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Synthesis and cytotoxicity evaluation of novel acylated starch nanoparticles

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ABSTRACT

Starch nanoparticles (StNPs) were acylated under ambient conditions to obtain various nanosized derivatives formed stable suspension in water and soluble in organic solvents. The degree of substitution (DS) was determined using ^{1}H NMR technique. The cytotoxicity potential of the derivatised StNPs was evaluated in mouse embryonic fibroblast (3T3L1) cells and A549 tumor cell line using MTT cell viability assay. Other parameters that determine the oxidative stress viz., reactive oxygen species (ROS) generation, intracellular reduced glutathione (GSH), superoxide generation and acridine orange/ethidium bromide staining were also investigated. The present study led to the conclusion that cytotoxic activity of acylated starch nanoparticles was dependent on their dosage, DS and type of substitution. The non-toxic nature in non-cancerous cells reveals that the nanoparticles (NPs) can be used for cancer therapy and drug delivery. The nanoparticles also offered reasonable binding propensity with CT-DNA.

1. Introduction

Cytotoxic drugs continue to play a major role in cancer therapy but often produce side effects, especially through the destruction of lymphoid and bone marrow cells [1]. Therefore, strategic improvements in cancer therapy are needed to improve efficacy while decreasing side effects. Over the past decades, nanoparticles (NPs) have been of great interest in applications for biological fields such as drug delivery systems [2,3] and anticancer applications [4]. The antitumor activities of natural biopolymer chitosan and its NPs are well reported [5]. Another abundant polysaccharide starch, is relatively, more competent than chitosan due to its low cost, easy availability and better solubility but suffers from drawback of hydrophilic nature. The synthesis of starch nanoparticles (StNPs) reported by Dufresne et al. [6] paved the way for the development of novel nanosized starch derivatives which can offer great potential for use in diverse medicinal applications. The evaluation of the *in vitro* cytotoxicity of a biomaterial is the initial step of biocompatibility study. Hence we investigated the cytotoxic potential of hydrophobic, nanosized, starch derivatives for two different applications viz. potential anticancer agents and promising biocompatible drug carriers. In the past our group has extensively studied the cytotoxicity evaluation of metal nanoparticles [4,7] as well as starch metal nanoconjugates [8] with prokaryotic as well as eukaryotic cells. In this paper we have reported the interesting interaction of nanosized acyl derivatives of starch with A549 human lung carcinoma cells as well as mouse embryonic fibroblast (3T3L1) cells and CT-DNA.

2. Experimental

Starch nanocrystals (StNPs) were prepared according to the procedure described elsewhere [6]. Acylation of StNPs was carried out by dispersion in aqueous alkali followed by room temperature reaction with various acid chloride or anhydride [9]. Attempts were made to carry out the synthesis under ambient conditions so as to preserve the nanosize. Four type of derivatives were synthesized viz. St-palmiate, St-benzoate, St-phthalate and St-cinnamate. The product was collected by centrifugation and purified (Supporting information).

3. Results and discussion

The acylation was confirmed by spectroscopic analysis. The characteristic peaks in the FT-IR spectra (Supporting information) of all the starch nanoparticles are the stretching and bending vibrations of hydrogen bonded –OH groups observed at 3400 and 1650 cm^{-1}. The FT-IR spectra after acylation, showed a carbonyl absorption band at 1736, 1740, 1742 and 1750 cm^{-1} for palmiate, benzoate, cinnamate and phthalate starch nanoparticles respectively. In case of aromatic derivatives the peaks due to stretching and bending of aromatic –C–H appear around 3000 and 800 cm^{-1} respectively.

The ^{1}H NMR data of the starch nanoparticles is given below.

