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## Characterization of vitamin–cisplatin-loaded chitosan nano-particles for chemoprevention and cancer fatigue

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### Abstract

**Context:** Vitamins have been shown to reduce chemotherapy-related fatigue (CRF) by conserving energy loss both during and after cancer treatment. However, it remains unknown whether this reduction of fatigue interferes with the cancer drugs or alters the effectiveness of these agents. **Objectives:** The objective was to synthesize vitamin–cisplatin-loaded chitosan nano-particles for chemoprevention and cancer fatigue. **Materials and methods:** Multi-vitamin (C, D3, and B12)–cisplatin composite nano-formulation called NanoCisVital (NCV) to overcome CRF. The interactions between vitamins and NCV were characterized using scanning electron microscopy (SEM), Fourier transform infrared (FT-IR) spectroscopy, and a particle size analyser. The chemo-preventive activity was performed by in vitro bio assays. **Results:** SEM analysis showed spherical shape and the size is ~225 nm. NCV inhibited the proliferating yeast cells as well as denaturation of bovine serum albumin, and it also reduced the sprouting of new blood vessels in dose-dependent manner. **Conclusion:** Collectively, these results demonstrate that the NCV particles can be used to reduce CRF without much affecting the anti-cancer properties of cisplatin.

### Introduction

Cancer is one of the leading causes of death worldwide (Anand et al., 2008). A number of chemotherapeutic agents have been used for chemoprevention. Chemoprevention is a protective intrinsic physiological mechanism of an organism against the growth of malignant cells using either natural or synthetic chemical agents. Several natural and synthetic chemicals have shown chemopreventive activity (Spoor, 1993; Dorai and Aggarwal, 2004; Swanson et al., 2010); however, they often produce fatigue and anaemia (Cella et al., 2002). For example, patients who receive platinum-based chemotherapy have double the risk of anaemia as compared to patients given non-platinum based chemotherapy (Coiffier et al., 2001; Chen et al., 2013). Anaemia is one of the most common and chronic disorders of cancer therapy; in this condition, the oxygen carrying capacity of red blood cells (RBCs) decreases, as does the absorption of vitamins (Cella, 1997; Balducci and Hardy, 1998; Bron et al., 2001; Balducci, 2003; Schrijvers, 2011). Vitamin deficiency has been shown to trigger an inflammatory response, which then further promotes tumour growth through the activation of the surrounding stroma, particularly neovascularization (Olivares et al., 2000; Ames and Wakimoto, 2002; Ananthakrishnan et al., 2014). Therefore, vitamins and dietary supplements that come as pills, tablets, or a liquid are very important for patients receiving cancer therapy (Cassileth and Deng, 2004). However, the use of nutritional supplements instead of conventional cancer treatment could be harmful to human health and greatly reduce the chance of curing or controlling the cancer. As a result, we hypothesize that the combinatorial regimens will increase anti-cancer properties while at the same time maintaining tissue homeostasis as compared to a single therapeutic treatment alone (Conklin, 2000; Bauer and Capra, 2005; Aggarwal et al., 2006).

