Novel alginate-enclosed chitosan–calcium phosphate-loaded iron-saturated bovine lactoferrin nanocarriers for oral delivery in colon cancer therapy

Aim: To develop polymeric-ceramic nanocarriers (NCs) in order to achieve oral delivery of the anticancer neuropeptide iron-saturated bovine lactoferrin (Fe-bLf) protein. **Materials & methods:** Fe-bLf or paclitaxel (Taxol®) were adsorbed onto calcium phosphate nanocores, enclosed in biodegradable polymers chitosan and alginate. The Fe-bLf or Taxol-loaded NCs indicated as AEC–CP–Fe-bLf or AEC–CP–Taxol NCs, respectively, were made by combination of ionic gelation and nanoprecipitation. Size distribution, morphology, internalization and release profiles of the NCs were studied along with evaluation of *in vitro* and *in vivo* anticancer activities and compared with paclitaxel. **Results:** AEC–CP–Fe-bLf NCs obtained spherical morphology and showed enhanced endocytosis, transcytosis and anticancer activity in Caco-2 cells *in vitro*. AEC–CP–Fe-bLf NCs were supplemented in an AIN 93G diet and fed to mice in both prevention and treatment human xenograft colon cancer models. AEC–CP–Fe-bLf NCs were found to be highly significantly effective when given orally, as a pretreatment, 1 week before Caco-2 cell injections. None of the mice from the AEC–CP–Fe-bLf NC-fed group developed tumors or showed any signs of toxicity, while the mice fed the control AIN 93G diet showed normal tumor growth. Fe-bLf or Taxol, when given orally in a diet as nanoformulations post-tumor development, showed a significant regression in the tumor size with complete inhibition of tumor growth later, while intratumoral injection of Taxol just delayed the growth of tumors. The pharmacokinetic and bioavailability studies indicated that nanoformulated Fe-bLf was predominantly present on tumor cells compared to non-nanoformulated Fe-bLf. Fe-bLf-loaded NCs were found to help in absorption of iron and thus may have utility in enhancing the iron uptake during iron deficiency without interfering with the absorption of calcium. **Conclusion:** With the promising results of our study, the future potential of NC-loaded Fe-bLf in chemoprevention and in the treatment of human colon cancer, deserves further investigation for translational research and preclinical studies of other malignancies.

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The combination of environmental and genetic factors cause carcinogenesis, a multistep process, which leads to a series of genetic and epigenetic changes at various stages in the development and progression of the fatal metastatic disease [1]. Cancer cells arise from a single transformed cell that overcomes regular cell death/apoptosis. Thus, a cancerous state is a result of several sequential events triggered by various factors including genetic predispositions, transformation by viruses, radiation and/or by certain chemicals. Angiogenesis, the development of new blood vessels from endothelium of the pre-existing blood vessels plays an important role in cancer metastasis [2–4]. Based on this basic property of cancer development, many anticancer drugs have been designed to target angiogenesis and to further inhibit metastasis via active and/or passive targeting [5].

A large spectrum of chemotherapeutics presently available in the market have a number of limitations, as well as imposing many side effects [6–10]. Extremely short plasma half-lives of the therapeutics cause cancer patients to suffer harsh management of prolonged parenteral administration [11,12]. In this regard, oral administration would be convenient, cost-effective and reduce patient compliance [13]. Harsh side effects of present day anticancer chemotherapeutics can be readily overcome by using nanotechnology of anticancer biomacromolecules, such as siRNA, miRNA and/or anticancer protein/peptides [14]. Progress in biotechnology and genetic engineering has led to an increased curiosity in the use of these biomacromolecules, but delivery of these molecules to the target site maintaining their structural integrity, poses challenges from the tough conditions in the GI tract. Recent

advances in nanotechnology can be explored to protect the active biological conformation and structure of macromolecules to overcome the sturdy barriers offered by the GI tract epithelium [15–18]. Nanotechnology offers benefits such as slow/controlled release, target specificity, improved efficiency, lower side effects and ease of delivery [19]. Uses of polymeric nanocarriers (NCs) include safe and effective delivery of anticancer biomacromolecules, the improvement in the biodegradability of the nanoformulation, after a time period [20–24]. Due to their minute size, these NCs can transport the cargo via very small pores, this property could offer additional benefits in cancer therapy, thereby exploiting the enhanced permeability retention effect (EPR) offered by pathological angiogenic vasculature in the cancer [22,25,26].

Modern developments in polymeric nanoformulations for the treatment of cancer have reported a number of homo- and co-polymer combinations, for sustained and controlled delivery of anticancer agents [15,27–34]. Recently, nanocarrier-based oral formulation for anticancer drug delivery has attracted considerable interest [28,29,35]. In this regard, poly(ethylene glycol)-poly(lactic acid) polymers have been employed for oral formulation of antiangiogenic TNP470, [28] and poly(lactide)–vitamin E derivative/montmorillonite NC-based formulations for oral delivery of docetaxel have been investigated [35]. NCs of 100–1000 nm size range based on natural proteins, for example by salting out silk fibroin [36,37], have also been reported.

Since, carcinogenesis is proposed to be a chronic inflammation [38–40], a number of natural anticancer compounds that can modulate the immune system against cancer, including curcumin, capsaicin, neem extracts and matrine, have been studied for their nontoxicity and specific effects on cancer cells [41–43]. This evidence regarding dietary nutraceuticals is fascinating and warrants greater attention. Milk and milk-based products were shown to contain components that include fatty acids and multifunctional proteins such as bovine lactoferrin (bLf) with reported anti-inflammatory and anticarcinogenic activities [44,45]. Lactoferrin (Lf), is an iron binding approximately 78–80 kDa glycoprotein, found to be present in mammalian milks and in most exocrine secretions [46]. Although bLfs play important physiological roles in the process of iron homeostasis, iron transportation and sequestration, it is also well-known for its

antimicrobial, antiviral, anti-inflammatory, antioxidant properties and immunomodulatory functions [47–49]. In the recent decade, bLf has gained significant attention for its future potential use as a safer natural food (bovine milk) derived anticancer chemopreventive/therapeutic [50–53]. In its natural form, bLf is normally only 15–20% saturated with iron, indicating that the lobes are not fully occupied by iron [54]. Furthermore, it has been shown earlier that orally-fed bLf resists degradation in the intestine, which bears direct relations to the levels of iron saturation [55]. Interestingly, we showed for the first time, in a recent study that iron content is a critical determinant in the antitumor activity of bLf and found that 100% iron-saturated bLf (Fe-bLf) acts as a potent natural adjuvant for augmenting cancer chemotherapy [53]. When fed orally to mice, Fe-bLf was preferentially taken up within the Peyer's patches and a few tumor infiltrating cells were also found to carry Fe-bLf [201]. Increased leukocytes (CD4⁺, CD8⁺, natural killer [NK] and dendritic cells) infiltration into tumors, lamina propria and the spleen was observed following the oral administration of Fe-bLf. More importantly, Fe-bLf showed no toxicity and restored both red and white blood cell numbers, which were otherwise depleted by chemotherapy, thus potentially fortifying the mice against cancer [53,201]. 'ReCharge', a novel ice-cream product containing Fe-bLf as its key ingredient, has shown promising results in Phase II clinical trials for treating chemotherapy induced immunosuppression, and gut damage in cancer patients [301]. The present study was aimed to further enhance the bioavailability of Fe-bLf to tumor sites, which could be achieved by making use of the aforementioned advances in nanotechnology. We hypothesized that Fe-bLf loaded on to NCs could eradicate the cancer cell population more efficiently, as the *in vivo* bioactivity of the protein would be dramatically enhanced by its nanoencapsulation. We designed and developed chitosan-coated calcium phosphate adsorbed Fe-bLf NCs (C-CP-Fe-bLf NCs) for enhanced anticancer activity. To provide protection, in the GI tract against degradation, these NCs were further encapsulated in alginate gel (termed as AEC-CP-Fe-bLf NCs).

All of the constituents chosen for this nanoformulation were biocompatible. For example, calcium phosphate is present in teeth and bones. Both chitosan and alginate are biodegradable polymers known to offer low immunogenicity and biocompatibility [56]. We further investigated the size and morphology

of these NCs for the analysis of their physical characterization. *In vitro* protein release profiles over a time period of 24 h were done in physiological pH and cross-checked with a gel retardation assay. The pH sustainability measurements were done in order to assess protection offered by the alginate gel to the inner C-CP-Fe-bLf NCs. Anticancer efficacy of the Fe-bLf-loaded NCs were analyzed *in vitro* in the colon cancer cells followed by cellular internalization and transcytosis studies. *In vivo* antitumor chemopreventive and treatment efficacies of nanoformulated Fe-bLf, were tested in nude mice (BALB/c nu/nu), bearing the xenograft human colon tumors, with human colon cancer epithelial Caco-2 (human colon adenocarcinoma) cells, as a proof-of-concept. In addition, we also loaded paclitaxel (Taxol®) as a model anticancer drug onto these novel NCs, to compare the aforementioned efficacies of nanoformulated Fe-bLf.

Materials & methods

■ Materials

Sodium alginate, calcium phosphate, chitosan with a 20–200 cp viscosity, average molecular weight of 200 kDa and deacetylation degree of 80% and sodium tri-polyphosphate (STPP) were purchased from Sigma Aldrich. Lissamine rhodamine B sulfonyl chloride was from Invitrogen, Australia. Antiactin antitubulin antibodies and horseradish peroxidase (HRP)-conjugated anti-goat IgG were procured from Santa Cruz Biotechnology Inc. and Sigma Aldrich. FITC-labeled antimouse IgG was purchased from Sigma-Aldrich. Caco-2, normal FHs 74 Int (adherent human primary fetal small intestinal) cell lines were obtained from the American Type Culture Collection (VA, USA). Polyvinylidene fluoride membranes for western blotting were from Amersham Biosciences. All remaining chemicals, including calcium chloride and acetic acid, were of analytical grade.

■ Preparation of endotoxin-free Fe-bLf & enzyme sensitivity studies

Endotoxin-free bLf was prepared from Australian bovine milk. Endotoxin was tested with Genscript ToxinSensor™ (NJ, USA), Chromogenic Limulus Amebocyte Lysate Endotoxin Assay Kit (Genscript ToxinSensor). Fe-bLf was prepared from the bLf according to the previously described method developed in our laboratory [53]. In brief, bLf after treatment with mild acidic solution (pH 2.6) was dialyzed for a period of 48 h in 0.1 M citric acid to get rid of the bound metal ions and then saturated with ferric [Fe(III)] nonahydrate

to form bLf-Fe³⁺-co-ordinate complexes for the development of deep-red colored Fe-bLf. The obtained Fe-bLf was loaded on to the C-CP NCs and covered with alginate coating.

In order to proceed with the omnizyme enzyme resistance studies, Fe-bLf samples were digested with a gastric enzyme cocktail (omnizyme) using a modified protocol [57]. Briefly, Fe-bLf was mixed with commercially available enzyme tablet cocktail. The reaction mixture was set in 1:50 (enzyme:protein) ratio and incubated at 37°C in a shaker with constant stirring; the digests were collected after 12, 24 and 48 h time intervals. The reaction was stopped by inactivating the enzymes at 42°C and aliquots were stored at -20°C until further analysis in SDS-PAGE (The mini PROTEAN 3 apparatus, BioRad, NSW, Australia).

