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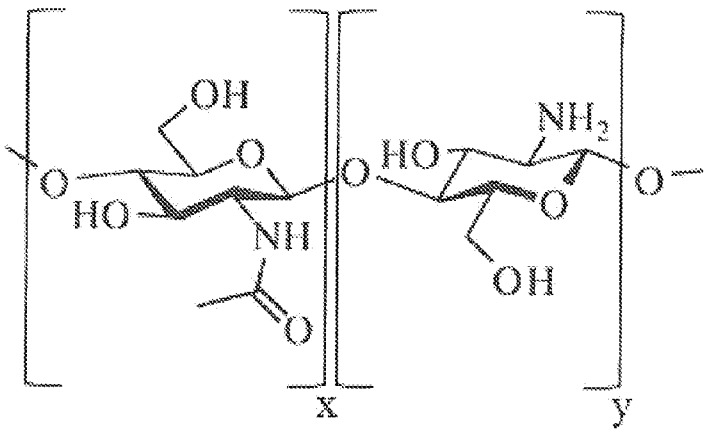
(54) Title: CYTOKINE-CHITOSAN BIOCONJUGATES AND METHODS OF USE THE SAME

Fig. 1



(57) Abstract: Compositions including chitosan covalently linked to a cytokine or growth factor are provided herein. The compositions can be used to produce pharmaceutical compositions and can be used in methods of treating a variety of diseases or disorders. The compositions are especially suitable for localized delivery and may allow for intraturooral delivery and treatment of cancers or stimulation of an immune response to a eo -administered antigen.

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CYTOKINE-CHITOSAN BIOCONJUGATES AND METHODS OF USING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

This patent application claims the benefit of priority of United States Provisional Patent Application No. 62/006,114, filed May 31, 2014, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

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INTRODUCTION

Cytokines are powerful modulators of immune function. For decades, scientists have hypothesized that exogenous cytokines can be administered to overcome immune dysfunction and treat a wide variety of diseases, including cancer. However, despite hundreds, if not thousands, of preclinical and clinical studies, only 2 of 40+ identified cytokines have been approved as single agent immunotherapies for a limited number of malignancies. Unintentional signaling and dose-limiting side effects due to systemic delivery of pleiotropic cytokines have prevented cytokine-based immunotherapies from fulfilling their clinical potential.

SUMMARY

Compositions comprising chitosan covalently linked to a cytokine or growth factor and methods of using these bioconjugates are provided herein. The cytokine or growth factor is biologically active in the bioconjugate. The cytokines include all of the interleukins and further include, but are not limited to, IL-2, IL-12, GM-CSF, IL-1, TNF-α, IFN-γ, IFN-α, IL-10, TGF-β, IL-15, IL-23, IL-27, IL-35 and IL-7. The cytokines or growth factors may be attached to the chitosan in a variety of ways and may contain mutations to allow covalent attachment to the chitosan. The chitosan may have a molecular weight between 10kDa and 500kDa, between 100kDa and 400kDa or between 200kDa and 300kDa. The compositions may be formulated into

pharmaceutical compositions. The pharmaceutical compositions may be formulated for local administration.

In another aspect, methods of treating a disorder in a subject are provided. The compositions are administered to the subject in an amount effective to treat the disorder. The compositions may be administered locally. In one aspect, the disorder is cancer and the composition is administered intratumorally. In other embodiments, the disorder is an autoimmune disease, allergy or infection and the composition is formulated to target these disorders.

In a still further aspect, methods of stimulating an immune response in a subject by administering the cytokine or growth factor-chitosan bioconjugate compositions provided herein with an antigen to the subject in an amount effective to stimulate an immune response directed to the antigen are also provided. The antigen may be a protein, polypeptide or vaccine. The cytokine or growth factor chitosan bioconjugate may act as an adjuvant to further stimulate an immune response to the antigen.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram showing the chemical structure of chitosan. Chitosan is an unbranched copolymer of N-acetylglucosamine (x) and glucosamine (y) units linked by β(1-4) glycosidic bonds where the ratio of y to x is greater than 1:5

Figure 2 is a set of photographs showing that attachment to chitosan enhances cytokine retention in a subject. Mice bearing 7-day-old s.c MC32a (colon adenocarcinoma) tumors were shaved and given a single i.t. injection of Alexa Fluor 660(AF660)-labeled IL-12 (5μg) in either phosphate buffered saline (PBS) or 1.5% (w/v) chitosan solution. IL-12 became undetectable between 24 and 48 hrs when administered with PBS. When mixed with chitosan solution, IL-12 persisted in the tumor for at least 5 to 6 days. Method adapted from ref. [6].

Figure 3 is a graph showing that chitosan/IL-12 immunotherapy improves survival following breast tumor resection. Mice bearing orthotopic 4T1 mammary carcinomas were given i.t. treatments on days 6 and 12 after tumor implantation with (▪) saline, (▲)chitosan, (●) IL-12 alone(1μg) or (○) chitosan/IL-12(1μg) admixture. Primary tumors were resected on day 15.

Figure 4 is a set of figures showing purification of recombinant human IL-12. Figure 4A is a set of isothermogram graphs depicting the heparin binding affinity of human IL-12. Figure

4B is a graph showing the protein purification profile on heparin-sepharose. Figure 4C and Figure 4D are photographs of SDS-PAGE gels of the 500 mM NaCl fraction stained with Coomassie Blue (Figure 4C) and detected with antibodies specific for the p70, p40 and p35 subunits of IL-12, respectively (Figure 4D). Lanes, **NR** and **R** represent the electrophoresis profile of IL-12 under non-reducing and reducing conditions, respectively. The minor additional bands represent different glycosylated products of IL-12.

Figure 5 is a set of graphs showing the bioactivity of IL-12-chitosan bioconjugates. Figure 5A is a graph showing proliferation of IL-12-sensitive 2D6 cells treated with recombinant IL-12, IL-12-chitosan bioconjugates or unreacted, dialyzed IL-12+chitosan or nothing (untreated). The proliferation was measured via a luminescence-based cell viability assay (CellTiter-Glo; Promega). Figure 5B is a graph showing the change in tumor volume over time in C57BL/6 mice (n=4) inoculated s.c. with 500,000 MB49 bladder carcinoma cells. The mice were treated i.t. on days 7, 10 and 14 with IL-12-chitosan bioconjugates containing an estimated 1μg IL-12. MB49-bearing control mice (n=5) were not treated.

Figure 6 is a graph showing the bioactivity of IL-12-chitosan non-specific conjugates as quantified through IL-12 dependent 2D6 T cell proliferation assays. Unmodified IL-12 was non-specifically conjugated to chitosan following various bio-conjugation methods. Methods followed include, 1) carbodiimide mediated peptide bonding where amine groups on chitosan are covalently bonded to carboxylic acid groups on IL-12. 2) Amine-to- sulfhydryl crosslinker (Sulfo –SMCC ester) mediated conjugation of thiolated chitosan to IL-12, and 3) Tyrosinase mediated catalytic conjugation of tyrosinase modified o-quinones on IL-12 to nucleophilic amine group on chitosan. As seen, nonspecific conjugation of IL-12 to chitosan is effective to varying degree with amine-to- sulfhydryl crosslinker mediated conjugation yielding a completely inactive product. Peptide bonding and tyrosinase catalysis methods yielded chitosan-IL-12 conjugates that are bioactive with up to 60% and 17% loss of IL-12 bioactivity observed with peptide bonding and tyrosinase catalysis respectively.

DETAILED DESCRIPTION

We hypothesized that a safer, more effective approach for the administration of cytokine therapeutics is through local, sustained delivery. Local delivery reduces cytokine-mediated toxicities and exploits the preferred paracrine mechanism of action of most cytokines.

