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Research Article

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Synthesis and Characterization of $[CoW_{11}O_{39}(CpTi)]^{7-}$-Chitosan Nano-Assembly, Cytotoxicity Assay and Release Profile

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ABSTRACT

Polyoxometalates (POMs) are inorganic metal–oxygen group anions class of compounds. They are distinctive in its topological and electronic adaptability and are significant in numerous disciplines. POMs are proved to be important for applications in medicine and in material science. An accumulative number of possible uses for POMs in human medicine are vividly described in the several recent literatures. Here, we represent nano-composite formation of tungsten containing anticancer polyanion, $K_6H[CoW_{11}O_{39}(CpTi)].13H_2O$ ($CoW_{11}CpTi$) with biocompatible ChitosanYC-100 (CSYC100). This synthesis was attained without the aid of any cross-linker through electrostatic interaction technique. This title anion $CoW_{11}CpTi$ is reported to be the most potent anti-cancer POM amongst the family of CpTi substituted POMs studied so far. This POM-chitosan nano-composite was characterized using Fourier Transform Infrared Spectroscopy (FTIR); dynamic light scattering (DLS), Transmission Electron Microscopy (TEM) and Thermo Gravimetric Analysis (TGA). The release profile recorded was slow and sustained at physiological pH. Cytotoxicity assays which shows an attribute to reduce the toxicity of these POM were performed on C2C12 (mouse myoblast cell line) and A-549 (lung cancer cell line), which proved the reduced toxicity of nano-composites as compared to the bare drugs. Thus, this study has designated the probability of using POM-chitosan nano-composite for less toxic and effective biomedicinal applications.

Keywords: *Anti*-tumour; Chitosan nano-assembly; *In-vitro* MTT assessment; Drug release

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INTRODUCTION

Polyoxometalates (POMs) can be defined as 'early transition metal oxygen clusters'. POMs have gained tremendous attention because of their mutable uses in the areas of novel drug development, material sciences and in industrial catalyst.[1] Use of POMs in latent applications for human medicine is growing exponentially and are recently published in the literatures.[2,3] The toxicity due to presence of metal ions is the major drawback of these precious medicinal molecules. To lower down the toxicity, it needs to be conjugate/encapsulate in some biopolymeric delivery vehicle.

Chitosan and its derivatives linger to attract noteworthy attention as a potent drug transporter with remarkable biocompatibility along with appreciable cellular uptake rates. [4-6] Chitosan is a carbohydrate heteropolymer composed of glucosamine and N-acetyl glucosamine linked by β 1-4 glucosidic bonds as a repeating unit. Chitosan is developed from chitin by its N-deacetylation. Chitin a structural polysaccharide found in the exoskeleton of shrimps and shells of crabs and the second most abundant polysaccharide found in nature after Cellulose.[7] This versatile feature has been used in this study to beat the significant biomedical potential of POMs by their encapsulation in Chitosan YC-100 (CSYC100) matrix. The chosen title anion $K_6H[CoW_{11}O_{39}(CpTi)].13H_2O$, hereafter denoted as ($CoW_{11}CpTi$) is reported to be the most potent anti-cancer

POM amongst the family of CpTi substituted POMs studied so far. It is reported that polyoxotungstate $CoW_{11}CpTi$ curiously decreased tumor weight of the rats bearing HLC (colon cancer cell), HL-60 (leukemia) and SSMC-7721 (liver cancer cell) where the experimental results were procured using the animal tumor implantation method. The *in-vivo* anti-cancer efficacy of $CoW_{11}CpTi$ is at par with the clinical anti-cancer drugs 5-FU (5-fluorouracil) and CP (abbreviation of cyclophosphamide), but the cytotoxicity of $CoW_{11}CpTi$ is reported to be less than them.[8]

The research described in this report is focused to prepare nano-composite of low molecular weight carbohydrate polymer CSYC100 and an anti-cancer POM $CoW_{11}CpTi$. The primary agenda of this research is to portray reduction in the metallic toxicity of POMs by its involvement in the formation of nano-composite with chitosan YC-100. To support this hypothesis, $CoW_{11}CpTi$-CSYC100 nano-composites were prepared and its toxicity was assessed *in vitro* on cell line C2C12 (normal myoblast cell line) and A-549 (adenocarcinomic human alveolar basal epithelial cells-lung cancer cell line). To the best of our knowledge, this the first report showing the toxicity reduction of $[CoW_{11}O_{39}(CpTi)]^{7-}$ by forming their nano-composite with CSYC100.