■ Preparation of alginate-enclosed chitosan-calcium phosphate-loaded Fe-bLf NCs

AEC-CP-Fe-bLf NCs were prepared by a combination of nanoprecipitation and ionic gelation methods. Calcium phosphate was prepared from its constituents as follows: disodium hydrogen orthophosphate (Na₂HPO₄) in a molar ratio of 4:1 was added to calcium chloride (CaCl₂) in a drop-wise manner, with continuous stirring. The obtained suspension was sonicated at 4°C to ensure a white precipitate of calcium phosphate was obtained. Acquired calcium phosphate (1% w/v) suspension was incubated for 24 h with 10% w/w Fe-bLf with constant stirring at 4°C, under a pH <8.0, the isoelectric pH of Lf, in order to adsorb Fe-bLf on to the NCs. After the electrostatic interaction of Fe-bLf on to the ceramic core, the obtained suspension was centrifuged and washed several times to remove traces of unbound protein and freeze dried. 0.01% w/w of chitosan solution in acetate buffer (pH 4) was added to the calcium phosphate under constant stirring and 0.01% w/w of crosslinking agent, STPP was added in a drop-wise manner. Constant stirring at 6000 rpm for 12 h was done to ensure that the nanoformulation obtained the size of 207 ± 39.1 nm, followed by freeze drying the sample to make sure that the sample has obtained spherical shape. These nanocores were then encapsulated in the alginate gel by using 2% w/v alginate solution and calcium chloride. The finally obtained nanoparticles were washed and freeze dried for further characterization. All these experiments were done at 4°C as to protect the polymeric and protein components in the formulation.

■ Preparation of alginate-enclosed chitosan–calcium phosphate–Taxol nanoparticles

Calcium phosphate (1% w/v) suspension was incubated for 24 h with 0.1% anticancer drug, Taxol, with constant stirring at 4°C; under a pH <8.0 in order to electrostatically adsorb Taxol on to the NCs. After the adsorption of Taxol on to the ceramic core, the obtained suspension was centrifuged and washed several times to remove traces of unbound drug and freeze dried. Rest of the procedure was followed as described above.

■ Encapsulation efficacy

Encapsulation efficacy of the nanoparticles was expressed as the ratio between bound and total protein added to the formulation. In other words, encapsulation efficacy (%) = (total protein loaded – total unbound protein)/total protein loaded × 100.

■ Characterization of

AEC–CP–Fe–bLf NCs

Dynamic light scattering was employed using a zetasizer, after 500-fold dilution with autoclaved milliQ water to determine the average size of the prepared NCs following various steps of their preparation viz, calcium phosphate core, Fe–bLf-loaded calcium phosphate core, chitosan formulation and alginate-enclosed NCs. Surface morphology of these particles was determined by scanning electron microscopy (SEM), at an accelerating voltage of 5–10 kV (Zeiss Supra). For SEM visualization, different preparations including C–CP NCs, C–CP–Fe–bLf NCs, and AEC–CP–Fe–bLf NCs were fixed on the stub by a double-sided sticky tape and then coated with a gold layer by a SC7620 sputter coater (Quorum technologies, UK) for 60 s.

■ Control release & pH sensitivity studies of AEC–CP–Fe–bLf NCs

In order to obtain the control release studies of the oral drug delivery system, *in vitro* release profile of the NC-entrapped Fe–bLf was carried out in phosphate-buffered saline (PBS) (pH 7.2). AEC–CP–Fe–bLf NCs (1% w/v) were suspended into PBS under constant stirring maintained at a temperature of 37°C. At different time intervals starting from 1–24 h, each sample was centrifuged, supernatant collected and the pellets were dispersed in fresh solution. Supernatants were analyzed for protein concentration. To estimate the protein concentration the Better Bradford™ assay (Pierce) was performed, with Fe–bLf protein standards. For immunoblotting,

15 µg of Fe–bLf, NCs were separated by SDS-PAGE, by using 10% gel. The separated proteins were then transferred to a polyvinylidene fluoride membrane for 90 min at 0.35 mA and 100 V (the mini PROTEAN 3 apparatus, BioRad). Following transfer, the membrane was removed, rinsed with Tris buffered saline and incubated overnight in 1:500 dilution of goat anti-bLf antibody, and later with anti-goat HRP-conjugated IgG at 1:60,000 dilution. The bands were developed with enhanced chemiluminescent detection kit.

■ Cell culture & cell viability

Caco-2 and FHs 74 Int cell lines were cultivated in T-75 flasks using their complete growth media. DMEM (American Type Culture Collection) with 10% heat-inactivated foetal bovine serum (Sigma-Aldrich), 1% v/v penicillin–streptomycin (Sigma) was used for Caco-2. FHs 74 Int cells were cultured routinely as described earlier [57], in Hybri-Care medium (American Type Culture Collection: 46-X), supplemented with 30 ng/ml human recombinant EGF (Affinity Bioreagents) with 10% fetal bovine serum. Cell culturing was carried out at 37°C in 95% relative humidity and 5% CO₂.

■ Lactate dehydrogenase cytotoxicity assay

The cytotoxic effect of different test treatments on Caco-2 and FHs 74 Int was studied by a colorimetric lactate dehydrogenase (LDH) release assay using a commercially available kit (Roche Applied Science). This assay quantifies cell death and lysis based on measuring the release of LDH, which is present within the supernatant following the loss of membrane integrity. The assay was carried out as per kit instructions. Briefly, 2×10^3 cells per well (total volume 100 µl) were plated into a 96-well plate and incubated for 24 h at 37°C in 5% CO₂. Following incubation, growth media was replaced with fresh media containing different test preparations, such as 1600 µg/ml Fe–bLf (previously determined optimal dose), 1 mg/ml of AEC–CP–Fe–bLf NCs, alginate-enclosed chitosan–calcium phosphate–Taxol nanoparticles (AEC–CP–Taxol NCs), with same concentration of void NCs and the cells were incubated further for 48 h. The amounts of LDH released in the supernatant were determined and calculated as per kit instructions. All tests were performed in triplicate and assay was repeated three times independently with similar results.

■ Apoptosis assays

The detection and quantification of apoptosis and its differentiation from necrosis was performed with the use of the Annexin-V-FLUOS staining kit (Roche Applied Science, NSW, Australia). This assay is based on the principle of plasma membrane alterations found during early stages of apoptotic cells, namely the translocation of phosphatidylserine exposed at the external surface of the cell. Annexin V is a Ca^{2+} dependent phospholipid-binding protein that has a high affinity for phosphatidylserine. However, necrotic cells also expose phosphatidylserine due to the loss of membrane integrity, therefore apoptotic cells are differentiated from necrotic cells by the use of propidium iodide that stains DNA of leaky necrotic cells only. The assay was carried out as per kit instructions. Caco-2 cells were seeded at a density of 10^5 onto six-well plates and treated with different preparations of NCs. Following appropriate incubations, with 1 mg/ml NCs, cells were harvested and 25 μl of Annexin-V-FLUOS labeling solution prepared immediately prior to use was added to the cell suspensions and incubated for 15 min at room temperature. The cells were further analyzed in a flow cytometer, using FITC and phycoerythrin filters. The total number of apoptotic or necrotic cells was counted. The apoptotic and the necrotic indices were calculated as follows: apoptotic index (A/I) or necrotic index = (number of apoptotic or necrotic cells / total number of nucleated cells) $\times$ 100.

Furthermore, apoptotic cell death was also measured by detecting the DNA strand breaks in individual cells by terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labelling (TUNEL) assay, using the *in situ* cell death detection assay kit (Roche). Nick labelling of free 3' OH ends in genomic DNA with fluorescein-dUTP was the principle behind the TUNEL assay. To perform the assay, cancer cells were treated with 1 mg/ml NCs and after a brief trypsinization, the cells were fixed with fixation solution (4% paraformaldehyde in PBS, pH 7.4) for 20 min at 15–25°C. After fixation, cells were washed with PBS and incubated with blocking solution (3% H_2O_2 in methanol) for 10 min at 15–25°C. Cells were then rinsed with PBS and permeabilized with 0.1% Triton-X-100 for 2 min. Labeling solution was mixed with enzyme solution to obtain the TUNEL reaction mixture. Cells were treated with 100 μl TUNEL reaction mixture and incubated for 60 min at 37°C in a humidified atmosphere. After the final wash, the amount of FITC fluorescence was quantified using FACS canto II flowcytometer.

■ Flow cytometry & immunofluorescence staining

Caco-2 cells were grown in six-well plates for 12 days for the expression of the surface oligosaccharide receptors and were treated with 50 $\mu\text{g}/\text{ml}$ rhodamine-loaded CP, C-CP NCs, C-CP-Fe-bLf, and AEC-CP-Fe-bLf NCs, for different time intervals from 1 to 12 h. After every successful treatment, cells were trypsinized, washed with PBS several times and permeabilized with 90% ethanol. The cells were then analyzed by using BD FACS[™] canto II flowcytometer. In addition, qualitative immunofluorescence detection of the NCs internalization was studied by confocal microscopy. Caco-2 cells following treatment with rhodamine-labeled NCs were stained with mouse antitubulin antibody.

The slides were observed under Lieca confocal microscope after staining with FITC-conjugated antimouse IgG and with 4',6-diamidino-2-phenylindole (nuclear stain). For determining the intracellular localization of Fe-bLf in the internalized NCs, HRP-conjugated anti-goat IgG was added to the cells prestained with goat anti-bLf antibody. The immune reactivity was detected by using 3,3'-diaminobenzidine substrate (Vector labs).

■ Assessment of *in vitro* transport of NCs across CaCo-2 cell monolayer

Caco-2 cells differentiate into enterocyte-like cells under normal culture conditions and form a polarized epithelial monolayer expressing several differentiation markers such as brush-border membrane enzymes. The cell line represents an appropriate model for the study of transport mechanisms related to the intestinal barrier [57]. Internalization of NCs was also analyzed by a transepithelial electric resistance (TEER) assay by the methods as described earlier [57], with slight modifications. Briefly, Caco-2 cells were grown in six-well Millicell[®] cell culture inserts (Millipore, MA, USA) for 24 days, with constant supply of fresh complete DMEM for every third day to the apical and basolateral sides of growing monolayer. After formation of monolayer, transwells were washed with Hank's balanced salt solution to remove any unattached and/or dead cells. Resistance of the monolayer was measured by using Millicell-ERS system Voltammeter (Millipore). Monolayers in which TEER values were higher than 500 ohms were selected for further processing. These monolayers were reincubated in fresh medium without phenol red for 1 h, before treatment with 50 $\mu\text{g}/\text{ml}$ of fluorescently-labeled NCs. TEER

values of the monolayers were measured for different time intervals ranging between 2, 4, 6, 12, 24 and 48 h. Periodically, 100 μ l of medium from the basolateral sides was withdrawn and fresh medium was supplied. Relative fluorescent units for all the samples after 48 h incubation were measured using a fluorescence plate reader (MTP 601F, Techomp), at excitation wavelength of 575 and 595 nm of emission wavelength. The fluorescent units were compared with standard initial apical concentrations of rhodamine-loaded NCs, to analyze the total percentage of cumulative transport to the basolateral side.

