Title: ENHANCEMENT OF POLYSACCHARIDE-MEDIATED NUCLEIC ACID DELIVERY

Abstract: The present disclosure provides compositions for enhanced delivery of therapeutic agents. The drug delivery vehicle compositions for delivering therapeutic nucleic acids or therapeutic anionic molecules include a modified polysaccharide (e.g., chitosan), with at least one secondary amine and/or at least one tertiary amine. The polysaccharide may also include an additional secondary amine. The compositions of the disclosure may provide efficient transfection of a therapeutic nucleic acid. Methods for synthesizing the drug delivery vehicle compositions are also set forth. The disclosure also provides methods for delivering/administering therapeutic agents using the drug delivery vehicle compositions of the disclosure to a patient in need thereof.
ENHANCEMENT OF POLYSACCHARIDE-MEDIATED NUCLEIC ACID DELIVERY

FIELD OF THE DISCLOSURE
The present disclosure generally relates to delivery of therapeutic agents. In particular, the present disclosure relates to drug delivery vehicle compositions for enhanced nucleic acid delivery and enhanced anionic therapeutic-agent delivery. Some embodiments of the disclosure relate to methods of making and using such compositions. The present disclosure also relates to methods of administering/delivering a nucleic acid or an anionic therapeutic agent to a patient using the drug delivery compositions of the disclosure.

BACKGROUND OF THE DISCLOSURE
From DNA vaccines for immunotherapy to gene knockdown through antisense oligonucleotides and siRNA, application of nucleic acids to more effectively treat a wide array of complex disorders are becoming more of a reality. Despite being a very powerful therapeutic agent, the efficacy of nucleic acids is limited by their stability, minimal cellular uptake in the native state, poor endosomal escape and inefficient cellular distribution. Over the past 15 years efforts have focused on the use of viral and non-viral carriers for delivering nucleic acids into cells. Although viral vectors are very efficient both in vitro an in vivo, substantial possibility of immunogenic, oncogenic, and cytotoxic consequences are present and has thus facilitated the study of synthetic or natural biomaterials as gene carriers. These materials offer a safer alternative to viral vectors, but suffer from low transfection efficiencies. Recent research has focused on the use of a variety of cationic molecules due to their inherent ability to complex with the negatively charged nucleic acids, providing a means for condensation and imparting a positive surface charge to the nanoparticles. These include cationic lipids as well as cationic polymers such as polyethyleneimine (PEI), dendrimers and polylysine, which have been demonstrated to be effective at enhancing transfection of the nucleic acids and retaining stability and bioactivity. However, cytotoxicity and in vivo systemic toxicity issues have significantly limited human applications of these materials. Thus, development of tailored biomaterials that allow for highly efficient delivery inside cells while
exhibiting minimal or acceptable toxicity is essential for the eventual clinical use of nucleic acids as drugs.

**SUMMARY**

Therefore a need has arisen for better compositions and methods for improved delivery of nucleic acids. The present disclosure provides compositions and methods for enhanced delivery of therapeutic nucleic acids as well as anionic therapeutic agents. Compositions of the disclosure may include a drug delivery vehicle made of a modified polysaccharide having a degree of substitution with at least one secondary amine and/or at least one tertiary amine. In some embodiments, the modified polysaccharide may also include at least one additional secondary amine. In some embodiments, the modified polysaccharide may also include at least two secondary amines. The drug delivery vehicle may also include at least one therapeutic nucleic acid and/or at least one anionic therapeutic agent. In some embodiments, the drug delivery vehicle may be comprised in a nanoparticle. The nanoparticle may be less than 1000 nm in diameter. In some embodiments, the nanoparticle may be about 50 nm, about 100 nm, about 150 nm, about 200 nm, about 250 nm, about 300 nm, about 350 nm, about 400 nm, about 450 nm, about 500 nm, about 550 nm, about 600 nm, about 650 nm, about 700 nm, about 800 nm, about 850 nm, about 900 nm, about 950 nm or about 999 nm, or any intermediate size less than 1000 nm in diameter.

Some exemplary polysaccharides that may be used include chitosans, modified chitosans, modified dextrans, glucosamines, hybrid polymers of any of the preceding polymers, and/or any combinations thereof. Any polysaccharide having at least one primary amine that is naturally present in its structure or having at least one primary amine that is added by one or more chemical reactions may be used as a polysaccharide for obtaining the compositions of this disclosure. Any polysaccharide having at least one primary amine that is naturally present in its structure and having at least one secondary amine that naturally present in its structure may be used as a polysaccharide that may be further modified by the addition of at least one more secondary amine and/or at least one tertiary amine through covalent conjugation of other amine carrying molecules for obtaining the compositions of this disclosure. In some embodiments, one or more secondary amine(s) or one or more additional secondary amine(s) and/or one or more tertiary amine(s) may be introduced into a
polysaccharide by a reactive compound conjugation method. Any covalent conjugation chemistry known to one of skill in the art, in light of this disclosure, may be used. For example, carbodiimide coupling or SH-maleimide coupling are some exemplary non-limiting methods that may be used. In some embodiments, the compositions and methods of the disclosure for enhanced delivery of nucleic acids may have increased solubility, enhanced buffering capacity in an aqueous solution, enhanced endosomal escape properties, may have no biological toxicity, may facilitate cytoplasmic release of a complexed nucleic acid or anionic therapeutic agent, may provide efficient transfection of the nucleic acid, may provide efficient delivery of the anionic therapeutic agent.

