



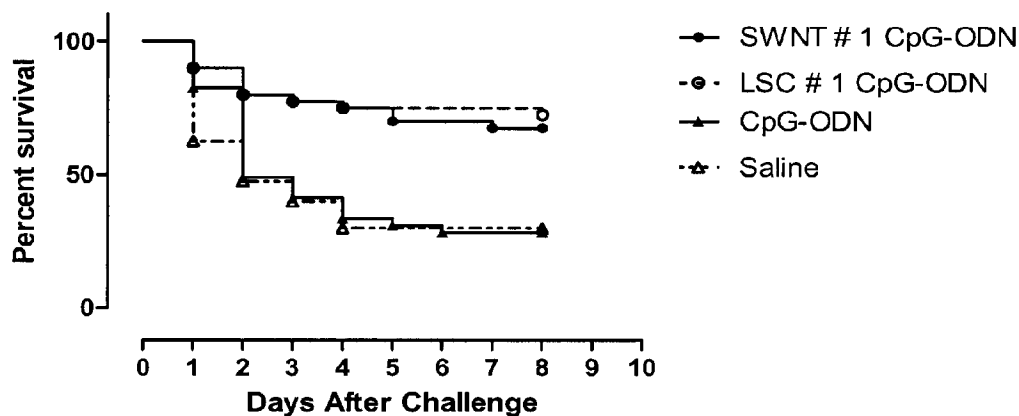
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(57) Abrégé/Abstract:

The present disclosure relates to formulations of CpG oligonucleotides with carbon nanotubes or lipid surfactants. Also disclosed are methods of promoting innate immunity in an avian subject comprising administering in ovo the formulations.

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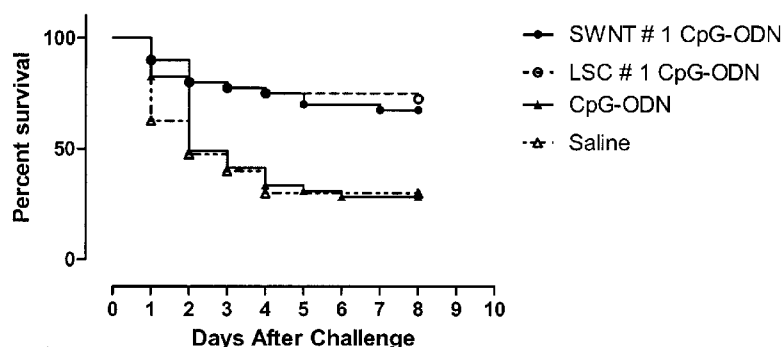


Figure 1

(57) Abstract: The present disclosure relates to formulations of CpG oligonucleotides with carbon nanotubes or lipid surfactants. Also disclosed are methods of promoting innate immunity in an avian subject comprising administering in ovo the formulations.

WO 2014/134698 A1

**TITLE: CPG OLIGONUCLEOTIDE FORMULATIONS AND METHODS AND
USES THEREOF**

FIELD

[0001] The disclosure relates to formulations of CpG-oligonucleotides
5 with carbon nanotubes or lipid surfactants. The disclosure further relates to
methods and uses of the formulations *in ovo* for promoting innate immunity.

BACKGROUND

[0002] An increasing emergence of diseases in animals, mainly food
animal species, has focused attention on immune-based methods of
10 protection from disease. Neonatal chickens are highly susceptible to diseases
since many pathogens enter through mucosal surfaces and the mucosal
immune system is not fully mature until several weeks post hatch¹.
Escherichia coli (*E. coli*) causes a variety of disease syndromes in poultry
including yolk-sac infection, omphalitis, respiratory tract infection, septicemia,
15 and cellulitis². Vaccines are a well-known method to stimulate the immune
system for improved disease protection, but they are not available for all
disease agents and are not effective when an immediate protective effect is
required. Additionally, the routine prophylactic use of antibiotics is
controversial because it can lead to the emergence of antibiotic-resistant
20 microbes. This may result in food allergies and soil or water contamination via
fecal excretion of antibiotics in poultry manure applied to the land. The
emergence and increasing spread of resistant bacteria are rendering current
antibiotics progressively less effective. Thus, there is pressure to restrict the
use of antibiotics in agriculture, while antibiotics are becoming less effective in
25 treating infections. Hence, these trends point to a need for development of
alternative strategies for infectious disease control³.

[0003] The vertebral innate immune system identifies pathogen-
associated molecular patterns (PAMPs) by pattern recognition receptors
(PRRs), inducing immune responses which prevent infections⁴. Non-vertebral
30 DNA acts as a PAMP which contains comparatively higher amounts of

unmethylated cytosine-phosphodiester-guanine (CpG) dinucleotides than vertebral DNA. These unmethylated CpG dinucleotides (CpG motifs), are recognized as the molecular pattern that contributes to the immunostimulatory activity of non-vertebral DNA⁵. Toll-like receptor 9 (TLR 9) in mammals, which is an intracellular PRR, recognizes these unmethylated CpG motifs and initiates immunostimulatory pathways⁵. The context of the unmethylated CpG dinucleotides including specific flanking sequences are responsible for the extent of immune stimulation⁶. Synthetic preparations of oligodeoxynucleotides containing CpG motifs (CpG-ODNs) simulating non-vertebral DNA have shown similar immunostimulatory activity⁷. This discovery has shown an important preventive immunotherapeutic treatment option which stimulates the host's immune system. Studies have revealed the immunostimulatory action of CpG-ODNs in many vertebral species including mice⁸, fish⁹, cattle and sheep¹⁰, and chicken^{11,12}. Other than the immunostimulatory action, CpG-ODNs are being used as vaccine adjuvants due to the ability to activate antigen presenting cells and B cells¹³. CpG-ODNs are also used for cancer therapy due to natural killer cell and cytotoxic T cell activation¹³. Furthermore, CpG-ODNs are being used for anti-allergic treatments due to the ability to promote Th1-type immune response and thus suppress Th2-type allergic responses¹³. The immunoprotective effect of CpG-ODN against several crucial bacterial diseases such as *E. coli*^{11,14,15} and *Salmonella typhimurium*¹², protozoan diseases like *Eimeria*¹⁶ and viral diseases such as infectious bursal disease¹⁷ and infectious bronchitis¹⁸ have been demonstrated in chickens.

25 [0004] There have been several drawbacks in using CpG-ODNs as immunostimulatory molecules such as cost, large doses needed for an effective response and CpG-ODN recognizing PRR which is located intracellularly and tough to reach. Considerations in drug delivery systems have been taken into account to overcome these problems. Studies have shown
30 that lipid based drug delivery systems and carbon nanotube associated delivery systems can improve the immunostimulatory effect as well as the

uptake of CpG-ODN in many species, including mice^{19,20,21}, and pigs²². However, little is known about the use of CpG-ODN delivery and formulation systems in neonatal chickens.

[0005] Extending the bioavailability and duration of action of CpG
5 ODNs may increase their therapeutic value. Unfortunately, CpG-ODNs used *in vivo* are rapidly eliminated from the circulation due to adsorption onto serum proteins and degradation by serum nucleases²³. A possible technique for protecting CpG-ODNs from degradation while increasing their uptake by cells of the immune system involves liposome encapsulation²¹ or fusion with
10 biphasic lipid vesicles²². Formulating CpG-ODN with polyphosphazenes showed an enhanced protection of chickens from *E. coli* infection²⁴.

[0006] Nanotechnology can be described as the technology involved in the manufacture, control, manipulation, and study of structures or devices whose size is of the nanometer scale. Carbon nanotubes (CNTs) can be, for
15 example single walled (SWNT) or multi walled (MWNT). CNTs have been investigated as multipurpose carriers for drug delivery and diagnostic applications. The organic functionalization of CNTs can significantly improve their solubility and biocompatibility profile; as a result, their manipulation and integration into biological systems has become possible. Functionalized CNTs
20 currently hold strong promise as novel systems for the delivery of drugs, antigens and genes²⁵. Use of carbon nanotubes to enhance uptake of CpG-ODN and increase antiglioma immunity in mice have been reported¹⁹.