In addition to chemotherapeutic agents, there is a growing body of evidence that supports the hypothesis that vitamins can reduce the incidence of cancer (Gaziano et al., 2012). Vitamins are among the most studied dietary ingredients, and they have been shown to prevent carcinogen formation by inactivating the action of free radicals (Hemekens, 1994). A large intake of antioxidant vitamins, such as C, E, and A, has been shown to reduce the risk of cancer by preventing DNA damage from reactive oxygen species (Hunter et al., 1993). Vitamin D is not an antioxidant, but the supplementation of 1100 IU/d of vitamin D3 (cholecalciferol) in conjunction with 1450 mg/d of calcium yielded a 60% reduction in the incidence of all invasive cancers (Garland et al., 2009). In contrast to antioxidant vitamins, vitamin D has been shown to reduce the incidence of cancer by up-regulating the expression of the principal epithelial intercellular adherence proteins, E-cadherins (Gniaidekyll et al., 1997; Pendas-Franco et al., 2007). At the same time, dietary intake of vitamin B12 (cobalamin) and B-complex vitamins has also been
shown to reduce the cervical cancer risk and contribute to the maintenance of proper nerve function, blood cell formation, and amino acid production (Mamede et al., 2011). Although vitamins protect tissue homeostasis from the long latency of cancer, some major issues in vitamin chemoprevention are its efficacy, potential toxicity, and any dose-related side effects. For example, the physiological concentration of vitamin C (ascorbic acid) in plasma is under 0.1 mM; however, the dose required to trigger apoptosis in cancer cells is 10 nM to 1 mM and can vary even further depending on the cell line (Park, 2013). A higher dose of vitamin C than the normal physiological concentration has been shown to trigger haemolysis in glucose-6-phosphate dehydrogenase (G6PD) deficiency variants (Ibrahim et al., 2006; Abdul-Razzak et al., 2008). Vitamin-induced side effects and toxicity can be minimized with the selective delivery of vitamins and chemotherapeutic agents using nano delivery approaches (Mu et al., 2003; Win et al., 2006; Chidambaram et al., 2011; Yu et al., 2012; Cucinotto et al., 2013). Chitosan nano-particles have shown good biocompatibility and absorption properties as well as enhanced permeability and retention (EPR) effects and increased blood circulation times, which enhance drug delivery to the tumour sites (Patel et al., 2010). The use of chemotherapeutic drugs and vitamin-loaded nano-particles may reduce the resistance commonly seen in cancerous cells and tumours. The combined effect of long-term treatment with cancer drugs in conjunction with vitamins is not known. Thus, the purpose of this research is to determine the combined effects of multivitamins and cisplatin-encapsulated chitosan nano-particles on chemopreventive properties, such as anti-inflammatory, anti-proliferative, and anti-angiogenic activities, in the progression of cancer. Towards this goal, we have developed a multi-vitamin (Nanovital or NV)-cisplatin composite nano-formulation called NanoCisVital (NCV), which displayed better chemopreventive properties than the drug alone. These results provide a new therapeutic approach that could reduce the incidence of chemotherapy-related fatigue (CRF).

Materials and methods

Materials

Chitosan powder (low-molecular weight) was purchased from India Sea Foods (Kochi, Kerala, India), and vitamin C, vitamin B12, and vitamin D3 were all purchased from Hi-Media (Mumbai, India). Sodium tri-poly phosphate (STPP), Triton X-100, and glacial acetic acid were all obtained from Sigma-Aldrich Chemicals Private, Ltd. (Bangalore, India). Cisplatin was purchased from Parenteral Drugs India, Ltd. (Indore, India). All other reagents were of analytical grade and were obtained from Merck Millipore (Mumbai, India).

Methods

Preparation of chitosan nano-particles

The chitosan nano-particles were prepared using the ionic gelation method (Avadi et al., 2010). The chitosan solution was obtained by dissolving low-molecular weight chitosan in 1% (v/v) acetic acid solution. Chitosan nano-particles were prepared spontaneously upon addition of the STPP solution into the chitosan solution under gentle magnetic stirring at room temperature for 1 h. The STPP was used as a cross-linking agent. The volume ratio of the chitosan solution 0.1% (w/v): STPP solution 0.04% (w/v) was 2:1, and the opaque suspension was assigned to nano-particles. For vitamins such as vitamin B12 (1 mg/1 ml), C (75 mg/ml), and anti-cancer drug, cisplatin (2 mg/ml) were dissolved in 0.2% (w/v) STPP in water. Vitamin D (1 mg/1 ml) was dissolved in 0.2% (w/v) STPP in ethanol. For the preparation of vitamin (and cisplatin)-loaded nano-particles, previously dissolved vitamins and cisplatin were sequentially added to aqueous chitosan solution and homogenized at 500 rpm with an interval of 6 h using magnetic stirrer. The nano-particles were centrifuged (Eppendorf, Germany) at 15 000 rpm in a 20 μl glycerol bed for 25 min. The supernatants were discarded, and the pellet was re-suspended in a 5% sucrose solution. This suspension was freeze-dried (Operon, Korea) and stored at 4 ºC until its use. For an easy representation, all synthesized nano-particles are shown in Table 1.