In some embodiments, the polysaccharide comprising the drug deliver vehicle may be chemically modified. For example, the natural structure of chitosan comprises primary amines as well as secondary amines with various degree of substitution in the N-acetyl monomeric units. Therefore, in some embodiments, compositions of the disclosure may comprise a modified chitosan having at least one additional secondary amine which has been introduced through covalent conjugation of other secondary-amine carrying molecules. In some embodiments, a composition of the disclosure may comprise a modified chitosan having at least one tertiary amine. In some embodiments, a composition of the disclosure may comprise a modified chitosan having at least one additional secondary amine and at least one tertiary amine that may be introduced through chemical conjugation of other secondary and/or tertiary amine carrying molecules. Exemplary secondary-amine and/or tertiary amine carrying molecules include imidazole-4-acetic acid, arginine, histidine, polyarginine, polyhistidine, or any other secondary and/or tertiary amine carrying molecules or combinations thereof. In some embodiments, the degree of substitution of the modified chitosan with at least one additional secondary amine and/or at least one tertiary amine is at least about 10%. In some embodiments, the degree of substitution of the modified chitosan with at least one additional secondary amine and/or at least one tertiary amine is at least about 19.9% to at least about 30.2%. In some embodiments, the degree of substitution of the modified chitosan with at least one additional secondary amine and/or at least one tertiary amine is at least about 5%, about 10%, about 15%, about 16%, about 17%, about 18%, about 19%, about 19.5%,
about 19.9%, about 20%, about 20.2%, about 20.4%, about 20.5%, about 20.7%, about 20.8%, about 20.9%, about 21%, about 22%, about 23%, about 25%, about 26%, about 27%, about 28%, about 29%, about 30%, about 30.2%, about 30.4%, about 30.6%, about 30.8%, about 31%, about 32%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 99%.

In some embodiments the therapeutic agent may be a nucleic acid, such as but not limited to, a polynucleotide, an oligonucleotide, a DNA, a plasmid DNA (pDNA), a RNA, an antisense molecule, an siRNA, an miRNA, a DNA triple-helix. Other therapeutic agents that may comprise the drug delivery vehicle of the disclosure may be anionic therapeutic agents, such as anionic proteins, anionic biomolecules, or anionic small molecule agents (collectively "anionic agents").

The drug delivery vehicle compositions of the disclosure may have an effective buffering capacity in an aqueous solution from about pH 4.5 to about pH 8.5. In some embodiments, the degree of substitution of the polysaccharide with a secondary amine and/or a tertiary amine changes the buffering capacity. For example, the buffering capacity of chitosan with a degree of substitution with an additional secondary amine and/or a tertiary amine of about 19.9% is between about pH 6.5 and pH 8, while the buffering capacity of chitosan with a degree of substitution of about 30.2% is between about pH 6.0 and pH 8.5. In some embodiments, the modified polysaccharide may be at least 90%, at least 95%, at least 99% or completely soluble in an aqueous solution at a pH greater than about 7, such as the physiological pH of about 7.4. In some embodiments, where the degree of substitution of chitosan is about 20%, the modified chitosan may be at least 90% soluble at above pH 8.

The disclosure also comprises methods for synthesizing the drug delivery vehicle compositions described herein. In some embodiments, a method of the disclosure may include reacting a polysaccharide and a reactive compound; to introduce at least one secondary amine and/or at least one additional secondary amine and/or at least one tertiary amine onto the polysaccharide and complexing an anionic therapeutic agent, such as a therapeutic nucleic acid or a therapeutic anionic small molecule agent to the modified polysaccharide. In some embodiments, complexing the therapeutic nucleic acids or other therapeutic agent may be via electrostatic
interaction. In some embodiments, the secondary and/or tertiary amines may be introduced into a polysaccharide by a reactive compound conjugation method. Therefore, in some embodiments, the polysaccharides of the disclosure, such as a chitosan, a modified chitosan, a dextran modified with primary amines, a glucosamine, a hybrid polymer of any of the preceding polymers, or any combinations thereof may be reacted with a reactive compound comprising an imidazole, such as imidazole-4-acetic acid. The imidazole-4-acetic acid may react with the polysaccharide by carbodiimide chemistry to obtain compositions of the disclosure comprising a polysaccharide comprising at least one secondary amine and/or at least one tertiary amine. In some embodiments, the polysaccharides of the disclosure may be reacted with arginine, histidine, polyarginine, polyhistidine, or any other secondary and/or tertiary amine carrying molecules or combinations thereof, to obtain compositions of the disclosure comprising a polysaccharide comprising at least one secondary amine or at least one additional secondary amine and/or at least one tertiary amine.

In some embodiments of the method the reacting step may continue until the degree of substitution of polysaccharide with a secondary amine, and/or an additional secondary amine; and/or a tertiary amine is about 10%, about 15%, about 16%, about 17%, about 18%, about 18.5%, about 19%, about 19.5%, about 19.9%, about 20%, about 21%, about 22%, about 25%, about 30%, about 31%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, the controlled reaction produces compositions wherein the buffering capacity in an aqueous solution of the polysaccharide modified with a secondary amine and/or a tertiary amine is in the range of from about pH 4.5 to about pH 8.5.

The disclosure also includes methods for therapeutic nucleic acid delivery and/or anionic therapeutic agent delivery comprising administering a drug delivery vehicle according to the disclosure to a patient in need thereof. The patient may be a human patient.

Other features and advantages of the present invention will be readily apparent to those skilled in the art upon a reading of the description of the embodiments that follows.
BRIEF DESCRIPTION OF THE DRAWINGS

Some specific example embodiments of the disclosure may be understood by referring, in part, to the following description and the accompanying drawings.

FIGURE 1 shows a schematic of the carbodiimide reaction for conjugation of IAA to chitosan according to an example embodiment of the disclosure;

FIGURES 2A, 2B & 2C show NMR Spectra for unmodified chitosan (FIGURE 2A), chitosan-IAA 19.9% (FIGURE 2B), and chitosan-IAA 30.2% (FIGURE 2C) according to an example embodiment of the disclosure;

FIGURES 3A & 3B show the characterization of modified polymers: FIGURE 3A shows the buffering capacity of modified chitosan as compared to unmodified chitosan and IAA; and FIGURE 3B shows the cytotoxicity of Chitosan-IAA at 0.25 mg/mL in HEK293T cells (n=3), according to an example embodiment of the disclosure;

FIGURES 4A & 4B show the transfection of HEK293T cells with modified chitosan with equal weight of polymer (FIGURE 4A) and various N/P ratios (FIGURE 4B). The three types of chitosan studied were Protasan UP CL113 (unmodified chitosan), chitosan-IAA 19.9%, and chitosan-IAA 30.2%. Exgen 500 was used as a positive control. All samples are n=3. * = p < 0.05 as compared to unmodified chitosan at same N/P ratio or equivalent weight using t-test, according to an example embodiment of the disclosure;

FIGURE 5 shows the transfection of GAP480 silencer siRNA and Negative Control (NC) siRNA with modified chitosans and siPORT Amine in HEK 293T cells (n=3), * = p < 0.05 as compared to unmodified chitosan at same N/P ratio or equivalent weight using t-test, according to an example embodiment of the disclosure.