[0007] Despite the widely demonstrated potential of CNTs in drug delivery, research indicates these particles can potentially cause adverse
25 effects because of their small size and extreme aspect ratio²⁹. However, controversy surrounds the interpretation ascribed to CNT toxicity data, and the state of the art is presently uncertain. To date, the toxicity of a particular CNT or CNT formulation is usually determined on a case-by-case basis. What is apparent however, is that toxicity is related to properties of the CNT
30 material, such as its structure (for example, SWNT vs. MWNT), length, aspect

ratio, surface area, chirality, degree of aggregation, extent of oxidation, surface topology, bound functional group(s), and method of manufacturing (which can, for example leave catalyst residues and/or produce impurities). Toxicity of CNTs is also related to their concentration in a formulation and the
5 dose to which cells or organisms are exposed³⁰.

SUMMARY

[0008] The present inventors have prepared novel formulations of CpG-ODN with carbon nanotubes and CpG-ODN with lipid surfactants and have demonstrated the delivery and immunostimulatory activity of these novel
10 formulations in chicken embryos.

[0009] Accordingly, the present disclosure provides a composition comprising (a) a CpG oligonucleotide noncovalently complexed with a non-functionalized carbon nanotube or (b) a CpG oligonucleotide complexed with a cationic lipid surfactant; and a pharmaceutically acceptable carrier.

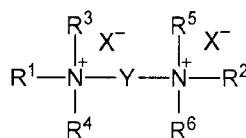
15 **[0010]** In an embodiment, the non-functionalized carbon nanotube of (a) is a single-walled carbon nanotube. In another embodiment, the non-functionalized carbon nanotube of (a) is a multi-walled carbon nanotube. The mean length of the non-functionalized single-walled or multi-walled carbon nanotube of (a) is from about 50 nm to about 5000 nm, optionally, about 100
20 nm to about 1000 nm. The mean diameter of the non-functionalized single-walled carbon nanotube of (a) is from about 0.6 nm to about 2.0 nm, optionally about 1.0 nm. The mean diameter of the non-functionalized multi-walled carbon nanotube of (a) is from about 2 nm to 50 nm.

[0011] In another embodiment, the CpG oligonucleotide complexed
25 with the cationic lipid surfactant in (b) is further formulated with a lipid phase to form a liposome. Optionally, the lipid phase comprises about 2 wt% of a phospholipid, about 0.2 wt% cholesterol and about 4 wt% propylene glycol.

[0012] In yet another embodiment, the cationic lipid surfactant is a dicationic gemini surfactant having the configuration *m-s-m* or *m-sN-m*, for

example, the dicationic gemini surfactant has the configuration 12-3-12 or 12-7N-12.

[0013] In yet another embodiment, the cationic lipid surfactant is at least one dicationic gemini surfactant having a structure of Formula (I):



(I)

wherein,

each of R^1 and R^2 is the same or different and is independently selected from C_{3-40} alkyl, C_{3-40} alkenyl and C_{3-40} alkynyl;

each of R^3 , R^4 , R^5 and R^6 is the same or different and is independently selected from C_{1-3} alkyl;

Y is selected from C_{2-12} alkylene, C_{3-12} alkenylene and $-\text{C}_{1-6}$ alkylene- NR^7 - C_{1-6} alkylene-, wherein R^7 is H, C_{1-4} alkyl, C_{2-4} alkenyl or C_{2-4} alkynyl; and

each X^- is the same or different and is a pharmaceutically acceptable counteranion.

[0014] In an embodiment, R^1 and R^2 are both $-(\text{CH}_2)_{11}\text{CH}_3$; each of R^3 , R^4 , R^5 and R^6 is $-\text{CH}_3$; Y is $-(\text{CH}_2)_3-$ and each X^- is the same and is Br^- .

[0015] In another embodiment, R^1 and R^2 are both $-(\text{CH}_2)_{11}\text{CH}_3$; each of R^3 , R^4 , R^5 and R^6 is $-\text{CH}_3$; Y is $-(\text{CH}_2)_3-\text{N}(\text{CH}_3)-(\text{CH}_2)_3-$ and each X^- is the same and is Br^- .

[0016] In one embodiment, the CpG oligonucleotide has a nucleotide sequence selected from TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO:1), TCGCGTGCGTTTTGTCGTTTTGACGTT (SEQ ID NO:2), TCGTCGTTTGTGTTTTGTCGTT (SEQ ID NO:3), and ggGGGACGATCGTCggggG (SEQ ID NO:4).

[0017] In a particular embodiment, the CpG oligonucleotide has the nucleotide sequence 5'- TCGTCGTTGTCGTTTTGTCGTT -3' (SEQ ID NO:1).

[0018] In another embodiment, the CpG oligonucleotide is
5 unmethylated.

[0019] Also disclosed herein is a method of promoting innate immunity in an avian subject comprising administering *in ovo* to the avian subject a composition of the present disclosure. Further disclosed herein is use of a composition of the present disclosure for promoting innate immunity in an
10 avian subject in need thereof. In another embodiment, the disclosure provides use of a composition of the present disclosure in the preparation of a medicament formulated for *in ovo* delivery for promoting innate immunity in an avian subject in need thereof. Further provided is a composition of the present disclosure for use *in ovo* in promoting innate immunity in an avian subject in
15 need thereof.

[0020] In an embodiment, the methods and uses of the present disclosure are for treating or preventing a bacterial, protozoan or viral disease or infection.

[0021] In an embodiment, the bacteria are *E. coli* or *Salmonella*;

20 [0022] In another embodiment, the protozoan disease or infection is *Eimeria*.

[0023] In yet another embodiment, the viral disease or infection is infectious bursal disease or infectious bronchitis.

[0024] In one embodiment, the administration *in ovo* to the subject
25 embryo is 2 to 3 days prior to hatching.

[0025] In an embodiment, the avian subject is a turkey or chicken.

[0026] Other features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples

while indicating embodiments of the disclosure are given by way of illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

5 **BRIEF DESCRIPTION OF THE DRAWINGS**

[0027] The disclosure will now be described in relation to the drawings in which:

[0028] Figure 1 shows the survival of broiler chickens following *E. coli* challenge. Groups of broiler chicken embryos at day-18 of incubation delivered with CpG-ODN formulated with SWNT # 1 or LSC # 1, CpG-ODN alone or saline by the *in ovo* route (n = 40 per group). Each formulation contains 50µg of CpG-ODN. 50% of birds in each group (n = 20) was subcutaneously inoculated with 1×10^4 *E. coli* and the remaining 50% of birds (n = 20) was subcutaneously inoculated with 1×10^5 cfu of *E. coli* at day-1 post-hatch or 24 h following hatch. Groups of birds that received CpG-ODN - SWNT # 1 or LSC # 1-CpG-ODN were significantly protected against *E. coli* infection compared to the control group (p < 0.0001).

[0029] Figure 2 shows the cumulative clinical scores (CCS) of broiler chickens following *E. coli* challenge. CCS of neonatal broiler chickens following challenge with 1×10^4 (n = 20) or 1×10^5 (n = 20) cfu of *E. coli*. Groups of birds that received 50µg CpG-ODN formulated with of SWNT # 1-CpG-ODN, or LSC #1-CpG-ODN had a significantly lower CCS compared to the CpG-ODN or saline group.