EXPERIMENTAL SECTION

Materials

CSYC100 is a low molecular weight chitosan (~10000 g/mol) and highly water soluble and purchased from Sigma–Aldrich, Steinheim, Germany. Other chemicals required for synthesis of POMs and other studies were bought commercially from Sigma–Aldrich.

Preparation of POMs

Polyoxotungstate $CoW_{11}CpTi$ was synthesised according to the procedure described by Wang et al. in 2003.[8]

Synthesis of CSYC100/ $CoW_{11}CpTi$ complexes

To synthesize CoW11CpTi-CSYC100 nano-composites, 50mg CSYC100 was dissolved in 70 ml double distilled water. The mixture was stirred until the CSYC100 complete dissolve and the clear solution is observed. The solution was then filtered to remove any suspended particles. CSYC100 filtrate was further used to prepare nano-composites of POMs. 170mg (0.39mM) $CoW_{11}CpTi$ was dissolved in 2 mL double distilled water separately. $CoW_{11}CpTi$ solution was then added drop wise with stirring in to the CSYC100 solution under controlled sonication, resulting in the formation of stable colloidal suspension of $CoW_{11}CpTi$-CSYC100 nano-composite. This suspension was centrifuged for for 20 min at 12,000 RPM (18,000g) to collect the $CoW_{11}CpTi$-CSYC100 nano-composites obtained as pellet. Supernatant was discarded and pellet of $CoW_{11}CpTi$-CSYC100 was recovered, dried and used for further studies.

Physicochemical characterization of POM-CSYC100 nano-composites

Morphology and particle size of the nano-composites was analysed by transmission electron microscopy (TEM) [Model-Philips Tecnai 20]. The particle size distribution and hydrodynamic diameter size were determined by Dynamic Light Scattering (DLS) method by means of particle size analyzer (Model: Malvern Zetasizer). Fourier Transformed Infra-Red (FT-IR) spectroscopy was used to recognize the signature absorption peaks, by recording the spectral scan ranging from 4000 to 400 cm^{-1} using the instrument Thermo scientific Nicolet-6700 class-1. Thermal strength of the nano-composites was analysed using the thermo gravimetric technique (Mettler-Toledo TGA/DSC-1 Star^e system) in nitrogen atmosphere from 25-800˚C temperature with a rate of 10˚C min^{-1}.

In-vitro release of $CoW_{11}CpTi$ from CSYC100 matrix

The *in vitro* release of $CoW_{11}CpTi$ from the nano-composite was carried out at 37°C in phosphate buffered saline (PBS) of pH 7.4 containing lysozyme (1.6 g mL^{-1}). The nano-composites were centrifuged for 15 min at 12,000 rpm. The supernatant was transferred to the fresh tube for recordicg its UV-Visible spectra using spectrophotometer and recovered pellet was re-dispersed in PBS. Similarly, the same procedure was repeated at the programmed time intervals, where the samples were centrifuged at 12,000 rpm (18,000 g) for 15 min and the supernatant was analysed using UV-Visible spectrophotometer to determine the amount of POM released from the nano-composite, after which the sample was swapped back into the solution. The absorbance obtained was compared with concentration dependent standard curve of $CoW_{11}CpTi$ was to determine its release at different time intervals.

Cell culture

Under present study we used C2C12 (Normal myoblast cells) and A-549 (adenocarcinomic human alveolar basal epithelial cells-lung cancer cell line), acquired from National Centre for Cell Sciences (NCCS), Pune,

India. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) complemented with 10% fetal bovine serum (FBS) (GIBCO) and 1% antibiotic-antimycotic (Anti-anti, GIBCO) at 37°C in humidified atmosphere with 5% CO_2 in an incubator. The media was refilled at the interval of 24 hrs and the cells were sub-cultured when 90% confluency was attained.

***In-vitro* cytotoxicity analysis using MTT assay**

Cytotoxicity on C2C12 mouse myoblast cells of the bare $CoW_{11}CpTi$ and $CoW_{11}CpTi$-CSYC100 nano-composites were analysed using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT assay. MTT assay is a colorimetric assay based on the selective ability of viable cells to reduce the tetrazolium component of MTT in to purple colored formazan crystals.[9] Cells were seeded into a 96 well plate at a seeding density of 10,000 cells/ml. MTT dye (5mg/mL) was added into each well after mixing and incubating the cell with various concentrations of $CoW_{11}CpTi$ -CSYC100 nano-composites and bare POMs (5mM-0.019mM) at specific intervals, in triplicates for 24 h. The % cell viability was determined using microplate reader (Microtek Power Wave XS US) by recording the optical absorbance at 570 nm comparative to the untreated cells.