While the present disclosure is susceptible to various modifications and alternative forms, specific example embodiments have been shown in the figures and are described in more detail below. It should be understood, however, that the description of specific example embodiments is not intended to limit the invention to the particular forms disclosed, but on the contrary, this disclosure is to cover all modifications and equivalents as illustrated, in part, by the appended claims.
DETAILED DESCRIPTION

The present disclosure comprises vehicles for delivering a therapeutic nucleic acid or a therapeutic anionic agent comprising a polysaccharide (e.g. chitosan), comprising at least one secondary amine or at least one additional secondary amine, and/or at least one tertiary amine. The present disclosure also includes methods for synthesizing the drug delivery vehicle compositions. In some embodiments, a method for synthesis of the delivery vehicle compositions of the disclosure comprise providing a polysaccharide and a reactive compound; allowing the reactive compound to react with the polysaccharide so as to introduce at least one secondary amine or at least one additional secondary amine, and/or tertiary amine onto the polysaccharide, thereby obtaining a modified polysaccharide comprising at least one secondary and/or at least one additional secondary amine, and/or at least one tertiary amine(s). In some embodiments, a therapeutic nucleic acid may be associated with a modified polysaccharide delivery vehicle composition of the disclosure. In some embodiments, a therapeutic anionic agent may be associated with a modified polysaccharide delivery vehicle composition of the disclosure.

The present disclosure also provides methods for delivering/administering therapeutic nucleic acids comprising the steps of providing a polysaccharide, and a reactive compound; allowing the reactive compound to react with the polysaccharide so as to introduce at least one secondary amine and/or at least one additional secondary amine and/or at least one tertiary amine onto the polysaccharide to create a modified polysaccharide; providing a therapeutic nucleic acid sequence; associating or complexing the nucleic acid sequence to the modified polysaccharide to create a drug delivery vehicle, and introducing the drug delivery vehicle into the patient. Therapeutic nucleic acids may include any nucleic acid known in the art and may comprise: a polynucleotide, a DNA sequence encoding a therapeutic protein, a DNA sequence, an RNA sequence, a small interfering RNA (siRNA), a micro RNA (miRNA), an antisense oligonucleotide, a triplex DNA, and plasmid DNA (pDNA).

The therapeutic nucleic acid may be treated or chemically modified. For example, the therapeutic nucleic acid may contain inter-nucleotide linkages other than phosphodiester bonds, such as phosphorothioate, methylphosphonate, methylphosphodiester, phosphorodithioate, phosphoramidate, phosphotriester, or
phosphate ester linkages, resulting in increased stability. Nucleic acid stability may also be increased by incorporating 3'-deoxythymidine or 2'-substituted nucleotides (substituted with, e.g., an alkyl group) into the nucleic acid during synthesis or by providing the nucleic acid as phenylisourea derivatives, or by having other molecules, such as aminoacridine or poly-lysine, linked to the 3' end of the nucleic acid. Modifications of the RNA and/or DNA may be present throughout the oligonucleotide or in selected regions of the nucleic acid, e.g., the 5' and/or 3' ends, for example by methylation.

The polysaccharide may comprise any polysaccharide that is capable of enduring the rigors of drug delivery vehicle synthesis and administration and is able to associate with a nucleic acid sequence. In some embodiments, the polysaccharide may be a chitosan, a dextran modified to comprise primary amines, a glucosamine, any polysaccharide that comprises a primary amine and optionally comprises one or more secondary amines, either naturally or by synthetic methods hybrid polymers of any of the preceding polymers, or any combinations thereof. Thus, the disclosure includes pharmaceutically acceptable drug delivery vehicles comprising therapeutic nucleic acids and methods for administering such compositions to a patient in need thereof. Therefore, the disclosure comprises methods for treatment and/or prevention and/or alleviation of certain pathological or physiological disease conditions wherein a therapeutic nucleic acid may be used. Such diseases and conditions are well known and include but are not limited to examples such as cancers, tumors, HIV, diabetes, genetic disorders, neurodegenerative diseases, inflammatory disorders, heart disease (e.g., cholesterol reduction), and bacterial, viral or fungal infections.

Chitosan as a Carrier of Nucleic Acids

Chitosan is a polysaccharide comprised of beta (1-4) linked units of glucosamine and N-acetyl glucosamine. Thus, naturally occurring chitosan comprises primary amines and has various degrees of substitution with secondary amines in its N-acetyl glucosamine subunits. Chitosan has been used as a non-viral, cationic carrier for gene delivery such as delivery of DNA vaccines via the oral route taking advantage of its mucoadhesive properties. Chitosan has also been shown to carry nucleic acids for intranasal, intrabiliary, and intravenous administrations. Chitosan
may also be used as a carrier for siRNA. Characterization of some chitosan-pDNA nanoparticles showed nanoparticle formations of 150-250 nm with zeta potentials up to +18 mV. The characterization also showed limitations of solubility and buffering capacity of chitosan-pDNA in aqueous solution when compared to other non-viral gene delivery vectors such as polyethylenimine. In addition, in spite of the applications described above, the inherent transfection efficacy of native chitosan is significantly less than other cationic polymers (e.g. PEI, dendrimers) or lipids. For example, *in vitro* studies have shown differences in protein expression by orders of magnitude when compared to PEI or lipofectamine based transfection. Lower transfection efficiencies may in part be due to low buffering capability and poor solubility of chitosan at neutral pH as well as incomplete dissociation of the nucleic acid in the cytoplasm. Some modifications of chitosan have been evaluated for their ability to deliver nucleic acids. Trimethylation of chitosan has been shown to enhance solubility at physiological pH, while thiolation has been shown to enhance mucoadhesive properties. Polyethylene glycol conjugation to chitosan provides enhanced stability and reduced nanoparticle aggregation with no reduction in transfection efficiency. Deoxycholic acid conjugation to chitosan has shown enhanced formation of self-aggregated nanoparticles with pDNA. Galactosylated chitosan-PEG conjugates have also shown enhanced targeting of nanoparticles with plasmid DNA to the liver. Polyethylenimine (PEI) grafted to chitosan showed enhanced transfection of plasmid DNA with improved endosomal escape, but the cytotoxicity of PEI poses a significant concern in real life applications. However, none of these efforts have been able to address specific transport barriers, solubility, buffering capacity, or improved intracellular nucleic acid delivery and none have provided efficient transfection.