Our recently published data demonstrate that intra-tumoral (i.t.) administration of IL-12 admixed with chitosan solutions (chitosan/IL-12) can retain IL-12 locally in the tumor and eradicate 80-100% of established colorectal, pancreatic and bladder tumors (Zaharoff et al. 2009; Zaharoff et al 2010; Yang et al 2013). Perhaps more important than primary tumor regression, was the finding that 47-100% of tumor-free mice rejected tumor rechallenge which implies the generation of tumor-specific immunity following chitosan/IL-12 immunotherapy. The use of intravesically administered chitosan/IL-12 admixtures for the treatment of superficial bladder cancer is under consideration.

Regarding breast cancer, recent preliminary data indicate that chitosan/IL-12 admixtures can control metastases and improve survival in an aggressive preclinical model. In these studies, mice bearing 4T1 tumors were given 3 i.t injections prior to primary tumor resection. Mice were followed for survival after resection. In a separate study, mice were euthanized five weeks after resection to document pulmonary metastases. In both cases, pre-resection treatment with chitosan/IL-12 improved surgical outcomes. Neoadjuvant immunotherapy with chitosan/IL-12 resulted in 67% durable cures. In contrast, none of the mice receiving tumor resection alone survived beyond 40 days after surgery.

Despite these promising results, systemic dissemination of IL-12 and the associated potential for toxicity remains a serious concern and a potential obstacle to clinical translation. The enhanced i.t. retention of IL-12 is due to the fact that highly viscous chitosan solutions hinder the diffusive transport of IL-12. Nevertheless, the majority of administered IL-12 is likely to reach the systemic circulation. In fact, elevated serum IL-12 and IFN-γ levels following chitosan/IL-12 injections have confirmed the leakage of IL-12 from a local injection site.

To overcome this limitation, we propose a novel delivery technology in which IL-12 is covalently conjugated to chitosan prior to intratumoral (i.t.) injection. Conjugation will increase the effective molecular weight of IL-12 and therefore reduce its diffusivity. In addition, polycationic chitosan is expected to interact electrostatically with negatively charged extracellular matrix proteins and cell membranes. Thus, IL-12-chitosan bioconjugates are effectively anchored to a local injection site. Similar methods may be useful with other cytokines or growth factors as well. Cytokine immunotherapy has been limited by significant toxicity or unintended side effects associated with systemic administration of the cytokines. Thus, conjugating the cytokine to chitosan to limit diffusion of the cytokine from the point of

administration or injection may limit side effects or toxicity associated with cytokine administration.

In the Examples, IL-12 was covalently linked to chitosan via a carbodiimide cross-linking between amine groups on the chitosan and carboxyl groups on the IL-12. While this form of non-specific linkage did reduce the activity of the IL-12, substantial IL-12 activity was maintained. Tryosinase-catalyzed tyrosine to amine linkage of the IL-12 to chitosan was also shown in the Examples to result in a bioconjugate with IL-12 activity maintained. Other more directed methods of linking IL-12 or another cytokine or growth factor to chitosan covalently may also be used and may have more limited effects on the bioactivity of the conjugated cytokine or growth factor. For example thioester linkage, a peptide linkage via a linking peptide, linkage via engineered lysines or cysteines on the cytokine positioned to limit effects on bioactivity of the cytokine or linkage between amines on the chitosan and the cytokine or growth factor may be used to covalently link chitosan to IL-12 or another cytokine or growth factor. Several of these alternatives are described in the Examples. The linkage via a lysine or cysteine on the cytokine or growth factor may entail making a lysine or cysteine substitution mutation in the cytokine or growth factor. Suitably these substitution mutations are on surface exposed amino acids to allow minimal steric hindrance for the linkage to chitosan and the mutations suitably have minimal effects on the biological activity of the cytokine or growth factor. For example, substitution mutations to position a lysine at one of positions 17, 18, 34, 35, 43, 44 or 248 of IL-12 may be suitable. The chemistry used to link the cytokine or growth factor to chitosan includes, but is not limited to click chemistry, periodate chemistry, maleimide thioether chemistry or thiol chemistry. See *Bioconjugate Chem.*, 2011, 22 (4), pp 551–555. Those of skill in the art will appreciate that other methods may be used to covalently link a cytokine or growth factor to chitosan while maintaining the biological activity of the cytokine or growth factor. Those of skill in the art are also capable of determining if the biological activity of the cytokine-chitosan bioconjugate is sufficient for the intended use of the conjugate.

The chitosan may also be modified prior to linkage to the cytokine or growth factor. The chitosan may be methylated or thiolated to make the linkage chemistry more straightforward. The cytokine or growth factor and the chitosan may be linked directly or through a linker. The linker may be a chemical linker or a peptide linker. The chitosan may be conjugated to the N-

terminal alpha amino group, C-terminal alpha carobxyl group, aspartic acid, glutamic acid or cysteine, lysine or tyrosine residues in the cytokine or growth factor.

IL-12 was conjugated to chitosan in the Examples, but a variety of cytokines or growth factors could be conjugated to chitosan to achieve a similar result. For example, cytokines or growth factors including all of the interleukins may be used in the bio-conjugates described herein. The cytokines and growth factors may specifically include, but are not limited to, IL-2, IL-12, GM-CSF, IL-1, TNF-α, IFN-γ, IFN-α, IL-10, TGF-β, IL-15, IL-23, IL-27, IL-35 and IL-7. These cytokines/growth factors have been suggested as potential immunotherapeutics but adoption of these cytokines into clinical practice has been limited by toxicity due to systemic administration or other off-target effects of these pleiotropic effectors. Each of these cytokines could be conjugated to chitosan using the methods described above and administered as a pharmaceutical composition similar to IL-12 chitosan in the Examples.

Each of the cytokines may be genetically engineered to add peptide linkers or modify amino acids without negatively impacting the function of the cytokine or growth factor to avoid having covalent linkages to chitosan that diminish or completely alter the activity of the cytokine or growth factor. For example thioester chemistry could be used to link the chitosan to lysine residues on the cytokine. Structural analysis of IL-12 suggested to us that lysine substitutions at amino acid 34 or 248 (R34K or S248K) or a combination thereof would be effective for linkage without altering the activity of the cytokine. Linking via di-sulfide bonds via cysteine substitution is also contemplated and may be used to link the cytokine or growth factor to chitosan.

The chitosan may have a molecular weight between 10kDa and 500kDa, between 100kDa and 400kDa or between 200kDa and 300kDa. Higher molecular weights are likely to lead to less diffusion after administration. In addition the chitosan can be modified and thus have different effects on the stability and diffusion rate of the cytokine-chitosan bioconjugates. The degree of deacetylation of the chitosan may also be modified in the compositions.

Methods of treating a disorder in a subject by administering an effective amount of the chitosan-cytokine/growth factor bioconjugates described herein are also provided. Cells, such as immune cells, may also be contacted with the cytokine/growth factor-chitosan bioconjugate compositions provided herein. In one embodiment, contacting immune cells with the cytokine-chitosan bioconjugate compositions results in an immune response against a cancer or tumor

cells and leads to reduced growth or reduced proliferation of the cancer or tumor cells. Suitably the cell is an immune cell, but also may be cells within a tumor. Suitably the compositions are administered locally to a tumor or to a site near cancer cells and the administration of the compositions effect the local immune response to the cancer or tumor. The cancer may be any cancer, including but not limited to, bladder, breast, colorectal, pancreatic, prostate, renal, lung, melanoma, lymphoma, brain, head and neck, or ovarian cancer. In an alternative embodiment the disorder is a disorder treatable by induction or inhibition of an immune response in a localized area by administration of the compositions described herein. The area could be the site of an infection or immune response such as an allergic or autoimmune response. In an alternative embodiment, the cytokine/growth factor-chitosan bioconjugate is administered with a vaccine or other antigen to act as an adjuvant and stimulate an immune response against one or more antigens. Suitably, the disorder can be treated by induction or repression of an immune response by administration of the compositions described herein. The disorder may further be selected from inflammation, arthritis, Multiple sclerosis, Crohn's disease or others. The subject may be a human or non-human animal such as a domestic animal or agricultural animal.