■ *In vivo* stability of Fe-bLf & AEC-CP-Fe-bLf NCs

In vivo stability of Fe-bLf and rhodamine-loaded AEC-CP-Fe-bLf NCs nanoparticles in mouse plasma after feeding was carried out according to the existing methodology [58,59] and by confocal microscopy. Fe-bLf and AEC-CP-Fe-bLf NCs were administered orally to two groups of mice at a dose fixed at 100 mg/kg bodyweight. The blood was harvested from the animal for a time period starting from 15 min to 10 days (i.e., 15 min, 30 min and 1 h, 2 h, 3 h, 6 h, 8 h, 10 h, 12 h, 24 h, 36 h, 72 h, 5 days, 7 days and 10 days) into heparinized microcentrifuge tubes (containing 20 μ l of 1000 IU heparin/ml of blood). Plasma was immediately separated by centrifugation at 1000 g for 15 min at 4°C. A thin smear of plasma sample was examined under confocal laser microscope.

■ Pharmacokinetic studies after oral administration of Fe-bLf & AEC-CP-Fe-bLf NCs

Female nude mice (age: 6 weeks), were randomly divided into two groups. The first group comprised the control animals and was given Fe-bLf only (Fe-bLf group). The second group was given AEC-CP-Fe-bLf NCs labeled with rhodamine/coumarin-6 as an oral administration. Fe-bLf dose in both cases was fixed at 100 mg/kg bodyweight. Prior to and at 30 min, 60 min, 1 h, 2 h, 3 h, 6 h, 8 h, 10 h, 12 h, 24 h, 36 h, 72 h, and 5 days, 7 days and 10 days after administration, blood was harvested from the tail vein into heparinized microcentrifuge tubes (containing 20 μ l of 1000 IU heparin/ml of blood). After each sampling, 0.25 ml of dextrose-normal saline was administered to prevent changes in the central compartment volume and electrolyte plasma was immediately prepared by centrifugation at 1000 g for 15 min at 4°C and stored at -80°C until use. The plasma

samples (100 μ l) were then acidified to pH 3.0 using 6 N HCl and Fe-bLf was extracted from it using twice the volume of a mixture of ethyl acetate and isopropanol (9:1; v/v) by shaking the mixture for 6 min. The samples were centrifuged at 5000 g for 20 min, and the upper ethyl acetate layer was removed. The extraction procedure was repeated twice. The combined extractions were evaporated to dryness in vacuum, and the residue was dissolved. An aliquot of this solution was analyzed for the content of Fe-bLf by HPLC.

■ Animal model of human colon cancer xenograft

A human colon cancer xenograft model was developed with Caco-2 cells. Tumors were established by subcutaneous injection of 2×10^6 tumor cells into the left flank of mice. All animal procedures were done in accordance with the guidelines from institutional animal ethics committee, PGIMER, Chandigarh, India. All animal experiments included six mice per treatment group and 5–6 week-old female (*Mus musculus*-nude mice-BALB/c nu/nu) were used in the study. The mice were fed either control AIN 93G diet or AIN 93G supplemented with either of the following treatments: Fe-bLf; AEC-CP-Fe-bLf NCs; or AEC-CP-Taxol NCs. Mice were weighed thrice weekly and assessed for signs of any physical or physiological distress. At the end of the experiments, mice were euthanized. Bovine casein in the control AIN93G diet was supplemented in the experimental diets with Fe-bLf, such that the protein content of the diet was unchanged. The diets contained 1.2% (w/w) Fe-bLf, unless otherwise indicated. The chemotherapeutic drug was injected intratumorally in a volume of 0.01–0.02 ml/g bodyweight once tumors reached ≈ 500 mm³. Diet intake and mice health was closely monitored. Tumor sizes were assessed weekly using vernier callipers. Blood was collected from the heart and serum was separated. Heparinized blood was used for red blood cell count.

■ *In vivo* natural killer cell depletion assay

Taking into consideration that the nanoencapsulated Fe-bLf-mediated antitumor effect was intended to be devoid of any NK cell responses, in the nude mice, NK-cell depletion assay was carried out. The *in vivo* NK-cell depletion assay was done as described earlier [60] by injecting anti-NK1.1 monoclonal antibodies in order to understand the role of NK cells in anticancer therapy in nude mice.

■ Estimation of iron by calorimetric method

The procedure for measuring iron saturation in the serum/tissues was modified from the method described [61]. Briefly, to 1 ml of serum sample or to the Fe-bLf, 50 μ l of ascorbic acid was added and mixed well to maintain iron in a reduced state. A sample without protein was taken as control and a series of standards were made with ferric nonahydrate. 100 μ l of 65% trichloroacetic acid was added into each tube after 5 min to digest the bound proteins. The tubes were immediately covered with parafilm and shaken vigorously for 30 s and allowed to stand for 10 min and centrifuged at 10,000 rpm for 20 min. Following centrifugation, 500 μ l of supernatant from each sample, iron standards and blank, were added into new eppendorf tubes containing 100 μ l of alkaline acetate solution followed by the addition of 75 μ l of tripyridyl solution, to obtain a colored product. The samples were mixed well and allowed to stand for a further 10 min. Absorbance was read at 550 nm with an Asys Expert Plus microplate reader (Asys Hitech, Cambridge, UK). Iron concentration was determined by comparing the resulting absorbance values taken with the iron standards.

■ Measurement of intracellular/serum calcium

Fura-2/acetoxymethyl ester (AM) was used to measure calcium concentration as previously described with modifications [62]. The samples were incubated with 3 mM Fura-2/AM for 30 min at 37°C in the dark and washed with Krebs/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (143.3 mM Na⁺, 4.7 mM K⁺, 2.5 mM Ca²⁺, 1.3 mM Mg²⁺, 125.6 mM Cl⁻, 25 mM HCO⁻³, 1.3 mM H₂PO⁻⁴, 1.2 mM SO₄⁻², 11.7 mM glucose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4) to remove extracellular Fura-2/AM. The loaded samples were further incubated at the room temperature for de-esterification of the Fura-2/AM. Excitation wavelength of 510 nm and an emission at 340 and 380 nm were recorded. The intracellular Ca²⁺ was calculated by ratio of fluorescence at 340/380 nm using Grynkiewicz method as represented below:

$$[Ca^{2+}] = K_d \times [(R - R_{min}) / (R_{max} - R)] \times Q$$

Here K_d is the Ca²⁺ binding constant (with Fura-2/AM at 37°C). R is the ratio of 340/380 during the experiment, R_{max} and R_{min} are the ratio of 340/380 under Ca²⁺ saturation conditions and

Ca²⁺ free conditions, and Q is the ratio of F_{min}/F_{max} at 380 nm. The calibration values were determined in the presence of 1 mM of Ca²⁺ or 10 mM ethylene glycol tetraacetic acid (R_{min}). Furthermore, the serum calcium concentrations were measured colorimetrically using ortho-cresolphthalein complexone method. Magnesium interference was prevented by the use of 8-hydroxyquinoline sulfonate in the color reagent.

■ Immunohistochemistry

Mice bearing xenograft tumors were sacrificed after the treatment period and the tumor tissues were collected. Tissues were snap frozen and stored in -80°C until further use. For immunohistochemical staining, the sections were fixed in acetone, rinsed in distilled water to remove optimal cutting temperature compound. The sections were rehydrated with TBS-Tween-20 and blocked with 3% hydrogen peroxide in 0.3% rabbit serum to overcome endogenous peroxidase activity. Goat anti-bLf antibody (Bethyl labs, TX, USA) (1:1000) was used for the detection of exogenous Fe-bLf with antigoat HRP-conjugated IgG secondary antibody. The sections were then washed in TBS-Tween-20 and developed with 3,3'-diaminobenzidine substrate (Sigma-Aldrich) and incubated for 10 min in the dark. After counterstaining with haematoxylin, slides were fixed with permanent mounting media and observed under 100 $\times$ oil immersion objective using bright field microscope (Zeiss axioscope 2 plus). Prussian blue staining was used to visualize the iron reactivity and the tissues were incubated for 20 min with Prussian blue stain and the slides were counterstained with neutral red stain for 1 min. After serial dehydration in gradients of ethanol, the slides were fixed with permanent mounting media and observed under the microscope for Prussian blue.

■ Statistical analysis

Results are expressed as mean values $\pm$ standard error of the mean of at least three experiments, unless otherwise stated. For the statistical evaluation of numerical data, one way analysis of variance and Student's t-test was used for evaluating the significance.

Results

■ Physical characterization of AEC-CP-Fe-bLf NCs

A schematic diagram of the predicted molecular structure of AEC-CP-Fe-bLf NCs is shown in FIGURE 1A. Fe-bLf was adsorbed on the calcium phosphate ceramic NCs and coated

with a thin chitosan film. STPP was used to make crosslinkages between positively charged amine groups within the chitosan as described previously. The entire polymeric-ceramic NCs were protected by alginate, as represented in the figure, in order to make an orally deliverable nanocarrier. Various parameters including the concentration of polymers and ceramic inner core along with spinning speed and time were standardized as represented in TABLE 1. Dynamic light scattering spectrometric results indicate an increase in the hydrodynamic size of NCs after coating with chitosan and further encapsulation using alginate. Size of the NCs was increased from 207 ± 39.1 nm for C-CP-Fe-bLf NCs to 344 ± 50.4 nm after complete formation of AEC-CP-Fe-bLf NCs (FIGURE 1C). SEM results further supported the DLS data and it also determined the spherical morphology of NCs (FIGURE 1B), which conveys the advantage in transporting the anticancer protein to the cancer tissue via mucosal, gastric and subsequent systemic circulation as exemplified in FIGURE 1D. Incorporation of Fe-bLf into AEC-CP-Fe-bLf NCs does not affect the size of the NCs as shown in FIGURE 1B and FIGURE 1D. Furthermore, morphological characteristics of the lyophilized Fe-bLf-loaded NCs were represented in the FIGURE 1C. As shown in this figure, a cherry red color – the characteristic of Fe (III) coordinate compounds, was observed in the case of Fe-bLf or CP NCs nanocores loaded with Fe-bLf. Chitosan-coated NCs and alginate-encapsulated NCs were transparent as the color was masked by the polymeric layers. Effect of different constituents/concentrations of chitosan, STPP, alginate and calcium phosphate and stirring time on the size and shape of the NCs varied was given in TABLE 1. In our study, we have optimized the formulation conditions with 10% w/w Fe-bLf with respect to calcium phosphate core NCs along with 0.01% chitosan, STPP and using 2% w/v alginate under 6000 rpm stirring for 12 h obtained spherical AEC-CP-Fe-bLf NCs of 350 ± 25 nm size (TABLE 1). The same conditions were used to prepare NCs for all our studies.