**Amine Modifications of Chitosan**

In some embodiments of the present disclosure, compositions comprising secondary amines, and/or additional secondary amines, and/or tertiary amine modifications of chitosan are described. The secondary and/or tertiary amine modifications of chitosan when used as nucleic acid delivery agents have a high transfection efficiency. In some embodiments, an increase in the degree of
substitution of the modified chitosan (i.e., increase in the number of secondary or tertiary amine residues on the chitosan polymer) may increase the transfection efficiency. Therefore, increase in expression of protein encoded by the nucleic acid and/or transcription or translation of the nucleic acid may be observed. The compositions of the disclosure may also have a high effective buffering capacity in aqueous solutions. In some embodiments, buffering capacity past pH 8 may be achieved at about 20% degree of substitution with secondary and/or tertiary amine residues of the chitosan. In some embodiments, the compositions of the disclosure may have a buffering capacity in aqueous solutions of from about pH 4.5 to about pH 8.5. In some embodiments, increased buffering is achieved at higher degrees of substitution of the chitosan with secondary and/or tertiary amine residues. In some embodiments, an increase in degree of substitution with secondary and/or tertiary amines may result in increased solubility of chitosan. In some embodiments, an increase in degree of substitution with secondary and/or tertiary amines may result in complete solubility of chitosan in an aqueous solution at pH of about 8.

In some embodiments, methods for preparing the compositions may include controlled conjugation of imidazole-4-acetic acid (IAA) to the primary amines of chitosan using carbodiimide chemistry. Secondary and/or tertiary amines may be introduced onto the polysaccharide structure using these methods. In some embodiments, the chitosan may be modified to have a degree of substitution of about 10-15% secondary amines prior to addition of additional secondary amines and/or tertiary amines using the above described methods to obtain the compositions of the disclosure. In some embodiments, the polysaccharide polymer may comprise one or more primary, secondary as well as tertiary amines.

**Pharmaceutical Formulations and Delivery**

The drug delivery vehicles of the present disclosure may be used in the manufacture of medicaments and for the treatment of humans and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions. While it is possible for a drug delivery vehicle of the disclosure to be administered alone, it may be preferable to present it as a pharmaceutical formulation comprising at least one active ingredient (e.g., an
amine-modified polysaccharide vehicle comprising a therapeutic nucleic acid/or a therapeutic anionic agent) together with one or more pharmaceutically acceptable carriers therefore and optionally other therapeutic agents. According to some embodiments, a carrier may be acceptable in the sense of being compatible with the other ingredients of the formulation and not injurious to the mammal. Pharmaceutical compositions, including drug delivery vehicles may comprise combinations of the polysaccharides of the disclosure with a therapeutic nucleic acid or therapeutic anionic agent and a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use in vitro, in vivo or ex vivo. In some embodiments, a therapeutic anionic agent may be a therapeutic anionic biomolecule such as an anionic protein may be delivered. A pharmaceutically acceptable carrier may encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also may include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)), incorporated by reference herein. An effective amount may be an amount sufficient to effect beneficial or desired results. An effective amount may be administered in one or more administrations, applications or dosages. The pharmaceutical compositions of the invention may be administered to a mammal such as a human patient in need thereof. Mammals may include, but are not limited to, humans, murines, simians, farm animals, sport animals, and pets.

Methods of Administration

Administration or delivery of a pharmaceutical composition of the present disclosure may comprise any method which ultimately provides the drug delivery vehicle of the disclosure comprising a therapeutic nucleic acid to cell/tissue/organ or site it is needed at. The drug delivery vehicles of the present disclosure may be delivered to a patient by a variety of means, including, but not limited to, oral ingestion, sublingual administration, intranasal, intramuscular injection, subcutaneous injection, parenteral administration, intrabiliary or topical application. In some embodiments parenteral administration may comprise intravenous administration,
intraperitoneal administration, subcutaneous administration, intrathecal administration, injection to the spinal cord, intramuscular administration, intraarticular administration, portal vein injection, or intratumoral administration. Topical administration may include administration to skin, eye, or any mucosal membranes. In some embodiments, a pharmaceutical composition of the disclosure may be contacted with a target tissue by direct application of the composition to the tissue. The drug delivery vehicles of the disclosure, comprising the amine modified polysaccharide carrying a therapeutic nucleic acid agent (or therapeutic anionic agent), may be introduced into a patient in an amount sufficient to produce a desired clinical effect, including, but not limited to, a change in gene expression, a change in gene transcription, a change in protein structure, a post-translational modification of a protein, or the treatment and/or prevention and/or alleviation of a medical condition. Administration in vivo may be effected in one dose, continuously or intermittently throughout the course of treatment. During the initial determination of dosage requirements, monitoring parameters that define the condition/disease may be advisable. Methods of determining the most effective means and dosage of administration are known to those of skill in the art, in light of this disclosure, and may vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the mammal being treated. Single or multiple administrations may be carried out with the dose level and pattern being selected by the treating physician. Suitable dosage formulations and methods of administering the pharmaceutical compositions may be empirically determined by those of skill in the art in light of this disclosure.

EXAMPLES

The following additional examples are offered to illustrate some embodiments of the invention, and should not be viewed as limiting the scope of the invention.

Materials

Protasan UP CL113 (Chitosan chloride salt) was purchased from Novamatrix, Norway (MW = 130,000 Da, Degree of Deacetylation = 86%). Imidazole-4-acetic acid monohydrochloride was purchased from AlfaAesar, Ward Hill, MA. EDC (1-
ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride) and Snakeskin pleated dialysis tubing (3,500 MW cut-off) were purchased from Pierce Biotechnology, Inc., Rockford, IL. Exgen500 was purchased from Fermentas, Hanover, MD. Ninhydrin reagent was from Sigma Aldrich, St. Louis, MO. Plasmid pgWiz Luciferase was purchased from Aldevron, LLC., ND. Silencer GAPDH siRNA (a gift) and SiPORT Amine were obtained from Ambion, Inc., Austin, TX. HEK293T cells were obtained from the American Type Culture Collection, Manassas, VA. Gibco modified DMEM was used for cell culture medium with all remaining cell culture reagents purchased through Invitrogen, Carlsbad, CA.

