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# Reduced toxicological manifestations of cisplatin following encapsulation in folate grafted albumin nanoparticles☆

Noor Alam [a], Ravindra Dhar Dubey [a], Ashok Kumar [b], Mytre Koul [b], Neelam Sharma [b], Parduman Raj Sharma [b], Bal Krishan Chandan [b], Shashank K. Singh [b], Gurdarshan Singh [a,c], Prem N. Gupta [a,c,*]

[a] Formulation & Drug Delivery Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India
[b] Cancer Pharmacology Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India
[c] PK-PD-Toxicology Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India

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ABSTRACT

*Aims:* Cisplatin is one of the most potent chemotherapeutic agents acting against a variety of tumors, however, its use is mainly limited due to the dose limiting toxicities and acquired resistance to cisplatin. Folate functionalized albumin nanoparticles were developed for targeted delivery of drug to limit the adverse effects of cisplatin.
*Main methods:* Cisplatin loaded nanoparticles functionalized with folate (CP-FA-BSA-NPs) were developed and characterized for various parameters. In order to investigate the targeting ability of folate conjugated nanoparticles, *in vitro* cellular uptake study was performed in folate receptor over expressing cells (MCF-7). Further, blood urea nitrogen (BUN) level, plasma creatinine level, body weight and kidney weight of the mice were measured followed by histopathological examination of various tissues to have an insight into the potential of developed formulation in the reduction of drug associated adverse effects.
*Key findings:* The cellular uptake studies demonstrated higher internalization of folate conjugated nanoparticles as compared to plain counterpart (CP-BSA-NPs). Following two cycles of cisplatin treatment, a week apart, BUN and plasma creatinine level were found to be significantly higher in case of free cisplatin as compared to saline, CP-BSA-NPs and CP-FA-BSA-NPs treated groups. Body weight and kidney weight of free cisplatin treated mice were significantly reduced as compared to other group. Histopathological examination of kidney from CP-BSA-NPs and CP-FA-BSA-NPs treated groups revealed no kidney damage, however, a sign of nephrotoxicity was observed in the case of free cisplatin.
*Significance:* The results demonstrated the potential of developed formulation in reducing the adverse effects of cisplatin.



## 1. Introduction

Cisplatin is an important component of antineoplastic regimens for the various solid tumors [1]. However, the use of cisplatin in chemotherapy is limited due to emergence of nephrotoxicity, neurotoxicity, and myelotoxicity [2,3]. Various nanosized drug carriers have been investigated in order to minimize side effects of cisplatin and enhance its antineoplastic efficacy including micelles [4], liposomes [5], solid lipid nanoparticle [6], polymeric nanoparticle [7,8] and drug-polymer conjugates [9,10].

In order to reduce cisplatin associated adverse effects and in turn improve the efficacy of this potent drug, the tumor should be selectively exposed to the drug while exposure to the kidney should be minimized. Nanocarrier based delivery systems appear to be a promising option for the cisplatin delivery because they possess potential to reduce toxicity, thereby improving the efficacy through more selective and controlled delivery of the drug in the tumor tissue [8]. The hydrophilic polymer based nanocarriers have possibly less opsonization by reticuloendothelial system through an aqueous steric barrier [11]. Albumin is nontoxic, biodegradable, biocompatible and hydrophilic polymer potentially useful in drug delivery [12]. The enhanced uptake of albumin-based drug delivery systems in solid tumors is mediated by enhanced permeation and retention effect. Additionally, accumulation of albumin nanoparticles into tumor tissue is due to transcytosis initiated by binding of albumin to a cell surface glycoprotein receptor (albondin) as well as due to binding of albumin to SPARC (secreted protein acid and rich in cysteine), which makes it an attractive carrier for drug delivery [13].

The tumor cells differ in their biology and physiopathological characteristics from normal cells and show high level expression of surface markers that may be exploited for drug targeting approaches in clinical settings. In order to target drugs to specific tumor tissue within the body, drug can be directly attached to a targeting agent, complexes

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* Corresponding author at: Formulation & Drug Delivery Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu-Tawi, 180001, India.
*E-mail addresses:* pngupta10@gmail.com, pngupta@iiim.ac.in (P.N. Gupta).

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with carrier or encapsulate into delivery system that contain targeting moieties [14]. Among the diverse kind of targeting agents, folic acid has become an emerging candidate for non-destructive targeting to membrane-bound tumor-associated receptors (*i.e.* folic acid receptor-α; FR-α) that are specifically over-expressed on the surface of many human tumor cells, including ovarian, breast, endometrial, colon and cervical cancer cells [15,16]. Further, folic acid binds with very high affinity (Kd ~ 1 nM) to its receptor at cell surfaces and internalized by receptor mediated-endocytosis [17].

In the present study, cisplatin-loaded bovine serum albumin nanoparticles (CP-BSA-NPs) were developed and surface modified by covalent attachment of folic acid. The developed formulations were extensively characterized *in vitro* for various parameters including particle size, zeta potential, drug loading, release profile and surface morphology. The differential scanning calorimetry was used to investigate physical state of cisplatin within the formulation. Subsequently, the developed formulations were evaluated for cellular uptake behavior. Further, *in vivo* toxicity profile of cisplatin loaded folic acid decorated bovine serum albumin nanoparticles (CP-FA-BSA-NPs) was investigated and compared with CP-BSA-NPs and free drug.