Methods of stimulating an immune response in a subject are also provided herein. The methods include administering the compositions comprising a chitosan-cytokine/growth factor bioconjugate to the subject in an amount effective to stimulate an immune response to the antigen. The antigen may be a protein or peptide antigen or may be part of a vaccine. The subject may be a human or non-human animal such as a domestic animal or agricultural animal and may be administered via any means available to those skilled in the art.

The increased immune response may include an enhanced B cell or T cell mediated immune response and may include increased antibody production, increased class switching or increased cell-mediated killing of infected or cancerous cells.

Cells may be contacted with the composition directly or indirectly *in vivo*, *in vitro*, or *ex vivo*. Contacting encompasses administration to a cell, tissue, mammal, patient, or human. Further, contacting a cell includes adding an agent to a cell culture. Other suitable methods may include introducing or administering the composition to a cell, tissue, mammal, or patient using appropriate procedures and routes of administration as defined below. Tissues include tumors or tissues including cancer cells. Subjects may be human or animal subjects and include cancer patients.

Treating cancer includes, but is not limited to, reducing the number of cancer cells or the size of a tumor in the subject, reducing progression of a cancer to a more aggressive form, maintaining the cancer or tumor in a less aggressive form for a longer period of time, reducing proliferation of cancer cells or reducing the speed of tumor growth, killing of cancer cells, reducing metastasis of cancer cells or reducing the likelihood of recurrence of a cancer in a subject. Treating a subject as used herein refers to any type of treatment that imparts a benefit to a subject afflicted with a disease or at risk of developing the disease, including improvement in the condition of the subject (e.g., in one or more symptoms), delay in the progression of the disease, delay the onset of symptoms or slow the progression of symptoms, etc.

The compounds may be used to make pharmaceutical compositions. Pharmaceutical compositions comprising the compositions described herein and a pharmaceutically acceptable carrier are provided. A pharmaceutically acceptable carrier is any carrier suitable for *in vivo* administration. Examples of pharmaceutically acceptable carriers suitable for use in the composition include, but are not limited to, water, buffered solutions, glucose solutions, oil-based or bacterial culture fluids. Additional components of the compositions may suitably include, for example, excipients such as stabilizers, preservatives, diluents, emulsifiers and lubricants. Examples of pharmaceutically acceptable carriers or diluents include stabilizers such as carbohydrates (e.g., sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein-containing agents such as bovine serum or skimmed milk and buffers (e.g., phosphate buffer). Especially when such stabilizers are added to the compositions, the composition is suitable for freeze-drying or spray-drying. The composition may also be emulsified.

The compositions described herein may also be co-administered with another agent such as an anti-cancer therapeutic. The two compositions may be administered in any order, at the same time or as part of a unitary composition. The two may be administered such that one composition is administered before the other with a difference in administration time of 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 16 hours, 20 hours, 1 day, 2 days, 4 days, 7 days, 2 weeks, 4 weeks or more.

An effective amount or a therapeutically effective amount as used herein means the amount of a composition that, when administered to a subject for treating a state, disorder or condition is sufficient to effect a treatment (as defined above). The therapeutically effective

amount will vary depending on the compound, formulation or composition, the disease and its severity and the age, weight, physical condition and responsiveness of the subject to be treated.

The compositions described herein may be administered by any means known to those skilled in the art, including, but not limited to, intratumoral, intravesical, oral, topical, intranasal, intraperitoneal, parenteral, intravenous, intramuscular, subcutaneous, intrathecal, transcutaneous, nasopharyngeal, or transmucosal absorption. Thus the compositions may be formulated as an ingestable, injectable, topical or suppository formulation. The compositions may also be delivered within a liposomal or time-release vehicle or vesicle. Administration of the compositions to a subject in accordance with the invention appears to exhibit beneficial effects in a dose-dependent manner. Thus, within broad limits, administration of larger quantities of the composition is expected to achieve increased beneficial biological effects than administration of a smaller amount. Moreover, efficacy is also contemplated at dosages below the level at which toxicity is seen.

It will be appreciated that the specific dosage administered in any given case will be adjusted in accordance with the compositions being administered, the disease to be treated or inhibited, the condition of the subject, and other relevant medical factors that may modify the activity of the composition or the response of the subject, as is well known by those skilled in the art. For example, the specific dose for a particular subject depends on age, body weight, general state of health, diet, the timing and mode of administration, the rate of excretion, medicaments used in combination and the severity of the particular disorder to which the therapy is applied. Dosages for a given patient can be determined using conventional considerations, e.g., by customary comparison of the differential activities of the compositions of the invention and of a known agent such as an unconjugated cytokine, such as by means of an appropriate conventional pharmacological or prophylactic protocol.

The maximal dosage for a subject is the highest dosage that does not cause undesirable or intolerable side effects. The number of variables in regard to an individual prophylactic or treatment regimen is large, and a considerable range of doses is expected. The route of administration will also impact the dosage requirements. It is anticipated that dosages of the composition will reduce symptoms of the condition at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% compared to pre-treatment symptoms or symptoms is left untreated. It is specifically contemplated that pharmaceutical preparations and compositions may palliate or

alleviate symptoms of the disease without providing a cure, or, in some embodiments, may be used to cure the disease or disorder.

Suitable effective dosage amounts for administering the compositions may be determined by those of skill in the art, but typically range from about 1 microgram to about 100,000 micrograms per kilogram of body weight weekly, although they are typically about 1,000 micrograms or less per kilogram of body weight weekly. Smaller doses may be required because the compositions are not diffusing into the surrounding and have a more targeted effect as compared to non-conjugated counterpart compositions. In some embodiments, the effective dosage amount ranges from about 10 to about 10,000 micrograms per kilogram of body weight weekly. In another embodiment, the effective dosage amount ranges from about 50 to about 5,000 micrograms per kilogram of body weight weekly. In another embodiment, the effective dosage amount ranges from about 75 to about 1,000 micrograms per kilogram of body weight weekly. The effective dosage amounts described herein refer to total amounts administered, that is, if more than one composition is administered, the effective dosage amounts correspond to the total amount administered. The composition can be administered as a single dose or as divided doses. For example, the composition may be administered two or more times separated by 4 hours, 6 hours, 8 hours, 12 hours, a day, two days, three days, four days, one week, two weeks, or by three or more weeks.

The present disclosure is not limited to the specific details of construction, arrangement of components, or method steps set forth herein. The compositions and methods disclosed herein are capable of being made, practiced, used, carried out and/or formed in various ways that will be apparent to one of skill in the art in light of the disclosure that follows. The phraseology and terminology used herein is for the purpose of description only and should not be regarded as limiting to the scope of the claims. Ordinal indicators, such as first, second, and third, as used in the description and the claims to refer to various structures or method steps, are not meant to be construed to indicate any specific structures or steps, or any particular order or configuration to such structures or steps. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to facilitate the disclosure and does not imply any limitation on the scope of the disclosure unless otherwise claimed. No language in the specification, and no structures shown in the drawings,

should be construed as indicating that any non-claimed element is essential to the practice of the disclosed subject matter. The use herein of the terms "including," "comprising," or "having," and variations thereof, is meant to encompass the elements listed thereafter and equivalents thereof, as well as additional elements. Embodiments recited as "including," "comprising," or "having" certain elements are also contemplated as "consisting essentially of" and "consisting of" those certain elements.

Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. For example, if a concentration range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1% to 3%, etc., are expressly enumerated in this specification. These are only examples of what is specifically intended, and all possible combinations of numerical values between and including the lowest value and the highest value enumerated are to be considered to be expressly stated in this disclosure. Use of the word "about" to describe a particular recited amount or range of amounts is meant to indicate that values very near to the recited amount are included in that amount, such as values that could or naturally would be accounted for due to manufacturing tolerances, instrument and human error in forming measurements, and the like. All percentages referring to amounts are by weight unless indicated otherwise.

No admission is made that any reference, including any non-patent or patent document cited in this specification, constitutes prior art. In particular, it will be understood that, unless otherwise stated, reference to any document herein does not constitute an admission that any of these documents forms part of the common general knowledge in the art in the United States or in any other country. Any discussion of the references states what their authors assert, and the applicant reserves the right to challenge the accuracy and pertinence of any of the documents cited herein. All references cited herein are fully incorporated by reference, unless explicitly indicated otherwise. The present disclosure shall control in the event there are any disparities between any definitions and/or description found in the cited references.

The following examples are meant only to be illustrative and are not meant as limitations on the scope of the invention or of the appended claims.

EXAMPLES

Our group has pioneered the use of chitosan-based solutions for the local delivery of recombinant cytokines and antigens [6, 38, 39, 88-91]. Chitosan is an abundant, natural polysaccharide derived primarily from the exoskeletons of crustaceans [92]. It is an unbranched copolymer of glucosamine and N-acetylglucosamine units linked by β(1-4) glycosidic bonds (**Figure 1**). The use of water soluble polysaccharides, like chitosan, avoids the use of cytokine-denaturating organic solvents. In vivo, chitosan is safely degraded into excretable glucosamine and N-acetylglucosamine fragments by lysozyme, glucosaminidase, lipase and other endogenous human enzymes. The rate of degradation can be controlled via chitosan concentration, molecular weight (MW), injection volume and N-acetylglucosamine: glucosamine ratio.

Chitosan Solution Enhances Local Retention and Activity of Delivered Cytokines: Our published studies demonstrated that simple, viscous chitosan solutions are able to significantly increase the local retention of co-formulated GM-CSF [88] and IL-12 [6]. In particular, i.t. injections of IL-12 alone dissipated quickly and became undetectable within 24 to 48 hrs (**Figure 2**). In contrast, by formulating IL-12 in chitosan solution, IL-12 was detectable for up to 6 days [6]. We believe that a combination of viscous and electrostatic interactions hinder the ability of cytokines to diffuse out of the chitosan solution and thus form a slow-release delivery system. The nature of non-covalent chitosan/cytokine interactions and the mechanism of cytokine release is the subject of ongoing studies in our lab.

Our data also showed that chitosan/cytokine depots also increased immunological activity versus saline-based cytokine injections. Specifically, a single chitosan/GM-CSF injection outperformed four daily injections of GM-CSF alone in terms of increasing the number and functionality of dendritic cells in draining lymph nodes. In vaccination experiments, chitosan/GM-CSF was superior to either chitosan or GM-CSF alone in enhancing antigen-specific $CD4^+$ proliferation, peptide-specific $CD8^+$ pentamer staining and cytotoxic T cell lysis [88]. Similarly, chitosan/IL-12 formulations outperformed IL-12 alone in eradicating 80-100% of aggressive, established solid tumors (MC38 and Panc02) [6] and 88-100% orthotopic, superficial bladder tumors (MB49) [38]. Interestingly, intravesical chitosan/IL-12 immunotherapy was found to induce systemic tumor-specific immunity which conferred complete protection from a distant s.c. tumor rechallenge. More recent unpublished data

demonstrate that i.t. injections of chitosan/IL-12 prior to tumor resection are capable of eliminating metastasis and extending survival (**Figure 3**) in an aggressive, highly metastatic model of breast cancer.

Limitations of chitosan/cytokine mixtures: Although we have successfully demonstrated that simple chitosan/cytokine mixtures are effective at increasing local cytokine retention and activity, we determined that most of the delivered cytokine leaches out of the chitosan depot and into the circulation. In fact, serum IL-12 and IFN-γ levels following i.t. injection of either IL-12 alone or chitosan/IL-12 mixtures were similar [38]. Because these data may limit the clinical application of our simple mixture platform due to concerns over IL-12-mediated toxicity, we developed a new delivery technology capable of preventing IL-12 dissemination.

Novel IL-12-chitosan bioconjugates: The amine functional group of chitosan's glucosamine residues (**Figure 1**) allows for facile chemical attachment of a variety of side chain moieties including proteins. As a result, we hypothesized that cytokines could be covalently conjugated to chitosan. Direct conjugation has two advantages over the previous simple chitosan/IL-12 mixtures. First, because the diffusion of macromolecules is inversely related to molecular size, by conjugating IL-12 (MW=75kDa) to a comparatively large chitosan molecule (MW=100-500kDa), the effective molecular weight of the cytokine can be increased up to 6-7 fold. As a result, diffusive transport and therefore systemic dissemination of cytokines would be drastically reduced if not eliminated altogether. Second, after i.t. injection, highly polycationic chitosan molecules interact electrostatically with negatively charged extracellular matrices and cell membranes [93]. Consequently, cytokines are effectively "anchored" to the injection site by chitosan's polycationic charge.

Our group has found that locally administered mixtures of chitosan and IL-12 can induce the complete regression of pancreatic adenocarcinomas [6], colon adenocarcinomas [6], bladder carcinomas [38], metastatic mammary carcinomas, renal cell carcinomas, melanomas and prostate adenocarcinomas. While this simple mixture platform is effective in retaining significant amounts of IL-12 in the tumor microenvironment, it does not prevent systemic dissemination of the majority of IL-12. It is estimated that as much as 75% of the injected IL-12 was absorbed into circulation and resulted in a concomitant spike in serum IFN-γ [38]. While a weekly dosing schedule of chitosan/IL-12 mixtures is not expected to induce clinical toxicity

[97], novel technologies which minimize or eliminate systemic IL-12 exposure are more translatable.

Thus, the proposed project will develop and evaluate a novel delivery platform based on the conjugation of IL-12 to chitosan. This strategy hinders IL-12 dissemination by: 1) increasing the effective size of IL-12; and 2) anchoring IL-12-chitosan bioconjugates to a local injection site through bioadhesive interactions. This approach has the potential to maintain high concentrations of IL-12 in the tumor following i.t. administration with minimal leakage into the circulation.

Overexpression and purification of recombinant human IL-12: The plans of our initial application to manufacture IL-12 using bacterial (*E. coli*) and yeast (*Pichia Pastoris*) expression systems were not successful due to the extensive glycosylation required for IL-12 expression and bioactivity. Fortunately, our collaborator, Dr. Barbara Felber, Senior Investigator, Vaccine Branch, NCI, has developed human embryonic kidney (HEK293) cells expressing murine IL-12 (mIL-12) and human IL-12 (huIL-12).

To avoid the potential generation of antigenic epitopes, none of the provided recombinant IL-12 constructs contains extrinsic affinity tags for purification. Careful examination of the amino acid sequence of IL-12 revealed that the p40 subunit of both mIL-12 and huIL-12 contain amino segments which can potentially bind to glycosaminoglycans such as, heparin. Interestingly, isothermal titration calorimetry (ITC) data showed that human IL-12 has strong binding affinity to heparin ($K_d \sim 70$ μM, **Figure 4A**). Based on the high binding affinity of IL-12 to heparin, an affinity chromatography (using heparin-sepharose) based purification protocol was designed to purify human IL-12. IL-12 was observed to elute as discrete peak at 500 mM NaCl (**Figure 4B**). SDS-PAGE gel analysis revealed that IL-12 was pure (>95%, **Figure 4C, D**). The yield of the purified protein is ~6.5 mg/20 mL of culture supernatant.

