; however, it has been shown to induce several side effects, including fatigue [47]. Vitamins are often recommended as a supplement to reduce fatigue [48]. Despite this, a recent study concluded that taking vitamins before and during chemotherapy was associated with breast cancer recurrence [49]. On the other hand, vitamin C has been shown to kill cancer cells in mice with ovarian cancer [50]. This ambiguity may be due to the direct interaction between drugs and vitamins, which prevent the effect of chemotherapy on rapidly dividing cells [51]. Therefore, in our study, we developed NCV for the sequential delivery of vitamins and cisplatin without much interference. To study the impact of NCV, we treated the breast cancer cell lines (MDA-MB-231 and MCF7) and the cervical cancer cell lines (HeLa and SiHa) with vitamins alone, drugs alone, and vitamins and drugs together.

The MCF7, MDA-MB-231, HeLa, and SiHa cell lines were treated with escalating concentrations of cisplatin and cisplatin-encapsulated nanoparticles to determine whether they could reduce the proportion of viable cancer cells as effectively as cisplatin. As shown in Fig. 6(a), cisplatin nanoparticles can decline cancer cell growth. The IC50 of cisplatin and vitamin–cisplatin nanoparticles was found to be distinct and cell type-specific in a concentration gradient experiment (Table 1). Blank nanoparticles did not show any significant cell death. In breast cancer cell lines, no significant IC50 difference is observed between cisplatin and cisplatin-encapsulated nanoparticles.

To measure the effects of vitamin B12, vitamin C,

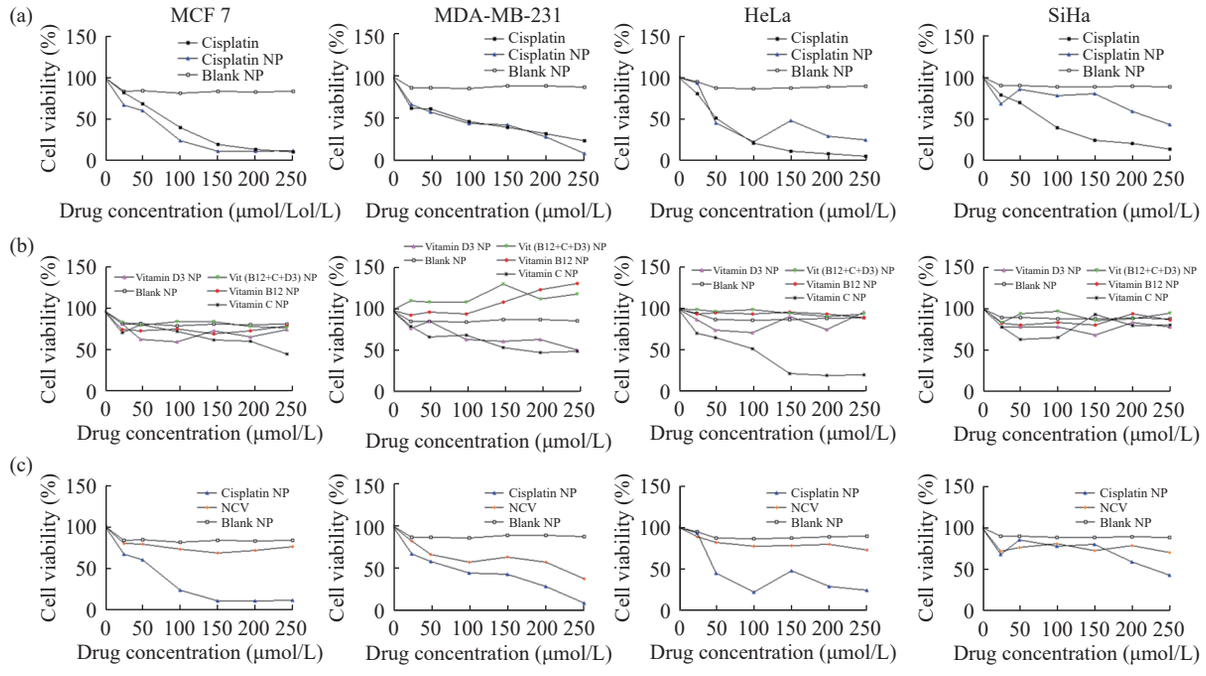

Fig. 6 *In vitro* cytotoxicity of **(a)** cisplatin, cisplatin NP, and blank NP; **(b)** vitamin B12 NP, vitamin C NP, vitamin D3 NP, vitamins-loaded NP, and blank NP; **(c)** cisplatin NP, NCV, and blank NP against MCF7, MDAMB231, HeLa, and SiHa cells incubated for 48 h. The cytotoxicity of formulations was evaluated by MTT assay. The data are presented as mean + SD (n=3).

