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particles for delivery of an active agent including drugs and vaccines.



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NANOPARTICLE

FIELD OF THE INVENTION

The present invention relates to methods of producing nanoparticles. In particular,
5 the invention relates to nanoparticles for delivery of an active agent including drugs
and vaccines.

BACKGROUND

Large spectrums of active agents presently available for the treatment of human
10 disease have a number of limitations and side effects. For example, the extremely
short plasma half-life of many cancer therapeutics causes cancer patients to suffer
harsh treatments of prolonged parenteral administration. Accordingly, oral
administration of active agents is particularly desired to provide a number of benefits
including convenience cost effectiveness and increased patient compliance to
15 treatment regimes.

While the harsh side effects of present day therapeutics, such as anti-cancer
chemotherapeutics, can be overcome by using bio-macromolecules, such as siRNA,
miRNA and/or anti-cancer protein/peptides, the delivery of these molecules to target
20 sites and the maintenance of active agent structural integrity poses a problem for
orally delivered that encounter the harsh conditions of the gastrointestinal tract.

Various technologies have been explored to provide controlled delivery formulations
for oral administration. However, there are drawbacks for several current delivery
25 systems.

There remains a continuing need in the art for improved controlled release or targeted
release formulations for oral administration. There also remains a need for improved
drug delivery systems to provide localized delivery of active agents, especially
30 chemotherapeutic agents, to reduce the potential for side effects while at the same
time providing the therapeutic benefit of the chemotherapeutic, or to act as an adjunct
to chemotherapy.

As will be apparent from the foregoing, there remained significant problems to be overcome in the provision of nanoparticles for the delivery of an active agent. It is an aspect of the present invention to overcome or ameliorate a problem of the prior art by providing nanoparticles for delivery of an active agent.

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A reference herein to a patent document or other matter which is given as prior art is not to be taken as an admission that that document or matter was known or that the information it contains was part of the common general knowledge as at the priority date of any of the claims.

10

SUMMARY OF INVENTION

In a first aspect, the present invention provides a method for producing a nanoparticle comprising calcium phosphate for delivery of an active agent, said method comprising; providing an aqueous mixture of calcium phosphate and at least one
15 active agent; and adsorbing the at least one active agent on the calcium phosphate.

In some embodiments, the calcium phosphate is a calcium phosphate core.

In some embodiments, the method further comprises forming an aqueous mixture of
20 an intestinal absorption enhancer and the calcium phosphate adsorbed with the at least one active agent; and adsorbing the intestinal absorption enhancer on the calcium phosphate adsorbed with the at least one active agent.

In some embodiments, the intestinal absorption enhancer is chitosan. In some
25 embodiments the adsorbing with chitosan is performed in the presence of a crosslinking agent. In some embodiments the adsorbing with chitosan is performed using ionic gelation.

In some embodiments the adsorbing with chitosan is performed with constant stirring
30 of the aqueous mixture to obtain a particle size of about 200 ± 25 nm to about 300 ± 25 nm. In some embodiments the adsorbing with chitosan is performed with constant stirring of the aqueous mixture at a speed and for a period that is sufficient to adsorb the chitosan and optionally to obtain a particle size of about 200 ± 25 nm to about 300 ± 25 nm. Optionally, the adsorbing is conducted at 6000rpm for 6 hours.

In another aspect, the method further comprises forming an aqueous mixture of an enteric coating and the chitosan adsorbed calcium phosphate which is adsorbed with the at least one active agent and adsorbing the enteric coating on the Chitosan
5 adsorbed calcium phosphate.

In some embodiments the enteric coating is alginate. In some embodiments the adsorbing with alginate is performed using ionic gelation.

10 In some embodiments, the calcium phosphate is freeze dried prior to forming the aqueous suspension and adsorbing. In another embodiment, the calcium phosphate is freeze dried prior to each adsorption.

In some embodiments the method comprises freeze drying the produced
15 nanoparticles and optionally at each adsorption step.

In some embodiments, the active agent is lactoferrin.

In some embodiments, the active agent is an anti-cancer agent. In some
20 embodiments the active agent is taxol or doxorubicin.

In some embodiments the nanoparticle is produced by the method according to any one of the methods described above.

25 In another aspect, the present invention provides a composition of nanoparticles for delivery of an active agent, each nanoparticle comprising a calcium phosphate core adsorbed with at least one active agent.

In one embodiment, the nanoparticles are further adsorbed with an intestinal
30 permeability enhancer. In some embodiments the intestinal permeability enhancer is chitosan.

In some embodiments the nanoparticles are further adsorbed with an enteric coating. In some embodiments the enteric coating is alginate.

In some embodiments the enteric coating adsorbed nanoparticles produced are freeze dried.

- 5 In some embodiments the enteric coating adsorbed nanoparticles have a mean particle size of about 200 ± 25 nm to about 300 ± 25 nm

In another aspect, the present invention provides a pharmaceutical composition comprising the composition described above and a pharmaceutically acceptable
10 carrier.

In one embodiment the pharmaceutical composition is formulated for oral administration.

- 15 In another aspect, the present invention provides a method of treating cancer in patient in need thereof comprising orally administering a therapeutically effective amount of a composition of nanoparticles described above.

Throughout the description and the claims of this specification the word “comprise”
20 and variations of the word, such as “comprising” and “comprises” is not intended to exclude other additives, components, integers or steps.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a characterisation of alginate adsorbed chitosan adsorbed lactoferrin
25 adsorbed calcium phosphate nanoparticles (“AC-CP NCs-Lf NCs”) and characterization.

Schematic representation of alginate enclosed NCs were shown in (a). Chitosan forms a thin film over cap ceramic cores, upon treatment with SODIUM TRI POLY
30 PHOSPHATE. Alginate forms an outer coating after gelation using CaCl_2 . The NCs were found to have spherical morphology when observed under SEM, scale bars indicates 200nm, 200nm, 300nm and 350 nm for calcium phosphate nanoparticles (cap), chitosan adsorbed calcium phosphate nanoparticles (‘C- CP NCs’), chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles (‘C-CP NCs-Lf’) and

alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ('AC-CP NCs-Lf NCs') respectively (b). (c) DLS spectrometric observation of various layers predicts the increase in size of calcium phosphate nanoparticles to alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ('AEC-CP NCs-Fe-bLF NCs') from 200 to 350 nm. Sizes of cap (dark blue line), C-CP NCs (red), C-CP NCs- Lf (green) and AC-CP NCs-Lf (light blue) were represented as according to light intensity and size of the nanoparticles ('NCs') respectively. Iron loaded bovine lactoferrin (Fe-bLf) loaded AC-CP NCs nanoparticles were found to have same dimensions as that of AC-CP NCs-Lf NCs, when assessed for morphology by SEM (d) or by DLS spectrometry (e).

Figure 2 shows a controlled release of alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ("AC-CP NCs-Lf NCs")

(a) Controlled release of lactoferrin from AC-CP NCs Lf NCs was observed by doing SDS PAGE gel retardation assay. Immunoblots indicate the integrity of the protein during the formulation process. Thick bands in the stacking gel indicates Lf mobilized on the NCs, this is evident from the immunoblots (b) Stability of nanoparticles in pH 1.2 and 7.4 was observed by treatment with HCl and NaOH for different time periods. Intensity of retarded bands in 10% SDS PAGE and immunoblots confirm an increase of degradation starting from 2-4 hours, in alkaline media. No intense bands were observed in HCl treated nanoparticles, except at 6 hours, indicating the integrity of nanoparticles in acidic media.

Figure 3 shows cell viability and endocytosis studies of chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles

(a) Viability of Caco2 cells were measured by TUNEL assay after treatment with alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ('AC-CP NCs-Lf NCs') and alginate adsorbed chitosan adsorbed Iron loaded-lactoferrin adsorbed calcium phosphate nanoparticles ('AEC-CP NCs-Fe bLf NCs'), with using 6 uM/ml of taxol as a negative control. The results obtained indicates there is no difference in fluorescence of alginate adsorbed chitosan adsorbed calcium phosphate nanoparticles ('AC-CP NCs') (green) compared to

control (red), but increase in cytotoxicity in alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ('AC-CP NCs-FebLf') (blue) and taxol (purple) treated cells, as indicated by relative fluorescence observed using flow cytometry (b) Endocytosis of C-CP NCs-Lf NCs was analyzed following cell lysis after treatment with 50ug/ml AEC-CP NCs-Fe-bLF NCs for different time intervals. Subsequent probing with Lf specific antibody in immunoblots shows the internalization of chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ('C-CP NCs-Lf NCs'). (b) Flowcytometric analysis shows an increase in internalization from 20% to 80% with time, up to 72 hrs.

10

Figure 4 shows a visualization of endocytosis

(a) Confocal microscopy shows the cytoplasmic and nuclear internalization of rhodamine labeled C-CP NCs Lf NCs. Green-actin filaments, Red- NCs and Blue-nucleus. Scale bars 10um. (b) immunocytochemistry using Lf specific antibodies further supports cytoplasmic and nuclear localization of Lf NCs. Black arrows in right panel indicate nuclear localization. Images were observed under 100X oil immersion objective.

15

20 Figure 5 shows Transcytosis studies

No significant difference in TEER measurements was observed when Caco-2 cell monolayer after treated with AC-CP NCs-Lf NCs (a), from 0 hours after treatment to 72 hrs. From the graph, a slight decrease in the TEER in case of monolayers treated with C-CP NCs and C-CP NCs Lf NCs can be observed at the end of 72 hrs. Right panel (b) shows transcytosis profiles of C-CP NCs NCs, C-CP NCs Lf NCs and AC-CP NCs Lf NCs for a time period of 2 hours to 72 hrs. The graph indicates the decrease in relative fluorescence units in apical side compared to the basolateral side with progression of time.

25

30

Figure 6 shows In vitro release profile of bLf from standardized and optimized of different nanoparticles in low 1.5 pH (stomach) to 8 pH (intestine) buffers.

Results are mean $\pm$ sem.

Figure 7 shows a Nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed nanoparticles ("AEC-CP-Lf NCs") diet inhibits tumour growth without causing any toxicity.

5

Effects of nanoformulated or normal diet were shown in xenograft colon cancer mouse model when fed one week prior to the injection of CaCo2 cells. Taxol was injected into the tumours established. *P<0.05, **P<0.001.

10 Figure 8 shows a Nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ("AEC-CP-Lf NCs") and taxol adsorbed calcium phosphate nanoparticles ("AEC-CP-Taxol NCs") diet inhibits tumour growth without causing any toxicity. AEC-CP-Lf NCs were fed one week prior to cancer cell injection.

15

Following CaCo2 cell injection, tumour bearing mice were fed either nanoformulated of Fe-bLf and Taxol loaded calcium phosphate nanoparticles ("AEC-CP-Taxol NCs") or normal diet. In this xenograft colon cancer mouse model, taxol was injected into established tumours while AEC-CP-Taxol NCs were given orally. *P<0.05, **P<0.001.

20

Figure 9 shows concentration of Fe-bLf in mice plasma following a single administration of Fe-bLf by oral gavage and AEC-CP-bLf NCs.

AEC-CP-bLf NCs were administered at a concentration of 100 mg Fe-bLf/kg body weight. Values are represented as means $\pm$ SEMs (n = 6).

25

Figure 10 showsiodistributionof Fe-bLf and AEC-CP-Fe-bLf NCs following oral administration.

30 Fluorescent signal of tissue extracts after 24 hours of oral administration in diet were analysed. Nanocarriers were labelled with coumarin-6 (60mg/Kg). The mean for representative experiment was calculated and presented as a mean $\pm$ SEM values. ** Indicates a highly significant P < 0.001 value relative to the normal control cell lines

and with media only. *Indicates a significant $P < 0.05$ value relative to the normal control cell lines and control with media only.

Figure 11 shows a schematic illustration of the proposed mechanism of AC-CP NCs-Lf NCs internalization.

(a) Without wishing to be bound by theory, the adsorbed alginate coating is degraded in the alkaline environment of the intestine, and the remaining chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ("C-CP NCs-Lf NCs") enter the circulation via endocytosis and/or transcytosis (b). (c) C-CP NCs-Lf NCs get released in the tumor site by the enhanced permeability and retention (EPR) effect. (d) Further uptake of C-CP NCs-Lf NCs into cancer cells occurs via oligosaccharide and/or Lf receptor mediated endocytosis.

Figure 12 shows nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ("AEC-CP-Lf NCs") increased clearance of *Giardia lamblia* parasites.

Graph shows cyst counts in the small intestine of mice after infection with $10^7/0.1\text{ml}$ of trophozoites of *Giardia lamblia* (Portland 1) belonging to different groups. Values are represented as mean $\pm$ SD.

Panel A shows a photomicrograph of higher magnification (H&E, 200X) of the small intestine of normal mice in the control group. The ileum shows long, normal villi, lining cells including the brush border & goblet cells are normal. Panel B shows a photomicrograph of higher magnification (H&E, 200X) of the small intestine of mice in the infected group. Higher magnification highlights the parasites. The villi show mild excess of LMN cells, occasional villous is swollen, lining cells are normal, some crypts show paneth cell hyperplasia, surface shows presence of parasites.

Figure 13 shows nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ("AEC-CP-Lf NCs") decrease *Giardia lamblia* trophozoite counts in the small intestine.

Figure 14 shows nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ("AEC-CP-Lf NCs") decrease *Salmonella* infection of the small intestine.

- 5 Efficacy of oral treatment with nanoformulated lactoferrin for reducing the number of bacteria in the small intestine of Balb/c mice infected with 200µl of 10⁸ CFU/ml of *Salmonella typhimurium* (wild strain) after 6-20 days of post infection/treatment as compared with the bovine lactoferrin treated mice; positive control, Ciprofloxacin treated mice and the normal mice. Values are represented as mean ± SD.

10

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based in part on methods of forming nanoparticles for delivery of active agents that are biocompatible and biodegradable.

- 15 The methods described herein have advantages including forming nanoparticles of a desired size for efficient delivery of active agents, optionally the formation of substantially spherical particles and an increased encapsulation efficiency.

- 20 The pharmacokinetic and bioavailability studies presented herein indicate that the nanoparticles formed increase the efficiency of delivery of active agents.

The nanoparticles are formed from calcium phosphate which is a natural chemical present in bone. The nanoparticles may further comprise chitosan and alginate, polymers which have low immunogenicity and are highly biocompatible.

25

In a first aspect, the present invention provides a method for producing a nanoparticle comprising calcium phosphate for delivery of an active agent, said method comprising; providing an aqueous mixture of calcium phosphate and at least one active agent; and adsorbing the at least one active agent on the calcium phosphate.

30

The term "nanoparticle", as used herein, generally refers to a polymer sphere or spheroid that can be produced to have a size of less than or equal to about 1000 nm in diameter, including, 5, 10, 15, 20, 30, 50, 100, 200, 250, 300, 350, 400, 500 and

750 nm. The term includes nanoparticles comprising a number of layers of polymer/adsorbed agents.

5 The calcium phosphate nanoparticles adsorbed with the molecules of the present invention can be prepared to have mean particle size diameters of about 10 to about 20,000 nanometers (nm), specifically about 20 to about 10,000 nm, more specifically about 50 to about 5000 nm, still more specifically about 100 to about 1000 nm, and yet more specifically about 200 to about 300 nm. The size of the calcium phosphate nanoparticles can be determined using known techniques in the art, such as laser
10 light scattering techniques, dynamic light scattering techniques, transmission electron microscopy, atomic force microscopy, scanning electron microscopy, the methods described in the Examples .

The term “calcium phosphate”, as used herein, generally refers to calcium phosphate
15 formed by known methods, including but not limited to wet precipitation methods using inorganic salts such as calcium salts and ammonium salts. For example, solutions of calcium nitrate and sodium bicarbonate/ammonium phosphate can be combined under rapid stirring to provide a calcium phosphate precipitate which can be isolated and optionally lyophilised.

20

The ratio of Ca to P can be chosen to form hydroxyapatite (OHyAp) or amorphous forms. In one embodiment, the calcium phosphate is amorphous calcium phosphate with a <150nm particle size (BET) and/or with a BET surface area of > 12 m²/g (typical). In one embodiment, the calcium phosphate is amorphous calcium
25 phosphate with the linear formula $\text{Ca}_2\text{O}_7\text{P}_2 \cdot \text{H}_2\text{O}$.

In some embodiments, the calcium phosphate is a calcium phosphate core.

The term “calcium phosphate core”, as used herein, generally refers to a calcium
30 phosphate nanoparticle suitable for adsorption of active agents, intestinal absorption enhancers, enteric coatings etc.

The term “active agent”, as used herein, generally refers to a therapeutic agent, including but not limited to chemotherapeutic agents, biologically active polypeptides,

radiotherapeutics, radiosensitising agents, other agents known to interact with an intracellular protein, a nucleic acid or insoluble ligand.