Synthesis of Modified Chitosan

Imidazole-4-acetic acid monohydrochloride conjugation to the primary amines of chitosan was achieved via use of carbodiimide chemistry. The reaction was performed at various ratios of IAA to the number of primary amines in chitosan. The general synthesis was as follows: A solution of 0.5% w/v chitosan PCL113 (Protasan) was prepared by dissolving the PCL113 in 0.1 M MES buffer, pH 5. An imidazole acetic acid (IAA) solution (2.0% w/v in 0.1 M MES buffer, pH 5.0) was also prepared. Both solutions were held on ice, and variable amounts of IAA solution were then added to the chitosan solution to achieve various degrees of primary amine modification. The chitosan-IAA solution was added to a 20 M excess of EDC (in relation to IAA) to promote addition of IAA to the chitosan backbone and immediately vortexed for 60 seconds. The final solution was then left to react overnight with end over end mixing. Dialysis was performed using the Snakeskin pleated dialysis tubing for 24 hours with the following cycles: 3 times for 2 hours against 3 mM HCl buffer followed by 3 cycles of 6 hours in length against de-ionized water. Samples were then left to lyophilize for 24 hours.

In a controlled manner, imidazole-4-acetic acid (IAA) was conjugated to the primary amines of chitosan using carbodiimide chemistry thereby introducing secondary and/or tertiary amines to the polysaccharide structure. A schematic of the conjugation scheme is shown in FIGURE 1. EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) reacts with the carboxylic acid of imidazole acetic acid and forms an intermediate that is amine-reactive. This
intermediate then reacts with the primary amines of chitosan forming an amide bond. The resulting polymer is a polysaccharide carrying primary, secondary as well as tertiary amines.

Ninhydrin Assay for Quantification of Degree of Modification

A ninhydrin assay was performed to determine the degree of IAA conjugation to the primary amines of chitosan. Briefly, a 0.2% w/v solution of chitosan was prepared by dissolving the PCL113 in 25 mM sodium acetate buffer, pH 5.5, and serially diluted to prepare the standards. A 0.05% w/v solution of each modified chitosan sample was prepared in the same sodium acetate buffer. The ninhydrin reagent was added to each solution in a 2:1 ratio and placed in a boiling water bath for 15-20 minutes. The optical density was then measured at 570 nm.

About 19.9% to 30.2% of chitosan primary amines were successfully modified with IAA (therefore the degree of substitution of chitosan with IAA was between 19.9% and 30.3%). Higher degrees of substitution (75% and 90%) were also achieved with increasing amounts of IAA. However, in order to preserve enough primary amines for nucleic acid condensation and for further ligand attachments to nanoparticles, the chitosan polymers with degrees of substitution of about 19.9% and about 30.2% were used for further analysis. The efficiency of the reaction ranged from about 60% to about 80% of the targeted modification.

$^1$H NMR Characterization

$^1$H NMR spectra of the experimental compounds were obtained with a Varian INOV-500 (500 MHz) at 80°C in D$_2$O and spectra were displayed in PPM. As shown in FIGURES 2A, 2B and 2C show the NMR spectra for unmodified chitosan (FIGURE 2A), chitosan-IAA 19.9% (chitosan with a 19.9% degree of substitution) (FIGURE 2B), and chitosan-IAA 30.2% (chitosan with a 30.2% degree of substitution) (FIGURE 2C). The NMR spectra indicates several characteristic peaks for chitosan including peak for the acetyl groups at 2.5 ppm, for C2 carbon at 3.6 ppm, and peaks for C1, C3, C4, C5, and C6 ranging from 4.2 ppm to 5.4 ppm. The C2 peaks in the modified chitosan samples show peak separation as a result of imidazole acetic acid addition to the primary amine resulting in a change of the
harmonic frequency for the C2 carbons. This NMR signature increases with increasing degree of modification of the polysaccharide. As shown, going from a 19.9% degree of substitution to a 30.2% degree of substitution, the peaks from 3.2 - 3.4 ppm increases in size while the peak at 3.6 ppm reduces in size. As calculated from the $^1$H NMR spectra, the degree of substitution for the two samples were 18.9% and 32.7% respectively.

**Buffering Capacity of Modified Chitosan**

Effective transfection of nucleic acids may depend on the efficiency of endosomal escape of the nanoparticle carriers. Endosomal escape properties have been associated to buffering capacity of the carrier polymer in aqueous solution within the pH ranges of 5.2 to 7 by a mechanism referred to as “proton sponge.” Tests of the buffering capacity of the modified polysaccharides and imidazole acetic acids in aqueous solution were performed following the method described by Tang and Szoka (1997). Each sample was put into solution in 150 mM NaCl to a concentration of 0.5 mg/mL. Solutions were then titrated incrementally with 0.01N NaOH from pH below 4.0 to pH 9.0 with pH read after each increment.

FIGURE 3A depicts the buffering capacity of modified chitosan as compared to unmodified chitosan and IAA. As seen in FIGURE 3A, limited buffering is achieved with unmodified chitosan, which may be due to the presence of primary amine alone as well as reduced solubility beyond pH 6.5. Imidazole acetic acid alone, although similar in buffering capacity from pH 5 to 6.5, demonstrated effective buffering at pH greater than 8 (data not shown). Chitosan-IAA was studied at various degrees of substitution (for example, 19.9%, 30.2%, 75%, and 90%). Effective buffering capacity was achieved past pH 8 with degrees of substitution of 19.9% and greater, with increased buffering at higher degrees of substitution. In some embodiments, increase in secondary and tertiary amine content allowed complete solubility of chitosan at pH > 7.