■ Controlled release of AEC-CP-Fe-bLf NCs & enzymatic sensitivity studies of Fe-bLf

Controlled release of AEC-CP NCs was analyzed by calorimetric assay in buffer at physiological pH over a time period of 24 h (FIGURE 2A). The result showed sustained release profiles of the encapsulated protein. Furthermore, to visualize this qualitatively, SDS-PAGE gel retardation

assay was done. Absence of bLf-specific bands in positive control indicates immobilization of AEC-CP-Fe-bLf NCs in the stacking gel. Furthermore, retardation of AEC-CP-Fe-bLf NCs in the stacking gel was indicated by intense bands, as well as bLf-specific bands in western blots, developed by using bLf specific antibody (FIGURE 2A inset). Since, the degree of iron saturation is known to play a role in bLf's ability to resist gut digestion [46,53,55], in order to understand the fate of Fe-bLf samples in the enzymatic milieu in the digestive tract, Fe-bLf samples were mixed with a commercially available enzyme tablet cocktail, Omnizyme®, for a period of 12, 24 and 48 h at 37°C. The resultant samples were then analyzed by SDS-PAGE, as is shown in FIGURE 2C; protein fragments of 15–50 kDa were observed in case of 48 h treated samples indicating the digestion of Fe-bLf in the enzyme cocktail.

To investigate the stability of AEC-CP-Fe-bLf NCs in the acidic pH in the stomach and alkaline pH in the intestine, AEC-CP-Fe-bLf NCs were treated with HCl pH 1.2 and with PBS at pH 7.4, for different time intervals from 1 to 6 h. The resultant samples were then analyzed by SDS-PAGE, as shown in the left panel of FIGURE 2B, more intense bands were observed from 4 h of incubation time in alkaline medium, representing the release of C-CP-Fe-bLf NCs by dissolution of outer alginate covering. Whereas in acidic medium, no Fe-bLf-specific bands were observed until 6 h of incubation time. Successive western blots indicate the clear bands of Fe-bLf confirm the findings observed by SDS-PAGE (FIGURE 2B). Similar results were observed calorimetrically as represented in FIGURE 3, where more cumulative release was observed in alkaline pH, compared with that of the acidic pH.

■ Pharmacokinetic study after oral administration of Fe-bLf & AEC-CP-Fe-bLf NCs

The feasibility of using AEC-CP-Fe-bLf NCs as an efficient carrier, to improve the bioavailability of Fe-bLf when delivered orally, was investigated. After oral administration of Fe-bLf and AEC-CP-Fe-bLf NCs at a concentration of 100 mg/kg of mice, the relevant pharmacokinetic parameters were determined. FIGURE 2D shows the mean Fe-bLf concentration in plasma at different time-points after oral administration. The concentration of Fe-bLf detected in the blood was significantly higher in mice, fed with AEC-CP-Fe-bLf NCs than those administered with equimolar concentration

Figure 1. Synthesis of alginate-enclosed calcium phosphate iron-saturated bovine lactoferrin nanocarriers and their characterization. (A) Schematic representation of alginate-enclosed NCs are shown. Chitosan forms a thin film over calcium phosphate ceramic cores, upon treatment with sodium tripoly phosphate. Alginate forms an outer coating after gelation using CaCl₂. (B) The NCs were found to have spherical morphology when observed under scanning electron microscope. (C) Lyophilized powders of (i) Fe-bLf, (ii) CP-Fe-bLf, (iii) void C-CP NCs, (iv) Fe-bLf-loaded C-CP NCs, (v) alginate-encapsulated C-CP-Fe-bLf NCs, and (vi) void AEC-CP NCs. The representative schematic diagrams are given in the inset of the corresponding image. (D) Dynamic light scattering spectrometric observation of various layers predicts increase in the size of ceramic to AEC-CP-Fe-bLf NCs from 200 to 350 nm. Sizes of CP NCs (dark blue line), CP-Fe-bLf NCs (red), C-CP NCs (orange), C-CP-Fe-bLf NCs (green) and AEC-CP-Fe-bLf NCs (light blue) were represented as according to light intensity and size of the NCs, respectively. AEC: Alginate-enclosed chitosan; C: Chitosan; CP: Calcium phosphate; Fe-bLf: Iron-saturated bovine lactoferrin; NC: Nanocarrier.

of Fe-bLf only, at all the time-points studied (FIGURE 2D). Oral delivery through AEC-CP-Fe-bLf ensured a sustained release of Fe-bLf over 6–8 h postfeeding, whereas, in the case of Fe-bLf,

the levels declined significantly after 3 h and were not detectable beyond 72 h. Nevertheless, plasma Fe-bLf concentration was prominent in case of AEC-CP-Fe-bLf NCs administered mice,

Table 1. Effect of different constituents and conditions on the preparation of nanocarriers of alginate-enclosed calcium phosphate iron-saturated bovine lactoferrin nanocarriers.

Sample number	Constituents (w/w) [†]	Conditions	Size	Morphology and observations	EE (%)
1	Lf: 5% Chitosan: 10% STPP: 10%	Agitation for 30 min	>1 μ m	Nonparticulate slurry	60
2	Lf: 5% Chitosan: 1% STPP: 1%	Stirring 6000 rpm $\times$ 30 min	>1 μ m	Nonparticulate slurry	60
3	Lf: 5% Chitosan: 1% STPP: 1%	Stirring for 6000 rpm $\times$ 1 h	>1 μ m	Rod-like consistency	73
4	Lf: 10% Chitosan: 1% STPP: 1%	Stirring for 6000 rpm $\times$ 4 h	800 nm–1 μ m	Nonhomogenous particles	80
5	Lf: 10% Chitosan: 1% STPP: 1%	Stirring 6000 rpm $\times$ 6 h	400–450 nm	Nonhomogenous particles	80
6	Lf: 10% Chitosan: 0.1% STPP: 0.1%	Stirring 6000 rpm $\times$ 6 h	250–300 nm	Spherical particles	80
7	Lf: 10% Chitosan: 0.1% STPP: 0.1% Lf: 10%	Stirring 6000 rpm $\times$ 6 h	250–300 nm	Spherical particles	85
8	Lf: 10% Chitosan: 0.01% STPP: 0.01%	Stirring 6000 rpm $\times$ 12 h	200 $\pm$ 25 –250 $\pm$ 25 nm	Spherical particles	80
9	Lf: 10% Chitosan: 0.01% STPP: 0.01% Alginate: 2% w/v	Stirring 6000 rpm $\times$ 12 h	350 $\pm$ 25 nm	Spherical particles	80

[†]With respect to the calcium phosphate core.

EE: Encapsulation efficiency; Lf: Lactoferrin; STPP: Sodium tri-polyphosphate.

with detectable levels of Fe-bLf up to 10 days post-treatment.

As shown in TABLE 2, on oral feeding of Fe-bLf at a dose of 100 mg/kg to mice the maximum concentration of Fe-bLf ($C_{\max}$) could be detected in the blood at 3 min post feeding. By contrast when the same amount of AEC-CP-Fe-bLf NCs was fed to another group of animals the maximum concentration of Fe-bLf was detected in the blood at 6–8 h.

Cell viability & *in vitro* transport of C-CP-Fe-bLf NCs via a Caco-2 cell monolayer

■ Cell viability

Since, the AEC-CP-Fe-bLf NCs were found to be dissolved in the intestinal pH releasing inner C-CP-Fe-bLf NCs, to proceed with these experiments, Caco-2 cells were treated with C-CP NCs (3 mg/ml) carrying Fe-bLf along with same concentration of void NCs without protein and the viability of the cells were measured

compared with Taxol (negative control) and/or untreated cells (positive control) (FIGURE 4A). As indicated in FIGURE 4B, a time dependent cell death by using C-CP-Fe-bLf NCs was observed. For example, an A/I of 35.3 ± 4.0 was observed at the end of 12 h whereas up to 24 h the percentage was increased to 44.7 ± 5.7 . We compared the cell cytotoxicity with LDH release assays and apoptosis with TUNEL assays on Caco-2 cells and normal FHs 74 Int cells, we found significant high cytotoxicity and A/I in the Caco-2 cells when treated with Fe-bLf (1600 μ g/ml optimal concentration to kill cancer cells) (FIGURE 4C). Similarly high cytotoxicity and A/I was observed in the AEC-CP-Fe-bLf NCs treated Caco-2 cells. However there was very little (<5%) cell death and cytotoxicity was present with both treatments for FHs 74 cells. Our control diets with void NCs (free from Fe-bLf) also have very low levels of cytotoxicity and A/I which is comparable to media alone in both Caco-2 and normal FHs 74 Int cells. These results indicated

Figure 2. In vitro and in vivo assays for controlled release of alginate-enclosed calcium phosphate iron-saturated bovine lactoferrin nanocarriers. (A) Controlled release of Fe-bLf from AEC-CP-Fe-bLf NCs was observed by spectroscopic analysis as well as by (i) SDS-PAGE gel retardation assay; (ii) immunoblots indicate the integrity of the protein during the formulation process. Thick bands in the stacking gel indicate bLf mobilized on the NCs, this is evident from the immunoblots. (B) Stability of NCs in pH 1.2 and 7.4 shows an increase of degradation starting from 2 to 4 h in alkaline media, whereas in acidic media the NCs were stable up to 6 h, which was represented in SDS gel as well as western blots. Lane 1: control, lanes 2, 4, 6 and 8 represent alkaline treatment in 1, 2, 4 and 6 h; lanes 3, 5, 7 and 9 represent acidic treatment in 1, 2, 4 and 6 h, respectively. (C) Fe-bLf fragmentation after 48 h treatment with Omnizyme cocktail gastric digestive enzymes. Lane 1: molecular weight marker; lane 2–4: Omnizyme treatment of Fe-bLf for 48, 24 and 12 h, respectively. (D) Concentration of Fe-bLf in mice plasma after a single administration of Fe-bLf and AEC-CP-Fe-bLf NCs by oral gavage (100 mg Fe-bLf/kg bodyweight). Values are represented as mean $\pm$ standard error of the mean ($n = 6$). AEC: Alginate-enclosed chitosan; CP: Calcium phosphate; Fe-bLf: Iron-saturated bovine lactoferrin; NC: Nanocarrier.

Table 2. Pharmacokinetic parameters of iron-saturated bovine lactoferrin and alginate-enclosed calcium phosphate iron-saturated bovine lactoferrin nanocarriers.

Formulation	Fe-bLf dose (mg/kg bodyweight of mice)	C _{max} (ng/ml)	T _{max} (h)
Fe-bLf	100	560 ± 40	3
AEC-CP-Fe-bLf NCs	100	1130 ± 80	8

AEC-CP-Fe-bLf NC: Alginate-enclosed chitosan-calcium phosphate-iron-saturated bovine lactoferrin nanocarrier; C_{max}: Maximum concentration; Fe-bLf: Iron-saturated bovine lactoferrin; T_{max}: Maximum time.

that Fe-bLf free form or nanoformulation is selectively and specifically killing Caco-2 cells and sparing the normal FHs 74 Int cells (FIGURE 4C).