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StNPs: 5.1 (w, 1H, H-1), 3.31 (s, 1H, H-2), 3.96 (w, 1H, H-3), 3.37 (s, 1H, H-4), 3.08 (w, 1H, H-4 end group), 3.60 (s, 1H, H-5), 3.65 (s, 2H, H-6,60), 5.42 (m, 1H, H-2), 5.44 (m, 1H, H-3) [2].
St-phthalate NPs (DS = 2.63): ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.0 (s, 1H), 7.89 (d, J = 6.8, 2H), 7.81 (d, J = 6.3, 2H), 4.95 (s, J = 2, 1H), 4.01 (s, J = 2.2, 1H), 3.57 (s, J = 2.1, 1H), 3.23 (s, J = 2.2, 1H).
St-cinnamate NPs (DS = 2.59): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (d, J = 6.4, 2H), 7.67 (d, J = 6.3, 2H), 6.45 (d, J = 2.2, 2H), 4.97 (s, J = 2.1, 1H), 4.45 (s, J = 2.3, 1H), 4.11 (s, J = 2.1, 1H), 3.44 (s, J = 2.2, 1H).
St-benzoate NPs (DS = 1.98): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (s, J = 6.5, 2H), 7.52 (s, J = 6.3, 2H), 5.5 (s, J = 2.3, 1H), 5.01 (s, J = 2.4, 1H), 4.45 (d, J = 2.5, 1H), 3.52 (s, J = 2.1, 1H).
St-palmiate NPs (DS = 2.49): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.98 (s, J = 2.2, 1H), 4.04 (s, J = 2.1, 1H), 3.47 (s, J = 2.3, 1H), 2.97 (s, J = 2.4, 1H), 2.34 (s, J = 2.1, 2H), 1.67 (s, J = 2.4, 2H), 1.21 (s, J = 2.3, 2H), 0.85 (t, J = 2.4, 3H).

Transmission electron micrographs (TEMs) (Fig. 1) showed that after acylation the size of the StNPs decreases. The platelet-like particles of the unmodified StNPs [6] (Supporting information) became somewhat spherical after modification with the diameter ranged from 40 to 50 nm, due to blocking of hydroxyl groups which leads to individualization of NPs [10]. The results of DLS (Supporting information) also showed a single narrow distribution with average particle size in the range of 30–50 nm. The results of thermal analysis (Supporting information) reveal that gelatinization temperature was lower while thermal stability was higher for acylated StNPs [3]. This suggests improved processibility which is necessary for drug delivery applications.

The *in vitro* cytotoxicity was evaluated at various doses by exposure of A549 cells as well as 3T3L1 cells to acylated StNPs. Upto 1000 μg/mL concentration the cell viability was recorded to be higher than 85% even after 72 h (data not shown). This biocompatibility was suggestive of the potential of these materials for use as nanocarriers of drug molecules. A dose range of 1000–10,000 μg/mL recorded moderate cytotoxicity in 3T3L1 cells (Supporting information) while StNPs induced practically no cell death. At this dose, in tumor cells interesting dose dependent cytotoxicity was observed within 24 h (Fig. 2a). Among all the NPs tested herein, St-cinnamate NPs showed highest cytotoxicity (Table 1 Supporting information) and its highest dose (10,000 μg/mL) recorded nearly 80% cytotoxicity (Fig. 2a). On the other hand, St-palmitate NPs recorded lowest cytotoxic potential, probably because of its completely aliphatic structure. Cytotoxicity differences between the acylated starch derivatives are probably due to the different hydrophobicity and charge density of StNPs. In addition, other parameters such as the degree of substitution and type of substitution also determine the extent of cytotoxic activity of StNPs. There may be a structure activity relationship which requires further investigation.

In biological activities, cell must interact with the extracellular environment which is generally through chemical, electrical or mechanical signaling. In the present studies, DS of acylated starch NPs are $\geqslant$2 which indicates the hydrophobic nature of acylated starch NPs. Cytotoxicity is reported to increase with increasing hydrophobicity [11]. Based on this it can be concluded that there is hydrophobic interaction between tumor cells and starch derivatives which is responsible for cytotoxicity.

Molecules at nanoscale may be able to help overcome the undesirable effects of traditional chemotherapeutic agents by maximiz-