The acquisition of IL-12-expressing HEK293 clones and the identification of heparin-binding motifs on IL-12 are helpful advances needed to move to mammalian expression systems. In addition, the newly identified heparin-binding motifs allow for facile purification of authentic IL-12, as well as planned IL-12 mutants, from culture supernatants without the involvement of affinity tags or laborious multi-step procedures. See Jayanthi et al. Protein Expr Purif (2014) 102: 76-84, incorporated herein by reference in its entirety.

Validation of IL-12-chitosan bioconjugates: Additional preliminary data demonstrate the successful non-specific conjugation of mIL-12 to chitosan via carbodiimide cross-linking. Briefly, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) was used to activate carboxyl groups on IL-12 which in turn reacted with N-hydroxysuccinamide (NHS) stabilized amine groups on chitosan to form a peptide bond. The conjugation reaction was carried out at pH 5.0 for 6 hours after which IL-12-chitosan bioconjugates were isolated via dialysis. In vitro bioactivity was verified by quantifying proliferation of IL-12-sensitive 2D6 cells (**Figure 5A**). As expected due to non-specific conjugation, a significant amount, approximately 35%, of IL-12 bioactivity was lost. Possible causes include loss of unreacted IL-12, direct inactivation of IL-12 by cross-linking reagents, or indirect inactivation by conjugation of IL-12 to chitosan in an inaccessible orientation. Nevertheless, it is reasonable to assume that any loss in IL-12 bioactivity can be counteracted by simply increasing doses of IL-12-chitosan used in subsequent immunotherapy studies. To control for the possibility that IL-12-chitosan-induced proliferation was due to unconjugated IL-12, a mixture of IL-12 and chitosan was subjected to dialysis but not the conjugation reaction. Unconjugated IL-12 and chitosan did not induce 2D6 proliferation indicating that the dialysis purification successfully removed free, unreacted IL-12.

In vivo bioactivity of IL-12-chitosan bioconjugates was verified by documenting tumor regression following i.t. administration (**Figure 5B**). I.t. IL-12-chitosan immunotherapy mediated tumor regression in 4 of 4 treated mice, including 2 complete regressions. These data demonstrate that first generation, non-specifically conjugated IL-12-chitosan constructs are not only active, but also promising antitumor agents. The proposed methods of specific conjugation of IL-12 to chitosan will attempt to improve upon the bioactivity of IL-12-chitosan bioconjugates through more controlled and site-specific conjugation protocols.

IL-12 maintains bioactivity when linked to chitosan in a variety of ways. Chitosan (degree of deacetylation >90%) was purchased from Primex (Siglufjordur, Iceland) and purified before use. Hydrochloric acid, sodium hydroxide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), N – hydroxysuccinimide (NHS), and mushroom tyrosinase were purchased from Sigma (St. Louis, MO). Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) was purchased from Life Technologies (Green Island, NY).

Carbodiimide mediated carboxyl-to-amine reactive group crosslinking:

Primary amine groups on chitosan were crosslinked with free carboxylic acid groups on proteins through carbodiimide mediated peptide bonding. Briefly, 1mg of chitosan was dissolved in 0.1M HCL and pH adjusted to 5.4 to 5.6 using 1M NaOH. 1mg of EDAC and NHS were added to 250µl of chitosan stock solution and mixed before adding 50µl of 1mg/ml IL-12 solution. The mixture was lest at room temperature for 6 hours for optimal conjugation efficiency. After 6 hours the chitosan-IL-12 conjugates were dialyzed using 100KD dialysis membrane to remove unreacted chemicals and freeze dried before further use.

Maleimide thioether mediated amine-to-sulfhydryl crosslinking:

Reactive amine groups on IL-12 were crosslinked with sulfhydryl groups on thiolated chitosan through maleimide NHS ester mediated amine-sulfhydryl crosslinking. Briefly, amine-to-sulfhydryl crosslinker Sulfo-SMCC was added to 50µg of IL-12 in 50µl deionized water at 80X molar access. After 30min incubation at room temperature the reaction mixture was desalted and added to 250µg of thiolated chitosan. After 30min, the chitosan-IL-12 conjugates were dialyzed using 100KD dialysis membrane to remove unreacted compounds and freeze dried before further use.

Tyrosinase-catalyzed tyrosine-to-amine crosslinking:

Reactive O-quinone created by tyrosinase catalyzed oxidation of tyrosine on IL-12 was used for crosslinking to chitosan via the primary amine group. Briefly, chitosan was dissolved in 20mM HCL and pH adjusted to 6.0 using 1M NaOH to get 0.1% (w/v) chitosan solution. Tyrosinase was diluted in PBs (pH 6.5) to achieve specific activity of 120U/ml. Equal volumes of tyrosinase and chitosan stock solutions were mixed and left at room temperature. 50µg of IL-12 was added to the reaction mixture and mixed at room temperature. After 8 hours of incubaton, the chitosan-IL-12 conjugates were dialyzed using 100KD dialysis membrane to remove unreacted compounds and freeze dried before further use.

Bioactivity of IL-12 chitosan conjugates:

Influence of non-specific conjugation or IL-12 onto chitosan on IL-12 bioactivity was determined by quantifying the proliferation of IL-12 responsive 2D6 cell line. In brief, cultured 2D6 T-cells were seeded in a 96 well plate at 20,000 cells/well. IL-12 was added to achieve final concentrations of 0.2 ng/mL, 0.04 ng/ml, and 0.008 ng/ml. Un-conjugated IL-12 and culture media alone served as controls. After a 24 hour incubation, IL-12-dependent proliferation of 2D6 cells was quantified via MTT based proliferation assay. The results are shown in Figure 6 and

demonstrate that the various means of linking the IL-12 to the chitosan result in various activity levels. Carboxyl to amine and tyrosine to amine linkage of the IL-12 to chitosan resulted in better IL-12 activity.

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CLAIMS

We claim:

1. A composition comprising chitosan covalently linked to a cytokine or a growth factor, wherein the cytokine or growth factor is biologically active.
2. The composition of claim 1, wherein the cytokine is an interleukin.
3. The composition of claim 1, wherein the cytokine or growth factor is selected from the group consisting of Il-2, IL-12, GM-CSF, IL-1, TNF-α, IFN-γ, IFN-α, IL-15, IL-10, TGF-β, IL-23, IL-27, IL-35 and IL-7.
4. The composition of claim 2, wherein the cytokine is IL-12.
5. The composition of any one of the preceding claims, wherein the covalent linkage is between carboxyl groups or amine groups on the cytokine or growth factor and the amine groups of the chitosan.
6. The composition of any one of claims 1-4, wherein the covalent linkage is between lysine residues in the cytokine or growth factor and the chitosan.
7. The composition of claim 6, wherein the cytokine or growth factor comprises at least one lysine substitution mutation.
8. The composition of claim 7, wherein the cytokine is IL-12 and the IL-12 comprises lysine at a position selected from the group consisting of positions 17, 18, 34, 35, 43, 44 or 248.
9. The composition of any one of claims 1-4, wherein the covalent linkage is between cysteine residues in the cytokine or growth factor and the chitosan.
10. The method of claim 9, wherein the cytokine or growth factor comprises at least one cysteine substitution mutation.
11. The composition of any one of claims 1-10, wherein click chemistry is used to generate the covalent linkage.
12. The composition of any one of claims 1-10, wherein periodate chemistry is used to generate the covalent linkage.
13. The composition of any one of claims 1-10, wherein the cytokine or growth factor is covalently linked to the chitosan using maleimide thioether chemistry.
14. The composition of any one of claims 1-10, wherein the cytokine or growth factor is covalently linked to the chitosan using thiol chemistry.