RESULTS AND DISCUSSION

Physicochemical characterization of POM-CSYC100 nano-composites

$CoW_{11}CpTi$ were prepared and further used to synthesize hybrid $CoW_{11}CpTi$-CSYC100 nano-composites with primary agenda to reduce their toxicity. *In-vitro* cytotoxicity of $CoW_{11}CpTi$-CSYC100 nano-composite and bare $CoW_{11}CpTi$ were examined on C2C12 (Normal myoblast cells). The nano-composites were prepared by an electro static interactions taking benefit of the negatively charged $CoW_{11}CpTi$ and positively charged CSYC100. Subsequently, $CoW_{11}CpTi$ possessing negative charge is capable of forming stable colloids with the biopolymer CSYC100 bearing positive charge.

Bare $CoW_{11}CpTi$ was characterized using FT-IR spectroscopy. Further, it was used to prepared nano-composite with CSYC100. Once the $CoW_{11}CpTi$-CSYC100 nano-composites were prepared, FT-IR spectroscopy was done to confirm the successful nano-composite development. Figure 1 describes the FT-IR spectrum of bare $CoW_{11}CpTi$, native CSYC100 and CoW11CpTi -CSYC100 nano-composites. FT-IR spectrum of CSYC100 is represented in Fig. 1A, which signifies all the characteristic peaks of chitosan at 3420 cm^{-1} appeared and this can be credited to the $–NH_2$ and –OH groups stretching vibration and a peak at 1620 cm^{-1} for the amide similar to as characterised by Anitha et al.[10] FTIR spectrum of bare $CoW_{11}CpTi$ denoted in Fig. 1C portrays characteristic peak sharp absorption at 1620 cm^{-1}. This feature is typical of the C–C stretching for η^5-C_5 H_5 ligand attached to Ti [11] which exhibits the presence of cyclopentadienyl ligand.

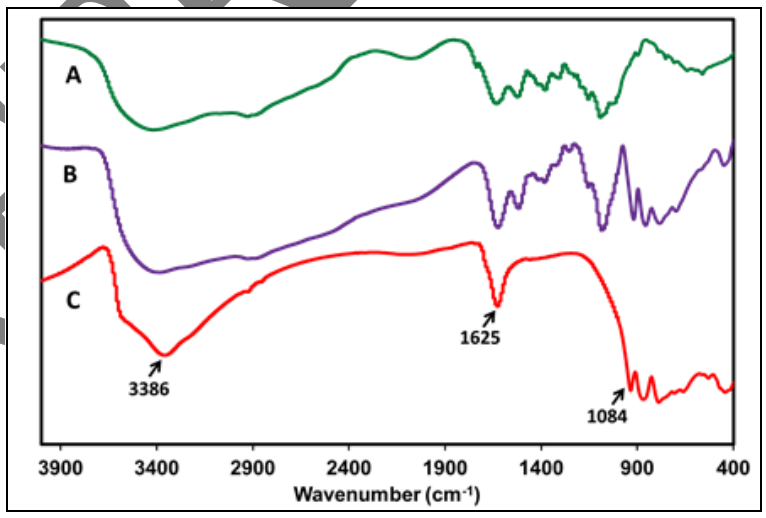

Figure 1: Comparative FT-IR spectrum of [A] CSYC100, [B] $CoW_{11}CpTi$-CSYC100 nano-composite, [C] bare $CoW_{11}CpTi$

The spectrum of $CoW_{11}CpTi$ -CSYC100 nano-composite (Fig. 1B) shows all the representative peaks existing in bare $CoW_{11}CpTi$ representing successful complex development. Also, the peak at 1060 cm^{-1} corresponds to the bridge oxygen (C–O–C) stretching bands.[10,12]

Figure 2 represents the TEM images of $CoW_{11}CpTi$ -CSYC100 nano-composite. It is a clear evident from the images that approximately all composites obtained by this technique were (1) mono dispersed (2) possessed spherical shape morphology and (3) had a sizes ranging from 35 to 80 nm in the diameter.