5 The term “aqueous mixture” as used herein, generally refers to water and mixtures comprising water including water and one or more water miscible solvent.

Applicant has characterised nanoparticles comprising iron saturated bovine lactoferrin (Fe-bLf) nanoparticles, and nanoparticles comprising taxol (paclitaxel) or doxorubicin.

10 In some embodiments, the active agent is lactoferrin (Lf).

The term “lactoferrin”, as used herein, generally refers to native or recombinant lactoferrin. Native lactoferrin can be obtained by purification from mammalian milk or colostrum or from other natural sources. Recombinant lactoferrin can be made by
15 recombinant expression of direct production in genetically altered animals, plants, fungi, bacteria or other prokaryotic or eukaryotic species, or through chemical synthesis.

In one embodiment the lactoferrin is any mammalian lactoferrin including but not
20 limited to sheep, goat, pig, mouse, water buffalo, camel, yak, horse, donkey, llama, elephant, bovine or human lactoferrin. Optionally the lactoferrin is bovine lactoferrin.

In one embodiment the lactoferrin is apo-lactoferrin. In one embodiment the lactoferrin, functional lactoferrin variant or functional lactoferrin fragment is free of
25 metal ions. In one embodiment the lactoferrin or functional variant or functional fragment thereof is at least about 5, 10, or 20% metal ion saturated on a stoichiometric basis.

In one embodiment the metal ion is an ion selected from the group comprising
30 aluminium, bismuth, copper, chromium, cobalt, gold, iron, manganese, osmium, platinum, ruthenium, and zinc ions, or any combination of any two or more thereof, or other ions that will coordinate specifically in a lactoferrin metal ion binding pocket.

Optionally the metal ion is an iron ion.

As used herein, the terms "iron-lactoferrin" and "iron-saturated lactoferrin" as used herein are intended to refer to a population of lactoferrin polypeptides providing a population of iron-binding pockets where at least about 25, 30, 35, 40, 45, 50, 55, 60, 5 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5, 99.9 or 100% of the metal ion-binding pockets present in the population have an iron ion bound.

In one embodiment, the lactoferrin, functional lactoferrin variant or functional lactoferrin fragment is involved in non-specific ion binding. Preferably, the ions that 10 may be non-specifically bound to the lactoferrin, functional lactoferrin variant or functional lactoferrin fragment are selected from aluminium, calcium, bismuth, copper, chromium, cobalt, gold, iron, manganese, osmium, platinum, ruthenium, selenium, and zinc ions, or any combination of any two or more thereof. The ion may be any ion or mixture of ions that will non-specifically bind to the lactoferrin, functional lactoferrin 15 variant or functional lactoferrin fragment, preferably calcium and selenium ions.

In one embodiment the metal ion lactoferrin or a metal ion functional variant or functional fragment thereof is at least about 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5 or 100% metal ion saturated on a 20 stoichiometric basis.

In one embodiment the metal ion lactoferrin or a metal ion functional variant or functional fragment thereof is at least about 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195 or 200% metal ion saturated 25 on a stoichiometric basis.

In one embodiment, iron saturated bovine lactoferrin is prepared using Bovine Lf; following alkaline treatment bovine Lf is dialysed for a period of 48 hours in 0.1M citric acid to get rid of the bound metal ions and then saturated with Fe(III) coordinate 30 compounds for the development of deep red coloured Fe-bLf.

Methods of preparing lactoferrin and iron-saturated lactoferrin are described in WO/2008/140335, WO/2008/079030 and WO/2006/054908 each of which are

incorporated herein by reference. Methods treating and preventing cancer are also described in WO/2008/140335, WO/2008/079030 and WO/2006/054908.

In some embodiments, the active agent is an anti-cancer agent.

5

The term "anti-cancer agent", as used herein, generally refers to molecules which inhibit or suppress the growth of cancer cells. Anti-cancer agents may also include compounds that destroy cancer cells or interfere with cell division, monoclonal antibodies that bind proteins on the cell surface, peptides that bind cell surface
10 receptors, interferons or cytokines which induce an immune response, vaccines which generate an immune response, hormones or compounds that block certain hormones involved in cancer, compounds that inhibit or prevent the growth of new blood vessels (e.g. angiogenesis inhibitors), agents that damage DNA (e.g. alkylating agents, for example, cisplatin, carboplatin, and oxaloplatin; anti-metabolites; and topoisomerase
15 inhibitors), and compounds with anti-cancer properties (e.g., taxanes, vinca alkaloids, and plant alkaloids). The term "anti-cancer agent" also includes radiation therapy. An anti-cancer agent may also include an agent specific for deregulated proteins of cancer cells, such as an inhibitor of receptor tyrosine kinases.

20 In one embodiment the chemotherapeutic agent is taxol (paclitaxel), doxorubicin, epirubicin, fluorouracil, cyclophosphamide or methotrexate.

In one embodiment the active agent is taxol or doxorubicin.

25 The term "encapsulation efficiency", as used herein, generally refers to the ratio of the amount of active agent adsorbed to the nanoparticles, to the amount of active agent added into the aqueous mixture. For example, encapsulation efficiency (EE) can be calculated as: $EE \text{ (in \%)} = (\text{amount of total active agent added into the aqueous mixture} - \text{amount of non-adsorbed active agent}) / \text{amount of total active agent added}$
30 into the aqueous mixture x 100.

In one embodiment, the encapsulation efficiency is at least 80%. In another embodiment, the encapsulation efficiency is at least 85%.

The term “adsorbing”, as used herein, generally refers to the binding or adhesion of a molecule to the surface of the nanoparticle. Adsorbing may be performed using known methods such as emulsion cross linking, coacervation/precipitation, spray-drying, emulsion-droplet coalescence methods, ionic gelation, reverse micellar methods, and sieving methods.

In some embodiments, the method further comprises forming an aqueous mixture of an intestinal absorption enhancer and the calcium phosphate adsorbed with the at least one active agent; and adsorbing the intestinal absorption enhancer on the calcium phosphate adsorbed with the at least one active agent.

The term “intestinal absorption enhancer”, as used herein, generally refers to a molecule, or a mixture of molecules, that enhances absorption of agents across biological mucosal epithelia such as the intestine. The term also encompasses enhancers that enhance the paracellular route of absorption, or by opening epithelial tight junctions.

In some embodiments, the intestinal absorption enhancer is chitosan. In some embodiment the adsorbing with Chitosan is performed in the presence of a crosslinking agent. In some embodiments the adsorbing with Chitosan is performed using ionic gelation.

The term “chitosan”, as used herein, generally refers to a linear co polymer polysaccharide consisting of β (1–4)-linked 2-amino-2-deoxy-d-glucose (d-glucosamine) and 2-acetamido-2-deoxy-d-glucose (N-acetyl-d-glucosamine) units. The structure of chitosan is very similar to that of cellulose (made up of β (1–4)-linked d-glucose units), in which there are hydroxyl groups at C2 positions of the glucose rings. Chitosan is poly[β -(1–4)-2-amino-2-deoxy-d-glucopyranose]. The term chitosan is used to describe a series of polymers of different degrees of deacetylation (DD), defined in terms of the percentage of primary amino groups in the polymer backbone, and average molecular weights (Mw). The DD of typical commercial chitosan is usually between 70% and 95%, and the Mw between 10 and 1000 kDa. The properties, biodegradability and biological role of chitosan is frequently dependent on the relative proportions of N-acetyl-d-glucosamine and d-glucosamine residues.

The term also encompasses modified forms of chitosan, such as thiolated chitosan, trimethylated chitosan, Carboxymethyl chitosan, N-(2-Hydroxyl) propyl-3-trimethyl ammonium chitosan chloride, etc.

5

The term "crosslinking agent", as used herein, generally refers to a molecule or mixture of molecules that can interact with the nanosphere via electrostatic forces to form ionic cross-linked networks. For example chitosan solution containing adsorbed onto nanoparticles upon contact with a cross-linking agent. One of the commonly used cross-linking agents for the ionic gelation of chitosan is tripolyphosphate (TPP). TPP is a non-toxic polyanion which can interact with chitosan via electrostatic forces to form ionic cross-linked networks. Covalently cross-linked chitosan coated nanospheres can be prepared by treating chitosan with various chemical reagents. The cross-linking procedure helps to reinforce the chemical and mechanical properties of chitosan, making it a more stable network. Thus it can perform controlled protein release at higher pH of intestine instead of rapidly releasing the protein drugs by rapid dissolution in the stomach. Other crosslinking agents for cross-linking chitosan are dialdehydes such as glyoxal and glutaraldehyde. The aldehyde groups form covalent imine bonds with the amino groups of chitosan, due to the resonance established with adjacent double ethylinic bonds via a Schiff reaction. Dialdehydes allow the cross-linking to happen by direct reaction in aqueous media and under mild conditions and it does not require the addition of auxiliary molecules such as reducers]. It also adds to retaining the biocompatibility of the polymer. Diethyl squarate and oxalic acid have also been found to act as direct cross-linkers for chitosan. The natural cross-linker like genipin is gaining wide acceptance for cross-linking chitosan.

In some embodiments the cross-linker is sodium tripolyphosphate. In one embodiment, the sodium tripolyphosphate is at the same concentration as the %w/v concentration of chitosan. In some embodiments, the sodium tripolyphosphate is at a concentration of 0.01 w/w%. In some embodiments, the sodium tripolyphosphate is at a concentration of 0.1 w/w%.

The term "ionic gelation", as used herein, generally refers to complexation between oppositely charged molecules to prepare adsorbed nanoparticles. For example, chitosan is dissolved in aqueous acidic solution to obtain the cation of chitosan. This aqueous solution is then added dropwise under constant stirring to polyanionic triolyphosphate solution. Due to the complexation between oppositely charged species, chitosan undergoes ionic gelation and precipitates to form spherical particles, adsorbing onto the calcium phosphate nanoparticle.

Without wishing to be bound by theory, Applicant has demonstrated that the size of chitosan adsorbed active agent adsorbed calcium phosphate nanoparticles can be determined using different amounts of chitosan and time of adsorption.

In some embodiments the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture to obtain a particle size of at least 200nm. In some embodiments the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture to obtain a particle size of between 250-300nm. In some embodiments the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture to obtain a particle size of between 200-250nm.

In some embodiments the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture at a speed sufficient to obtain a particle size of at least 200nm. In some embodiments the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture at a speed sufficient to obtain a particle size of between 250-300nm. In some embodiments the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture at a speed sufficient to obtain a particle size of between 200-250nm.

In some embodiments the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture for a time sufficient to obtain a particle size of at least 200nm.

In some embodiments the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture for a time sufficient to obtain a particle size of between 250-300nm. In some embodiments the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture for a time sufficient to obtain a particle size of between 200-250nm.

In some embodiments the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture at 6000rpm for 6 hours. In some embodiments the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture at 6000rpm for 12 hours. In some embodiments the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture at 6000rpm for 24 hours.

In some embodiments the adsorbing with Chitosan is performed at a concentration of 0.01% w/v. In some embodiments the adsorbing with Chitosan is performed at a concentration of 0.1% w/v.

In some embodiments the adsorbing with Chitosan is performed at a concentration of 0.01% w/w, with constant stirring of the aqueous mixture at 6000rpm for 6 hours. In some embodiments the adsorbing with Chitosan is performed at a concentration of 0.01% w/w, with constant stirring of the aqueous mixture at 6000rpm for 12 hours.

In some embodiments the adsorbing with Chitosan is performed at a concentration of 0.1% w/w, with constant stirring of the aqueous mixture at 6000rpm for 6 hours to form nanoparticles of about $250 \pm \text{nm}$ to about $300 \pm 25 \text{ nm}$. In some embodiments the adsorbing with Chitosan is performed at a concentration of 0.01% w/w, with constant stirring of the aqueous mixture at 6000rpm for 12 hours to form nanoparticles of about $200 \pm 25 \text{ nm}$ to about $250 \pm 25 \text{ nm}$.

In some embodiments, the nanoparticles formed are spherical.

In some embodiments the chitosan is chitosan with a 20-200 cP viscosity, average molecular weight (MW) of 200kDa and deacetylation degree of 80%.

In another aspect, the method further comprises forming an aqueous mixture of an enteric coating and the Chitosan adsorbed at least one active agent adsorbed calcium phosphate; and adsorbing the enteric coating on the Chitosan adsorbed at least one active agent adsorbed calcium phosphate.

The term “enteric coating”, as used herein, generally refers to a molecule or mixture of molecules to prevent early digestion or degradation of a nanoparticle.

In some embodiments the enteric coating is alginate.

5

The term “alginate”, as used herein, generally refers to a water-soluble linear polysaccharide extracted from brown seaweed and is composed of alternating blocks of 1–4 linked α -l-guluronic and β -d-mannuronic acid residues. Because of the particular shapes of the monomers and their modes of linkage in the polymer, the geometries of the G-block regions, M-block regions, and alternating regions are substantially different. Specifically, the G-blocks are buckled while the M-blocks have a shape referred to as an extended ribbon. If two G-block regions are aligned side by side, a diamond shaped hole results. This hole has dimensions that are ideal for the cooperative binding of calcium ions. The homopolymeric regions of β -d-mannuronic acid blocks and α -l-guluronic acid blocks are interdispersed with regions of alternating structure (β -d-mannuronic acid– α -l-guluronic acid blocks). The composition and extent of the sequences and the molecular weight determine the physical properties of the alginates.

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15

20 The term also encompasses modified forms of alginate, such as thiolated alginate, hydrophobically modified alginate, or complexes of alginate etc.

In some embodiments the adsorbing with alginate is performed at a concentration of 1% w/v. In some embodiments the adsorbing with alginate is performed at a concentration of 2% w/v.

25

In some embodiments, the adsorbing with alginate is performed with a 0.6% mass ratio of Ca/alginate.

30 In some embodiments the adsorbing of alginate increases the nanoparticle size by between about $100 \pm \text{nm}$ to about $150 \pm 25 \text{ nm}$.

In some embodiments the adsorbing with alginate is performed using ionic gelation.

In one embodiment, an aqueous solution of calcium phosphate solution is prepared and incubated for 24 hours with 10% w/w lactoferrin (Lf) with constant stirring at 4 degrees C for adsorption of Lf. The calcium phosphate nanoparticles adsorbed with Lf are centrifuged and washed several times to remove traces of unbound protein and freeze dried. In another embodiment, 0.01% w/w chitosan, dissolved in acetate buffer is added to calcium phosphate adsorbed with lactoferrin under constant stirring and 0.01% of cross linking agent (sodium tri polyphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$)) added drop wise with constant stirring at 6000rpm for 6 h to result in a nanoparticle size of about 200 ± 25 to about 250 ± 25 nm.

In one embodiment, an aqueous solution of calcium phosphate solution is prepared and incubated for 24 hours with 10% w/w lactoferrin (Lf) with constant stirring at 4 degrees C for adsorption of Lf. The calcium phosphate nanoparticles adsorbed with Lf are centrifuged and washed several times to remove traces of unbound protein and freeze dried. 0.01% w/w chitosan, dissolved in acetate buffer is added to the lactoferrin adsorbed calcium phosphate nanoparticles with constant stirring and 0.01% of sodium tri polyphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$) added drop wise and constantly stirred at 6000rpm for 12 h to result in a nanoparticle size of about 200 ± 25 to about 250 ± 25 nm. The formed nanoparticles were freeze dried. The aqueous solution of chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles is prepared and incubated for 24 hours with 10% w/w lactoferrin (Lf) with constant stirring at 4 degrees C for adsorption of Lf and the nanoparticles formed were freeze dried.

In one embodiment, an aqueous solution of calcium phosphate solution is prepared and incubated for 24 hours with 10% w/w lactoferrin (Lf) with constant stirring at 4 degrees C for adsorption of Lf, and freeze drying of the nanoparticles formed. The calcium phosphate nanoparticles adsorbed with Lf are centrifuged and washed several times to remove traces of unbound protein. 0.01% w/w chitosan, dissolved in acetate buffer is added to the lactoferrin adsorbed calcium phosphate nanoparticles with constant stirring and 0.01% of sodium tri polyphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$) added drop wise and constantly stirred at 6000rpm for 12 h to result in a nanoparticle size of 200 ± 25 to 250 ± 25 nm.

In another embodiment an aqueous solution of chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles is prepared and incubated for 24 hours with 10% w/w lactoferrin (Lf) with constant stirring at 4 degrees C for adsorption of Lf and freeze drying of the nanoparticles formed.

5

In another embodiment, the chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles are adsorbed with alginate gel by using 1% w/v alginate solution and calcium chloride, with 0.6% mass ratio of Ca/alginate. The nanoparticles produced are washed and freeze dried.

10

In another embodiment adsorption is performed at 4 degrees C to protect the polymeric and protein components in the formulation.