Characterization of nano-particles

The diameters of the F1 to F6 nano-particles were analysed with dynamic light scattering using a particle size analyser (Beckman Coulter, Delsa Nano C, Brea, CA). The dynamic light scattering was carried out at a wavelength of 633 nm and an angle detection of 90 ºC. The zeta potential of these nano-particles was determined by using the Zetasizer (Beckman Coulter). The morphology of the nano-particles was observed using a scanning electron microscope (SEM; Supra-55, Carl Zeiss, Germany). The samples were lyophilized and mixed with potassium bromide then pressed into a pellet form to investigate the chemical reactions between the drug and nano-particles using Fourier transform infrared (FT-IR) spectroscopy (Bruker, France) at 4 cm⁻¹ resolution.

Evaluation of encapsulation efficiency (EE)

The encapsulation efficiency (EE) was assessed by measuring the amount of remaining drugs and vitamins that could be collected after centrifugation. The levels of vitamin B12, vitamin C, vitamin D3, and cisplatin present in the medium were determined spectrophotometrically by reading the absorbance at 230 nm, 265 nm, 260 nm, and 310 nm, respectively (Biospectrophotometer, Eppendorf, Germany). The microgram of drug present in the milligram of nano-formulation was determined as the difference between the total microgram of drug used to prepare the nano-formulation and the amount of free drug present in the aqueous medium. The EE scores of the nano-particles were calculated using the following equation:

\[
EE(\% \text{, w/w}) = \frac{\text{Mass of the total drug} - \text{Mass of free drug}}{\text{Mass of total drug}} \times 100.
\]

Drug release studies

Four milligrams of F1-F6 nano-particles were dispersed in a phosphate-buffered saline solution (PBS; pH = 7.4) as a release medium in a dialysis membrane sac (12 kDa; Sigma Aldrich). The dialysis sac was placed in a beaker containing PBS (50 ml) and incubated at 37 ºC in a continuous shaking incubator under mild agitation (80–100 rpm). For each sample, 2 ml of releasing medium was withdrawn at predetermined time intervals and replaced with
the same medium. The samples of vitamin B12, vitamin C, vitamin D3, and cisplatin were investigated for drug content by ultraviolet spectrophotometry (Dynamica, Newport Pagnell, UK) at 230 nm, 265 nm, 260 nm, and 310 nm, respectively. The drug release study was carried out in triplicates (n = 3) for 60 h.

**Anti-proliferative activity assay**

The anti-proliferative activity of the F1-F6 nano-particles was evaluated with the help of Saccharomyces cerevisiae. The S. cerevisiae BY4741 strain (wild type) was used for this assay. A single healthy colony from the strain was inoculated into the “seed broth,” which contained yeast, peptone, and dextrose and incubated at 37°C overnight. The next day, the seeded broth was diluted with the blank culture media until the absorbance (OD) reached 0.1 at 600 nm. To validate the action of the vitamins and drug-loaded nano-particles, 1 ml of the overnight yeast culture was transferred into several tubes. The first tube was kept as a control without any formulation. The other tubes were distributed with vitamins (C, B12, and D) alone and F1-F6 nano-particles. The standard vitamins (C, B12, and D) and cisplatin were also placed into respective tubes without any nano-formulation. All tubes were incubated at 37°C, and their absorbance was observed at 600 nm for predetermined time intervals (2, 4, 6, 12, 24, 48, and 72 h) using a UV–Vis spectrophotometer (Dynamica Halo DB20).