## 2. Materials and method

### 2.1. Materials

Cisplatin, folic acid, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), bicinchoninic acid (BCA) reagents, fluorescence 5(6) isothiocyanate (FITC) and trypsin were purchased from Sigma-Aldrich (India). Glutaraldehyde (25%) was supplied from Alfa Aesar (England). Sodium diethyl dithiocarbamate (DDTC) was purchased from Himedia. Bovine serum albumin (BSA) was procured from Loba Chemie (India). High purity ethanol was commercially supplied by Merck (India). All other chemicals and reagents were of analytical grade.

### 2.2. Preparation and optimization of bovine serum albumin nanoparticles

The desolvation technique was used for the preparation of BSA nanoparticles as described previously [18], with slight modifications. Aqueous solution of BSA (4%, *w*/*v*) was prepared and the pH of the solution was adjusted to 7, 8.5, and 10. Subsequently, ethanol was added intermittently (0.5 ml/min with 2 min interval) to the protein solution under constant magnetic stirring (600 rpm) until the solution became turbid. The resulting coacervates were then hardened with 8% glutaraldehyde (1.17 μl/mg BSA) over a period of 6 h at room temperature under constant magnetic stirring (600 rpm). The resulting nanoparticles were purified by two cycles of centrifugation (20,000 rpm, 20 min, 12 °C; Sigma, Germany) and washed with phosphate buffer saline (PBS, pH 7.4). After that, developed particles were lyophilized (Advantage freeze dryer, VirTis, Gardiner, NY, USA) using sucrose as cryoprotectant and stored in desiccators till further use.

### 2.3. Preparation of cisplatin loaded bovine serum albumin nanoparticles

Cisplatin was incubated overnight in aqueous solution of BSA (4% *w*/*v*) at 37 °C with constant stirring on magnetic stirrer and then pH adjusted to 8.5 with 0.1 N NaOH. Nanoparticles were prepared using the procedure described above.

### 2.4. Preparation of folic acid decorated bovine serum albumin nanoparticles

The surface of BSA-NPs and CP-BSA-NPs were modified with folic acid by method described earlier [19]. Briefly, folic acid, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were taken in PBS, pH 7.4 (1:2:2 M ratio) and stirred for 15 min at room temperature. Subsequently, BSA-NPs (or CP-BSA-NPs; 20 mg/ml) was added to the mixture and then stirred for 4–5 h at room temperature. The resulting FA-BSA-NPs (or CP-FA-BSA-NPs) were centrifuged and the supernatants were stored for further analysis. Harvested samples were washed with PBS, lyophilized, and then stored. The unreacted folic acid remaining in the supernatant was quantified using a UV-spectrophotometer (Thermo Fisher Scientific, Finland) at 358 nm. The folic acid content of the samples was calculated by comparing the absorbance of the supernatant to reference samples which contained different concentration of folic acid standard.

### 2.5. Characterization of nanoparticles

#### 2.5.1. Particle size distribution and zeta potential measurement

Particle size distribution and zeta potential of the nanoparticles were measured by dynamic light scattering using Zetasizer (Nano ZS90, Malvern Instruments, UK). In order to determine the particle size and zeta potential, a suitable dilute suspension of particles was prepared in PBS (pH 7.4) and the samples were analyzed in triplicate.

#### 2.5.2. Confirmation of folic acid conjugation with nanoparticles

In order to confirm the successful conjugation of folic acid with the nanoparticles, the lyophilized FA-BSA-NPs were digested by trypsin (0.05 mg/mg BSA) overnight at 37 °C by using method described earlier [20]. The resulting tryptic hydrolysis product of FA-BSA-NPs was scanned in the range 250–450 nm by UV–VIS spectrophotometer (Shimadzu, US) using tryptic hydrolysis of BSA-NPs as a blank control.

#### 2.5.3. Determination of CP-BSA nanoparticle yield

The desolvated BSA contributed in the nanoencapsulation process. In order to know the amount of BSA transformed into nanoparticles, the amount of protein in supernatant was calculated. For the determination of the nondesolvated BSA present in supernatant, the nanoparticles were separated from the supernatant by centrifugation (20,000 rpm, 20 min). An aliquot of the supernatant was diluted with distilled water and the amount of the protein dissolved in the supernatant was determined using bicinchoninic acid (BCA) protein assay [21]. Briefly, BCA working reagent (200 μl) was added to supernatant (25 μl), incubated the mixture at 37 °C for 30 min and the samples were analyzed by UV-spectrophotometer (Thermo Fisher Scientific, Finland) at 562 nm. The protein content of the samples was calculated by comparing to reference samples which contained different amount of a BSA standard and were treated as described before.

#### 2.5.4. Morphological examination

The surface morphology of CP-BSA nanoparticles and CP-FA-BSA nanoparticles was observed using atomic force microscopy (AFM). The aliquots of CP-BSA-NPs and CP-FA-BSA-NPs suspension were diluted with water and then deposited on freshly cleaved mica. Following incubation for 3–4 min., samples were washed and dried. The morphological examination of samples was performed in tapping mode using a Bioscope Catalyst AFM (Bruker Corporation, Billerica, MA) having a Nanoscope V controller and images were processed using Nanoscope analysis, v.1.4.

#### 2.5.5. Drug loading

The amount of cisplatin entrapped into the nanoparticles was calculated by the difference between the total amount of initial drug used and the amount of cisplatin present in the supernatant obtained during the purification step of the nanoparticle preparation processes. The non-entrapped cisplatin in the supernatant obtained after centrifugation of nanoparticles was quantified by high performance liquid chromatography (HPLC) method [22]. An aliquot of the supernatant was placed in a 2 mL Eppendorf tube and mixed with 50 μl of a solution of sodium diethyl dithiocarbamate (DDTC, 10% *w*/*v* prepared with 0.1 N NaOH). Samples were incubated in a water bath (New Brunswick Scientific,