In one embodiment, the present invention provides a method of producing alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles using a combination of nanoprecipitation and ionic gelation, as described below. Calcium phosphate (1%w/v solution) is prepared and incubated for 24 hours with 10% w/w lactoferrin (Lf) with constant stirring at 4 degrees C, at a pH below 8.0 (the isoelectric pH of lactoferrin) in order to adsorb bLf on the calcium phosphate.

Following electrostatic interaction of Lf on to the ceramic core, the obtained solution is centrifuged and washed several times to remove traces of unbound protein and freeze dried. 0.01%w/w of chitosan solution in acetic acid is added to the calcium phosphate under constant stirring and 0.01%w/v of cross linking agent (sodium tri polyphosphate) is added drop wise, with constant stirring to result in nanoparticles in the size range of 200 ± 25 - 250 ± 25 nm. The chitosan adsorbed lactoferrin adsorbed calcium phosphate adsorbed nanoparticles are freeze dried, and incubated for 48 hours with 0.2% lactoferrin (Fe-bLf/bLf) with constant stirring at 4 degrees C, at pH below 8.0, the isoelectric pH of lactoferrin in order to adsorb Lf on the nanoparticles. After the electrostatic interaction of Lf on to the NCs, the obtained solution is centrifuged and washed several times to remove traces of unbound protein. The formed nanoparticles are then adsorbed with alginate using 2% w/v alginate solution and calcium chloride. The nanoparticles formed are washed and freeze dried.

The term "freeze drying", as used herein, generally refers to a means of drying achieved by freezing a wet substance at a temperature from about -172 degrees celsius to about -2 degrees Celsius followed by rapid dehydration by sublimation under a vacuum level down to the lower level of a diffusion pump. A useful pressure
5 range is from about 0.1 mTorr to about 0.5 Torr. The term "freeze drying" may be used interchangeably with the term "lyophilisation".

Freeze drying can be incorporated at any stage of the method to increase the surface area for adsorption of the active agent. Without being limited by theory, the use of
10 freeze drying can cause cracking and fissures in the surface of the nanoparticles to increase the surface area for adsorption of the active agent.

In another embodiment, the present invention provides a method of producing alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate
15 nanoparticles using a combination of nanoprecipitation and ionic gelation.

In contrast to the teachings of the prior art, the present inventors have surprisingly found no differences in TEER, when the nanoparticles of the present invention are administered. Without wishing to be bound by theory, the methods of the present invention comprise adsorption of chitosan onto the calcium phosphate nanoparticles
20 using the poly anion sodium tripolyphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$), which is predicted to react with reacting amine groups of chitosan thereby changing the charge of the nanoparticles formed. Consequently the nanoparticles of the present invention would not interfere with tight junction proteins unlike other nanoparticles. Therefore, the nanoparticles are able enter the circulation without effecting or with little damage to
25 the epithelial cell layer.

The amount of relative fluorescence units was measured as a function of transcytosis on both apical and basolateral sides. As shown in Figure 5b there is an increase in transcytosis in C-CP NCs-Lf and C-CP NCs from 2 hours to 72 hours compared to
30 AEC-CP NCs-Fe bLf NCs, indicating transcytosis increased with a decrease in the size of nanoparticles.

Without wishing to be bound by theory, a proposed model for delivery of active agent from orally administered nanoparticles is shown in Figure 6. The nanoparticles after being delivered orally reach the intestine protecting the active agent adsorbed to the nanoparticle from variations in pH and enzyme activity. The adsorbed alginate is degraded in the intestine in alkaline pH, releasing chitosan adsorbed nanoparticles into the bloodstream and entry into tissues such as malignant cancer tissue. Accumulation of nanoparticles into the tumor tissue may occur via the Enhanced permeability and retention (EPR) effect, and taken up into cells by endocytosis, which upon further degradation (in endolysosomes) releases the active agent inside the cell.

Further experiments done with Iron saturated bovine lactoferrin (Fe-bLf) when loaded on to the AEC-CP NCs-Fe-bLF NCs demonstrate there was not much increase in size of nanoparticles, as observed by DLS measurements and SEM. Accordingly, using the relationship between the present methods and the size of the particles formed, the Applicants can predict the efficacy of AC-CP NCs-Lf NCs for carrying and delivery of active agents (such as the anti-cancer Lf or Fe-Lf peptides).

In one embodiment, the present invention provides methods of preparing alginate adsorbed chitosan adsorbed taxol or doxorubicin adsorbed calcium phosphate nanoparticles.

Alginate adsorbed chitosan adsorbed taxol or doxorubicin adsorbed calcium phosphate nanoparticles are prepared by a combination of nano precipitation and ionic gelation methods as described below. Calcium phosphate (1%w/v) solution is incubated for 24hours with 0.1% taxol or doxorubicin with constant stirring at 4 degrees C; at a pH below 8.0 in order to electrostatically adsorb taxol or doxorubicin on to the nanoparticles. Following adsorption of taxol or doxorubicin on to calcium phosphate nanoparticle, the obtained nanoparticles are centrifuged and washed several times to remove traces of unbound drug and freeze dried. 0.1%w/w of chitosan solution in acetate buffer (pH4) is added to the taxol or doxorubicin adsorbed calcium phosphate nanoparticles, and 0.01%w/w of cross linking agent, sodium tripolyphosphate added in a drop wise manner, constantly stirring at 6000rpm for 12h to result in a nanoparticle size range of 200 ± 25 - 250 ± 25 nm. The nanoparticles formed are freeze dried.

In another embodiment, the chitosan adsorbed taxol or doxorubicin adsorbed calcium phosphate nanoparticles are coated with alginate optionally using 2% w/v alginate solution and calcium chloride. The formed nanoparticles are washed and freeze dried
5 for further characterization.

In another embodiment adsorption is performed at 4 degrees C to protect the polymeric and protein components in the formulation

10 Without wishing to be bound by theory, Applicant has found that freeze drying the calcium phosphate can affect active agent loading and/or in vitro active agent release. For Example, Applicant has found that freeze drying the calcium phosphate prior to forming the aqueous mixture results in nanoparticles that adsorb higher concentrations of active agent versus particles that have not been freeze dried prior to
15 active agent adsorption.

In some embodiments the method comprises freeze drying the produced nanoparticles.

20 Applicant has also found that freeze drying the nanoparticles formed by the present methods increases the encapsulation efficiency relative to those particles that were not freeze dried.

Applicant has also found that freeze drying the nanoparticles formed by the present
25 methods increases the efficacy of the active agent relative to those particles that were not freeze dried.

As discussed above, the present invention is based in part on methods of forming nanoparticles for delivery of an active agents that are biocompatible and bio
30 degradable. The nanoparticles are formed from calcium phosphate which is a natural chemical present in bone. The nanoparticles may further comprise chitosan and alginate, polymers which have low immunogenicity and are highly biocompatible.

In another aspect, the present invention provides a composition of nanoparticles for delivery of an active agent, each nanoparticle comprising a calcium phosphate core adsorbed with at least one active agent.

- 5 In one embodiment, the nanoparticles are further adsorbed with an intestinal permeability enhancer. In some embodiments the intestinal permeability enhancer is Chitosan.

In some embodiments the nanoparticles are further adsorbed with an enteric coating.

- 10 In some embodiments the enteric coating is alginate.

In some embodiments the nanoparticles are freeze dried.

In some embodiments the nanoparticles have a mean particle size of between about

- 15 200 and about 300 nm

In another aspect, the present invention provides a pharmaceutical composition comprising the composition described above and a pharmaceutically acceptable carrier.

20

In one embodiment the pharmaceutical composition is formulated for oral administration.

Applicant has demonstrated the nanoparticles of the present invention increase delivery of active agents. For example, in one embodiment shown herein the nanoparticles of the present invention increase Fe-bLf on tumour cells as compared to non-nanoformulated Fe-bLf.

25

Applicant has demonstrated the nanoparticles of the present invention surprisingly increase clearance of pathogens infecting the gastrointestinal tract, including protozoal and bacterial pathogens. For example, in one embodiment shown herein the nanoparticles of the present invention increase clearance of Salmonella from the small intestine. In another embodiment shown herein, the nanoparticles of the

30

present invention increase clearance of Giardia from the host, and decrease the amount of Giardia cysts in feces.

Accordingly, in another aspect, the present invention provides a method of preventing or treating gastrointestinal disease in patient in need thereof comprising orally
5 administering a therapeutically effective amount of a composition of nanoparticles described above.

In one embodiment the gastrointestinal disease is caused by a pathogen. In one
10 embodiment the pathogen is a bacteria or a protozoan parasite.

Gastrointestinal diseases and/or disorders are those caused by a bacterial pathogen, protozoan parasite viral pathogen and/or toxin, including a toxin from a pathogen. In some embodiments, the gastrointestinal disease is caused by a bacterium commonly
15 found in the gastrointestinal tract, including but not limited to, Escherichia coli, Campylobacter jejuni, Cryptosporidium spp., Giardia lamblia, Yersinia enterocolitica, Helicobacter pylori, all Clostridium spp., C. difficile and Vibrio cholera. In some embodiments, the gastrointestinal disease is caused by a bacterial pathogen that is ingested, for example, from consuming air, water and/or food. Exemplary bacteria
20 include, but not limited to, Salmonella, Shigella and Listeria spp. In some embodiments gastrointestinal disease is caused by a virus, including, but not limited to rotavirus, enteroviruses, adenoviruses, caliciviruses, reoviruses, coronaviruses, Norwalk-type viruses, coxsackieviruses, poliovirus and hepatitis A virus.

25 In another embodiment, the pathogen is a bacteria is selected from the group consisting of: Salmonella spp., Shigella spp., Listeria spp. enterotoxigenic Escherichia coli, Campylobacter jejuni, Yersinia enterocolitica, Helicobacter pylori, all Clostridium spp. and Vibrio cholera.

30 In another embodiment, the pathogen is a protozoan parasite is selected from the group consisting of: Giardia lamblia, Cryptosporidium spp. and Entamoeba histolytica.

In some embodiments the pathogen is a virus. In further embodiments, the virus is selected from the group consisting of: rotavirus, enteroviruses, adenoviruses,

caliciviruses, reoviruses, coronaviruses, Norwalk-type viruses, coxsackieviruses, poliovirus and hepatitis A virus.

5 In one embodiment the pathogen colonisation is decreased. In another embodiment pathogen clearance is increased. In another embodiment, pathogen excretion from the gastrointestinal tract (e.g. in feces) is decreased.

10 In some embodiments, the methods and/or compositions used herein reduce the severity of one or more symptoms associated with gastrointestinal disease caused by a pathogen by at least about any one of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% compared to the corresponding symptom in the same individual prior to treatment or compared to the corresponding symptom in other individuals not receiving the methods and/or compositions of the present invention.

15 Applicant has also demonstrated Fe-bLf loaded nanoparticles of the present invention increase absorption of iron and calcium. Accordingly, the nanoparticles of the present invention may be used to increase iron and/or calcium uptake during iron and/or calcium deficiency, without interfering with the absorption of other divalent trace metals.

20

Applicant has characterised the role of nanoparticles of the present invention in cancer therapy using a xenograft colon cancer model. Fe-bLf was adsorbed on to calcium phosphate nanoparticles adsorbed with bLF, chitosan and alginate, and supplemented in control diet in such a way that final nanoformulated diet has 1.2% of lactoferrin protein. In contrast, control AIN93G formulated diet comprises casein. Nanoformulated diet with Fe-bLf loading was started 7 days before CaCo2 cancer cell injections. Mice fed with Fe-bLf loaded calcium phosphate nanoparticles adsorbed with bLF, chitosan and alginate diet did not develop any tumours. In contrast, all mice in the normal control diet develop tumours.

30

Applicant has also demonstrated nanoparticles comprising taxol when given orally to tumour bearing mice regressed tumours, a surprising result since a single injection of taxol only delayed the growth of tumours. In contrast, 65% of control mice fed with Fe-bLf normal (not nanoformulated) diet develop tumours.

Accordingly, in another aspect, the present invention provides a method of preventing or treating cancer in patient in need thereof comprising orally administering a therapeutically effective amount of a composition of nanoparticles described above.

5

In one embodiment, the nanoparticles are alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles.

10

In one embodiment the cancer is a solid tumour, a leukemia, lymphoma, multiple myeloma, a hematopoietic tumor of lymphoid lineage, a hematopoietic tumor of myeloid lineage, a colon carcinoma, a breast cancer, a melanoma, a skin cancer or a lung cancer.

15

In one embodiment the cancer is a leukemia such as but not limited to, acute leukemia, acute lymphocytic leukemia, acute granulocytic leukemia, acute myelocytic leukemia such as myeloblastic, promyelocytic, myelomonocytic, monocytic, erythroleukemia leukemia and myelodysplastic syndrome, chronic leukemia such as but not limited to, chronic myelocytic leukemia, chronic granulocytic leukemia, chronic lymphocytic leukemia, and hairy cell leukemia.

20

In one embodiment the cancer is a lymphoma such as but not limited to Hodgkin's disease and non-Hodgkin's disease.

25

In one embodiment the cancer comprises a hematopoietic tumor of myeloid lineage such as but not limited to acute and chronic myelogenous leukemia, smoldering multiple myeloma, nonsecretory myeloma and osteosclerotic myeloma.

30

In one embodiment the cancer comprises a hematopoietic tumor of lymphoid lineage, including leukemia, acute and chronic lymphocytic leukemia, acute and chronic lymphoblastic leukemia, B-cell lymphoma, T-cell lymphoma, Burkitts lymphoma. In one embodiment the cancer comprises a hematopoietic tumor of B lymphoid lineage. In one embodiment the cancer comprises a hematopoietic tumor of T lymphoid lineage.

In one embodiment the cancer is colon cancer or colorectal cancer.

In one embodiment the cancer comprises (a) a tumour that is at least about 0.3, 0.4 or 0.5 cm in diameter, or (b) a tumour that is refractory to therapy with one at least
5 one immunotherapeutic, anti-angiogenic or chemotherapeutic agent.

In one embodiment one or more of the white blood cell count, the red blood cell count, or the myeloid cell count of the subject is maintained or improved.

10 In one embodiment the tumour is reduced in size or substantially eradicated.

In some embodiments, the methods and/or compositions used herein reduce the severity of one or more symptoms associated with cancer by at least about any one of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% compared to the
15 corresponding symptom in the same individual prior to treatment or compared to the corresponding symptom in other individuals not receiving the methods and/or compositions of the present invention.

As used herein, an "effective amount" or a "therapeutically effective amount" of an
20 active agent means a sufficient amount of the active agent to provide the desired effect. The amount that is "effective" will vary from subject to subject, depending on the age and general condition of the individual, the particular active agent or agents, and the like. Thus, it is not always possible to specify an exact "effective amount." However, an appropriate "effective" amount in any individual case may be determined
25 by one of ordinary skill in the art using routine experimentation.

In one embodiment, the nanoparticles can be used to treat cancer or to treat or prevent cancer metastasis.

30 In another aspect, the present invention provides a method of preventing or treating cancer in patient in need thereof comprising orally administering a therapeutically effective amount of a composition of nanoparticles described above and an anti-cancer agent.

In another aspect, the present invention provides methods of treatment, wherein the composition comprises or is administered separately, simultaneously or sequentially with, at least one additional active agent. In one embodiment the at least one additional active agent is an anti-cancer agent.

5

In one embodiment, the therapeutically effective amount of a composition of nanoparticles described above and an anti-cancer agent are co-administered or administered simultaneously.

10 In another embodiment, the therapeutically effective amount of a composition of nanoparticles described above and an anti-cancer agent are administered sequentially.

In another embodiment, the therapeutically effective amount of a composition of
15 nanoparticles described above anti-cancer agent is administered prior to administration of an anti-cancer agent.

In one embodiment, the therapeutically effective amount of a composition of nanoparticles described above is administered for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12,
20 13, 14, 21, 28, 35, or 42 days prior to administration of an anti-cancer agent.

In one embodiment, the therapeutically effective amount of a composition of nanoparticles described above is administered for 7 days prior to administration of an anti-cancer agent.

25

Methods treating and preventing cancer are also described in WO/2008/140335, WO/2008/079030 and WO/2006/054908 each of which are incorporated herein by reference.

30 In one embodiment, the system can be used for targeted delivery of active agent delivery. For example, as many cancers disseminate through the lymphatic route, the nanoparticles described herein can not only treat primary tumours but can target the lymphatic tissue to destroy metastatic cells.

In another embodiment, the nanoparticles may be administered orally.

Size of the nanoparticle has an influence of lymph node accumulation as smaller particles accumulate more quickly than larger particles. For lymphatic delivery of an active agent, the size of the calcium phosphate nanoparticle conjugates, which may be in the form of agglomerated particles, can be about 10 micrometers or less, specifically about 1 micrometer or less, more specifically about 500 nanometers or less, and yet more specifically about 250 nanometers or less. Exemplary ranges include about 10 nanometers to about 10 micrometers, specifically about 25 nanometers to about 1 micrometer, more specifically about 50 nanometers to about 500 nanometers, and yet more specifically about 200 nanometers to about 300 nanometers.