■ Internalization of the NCs

After analyzing the cytotoxicity of the Fe-bLf loaded NCs, the study was focused in finding the real-time functional mechanism of internalization. Rhodamine-labeled C-CP NCs, C-CP-Fe-bLf NCs and AEC-CP-Fe-bLf NCs were used for the quantification of endocytosis percentage of the NCs in Caco-2 cells, using flow cytometry (FIGURE 4D). Caco-2 cells were treated with rhodamine-labeled NCs, for up to 12 h, starting from 1 h. Flow cytometric analysis shows an increase in cellular uptake of void C-CP NCs from $16.65 \pm 0.45\%$ in 1 h to $62.2 \pm 2.4\%$ by the end of 12 h, whereas increase was observed to be prominent in C-CP-Fe-bLf NCs, which was $17.75 \pm 1.16\%$ initially at 1h and increased to $72.2 \pm 7.2\%$ by 12 h. In case of AEC-CP-Fe-bLf NCs the percentage of endocytosis was found to be $16.1 \pm 0.7\%$ at the end of 12 h as compared with $6 \pm 0.35\%$ in 1 h (FIGURE 4E). AEC-CP-Fe-bLf NCs. To determine the qualitative endocytosis, rhodamine-labeled C-CP-Fe-bLf NCs were added to Caco-2 cell monolayers grown in eight-well chamber slides. Cytoskeleton was stained with antiactin antibody and nuclear staining was done with 4',6-diamidino-2-phenylindole (FIGURE 5A). The micrographs indicate cytoplasmic and nuclear localization of C-CP-Fe-bLf NCs. Increased cell death was prominent in the case of cells treated with Fe-bLf-loaded NCs compared with NCs loaded with natural bLf. These results are even evident from immunocytochemical observation for Fe-bLf-specific immunoreactivity in the Caco-2 cell monolayer after treatment with C-CP-Fe-bLf NCs (FIGURE 5B).

■ Transcytosis of AEC-CP-Fe-bLf NCs

Transwell experiment is a good replacement for a real time analysis of *in vivo* situation for the absorption and/or transcytosis of the NCs or drug moieties. We have reported the assay for transcytosis efficacy of commercially available elderly supplement Enprocal [57]. TEER was

measured at regular time intervals after the treatment for 2, 4, 6, 12, 24 and 48 h, to check for any difference compared with initial treatment (0 h). TEER values were found to be unaffected (FIGURE 6A), indicating the NCs does not affect the integrity of monolayer by opening tight junctions. However, there has been a slight decrease in TEER values in monolayers treated with C-CP NCs, C-CP-Fe-bLf NCs was observed at 48 h of treatment, where as monolayers treated with AEC-CP-Fe-bLf NCs have not shown any decrease in TEER values. Amount of relative fluorescence units was measured as a function of transcytosis in both apical and basolateral sides. As shown in FIGURE 6B, an increase in the cumulative release of NCs in the basolateral side, which corresponds to transcytosis of C-CP-Fe-bLf NCs and C-CP NCs from 2 to 48 h compared with AEC-CP-Fe-bLf NCs, was observed indicating an improved transcytosis with decrease in size of NCs.

■ Xenograft colon cancer model

AEC-CP-Fe-bLf NCs were supplemented to the AIN 93G diet in such a way that the final nanoformulated diet has 1.2% of Lf protein of the total protein casein, present in the control AIN 93G diet. The nanoformulated diet with Fe-bLf loading was started 7 days before cancer cell injections in the prevention xenograft colon cancer model. As shown in the FIGURE 7A, mice that were fed with Fe-bLf-loaded AEC-CP-Fe-bLf NCs diet did not develop any tumor (n = 6). However, all mice in the normal control diet developed tumors of approximately 1 cm³ volume (FIGURE 7A). Taxol in the nanoformulation, when given orally to the tumor-bearing mice, regressed the tumors while normal single injection of Taxol delayed the growth of tumors. 65% of mice fed with normal Fe-bLf (not nanoformulated) diet, developed tumors, while the rest of the mice were unable to develop tumors (FIGURE 7A).

In vivo antitumor efficacies of AEC-CP-Fe-bLf NCs and AEC-CP-Taxol NCs were tested as a post-treatment on the established tumors (treatment model) and represented with respect to tumor volumes of Caco-2 tumor-bearing mice. Other combinations such as void AEC-CP

Figure 3. The *in vitro* release profile of bovine lactoferrin. Release profiles of Fe-bLf in various buffers at (A) low pH 1.5 (as observed in the stomach) to (B) pH 8 (as in the intestine) were analyzed. Percentage cumulative release of Fe-bLf was low in acidic buffer, whereas the amount of protein released from the NCs in the alkaline pH was increased. Data represents mean $\pm$ standard error of the mean; experiments were repeated three times independently with similar results. AEC: Alginate-enclosed chitosan; CP: Calcium phosphate; Fe-bLf: Iron-saturated bovine lactoferrin; NC: Nanocarrier.

Figure 4. Cellular apoptosis and endocytosis studies of chitosan-enclosed calcium phosphate iron-saturated bovine lactoferrin nanocarriers. (A) Viability of Caco-2 cells were measured by Annexin-V assay after treatment with C-CP-Fe-bLf NCs using Taxol® as a negative control. The results obtained indicate there was no cellular apoptosis with void C-CP NCs, but an increase in cytotoxicity in C-CP-Fe-bLf NCs and Taxol-treated cells, as indicated by relative fluorescence observed using flow cytometry measurements.

(B) Representative histogram for the percentage of apoptosis after treatment with C-CP-Fe-bLf NCs. (C) Cell cytotoxicity and cell death were determined by lactose dehydrogenase release assay and cell death (apoptosis) by terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling assay of colon cancer cells and compared with normal FHs 74 Int cells following treatment with 1600 µg/ml (optimal dose to cause cancer death) of Fe-bLf and AEC-CP-Fe-bLf NCs nanoformulations. Cells were treated for 24 h with different nanoformulations and stained by terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling analysis for apoptotic cells. Cell death is shown here in terms of apoptotic index (% apoptosis) and lactose dehydrogenase release (% cytotoxicity). All treatments were performed in triplicate and assay was repeated three times independently with similar results. The mean for representative experiment was calculated and presented as a mean ± standard error of the mean values. *Indicates a highly significant $p < 0.001$ value from the normal control cell lines and with media only. (D) Flow cytometric analysis shows an increase in internalization profiles of fluorescently labeled void C-CP NCs, C-CP-Fe-bLf NCs and AEC-CP-Fe-bLf NCs. (E) Histogram corresponding to the flow cytometric analysis shows an increase in internalization of NCs from 20 to 80% with time, from 1 up to 12 h; AEC-CP-Fe-bLf NCs have showed not much increase compared with other combinations. Data represent mean ± standard error of the mean; experiments were repeated three times independently with similar results.

AEC: Alginate-enclosed chitosan; C: Chitosan; CP: Calcium phosphate; Fe-bLf: Iron-saturated bovine lactoferrin; FHs 74 Int: Human fetal small intestine; NC: Nanocarrier.

NCs, and Taxol were taken as controls (FIGURE 8A). Tumors (~500 mm³) were developed in mice fed with the control AIN 93G diet within 45 days

after the injection of 10⁶ cancer cells. Treatments of AEC-CP-Fe-bLf NCs and AEC-CP-Taxol NCs were given along with void AEC-CP NCs,

and intratumoral injection of Taxol. In the case of the positive control group (intratumoral injection of Taxol) tumors regressed to half of their volumes in 2 weeks after the injection, but continued to grow thereafter. Mice receiving void AEC–CP NCs, showed no regression of tumor volume. By the end of the treatment regime, approximately 1000 mm³ tumors were observed in the case of the control diet, void NC-fed mice and intratumoral Taxol-treated groups (FIGURE 8A). More interestingly, a dramatic reduction of tumors within a time period of 10 days was seen in case of mice groups that received an oral diet of AEC–CP–Fe-bLf NCs and AEC–CP–Taxol NCs. After 45 days the tumors in these groups regressed completely, though nanoformulated Taxol showed quick response.

■ Biodistribution studies of Fe-bLf & AEC–CP–Fe-bLf NCs upon oral feeding

Apart from other physiochemical properties, all drug delivery systems intended for oral administration need to bypass two main barriers: the enzymatic barrier of the mucosal surfaces and the gut physical barriers [63,64]. Therefore, a simple experiment was performed to establish whether AEC–CP–Fe-bLf NCs following oral feeding can cross the mucosal barriers and retain Fe-bLf, by examination of Fe-bLf concentration in different tissues. Also, since the strategy employed in the administration of AEC–CP–Fe-bLf NCs was to ensure passive targeting followed by uptake of the NCs in the cancer cells, based on cell surface receptors specific for Fe-bLf, Fe-bLf concentration in various tissues could give a quantitative insight of the NCs internalization. As seen in FIGURE 7B a higher amount of Fe-bLf was detected in the tumor tissue, followed by the bone, blood and intestine. Organs of the reticuloendothelial system such as the liver, showed same amount of Fe-bLf as that of intestine. Nearly half the amount of Fe-bLf as that of the liver was observed in the

spleen and kidney. In order to detect the presence of NCs in the systemic circulation, blood samples that were collected 5 days postadministration of rhodamine-labeled NCs were analyzed. As shown in FIGURE 7C, a strong fluorescent signal was detected in the blood of AEC–CP–Fe-bLf NC-administered mice. These results show the positive influence of chitosan on transport across mucosal surfaces along with protecting Fe-bLf from hydrolytic and proteolytic degradations.

■ Presence of Fe-bLf on tumors & other body tissues

In order to assess the internalization of AEC–CP–Fe-bLf NCs in the tumor tissues, immunohistochemical observation for the Fe-bLf positivity in the tumor tissues was carried out. The goat antibody specific for bLf was used for the detection of exogenous Fe-bLf and further probed with anti-goat HRP-conjugated IgG secondary antibody. Immunohistochemical observations using 3,3'-diaminobenzidine substrate indicated the presence of Fe-bLf (positive immunoreactivity) in the tumor tissues from the treatment efficacy group (FIGURE 8B). The control group showed no immunoreactivity. These observations imply the tumor site bioavailability of nanoformulated Fe-bLf. As an additional means of the confirmation, iron levels in the tumor tissues were analyzed by Prussian blue color staining. A significant amount of Prussian blue staining indicating the presence of iron due to Fe-bLf-loaded NCs, was still clearly observed around the region of the serial tumor tissue slices, where Fe-bLf-positive immunostaining was observed (FIGURE 8C). Additionally, studies including measurement of bodyweight, organs of mice indicated normal weight of these organs. Furthermore, blood hematological profile of the control and experimental mice with nanodiet showed no morphological abnormalities in RBCs and significantly high increases in RBC counts, serum iron and serum calcium levels (TABLE 3).

Table 3. Weight of mice, organs, serum iron, serum calcium and blood hematological profile of control and experimental mice with nanoformulated diet and control diets.

Organ/cells	Fe-bLf	Taxol®	AEC–CP–Fe-bLf	Control nano diet
Body (g)	22.5 ± 5.6–28.5 ± 6.5	22.3 ± 4.5–24.0 ± 5.8	22.5 ± 5.1–26.5 ± 6.0	23.2 ± 4.5–32.0 ± 5.8
Spleen (mg)	95.5 ± 11.2*	72.0 ± 5.8	96.5 ± 10.2*	80.0 ± 10.2
Thymus (mg)	80.5 ± 12.4	70.5 ± 8.9	81.5 ± 15.5	72.5 ± 8.5
Liver (mg)	1200 ± 72.2	1130.5 ± 60.4	1234 ± 75.8	1160.5 ± 65.5
Red blood cells (×10 ⁶ /mm ³)†	8.2 ± 2.5**	2.1 ± 1.5	9.5 ± 2.5**	4.5 ± 1.5
Serum iron (µg/g)	175 ± 25**	95 ± 15	215 ± 35**	155 ± 20
Serum calcium (mg/dl)	8.9 ± 0.8	6.5 ± 0.5	12.9 ± 1.2**	8.5 ± 0.8

†Mean values of red blood cell counts were recorded in blood samples collected directly from the heart at the time of autopsy.

