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Full Length Article

# Bio-conjugation of curcumin with self-assembled casein nanostructure via surface loading enhances its bioactivity: An efficient therapeutic system

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ABSTRACT

In this study, self-assembled nanostructure of casein ($NS_{CS}$) of spherical shape was prepared using an optimized desolvation method with a mean size of 45 nm. Curcumin (Cur), a potent natural antioxidant, an anticancer and poorly soluble agent in aqueous solution, was conjugated to protein nanostructure ($NS_{CS}$-Cur) using CDI chemical activation method. We further, evaluated the physicochemical stability as well as the biological activity of $NS_{CS}$-Cur *in vitro*. When the antioxidant activity (free radical scavenging activity) and cellular antioxidant activity (CAA) assay of nano-formulated curcumin ($NS_{CS}$-Cur) was evaluated, it revealed that antioxidant activity of $NS_{CS}$-Cur was higher than that of free Cur in both. Furthermore, we showed that the cytotoxicity of nano-formulated curcumin ($NS_{CS}$-Cur) was found higher in four different cancer cells (breast cancer; MCF-7 and MDAMB231, cervical cancer; HeLa, osteosarcoma; MG 63) than that of free curcumin. Moreover, the conjugation with folic acid with $NS_{CS}$-Cur ($NS_{CS}$-Cur–FA) demonstrated enhanced cytotoxicity in cancer cells than un-conjugated one. When we studied the storage stability and bioactivity, $NS_{CS}$-Cur formulation showed significant stability (up to 30 days) with ignorable loss in its bioactivity. Hence, we concluded from our findings that curcumin loaded protein nanostructure ($NS_{CS}$-Cur) might be used to achieve higher stability, enhanced dispersibility in suspension as well as improved bioactivity of curcumin which also can be used in the pharmaceutical application.

## 1. Introduction

Cancer is now the one of the leading cause of death. Recent evidences suggest that cancer is fundamentally initiated due to the DNA damage and epigenetic alterations commonly induced by oxidants [1,2]. The experimental findings demonstrated that naturally occurring phytochemicals play as anti-carcinogenic factors via activating anti-oxidative stress signals both *in-vitro* and *in-vivo* [3]. Furthermore, several epidemiologic studies have demonstrated that intake of antioxidant nutrients present in the diet can reduce the risk of pancreatic carcinogenesis [4,5]. Hence in recent years, bioactive compounds present in natural dietary teamed as nutraceutical have gained a great interest due to their ability to reduce the risk of cancer development [6].

Among nutraceuticals, one of the most promising chemo-preventive agents is curcumin (Cur), commonly found in *Curcuma longa* (turmeric) and regularly used in Indian food as coloring agent and spice. It possesses a wide range of pharmacological potential that includes anticancer, antioxidant, antiulcer, immunomodulatory, anti-inflammatory, neuroprotective, wound healing and anti-aging activity [7–11]. Despite having great pharmacological potential, its application has been restricted as a pharmaceutical agent due to its poor aqueous dispersibility, inadequate tissue absorption, rapid systemic elimination, and degradation at alkaline pH, all of which severely curtails its bioavailability [8,12,13].

However, problems associated with the bioavailability of Cur such as rapid metabolism, limited tissue distribution, low serum levels and short half-life can be overcome by employing efficient nano-delivery systems without affecting its bioactivity [14]. Recently, biopolymers and biopolymers based composite nanostructure have been used for controlled and targeted drug delivery as well as tissue engineering application due to their biocompatibility to the biological system [15–18]. Although, different carrier molecules such as polymeric nanoparticles, polymeric micelles, hydrophilic polymers, liposomes or hydrogels have been reported as delivering agents of Cur [19]. But, importantly protein-based nanocarriers have particularly gained much interest as it meets the requirements of good drug carrier such as biodegradability, non-antigenicity, abundant and extraordinary binding capacity of various drugs as well as the possibility of less opsonization

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via the reticuloendothelial system [20–24]. The protein-based nanocarriers also demonstrate improved stability and extended shelf life, prolonged blood residence time, enhanced intestinal absorption and cellular uptake, and reinforced anti-cancer efficacy [21,24,25].

Milk proteins serve as natural vehicles for delivering essential materials, building blocks and immune system components from mother to the new-born. Casein is abundant and cheap as it contributes about 80% of the protein content of bovine milk and has the ability to bind a variety of small molecules [26,27]. It has been established as a green and safe as well as cheap material for the preparation of nano-carrier for drug delivery [20]. Nevertheless, the application of casein as drug carrier is associated with several limitations such as its tendency to aggregate near to its isoelectric point in aqueous solution due to its high hydrophobicity which can cause destabilization of the delivery system [28]. Furthermore, it is very sensitive to the presence of pepsin due to its open tertiary structure and high proline content which causes sudden release and its degradation of encapsulated bioactive compounds [29].

To overcome the above limitations of native casein as a delivery agent, we prepared stable self-assembled casein nanostructure ($NS_{CS}$) using glutaraldehyde as crosslinker. Nanostructure prepared with various crosslinker concentrations were optimized for obtaining minimal polydispersity and residual glutaraldehyde toxicity. Curcumin-loaded casein nanostructure was also prepared by chemical activation using1,1′-carbonyl diimidazole (CDI). Also, the Cur retention capacity and loading efficiency of $NS_{CS}$ and *in-vitro* drug release was also studied. Also, the storage stability or shelf-life of $NS_{CS}$-Cur was assessed. The anti-cancer activity and cellular antioxidant activity of curcumin-loaded nanostructure were also evaluated and compared with free curcumin.