15. The composition of any one of claims 1-14, wherein the chitosan has a molecular weight between 10 kDa and 500 kDa.

16. The composition of claim 15, wherein the chitosan has a molecular weight of between 100 kDa and 400 kDa.

17. The composition of claim 15, wherein the chitosan has a molecular weight between 200 kDa and 300 kDa.

18. The composition of any one of the preceding claims, wherein the cytokine and the chitosan are linked via a peptide linker.

19. The composition of any one of the preceding claims, wherein the chitosan is modified.

20. The composition of claim 19, wherein the chitosan is thiolated.

21. The composition of claim 19, wherein the chitosan is methylated.

22. A pharmaceutical composition comprising the composition of any one of claims 1-21 and a pharmaceutically acceptable carrier.

23. A method of treating a disorder in a subject comprising administering the composition of any one of claims 1-22 to the subject in an amount effective to treat the disorder.

24. The method of claim 23, wherein the disorder is localized to an area and the composition is administered locally.

25. The method of claim 23 or 24, wherein the composition is administered via a method selected from intratumoral, intravesicular, oral, topical, intranasal, intraperitoneal, parenteral, intravenous, intramuscular, subcutaneous, intrathecal, or transcutaneous administration.

26. The method of any one of claims 23-25, wherein the disorder is cancer.

27. The method of claim 26, wherein the composition is administered intratumorally.

28. The method of claim 26 or 27, wherein the cancer is bladder, breast, colorectal, pancreatic, prostate, renal, lung, melanoma lymphoma, brain, head and neck, or ovarian cancer.

29. The method of any one of claims 23-28, wherein the composition is administered in combination with a second composition to treat the disorder.

30. The method of claim 29, wherein the two compositions are administered in any order, at the same time or as part of a unitary composition.

31. The method of any one of claims 23-30, wherein the disorder can be treated by induction or repression of an immune response by administration of the composition.

32. The method of any one of claims 23-31, wherein the disorder is selected from the group consisting of allergy, autoimmune disease, inflammation, arthritis, Multiple sclerosis, or Crohn's disease.

33. The method of any one of claims 23-32, wherein the subject is a human.

34. A method of stimulating an immune response in a subject comprising administering the composition of any one of claims 1-22 and an antigen to the subject in an amount effective to stimulate an immune response to the antigen.

35. The method of claim 34, wherein the antigen is part of a vaccine.

36. The method of any one of claims 34-35, wherein the subject is a human.

37. The method of any one of claims 34-36, wherein the composition is administered via a method selected from intratumoral, intravesicular, oral, topical, intranasal, intraperitoneal, parenteral, intravenous, intramuscular, subcutaneous, intrathecal, or transcutaneous administration.