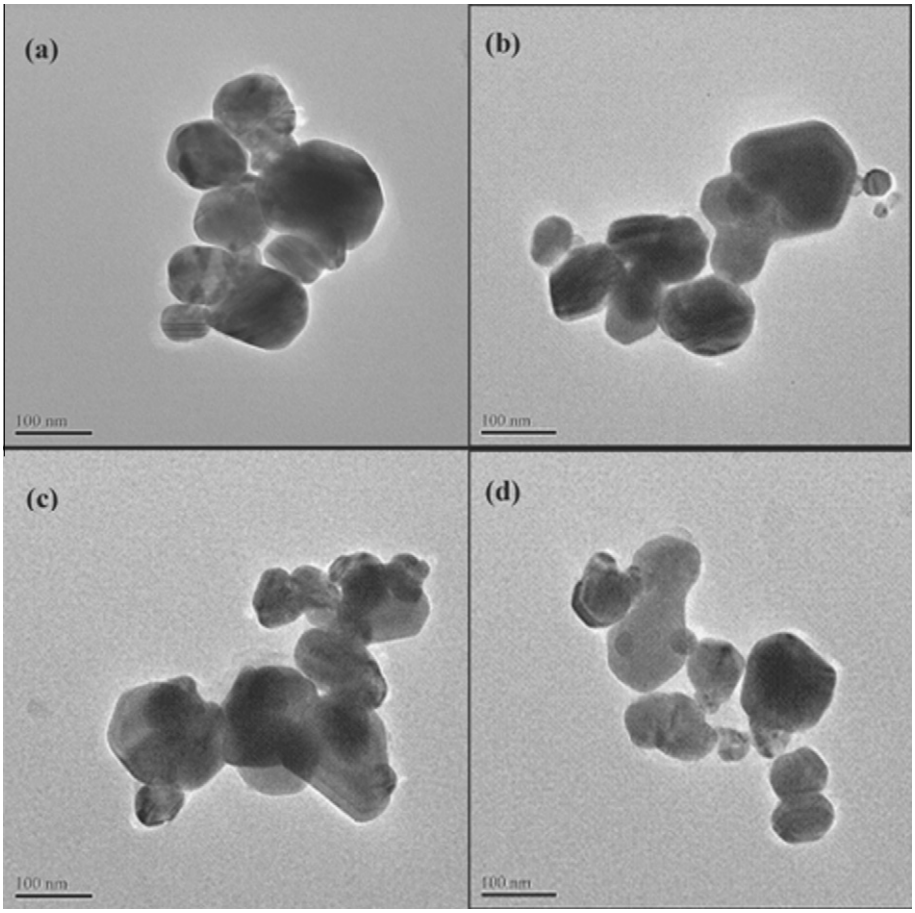

Fig. 1. TEM images of (a) St-phthalate, (b) St-cinnamate, (c) St-benzoate and (d) St-palmiate nanoparticles.

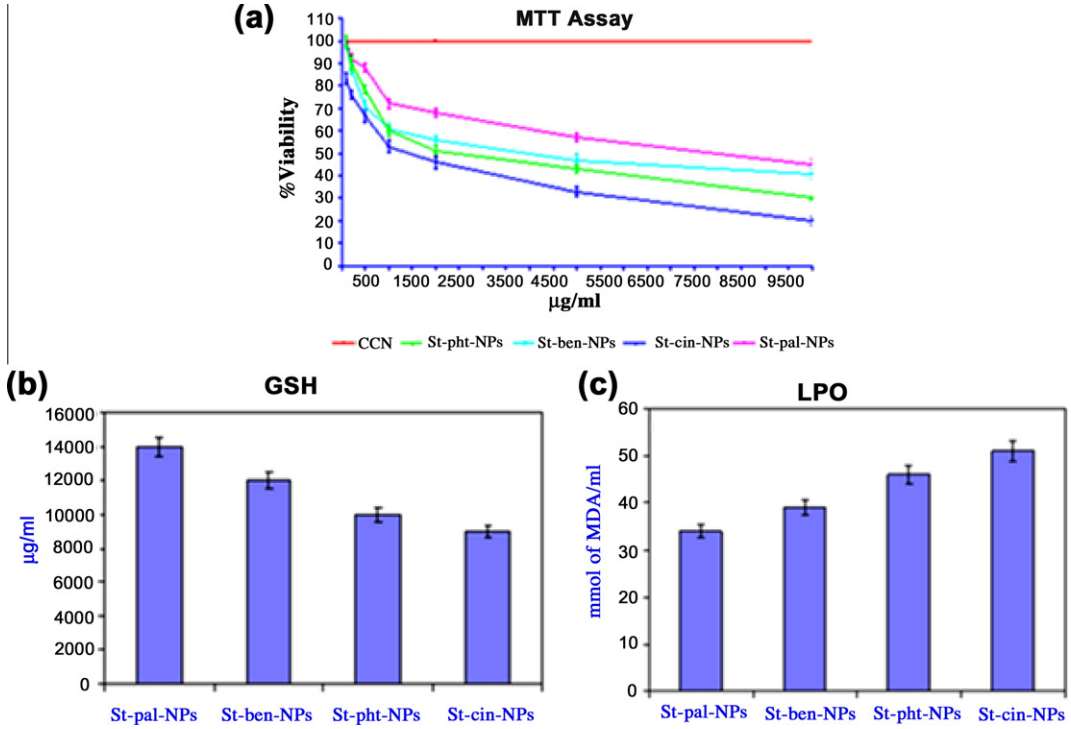

Fig. 2. Effect of modified starch nanoparticles exposure on cell viability (a) MTT assay, (b) reduced glutathione content and (c) lipid peroxidation in A549 cells. Results are expressed as mean ± SEM for $n = 3$ (replicates). Where NS = non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to untreated cells.