**Anti-inflammatory assay**

Anti-denaturation bovine serum albumin (BSA) assay was employed (Chatterjee et al., 2012) to evaluate the anti-inflammatory activity of F1-F6 nano-particles. In this assay, the reaction mixture consists of 0.2 ml (10 mg/ml) of BSA, 2.8 ml of PBS (pH 6.4), and 2 ml of varying concentrations of nano-formulation (100, 250, 500, 750, and 1000 µg/ml) to a final volume of 5 ml. The PBS lacking BSA served as control. These samples were incubated at 37°C for 15 min and then transferred into a 70°C water bath for 5 min. After cooling, the sample turbidity was measured at 620 nm using a spectrophotometer (UV-Dynamica). The anti-inflammatory activity of the F1-F6 nano-particles was determined by plotting the percentage of inhibition with respect to the control against the treatment conditions. In the present study, cisplatin and vitamins B12, C, and D alone were used as a positive anti-inflammatory drug. The percentage inhibition of protein denaturation was calculated with the following formula:

\[
\text{Percentage} \% \text{ of inhibition} = 100 \times \left[ \left( \frac{\text{VT}}{\text{VC}} \right) - 1 \right]
\]

where VT = absorbance of the test sample; VC = absorbance of the control.

**Blood compatibility studies**

Blood compatibility studies are conducted with the help of a haemolytic assay (Mathi et al., 2014). The 5 ml of blood samples were obtained from two healthy individuals (25–30 years old) and added with ethylenediaminetetraacetic acid (EDTA). The contents were mixed properly by centrifugation at 1500 rpm for 10 min, and then the serum was discarded. RBCs were washed three times with PBS for 7 min at 1000 rpm. The washed cells were re-suspended in PBS and diluted to prepare an erythrocyte stock solution. The freeze-dried samples were re-dispersed and sonicated in PBS to yield 0.2% suspensions. The nano-formulation suspensions that contained the drugs were added to the erythrocyte stock solution. Then the mixtures were incubated at 37°C in a continuously shaking water bath for 1 h. The mixtures were then centrifuged at 1000 rpm for 5 min, after which the supernatant was read in a spectrophotometer at 540 nm. The saline solution was used as a negative control (0% lysis), and 0.1% triton in PBS was used as a positive control (100% lysis). The amount of hemoglobin released was monitored using spectrophotometry (Biospectrophotometer, Dynamica) at 540 nm. The percentage of haemolysis was calculated using the following formula:

\[
\text{Haemolysis} \% = \left( \frac{\text{Absorbance of the sample}}{\text{Absorbance of the positive control}} \right) \times 100
\]

**Anti-angiogenesis assay**

An anti-angiogenesis activity was conducted using the chick chorioallantoic membrane (CAM) assay (Foubert et al., 2012). The 5-d-old fertilized eggs were purchased, disinfected with 70% ethanol, and transferred to an incubator (GENEI, Bangalore, India) at 37°C with 75% humidity; they were kept in these optimal growth conditions for 24 h. To ensure equal distribution of the blood vessels, the eggs were turned over every 6 h. After 24 h, the eggs were wiped with ethanol (70%) to prevent any mild infections, and then the eggshells were sawed with a metal saw blade. After sawing, the square of the eggshell was carefully removed with a pair of fine-tip forceps. Then the shell membrane was carefully peeled off with a focus on avoiding dropping any shell particles onto the membranes of the developing embryo. The F4 and F6 nano-particles were dissolved in dimethyl sulphoxide (DMSO) at variable (50 and 100 µg/ml) concentrations and applied to sterile Whatman membrane sheet discs. These variable concentration discs were dried in a disinfected environment and implanted on the outer third of the growing CAM blood vessels. The controls were treated with the blank DMSO discs. The zones developing around the DMSO discs were examined 5 and 30 min after implantation. The angiogenic responses were captured using a CANON 550D SLR camera.

**Statistical analysis**

All experiments were repeated three times and the results are shown as mean ± standard deviation. The statistical significance of the data was analysed with a one-way analysis of variance and the significance level was set at p < 0.05.