Fig. 1

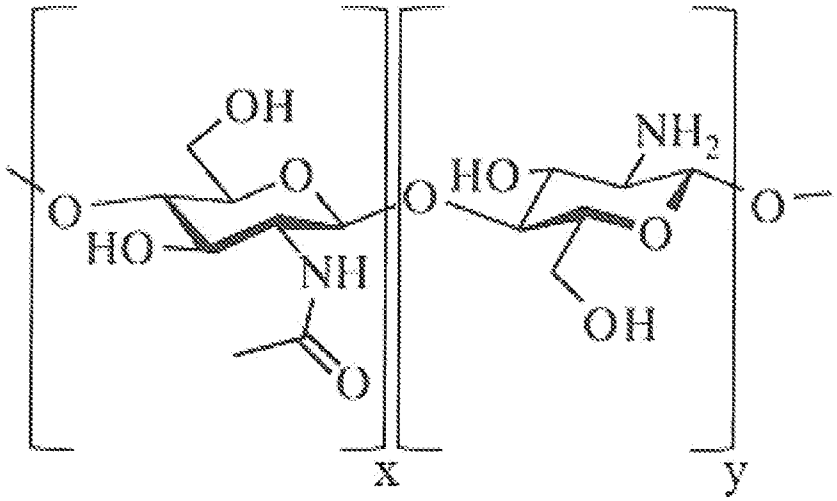

OH
O
HO
NH
O
O
HO
NH2
O
O
OH
x
y

Fig. 2

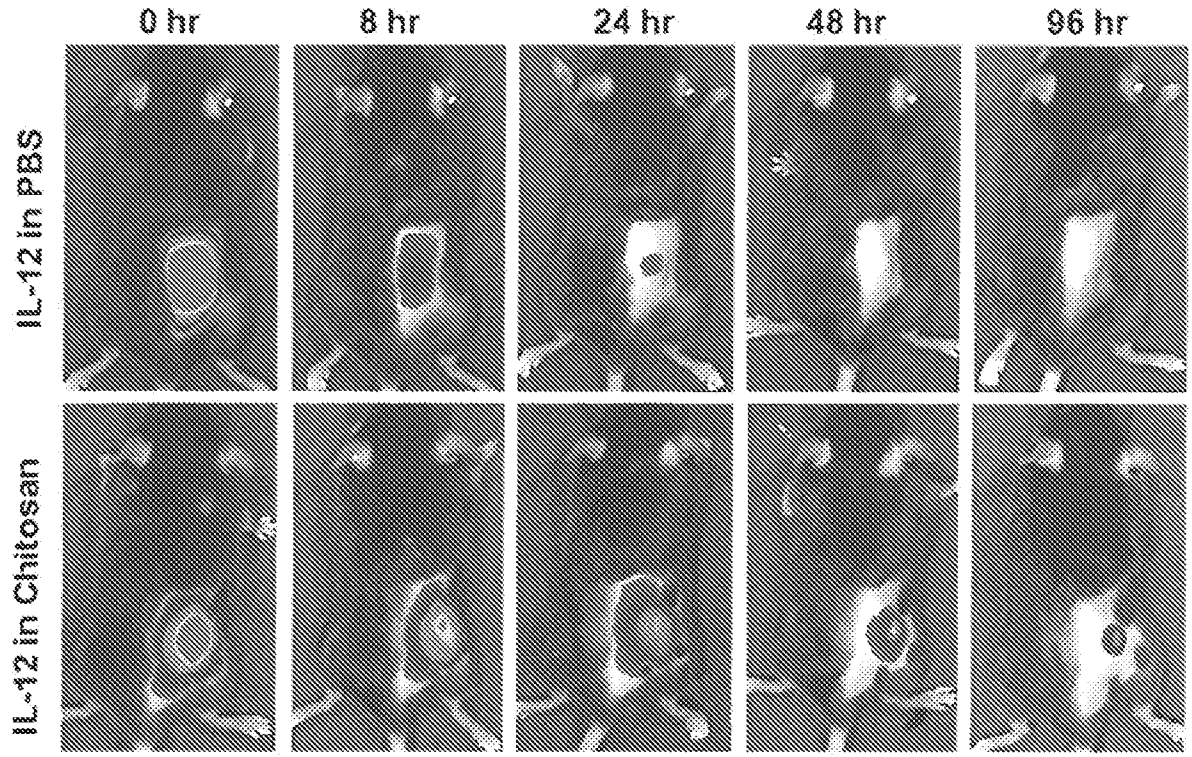

0 hr
8 hr
24 hr
48 hr
96 hr
IL-12 in PBS
IL-12 in Chitosan

Fig. 3

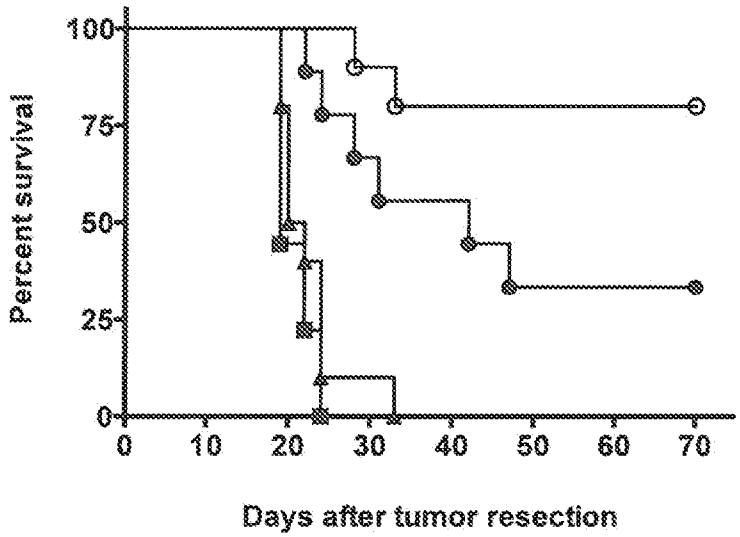

Percent survival
100
75
50
25
0
0
10
20
30
40
50
60
70
Days after tumor resection

Fig. 4A

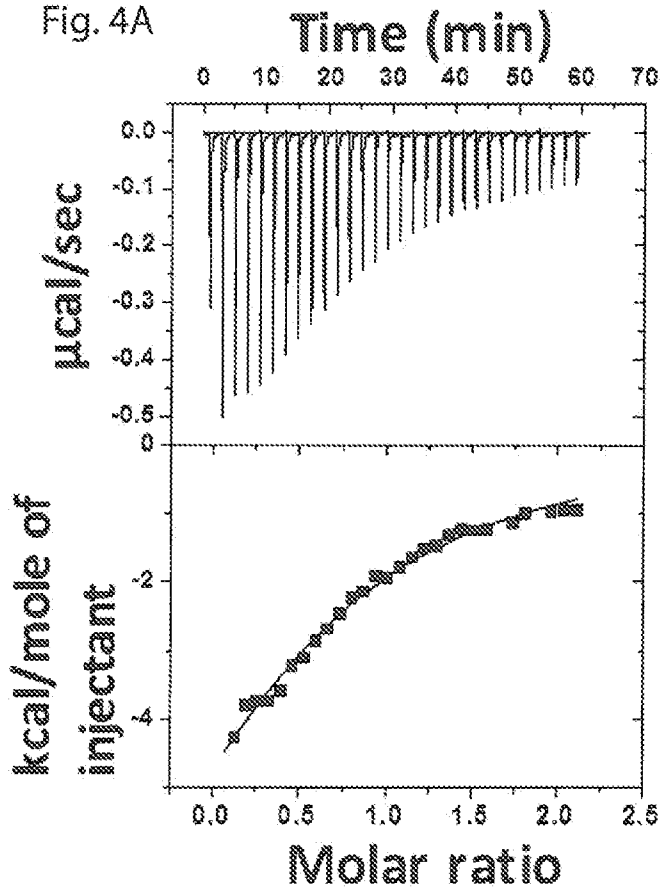

Time (min)
0
10
20
30
40
50
60
70
µcal/sec
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
kcal/mole of injectant
-2
-4
0.0
0.5
1.0
1.5
2.0
2.5
Molar ratio