[0030] Figure 3 shows the bacterial score per each bird according to bacterial growth on blood agar. Bacterial swabs were taken from air sacs. *E. coli* colonies were enumerated on sheep blood agar by streaking sequentially in four distinct areas on agar plates. The enumeration was expressed on a scale of 0 to 4+ (0, no growth; 1+, growth of bacteria on area 1; 2+, growth of the bacteria on areas 1 and 2; 3+, growth of bacteria on areas 1,2, and 3; 4+, growth of bacteria on areas 1, 2, 3, and 4). Heavy bacterial growth was

observed more frequently in lesions from birds in the control group or groups treated with CpG-ODN alone than in the groups treated with SWNT # 1-CpG-ODN or LSC # 1-CpG-ODN by the *in ovo* route.

[0031] Figure 4 shows the survival of broiler chickens following *E. coli* challenge. Groups of broiler chicken embryos at day-18 of incubation delivered with CpG-ODN formulated with SWNT#2 or gemini surfactant-CpG-ODN (LSC#2), CpG-ODN alone or saline by the *in ovo* route (n = 40 per group). Each formulation contains 50µg of CpG-ODN. Fifty % of birds in each group (n = 20) was subcutaneously inoculated with 1×10^4 *E. coli* and the remaining 50% of birds (n = 20) was subcutaneously inoculated with 1×10^5 cfu of *E. coli* at day-1 post-hatch or 24 h following hatch. Groups of birds received CpG-ODN - SWNT#2 or gemini surfactant-CpG-ODN (LSC#2- CpG-ODN) were significantly protected against *E. coli* infection compared to the saline control group (p < 0.0001). The group that received CpG-ODN alone had increased survival but protection was not statistically significant (p = 0.0573).

[0032] Figure 5 shows cumulative clinical scores (CCS) of broiler chickens following *E. coli* challenge. CCS of neonatal broiler chickens following challenge with 1×10^4 (n = 20) or 1×10^5 (n = 20) cfu of *E. coli*. Groups of birds received 50µg CpG-ODN formulated with of SWNT#2-CpG-ODN, or surfactant (gemini surfactant-CpG-ODN or LSC#2 CpG-ODN) had a significantly lower CCS compared to the CpG-ODN or saline group. [Bird Numbers per group; SWNT#2-CpG = 33; gemini surfactant-CpG-ODN (LSC#2-CpG) = 40; CpG ODN = 26; and Saline = 40].

[0033] Figure 6 shows *E. coli* colonies were enumerated on sheep blood agar by streaking sequentially in four distinct areas on agar plates. The enumeration was expressed on a scale of 0 to 4+ (0, no growth; 1+, growth of bacteria on area 1; 2+, growth of the bacteria on areas 1 and 2; 3+, growth of bacteria on areas 1,2, and 3; 4+, growth of bacteria on areas 1, 2, 3, and 4). Heavy bacterial growth was observed more frequently in lesions from birds in

the control group than in the groups treated with SWNT#2-CpG-ODN, gemini surfactant-CpG-ODN (LSC#2 CpG-ODN) or CpG-ODN alone by the *in ovo* route.

DETAILED DESCRIPTION

- 5 **[0034]** Unless otherwise indicated, the definitions and embodiments described in this and other sections are intended to be applicable to all embodiments and aspects of the present disclosure herein described for which they are suitable as would be understood by a person skilled in the art.

Definitions:

- 10 **[0035]** As used in the present disclosure, the singular forms “a”, “an” and “the” include plural references unless the content clearly dictates otherwise. For example, an embodiment including “a dicationic gemini surfactant” should be understood to present certain aspects with one dicationic gemini surfactant, or two or more additional dicationic gemini
15 surfactants.

- [0036]** In embodiments comprising an “additional” or “second” component, such as an additional or second dicationic gemini surfactant, the second component as used herein is chemically different from the other components or first component. A “third” component is different from the
20 other, first, and second components, and further enumerated or “additional” components are similarly different.

- [0037]** In understanding the scope of the present disclosure, the term “comprising” and its derivatives, as used herein, are intended to be open ended terms that specify the presence of the stated features, elements,
25 components, groups, integers, and/or steps, but do not exclude the presence of other unstated features, elements, components, groups, integers and/or steps. The foregoing also applies to words having similar meanings such as the terms, “including”, “having” and their derivatives. The term “consisting” and its derivatives, as used herein, are intended to be closed terms that
30 specify the presence of the stated features, elements, components, groups,

integers, and/or steps, but exclude the presence of other unstated features, elements, components, groups, integers and/or steps. The term “consisting essentially of”, as used herein, is intended to specify the presence of the stated features, elements, components, groups, integers, and/or steps as well
5 as those that do not materially affect the basic and novel characteristic(s) of features, elements, components, groups, integers, and/or steps.

[0038] Terms of degree such as “about” and “approximately” as used herein mean a reasonable amount of deviation of the modified term such that the end result is not significantly changed. These terms of degree should be
10 construed as including a deviation of at least $\pm 5\%$ of the modified term if this deviation would not negate the meaning of the word it modifies.

[0039] The term “suitable” as used herein means that the selection of the particular compound, composition and/or conditions would depend on the specific synthetic manipulation to be performed, and the identity of the
15 molecule(s)/material(s) to be transformed, but the selection would be well within the skill of a person trained in the art. All process/method steps described herein are to be conducted under conditions sufficient to provide the desired product. A person skilled in the art would understand that all conditions, including, for example, solvent, time, temperature, pressure,
20 reactant ratio and whether or not the process/method should be performed under an anhydrous or inert atmosphere, can be varied to optimize the yield and/or properties of the desired product and it is within their skill to do so.

[0040] The term “compositions of the present disclosure” and the like as used herein refers to a composition comprising a CpG oligonucleotide
25 noncovalently complexed with a non-functionalized carbon nanotube, for example a non-functionalized single-walled carbon nanotube and a carrier; a CpG oligonucleotide complexed with a cationic lipid surfactant and a carrier; or a CpG oligonucleotide complexed with a cationic lipid surfactant further formulated with a lipid phase to form a liposome and a carrier. In an
30 embodiment, the carrier is a pharmaceutically acceptable carrier.

[0041] The term "alkyl" as used herein means straight or branched chain, saturated alkyl groups. The term C₁₋₃alkyl means an alkyl group having 1, 2 or 3 carbon atoms.

[0042] The term "alkenyl" as used herein means straight or branched chain, unsaturated alkenyl groups. The term C₂₋₄alkenyl means an alkenyl group having 2, 3 or 4 carbon atoms and at least one double bond.

[0043] The term "alkynyl" as used herein means straight or branched chain, unsaturated alkynyl groups. The term C₂₋₄alkynyl means an alkynyl group having 2, 3 or 4 carbon atoms and at least one triple bond.

[0044] The term "alkylene" as used herein refers to a bivalent alkyl group. The term C₂₋₁₂alkylene means an alkylene group having 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 carbon atoms.

[0045] The term "alkenylene" as used herein refers to a bivalent alkenyl group. The term C₃₋₁₂alkenylene means an alkenylene group having 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 carbon atoms and at least 1, for example 1-4, 1-3, 1-2 or 1 double bond(s).

[0046] The term "carbon nanotube" as used herein refers to an allotrope of carbon comprising a graphene sheet rolled into a cylindrical nanostructure. A carbon nanotube may be single-walled, in which case it is referred to herein as a "SWNT" or a "single-walled carbon nanotube" or the like. A carbon nanotube may also be multi-walled, in which case it is generally referred to as a "MWNT" or a multi-walled carbon nanotube or the like. A carbon nanotube, for example, a SWNT may vary in respect one or more of a number of physical properties such as length, diameter, aspect ratio, surface area, molecular weight, surface topology, the way the graphene sheet is rolled (denoted by indices (*n,m*)), degree of aggregation, morphology, extent of oxidation, and purity. For example, a person skilled in the art would understand that a high purity carbon nanotube is expected to be less toxic than a carbon nanotube of lower purity. A carbon nanotube, for example a SWNT can be functionalized; i.e. a surface of the carbon nanotube has been modified with

at least one functional group. A carbon nanotube, for example a SWNT can also be non-functionalized; i.e. the carbon nanotube surfaces have not been modified. A SWNT that has not been functionalized is referred to herein as a "non-functionalized single-walled carbon nanotube". A carbon nanotube, for example a SWNT may be available from a commercial source (for example, from UnidymTM, Inc. or from NanoIntegris, Inc.) and/or it may be prepared by a suitable synthetic method. A number of synthetic routes are known in the art, for example those that use arc discharge, laser ablation, plasma torch or chemical vapor deposition (CVD) techniques and the like. The choice of a suitable commercial source or synthetic route for the required carbon nanotube, for example a SWNT can be made by a person skilled in the art.