**Results**

**Characterization of nano-particles**

The size and zeta potential of the prepared nano-particles are shown in Table 2. The mean diameter of the various nano-particles ranged from 98 ± 5.27 to 208 ± 4.22 nm. The B12+C+D3+cisplatin-loaded F6 chitosan nano-particles (NanocisVital or NCV) were greater than 200 nm in diameter. Zeta potential values were recorded to determine the stability of the nano-particles. The values ranged from 22 ± 6 mV. SEM images showed that blank nano-particles were spherical in shape and are well dispersed, whose size is consistent with the SEM observation (100–500 nm). No severe agglomeration of the particles was found. NCV nano-particles were separated from each other, with a size of 105–215 nm. Normally, chitosan nanoparticles with this size tend to aggregate, leading to the formation of larger aggregates due to strong inter-particle interaction. However, as shown in Figure 1, the NCV nano-particles had a rather good dispersity, which is crucial for optimal drug delivery.

**Fourier transform infrared spectroscopy (FT-IR) analysis**

To identify the molecular distribution of the vitamins and cisplatin in the molecular scaffold of the polymeric nano-particles, we recorded the infrared spectra using a Fourier
cisplatin from C0 chitosan nano-particles with C changes the characteristic peak of exactly cancelled out by the two negative charges supplied by the because the two positive charges of the platinum (II) ion are nano-particles (Figure 3C and D). Cisplatin is a neutral complex nano-formulation matrix (Figure 5A versus 5B).

The anti-inflammatory effect of various nano-particles was evaluated using RBCs. The RBCs were treated with increasing concentrations of drug and drug-loaded chitosan nano-particles. A significant difference in the percentage of haemolysis was observed in cisplatin versus cisplatin encapsulated F4 nano-formulation (Figure 5A). Similarly, drug- and vitamin-loaded F6 nano-particles also showed significant reduction in the haemolytic activity as compared to drug alone and blank nano-particles (Figure 5A versus 5B).

These results suggest that vitamin- and drug-loaded F6 nano-particles show good biocompatibility and they can be used for drug delivery purposes. Next we validated the encapsulation efficiency (EE) of cisplatin and vitamins (B12, C, and D3) in the chitosan nano-particles, which are represented in Table 3.

The drug release studies were carried out at pH 7.4, and the drug- and vitamin-loaded F6 nano-particles were compared with the drugs (F4) and vitamins alone (F1, F2, F3 and F5). As shown in Figure 5(C), the releasing pattern of free cisplatin was very rapid and had finished after only 12 h. However, the cisplatin-loaded F4 nano-formulation showed a sustained release until 60 h had passed. At 12 h, 99.54% of the drug was released for the cisplatin, but only 52.66% had been released from the loaded nano-particles. The release rate for the vitamins alone was 13% during the initial 30 min and 54.67% after 60 h. But this was reduced to the synergistic combination of vitamins to 5.63% and 45.13%. The micellar nature of chitosan delays the percentage of release of vitamins from the nano-particles. The synergistic combinations of vitamins with cisplatin (F6 nano-particles) showed a somewhat rapid release of 16.25% at 30 min due to the presence of cisplatin. Then the release continued in a sustained manner to reach 49.59% at 60 h. The rapid release was the result of the surface adsorbed drugs; however, the initial burst release was not severe compared with other systems (He et al., 1999; Zhou et al., 2001; Wu et al., 2005).

The role of vitamin and cisplatin-loaded chitosan nano-particles on chemopreventive properties

To evaluate the importance of vitamin- and cisplatin-loaded F6 nano-particles, we performed anti-proliferative, anti-inflammatory, and anti-angiogenic assays. The anti-proliferative activity was evaluated using actively proliferating yeast (BY4741 strain) as a model system for investigating anti-proliferative activities of eukaryotes (Simon, 2001). NCV delayed the proliferation of yeast cells as compared to blank nano-particles (Figure 6A). Our results showed that, F4, F5 and F6 nano-particles inhibited cell growth proliferation during a 72-h period, with respect to F1-, F3- and control-treated samples. However, F1 and F3 nano-particles delayed the proliferation rate until 12 h and continue to grow until 72 h. The F3 nano-particles had little or no effect on proliferation at 72 h.