Fig. 4B

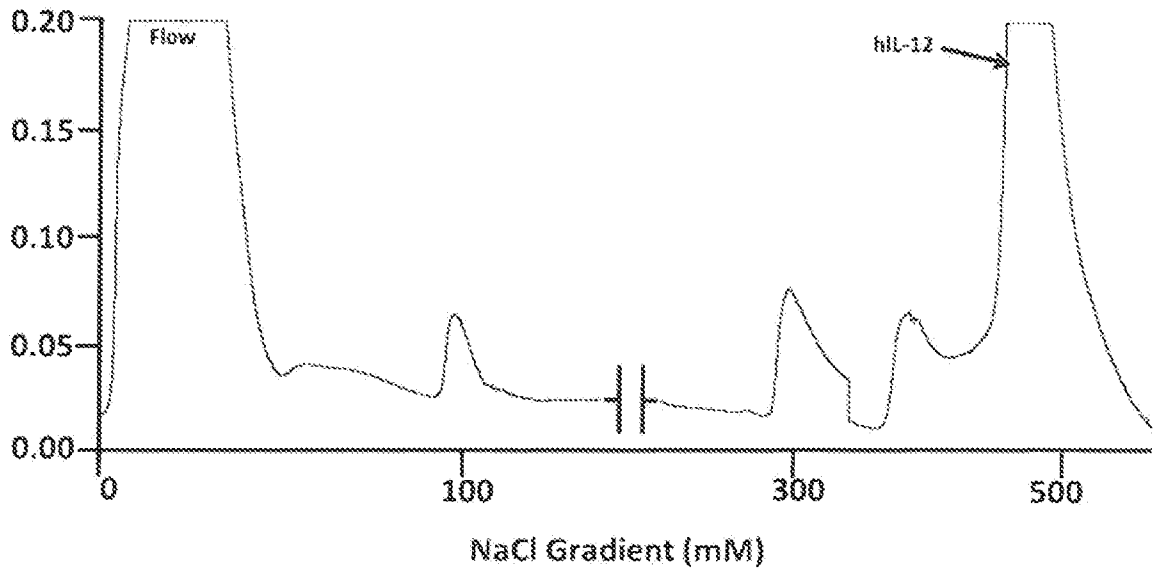


Fig. 4C

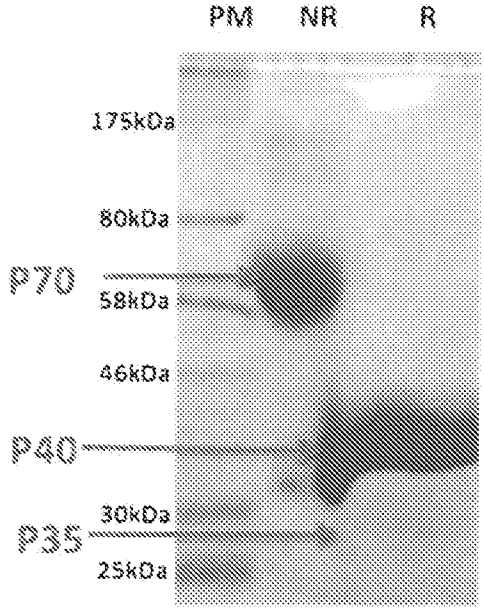


Fig. 4D

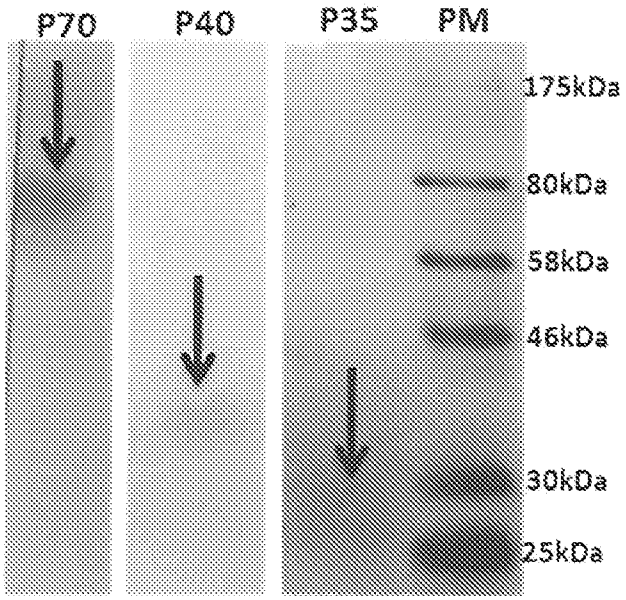


Fig. 5A

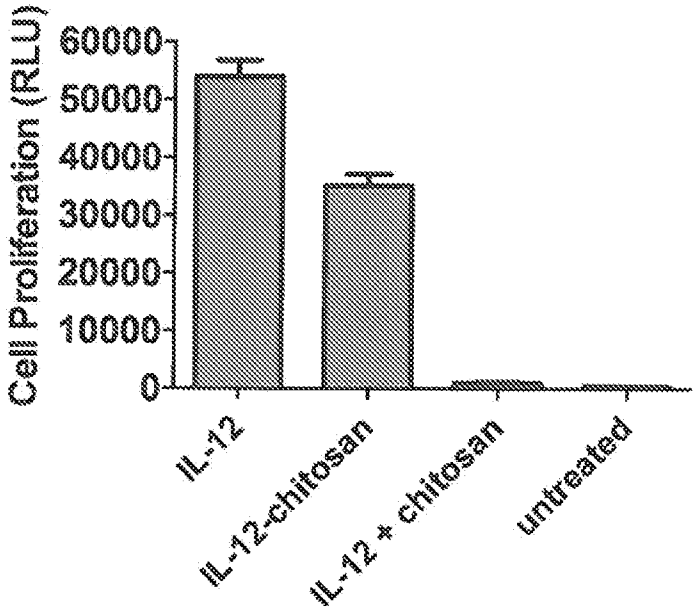

Cell Proliferation (RLU)
60000
50000
40000
30000
20000
10000
0
IL-12
IL-12-chitosan
IL-12 + chitosan
untreated

Fig. 5B

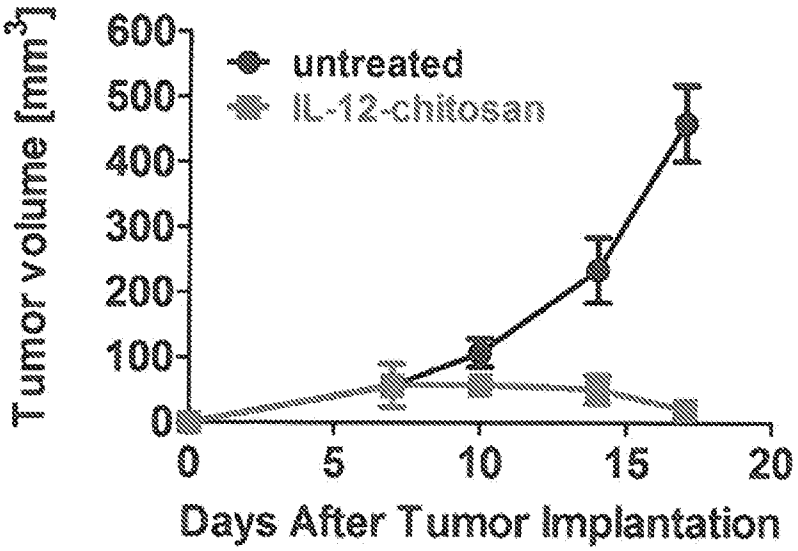
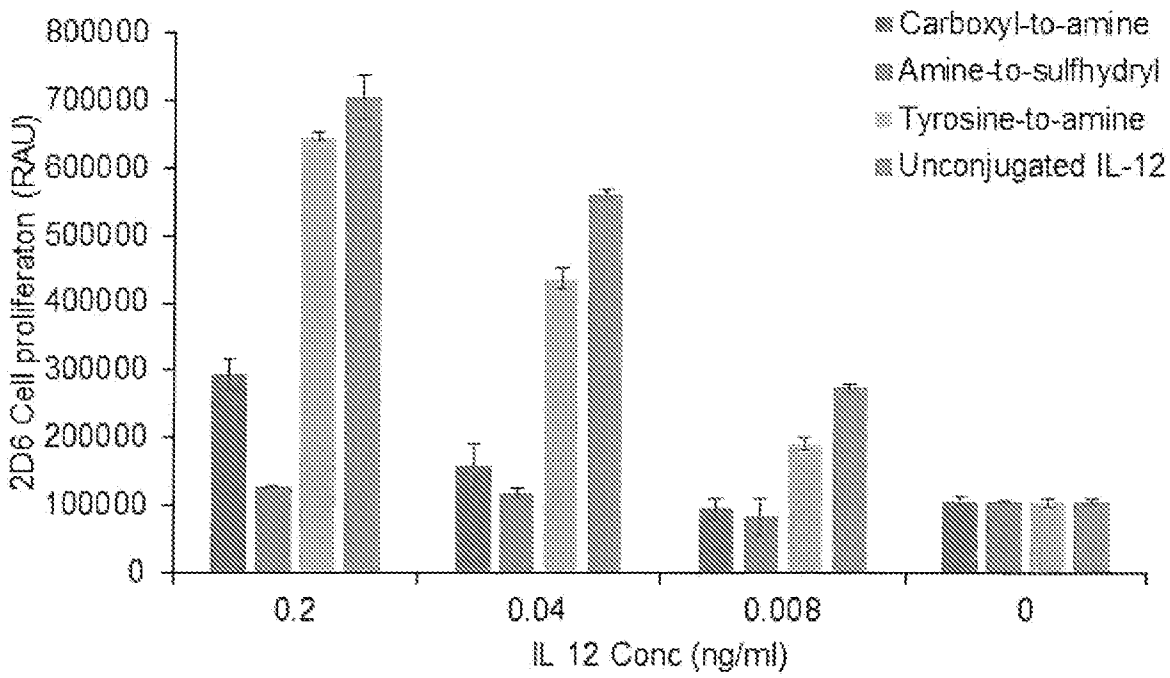

Tumor volume [mm3]
600
500
400
300
200
100
0
untreated
IL-12-chitosan
0
5
10
15
20
Days After Tumor Implantation

Fig. 6


800000
700000
600000
500000
400000
300000
200000
100000
0
2D6 Cell proliferaton (RAU)
0.2
0.04
0.008
0
IL 12 Conc (ng/ml)
Carboxyl-to-amine
Amine-to-sulfhydryl
Tyrosine-to-amine
Unconjugated IL-12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/33541

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01N 43/04, A61K 31/715 (2015.01)
CPC - A61K 31/715, C08B 37/003
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)
IPC(8) - A01N 43/04, A61K 31/715 (2015.01)
CPC - A61K 31/715, C08B 37/003

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 514/55, 514/54

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
pubWEST; PatBase; Google Scholar
search terms - Chitosan, cytokine, growth factor, growth factors, interleuki$, IL-, tnf, ifn, interfer$, CSF, tgf, link$, covalen$, fused, fusion, conjug$, fusing, complex$, cysteine, lysine, disulfide

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 2007/0292387 A1 (JON et al.) 20 December 2007 (20.12.2007) claims 1-3, 14; para [0042]; [0043].	1-3, 5/(1-3) ---------- 4, 5/4, 6-10
Y	Vinsova et al., 'Recent Advances in Drugs and Prodrugs Design of Chitosan' Current Pharmaceutical Design, 2008, 14, 1311-1326 1311. entire document, especially p. 1321, last para to p. 1322, para 1; p. 1317, para 3.	4, 5/4, 6-8, 9/4, 10/4
Y	US 2013/0129674 A1 (BASILE) 23 May 2013 (23.05.2013) para [0187]; [0196].	9, 10
Y	US 2010/0291043 A1 (MEDIN et al.) 18 November 2010 (18.11.2010) para [0009]; [0106]-[0107].	7, 8

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"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)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"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
"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
"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 August 2015 (05.08.2015)	25 AUG 2015
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/33541

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 11-37
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.