and vitamin D3 on cancer cell viability, the cells were treated with increasing concentrations of vitamins, and the percentage of cell viability was measured by the MTT assay. The MCF7 cells treated with varying amounts of vitamin D3 and vitamin B12 (Fig. 6(b)) retain their viability. In contrast, at a concentration of 250 μmol/L, vitamin C inhibited the proliferation of MCF7 cells by approximately 50% compared to control nanoparticles. Vitamin C and vitamin D3 at a concentration of 250 μmol/L inhibited the development of MDA-MB-231 cells by approximately 50%. Vitamin B12 alone and/or together with other vitamins (C, D3, and B12) increased the proliferation of the MDA-MB-231 cell line. To validate that the effects of vitamins is specific to cell type, we performed a similar study using HeLa and SiHa cervical cancer cell lines. Vitamin C reduced the cell growth in HeLa cells similar to the MCF7 cell line. The addition of a specific vitamin has no detectable influence on the viability of SiHa cells. Together, they suggest that multivitamin complexes have a minimal effect on cell growth.

To conclude the synergistic effect of cisplatin and vitamins in drug-loaded nanoparticles, cisplatin and vitamins were added at a constant ratio of 1:3 based on their IC50 values. The cytotoxicity of the free drugs and different NPs was tested *in vitro* using MTT assays on MCF7, MDA-MB-231, HeLa, and SiHa cells. The anticancer activities of nano-encapsulated forms and free drugs were concentration-dependent (Fig. 6(c)). Most importantly, NCV significantly arrested the growth of cancer cells, similar to cisplatin nanoparticles in MDA-MB-231 cell lines. The action of NCV on cellular toxicity is minimal as compared to that of cisplatin nanoparticles in a weekly tumorigenic breast cancer cell line, MCF7, HeLa, and SiHa cervical cancer cell lines. Taken together, NCV can be used to treat triple-negative breast cancer with few side effects.

Results showed that cisplatin nanoparticles effectively killed both MDA-MB-231 and MCF7. The cell death response is delayed in HeLa as compared to breast cancer cells, and it is further extended in SiHa cell lines. This may be due to genetic heterogeneity between cervical cancer cell lines. For example, it is well known that these cell lines differ based on HPV copy number, and SiHa is known to be resistant to cisplatin drug treatment [52]. Nanoparticles encapsulated with a multivitamin were unable to trigger cell death in MDA-MB-231, MCF-7, HeLa, or SiHa cell lines. Vitamin C-encapsulated nanoparticles can induce 50% and 80% cell death in MCF7 and HeLa cells at 250 and 150 μmol/L concentrations, respectively. Similarly, vitamin C and vitamin D3-encapsulated nanoparticles triggered 50% cell death

in MDA-MB-231 cell lines at a concentration of 250 μmol/L, whereas no cell death was observed in SiHa cells following vitamin treatment.

However, compared to other cell lines, MCF7 is the least sensitive to treatment with NCV. NCV affects cancer cells through the physicochemical interaction of cellular proteins and nanoparticles. *In vitro* cytotoxicity of NCV and cisplatin investigations identified that the rate of cell viability decreases while increasing the treatment time with nanoparticles. Results of IC50 revealed a reduced viable cell percentage upon treatment for a longer duration. The drug is covalently attached to the chitosan polymer. Consequently, the drug's immediate action is delayed due to the bond cleavage time, and that further inhibits the short-term release of the drug. This can explain the delayed increase in NCV toxicity observed in this study. The MTT assay results on human fibroblasts exhibited that nanoparticles showed lower cytotoxicity than free drugs, which indicates the lesser and inaccurate toxicity of drug and polymer mixes when treated on normal fibroblast cells [53].