[0047] Samples of non-functionalized carbon nanotubes such as single-walled carbon nanotubes may have a polydisperse distribution in terms of the length and/or diameter of the non-functionalized carbon nanotubes therein. Accordingly, the term "length range" as used herein refers to the range of lengths present in a sample of carbon nanotubes, and the term "diameter range" as used herein refers to the range of diameters present in a sample of carbon nanotubes. The term "mean length" as used herein refers to the mean length of the carbon nanotubes in a sample of carbon nanotubes, and the term "mean diameter" as used herein refers to the mean diameter of the carbon nanotubes in a sample of carbon nanotubes. While the mean length and/or mean diameter between different samples of carbon nanotubes such as single-walled carbon nanotubes may differ, a person skilled in the art would understand that there can be a range of mean lengths and/or diameters that may have utility, for example in the methods and uses herein.

[0048] The term "amphiphilic" as used herein refers to a compound comprising both hydrophilic (water loving) and lipophilic (fat loving) portions.

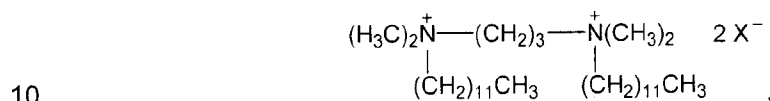
[0049] The term "liposome" as used herein refers to artificially prepared vesicles, the surface of which is a bilayer comprising amphiphilic structures.

[0050] The term "cmc" as used herein refers to the "critical micelle concentration" which is the concentration of a surfactant, for example a gemini surfactant, above which monomeric surfactants assemble into micelles.

[0051] The term "gemini surfactant" as used herein refers to an
5 amphiphilic structure also known as a "bis-surfactant" which comprises at least two lipophilic tails and two polar groups linked by a spacer. In an embodiment of the present disclosure, the gemini surfactant has two lipophilic tails and two polar groups linked by a spacer in the sequence: lipophilic tail–polar group–spacer–polar group–lipophilic tail. Examples of gemini surfactant
10 spacers are known which are, for example, short (e.g. two methylene groups) or long (e.g. 12 methylene groups); rigid (e.g. a 1,2-diphenylethylene moiety) or flexible (e.g. a methylene chain); and polar (e.g. a polyether) or nonpolar (e.g. an aliphatic or aromatic moiety). The polar group can be nonionic (for example, a polyether or a sugar), negatively charged (for example, a
15 phosphate, a sulfate or a carboxylate moiety) or positively charged (for example an ammonium moiety). Gemini surfactants with two positively charged polar groups are referred to herein as "dicationic gemini surfactants". For example, PCT Application Publication No. WO 2010/055416 discloses certain possible variations of the structure of a dicationic gemini surfactant.
20 The selection of a suitable dicationic gemini surfactant for the compositions of the present disclosure can be made by a person skilled in the art. Gemini surfactants having either a symmetrical or an unsymmetrical set of polar groups and/or lipophilic tails are known. Gemini surfactants, for example dicationic gemini surfactants may be commercially available and/or they may
25 be prepared by a suitable synthetic method. A number of synthetic routes are known in the art, for example as described in F.M. Menger & J.S. Keiper, "Gemini Surfactants" *Angew. Chem. Int. Ed.* 2000, 39, 1906-1920; in D. Shukla & V.K. Tyagi, "Cationic Gemini Surfactants: A Review" *Journal of Oleo Science* 2006, 55, 381-390 and/or in B.S. Sekhon, "Gemini (dimeric)
30 Surfactants: The Two-Faced Molecules" *Resonance*, 2004, 42-49. The

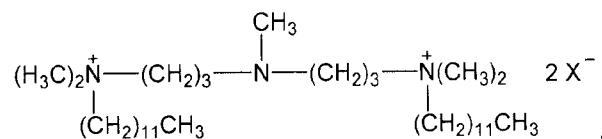
selection of a suitable synthetic route to the required gemini surfactant can be made by a person skilled in the art.

- [0052]** Dicationic gemini surfactants having lipophilic tails comprising alkyl groups, polar groups comprising a dimethyl ammonium moiety, and a spacer comprising an alkylene group may be referred to as having an *m-s-m* configuration, wherein *m* is the number of alkyl carbon atoms in the lipophilic tail and *s* is the number of carbon atoms in the spacer. For example, the term “12-3-12” as used herein refers to a dicationic gemini surfactant having the following structure:



wherein X^- is a counteranion, for example a pharmaceutically acceptable counteranion. In the studies of the present disclosure, X^- is Br^- . A dicationic gemini surfactant having the configuration 12-3-12 can be synthesized using a methodology which is low in cost, and its synthesis can also be scaled up.

- [0053]** Dicationic gemini surfactants having lipophilic tails comprising alkyl groups, polar groups comprising a dimethyl ammonium moiety, and a spacer comprising an $\text{---alkylene---N(CH}_3\text{)---alkylene---}$ group may be referred to as having an *m-sN-m* configuration, wherein *m* is the number of alkyl carbon atoms in the lipophilic tail and *s* is the number of atoms in the main chain of the spacer. For example, the term “12-7N-12” as used herein refers to a dicationic gemini surfactant having the following structure:



wherein X^- is a counteranion, for example a pharmaceutically acceptable counteranion. In the studies of the present disclosure, X^- is Br^- .

[0054] The term "pharmaceutically acceptable" as used herein means compatible with the treatment of subjects, in particular birds.

[0055] The term "counteranion" as used herein refers to a negatively charged species consisting of a single element, or a negatively charged species consisting of a group of elements connected by ionic and/or covalent bonds. For example, the counteranion can be an inorganic counteranion or an organic counteranion. In an embodiment of the present disclosure, the counteranion has a single negative charge. In another embodiment, the counteranion is a pharmaceutically acceptable counteranion.

10 [0056] The term "pharmaceutically acceptable counteranion" means a counteranion that is suitable for, or compatible with, the treatment of subjects. In an embodiment of the present disclosure, the pharmaceutically acceptable counteranion is Cl^- or Br^- . It is an embodiment that the pharmaceutically acceptable counteranion is Br^- .

15 [0057] In embodiments of the present disclosure, the dicationic gemini surfactants described herein have at least one asymmetric center. Such compounds exist as enantiomers. Where compounds possess more than one asymmetric center, they may exist as diastereomers. It is to be understood that all such isomers and mixtures thereof in any proportion are encompassed within the scope of the present disclosure. It is to be further understood that while the stereochemistry of the compounds may be as shown in any given compound listed herein, such compounds may also contain certain amounts (e.g. less than 20%, suitably less than 10%, more suitably less than 5%) of compounds having alternate stereochemistry. For example, the dicationic gemini surfactants that are shown without any stereochemical designations are understood to be racemic mixtures (i.e. contain an equal amount of each possible enantiomer or diastereomer). However, it is to be understood that all enantiomers and diastereomers are included within the scope of the present disclosure, including mixtures thereof in any proportion.

[0058] The term "treating" or "treatment" as used herein and as is well understood in the art, means an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, diminishment of the reoccurrence of disease, and remission (whether partial or total), whether detectable or undetectable. "Treating" and "treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. "Treating" and "treatment" as used herein also include prophylactic treatment. For example, a subject with an early bacterial infection can be treated to prevent progression, or alternatively a subject in remission can be treated with a composition described herein to prevent recurrence.