*p < 0.05 denotes statistical significance; **p < 0.001 denotes results that are highly significant.

AEC–CP–Fe-bLf: Alginate-enclosed chitosan–calcium phosphate–iron-saturated bovine lactoferrin; Fe-bLf: Iron-saturated bovine lactoferrin.

Figure 5. Visualization of endocytosis. (A) Confocal microscopy shows the cytoplasmic and nuclear internalization of rhodamine-labeled NCs in the Caco-2 cells. Actin filaments are stained green, NCs are stained red and the nuclei are stained blue. **(iii)** Control cells show no red fluorescence; rhodamine-loaded C-CP-Lf NCs and C-CP-Fe-bLf NCs shows fluorescence corresponding to the rhodamine. **(B)** Immunocytochemistry using bLf-specific antibodies further supports cytoplasmic and nuclear localization of Lf NCs. **(i)** Control cells show no reactivity with 3,3'-diaminobenzidine. **(ii)** C-CP-Lf show internalization of NCs with moderate cell death compared with **(iii)** C-CP-Fe-bLf NCs with increased cell detachment due to cell death. Arrows indicate nuclear localization. Images were seen under **(i & iii)** 63x and **(ii)** 100x oil immersion objectives.
C: Chitosan; CP: Calcium phosphate; DAPI: 4',6-diamidino-2-phenylindole; Fe-bLf: Iron-saturated bovine lactoferrin; FITC: Fluorescein isothiocyanate; NC: Nanocarrier.

■ NK cells have no role in antitumor activities

In vivo, the NK cell depletion assay through injection of anti-NK1.1 monoclonal antibodies in the nude mice, revealed no further increase in

the tumor sizes in antibody-treated group of mice. Mice receiving AEC-CP-Fe-bLf NCs when injected with anti-NK1.1 monoclonal antibodies showed no visible tumors (FIGURE 9). This indicates that NK cells have no role in the antitumor activity observed in case of AEC-CP-Fe-bLf NCs.

Discussion

In order to be able to overcome the side effects of present-day's toxic chemotherapeutics, using natural source-derived nontoxic, anticancer proteins to target solid tumors, can be a better approach in the future. To date, a number of anticancer and/or antiangiogenic proteins have been identified and tested successfully for the development of effective anticancer treatments [5]. Various tumor suppressor peptides and antagonists to antiapoptotic genes including survivin antagonists and other antiangiogenic peptides such as the RGD peptide have been effectively shown to perk up antiangiogenic and/or antiapoptotic tumor therapy [5,64–66]. However, these molecules are very unstable and show rapid renal clearance in rodents and humans [67,68]. NC-based encapsulated system to deliver anticancer peptide and proteins could be a promising strategy for the better protection and controlled delivery of the protein/drug molecules to the target site [21]. In recent studies, anticancerous protein targeting to the tumor by using polymeric nanoparticles has been reported [15,69–71]. Nevertheless, many of the studies aimed at the development of a parenteral administration are invasive procedures and impose painful regimes. Therefore, we aimed to develop a nanoplatform that could carry anticancer proteins and drugs for oral administration, so that patient compliances against the painful intravenous infusions or any other invasive procedures in the clinic could be effectively reduced. We have demonstrated the antitumor efficacy of a novel nanoformulation of anticancer protein Fe-bLf (AEC-CP-Fe-bLf NCs), for oral administration. The nanoformulation was designed for protection against pH variations, microflora, proteolytic and gastric enzymes in the intestinal milieu, thereby increasing Fe-bLf's bioavailability at the tumor sites.

Iron-saturated (100%) form of natural Fe-bLf was incorporated into the nanoparticles to improve its oral bioavailability. Lf, a natural secretory-defence protein present in bodily fluids is a single chain approximately 80 kDa metal binding glycoprotein [46]. It is a multifunctional protein and plays a key role in iron absorption in the intestine, promotes intestinal cell growth and protects against microbial infections [47], alongside acting as an anticancerous agent [50–53]. Through receptor mediated endocytosis, Fe-bLf could help in increased intestinal absorption of the inner

Figure 6. Transcytosis studies. (A) TEER measurements for a time period of 48 h showed no significant difference from initial value than after when the Caco-2 cell monolayer was treated with AEC-CP-Fe-bLf NCs. From the graph, a slight decrease in the TEER, in the case of monolayers treated with C-CP NCs and C-CP-Fe-bLf NCs at the end of 48 h, can be observed. (B) Transcytosis profiles of C-CP NCs, C-CP-Fe-bLf NCs and AEC-CP-Fe-bLf NCs for a time period of 2–48 h. The graph indicates the cumulative release of rhodamine-labeled NCs in the basolateral side. Data represents mean $\pm$ standard error of the mean. Experiments were repeated three times independently with similar results. AEC: Alginate-enclosed chitosan; C: Chitosan; CP: Calcium phosphate; Fe-bLf: Iron-saturated bovine lactoferrin; NC: Nanocarrier; TEER: Transepithelial electric resistance.

core comprising C-CP-Fe-bLf NCs – released after degradation of alginate outer layer. The NC-entrapped protein Fe-bLf can be protected in gastric lower pH (acidic) milieu in the stomach. The predominant unionized form of alginate forms the matrix around the inner core in such lower pH [72]. When the pH of the milieu changes to the alkaline range in the intestine, sodium alginate is formed releasing the contents present inside the alginate layer [73].

We have shown earlier the augmentation of chemotherapy with non-nanoformulated Fe-bLf, when given in the AIN 93G diet, prior to chemotherapy [53,201] and eradication of the large tumors in combination with anticancer drugs. However, 20% iron-saturated or apo form of bLf were found to be ineffective in eradicating these tumors, owing to their high degradation compared with iron-saturated form of the bLf. In the present study, omnizyme

Figure 7. Antitumor efficacy of the nanocarriers and biodistribution. (A) Pretreatment of AEC-CP-Fe-bLf NCs in comparison with the void AEC-CP NCs (no Fe-bLf) or mice treated with control diet or intratumoral injection of Taxol® or a non-nanoformulated Fe-bLf protein are shown in the graph (prevention model). Upon subcutaneous challenge with the 2×10^6 colon cancer cells, mice that were given a diet containing void NCs and control diet developed tumors (~1 cm) in 6 weeks, whereas Fe-bLf-treated mice showed a notable regression in tumorigenesis; AEC-CP-Fe-bLf NC-treated mice showed complete abrogation of tumorigenesis. Data represents mean $\pm$ standard error of the mean. Experiments were repeated three times. **(B)** Biodistribution study of Fe-bLf and AEC-CP-Fe-bLf NCs after oral administrations in diet. Fluorescent signals of tissue extracts after 24 h of oral administration were analyzed. Nanocarriers were labeled with coumarin-6 (60 mg/kg). The mean for the representative experiment was calculated and presented as mean $\pm$ standard error of the mean values.

AEC: Alginate-enclosed chitosan; CP: Calcium phosphate; Fe-bLf: Iron-saturated bovine lactoferrin; NC: Nanocarrier.

Figure 7. Antitumor efficacy of the nanocarriers and biodistribution (cont.). (C) Analysis for the rhodamine-labeled NCs in the blood of mice are represented in the figure. Blood samples from AEC-CP-Fe-bLf treated mice (**i-iii**) were positive for rhodamine fluorescence compared with control (**iv-vi**) samples.

AEC: Alginate-enclosed chitosan; CP: Calcium phosphate; Fe-bLf: Iron-saturated bovine lactoferrin; NC: Nanocarrier.

digestion of the Fe-bLf samples, over varying time periods starting from 12 to 48 h, showed complete resistance of the Fe-bLf up to 24 h of the digestion, after which it started to fragment from 24 to 48h. Thus, this study was aimed at the incorporation of gut enzyme-resistant Fe-bLf in NCs for improving its anticancer efficacy by increasing its bioavailability at the tumor sites through enhanced permeability retention effect (FIGURE 10).

The nanoplatform was developed to carry Fe-bLf embedded in the core, formed by ceramic calcium phosphate and chitosan. Dynamic light scattering spectrometric data represents the formation of layers, one over the other, throughout the process of final AEC-CP-Fe-bLf NCs formation. The range of Fe-bLf-loaded NCs was found to increase from 207 ± 39.1 nm for ceramic core to 350 ± 25 nm after the formation of final AEC-CP-Fe-bLf NCs (TABLE 1). SEM indicated a stable spherical morphology of AEC-CP-Fe-bLf NCs. In addition, alginate and chitosan being biodegradable polymers, solubilise in aqueous environment and release the loaded Fe-bLf in a controlled manner as indicated by SDS-PAGE (FIGURE 2B). Retardation of NCs in the stalking gel could be attributed to their large molecular weight of the NCs, compared with proteins, as well as their net positive surface charge. Presence

of Fe-bLf-specific bands in an immunoblot indicates the protection and presence of the Fe-bLf, thereby confirming the success of formulation procedure. The controlled release experiments using SDS-PAGE gel retardation assays and subsequent immunoblots indicated the controlled release of Fe-bLf from AEC-CP-Fe-bLf NCs.

Residence time of a drug through the GI tract is predicted to be in between 9 and 12 h. Thus, our nanoformulated AEC-CP-Fe-bLf NCs should resist the enzymatic and pH variations posed by GI tract, in order to transport protein to cancer cells. Taking in to consideration that maximum transit time of a drug in stomach and intestine is 4 h, we have determined the stability of AEC-CP-Fe-bLf NCs in acidic media (HCl: pH 1.6), alkaline media (PBS: pH 7.4) and plasma for a time period of 6 h and longer. The results indicate the release of C-CP-Fe-bLf nanocores after the dissolution of outer alginate layer from AEC-CP-Fe-bLf NCs from 4 to 6 h in alkaline medium, which is similar to the intestinal milieu. Absence of prominent bands of Fe-bLf in HCl-treated NCs for a time period of 1–4 h indicates the intactness of AEC-CP-Fe-bLf NCs in acidic media due to the presence of encapsulating layer of alginate known to provide stability in the acidic conditions of the stomach pH [72].

Figure 8. Tumor inhibition and nanocarrier internalization studies. (A) Nanoformulated AEC-CP-Fe-bLf NCs diet inhibits tumor growth without causing any toxicity. Effects of nanoformulated Fe-bLf and Taxol®-loaded NCs (AEC-CP-Taxol NCs or AEC-CP-Fe-bLf NCs) or normal diet are shown in xenograft colon cancer mouse model when fed in the established tumors (treatment model) in tumor-bearing mice with Caco-2 cells injection. Taxol was injected in established tumors, while AEC-CP-Taxol NCs were given orally. It is evident from the figure that AEC-CP-Taxol NCs completely regressed the tumors followed by AEC-CP-Fe-bLf NCs. Taxol intratumoral injection reduced the tumors only for a period of 2 weeks and the tumor growth was observed thereafter. Void NCs and control AIN-93G diets did not show any effect on the established tumors. Data represents mean $\pm$ standard error of the mean. Experiments were repeated three times. **(B)** Immunohistochemical localization of AEC-Fe-bLf NCs with antibovine lactoferrin antibody is shown in **(i & ii)** control versus NC-treated mice, **(iii & iv)** represent Prussian blue staining for the tumor tissues to confirm Fe-bLf internalization of the tumor tissues. Images are represented as observed under 100 $\times$ oil immersion objective. AEC: Alginate-enclosed chitosan; CP: Calcium phosphate; Fe-bLf: Iron-saturated bovine lactoferrin; NC: Nanocarrier.