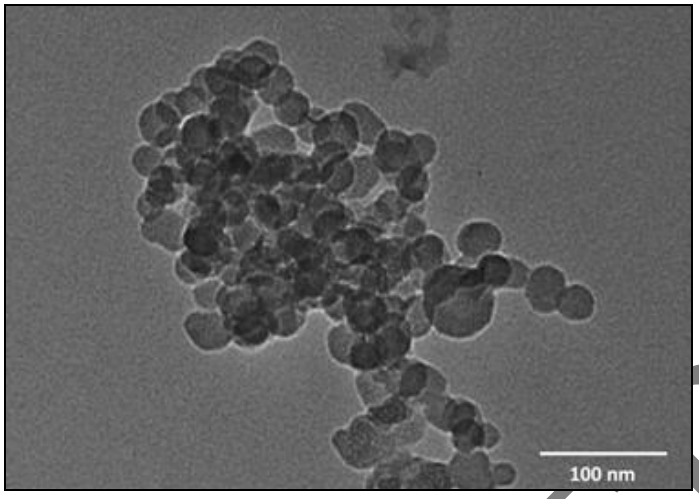

Figure 2: TEM images of CpTi-CSYC100 nano-composite

DLS analysis portrayed hydrodynamic size of the nano-composites (Fig. 3). DLS anaysis unveiled that 98% or more particles possess hydrodynamic diameter size less than 100nm (Number % distribution). $CoW_{11}CpTi$-CSYC100 nano-composite, size distribution of particles ranges from 42.82 to 72.82 d nm and 35.9% (highest) particles are of 50.75 d nm size.

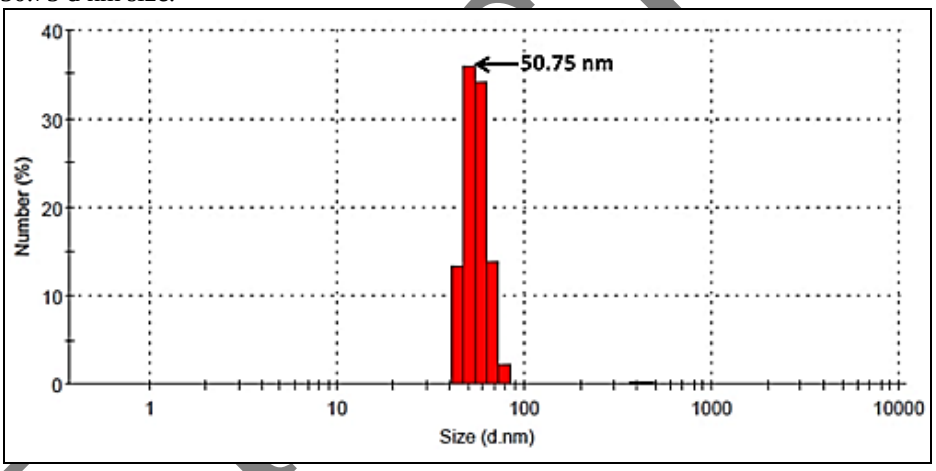

Figure 3: Particle size distribution (number %) using DLS analysis of CoW11CpTi-CSYC100 nano-composite

Thermograms of CSYC100, $CoW_{11}CpTi$-CSYC100 nano-composite and bare $CoW_{11}CpTi$, shown in Figure 4 describes that the thermal strength of the nano-composite is better when compared to the bare CSYC100. This may be credited due to the formation of a complex by CSYC100 with thermally stable $CoW_{11}CpTi$ having lesser degradation as those are heavy metal salts. From the thermogram of CSYC100 in Fig. 4, the degradation observed up to 100ºC can also be due to the loss in moisture and then polymer remains stable till 228ºC as there is minimal change in the weight loss from 100 to 228 ºC. Afterwards, an exponential degradation of was observed up to 380ºC which may be due to degradation of CSYC100 polymeric structure.

In case of nano-composite, thermogram showed the degradation starting at 180ºC, further with a slow degradation till 320ºC. The % weight residual at 900ºC for the nano-composite was more when related to CSYC100. Also, the sharpness of the curve was reduced for nano-composite, representing its debilitated degradation in comparison to that of bare CSYC100. $CoW_{11}CpTi$ exhibited a small degradation due to the moisture loss at around 100°C. Further, there was no degradation observed up to 900°C which represents ~90% of the particles didn't under go any degradation and stayed stable. Basically, the thermal stability of CSYC100 is significantly improved when developing composites with the thermally stable $CoW_{11}CpTi$.

To study the release of $CoW_{11}CpTi$ from nano-composites, lysozyme was dissolved in PBS at pH 7.4 and this solution was used as release medium. Lysozyme was used as it enzymatically degradation of the glycosidic bonds in CSYC100 polymer. Lysozyme concentration was adjusted to mimic the *in vivo* system. Here lysozyme weakens the electrostatic interaction between $CoW_{11}CpTi$ and CSYC100 which would cause its controlled

release from composites. It was seen that, there was a slow and steady release of $CoW_{11}CpTi$ for 11 hours, with the 98% of collective release at the end (Figure 5).