ing their availability at the target tumor site and minimizing noxious effects on healthy tissue [12]. Tumor cells overexpress many receptors and biomarkers, which can be used as targets to deliver cytotoxic agents into tumors [13]. Cancer cells are highly metabolic and porous in nature and are known to internalize solutes rapidly compared to normal cells. Therefore, we hypothesized that acyl StNPs will also show preferential internalization within cancer cells. Tumor sites possess unique vasculature characteristics that can be easily exploited by nanoscale systems to efficiently reach cancer targets. The endothelial cells in tumor vasculature have loose interconnections and focal intercellular openings. These breaks in the endothelial cell lining range in size between 100 and 1000 nm, and nanoparticles (NPs) can easily extravasate these openings. This phenomenon of localizing NPs in the leaky vasculature of tumor tissues is an example of passive targeting [12].

The cytotoxicity of chitosan NPs and derivatives has been attributed to positive surface charge [5]. However zeta potential measurements of acylated StNPs (Table 1 Supporting information) showed that they have negative surface charge. This is because, neither size nor zeta potential alone determine the optimal cellular response induced by NPs [14]. It has also been proposed that, in regions where the columbic repulsion of similar charges is not too pronounced, the presence of a high electric field may cause local electroporation. So, high electrical fields of the NPs of about 50 nm, which may succeed even with negative zeta potential, may eventually lead to cytotoxicity. This phenomenon is known to facilitate permeation of various nanoscale objects through biological membranes. It is reported in literature, that the proteins from the growth media adsorb to the surfaces of both cationic and anionic NPs, increase their hydrodynamic radius, and flips their charge immediately to similar negative value of the serum proteins in the original media [15]. Thus size, aggregation state, surface charge and surface chemistry would be significantly modified via electrostatic screening which in turn could influence their ability to interact with or enter cells [16,17]. Therefore, NPs that had a positive effective surface charge upon preparation are no longer cationic in the cellular media. This is important when considering the molecular effect of charge on toxicity and cellular uptake, and argues against the simple picture, still propagated in the literature, that cationic nanoparticles disrupt the negatively charged cellular membrane by electrostatic interactions. Protein adsorption to the NPs surface can mediate the uptake of the nanomaterial via receptor mediated endocytosis [18,19]. This is believed to be the reason for the interaction of the nanosized derivatives with biological systems.

Oxidative stress is often considered as the main cause for stimulation of cytotoxicity that is induced by natural or synthetic toxic agents [20]. Toxicity of NPs is exerted in form of transferring electron from molecular oxygen or by blockade of electron transport chain through an unknown mechanism [21,22]. Cellular response to increased oxidative stress results in a compromised status of endogenous antioxidants such as reduced glutathione (GSH). Also, free radical induced membrane damage caused due to lipid peroxidation (LPO) results in leakage of cellular enzymes [23]. Hence, biochemical estimation of GSH and LPO are reliable markers of cell damage caused due to production of free radicals. GSH is considered to be the first line of cellular defense for cells under various conditions of oxidative stress [24]. GSH is used as a co-substrate by enzymes; GPX and GR for reduction of hydrogen peroxide and lipid peroxides [24]. It is evident from Fig. 2b that 10,000 µg/mL of acylated starch NPs dose shows significantly low content of cellular GSH. This indicates that at high concentration of 10,000 µg/mL these NPs show cytotoxic nature (Fig. 2b).

Damage of cellular membrane due to LPO is the major cause of cell death and hence, its integrity is pivotal for its survival. In the present study, a 10,000 µg/mL dose of acylated starch NPs recorded higher LPO indices (Fig. 2c). Significantly elevated indices of LPO and lower activity of GSH showed that acylated starch NPs were cytotoxic in nature.