Discrete bands after 4 h treatment in the alkaline media showed the release of Fe-bLf from the C-CP-Fe-bLf NCs (FIGURE 4B). Western blot with bLf-specific antibody revealed that the Fe-bLf released from NCs in the alkaline medium was in its intact form as natural and as that of the non-nanoformulated Fe-bLf. Thus, alkaline pH sensitivity of the alginate gel, as discussed above, has helped the protection of the C-CP-Fe-bLf NCs in the GI milieu [74]. Moreover, the ability of nanoformulated Fe-bLf to resist the omnizyme treatment for longer time intervals (48 h) indicate the released Fe-bLf would have survived during its residence time in the gut.

Furthermore, *in vitro* cellular internalization assays for C-CP-Fe-bLf NCs by Caco-2 cells, shows internalization of NCs in 1–12 h. Flow cytometric analysis indicate increase in uptake of the C-CP-Fe-bLf NCs (without an alginate layer) by cells compared with void C-CP NCs (without Fe-bLf) or AEC-CP-Fe-bLf NCs, this can be explained on the basis that C-CP-Fe-bLf NCs have exposed epitopes of Fe-bLf, which may help in improved internalization due to the presence of Lf cell surface receptors (LFR) and probably by receptor-mediated endocytosis. It has been extensively investigated that the endocytosis of bLf is through LfR [73,75]. In this regard, LFRs and low-density lipoprotein (LRP) receptors have attracted the attention of many researchers in the recent past [76–78]. LRP receptor-specific antibodies were used to show abrogation of bLf internalization in osteoblasts [79] and human fibroblast cells [80]. Mitogen-activated lymphocytes were found to overexpress LfR [81]. More recently, siRNA to LfR was used to confirm the internalization of bLf through LfR using clatherin-mediated endocytosis in Caco-2 cells [75,82]. Thus, the Fe-bLf-loaded NCs might probably undergo internalization more preferably based on the clatherin-mediated endocytosis using LfR and/or LRP receptors.

In the *in vivo* stability and biodistribution assays, we have seen that the concentration of Fe-bLf detected in the blood/plasma was significantly higher in mice fed with nanoformulated AEC-CP-Fe-bLf NCs, than those administered with an equimolar concentration of non-nanoformulated Fe-bLf only, at all the time-points studied (FIGURE 2D). Oral delivery of Fe-bLf through AEC-CP-Fe-bLf NCs ensured a sustained release of Fe-bLf over 6–8 h postfeeding, whereas, in case of Fe-bLf, the levels declined significantly after 3 h and was not detectable beyond 72 h. However,

Figure 9. Natural killer cells have no role in antitumor activity of alginate-enclosed chitosan–calcium phosphate–iron-saturated nanocarriers.

Depletion of NK cells in the nude mice by using anti-NK1.1 monoclonal antibodies showed no inhibition or growth of tumorigenesis using the nanoformulated Fe-bLf diet. This showed that regulation of a tumor is purely based on the anticancerous nature of Fe-bLf. Control tumors in nude mice have shown the same amount of tumor volume as that of NK cell antibody administered mice, showing that there is no effect of NK cells on tumorigenesis. Data represent mean $\pm$ standard error of the mean. Experiments were repeated three times.

ab: Antibody; AEC: Alginate-enclosed chitosan; C: Chitosan; CP: Calcium phosphate; Fe-bLf: Iron-saturated bovine lactoferrin; NC: Nanocarrier.

the high levels of Fe-bLf were present in plasma after 72 h, and even after 10 days low levels of Fe-bLf were detected in case of animals receiving NCs in a separate experiment. This showed an interesting result with the sustained release of Fe-bLf from the AEC-CP-Fe-bLf NCs present in diets.

Assessment of *in vivo* antitumor efficacy of nanocapsulated Fe-bLf in xenograft colon cancer mice model has revealed a highly significant antitumorigenicity in our prevention studies; while void and normal diets were unable to inhibit the tumorigenesis (FIGURE 7A). Similarly *in vitro* results with Caco-2 cancer cells showed an enhanced anticancer efficacy with higher A/I and cell cytotoxicity was observed after treatment with Fe-bLf-loaded NCs. We have previously demonstrated the presence of Fe-bLf at the Peyer's patches and few tumor sites/cells, in the mice fed with non-nanoformulated Fe-bLf supplemented AIN-93G diet [53,201]. Our present results showed that the Fe-bLf is resistance to omnizyme, cocktail gastric proteolytic enzymes (FIGURE 2C). These results confirm that Fe-bLf is resistant to gastric proteolytic enzymes due to the presence of high levels of iron saturation

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Figure 10. Schematic illustration of the proposed mechanism of alginate-enclosed chitosan-calcium phosphate-iron-saturated bovine lactoferrin nanocarrier internalization and action.

(A) The alginate coating of orally administered AEC-CP-Fe-bLf NCs is degraded in the alkaline environment offered in the small intestine and the alginate coating free C-CP-Fe-bLf NCs enter the blood circulation via endocytosis and/or transcytosis (B). (C) C-CP-Fe-bLf NCs are released in the tumor site by making use of the enhanced permeability retention effect. (D) Furthermore, uptake of C-CP-Fe-bLf NCs in to the cancer cells is based on oligosaccharide and/or lactoferrin receptor-mediated endocytosis.

AEC: Alginate-enclosed chitosan; C: Chitosan; CP: Calcium phosphate; Fe-bLf: Iron-saturated bovine lactoferrin; NC: Nanocarrier.

and further got internalized through gut and transported to the tumor sites [55]. We believe that being more bioavailable, Fe-bLf may have been more effective in this study, which is why no tumors were developed in mice fed with nanoformulated Fe-bLf. However, xenograft nude mice in the present study do not have immune cells and so do not have Peyer's patches. Thus the route of transportation of Fe-bLf is through endocytosis or LRP- and/or LfR-mediated internalization. Moreover, nanoformulated Fe-bLf seems to follow an additional internalization mechanism by directly invading the tumor vasculature, which needs to be further elucidated (FIGURE 10). We have also shown through the immunohistochemical observations, the bLf-specific immunoreactivity in the tumor tissues, which was confirmed further for the presence of iron using Prussian blue staining. This shows the tumor-specific internalization/delivery of Fe-bLf NCs. The anti-bLf antibody used in this study was specific to bLf, thus, ruling out any crossreactivity of human and mice Lfs. The presence of strong Fe-bLf specific immunoreactivity in tumor tissue sections from the treatment efficacy group (fed to mice with ~500 mm³ tumors) further proved the efficacy of internalization or transport of Fe-bLf to the tumor tissues following oral feeding. Serial tumor sections showed a strong reaction with the Prussian blue staining for Fe³⁺ in the tumor cells (FIGURE 8D). These results thus confirm the presence of Fe-bLf at the tumor site.

bLf and Fe-bLf was found to exert *in vitro* and *in vivo* antitumoral activities, through several suggested mechanisms such as inhibition of tumor cells proliferation [83], regulation of NK cells activity and inhibition of VEGF-mediated angiogenesis [53,84,85] and enhancement of apoptosis [86]. It was shown that bLf enhances antiapoptotic activity by modulation of apoptosis-related genes such as TNF- α , Bcl-2, Bcl-xL, Bax and Bid in the colon of azoxymethane-induced colon cancer model in rats [87]. In addition to this, bLf also seems to downregulate inhibitors of apoptosis protein family members known to play an important role in the inhibition of apoptosis [46,66]. Recently, we found that Fe-bLf downregulates the gene expression of survivin [46], an antiapoptotic protein, which is known to upregulate the growth of cancer cells and is always overexpressed in all tumor types [10,88–93]. It is also upregulated as the size of tumors grow [89,90,88–93]. This novel finding needs further investigation and also raises a possibility of targeting the apoptotic pathway

to induce cell death due to downregulation of survivin expression after treatment with Fe-bLf in cancer cells, which always overexpress survivin protein. Fe-bLf did not show any toxicity to normal body cells, which have normal/very low expression of the survivin.

In our previous study, we observed the anticancer effect of Fe-bLf, particularly owing to the significantly increased numbers of immune subsets of CD4⁺, CD8⁺ T cells, dendritic cells and NK cells in the spleen, as well as in the intestinal mucosal sites in the wild-type C57 BALB-C mice [53]. The results of the this study implies that immune cells including NK cells have a role in tumor rejection through antitumor immunity in the mice. In the present study, we have used nude mice (with no T lymphocytes but known to have NK cells) for the development of standard xenograft colon cancer model. Therefore, considering that the observed antitumor effects of the present study could be via NK immune cells, the objective of NK-cell depletion experiment was deemed important. In the present xenograft colon cancer model, we depleted NK cells in nude mice by the administration of anti-NK1.1 monoclonal antibodies (FIGURE 9). No further growth of tumors was observed in the absence of NK cells (depleted group of mice), indicating that nanoformulated Fe-bLf targets tumor cell growth through non-NK cell-mediated mechanisms. This needs further investigation, as the regression of tumors in nude mice devoid of NK cell response indicates possible involvement of some other nonimmunological targets of Fe-bLf inducing promising antitumor effects as observed in this study.

In order to validate the treatment based antitumor efficacy of the nanoformulations, AEC-CP-Fe-bLf NCs and AEC-CP-Taxol NCs were orally administrated when tumor volume reached approximately 500 mm³. Interestingly, nanodiets with Fe-bLf and Taxol were able to completely abolish the established tumors, while tumors of control or control nanodiet-fed mice continued to grow (FIGURE 8A). Increase in antitumor efficacies of nanoencapsulated Fe-bLf and Taxol, could be attributed to the enhanced GI resistibility and more antitumor bioavailability offered by nontoxic AEC-CP NCs either to the entrapped Fe-bLf or Taxol. These results further indicate the use of AEC-CP-Fe-bLf NCs for efficient delivery of the anticancer proteins with the enhanced efficiency compared with non-nanoformulated Fe-bLf protein.

Measurement of bodyweight and other vital organs of mice indicated normal weight of these organs, when fed with nanoformulated diets. Experimental mice fed with nanoformulated Fe-bLf diets showed no morphological abnormality in RBC morphology, increases RBC counts, serum iron and serum calcium levels compared with control and Taxol diet fed mice, which proves the safe and biocompatible nature of NCs (TABLE 3).