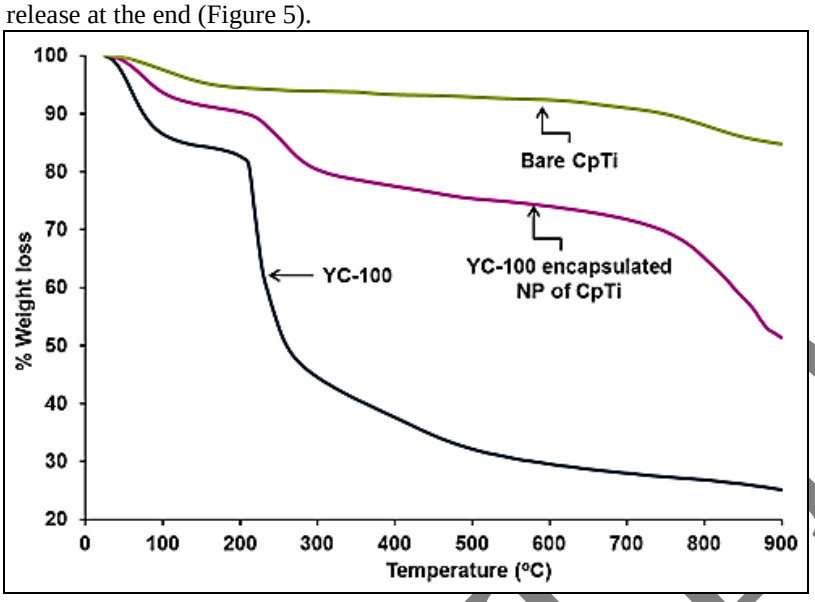

Figure 4: Comparative thermo gravimetric analysis of CSYC100, CoW11CpTi-CSYC100 nano-composite, Bare CoW11CpTi