AO/EB staining for cell viability depicted a cell death with maximum number of EB positive cells (red[1] colored) recorded in high-

[1] For interpretation of color in Fig. 3, the reader is referred to the web version of this article.

est dose (10,000 μg/mL) of acylated starch NPs (Fig. 3). Previous studies have reported polysaccharide NP induced cytotoxicity against various tumor cell resulting due to oxidative damage to the cell membrane and mitochondrial dysfunction [22,25]. Similar alterations observed in our study are in accordance with these reports and thus establish the doses and the resultant cellular damage caused by acylated StNPs.

The binding interaction of many organic carcinogens such as polycyclic aromatic hydrocarbons with DNA is the key step in their genotoxic effect. Titration with UV absorption spectroscopy is an effective method to examine the binding mode of DNA with the molecules [26]. In general, the spectra of the compounds show UV absorption bands that are usually symmetrical with no obvious splitting. In the UV region, compounds exhibited bands between 240 and 320 nm, which are assigned to the $\pi \rightarrow \pi^*$ transitions, due to long living triplet excited state. Hypochromism results from the contraction of DNA helix axes as well as the conformational changes on molecule of DNA, while hyperchromism results from the secondary damage of DNA double helix structure [27,28]. Upon increasing concentration of CT DNA, the UV region exhibited an increase in absorption intensity 'hyperchromic' effect with a blue shift of 2–10 nm in $\pi \rightarrow \pi^*$ region (Fig. 4a and Supporting information). The strong hyperchromic effect with a blue shift is suggestive of higher binding propensity to CT DNA due to stabilization of the nanoparticle–DNA adduct. These changes are typical of compounds bound to double stranded DNA through non-covalent interaction [29]. In the present case, the complete intercalation of the compounds between a set of adjacent base pairs seems sterically impossible, but some partial intercalation can be envisioned [30].

Enhancement of the fluorescence emission when binding with the biomacromolecules (such as DNA and proteins), is a very useful fluorescent probe in genomics and proteomics [31]. In present study we found that luminescence was not observed for compounds either in DMSO or in presence of DNA. Hence, competitive binding studies using ethidium bromide (EB) bound DNA was carried out. EB is a conjugate planar molecule. Its fluorescence intensity is very weak but it is greatly enhanced when EB is specifically intercalated into the adjacent base pairs of double stranded DNA. The enhanced fluorescence can be quenched by the addition of a second molecule [32]. The addition of compounds to DNA-EB sys-