In one embodiment, the nanoparticles can include a targeting ligand leading to selective cancer cell uptake and active agent release.

In another embodiment, the nanoparticles can be used as a combination therapy with radiotherapy, surgery, systemic chemotherapy, or a combination comprising at least one of the foregoing.

In one embodiment, the nanoparticles overcome drug resistance, as compared to the active agent used alone, via intracellular conjugate uptake. Specifically, the nanoparticles are prepared with an anticancer agent.

In another aspect, the present invention provides methods of treatment, wherein the composition comprises or is administered separately, simultaneously or sequentially with, at least one additional active agent. In one embodiment the at least one additional active agent is an anti-cancer agent.

In another aspect, the present invention provides the use of a composition of nanoparticles described above for the manufacture of a medicament for the treatment or prevention of cancer.

In another aspect, the present invention provides bio-distribution of biomolecules including drugs to tumour sites, eye, brain, kidney, brain, bone, spleen, blood, lung, muscle and heart – which could be employed in delivery of therapeutic molecules including drug molecules, siRNA, miRNA, protein and peptides to target these organ
5 related diseases or disorders.

In another aspect, the present invention provides a tools for the development of neutraceutical based nano delivery system for the delivery of anti-cancer bioactives and other bio-macromolecules including drugs, siRNA, miRNA, peptides, genes and
10 proteins.

In some embodiments the nanoparticle is produced by the method according to any one of the methods described above.

15 To examine the delivery of active agents, Applicant formulated nanoparticles using a number of methods described herein.

Throughout the description and the claims of this specification the word “comprise” and variations of the word, such as “comprising” and “comprises” is not intended to
20 exclude other additives, components, integers or steps.

EXAMPLES

EXAMPLE 1: Materials and methods:

25 Chitosan with a 20-200 cP viscosity, average molecular weight (MW) of 200kDa and deacetylation degree of 80%, sodium tri poly phosphate, sodium alginate and calcium phosphate nanoparticles were purchased from Sigma Aldrich, or produced using the methods described herein. Lissamine rhodamine B sulfonyl chloride was purchased from Invitrogen. Anti actin antibody was procured from Santa Cruz Biotechnology Inc,
30 HRP conjugated anti-lactoferrin antibody and FITC labelled anti-actin antibody were purchased from Sigma. Caco-2 Cell lines were obtained from ATCC. Nitrocellulose membranes for western blotting were from Amersham. All remaining chemicals including calcium chloride and acetic acid are of analytical grade.

EXAMPLE 2: Preparation of alginate adsorbed chitosan adsorbed lactoferrin (LF) adsorbed calcium phosphate nanoparticles ('ACSC-Lf NC').

ACSC-Lf NC were prepared by a combination of nanoprecipitation and ionic gelation, as described below. Calcium phosphate solution was prepared and incubated for 5 24 hours with 10% w/w lactoferrin (Lf) with constant stirring at 4 degrees C, for the proper adsorption of the protein on the NC and freeze dried. The obtained solution was centrifuged and washed several times to remove traces of unbound protein. 0.01% w/w chitosan, dissolved in acetate buffer was added to the calcium phosphate – lactoferrin nanocore under constant stirring and 0.01% of cross linking agent, 10 sodium tri polyphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$) was added drop wise. Constant stirring at 6000rpm for 12 h was performed to obtain a nanoparticle size of 200 ± 25 - 250 ± 25 nm. These nanoparticles were freeze dried and then adsorbed with alginate gel by using 1% w/v alginate solution and calcium chloride, with 0.6% mass ratio of Ca/alginate. The finally obtained nanoparticles were washed and freeze dried for further 15 characterization. Experiments were done at 4 degrees C to protect the polymeric and protein components in the formulation.

EXAMPLE 3: Preparation of alginate adsorbed chitosan adsorbed iron saturated LF adsorbed calcium phosphate nanoparticles ('AEC-CP NCs-Fe bLf NCs'). 20

Alginate adsorbed chitosan adsorbed iron saturated LF adsorbed calcium phosphate nanoparticles were prepared by a combination of nanoprecipitation and ionic gelation, as described below. Calcium phosphate (1%w/v solution) was prepared and incubated for 24 hours with 10% w/w lactoferrin (bLf) with constant stirring at 25 degrees C, at pH below 8.0, the isoelectric pH of lactoferrin in order to adsorb Lf on the nanoparticles. Following the electrostatic interaction of Lf onto the calcium phosphate, the obtained solution was centrifuged and washed several times to remove traces of unbound protein and then freeze dried. 0.01% w/w of chitosan solution in acetic acid was added to the calcium phosphate under constant stirring 30 and 0.01% w/w of cross linking agent, sodium tri polyphosphate was added drop wise with constant stirring has been result in nanoparticles in the size range of 200 ± 25 - 250 ± 25 nm. Nanoparticles were then freeze dried and incubated for 48 hours with 0.2% lactoferrin (Fe-bLf/bLf) with constant stirring at 4 degrees C, at pH below 8.0, the isoelectric pH of lactoferrin in order to adsorb Lf on the nanoparticles. After the

electrostatic interaction of Lf on to the NCs, the obtained solution was centrifuged and washed several times to remove traces of unbound protein. The formed nanoparticles were then adsorbed with alginate gel by using 2% w/v alginate solution and calcium chloride. The finally obtained nano particles were washed and freeze dried for further
5 characterization. All these experiments were done at 4 degrees C to protect the polymeric and protein components in the formulation.

EXAMPLE 4: Preparation of alginate adsorbed chitosan adsorbed LF adsorbed calcium phosphate nanoparticles.

10 AEC-CP NCs-Fe bLf NCs was prepared by a combination of nanoprecipitation and ionic gelation methods. Calcium phosphate was prepared from its constituents as follows – disodium hydrogen orthophosphate (Na_2HPO_4) in a molar ratio of 4:1 was added to calcium chloride (CaCl_2) in a drop wise manner, with continuous stirring. The obtained solution was sonicated at 40 degrees C to ensure a white precipitate of
15 calcium phosphate was obtained. Acquired calcium phosphate nanoparticle (1%w/v) solution was incubated for 24 hours with 10% w/w lactoferrin (Lf) with constant stirring at 4 degrees C, under a pH below 8.0, the isoelectric pH of lactoferrin in order to adsorb Lf on to the NC. After the electrostatic interaction of Lf on to the ceramic core, the obtained nanoparticles were centrifuged and washed several times to remove
20 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 cross linking agent, sodium tri polyphosphate was added in a drop wise manner. Constant stirring at 6000rpm for 12h was performed to result in nanoparticles in the size range of 200 ± 25 - 250 ± 25 nm, and the particles formed freeze
25 dried to ensure that the sample obtained spherical shape. The nanoparticles were then adsorbed with alginate gel by using 2% w/v alginate solution and calcium chloride. The obtained nanoparticles were washed and freeze dried for further characterization. All experiments were performed at 4 degrees C as to protect the polymeric and protein components in the formulation.

EXAMPLE 5: Preparation of Alginate enclosed chitosan coated hydroxyapatite (OHyAp) nanoparticles

Alginate adsorbed chitosan adsorbed hydroxyapatite (OHyAp) nanoparticles were prepared as described below. Calcium nitrate tetra hydrate ($\text{Ca}(\text{NO}_3)_4\text{H}_2\text{O}$) and

phosphoric pentoxide (P_2O_5) in 10:3 molar ratio were added. This solution was dissolved in ethanol and stirred for 24h at room temperature, to obtain a gel-like substance. This substance was then allowed to set at room temperature for 24h and dried in oven for at 80 degrees C for a same time period. Sintering process was carried out in a muffle furnace up to 600 degrees C at a rate of 5 degrees C/min to obtain OHyAp nanoparticles. (1%w/v) solution containing OHyAp nanoparticles was incubated for 24hours with 0.1% lactoferrin (Lf) with constant stirring at 4 degrees C, under a pH below 8.0 (the isoelectric pH of lactoferrin, where the protein will have a net positive charge), in order to adsorb Lf on to the nanoparticles. After the Lf has electrostatically and physically bound on to the OHyAp nanocore, the obtained solution was centrifuged and washed several times to remove traces of unbound protein and the nanoparticles formed freeze dried. Chitosan at a concentration of 0.01% w/w in acetate buffer (pH 4) was added to the OHyAp under constant stirring and 0.01% w/w of cross linking agent, sodium tri polyphosphate was added in a drop wise manner. Constant stirring at 6000rpm for 12 h was performed to result in nanoparticles in the size range of 200 ± 25 - 250 ± 25 nm, and were subsequently freeze dried to make sure that the nanoparticles obtained a spherical shape. The nanoparticles were then adsorbed with alginate gel by using 2% w/v alginate solution and calcium chloride. The obtained nanoparticles were washed and freeze dried for further characterization. All experiments were performed at 4 degrees C as to protect the polymeric and protein components in the formulation.

EXAMPLE 6: Preparation of alginate adsorbed chitosan adsorbed taxol or doxorubicin adsorbed calcium phosphate nanoparticles ('AEC-CP NCs-taxol or doxorubicin NCs')

AEC-CP NCs-taxol or doxorubicin NCs were prepared by a combination of nanoprecipitation and ionic gelation methods as follows. Calcium phosphate (1%w/v) solution was incubated for 24hours with 0.1% anti cancer drug taxol or doxorubicin with constant stirring at 40C; at a pH below 8.0 in order to electrostatically adsorb taxol or doxorubicin on to the nanoparticle. Following the adsorption of taxol or doxorubicin on to the calcium phosphate nanoparticle, the obtained nanoparticles were centrifuged and washed several times to remove traces of unbound anti-cancer agent and freeze dried. 0.1% w/w of chitosan solution in acetate buffer (pH4) was added to the calcium phosphate under constant stirring and 0.01% w/w of cross

linking agent, sodium tri polyphosphate was added in a drop wise manner. Constant stirring at 6000rpm for 12 h was performed to result in nanoparticles in the size range of 200 ± 25 - 250 ± 25 nm. The samples were then freeze dried to ensure the nanoparticles are of spherical shape. The formed nanoparticles were then adsorbed with alginate gel by using 2% w/v alginate solution and calcium chloride. The obtained nanoparticles were washed and freeze dried for further characterization. . All experiments were done at 4 degrees C as to protect the polymeric and protein components in the formulation.

EXAMPLE 7: Preparation of iron saturated bovine lactoferrin (Fe-bLf):

Alginate adsorbed chitosan adsorbed iron saturated bovine lactoferrin adsorbed calcium phosphate nanoparticles ('AEC-CP NCs-Fe bLf NCs") were prepared as follows. Iron saturated bovine lactoferrin was prepared according to the methods disclosed in PCT/NZ2008/000105, PCT/NZ2007/000389 and PCT/NZ2005/000305.

In brief, bovine Lf, after alkaline treatment, was dialysed for a period of 48 hours in 0.1M citric acid to get rid of the bound metal ions and then saturated with Fe(III) coordinate compounds for the development of deep red coloured Fe-bLf. The obtained Fe-bLf was adsorbed on to the AEC-CP NCs-Fe bLf NCs and subsequently adsorbed with alginate to examine size of the particles produced and effects on cancer cells.

EXAMPLE 8: Characterization of AEC-CP Lf NCs

The average size of the nanoparticles, in various steps of their preparation compared to calcium phosphate, Lf adsorbed calcium phosphate, chitosan adsorbed, and alginate adsorbed nanoparticles was determined by Dynamic light scattering, using zetasizer, after 500 fold dilution with autoclaved milliQ water. Surface morphology of these particles was determined by Secondary electron microscopy (supra), at an accelerating voltage of 5-10 kV. To prepare samples for SEM; C-CP NCs, C-CP NCs-Lf, and AEC-CP NCs-Fe-bLF NCs were fixed on the stub by a double-sided sticky tape and then coated with gold layer by SC7620 sputter coater (Quorum technologies, UK) for 60s.

EXAMPLE 9: Cell culture and cell viability

Caco2 cell lines were cultivated in T-75 flasks using Dulbecco's modified eagle's medium (ATCC) with 10% heat inactivated foetal bovine serum (sigma), 1%v/v penicillin–streptomycin (sigma) and with a 1% glutamex supplement. Cell culturing was carried out at 37 degrees C in 95% relative humidity and 5% CO₂. Cultures were seeded at 10⁵ cells per well in multi well plates, and were grown for 12 days for expression of oligosaccharide and/or lactoferrin receptors with a nutrient supplement, once in 3 days. Viability of Caco2 cells after treatment with 50ug/ml AC-CP NCs-Lf NCs and Fe-bLf containing AEC-CP NCs-Fe-bLF NCs were determined by using TUNEL assay, in situ cell death detection kits, Roche, according to manufacturer's instructions. Briefly, Caco2 cells were treated for 24, 48 hr time intervals, fixed using 4% PFA and resuspended in TUNEL reaction solution containing label solution and enzyme solution, after permeabilization. Cells were later washed and observed for fluorescence using flow cytometry, using FITC detector.

EXAMPLE 10: Apoptosis versus necrosis.

In order to determine the nature of cell death following treatment with different preparations of NCs and difference between apoptosis and necrosis was determined by TUNEL staining using an in situ apoptosis detection kit from Boehringer Mannheim as per kit instructions. Slides were also stained with propidium iodide (Sigma-Aldrich) to distinguish necrotic cells from those undergoing apoptosis. Same slides were counterstained with methylene blue-staining and mounted. 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 (N/I) = number of apoptotic or necrotic cells x 100/total number of nucleated cells. Following treatments, another kit, Annexin-VFLUOS staining (Roche Applied Science), was also used to differentiate apoptotic cells from necrotic cells, as per manufacturer's instructions. This assay is based on principle that phosphatidyl serine, located in the inner leaflet of the cell membrane, is exposed at the cell surface in the early stage of apoptosis. Annexin V shows high-affinity for phosphatidyl serine-binding that makes it a useful selective and powerful tool for detection of apoptotic cells.

EXAMPLE 11: Endocytosis studies of the nano particles.

Nanoparticle formulation (50ug/ml) prepared in sterile phosphate buffered saline (PBS pH 7.4) was treated on to the wells, having Caco2 cells grown for 12 days. The cells

were incubated for 6 hours, washed and lysed by using Radio Immunoprecipitation Assay (RIPA) buffer. The obtained lysates were preserved for further studies. For Immunoblotting, 15 µg of Lf, lysates from the Caco2 cells was separated by SDS PAGE, by using 10% gel. The separated proteins then were transferred to a PVDF membrane after proper charging, for 90 min at 0.35 mA and 100 V. following transfer, the membrane was removed, rinsed with TBS and incubated overnight in 1:500 dilution of goat anti Lf antibodies, and later with anti goat HRP conjugated antibodies at 1:60000 dilution. Chromatogram was developed by using ECL (Invitrogen) chemiluminescence detection kit.

EXAMPLE 12: Flowcytometry and immunofluorescence staining.

Caco-2 cells were grown in 6 well plates for 12 days for the expression of the surface oligosaccharide receptors and were treated with 50 µg/ml rhodamine loaded cap, C-CP NCs, C-CP NCs-Lf, and AEC-CP NCs-Fe-bLf NCs, for different time intervals from 12 hours to 72 hrs. After every successful treatment, cells were trypsinized, washed with PBS several times and permeabilized with 90% ethanol. The cells then were acquired by using BD FACS canto II flowcytometer. For immunofluorescence measurements by using confocal microscopy, we have grown the Caco-2 cells in sterile 8 well chamber slides (BD) and stained with anti actin and anti lactoferrin antibodies. The slides were observed under Leica confocal microscopy after staining with fluorescent labelled secondary anti bodies and nuclear staining with DAPI. For intracellular localization of Lf loaded nanoparticless, HRP conjugated anti Lf antibody was added to the slide pre stained with goat anti Lf antibody. The immuno complexes were detected by using DAB substrate.

EXAMPLE 13: Assessment of in vitro transport of NCs across CaCo-2 cell monolayer.

Internalization of NCs were analysed by transepithelial electric resistance (TEER) assay using standard methods with slight modifications. Briefly, Caco-2 cells were grown in 6 well transwell inserts (millipore) for 24 days, with constant supply of fresh DMEM for every third day. After formation of monolayer, transwell plates were washed with HBSS for removal of unattached and/or dead cells, resistance of the monolayer was measured by using millicell ERS instrument (Millipore). Monolayers in which TEER values were higher than 500 have been selected for further processing.