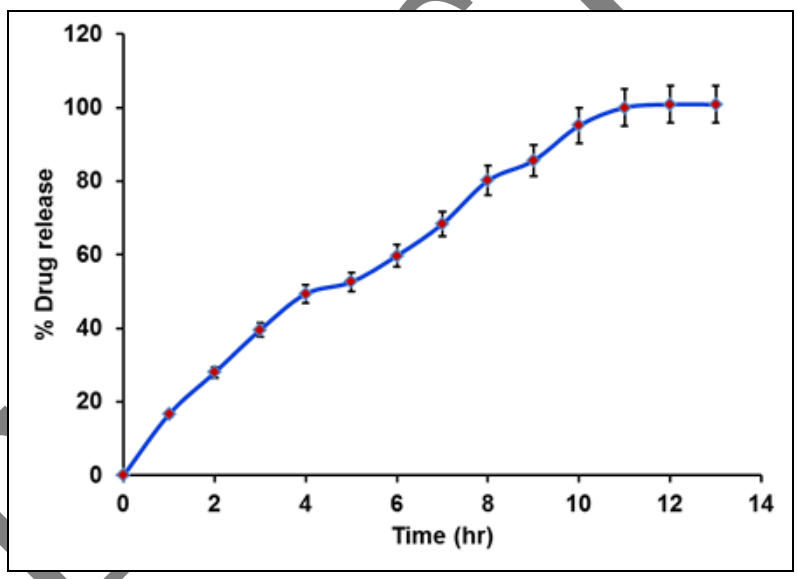

Figure 5: Release of CoW11CpTi from CoW11CpTi -CSYC100 nano-composite

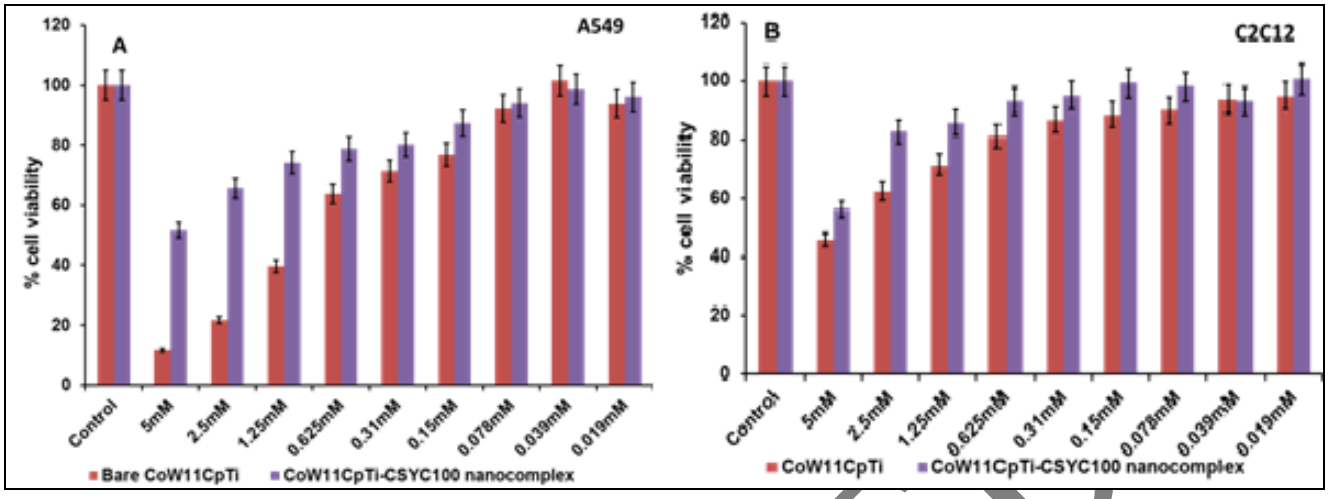

Figure 6: In-vitro cytotoxicity investigation of CoW11CpTi and CoW11CpTi-CSYC100 nano-composite on [A] A549 (adenocarcinomic human alveolar basal epithelial cells) and [B] C2C12 (mouse myoblast cell line) by 24 hrs MTT assay

Results of MTT assay was performed to study the toxicity of bare $CoW_{11}CpTi$ and hybrid $CoW_{11}CpTi$ -CSYC100 nano-composites on C2C12 and A549 cell line is represented in Fig. 6. Concentrations of bare $CoW_{11}CpTi$ and $CoW_{11}CpTi$ -CSYC100 were varied from 0.019 mM to 5mM range. At very high concentration of 5mM, $CoW_{11}CpTi$ and hybrid $CoW_{11}CpTi$-CSYC100 nano-composite were toxic to the C2C12 and A549 cells. However, there was significant variation in the percent (%) viability of C2C12 cells and A549 cells at their lower concentrations. Calculated IC_{50} values for bare $CoW_{11}CpTi$ and hybrid $CoW_{11}CpTi$-CSYC100 nano-composite on C2C12 cells were 4.8 mM and 5.3 mM, similarly IC_{50} values for these compounds on A549 were 0.9 and 4.9 mM respectively. This show Hybrid POM was less toxic at higher concentrations as compared to bare POM. Also, cancerous cells A459 can easily be inhibited by $CoW_{11}CpTi$-CSYC100 nano-composite at very low concentrations where normal C2C12 cells remain viable and unaffected.

CONCLUSIONS

Under present study, cytotoxicity of an anticancer POM, $CoW_{11}CpTi$ was reduced by preparing their nano-composite using CSYC100. To start with, bare $CoW_{11}CpTi$ was prepared and their purity was analyzed using FT-IR. These POM was then used to prepare nano-composites namely $CoW_{11}CpTi$-CSYC100 which was characterized using DLS, FT-IR, TEM and TGA analysis. The release profile of $CoW_{11}CpTi$ from $CoW_{11}CpTi$-CSYC100 nano-composite was recorded at physiological pH conditions which showed $CoW_{11}CpTi$ was released from CSYC100 matrix at slow rate and thus we concluded that the release was sustained. Lastly, the cytotoxicity assays of $CoW_{11}CpTi$ -CSYC100 nano-composite was performed on normal myoblast cell lines C2C12 and A549 lung cancer cell line. $CoW_{11}CpTi$ -CSYC100 nano-composite at the concentrations of 1.25mM and lower, did not exhibit toxic effect on C2C12 cells as 95% total C2C12 cell mass remained viable. While in case of A549 cells highest 5mM concentration of bare $CoW_{11}CpTi$ is toxic to the cancer cells and after encapsulation cell viability increases from 10% to 55%. Cytotoxicity assays shows that $CoW_{11}CpTi$ in complex with CSYC100 had reduced toxic effect on C2C12 mouse myoblast cells. Thus, the present study has designated the feasibility of consuming $CoW_{11}CpTi$-CSYC100 chitosan nano-composite for lesser toxic biomedicinal applications.

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