Treatment methods comprise administering to a subject a therapeutically effective amount of one or more of the compositions of the present disclosure, and optionally consists of a single administration, or alternatively comprises a series of administrations. As used herein, the term "effective amount" or "therapeutically effective amount" means an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example in the context of treating a bacterial infection, an effective amount is an amount that, for example, reduces the bacterial infection in comparison to the bacterial infection without administration of the composition. Effective amounts may vary according to factors such as the disease state, age, sex and/or weight of the subject. The amount of a given composition that will correspond to such an amount will vary depending upon various factors, such as the given composition, the pharmaceutical formulation, the route of administration, the type of condition, disease or disorder, the subject being treated, and the like, but can nevertheless be routinely determined by one skilled in the art.

Compositions:

[0059] The CpG oligonucleotide noncovalently complexed with a non-functionalized carbon nanotube and/or the CpG oligonucleotide complexed with a cationic lipid surfactant are suitably formulated into compositions.

5 Accordingly, the present disclosure includes a composition comprising (a) a CpG oligonucleotide noncovalently complexed with a non-functionalized carbon nanotube or (b) a CpG oligonucleotide complexed with a cationic lipid surfactant and a carrier. In another embodiment, the composition is a pharmaceutical composition and the carrier is a pharmaceutically acceptable

10 carrier. In an embodiment, the carbon nanotube is a single-walled carbon nanotube.

[0060] The term "CpG oligonucleotide" or "CpG-ODN" as used herein refers to single stranded DNA molecules that contain a cytosine followed by a guanine, also called a CG motif, and a phosphodiester backbone or a

15 modified phosphorothioate (PS) backbone. In an embodiment, the CpG oligonucleotide comprises a phosphorothioate (PS) backbone. In an embodiment, the CpG oligonucleotide or CpG-ODN is unmethylated. Vertebrate, for example avian immune systems, recognize unmethylated CpG oligonucleotides as foreign.

20 **[0061]** The CpG oligonucleotide is at least 6 nucleotides long. In one embodiment, the CpG-ODN is between 8 and 100 nucleotides in length, optionally 8-40 nucleotides in length. In another embodiment, the CpG-ODN is at least 20 nucleotides in length, at least 24 nucleotides in length, at least 27 nucleotides in length or at least 30 nucleotides in length.

25 **[0062]** The CpG oligonucleotide can be isolated or synthetic in nature. A person skilled in the art would readily be able to isolate or synthesize the CpG oligonucleotides disclosed herein.

[0063] In an embodiment, the CpG ODN comprises the following formula: 5'-n₁n₂CGn₃n₄-3' (SEQ ID NO:5), wherein C is unmethylated and n₁,

n_2 , n_3 and n_4 are nucleotides. The CpG ODN may be a non-palindromic or a palindromic sequence.

[0064] In an embodiment, n_1n_2 are selected from GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG; and n_3n_4 are selected from TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA and CpA. In another embodiment, n_1n_2 are GpA or GpT and n_3n_4 are TpT. In other embodiments, the n_1n_2 are purines and the n_3 or n_4 are pyrimidines, or n_1n_2 is GpA and n_3 or n_4 or both are pyrimidines.

[0065] In another embodiment, the CpG oligonucleotides are of the formula: 5'-N₁n₁CGn₂N₂-3' (SEQ ID NO:6), wherein n_1 and n_2 are nucleotides and N₁ and N₂ are nucleic acid sequences composed of from about 0-25 nucleotides each. In an embodiment, n_1 is A, G or T and n_2 is C, A or T, optionally wherein n_1 is C and/or n_2 is G.

[0066] In yet another embodiment, the CpG oligonucleotides are of the formula: 5'-N₁n₁n₂CGn₃n₄N₂-3' (SEQ ID NO:7), wherein n_1 , n_2 , n_3 and n_4 are nucleotides and N₁ and N₂ are nucleic acid sequences composed of from about 0-25 nucleotides each. In some embodiments, n_1n_2 are selected from GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG; and n_3n_4 are selected from TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA and CpA. In another embodiment, n_1n_2 are GpA or GpT and n_3n_4 are TpT. In other embodiments, the n_1n_2 are purines and the n_3 or n_4 are pyrimidines, or n_1n_2 is GpA and n_3 or n_4 or both are pyrimidines.

[0067] In an embodiment, the N₁ and N₂ do not contain a CCGG or CGCT sequence or more than one CCG or CGG trimer. For example, if the nucleic acid has a phosphodiester backbone or a chimeric backbone, such quadramers or trimers will only have minimal biological activity. However, if the backbone has a primarily phosphorothioate backbone, inclusion of these quadramers and trimers would be useful for biological activity.

[0068] In yet a further embodiment, the CpG oligonucleotides are of the formula: 5'-TCN₁Tn₁n₂CGn₃n₄-3' (SEQ ID NO:8).

[0069] Suitable CpG oligonucleotides comprise a nucleotide sequence selected from the group consisting of TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO:1), TCGCGTGCGTTTTGTCGTTTTGACGTT (SEQ ID NO:2), TCGTCGTTTGTCTGTTTTGTCGTT (SEQ ID NO:3), and
5 ggGGGACGATCGTCggggG (SEQ ID NO:4). In the last sequence, the lower case letters represent nucleotides linked together by phosphorothioate linkages, while the upper case G's represent nucleotides linked together by phosphodiester linkages.

[0070] Further examples of CpG oligonucleotides that are suitable for
10 the present formulations are described in WO01/22972, published on April 1, 2001 and in WO03/03656, published on April 17, 2003.

[0071] In a particular embodiment, the CpG oligonucleotide for use in the present formulations is 5'- TCGTCGTTGTCGTTTTGTCGTT -3' (SEQ ID NO:1).

15 [0072] The term "isolated" refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized.

[0073] The term "nucleic acid molecule" is intended to include
20 unmodified DNA or RNA or modified DNA or RNA. For example, the nucleic acid molecules of the disclosure can be composed of single- and double stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is a mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that
25 may be single-stranded or, more typically double-stranded or a mixture of single- and double-stranded regions. In addition, the nucleic acid molecules can be composed of triple-stranded regions comprising RNA or DNA or both RNA and DNA. The nucleic acid molecules of the disclosure may also contain one or more modified bases or DNA or RNA backbones modified for stability
30 or for other reasons. "Modified" bases include, for example, tritiated bases

and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA; thus "nucleic acid molecule" embraces chemically, enzymatically, or metabolically modified forms. The term "polynucleotide" shall have a corresponding meaning.

- 5 [0074] CpG oligonucleotides are synthetic oligonucleotides and are readily available, for example, from OPERON.

[0075] A nucleic acid molecule of the disclosure may also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-
10 phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See e.g., Itakura et al. U.S. Patent No. 4,598,049; Caruthers et al. U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071).

[0076] An isolated nucleic acid molecule of the disclosure which
15 comprises DNA can be isolated by preparing a labelled nucleic acid probe based on all or part of the nucleic acid sequences and using this labelled nucleic acid probe to screen an appropriate DNA library.

[0077] The composition comprising a CpG oligonucleotide noncovalently complexed with a non-functionalized carbon nanotube, such as
20 a single-walled carbon nanotube, and a carrier can be prepared by adding a solution comprising a CpG oligonucleotide to non-functionalized carbon nanotubes under conditions to form the composition. The particular conditions, for example, the relative concentrations of the CpG oligonucleotide to the non-functionalized carbon nanotubes, the time and the
25 temperature can vary depending, for example, on the particular CpG oligonucleotide and/or the particular non-functionalized carbon nanotube used but can be determined by a person skilled in the art.