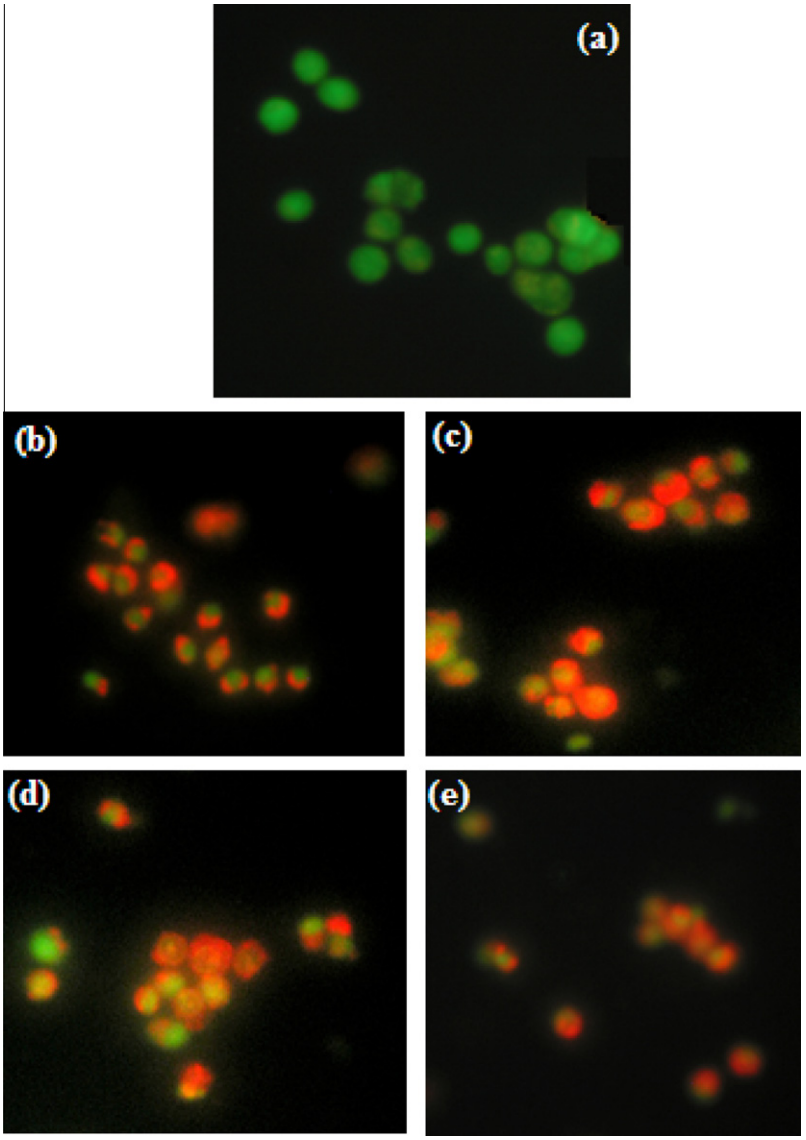

Fig. 3. AO/EB photomicrographs of stained A549 cells exposed to (a) 0 mg/mL and 10 mg/mL concentration of (b) St-palmiate, (c) St-benzoate, (d) St-phthalate and (e) St-cinnamate nanoparticles. Results are expressed as mean ± SEM for $n = 3$ (replicates). Where NS = non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to untreated cells.

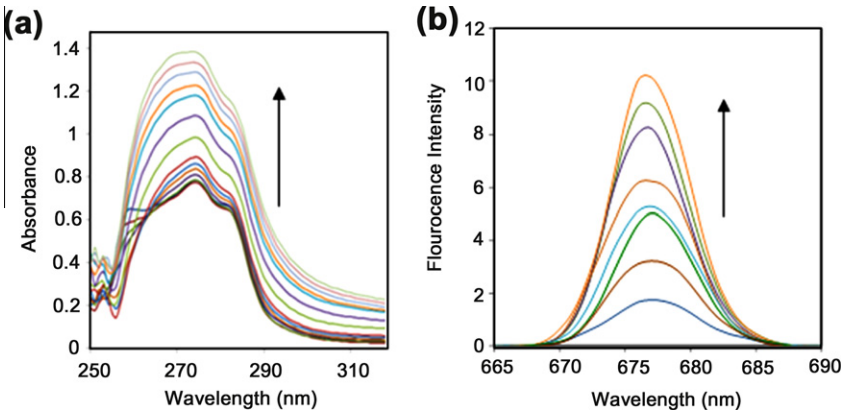

Fig. 4. (a) Absorption spectra of the St-benzoate NPs (0.1 mg/mL) without and with CT-DNA at different concentrations (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55 and 0.60 mL of stock solution). The arrow shows the intensity changes on increasing the acylated StNPs concentration, (b) emission spectra of EB bound to DNA in the absence and presence of the St-benzoate NPs. [EB] = 40 μM, [DNA] = 50 mL, [St-benzoate NPs] = 2, 4, 6, 8, 10, 12 and 14 μL, respectively; λ_{mex} = 340 nm. The arrow shows the intensity changes on increasing the acylated StNPs concentration.

tem displayed an increase in emission intensity of the DNA-EB system (Fig. 4b and Supporting information). Increase in fluorescent intensity indicates that the acylated StNPs has not completely intercalated into the DNA helix, as complete intercalation would decrease the emission intensity due to the replacement of the intercalated EB from DNA. The observed results suggest that the nanoparticles can make a contraction in the helix axis of DNA [31,33]. The binding affinity seems to follow the order St-Pal-NPs ⩾ St-Pht-NPs > St-Cin-NPs > St-Ben-NPs. Aliphatic derivative has greater binding probably due to more flexible structure. The presence of additional carboxyl group as in phthalate or extension of conjugation as in cinnamate both increase the binding affinity.

4. Conclusions

The present study reports for the first time, cytotoxic potential of the acylated starch nanoparticles along with its biocompatible nature and warrants further evaluation at preclinical and clinical levels. The noncytotoxicity to non-cancerous cells and useful thermal properties suggest promising drug delivery applications of these materials at lower concentrations while higher doses would be useful as anticancer agents. The cytotoxicity of derivatives with aromatic groups and high degree of substitution was higher relative to those containing aliphatic group. The details of the mechanism of action, especially to clarify the mode of interaction with tumor cells, effect of degree of substitution and particle size are now under investigation. Despite negative zeta potential the nanoparticles exhibited reasonable biding propensity with CT-DNA, although complete intercalation was not observed.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2012.10.001.

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