Iron metabolism in the body is tightly controlled as there is no specific mechanism for iron excretion and is influenced by many nutritional factors [94]. Lf plays a crucial role synergistically in combination with other iron-binding proteins such as transferrin, in maintaining iron homeostasis [95]. Highly significantly elevated iron levels in the AEC-CP-Fe-bLf NC treatment group was observed compared with the control fed group. This increase in iron levels in the serum supports the novel nutraceutical benefits of using Fe-bLf in a nanoformulation for iron deficient patients. Since calcium is one of the ingredients in our NCs, this could question the toxicity and/or inhibition of iron uptake by using these NCs. Moreover, it was reported that iron absorption could be inhibited by calcium in nonheme and heme iron forms during the intestinal absorption [96]. In this regard, it has been reported that the iron uptake decreased only if double the amount of calcium is present in a meal [97]. However, in our study we found that there was a significant increase in the levels of both these micronutrients (iron and calcium) in the serum, showing that the levels of calcium in our NCs did not interfere or alter the absorption of free iron from the diet. We feel that with the help of endocytosis through LfR and/or LRP receptors, these Fe-bLf-loaded NCs help in absorption of more iron and this may aid in enhancing the iron uptake in the iron deficient mice/patients, without interfering with the absorption of calcium. Since serum levels of iron and calcium were found to be under the recommended limit in treated animals, this indicates that the iron and calcium phosphate form of the NCs are metabolized and assimilated inside the body. Thus, our NCs can be used safely for iron- and calcium-deficient patients or patients on anticancer drugs which deplete the calcium and iron absorption [48,53]. With the inclusion of 1.2% (w/w) Fe-bLf in the diet, increases in the iron level in the nanoformulated diet was found to be increased by a fraction (i.e.,

from 0.0053 to 0.0069%). Furthermore, according to the formulation of the AIN 93G diet, it contains 0.107% calcium, which again increases by a fraction to 0.111% after the addition of NCs. Thus, the concerns related to the toxicity of the calcium phosphate NCs could be eliminated. Furthermore, as shown in FIGURE 4C, AEC-CP-Fe-bLf NC treatment led to the selective and specific apoptosis and cytotoxicity towards cancer cells (Caco-2) with no effect on normal intestinal FHs 74 Int cells (FIGURE 4C). This reveals the nontoxic nature of the prepared NCs. The absence of any nanotoxicity as a result of oral administration of AEC-CP-Fe-bLf NC formulation was later confirmed in the *in vivo* study by measuring the bodyweight and the weight of various organs, as represented in TABLE 3, and also by routine health checkup and normally displayed behavior of mice fed on nanodiets. In addition, we did not observe any signs of inflammation or pathological abnormalities in the collected tissues of NC-fed mice [KANWAR J ET AL., UNPUBLISHED DATA]. This again confirms that the nanoformulated diet is not toxic to normal body cells. bLf is not immunogenic to humans [98]. Human Lf is found to be a 69% similar to bLf [84]. However, bLf never produces humoral and cellular immune responses in humans and mice [99].

Many toxic anticancer chemotherapeutics, including presently used Taxol, are cytotoxic or impair hematopoiesis and hence reduce RBC count and usually cause iron deficiency [100–102]. The data presented here indicated that Fe-bLf or nanodiets with Fe-bLf have the potential ability to reverse the RBC count in the immunocompromised mice. This data is in accordance with previous studies of Fe-bLf's role in accelerating and reconstitution of lymphopoiesis, myelopoiesis and humoral immunity [53,103,104,201]. Oral administration of Lf has been shown to increase hemoglobin and total serum iron in pregnant women [105,106]. Taking this fact into consideration, administration of AEC-CP-Fe-bLf NCs was found to be safe, as confirmed by the aforementioned bodyweight index, RBC count and serum iron and calcium levels in control versus treated mice.

It has been reported that surface modification with chitosan can enhance the uptake of NCs by cells [5,107]. In the present study, we demonstrated the increase in transcytosis of NCs containing chitosan and Fe-bLf. Here we observed that there was no significant difference

in TEER values before and after treatment until 48 h. A previous study has reported a decrease in TEER values after treatment of Caco-2 cell monolayer with chitosan NCs [29]. Furthermore, the authors described that the decrease in TEER values was due to recessively opening tight junctions by chitosan [29]. However, we observed a different scenario, wherein no differences in TEER values were observed. This could possibly be because of a different nanoformulation used in the preparation of these NCs. We have primed chitosan film over the nanocore by using a poly-anion, $\text{Na}_3\text{P}_3\text{O}_{10}$, which would react with reacting amine groups of chitosan, because of which these NC proteins open the tight junctions in a reversible manner causing no damage to the cell membrane, as reported previously [108]. Combination of mucoadhesion and a transient opening of the tight junctions of chitosan were also reported as a transport mechanism for chitosan in the mucosal cell membrane [109]. In the present study, Caco-2 cells were grown over a period of 24 days in the transwell chambers in a monolayer resembling that of the human intestinal epithelium gut wall and known to express mucin [110,111]. Nanoparticles are internalised through the transcytosis because chitosan is known to be absorbed by reversible opening of tight junctions [108,109]. It has been suggested that positive charges on chitosan and the negative charges on the sialic-acid groups in mucin, located on the gut epithelial cells, may have provided a prolonged contact time between the Fe-bLf/Taxol drug-loaded NCs and the absorptive surface, promoting its sustained release and slow absorption over a period of time in the gut. It can be inferred that the AEC-CP-Fe-bLf NCs reach the tumor site along with Fe-bLf via an enhanced permeability retention effect, similar to other studies [112]. In addition to this, biodistribution studies confirmed that the NCs were also found in blood, tumor and other tissues (FIGURE 7B & 7C). Thus, the AEC-CP-Fe-bLf NCs could enter the blood circulation without affecting the gut epithelial cell layer. In total, we have proposed a mechanism for the localization, internalization and transportation of these NCs as described in FIGURE 10. After being delivered orally, the NCs reach the intestine and are protected from the gastric proteolytic enzymes and microflora in the gut, as well as from variations in pH in the GI tract. The alginate covering is degraded in the intestinal alkaline pH, releasing C-CP-Fe-bLf NCs (free from alginate) into the main blood stream.

Due to high vascularisation and blood flow at the tumor sites, these NCs internalize into the tumor cells. The finally formed endosomes containing C-CP-Fe-bLf NCs then become endolysosomes, which upon further degradation release the anticancer Fe-bLf or Taxol inside the tumor cells, to induce apoptosis.

Conclusion

In this study, we developed and validated the preclinical efficacies of novel nano oral formulations for the delivery of the natural anticancer Fe-bLf protein and compared with anticancer chemotherapeutics such as Taxol. In order to protect Fe-bLf and increase its bioavailability to the tumor sites, various biodegradable polymer layers were integrated on to the ceramic cores, which was confirmed and characterized by DLS spectrometry and SEM. A number of *in vitro* and *in vivo* assays performed indicate the potentials of use of these NCs for the controlled release of Fe-bLf or Taxol and their protection from the challenging gut environment. The NCs undergo endocytosis possibly by utilizing different LfR or LRP receptors. AEC-CP-Fe-bLf NCs were shown to enhance the bioavailability and metabolic stability of Fe-bLf. These NCs prevented the degradation of entrapped Fe-bLf in mouse plasma in comparison with non-nanoformulated Fe-bLf. Oral delivery of these NCs to normal mice showed that they can cross the mucosal barrier undamaged, and confocal microscopy detected the AEC-CP-Fe-bLf NCs in the blood/plasma after feeding. Oral administrations of AEC-CP-Fe-bLf NCs showed very high anticancer activities to colon tumors when tested as a proof-of-concept in a xenograft colon cancer model. We believe that the improved bioavailability of Fe-bLf through the nanoformulation resulted in the prevention of tumor development, and in the treatment model, it was able to regress the tumors faster and effectively, causing specific death of the tumor cells, and sparing the normal body cells. Taken together, our results conclude that the NCs can be valuable tools in the development of neutraceutical-based nano delivery system for the delivery of anticancer bioactives and other biomacromolecules including drugs, siRNA, miRNA, peptides, genes and proteins.

Future perspective

This article tackled several issues in the process of oral administration-based nanodrug

delivery systems to deliver biologically active natural source derived nontoxic anticancer Fe-bLf to the tumor site. Herein, a number of interesting results were observed with respect to the internalization profiles and enhanced bioavailability of macromolecules, absorption of iron and calcium and anticancer efficacy of the nanoformulated Fe-bLf, compared with its free non-nanoformulated form. However, more detailed insights into this proof-of-concept study are required to further decipher the mechanistic action of AEC-CP-Fe-bLf NCs and its efficacy in different tumor types. Thus, the questions raised by the present study in relation to the endocytosis mechanism and promotion of apoptosis by Fe-bLf could be addressed in detailed follow-up systematic studies. The antiangiogenic nature of the NCs in reducing the tumor vasculature along with up/downregulations of key molecules involved in apoptotic pathways, could be studied in parallel to reveal the indirect antitumor effects caused by Fe-bLf, by priming the immune system. Furthermore, biodistribution data revealed mild distribution of the Fe-bLf through NCs in the eye, brain, kidney, lung, muscle and heart – which could be employed in delivery of therapeutic molecules to target these organ-related diseases or disorders, by proper surface modifications or conjugation with disease- or organ-specific biomarkers. These NCs could further be used as targeted delivery vehicles for active targeting by additionally tagging the

tumor-specific ligands, such as antibodies or aptamers to accomplish enhanced specificity. These nanoformulations can be used for better absorption and sustained release of drugs, oral vaccines and may be useful for iron and calcium deficient patients. The promising preventive and treatment efficacies of nanoformulated nontoxic natural Fe-bLf deserves further follow-up for translational research.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Oral administration of biomolecules is an uphill task to deliver at tumor sites, when the challenges in gut milieu are considered. However, nanotechnology promises a new hope and offers advantages such as controlled release, target-specific delivery, lower toxic drug delivery and toxicity. Earlier we showed for the first time that the iron content is a critical determinant in the antitumor activity of bovine lactoferrin (bLf).

Aim

- To solve the puzzle, whether the iron-saturated bLf (Fe-bLf) preserves its anticancer activity in nanoformulation when delivered orally. In this study, using human xenograft colon cancer model as a proof-of-concept, we studied the efficacy of nanoformulated Fe-bLf in prevention and treatment of tumors.

Outcomes of the study

- Both *in vitro* and *in vivo* assays performed indicate the potentials of use of the alginate-enclosed chitosan-calcium phosphate-loaded (AEC-CP)-Fe-bLf nanocarriers (NCs) for the controlled release of Fe-bLf and its protection from the challenging gut environment.
- The NCs undergo endocytosis by possibly utilizing different Lf or LRP receptors. AEC-CP-Fe-bLf NCs were shown to, very promisingly, enhance the bioavailability and metabolic stability of Fe-bLf.
- AEC-CP-Fe-bLf NCs prevented degradation of entrapped Fe-bLf in mouse blood in comparison with non-nanoformulated Fe-bLf.
- AEC-CP-Fe-bLf NCs were found to help in absorption of iron and thus may have utility in enhancing the iron uptake in iron deficient/anemic patients. Calcium phosphate in the inner core of NCs helps in the absorption of calcium without interfering with the absorption of iron.

Conclusion

- Our results conclude that the developed NCs protect and deliver Fe-bLf effectively to the tumor sites through enhanced permeability and retention effect. NCs were found to be safe, nontoxic and the increased bioavailability to the tumor sites has thus led to the complete inhibition of tumorigenesis in a prevention model and complete tumor rejection through regression in treatment studies.

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