[0078] For example, a person skilled in the art would understand that a composition comprising a CpG oligonucleotide noncovalently complexed with
30 a non-functionalized carbon nanotube, such as a single-walled carbon

nanotube, would be a substantially uniformly black solution without any appreciable evidence of sedimentation. For example, if there is too low a concentration of the CpG oligonucleotide in comparison to the non-functionalized carbon nanotube, the composition would not be fully monodisperse and sedimentation would be observed even after, for example an extended time of sonication. Increasing the temperature and/or the intensity of sonication may, in certain cases be used to prepare a monodisperse composition where the concentration of the CpG oligonucleotide in comparison to the non-functionalized carbon nanotube was initially too low to prepare a monodisperse composition. On the other hand, if there is a high concentration of the CpG oligonucleotide in comparison to the non-functionalized carbon nanotube, the composition would be monodisperse (i.e. the non-functionalized carbon nanotubes therein would have a CpG oligonucleotide noncovalently complexed) but there would also be free CpG oligonucleotide in the composition. Such compositions would have utility in the methods and uses of the present disclosure, but, for example may be wasteful as they contain excess CpG oligonucleotide that is not complexed.

[0079] For example, an about 0.5 mg/mL solution of CpG-ODN in sterile water can be added to a non-functionalized single-walled carbon nanotube powder (for example, 0.1 mg HiPco SWNTs per mL of CpG-ODN solution or 0.1 mg Puretubes SWNTs per mL of CpG-ODN solution) in a suitable vessel, for example a glass vial, and sonicating, for example bath sonicating at low shear the resulting suspension for a time and at a temperature that allows for the formation of a composition comprising a CpG oligonucleotide noncovalently complexed with a non-functionalized single-walled carbon nanotube, for example for about 5 hours at a temperature not exceeding about 40 °C to prepare a composition comprising about 10 µg non-functionalized single-walled carbon nanotubes and about 50 µg CpG oligonucleotide, for example CpG-ODN per about 100 µl carrier.

[0080] In an embodiment of the present disclosure, the compositions comprise a ratio by weight of the CpG oligonucleotide, for example CpG-ODN

to the non-functionalized carbon nanotube such as a single-walled carbon nanotube of about 4:1, about 5:1, about 6:1, about 7:1, about 8:1, about 9:1, about 10:1, about 11:1, about 12:1, about 13:1, about 14:1, about 15:1, about 16:1, about 17:1, about 18:1, about 19:1, about 20:1, about 25:1, about 30:1, about 35:1, about 40:1, about 45:1 or about 50:1. In another embodiment, the compositions comprise a ratio by weight of CpG-ODN to the non-functionalized single-walled carbon nanotube of about 5:1, about 7.5:1 or about 10:1. It is an embodiment that the compositions comprise a ratio by weight of CpG-ODN to the non-functionalized single-walled carbon nanotube of about 5:1, for example, the composition of the present disclosure comprises about 50 µg CpG oligonucleotide, for example CpG-ODN and about 10 µg non-functionalized single-walled carbon nanotubes in a pharmaceutically acceptable carrier.

[0081] For example, the composition comprising the CpG oligonucleotide complexed with a cationic lipid surfactant and a carrier can be prepared by adding a solution comprising a CpG oligonucleotide to a cationic lipid surfactant, for example a dicationic gemini surfactant under conditions to form the composition. The particular conditions, for example the relative concentrations of the CpG oligonucleotide to the cationic lipid surfactant, the time and the temperature can vary depending, for example, on the particular CpG oligonucleotide and/or the particular cationic lipid surfactant used but can be determined by a person skilled in the art.

[0082] For example, a person skilled in the art would understand that there must be an excess of the cationic lipid surfactant, for example, the dicationic gemini surfactant in comparison to the CpG oligonucleotide so that the negative charges on the CpG oligonucleotide are neutralized by the cationic lipid surfactant, for example, the dicationic gemini surfactant, and there is a further excess of the cationic lipid surfactant, for example, the dicationic gemini surfactant, so that the final complex between the CpG oligonucleotide and the cationic lipid surfactant is positively charged.

[0083] For example, an about 0.5 mg/mL solution of CpG-ODN in sterile water can be added to 1.1 mg of a dicationic gemini surfactant such as 12-7N-12 per mL of CpG-ODN solution in a suitable vessel, for example a glass vial to give a composition having a concentration of CpG-ODN of about 0.5 mg/mL and a concentration of the dicationic gemini surfactant such as 12-7N-12 of about 1.1 mg/mL.

[0084] In another embodiment of the present disclosure, the compositions comprise a cationic lipid, for example, a dicationic gemini surfactant and a CpG oligonucleotide, for example CpG-ODN in a charge ratio of about 40:1, about 37.5:1, about 35:1, about 32.5:1, about 30:1, about 27.5:1, about 25:1, about 22.5:1, about 20:1, about 19:1, about 18:1, about 17:1, about 16:1, about 15:1, about 14:1, about 13:1, about 12:1, about 11:1, about 10:1, about 9:1, about 8:1, about 7:1, about 6:1, about 5:1, about 4:1, about 3:1 or about 2.5:1. In a further embodiment, the compositions comprise a dicationic gemini surfactant, for example 12-7N-12 and CpG-ODN in a charge ratio of about 2.5:1, for example, the composition comprises about 1.1 mg/mL of 12-7N-12 and about 0.5 mg/mL CpG-ODN in a pharmaceutically acceptable carrier.

[0085] In another embodiment, the compositions comprise a cationic lipid, for example, a dicationic gemini surfactant such as 12-3-12 at a percentage of about 0.1% (w/v) to about 0.5% (w/v) or about 0.125% (w/v) and a CpG oligonucleotide, for example CpG-ODN at a concentration of about 0.4 mg/mL to about 1.0 mg/mL or about 0.5 mg/mL.

[0086] In a further embodiment, the composition comprising the CpG oligonucleotide complexed with the cationic lipid surfactant and a carrier can be further formulated with a lipid phase to form a composition comprising a liposome. For example, the composition comprising the CpG oligonucleotide complexed with the cationic lipid surfactant and a carrier can be added to a lipid phase under conditions to form the composition comprising a liposome.

[0087] The particular conditions, for example the relative concentrations of each of the components, the time and the temperature can vary depending, for example on the particular CpG oligonucleotide and/or the particular cationic lipid surfactant used and/or the particular lipid phase used, but can be determined by a person skilled in the art. In an embodiment, the lipid phase comprises a phospholipid, for example a phosphatidylcholine such as Phospholipon™ 90H or a similar lipid, and optionally cholesterol and/or a hydrophilic solvent other than water, for example a glycol, such as propylene glycol or butylene glycol or a polyol such as glycerol or mixtures thereof. It is an embodiment that the phospholipid is a pharmaceutical grade phospholipid. For example, cholesterol can be used to put the lipid bilayer of the liposome into a liquid crystalline intermediate phase, thus lowering the transition temperature of the lipid bilayer and making the liposome more flexible so that it can, for example, be taken up more easily by a cell. For example, the transition temperature of the lipid bilayer can be lowered so that it is closer to body temperature (about 37 °C). The hydrophilic solvent other than water can be added to assist in the manufacturing process.

[0088] For example, the composition comprising the CpG oligonucleotide, for example CpG-ODN complexed with the cationic lipid surfactant, for example a dicationic gemini surfactant such as 12-7N-12 and a carrier such as sterile water, the composition having a concentration of CpG-ODN of about 0.5 mg/mL and a concentration of the 12-7N-12 of about 1.1 mg/mL can be warmed, for example to about 40 °C and added to a lipid phase comprising, for example about 2 wt% Phospholipon 90H, about 0.2 wt% cholesterol and about 4 wt% propylene glycol which has been melted, for example at about 70 °C in, for example, a water bath to give a substantially clear liquid, and the resulting mixture mixed, for example by intermittent vortexing/heating until a substantially homogeneous formulation is formed, then sonicating, for example using a cup-horn Sonicator 4000 on amplitude 11 for about 30 minutes to give the composition comprising the liposome.