The anti-inflammatory effect of various nano-particles was evaluated by measuring the percentage of inhibition of protein denaturation using BSA. Drug-loaded nano-particles inhibited the

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Nano-particles</th>
<th>Mean diameter (nm)</th>
<th>Polydispersity index</th>
<th>Zeta potential (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank nano-particles</td>
<td>98 ± 5.27</td>
<td>0.173 ± 0.044</td>
<td>24.12 ± 0.52</td>
</tr>
<tr>
<td>2</td>
<td>Vitamin B12-loaded nano-particles</td>
<td>107 ± 2.16</td>
<td>0.187 ± 0.012</td>
<td>26.23 ± 1.24</td>
</tr>
<tr>
<td>3</td>
<td>Vitamin C-loaded nano-particles</td>
<td>192 ± 3.61</td>
<td>0.184 ± 0.036</td>
<td>24.37 ± 0.47</td>
</tr>
<tr>
<td>4</td>
<td>Vitamin D3-loaded nano-particles</td>
<td>103 ± 4.04</td>
<td>0.213 ± 0.021</td>
<td>22.44 ± 0.65</td>
</tr>
<tr>
<td>5</td>
<td>Cisplatin-loaded nano-particles</td>
<td>148 ± 2.05</td>
<td>0.221 ± 0.046</td>
<td>25.18 ± 0.27</td>
</tr>
<tr>
<td>6</td>
<td>B12+C+D3-loaded nano-particles</td>
<td>200 ± 2.58</td>
<td>0.273 ± 0.025</td>
<td>26.34 ± 0.36</td>
</tr>
<tr>
<td>7</td>
<td>B12+C+D3+Cisplatin-loaded nano-particles</td>
<td>202 ± 4.22</td>
<td>0.296 ± 0.043</td>
<td>25.26 ± 0.39</td>
</tr>
</tbody>
</table>

Note: Chitosan = 0.1% w/v, TPP = 0.04% w/v.

Blood compatibility and in vitro drug release

The blood compatibility of the nano-particles (F1–F6) was investigated by measuring the percentage of haemolysis transform spectrometer. The FT-IR spectra showed several characteristic absorption peaks and variable stretching and bending for different particles (Figure 2A).
denaturation of the BSA in a dose-dependent manner (Figure 6B). We performed in ovo chick CAM assay to validate the anti-angiogenic activity. As shown in Figure 6(C), the cisplatin-treated chick embryo demonstrated avascular zones reflecting anti-angiogenic activity. The vitamin and cisplatin-treated embryo showed wide avascular zones as compared to the control, indicating anti-angiogenic activity as well as maintenance of the vascular activity around the disc (Figure 6C). All experiments

Figure 1. Characterization of multi-vitamin and drug-loaded chitosan nano-formulation. Note: Photographic images and the SEM micrographs showing the colour and morphology of various drug-loaded chitosan nano-formulation (A and H) blank (B and I) Vitamin B12 (C and J) Vitamin C (D and K) Vitamin D3 (E and L) cisplatin (F and M) B12+C+D3 and (G and N) B12+C+D3+cisplatin.
were repeated three times and mean values were shown ± SD. These data suggest that vitamin and cisplatin-loaded nanoparticles destroyed the blood vessel at the target site, but did not affect any of the neighboring vessels. Overall, these findings suggest that the combinatorial action of vitamins and cisplatin had a better impact than the single drug.