Effect of NCV on *DDX3X* and *Ki-67* gene expression

Breast and cervical cancer cells were treated with the IC50 concentration for 24 h to examine if the expression of the cisplatin-sensitive gene *DDX3X* remained constant between the free drug and the encapsulated form. We evaluated *Ki-67* and *DDX3X* transcript levels following treatment using qRT-PCR (Tables (2) and (3)). *DDX3X* mRNA levels were decreased in MCF7 and MDA-MB-231 cells treated with cisplatin and cisplatin nanoparticles (Fig. 7(a)). On the other hand, *DDX3X* expression levels in HeLa cells were unaffected. The difference in *DDX3X* expression between cisplatin and its encapsulated form indicates that SiHa cells are responsive to cisplatin but not to cisplatin-encapsulated nanoparticles. The expression of *Ki-67*, a cell proliferation marker, was then investigated to see if the expression of *DDX3X* was connected to cell proliferation. Cisplatin and its encapsulated form reduced *Ki-67* expression in both the MDA-MB-231 and MCF7 cell lines. However, HeLa cells had higher levels of *Ki-67* mRNA expression than controls, whereas SiHa cells did not. These data suggest that when cisplatin nanoparticles are combined, they are sensitive to breast cancer cells but not to cervical cancer cells.

Vitamins are essential for maintaining cellular health. Real-time PCR analysis demonstrated that vitamins and their nanoparticles reduced the expression of *DDX3X* and *Ki-67* in MCF7 cells. MDA-MB-231 and SiHa cell lines, in contrast, have elevated *DDX3X* and *Ki-67* mRNA levels. In comparison, HeLa cells treated with vitamins or

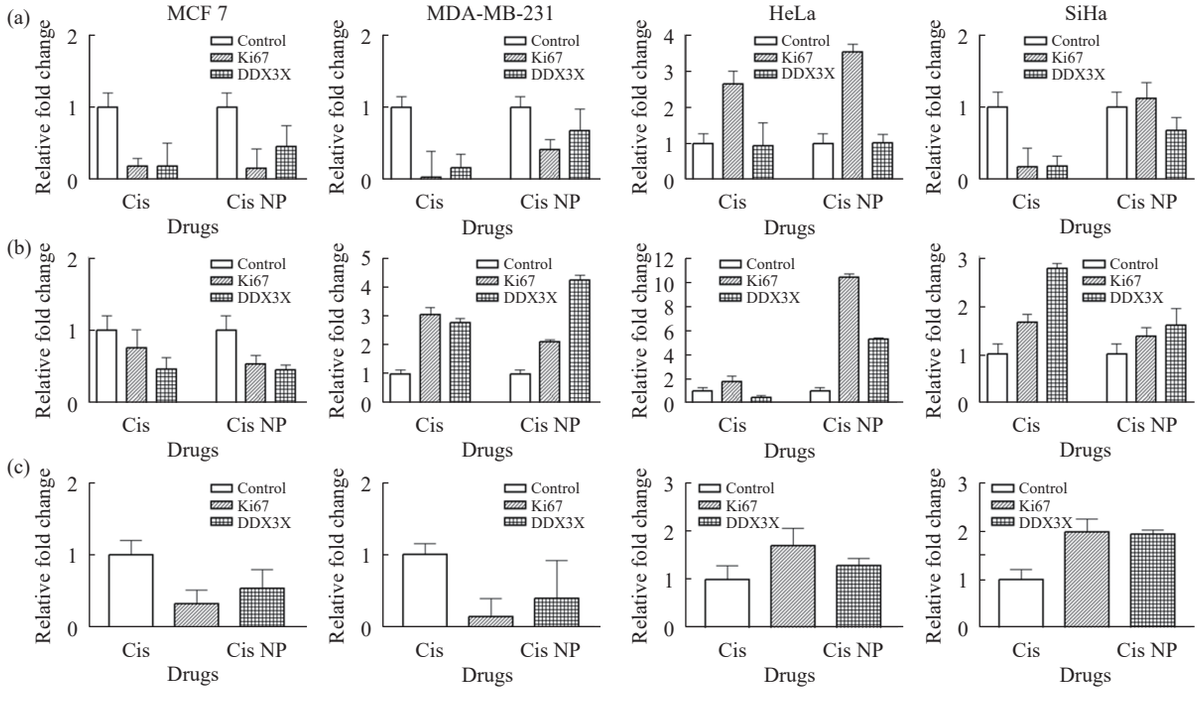

Fig. 7 Real-time PCR analysis of **(a)** Cisplatin (Cis) and Cis NP; **(b)** vitamins alone and vitamins-loaded NP; **(c)** NCV ($P < 0.05$).