**Determination of Cytotoxicity.**

HEK293T cells were seeded in 96-well plates at a density of 10,000 cells per well in 0.2 mL of cell growth medium. After 24 hr, the medium was removed and
replaced with 0.1 mL of serum free medium containing chitosan (modified and unmodified) and grown for an additional 48 hr. Each sample of chitosan was added in concentrations of 0.25 mg/mL (10-fold of transfection concentration) and performed in triplicate. The metabolic activity of each well was determined relative to control wells using the MTT assay. After incubation, 100 µL of Hank’s Balanced Salt solution and 20 µL of MTT solution (5 mg/mL in Hank’s Balanced Salt solution) were added to each well. After 3 hr at 37°C, 120 µL of MTT solubilization solution was added to break up the formazan crystals. The optical density of each well was then measured at 570 nm.

Minimal to no cytotoxicity was observed for the chitosan polymers with degrees of substitution of 19.9% and 30.2% as compared to untreated cells (FIGURE 3B). Cell viability remained similar to the unmodified chitosan thus demonstrating no change in polymer toxicity following introduction of secondary and tertiary amines.

**Nanoparticle Formation**

Separate nanoparticle formulations were prepared for both pDNA (pgWiz Luciferase) and siRNA (Silencer GAPDH siRNA) with chitosan and chitosan-IAA at various nitrogen to phosphate (N/P) ratios. For pDNA nanoparticles, chitosan and chitosan-IAA solutions (0.01–0.06% in 25 mM sodium acetate buffer, pH 5.5) and a pDNA solution of 4 µg/mL in 25 mM sodium sulfate were preheated to 50–55°C separately. An equal volume of chitosan solution was added drop-wise to a pDNA solution and vortexed for 20–30 seconds. The final volume of the mixture in each preparation was 200 µL. Nanoparticle preparation for siRNA was performed identically with RNAse free solutions, 200 mM sodium acetate, and with siRNA in RNAse free water rather than sodium sulfate. The nanoparticles were used for transfection, cytotoxicity studies, particle sizing, zeta potential, and gel retardation assays.

**Nanoparticle Characterization**

For particle-mediated delivery of nucleic acids (in which the particles form the drug delivery vehicle), appropriate ranges of size and surface charge may result in effective cellular uptake. In some embodiments, cationic particles as well as particles
below 500 nm may be used to enhance endocytosis. Modifications of the polymer and nucleic acid ratio as reflected in the N/P ratio) may significantly influence both size and zeta potential of the resulting nanoparticles. Therefore, particle size distribution was determined by Dynamic Light Scattering with a ZetaPlus system (Brookhaven Instruments Corporation, Holtsville, NY). Zeta potential was also analyzed using the ZetaPlus system. Nanoparticle solutions of 1 mg/mL concentration were diluted in 1mM KCl, and then read in the ZetaPlus system. Ten readings were taken for each sample. Nanoparticle formulations were prepared at different N/P ratios to study the effects on size and zeta potential.

As shown in Table 1, particles formed with the modified chitosan varied in size based on the N/P ratio and degree of substitution. Particles formed with the modified chitosans and pDNA were significantly larger in size compared to particles formed using the unmodified polymer. In addition, the particle size decreased with increasing N/P ratio for a given polymer. However, no significant difference in size was observed between the two modified polymers at a given N/P ratio. For example, for unmodified chitosan/DNA nanocomplexes, average particle size ranged from 161-311 nm, while the chitosans with degrees of substitution of 19.9% and 30.2% produced particles with average sizes from 267-861 nm and 324-831 nm, respectively (at various N/P ratios). Similar results were demonstrated with siRNA, however with smaller effective diameters than with pDNA (Table 1). Zeta potential was also significantly affected by both the N/P ratio and degree of substitution (Table 2). Average zeta potentials for the unmodified chitosan/DNA complexes ranged from 0.11 to 12.07 mV and increased with increasing N/P ratio. This significantly increased for both the chitosan-IAA 19.9% (19.88-26.05 mV) and chitosan-IAA 30.2% (23.06-24.9 mV). A similar trend was observed with siRNA nanocomplexes. However, no significant difference in zeta potential was observed between the two different modified chitosans.
Table 1

<table>
<thead>
<tr>
<th>N/P Ratio</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified Chitosan</td>
<td>311.9 ± 4.5</td>
<td>225.2 ± 0.8</td>
<td>219.9 ± 6.2</td>
<td>161.8 ± 30.4</td>
</tr>
<tr>
<td>Chitosan-IAA 20</td>
<td>861.1 ± 75.2</td>
<td>687.8 ± 34.6</td>
<td>472.3 ± 7.9</td>
<td>267.3 ± 1.7</td>
</tr>
<tr>
<td>Chitosan-IAA 30</td>
<td>831.4 ± 27.8</td>
<td>712.5 ± 55.9</td>
<td>511.2 ± 17</td>
<td>324.1 ± 11.8</td>
</tr>
</tbody>
</table>

Effective Diameter (nm) of Nanoparticles with siRNA

<table>
<thead>
<tr>
<th>N/P Ratio</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified Chitosan</td>
<td>218.1 ± 14.8</td>
<td>137.8 ± 4.4</td>
<td>111.2 ± 19.2</td>
<td>73.4 ± 23.5</td>
</tr>
<tr>
<td>Chitosan-IAA 20</td>
<td>242.1 ± 9.6</td>
<td>206.7 ± 9.3</td>
<td>128.8 ± 4.9</td>
<td>152.9 ± 13.9</td>
</tr>
<tr>
<td>Chitosan-IAA 30</td>
<td>295.7 ± 8.7</td>
<td>176.1 ± 5.7</td>
<td>163.1 ± 4.7</td>
<td>156.2 ± 4.7</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>N/P Ratio</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified Chitosan</td>
<td>0.11 ± 0.64</td>
<td>10.76 ± 2.36</td>
<td>11.90 ± 3.05</td>
<td>12.07 ± 1.12</td>
</tr>
<tr>
<td>Chitosan-IAA 20</td>
<td>19.88 ± 1.06</td>
<td>21.02 ± 0.85</td>
<td>21.94 ± 2.5</td>
<td>26.05 ± 0.87</td>
</tr>
<tr>
<td>Chitosan-IAA 30</td>
<td>23.06 ± 0.82</td>
<td>24.21 ± 0.79</td>
<td>24.35 ± 0.47</td>
<td>24.9 ± 1.38</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N/P Ratio</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified Chitosan</td>
<td>15.73 ± 4.10</td>
<td>17.63 ± 3.76</td>
<td>20.28 ± 1.82</td>
<td>34.07 ± 2.28</td>
</tr>
<tr>
<td>Chitosan-IAA 20</td>
<td>29.85 ± 3.64</td>
<td>29.85 ± 3.30</td>
<td>32.45 ± 2.98</td>
<td>38.28 ± 2.92</td>
</tr>
<tr>
<td>Chitosan-IAA 30</td>
<td>30.63 ± 0.15</td>
<td>34.66 ± 8.05</td>
<td>33.89 ± 4.49</td>
<td>37.58 ± 1.90</td>
</tr>
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</table>