[0089] In an embodiment of the present disclosure, the compositions further formulated with the lipid phase to form the composition of the present disclosure comprising a liposome have a lipid phase comprising about 1 wt% to about 5 wt% or about 2 wt% of a phospholipid such as Phospholipon 90H or a similar lipid, about 0 wt% to about 0.5 wt% or about 0.2 wt% of cholesterol and about 0 wt% to about 10 wt% or about 4 wt% of a hydrophilic solvent other than water for example a glycol, such as propylene glycol or butylene glycol or a polyol such as glycerol or mixtures thereof. It is an embodiment that the lipid phase comprises about 2 wt% Phospholipon 90H, about 0.2 wt% cholesterol and about 4 wt% propylene glycol.

[0090] In an embodiment of the present disclosure, the non-functionalized single-walled or multi-walled carbon nanotube has a length range of about 50 nm to about 5000 nm, about 100 nm to about 4000 nm, about 65 nm to about 2000 nm, about 80 nm to about 1500 nm or about 100 nm to about 1000 nm. In another embodiment, the non-functionalized single-walled carbon nanotube has a length range of about 100 nm to about 4000 nm. In a further embodiment, the non-functionalized single-walled carbon nanotube has a length range of about 65 nm to about 2000 nm, about 80 nm to about 1500 nm or about 100 nm to about 1000 nm. It is an embodiment of the present disclosure that the non-functionalized single-walled carbon nanotube has a length range of about 100 nm to about 1000 nm.

[0091] In another embodiment, the non-functionalized single-walled or multi-walled carbon nanotube has a mean length of about 50 nm to about 5000 nm, about 250 nm to about 2500 nm, about 500 nm to about 1500 nm, about 100 nm to about 1000 nm or about 1000 nm. It is an embodiment that the non-functionalized single-walled or multi-walled carbon nanotube has a mean length of about 100 nm to about 1000 nm.

[0092] In an embodiment of the present disclosure, the non-functionalized single-walled carbon nanotube has a diameter range of about 0.6 nm to about 2.0 nm, about 0.65 nm to about 1.5 nm, about 0.7 nm to

about 1.3 nm, about 0.8 nm to about 1.2 nm or about 1.2 nm to about 1.7 nm.

In another embodiment, the non-functionalized single-walled carbon nanotube has a diameter range of about 0.65 nm to about 1.5 nm, about 0.7 nm to about 1.3 nm or about 0.8 nm to about 1.2 nm. In a further embodiment, the
5 non-functionalized single-walled carbon nanotube has a diameter range of about 1.2 nm to about 1.7 nm. It is an embodiment of the present disclosure that the non-functionalized single-walled carbon nanotube has a diameter range of about 0.8 nm to about 1.2 nm.

[0093] In an embodiment of the present disclosure, the non-
10 functionalized multi-walled carbon nanotube has a diameter range of about 2 nm to about 50 nm.

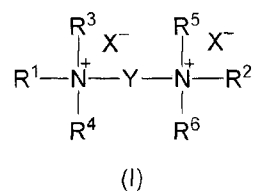
[0094] In another embodiment of the present disclosure, the non-functionalized single-walled carbon nanotube has a mean diameter of about 0.6 nm to about 2 nm, about 0.65 to about 1.4 nm, about 0.7 to about 1.3 nm,
15 about 0.8 nm to about 1.2 nm, about 1.2 nm to about 1.6 nm, about 1.0 nm or about 1.4 nm. In another embodiment, the non-functionalized single-walled carbon nanotube has a mean diameter of about 0.65 to about 1.4 nm, about 0.7 to about 1.3 nm, about 0.8 nm to about 1.2 nm or about 1.0 nm. In a further embodiment, the non-functionalized single-walled carbon nanotube
20 has a mean diameter of about 0.8 nm to about 1.2 nm or about 1.0 nm. It is an embodiment that the non-functionalized single-walled carbon nanotube has a mean diameter of about 1.0 nm. In another embodiment of the present disclosure, the non-functionalized single-walled carbon nanotube has a mean diameter of about 1.4 nm.

25 **[0095]** In an embodiment of the present disclosure, the non-functionalized multi-walled carbon nanotube has a mean diameter of about 2 nm to about 50 nm.

[0096] In an embodiment, the non-functionalized single-walled carbon nanotube is a PureTubesTM carbon nanotube from NanoIntegris having a
30 length range of about 100 nm to about 4000 nm, a mean length of about 1000

nm, a diameter range of about 1.2 nm to about 1.7 nm and a mean diameter of about 1.4 nm. In another embodiment, the non-functionalized single-walled carbon nanotube is a HiPco™ carbon nanotube from Unidym having a length range of about 100 to about 1000 nm, a diameter range of about 0.8 nm to about 1.2 nm and a mean diameter of about 1.0 nm.

[0097] In another embodiment of the present disclosure, the cationic lipid surfactant is a dicationic gemini surfactant having, for example, a low cmc. It is an embodiment that the cationic lipid surfactant is at least one dicationic gemini surfactant having a structure of Formula (I):



wherein,

each of R^1 and R^2 is the same or different and is independently selected from C_{3-40} alkyl, C_{3-40} alkenyl and C_{3-40} alkynyl;

each of R^3 , R^4 , R^5 and R^6 is the same or different and is independently selected from C_{1-3} alkyl;

Y is selected from C_{2-12} alkylene, C_{3-12} alkenylene and $-\text{C}_{1-6}$ alkylene- NR^7 - C_{1-6} alkylene-, wherein R^7 is H, C_{1-4} alkyl, C_{2-4} alkenyl or C_{2-4} alkynyl; and

each X^- is the same or different and is a counteranion.

[0098] In an embodiment, each of R^1 and R^2 is the same or different and is independently selected from C_{5-20} alkyl, C_{5-20} alkenyl and C_{5-20} alkynyl. In another embodiment, each of R^1 and R^2 is the same or different and is independently selected from C_{5-20} alkyl. In a further embodiment, R^1 and R^2 are the same and are selected from C_{5-20} alkyl. It is an embodiment that R^1 and R^2 are both $-(\text{CH}_2)_{11}\text{CH}_3$.

[0099] In another embodiment, each of R^3 , R^4 , R^5 and R^6 is the same or different and is independently selected from C_{1-2} alkyl. It is an embodiment that each of R^3 , R^4 , R^5 and R^6 is $-CH_3$.

[00100] In an embodiment, Y is selected from C_{2-12} alkylene and $-C_{1-6}$ alkylene- NR^7 - C_{1-6} alkylene-, wherein R^7 is H or C_{1-4} alkyl. In another embodiment, Y is C_{2-12} alkylene. It is an embodiment that Y is $-(CH_2)_3-$. In another embodiment, Y is $-C_{1-6}$ alkylene- NR^7 - C_{1-6} alkylene-, wherein R^7 is C_{1-4} alkyl. It is an embodiment that Y is $-(CH_2)_3-N(CH_3)-(CH_2)_3-$. When R^7 is H, i.e. Y is $-(CH_2)_3-N(H)-(CH_2)_3-$, the N-H moiety of the spacer can be protonated in use.

[00101] In an embodiment, each X^- is the same or different and is a pharmaceutically acceptable counteranion. In another embodiment, each X^- is the same and is selected from the group consisting of Br^- and Cl^- . It is an embodiment that each X^- is the same and is Br^- .

[00102] In an embodiment, the dicationic gemini surfactant has the configuration m - s - m , wherein m is an integer from 3 to 40 and s is an integer from 2 to 16. In another embodiment, m is an integer from 5 to 20. In a further embodiment, s is an integer from 2 to 12. It is an embodiment that m is 12. In another embodiment, s is an integer from 2 to 8. It is an embodiment that s is 3. In an embodiment, the dicationic gemini surfactant has the configuration 12-2-12, 12-3-12, 12-7-12, 12-16-12, 16-3-16 or 18-3-18. In another embodiment, the dicationic gemini surfactant has the configuration 12-3-12.