These monolayers were incubated in fresh medium without pH indicator, before treatment with 50ug/ml of fluorescent nanoparticles. TEER values of the monolayers were measured for different time intervals ranging from 0hr, 2hr, 6hr, 24hr, 48hr and 72 hrs. Periodically, 100ul of medium from the apical and basolateral sides was withdrawn and fresh medium was supplied. Relative fluorescent units (RFU) for all the samples after 72hours incubation were measured using a fluorescence plate reader (MTP 601F, Techomp), at excitation wavelength of 490nm and 530nm of emission wavelength.

EXAMPLE 14: Animal model of human xenograft colon cancer

A human cancer xenograft model of colon cancers was developed with CaCo2 cancer cells. Tumours were established by s.c. injection of 2×10^6 tumour cells into the left flank of mice. All animal procedures were done in accordance with the guidelines from Deakin University, Australia. All animal experiments were including 6 mice per treatment group and five to six week old female BALB/c SCID (immunocompromised) in the study. The mice were fed either control AIN 93G diet or AIN 93G supplemented with either or following nanoparticle preparations: i) CEC-CP-Lf NCs, ii) CEC-CP-Taxol/doxorubicin nanoparticles. Chemotherapeutic drugs were injected in a volume of 0.01–0.02ml/g body weight once tumours reached $\cong 400\text{mm}^3$. Diet intake was monitored. Tumour size was assessed weekly using Vernier callipers. Mice health was closely monitored. Mice were weighed thrice weekly and assessed for signs of any physical or physiological distress. At the end of experiments mice were euthanized. Bovine casein in the control AIN93G diet was supplemented in the experimental diets with bLf such that the protein content of the diet was unchanged. The diets contained 1.2% (w/w) bLf, unless otherwise indicated.

EXAMPLE 15: Physical characterisation of calcium phosphate nanoparticles adsorbed with iron loaded bovine lactoferrin (Fe-bLF), chitosan and alginate.

A schematic diagram of the predicted molecular structure of calcium phosphate nanoparticles adsorbed with Fe-bLF, Chitosan and alginate is shown in Figure 1a. Lactoferrin was filtered through a 100nm filter to trounce the agglomerates and adsorbed onto the calcium phosphate nanoparticles. The calcium phosphate nanoparticles then were adsorbed with a thin chitosan (0.1%w/v in acetic acid) film. A

non-toxic poly anion, sodium tripolyphosphate was used to make cross linkages between positively charged amine groups within the chitosan. DLS spectrometric results indicated an increase in size of nanoparticles after adsorbing with chitosan and/or alginate, from 200 ± 25 nm for calcium phosphate nanoparticles (ceramic 'cores') to 350 ± 25 nm following complete formation of calcium phosphate nanoparticles adsorbed with Fe-bLF, chitosan and alginate ('AEC-CP NCs-Fe-bLF NCs'; Figure 1c). SEM results further supported the DLS data and it also determines spherical morphology of nanoparticles (Figure 1b), demonstrating the advantage in transporting the anti-cancer agents to the cancer tissue via mucosal, gastric and subsequent systemic circulation as exemplified in Figure 6. Incorporation of iron saturated bovine lactoferrin (Fe-bLf) into calcium phosphate nanoparticles adsorbed with chitosan and alginate does not affect the size of the nanoparticles as shown in Figure 1d and Figure 1e.

EXAMPLE 16: Controlled release of calcium phosphate nanoparticles adsorbed with Fe-bLF.

Chitosan and alginate release was analysed by SDS PAGE gel retardation assay. Absence of Lf specific bands in positive control indicates immobilization of calcium phosphate nanoparticles adsorbed with Fe-bLF, Chitosan and alginate in the stacking gel. Further, retardation of calcium phosphate nanoparticles adsorbed with Fe-bLF, Chitosan and alginate in the stacking gel were indicated by intense bands in stacking gel as well as Lf specific bands in western blots, developed by using Lf specific antibodies (Figure 2a). To investigate the stability of calcium phosphate nanoparticles adsorbed with Fe-bLF, Chitosan and alginate in the acidic pH in the stomach and alkaline pH in the intestine, calcium phosphate nanoparticles adsorbed with Fe-bLF, Chitosan and alginate were treated with HCl pH 1.2 and with phosphate buffered saline at pH 7.4, for different time intervals from 1hr-6 hrs. The resultant samples were then analysed by SDS PAGE, as shown in the left panel of Figure 2b, more intense bands were observed from 4 hours of incubation time in alkaline medium, representing the release of calcium phosphate nanoparticles adsorbed with Chitosan by dissolution of outer alginate covering. In contrast, in acidic medium there were no bands until 6 hrs. Successive western blots indicate the clear bands of Lf confirm the findings observed by SDS-PAGE (Figure 2b).

EXAMPLE 17: Cell viability of cells exposed to calcium phosphate nanoparticles adsorbed with Fe-bLF

Chitosan and alginate at a concentration of 50ug/ml, were used to test the cell viability by doing TUNEL assay. As indicated in Figure 3a, there was no difference in the amount of fluorescent signal in calcium phosphate nanoparticles adsorbed with Fe-bLF, Chitosan and alginate treated cell lines (green) was observed in comparison to control (red), which indicates no toxicity of calcium phosphate nanoparticles adsorbed with Fe-bLF, Chitosan and alginate. The same concentration was used for further experiments done to characterize the nanoparticles in vitro.

EXAMPLE 18: Internalization of nanoparticles by endocytosis.

To assess internalization of calcium phosphate nanoparticles adsorbed with LF and chitosan, Caco-2 cells were treated with 50ug/ml calcium phosphate nanoparticles adsorbed with LF and chitosan for a time period of 24 hours and lysed using RIPA buffer. The obtained lysates were analysed by 10% SDS PAGE (Figure 3b). Lf specific bands were confirmed by doing immunoblotting with anti Lf antibodies (Figure3b). Rhodamine labelled calcium phosphate nanoparticles adsorbed with chitosan ('C-CP NCs'), calcium phosphate nanoparticles adsorbed with LF and chitosan ('C-CP NCs-Lf') and calcium phosphate nanoparticles adsorbed with LF, chitosan and alginate ('AC-CP NCs Lf NCs') were used for the quantification of endocytosis of the nanoparticles in Caco-2 cell lines.

Caco-2 cells were treated with 50ug/ml of the rhodamine labelled nanoparticles for a time period ranging from 12 hours to 72 hrs. Flowcytometric analysis shows an increase in cellular uptake of calcium phosphate nanoparticles adsorbed with chitosan from 17.5% in 12 hours to 72.2% in 72 hours, whereas increase was observed to be prominent in calcium phosphate nanoparticles adsorbed with Lf and chitosan, which is 16.35% initially in 12hours and increases to a massive 89.4% at the time period of 72 hrs. In the case of calcium phosphate nanoparticles adsorbed with Lf, chitosan and alginate the percentage of endocytosis was increased to 58-62% from 6% in 12 hours (Figure 3c). To determine qualitative endocytosis, rhodamine-labelled calcium phosphate nanoparticles adsorbed with Lf and chitosan were added to Caco-2 cell monolayers grown 8 well chamber slides. The cytoskeleton was stained with anti actin antibody and nuclear staining was done with DAPI (Figure 4a). The micrographs

indicate cytoplasmic and nuclear localization of calcium phosphate nanoparticles adsorbed with Lf and chitosan. Cytoplasmic and nuclear localization is also evident from immunocytochemical observation for Lf specific immunoreactivity in Caco-2 cell monolayer after treatment with calcium phosphate nanoparticles adsorbed with Lf and chitosan (Figure 4b).

EXAMPLE 19: Transcytosis of calcium phosphate nanoparticles adsorbed with Fe-bLf, chitosan and alginate

Caco-2 cells were grown in transwell plates for a time period of 24 days to form a monolayer and were treated with 50ug/ml Rhodamine labelled calcium phosphate nanoparticles adsorbed with chitosan ('C-CP NCs'), calcium phosphate nanoparticles adsorbed with LF and chitosan ('C-CP NCs-Lf') and calcium phosphate nanoparticles adsorbed with LF, chitosan and alginate ('AC-CP NCs Lf NCs'), in different transwells respectively. Trans-epithelial electrical resistance (TEER) was measured at regular time intervals after the treatment for 2hr, 4hr, 6hr, 24hr, 48hr and 72 hrs; for any difference compared to initial (0hr) treatment. TEER values were found to be unaffected (Figure 5a) until 72 hours, indicating the nanoparticles do not affect the integrity of monolayer by opening tight junctions. However, the results indicate a slight decrease in TEER values in monolayers treated with calcium phosphate nanoparticles adsorbed with chitosan ('C-CP NCs') and calcium phosphate nanoparticles adsorbed with Lf and chitosan ('C-CP NCs-Lf') was observed at 72 hours of treatment. In contrast, monolayers treated with calcium phosphate nanoparticles adsorbed with Lf, chitosan and alginate have not shown any decrease in TEER values even after 72 hrs.

The amount of relative fluorescence units was measured as a function of transcytosis on both apical and basolateral sides. As shown in Figure 5b there is an increase in transcytosis in C-CP NCs-Lf and C-CP NCs from 2hr to 72 hours compared to AEC-CP NCs-Fe bLf NCs, indicating an improved transcytosis with decrease in size of nanoparticles.

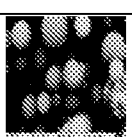
EXAMPLE 20: Prevention and regression of cancer using nanoparticles

To examine the role of nanoparticles in cancer therapy, a xenograft colon cancer model was used. Fe-bLf was adsorbed on to calcium phosphate nanoparticles

adsorbed with bLF, chitosan and alginate, and supplemented in control diet in such a way that final nanoformulated diet has 1.2% of lactoferrin protein. While control AIN93G formulated diet have casein. Nanoformulated diet with Fe-bLf loading was started 7 days before CaCo2 cancer cell injections. Mice fed with Fe-bLf loaded calcium phosphate nanoparticles adsorbed with bLF, chitosan and alginate diet did not develop any tumours. In contrast, all mice in the normal control diet develop tumours. Furthermore, nanoparticles comprising taxol when given orally in the tumour bearing mice regressed tumours while normal single injection of taxol only delayed the growth of tumours. In contrast, mice fed with Fe-bLf normal (not nanoformulated) diet, develop tumour in 65% mice while the remaining mice do not develop tumours. Figure 7 shows a nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed nanoparticles ("AEC-CP-Lf NCs") diet inhibits tumour growth without causing any toxicity. Figure 8 shows a Nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ("AEC-CP-Lf NCs") and taxol adsorbed calcium phosphate nanoparticles ("AEC-CP-Taxol NCs") diet inhibits tumour growth without causing any toxicity. AEC-CP-Lf NCs were fed one week prior to cancer cell injections.

EXAMPLE 21: Effect of different constituents on the preparation of nanocarriers of AEC-CP-Lf NCs

S. No.	Constituents (w/w) [#]	Conditions	Size	Morphology and observations		E.E [*]
1.	Lf – 5% Chitosan – 10% STPP – 10%	Agitation for 30min	> 1 μ m		Non particulate slurry	60%
2.	Lf – 5% Chitosan – 1% STPP – 1%	Stirring 6000 rpmX 30min	> 1 μ m		Non particulate slurry	60%
3.	Lf – 5% Chitosan – 1% STPP – 1%	Stirring for 6000rpmX 1h	> 1 μ m		Rod like consistency	73%
4.	Lf-10% Chitosan -1% STPP – 1%	Stirring for 6000rpmX 4h	800nm -1 μ m		Non homogenous particles	80%

5.	Lf -10% Chitosan - 1% STPP – 1%	Stirring 6000rpm X 6h	400- 450nm		Non homogenous particles	80%
6. Method 1	Lf – 10% Chitosan - 0.1% STPP - 0.1%	Stirring 6000rpmX 6 h	250- 300nm		Spherical particles	80%
7. Method 2	Lf – 10% Chitosan - 0.1% STPP - 0.1%- Lf – 10%	Stirring 6000rpmX 6 h	250- 300nm		Spherical particles	85%
8.	Lf-10% Chitosan- 0.01% STPP-0.01%	Stirring 6000rpmX1 2 h	200±2 5 - 250±2 5 nm		Spherical particles	80%
9	Lf-10% Chitosan- 0.01% STPP- 0.01% Alginate 2%w/v	Stirring 6000rpmX 12 h	350±2 5nm Followi ng coating with alginat e		Spherical particles	80%

(#= with respect to Cap core * =encapsulation efficiency)

EXAMPLE 22: Pharmacokinetic studies after oral administration of Fe-bLf and 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 body weight of mice, the relevant pharmacokinetic parameters were determined. 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 labelled with rhodamine/ coumarin-6 as an oral administration. Fe-bLf dose in both cases was fixed at 100 mg/kg body weight. Prior to and 30 min, 60 min, 1 h, 2 h, 3 h, 6 h, 8 h, 10 h, 12 h, 24 h, 36 h, 72 h, 5 D, 7 D and 10 days after administration, blood was harvested from the tail vein into heparinized microcentrifuge tubes (containing 20 µl 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
 5 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.

EXAMPLE 23: Estimation of intracellular iron by calorimetric method.

The procedure for measuring iron saturation in the tissues was modified as follows. To 1 ml
 10 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 sec and allowed to stand for 10 min and
 15 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 coloured 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,
 20 Cambridge, UK). Iron concentration was determined by comparing the resulting absorbance values taken with the iron standards.

EXAMPLE 24: Measurement of intracellular calcium $[Ca^{+2}]_i$:

Fura-2/acetoxymethyl ester (AM) was used to measure calcium concentration. In brief,
 25 serum samples were incubated with 3mM Fura-2/AM for 30 min at 37 $^{\circ}$ C in the dark and washed with Krebs/HEPES buffer (143.3 mM Na^{+} , 4.7 mM K^{+} , 2.5 mM Ca^{+2} , 1.3 mM Mg^{+2} , 125.6 mM Cl^{-} , 25 mM HCO_3^{-} , 1.3 mM $H_2PO_4^{-}$, 1.2 mM SO_4^{-2} , 11.7 mM glucose and 10 mM HEPES, pH 7.4) to remove extracellular Fura-2/AM. The loaded serum samples were further incubated at the room temperature for de-esterification of the Fura-2/AM. Excitation
 30 wavelength of 510 nm and an emission at 340 and 380 nm were recorded. The $[Ca^{+2}]_i$ was calculated by ratio of fluorescence at 340/380 nm using Grynkiewicz method as represented below:

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

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

and Ca^{+2} free conditions and Q is the ratio of $F_{\min}/F_{\max}$ at 380nm. the calibration values were determined in the presence of 1mM of Ca^{+2} or 10mM EGTA ($R_{\min}$).

EXAMPLE 25: Pharmacokinetic parameters of Fe-bLf and AEC-CP-Fe-bLf NCs

Formulation	Fe-bLf Dose (mg/Kg body Wt of mice)	$C_{\max}$ (ng/ml)	Tmax (h)
Fe-bLf	100	560 ± 40	3
AEC-CP-Fe-bLf NCs	100	1130 ± 80	8

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

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EXAMPLE 26: Weight of mice, organs, serum iron, serum calcium and blood haematological 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 to 28.5 ± 6.5	22.3 ± 4.5 to 24.0 ± 5.8	22.5 ± 5.1 to 26.5 ± 6.0	23.2 ± 4.5 to 32.0 ± 5.8
Spleen (mg)	$95.5 \pm 11.2^*$	72.0 ± 5.8	$96.5 \pm 10.2^*$	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
RBCs ($\times 10^6/\text{mm}^3$) ^a	$8.2 \pm 2.5^{**}$	2.1 ± 1.5	$9.5 \pm 2.5^{**}$	4.5 ± 1.5
Serum iron ($\mu\text{g/g}$)	$175 \pm 25^{**}$	95 ± 15	$215 \pm 35^{**}$	155 ± 20
Serum calcium (mg/dl)	8.9 ± 0.8	6.5 ± 0.5	$12.9 \pm 1.2^{**}$	8.5 ± 0.8

^aMean values of red blood cell (RBC) counts were recorded in blood samples collected directly from the heart at the time of autopsy.

EXAMPLE 27: Fe-bLf concentration in plasma at different time points following oral administration

Figure 9 shows the mean Fe-bLf concentration in plasma at different time points following 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 of Fe-bLf only, at all the time points studied (Figure 9). Oral delivery of AEC-CP-Fe-bLf ensured a sustained release of Fe-bLf over 6-8 h post feeding, whereas, in case of Fe-bLf the levels declined significantly after 3 h and were not detectable beyond 72 h. Plasma Fe-bLf concentration was increased in AEC-CP-Fe-bLf NCs administered mice, with detectable levels of Fe-bLf up to 10 days post treatment. As seen in Figure 10, increased levels of Fe-bLf was detected in the tumour tissue followed by intestine and blood. Organs involved in biodistribution of biomolecules including liver, spleen, kidney have also showed increased Fe-bLf concentration. In order to detect the presence of NCs in the systemic circulation, blood samples that were collected 5 day post administration of rhodamine labelled NCs were analysed.