Table 2 Primers used for relative expression of *GAPDH*, *DDX3Y*, *DDX3X*, and *Ki-67* genes

Name	Primer sequence
GAPDH F	TGACATCAAGAAGGTGGTGA
GAPDH R	TCCACCACCCTGTTGCTGTA
DDX3Y F	AACCCTGTCAAGTCTGTCGAGCCTC
DDX3Y R	GCGATCCACGGTGGTTTGAATACA
DDX3X F	GGAGGAAGTACAGCCAGCAAAG
DDX3X R	CTGCCAATGCCATCGTAATCACTC
Ki67 F	TCCTTTGGTGGGCACCTAAGACCTG
Ki67 R	TGATGGTTGAGGTCGTTCCTTGATG

Table 3 qRT-PCR cycling conditions

Name of the step	Temperature (ºC)	Time (s)
Initial denaturation	95	60
Denaturation	95	5
Annealing	56	30
Extension	72	30

vitamin nanoparticles expressed DDX3X at different levels (Fig. 7(b)). Overall, the evidence suggests that several cell types are required for the chemical reaction caused by vitamins. To investigate the action of cisplatin in the presence of vitamins, we combined vitamins and cisplatin into a capsule known as NCV. *DDX3X* and *Ki-67* expression levels were downregulated in MCF7 and MDA-MB-231 cells, although they were upregulated in HeLa and SiHa cell lines (Fig. 7(c)). All things considered, we assume that NCV can be used to treat breast cancer.

DDX3X has previously been identified as a cisplatin-sensitive gene [54]. To assess the potential function of NCV in chemosensitivity, we validated the expression of DDX3X and Ki-67, a cell proliferation marker, in both breast cancer cell lines and cervical cancer cell lines (Table 4). Cisplatin and cisplatin nanoparticles, as expected, reduced DDX3X mRNA expression in both breast cancer cell lines. In cervical cancer cell lines, cisplatin nanoparticles did not affect DDX3X expression. This information is based on cell viability tests. In contrast to MCF7, vitamin nanoparticles boosted the expression of DDX3X and Ki-67 mRNA in MDA-MB-231, HeLa, and SiHa cell lines when compared to vitamin supplementation alone. It implies that vitamin supplements can prevent cancer cell death induced by cisplatin. The NCV successfully downregulated the *DDX3X* gene expression in breast cancer cell lines, but not in cervical cancer cell lines.

Table 4 Annealing temperatures for *GAPDH*, *Ki-67*, and *DDX3X*

Cell line	Annealing temperature (°C)		
	GAPDH	*Ki-67*	*DDX3X*
HeLa	60.6	56.2	61
MDAMB231	59.7	56.2	61
MCF7	61	59.7	59.7
SiHa	59.7	59.7	59.7

Conclusion

NCV is a multivitamin and cisplatin-encapsulated nanoparticle with the potential to be used in the treatment of cancer. The MTT assay revealed that NCV has a superior cytotoxicity in MDA-MB-231 breast cancer cells, indicating that it possesses important physicochemical properties. NCV significantly reduced the mRNA levels of *DDX3X* and *Ki-67* in breast cancer cells, whereas it had little effect on these genes in cervical cancer cells. It could be due to increased cellular internalisation and release of encapsulated components in the MDA-MB-231 breast cancer cell line compared to cervical cancer cell lines. Taken together, our results suggest that NCV could be an improved treatment option for breast cancer, and these findings can be applied to *in vivo* studies to verify the effectiveness of drug-multivitamin-loaded chitosan-based nanomaterial therapy.

CRediT Author Statement

Mahendran Botlagunta conceived the idea for the project. **Rajath Othayoth**, **Karishma Khatri**, **Ramya Gadicherla**, and **Suseela Kodandapani** performed all the experiments, analyzed the data, and prepared the manuscript. **Rajath Othayoth** and **Mahendran Botlagunta** reviewed and analyzed the interpreted data and the manuscript.

Acknowledgement

We would like to express our sincere gratitude to Indo-American Cancer Research Foundation, Basavatarakam Indo-American Cancer Hospital and Research Institute, Hyderabad, India for their support to conduct this study and Dr. Nandita Tanneru, Atal Incubation Centre, Centre for Cellular & Molecular

Biology, Hyderabad, India in providing critical inputs and suggestions. This work was supported by DST-SERB, India under grant No. EMR/2017/001294.

Conflict of Interest

The authors declare that no competing interest exists.

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