In Vitro Transfection of HEK293T Cells with Chitosan-DNA Nanoparticles

In vitro transfection efficiency was studied on HEK293T using the pgWiz Luciferase plasmid. HEK293T cells were seeded 24 hr prior to transfection in a 24-well plate at a density of 5x10⁴ cells per well in 1.0 mL of complete medium (DMEM containing 10% FBS, supplemented with 1% penicillin and streptomycin). Nanoparticles were prepared with the modified and unmodified chitosan with varying concentrations of chitosan and amounts of DNA. The three types of chitosan studied were Protasan UP CL113 (unmodified chitosan), chitosan-IAA 19.9%, and chitosan-IAA 30.2%. Exgen 500 was used as a positive control. The amount of nanoparticles equivalent to 1.0 µg of DNA was added to each well and incubated with the cells for 4 hr in 0.5 mL of serum-free medium. After 4 hours, 0.5 mL of DMEM medium (20%
FBS 2% penicillin and streptomycin) was then added each well to bring total volume with 1 mL and cells were further incubated for 44 hr at 37°C before being analyzed for transfection efficiency. As a positive control, transfection with PEI-DNA complexes (Exgen 500) was performed. All transfection experiments were performed in triplicate.

Transfection results (FIGURE 4A) demonstrated a substantial increase in efficiency based on the degree of modification when equivalent amounts of chitosan or chitosan-IAA were used to prepare the nanoparticles. In FIGURE 4A, the N/P ratios for unmodified chitosan, chitosan-IAA 19.9%, and chitosan-IAA 30.2% are 19, 24, and 26, respectively. All samples are n=3. * = p < 0.05 as compared to unmodified chitosan at same N/P ratio or equivalent weight using t-test, † = p < 0.05 as compared to unmodified chitosan at same N/P = 5 sample within each chitosan polymer degree of modification using t-test. Greater than 10-fold increase in average luciferase expression was observed for the chitosan-IAA 19.9% and approximately 100-fold increase for the chitosan-IAA 30.2%.

When nanoparticles were prepared at various N/P ratios (FIGURE 4B), some variations were seen in the transfection efficiencies. With an increase in N/P ratio, a significant increase in transfection was noted, for all polymers. However, cytotoxicity limitations were evident with Exgen 500 (PEI), which was used as a positive control for transfection efficacy. At lower N/P ratios (N/P = 5, 10), both type of modified chitosans provided over 100-fold greater luciferase expression as compared to unmodified chitosan and Exgen 500 (a linear 25 kDa polyethyleneimine). The higher transfection efficiency compared to Exgen 500 is likely due to the higher number of protonated amine groups in the chitosans, whereas only 1 out of every 3 amines in Exgen 500 is protonated. At higher N/P ratios, the Exgen 500 was most effective; however cytotoxic effects of PEI, especially at high concentrations are well documented. The modified chitosans both showed greater than 10 to 100–fold higher efficiency than the unmodified chitosan, while retaining minimal cytotoxicity.

**Luminometric Assay for Luciferase**

Following transfection for 48 hours, the cells were permeabilized with 250 μL of Glo- Lysis buffer to release the luciferase protein for analysis. Luciferase activity
in cell extracts was measured using a Bright-Glo luciferase assay (Promega, Madison, WI) on a luminometric plate reader. Normalization of RLU levels for each well was done by determination of overall protein content in each well using a Micro BCA protein assay kit (Pierce Biotechnologies, Inc., Rockford, IL).

_In Vitro Transfection of HEK293T Cells with Chitosan-siRNA Particles_

HEK293T cells were seeded 24 hr prior to transfection in a 96-well plate at a density of $1 \times 10^4$ cells per well in 0.2 mL of complete medium (DMEM containing 10% FBS, supplemented with 1% penicillin and streptomycin). Nanoparticles formulations at various N/P ratios were studied. An amount of nanoparticles carrying 50 nM of siRNA was added to each well and incubated with the cells for 4 hr in 0.1 mL of serum-free medium. After 4 hours, 0.1 mL of DMEM medium (20% FBS 2% penicillin and streptomycin) was added to each well to bring total volume with 0.2 mL and cells were further incubated for 44 hr at 37°C before being analyzed for transfection efficiency. As a positive control, transfection of the siRNA was performed using siPORT Amine. All transfection experiments were performed in triplicate.

_Analysis of GAPDH Knockdown with Chitosan-IAA/siRNA_

_Nanoparticles_

GAPDH knockdown was analyzed using the KDalert GAPDH assay kit (Ambion, Inc., Austin, TX). After transfection for 48 hours, media was removed from each well and replaced with 0.2 mL of KDalert lysis buffer and incubated at 4°C for 20 minutes. 10 μL of each lysate was then transferred to a new 96 well plate and 90 μL of KDalert master mix was then added to each well. Fluorescence levels for each well were then read immediately after addition of the master mix ($λ_{Exc}= 560$ nm and $λ_{Emm} = 590$ nm). After 4 minutes, fluorescence of the plate was measured again, and the difference of the two readings was used to determine GAPDH activity.