Studies including measurement of body weight, organs of mice indicated normal weight of these organs. Furthermore blood haematological profile of control and experimental mice with nanodiet have shown no abnormality in RBC, serum iron and serum calcium levels (Example 26). Interestingly nanoformulated diets increased the RBCs, WBCs, serum calcium and serum iron as compared to control mice fed with normal diet. Confirming that nanodiet help in absorption of calcium and iron.

EXAMPLE 28: Nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ("AEC-CP-Lf NCs") increased clearance of *Giardia lamblia* parasites.

Enumeration of *Giardia* trophozoites and cysts in Feces

The parasite load in suckling BALB/C mice infected with trophozoites of the standard strain, Portland 1 strain of *Giardia lamblia* reached a peak of around 10^7 trophozoites per mouse at 7 days of post infection and 10^5 at 9 days of post infection whereas no

trophozoites were found in the mice after 2 days of treatment with nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ("AEC-CP-Lf NCs") or 9 days of post infection. The mice were cleared of *Giardia* subsequently after drug treatment but the infection persisted in mice in the untreated group for 27 days.

Figure 12 shows nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ("AEC-CP-Lf NCs") increased clearance of *Giardia lamblia* parasites. Graph shows cyst counts in the small intestine of mice after infection with $10^7/0.1\text{ml}$ of trophozoites of *Giardia lamblia* (Portland 1) belonging to different groups. Values are represented as mean $\pm$ SD. Panel A shows a photomicrograph of higher magnification (H&E, 200X) of the small intestine of normal mice in the control group. The ileum shows long, normal villi, lining cells including the brush border & goblet cells are normal. Panel B shows a photomicrograph of higher magnification (H&E, 200X) of the small intestine of mice in the infected group. Higher magnification highlights the parasites. The villi show mild excess of LMN cells, occasional villous is swollen, lining cells are normal, some crypts show paneth cell hyperplasia, surface shows presence of parasites. Figure 13 shows nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ("AEC-CP-Lf NCs") decrease *Giardia lamblia* trophozoite counts in the small intestine.

The cyst load was least from the beginning, i.e., day 3 onwards, in mice (*Giardia*-infected). Excretion of cysts in feces (Fig. 13) increased gradually on day 7 post inoculation in all the groups of mice. The period of maximum cyst release was between days 7 & 11 of infection & after day 11 decline in no. of cysts was observed in the infected group.

Mice became *Giardia* free by day 9 p.i or after 2 days of treatment with nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles (AEC-CP-Lf NCs) whereas the infectivity period was much more prolonged (Fig.12) up to 18 days more and cyst count was much higher from the beginning in the *Giardia*-infected group. But in the bLf treated and metronidazole

treated group the trophozoite and cyst score declined only after 7 days of treatment or 14 days post infection.

Maximum colonization of the parasite was seen in the middle segment, jejunum of the small intestine. Accordingly, the nanoparticles of the present invention result in increased elimination of cysts and shortening the period of infectivity. Moreover no toxic effect of the nanoparticles was observed.

Histopathological changes:

The results of histopathological examination of the intestinal tissue sections of infected mice suggest that *Giardia lamblia* can alter the architecture of the intestinal mucosa through hyperplasia of the crypts and atrophy of the villi. Mild infiltration of lymphomononuclear in the lamina propria alongwith the vacuolated epithelial cells and reduced goblet cells is also observed (Fig. 12 B). As compared to normal mice in control group ileum shows normal villi and normal brush border cells and goblet cells (Fig. 12 A).

EXAMPLE 29: Nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles ("AEC-CP-Lf NCs") decrease *Salmonella* infection of the small intestine.

Balb/c mice were inoculated with about 200 μ l of 10^8 CFU/ml of *Salmonella typhimurium* (wild type strain). After 3 days of infection, on the 4th day mice were treated with nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticle diet, bovine lactoferrin (bLf) or Ciproflaxacin.

Efficacy of oral treatment with nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles for reducing the number of bacteria in the small intestine of Balb/c mice infected with 200 μ l of 10^8 CFU/ml of *Salmonella typhimurium* (wild strain) after 6-20 days of post infection/treatment as compared with the bovine lactoferrin treated mice; positive control, Ciprofloxacin treated mice and the normal mice. Values are represented as mean $\pm$ SD.

Figure 14 shows after 6 days post infection or two days of drug treatments, bacterial load reached peak in the small intestine of infected mice but infection was almost cleared in mice fed with nanoparticles of the present invention. Bacterial load was also observed in the small intestine in ciprofloxacin and bovine Lf treated groups.

5

EXAMPLE 30: Presence of *Salmonella* bacteria in fecal samples of mice treated with nanoformulated alginate adsorbed chitosan adsorbed lactoferrin adsorbed calcium phosphate nanoparticles.

- 10 The presence of bacteria was checked in the fecal samples of mice in the infected and drug treated groups to check the persistence of infection on 3rd, 6th and 10th and 20th day of infection:

p.i. days	Infected group (Balb/c mice inoculated 10^6 CFU/ml of <i>S. typhimurium</i>)	Mice fed with Lf Np's diet	Mice fed with Oral bovine Lf	Mice fed with antibiotic (ciprofloxacin)
3 rd day	+	+	+	+
6th	+	-	+	+
10th	-	-	-	-
20th	-	-	-	-

+ sign indicates fecal sample of bacteria positive for presence of infection with *Salmonella typhimurium* in mice

- sign indicates fecal sample of bacteria negative for absence of infection with

5 *Salmonella typhimurium* in mice

After 10 days of infection or 6 days of treatment, no bacterial count was observed in the bovine Lf treated group and the group treated with nanoparticles of the present invention, small count of bacteria was seen in the ciprofloxacin treated group and large numbers in the infected group with no drug treatment. After 20 days of infection of *Salmonella typhimurium* in mice, infection was resolved in all the infected & treated groups.

15 While the foregoing written description of the invention enables one of ordinary skill to make and use what is considered presently to be the best mode thereof, those of ordinary skill will understand and appreciate the existence of variations, combinations, and equivalents of the specific embodiment, method, and examples herein. The

invention should therefore not be limited by the above described embodiment, method, and examples, but by all embodiments and methods within the scope and spirit of the invention as broadly described herein.

CLAIMS

1. A method for producing a nanoparticle comprising calcium phosphate for delivery of an active agent, said method comprising;

5 providing an aqueous mixture of calcium phosphate and at least one active agent; and

adsorbing the at least one active agent on the calcium phosphate.

10 2. A method according to claim 1 wherein the calcium phosphate is a calcium phosphate core.

3. A method according to claim 1 or claim 2 further comprising forming an aqueous mixture of an intestinal absorption enhancer and the calcium phosphate adsorbed with the at least one active agent; and

15 adsorbing the intestinal absorption enhancer on the calcium phosphate adsorbed with the at least one active agent

4. A method according to claim 3 wherein the intestinal absorption enhancer is chitosan

20

5. A method according to claim 4 wherein the adsorbing with Chitosan is performed in the presence of a crosslinking agent

25 6. A method according to claim 4 or claim 5 wherein the adsorbing with Chitosan is performed using ionic gelation

7. A method according to any one of claim 4 to 6 wherein the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture to obtain a particle size of at least 200nm.

30

8. A method according to claim 7 wherein the adsorbing with Chitosan is performed with constant stirring of the aqueous mixture at 6000rpm for 6 hours.

9. A method according to any one of claims 1 to 8, further comprising

forming an aqueous mixture of an enteric coating and the Chitosan adsorbed at least one active agent adsorbed calcium phosphate; and

adsorbing the enteric coating on the Chitosan adsorbed at least one active agent adsorbed calcium phosphate

5

10. A method according to claim 9 wherein the enteric coating is alginate

11. A method according to claim 10 wherein the adsorbing with alginate is performed using ionic gelation

10

12. A method according to any one of claims 1 to 11, wherein the calcium phosphate is freeze dried prior to forming the aqueous suspension

15

13. A method according to any one of claims 1 to 12, further comprising freeze drying the produced nanoparticles.

14. A method according to any one of claims 1 to 13, wherein the active agent is lactoferrin.

20

15. A method according to any one of claims 1 to 13, wherein the active agent is an anti-cancer agent

16. A method according to claim 15, wherein the active agent is taxol.

25

17. A nanoparticle produced by the method according to any one of claims 1 to 16.

18. A composition of nanoparticles for delivery of an active agent, each nanoparticle comprising:

a calcium phosphate core adsorbed with at least one active agent.

30

19. A composition according to claim 18 wherein the nanoparticles are further adsorbed with an intestinal permeability enhancer.

20. A composition according to claim 19 wherein the intestinal permeability enhancer is Chitosan

21. A composition according to claim 19 or claim 20 wherein the nanoparticles are
5 further adsorbed with an enteric coating.

22. A composition according to claim 21 wherein the enteric coating is alginate

23. A composition according to any one claims 18 to 22 wherein the nanoparticles
10 are freeze dried.

24. A composition according to any one claims 18 to 22 wherein the nanoparticles have a mean particle size of between 200 and 250 nm

15 25. A pharmaceutical composition comprising the composition of any one of claims 18 to 24 and a pharmaceutically acceptable carrier.

26. A pharmaceutical composition according to claim 25 wherein the pharmaceutical composition is formulated for oral administration.

20

27. A method of treating cancer in patient in need thereof comprising orally administering a therapeutically effective amount of a composition of nanoparticles according to any one of claims 18 to 25.

25 28. A method of preventing or treating gastrointestinal disease in patient in need thereof comprising orally administering a therapeutically effective amount of a composition of nanoparticles, according to any one of claims 18 to 25.

29. A method according to claim 28 wherein the gastrointestinal disease is caused
30 by a bacterial pathogen or a protozoan pathogen.