**Discussion**

CRF: sometimes simply called “cancer fatigue” is one of the most common side effects of cancer and its treatments (Stone et al., 2000). Based on published reports, it is clear that cancer drugs induce cell death in rapidly growing cancer cells, but they have also been found to harm healthy cells by suppressing the immune system and causing cancer-related fatigue (Ahles and Saykin, 2007; Apetoh et al., 2007; Gascoigne et al., 2009; Pecqueur et al., 2013). For example, non-small-cell lung cancer (NSCLC) patients treated with pemetrexed showed serious life-threatening infections caused by a combination of several side effects, such as myelosuppression, mucositis, and diarrhea (Hanna, 2004; Eguia et al., 2011). Several different approaches, including dietary changes and nutritional supplements, can be effective in increasing energy levels in cancer patients (Grober, 2009). Supplementation of vitamins, such as vitamin C, vitamin D3, folic acid, and vitamin B$_{12}$, 1 week prior to treatment has been reported to reduce gastrointestinal and haematological complications, and the incidence of treatment-related number of deaths decreased (Chlebowski et al., 2008; Chlebowski, 2011). However, large doses of vitamins have also been shown to interfere with chemotherapeutic drugs. The question remains whether the vitamins can reduce unwanted side effects without altering the effectiveness of these drug agents (Sak, 2012; Tsai et al., 2012). To address this issue, we encapsulated vitamins C, D3, and B12 and the anti-cancer drug cisplatin together using chitosan nano-particles designated as NanoCisVital or NCV nanoparticles. These NCV nano-particles were prepared by the sequential addition of B12, C, and D3 vitamins under continuous stirring at 500rpm. The morphology and size of the nano-particles were observed using a SEM and a Zetasizer. The NCV nano-particles showed a higher mean diameter ($208 ± 2.22$ nm) than various combinations of drugs and blank nano-particles ($98 ± 5$ nm). The different sizes and zeta potentials are due to the electrostatic interaction of amine groups in the chitosan with the negative charges of STPP ions and the concentration of the therapeutic agents. SEM images showed that all the particles were spherical in shape and had a smooth surface without any aggregation, which may be due to the strong interaction of the drugs and vitamins with the various nanoparticles. The average particle sizes of the particles varied from $105$ nm to $215$ nm.
Figure 3. FTIR spectra of (A) Vitamin C (B) Vitamin C-loaded nano-formulation (C) Vitamin D3 (D) Vitamin D3-loaded nano-formulation.

Figure 4. FTIR spectra of (A) cisplatin (B) cisplatin loaded nano-formulation (C) B12+C+D3 loaded nano-formulation (D) B12+C+D3+cisplatin loaded nano-formulation.
FT-IR spectroscopy was used to determine the nature of interaction between the drugs, vitamins, chitosan, or STPP. The various physiochemical interactions between drugs, vitamins, chitosan, and STPP can alter or broaden the absorption peaks. The nano-particles are formed in the medium, which produces a positive charge for the chitosan due to electrostatic interaction between its primary amino group and STPP. The negative charges show an affinity towards the chitosan, so the electrostatic interaction and the chemical reactions occur between the drugs and the chitosan. The FT-IR spectra of the physical mixture of cisplatin and the vitamins were compared, which identified the shifting of predominant peaks of the drug and the vitamins, suggesting that a chemical interaction had taken place. The physical interactions between the cisplatin and the vitamins are due to the formation of hydrogen bonds, Van der Waals forces of attraction, or dipole–dipole interactions. The strong positive charge of chitosan nano-particles indicates good interaction with the cisplatin and vitamins. The encapsulation efficiency shows that higher amounts of cisplatin and vitamins increased the drug loading and decreased the efficiency. This may be due to the ability of the polymer matrix to accommodate a large amount of vitamins and cisplatin molecules in the polymeric network to its saturation point. The percentage of encapsulation efficiency is reduced when it overcomes these saturation points. The saturation points determine the maximum amount of drug molecules that can be accommodated in the polymer matrix of a definite quantity. These points are varied for different therapeutic molecules depending upon their chemical nature and are also evident from the drug release studies. The percentage of drug released from F6 nano-particles is gradually increased as compared to the free cisplatin. This percentage is time-dependent; initially, there is a lag phase followed by a log phase. These characteristics may be due to the fact that the dissolved drugs and vitamins may rapidly diffuse into the release medium near the surface of the micelle and show a rapid burst release. The initial rapid release may cause an aggregation of the polymer into the micelles at physiological temperature; this micellar nature prolongs the release rate of drugs and vitamins from the micelles. This type of rapid release followed by the slow and sustained release of drugs and vitamins alone indicates a biphasic release pattern of loaded nano-particles (Koocheki et al., 2011). This time-dependent release of vitamins over the cisplatin helps to reduce the signs of CRF. The anti-proliferative assay of vitamins and cisplatin-loaded nano-particles indicates the action of nanoparticles on the proliferation of yeast cells. The cells treated with F4 nano-particles showed better growth arrest than the cisplatin throughout the entire 72-h period. The synergistic combinations of vitamins and also of vitamins and cisplatin showed almost the same amount of growth arrest due to the presence of vitamin C and cisplatin, indicating that the synergistic combinations positively inhibited the yeast cell proliferation. Next, we validated the anti-inflammatory activity of nanoparticles using a protein denaturation assay. The denaturation of tissue proteins has been extensively studied to determine the causes of inflammatory diseases (Chatterjee et al., 2012). The increase in percentage of inhibition with respect to any increase in the concentration of the sample indicates the stabilization of and good inhibition on the heat-induced proteins (BSA) (Mizushima et al., 1968). All the vitamins positively inhibited the denaturation of proteins with respect to the concentrations compared to cisplatin. This behaviour indicates that the anti-inflammatory action of vitamins is much higher than the cisplatin. The synergistic combinations of vitamins and vitamins with cisplatin showed almost the same results. This trend suggests that the synergistic combination enhances the percentage of inhibition of BSA denaturation by