The results, shown in FIGURE 5, demonstrate an increase in gene knockdown with increased N/P ratio for all three chitosan samples. Little gene silencing was observed at low N/P ratios (5 and 10) with any of the chitosan samples, however substantial increase, specifically with the chitosan-IAA 30.2% was seen at N/P ratios
at or above 25. At N/P = 25, the gene knockdowns for chitosan, chitosan-IAA 19.9\%, and chitosan-IAA 30.2\% were 35\%, 40\% and 64\% respectively. These values increased to 41\%, 52\%, and 87\% respectively at N/P = 50. The 30.2\% modified polymer matched the gene knockdown of the siPORT Amine positive control at N/P = 50, while still well within the nontoxic dose range of the polymer. A negative control (NC) siRNA delivered by all three variations of the polymer at N/P = 50 demonstrated no gene knockdown due to the polymer itself.

While embodiments of this disclosure have been depicted, described, and are defined by reference to specific embodiments of the disclosure, such references do not imply a limitation of the disclosure, and no such limitation is to be inferred. The subject matter disclosed is capable of considerable modification, alteration, and equivalents in form and function, as will occur to those ordinarily skilled in the pertinent art and having the benefit of this disclosure. The depicted and described embodiments of this disclosure are examples only, and are not exhaustive of the scope of the disclosure. For example, one skilled in the art may readily imagine a variety of combinations of different types of nucleic acids or anionic agents and polysaccharides and reactive compounds and assembly of nanoparticles and perhaps also additional materials. Treatment of a variety of conditions may be also envisaged.
What is claimed is:

1. A drug delivery vehicle comprising:
   a nanoparticle comprising:
   a modified polysaccharide having a degree of substitution with
   at least one secondary amine or at least one tertiary amine; and
   at least one therapeutic nucleic acid and or at least one
   therapeutic anionic agent.

2. The drug delivery vehicle of claim 1, wherein the modified
   polysaccharide comprises at least one secondary amine and at least one tertiary amine.

3. The drug delivery vehicle of claim 1, wherein the modified
   polysaccharide comprises at least two secondary amines.

4. The drug delivery composition of claim 1, wherein the therapeutic
   anionic agent is a therapeutic anion small molecule agent or a therapeutic anionic
   biomolecule.

5. The drug deliver vehicle of claim 1, wherein the polysaccharide is
   selected from a group consisting of a chitosan, a dextran modified to comprise one or
   more primary amines, a glucosamine, hybrid polymers of any of the previous
   polymers, and any combinations thereof.

6. The drug deliver vehicle of claim 1, wherein the polysaccharide is a
   chitosan.

7. The drug delivery vehicle of claim 6, wherein the degree of
   substitution of the chitosan with at least one secondary amine or at least one tertiary
   amine is at least about 10%.
8. The drug delivery vehicle of claim 6, wherein the degree of substitution of the chitosan with at least one secondary amine or at least one tertiary amine is about 19.9% to about 30.2%.

9. The drug delivery vehicle of claim 1, having an effective buffering capacity in aqueous solution from about pH 4.5 to about pH 8.5.

10. The drug delivery vehicle of claim 1, wherein the polysaccharide is at least 90% soluble in an aqueous solution at a pH greater than about 7.

11. A method for synthesizing a drug delivery vehicle comprising:
    reacting at least one reactive compound and a polysaccharide to introduce at least one secondary amine or at least one tertiary amine onto the polysaccharide, thereby obtaining a modified polysaccharide having a degree of substitution with the secondary amine or the tertiary amine; and
    complexing a therapeutic nucleic acid or a therapeutic anionic agent to the modified polysaccharide to form a drug delivery vehicle.

12. The method of claim 11, wherein reacting comprises reacting the at least one reactive compound and a polysaccharide to introduce both at least one secondary amine and at least one tertiary amine onto the polysaccharide.

13. The method of claim 11, further comprising reacting at least one reactive compound and a polysaccharide to introduce at least two secondary amines onto the polysaccharide.

14. The method of claim 11, wherein the therapeutic anionic agent is a therapeutic anionic biomolecule or a therapeutic anionic small molecule agent.

15. The method of claim 11, wherein the polysaccharide is selected from a group consisting of a chitosan, a dextran modified to comprise one or more primary
amines, a glucosamine, hybrid polymers of any of the previous polymers and any combinations thereof.

16. The method of claim 11, wherein the polysaccharide is a chitosan.

17. The method of claim 11, wherein the reactive compound comprises an imidazole.

18. The method of claim 15, wherein the reactive compound is selected from a group consisting of imidazole-4-acetic acid, arginine, histidine, polyarginine, polyhistidine, and any combinations thereof.

19. The method of claim 11, wherein reacting continues until the degree of substitution of the polysaccharide with the at least one secondary amine or the at least one tertiary amine is at least about 10%.

20. A method for nucleic acid delivery comprising administering to a patient in need thereof a drug delivery vehicle comprising:
   a nanoparticle comprising:
   a modified polysaccharide having a degree of substitution with at least one secondary amine or at least one tertiary amine; and
   at least one therapeutic nucleic acid and or at least one therapeutic anionic agent.
IMIDAZOLE ACETIC ACID (IAA) + REACTIVE O-ACYLISOURIEA ESTER

REACTIVE O-ACYLISOURIEA ESTER + CHITOSAN

EDC

RT

RT

IAA MODIFIED CHITOSAN

FIG. 1
### INTERNATIONAL SEARCH REPORT

**International application No.**

PCT/US 08/75799

#### A. CLASSIFICATION OF SUBJECT MATTER

**IPC**: H01M 2/00 (2008.04)

**USPC**: 514/54

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)

USPC - 514/54

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

USPC - 514/54, 777, 860, 536/1.11, 123.12, 424/278.1

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

PubWest(PGDB, USPT, EPAB, JPAB); Google

Search Terms: chitosan, drug, pharmaceutical, delivery, vehicle, nucleic acid, dna, ma, nucleoside, nucleotide, deoxyribose, ribonucleic, adenine, guanine, cytosine, uracil, polysaccharide, glucosamine, d-acetylglucosamine, dextran, buffer, buffer, nanoparticle, nanocrystal.

#### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>US 2006/0194843 A1 (Berdini et al.) 31 August 2006 (31.08.2006) entire document</td>
<td>18</td>
</tr>
</tbody>
</table>

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 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

06 November 2008 (06.11.2008)

Date of mailing of the international search report

19 NOV 2008

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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Form PCT/ISA/210 (second sheet) (April 2007)

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PCT OSP: 571-272-7774