Figure 1

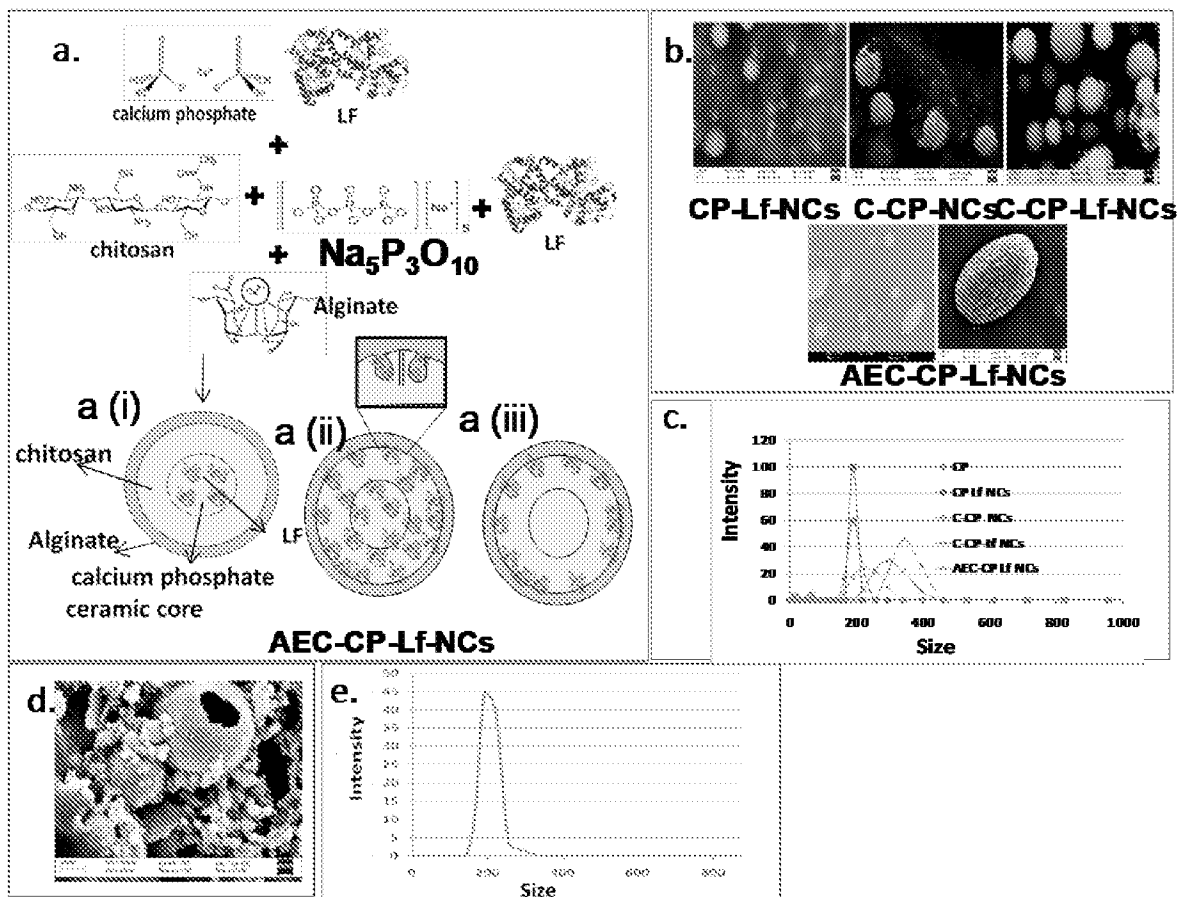


Figure 2

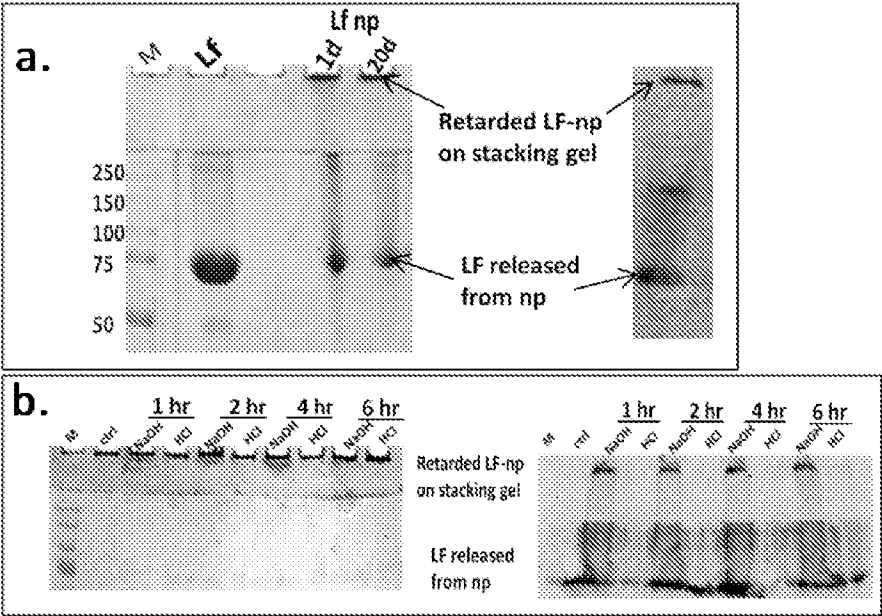


Figure 3

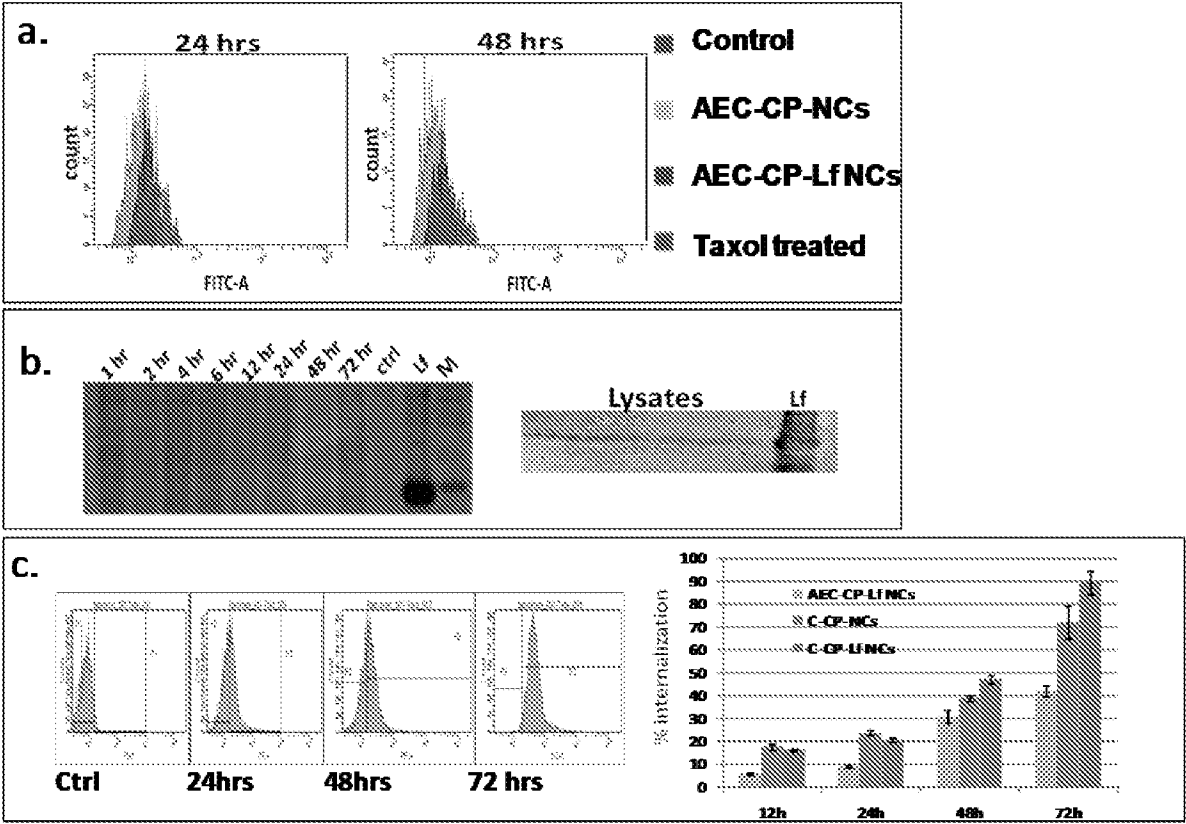


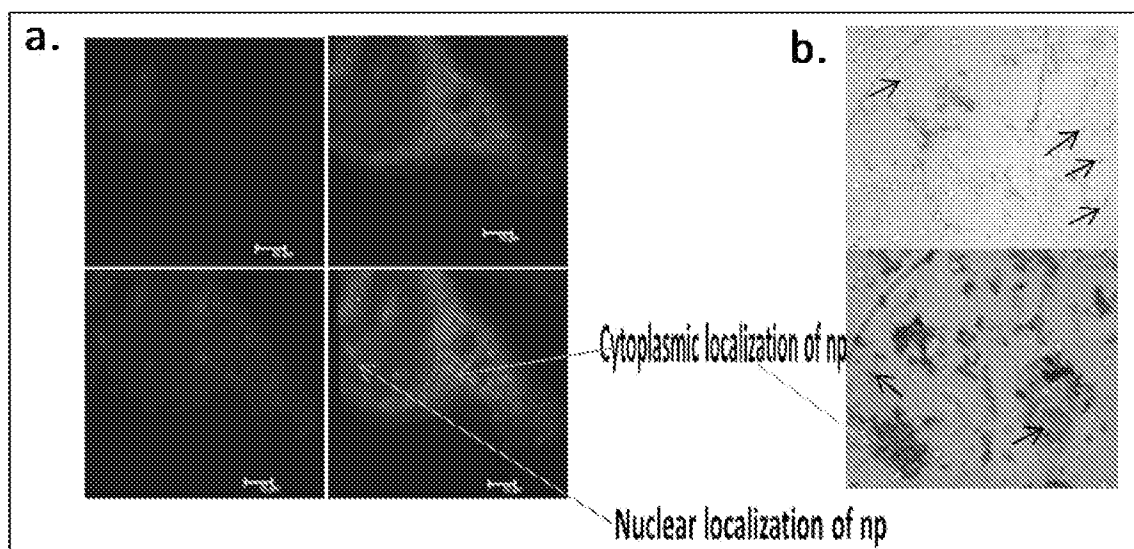
Figure 4

Figure 5

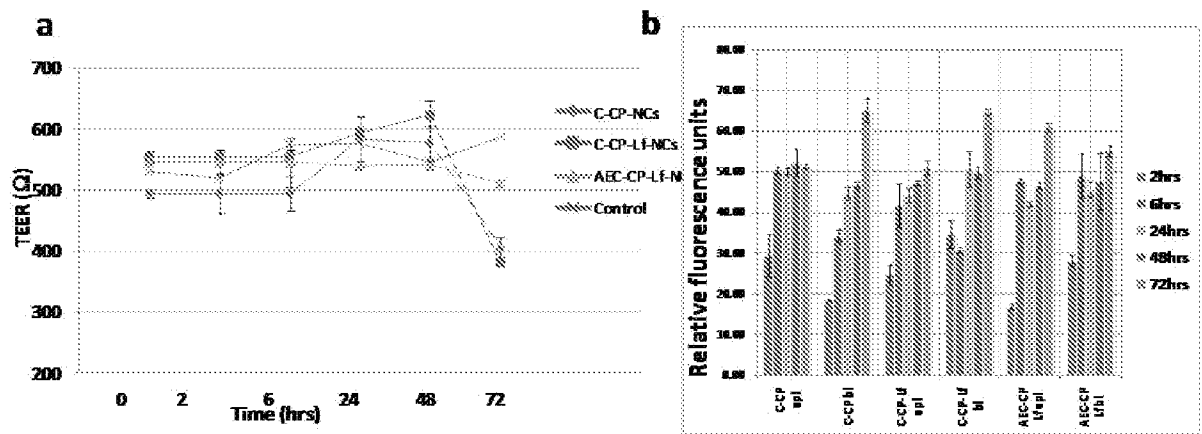


Figure 6

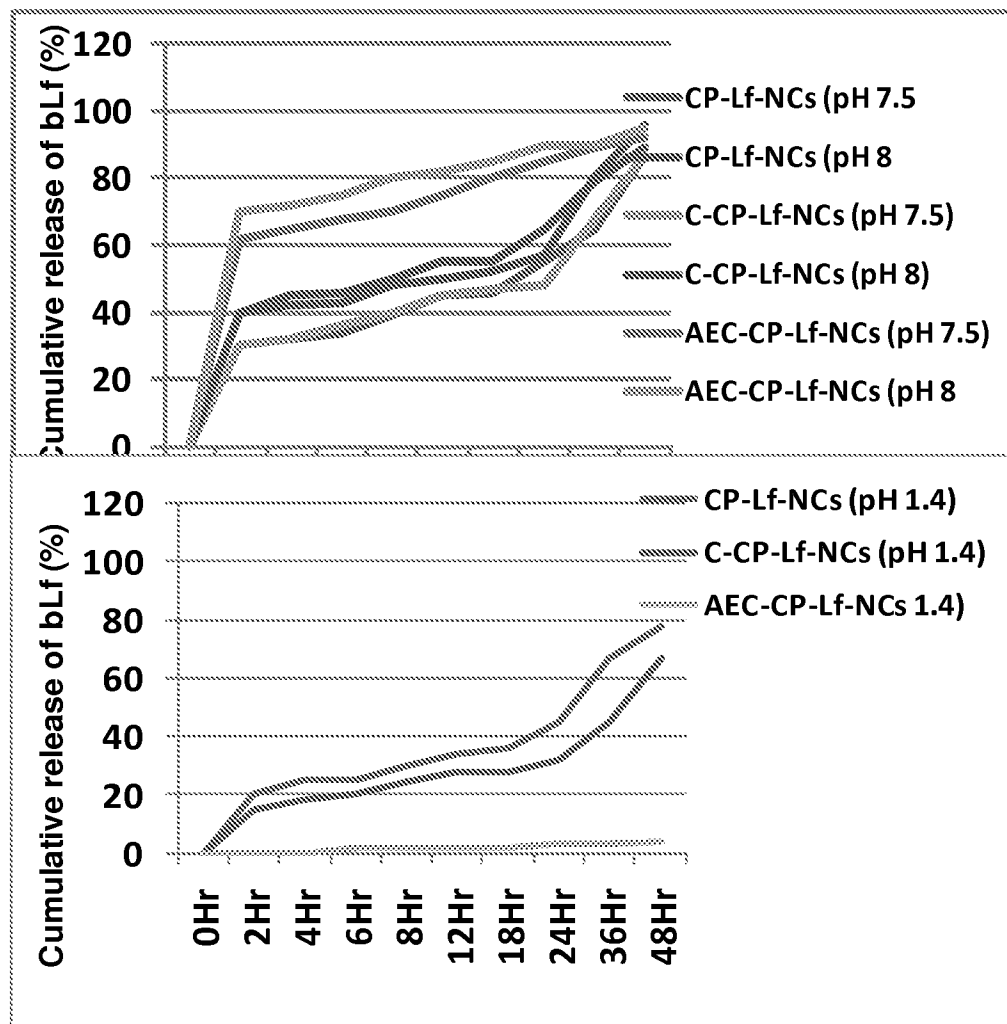


Figure 7

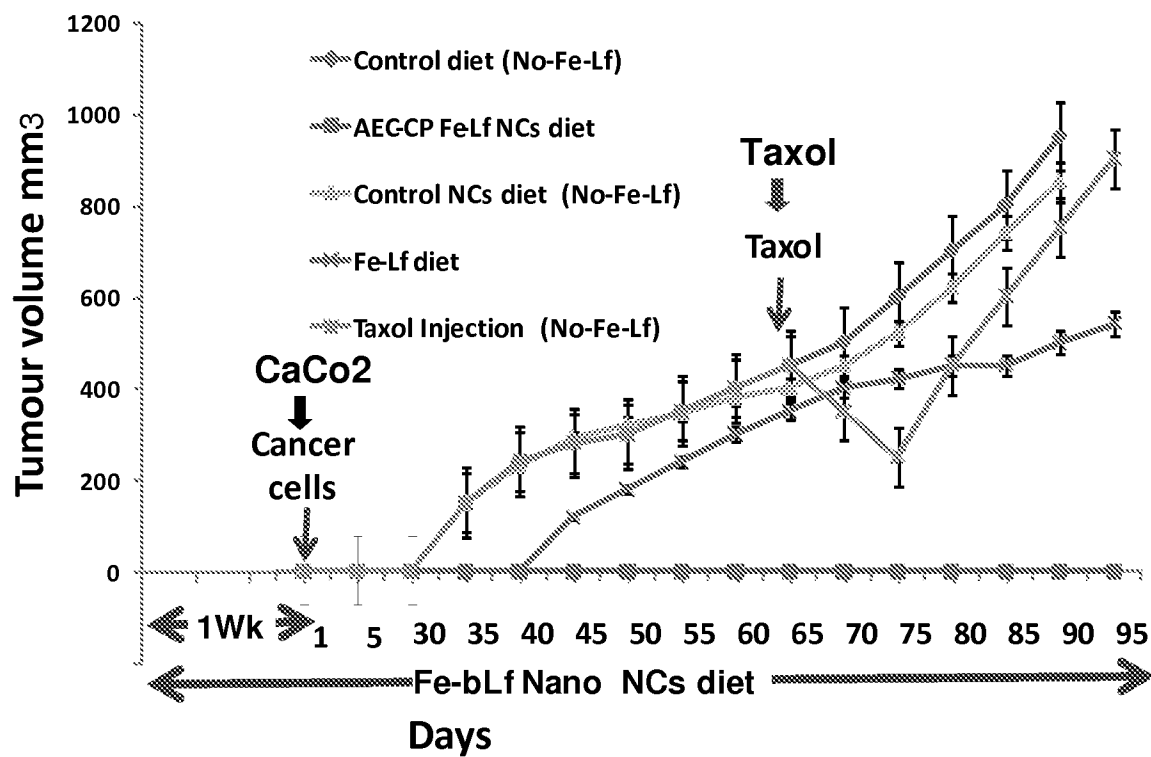


Figure 8

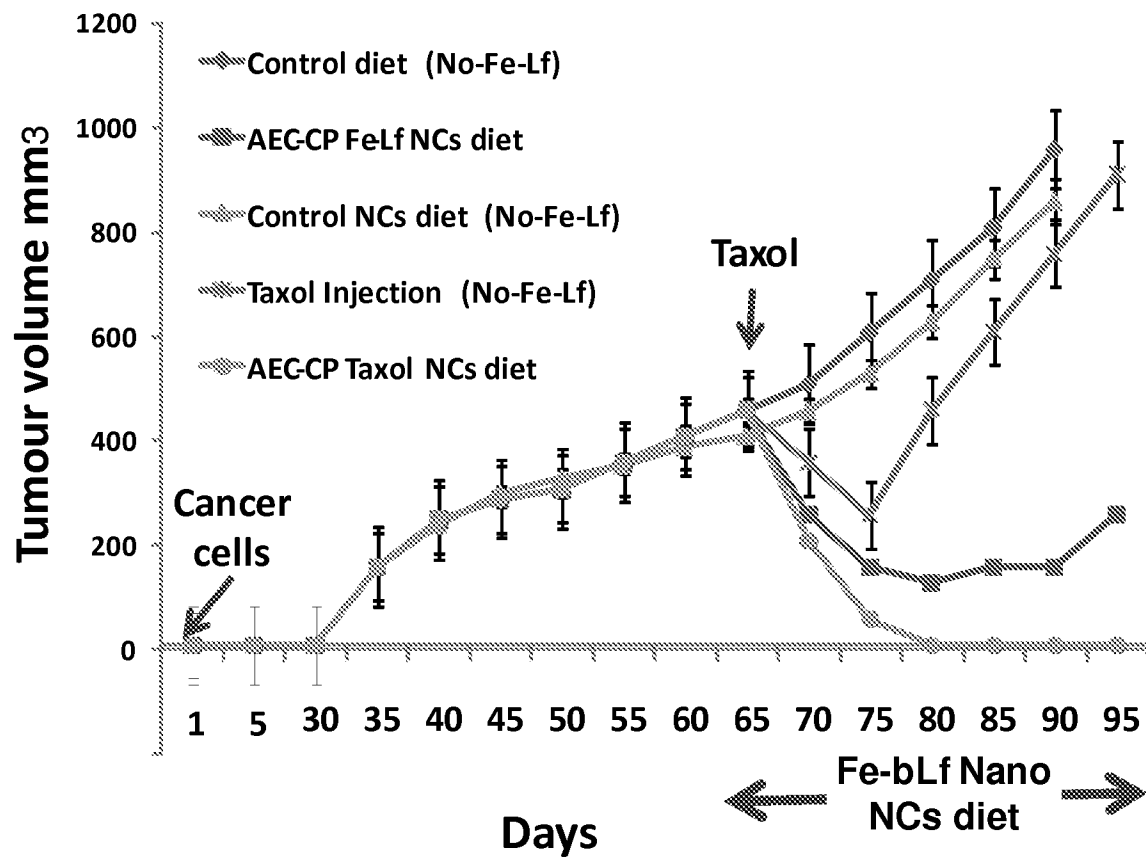


Figure 9

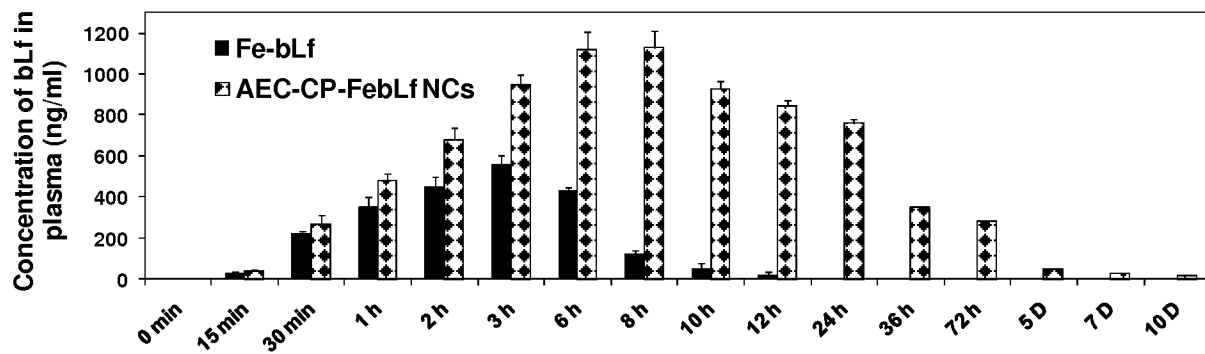


Figure 10

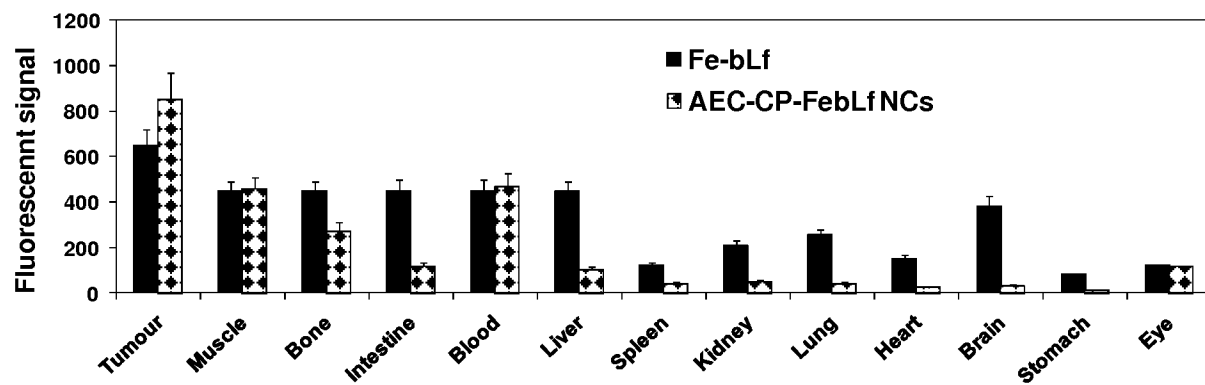


Figure 11

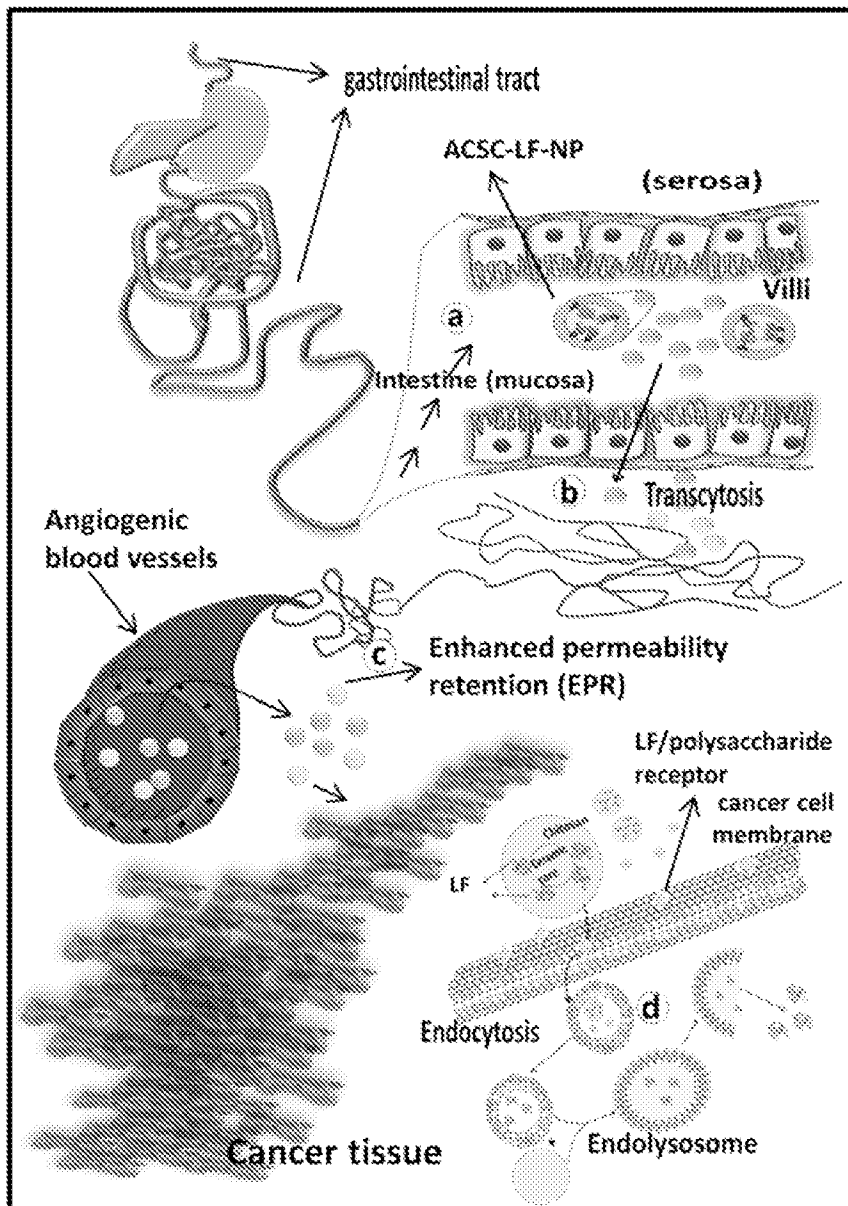


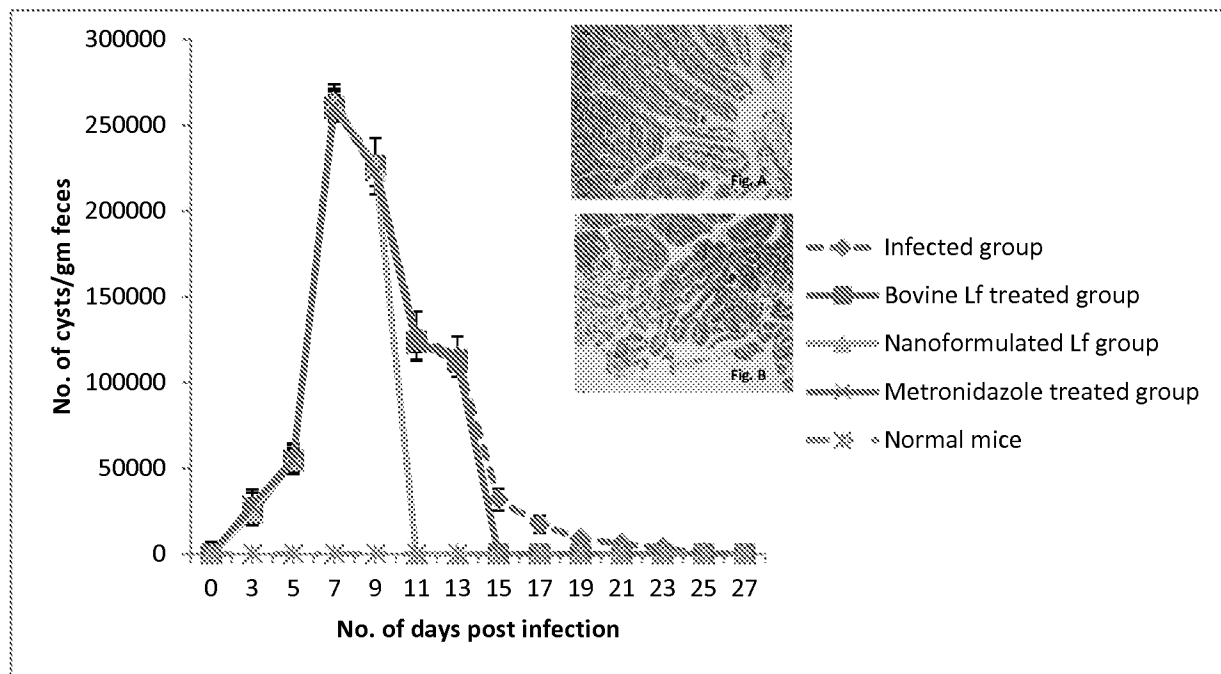
Figure 12

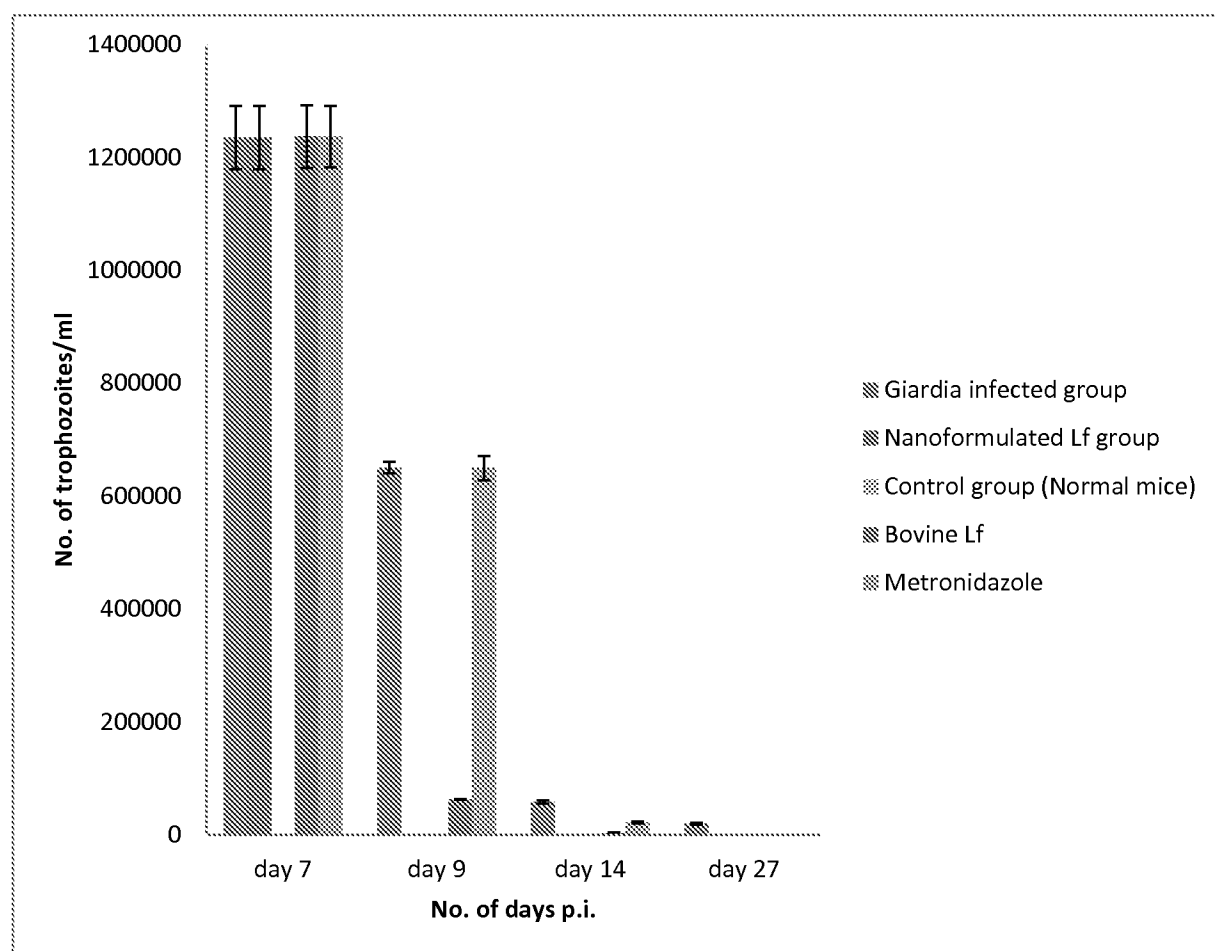
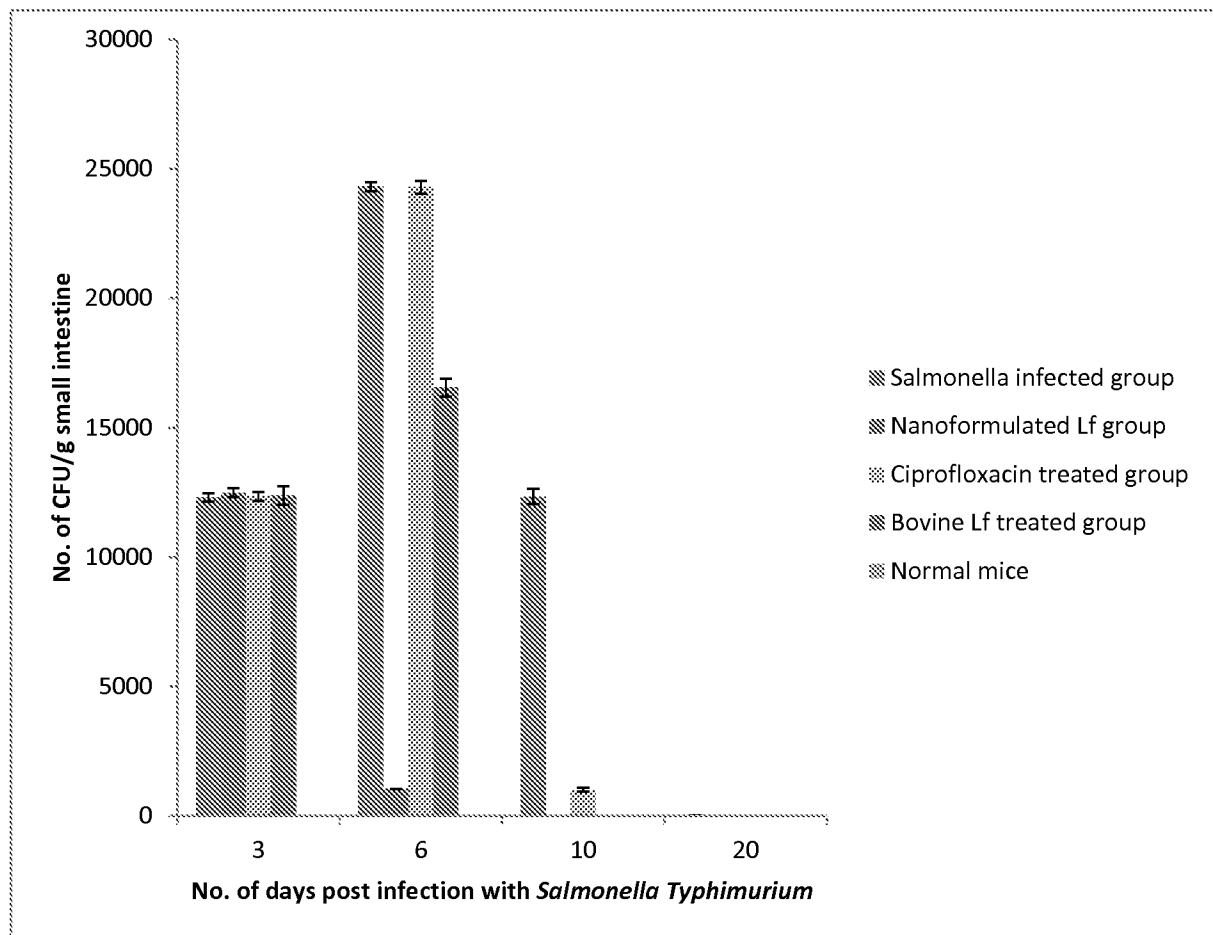
Figure 13

Figure 14

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2012/000456

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

A61K 9/51 (2006.01) **A61K 47/02** (2006.01) **A61P 1/04** (2006.01)
A61K 31/337 (2006.01) **A61K 47/36** (2006.01) **A61P 31/00** (2006.01)
A61K 31/365 (2006.01) **A61K 31/722** (2006.01) **A61P 35/00** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, MEDLINE

NANOSPHERE, NANOPARTICLE, NANOSHEET, NANOFIBRE, NANOCRYSTAL, NANOSHELL, NANOBUBBLE,
 CALCIUM PHOSPHATE, CAPO4, HYDROXYAPATITE, INTESTINAL ABSORPTION ENHANCER, CHITOSAN,
 ENTERIC COAT, ALGINATE, LACTOFERRIN, TAXOL, PACLITAXEL, ADSORB, BIND, ADHERE, CROSS LINK, IONIC
 GELATION, AND LIKE TERMS.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Rawat M et al, 'Development and In Vitro Evaluation of Alginate Gel-Encapsulated, Chitosan-Coated Ceramic Nanocores for Oral Delivery of Enzyme' <i>Drug Development and Industrial Pharmacy</i> (2008) Vol 34(2) pp 181-188 (See abstract, page 182 adjoining paragraphs at column 1-2, page 182 column 2, page 183 column 2, page 186 column 2, page 187 column 2)	1-29