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Nano-particles</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vitamin B12-loaded nano-particles</td>
<td>65.05 ± 0.44</td>
</tr>
<tr>
<td>2</td>
<td>Vitamin C-loaded nano-particles</td>
<td>56.9 ± 1.12</td>
</tr>
<tr>
<td>3</td>
<td>Vitamin D3-loaded nano-particles</td>
<td>64.6 ± 0.56</td>
</tr>
<tr>
<td>4</td>
<td>Cisplatin-loaded nano-particles</td>
<td>62.7 ± 1.25</td>
</tr>
<tr>
<td>5</td>
<td>B12+C+D3-loaded nano-particles</td>
<td>61.6 ± 1.64</td>
</tr>
<tr>
<td>6</td>
<td>B12+C+D3+Cisplatin-loaded nano-particles</td>
<td>61.2 ± 1.43</td>
</tr>
</tbody>
</table>

Note: Chitosan = 0.1% w/v, TPP = 0.04% w/v.

Figure 5. Haemolytic activity and drug release of various nano-formulation at 37°C. Note: (A) Top panel showing the percentage of haemolytic activity at various concentrations. (B) Bottom panel showing in vitro release of cisplatin from cisplatin-loaded chitosan nano-formulation at 37°C.
increasing its concentration. The haemolysis assay was used for the blood compatibility studies. The vitamin B12, vitamin C, and vitamin D3 loaded F1, F2 and F3 nano-particles showed less lysis than the cisplatin drug (data not shown). However, the activity of cisplatin-loaded F4 nano-particles on RBCs was much lower due to the time-dependent release of the cisplatin from the nano-particles. In addition, the synergistic combinations of vitamins produced better lysis than the synergistic combinations of cisplatin and vitamins, indicating that the synergistic combinations effectively inhibited the lysis of RBCs. According to
previous studies, less than 5% of haemolysis does not produce a significant effect on the human body. Therefore, the synergistic combinations can be used for advanced drug delivery purposes. Anti-angiogenesis activity along with CAM assay has also been used to study the action of nano-particles on blood vessels formation (Foubert et al., 2012). The formation of wide, thick vascular zones indicates the anti-angiogenic activity of F4 nano-particles on the blood vessels. The synergistic combination of cisplatin and vitamins showed almost the same results as cisplatin alone, which indicates that NCV strongly inhibited the blood vessels’ growth and size in a dose-dependent manner. The F6 nano-particles loaded with cisplatin and vitamins effectively controlled microvesSEL formation in a chick embryo model.

Conclusion
NCV showed a particle size of less than 225 nm and good encapsulation efficiency, anti-inflammatory, anti-angiogenic activity and less haemolytic activity without much interference with the function of the therapeutic agent. The synergistic action of vitamins along with cisplatin can be better used as drug delivery system in cancer therapy while maintaining the tissue homeostasis.

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Declaration of interest
The authors disclose no conflict of interests.

References