## 2. Materials and methods

### 2.1. Materials

Curcumin (Cur), *N-acetylcysteine* (NAC), sodium cacodylate, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS+), 1-anilinonapthalene-8-sulfonic acid (ANS) and 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) were purchased from Sigma-Aldrich, India. MTT assay kit, glutaraldehyde (GTD, 25%), Casein, DMEM media, fetal bovine serum (FBS), antibiotics, ethanol, disodium EDTA, DAPI, acridine orange, potassium persulfate and phosphate buffer saline (PBS) were purchased from HiMedia India Pvt. Ltd. Annexin V/7-AAD staining kit and ROS quantification kit (2′,7′-dichlorodihydrofluorescein diacetate *$H_2$DCFDA*) were purchased from BD Bioscience, India. All cell lines were obtained from NCCS, Pune, India. All plastic wares were purchased from Tarsons, India. Triton-X was obtained from Calbiochem, India. All other reagents were of analytical grade. A medical practitioner (Dr. Bibhukalyan Prasad Nayak) freshly collected human blood ($B^{+\ ve}$) from the donor before the experiments. All glassware used in the present study was purchased from Borosil, India. Milli-Q water was

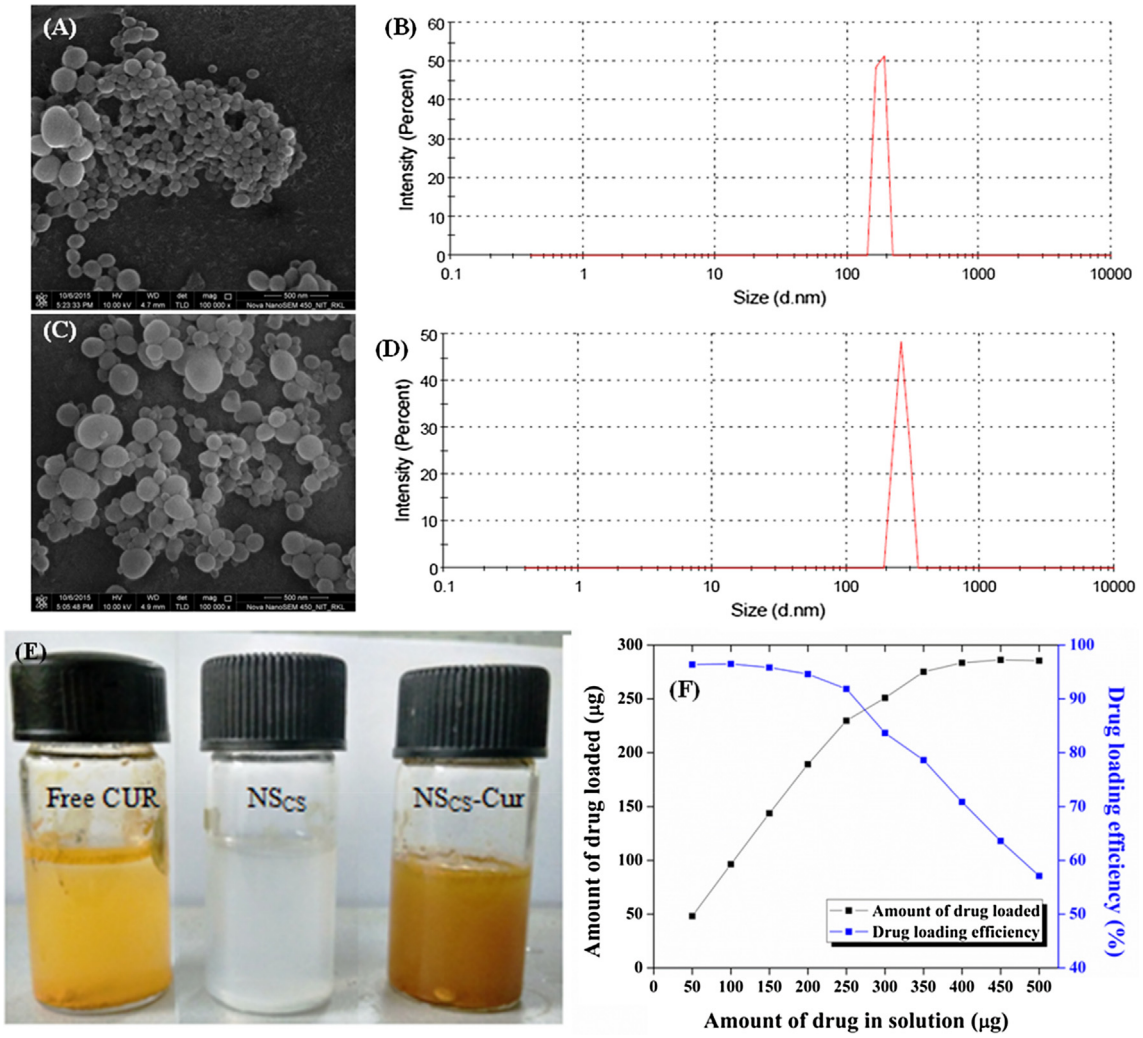


**Fig. 1.** Characterization of $NS_{CS}$ (A) FESEM image, (B) DLS. Characterization of $NS_{CS}$-Cur (C) FESEM image, (D) DLS. (E) Photographic image of free curcumin, $NS_{CS}$, and $NS_{CS}$-Cur. (F) Drug loading capacity and efficiency of $NS_{CS}$ for curcumin as a model drug.