X	WO 2009/135190 A2 (NOD PHARMACEUTICALS INC) 5 NOVEMBER 2009 (See [0012], [0014], [0015], [0032], [0035], [0048] -[0051])	1-29

☒ Further documents are listed in the continuation of Box C☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"E" earlier application or patent but published on or after the international filing date

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"O" document referring to an oral disclosure, use, exhibition or other means

"&" document member of the same patent family

"P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search
22 May 2012

Date of mailing of the international search report
22 May 2012

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2012/000456

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2008/0241256 A1 (KUHN) 2 OCTOBER 2008 (see abstract, [0007], [0026], [0027], [0029], [0039], [065]-[0068], [0085], claim 15, claim 16)	1, 2, 12-18, 23-29
X	WO 2010/078941A1 (BIOLITEC AG) 15 JULY 2010 (See page 4 lines 28-30, adjoining paragraphs at pages 4-5, adjoining paragraphs at pages 8-9, pages 10-12, claim 1, claim 8)	1, 2, 12-18, 23-29
X	Iafisco M et al 'Adsorption and spectroscopic characterization of lactoferrin on hydroxyapatite nanocrystals' <i>Dalton Transactions</i> (28 January 2011) Vol 40(4) pp 820-827 (See abstract, page 821 column 2)	1, 2, 12-18, 23-29
A	CN 101822961 A (WUHAN UNIVERSITY OF TECHNOLOGY) 8 SEPTEMBER 2010	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2012/000456

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report :				Patent Family Member			
WO	2009135190	CN	102014880	EP	2285361	JP	2011519867
US	2008241256	US	2011318422				
WO	2010078941	CN	102256622	EP	2198885	US	2011257586
CN	101822961	NONE					
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.							
END OF ANNEX							