[00103] In another embodiment, the dicationic gemini surfactant has the configuration m - sN - m , wherein m is an integer from 3 to 40 and s is an integer from 2 to 12. In another embodiment, m is an integer from 5 to 20. It is an embodiment that m is 12. In another embodiment, s is an integer from 5 to 10. It is an embodiment that s is 7. In an embodiment, the dicationic gemini surfactant has the configuration 12-7N-12.

[00104] In an embodiment, the lipid phase comprises a phospholipid. In another embodiment, the phospholipid is a phosphatidylcholine such as

Phospholipon 90H or a similar lipid. In another embodiment, the lipid phase further comprises cholesterol. In a further embodiment, the lipid phase further comprises a hydrophilic solvent other than water. It is an embodiment that the hydrophilic solvent other than water is a glycerol or a polyol or mixtures thereof. In an embodiment, the glycol is propylene glycol or butylene glycol. In another embodiment, the glycol is propylene glycol. In a further embodiment, the polyol is glycerol. It is an embodiment that the lipid phase comprises a phospholipid, cholesterol and propylene glycol.

Methods and Uses

10 **[00105]** The compositions disclosed herein are useful for promoting innate immunity in a subject. In particular, the compositions are useful for *in ovo* delivery to an avian embryo for promoting innate immunity to prevent and/or treatment infection.

15 **[00106]** Accordingly, in one embodiment, the present disclosure provides a method of promoting innate immunity in an avian subject in need thereof comprising administering *in ovo* a composition disclosed herein to the animal in need thereof. Also provided is use of a composition disclosed herein for promoting innate immunity *in ovo* in an avian subject in need thereof. Further provided is use of a composition disclosed herein in the preparation of
20 a medicament formulated for *in ovo* delivery for promoting innate immunity in an avian subject in need thereof. Even further provided is a composition disclosed herein for use in promoting innate immunity *in ovo* in an avian subject in need thereof.

25 **[00107]** The phrase "promoting innate immunity" as used herein refers to promoting immunity that is not antigen specific and involves phagocytosis, cytokine release and cytolytic functions. In one embodiment, promoting innate immunity is effective at preventing, reducing or eliminating a microbial infection. Promoting innate immunity includes, without limitation, increasing interferon-gamma production and increasing natural killer cell activity.

[00108] In an embodiment, the methods and uses disclosed herein are used for treating or preventing an infection, including without limitation, a bacterial infection, such as an *E. coli* infection or a *Salmonella* infection, a viral infection, such as infectious bursal disease or infectious bronchitis, or a
5 protozoan infection, such as *Eimeria*.

[00109] The term "avian subject" as used herein refers to all avian species. In an embodiment of the present disclosure, the bird is poultry such as a fowl (for example, a chicken, a turkey or a quail), a waterfowl (for example, a domestic duck or a domestic goose), a pigeon, a dove or a
10 pheasant. It is an embodiment that the bird is a chicken or a turkey. For *in ovo* delivery, the formulation is delivered to an egg at an embryonic stage of development of the avian subject.

[00110] The compositions of the present disclosure may be administered to a subject in a variety of forms as will be understood by those
15 skilled in the art.

[00111] In an embodiment, the *in ovo* administration is at 2 to 3 days before hatching of a subject avian species. For example, if the subject is a chicken, the *in ovo* administration is at 17 to 19 days, for example at 18 days of incubation of the chicken embryo.

20 [00112] A person skilled in the art would readily be able to determine the appropriate amounts and dosing for *in ovo* delivery. In the present examples, *in ovo* delivery was conducted by administering 100 µl volume by the *in ovo* route into the amniotic cavity through the air cell of the egg using a 20 gauge, 1 inch needle.

25 [00113] Conventional procedures and ingredients for the selection and preparation of suitable compositions are described, for example, in Remington's Pharmaceutical Sciences (2000 - 20th edition) and in The United States Pharmacopeia: The National Formulary (USP 24 NF19) published in 1999.

[00114] Compositions of the present disclosure may be used alone or in combination with other known agents useful for promoting innate immunity in a subject. When used in combination with other agents useful for promoting innate immunity in a subject, it is an embodiment that the compositions of the present disclosure are administered contemporaneously with those agents. As used herein, "contemporaneous administration" of two substances to a subject means providing each of the two substances so that they are both biologically active in the individual at the same time. The exact details of the administration will depend on the pharmacokinetics of the two substances in the presence of each other, and can include administering the two substances within a few hours of each other, or even administering one substance within 24 hours of administration of the other, if the pharmacokinetics are suitable. Design of suitable dosing regimens is routine for one skilled in the art. In particular embodiments, two substances will be administered substantially simultaneously, i.e., within minutes of each other, or in a single composition that contains both substances. It is a further embodiment of the present disclosure that the composition of the present disclosure and the other agent(s) is administered to a subject in a non-contemporaneous fashion.

[00115] The dosage of compositions of the present disclosure can vary depending on many factors such as the pharmacodynamic properties of the composition, the mode of administration, the age, health and weight of the recipient, the nature and extent of the symptoms, the frequency of the treatment and the type of concurrent treatment, if any, and the clearance rate of the composition in the subject to be treated. One of skill in the art can determine the appropriate dosage based on, for example the above factors. Compositions of the present disclosure may be administered initially in a suitable dosage that may be adjusted as required, depending on the clinical response, and may be administered in a single daily dose or the total daily dose may be divided into two, three or four daily doses.

[00116] As a representative example, *in-ovo* dosages of one or more compositions of the present disclosure are administered in a volume of about

1.0 µl, about 5.0 µl, about 10.0 µl, about 20.0 µl, about 25.0 µl, about 30.0 µl, about 40.0 µl, about 50.0 µl, about 60.0 µl, about 70.0 µl, about 75.0 µl, about 80.0 µl, about 85.0 µl, about 90.0 µl, about 95.0 µl, about 100.0 µl, about 105.0 µl, about 110.0 µl, about 115.0 µl, about 120.0 µl, about 125.0 µl, about 150 µl, about 200 µl, about 250 µl, about 300 µl, about 350 µl, about 400 µl, about 450 µl, about 500 µl, about 550 µl, about 600 µl, about 650 µl, about 700 µl, about 750 µl, about 800 µl, about 850 µl, about 900 µl, about 950 µl or about 1000 µl. In an embodiment, *in-ovo* dosages of one or more compositions of the present disclosure are administered in a volume of about 50 µl to about 150 µl, about 75 µl to about 125 µl, about 90 µl to about 110 µl or about 100 µl. In a further embodiment, *in-ovo* dosages of one or more compositions of the present disclosure are administered in a volume of about 100 µl.

[00117] Treatment or prevention methods comprise administering to a subject, a therapeutically effective amount of one or more of the compositions of the present disclosure, and optionally consists of a single administration, or alternatively comprises a series of administrations. For example, the one or more compositions of the present disclosure are administered in a single administration any time prior to or following the onset of the disease, disorder or condition. For example, the one or more compositions of the present disclosure are administered immediately prior to the onset of the disease, disorder or condition or about 24 hours prior to the onset of the disease, disorder or condition, or any time in between. Alternatively, the one or more compositions of the present disclosure are administered immediately following the onset of the disease, disorder or condition or about 48 hours following the onset of the disease, disorder or condition, or any time in between, for example about 1 minute, about 10 minutes, about 30 minutes, about 1 hour, about 5 hours, about 10 hours, about 20 hours, about 24 hours, about 30 hours or about 36 hours, following the onset of the disease, disorder or condition.

[00118] However, in another embodiment, the compositions may be administered to the subject in a series of administrations, for example about 1, 2, 3, 4, 5 or 6 times daily for 1 day to about 10 days either before or after

the onset of the disease, disorder or condition. The length of the treatment period depends on a variety of factors, such as the cause of the disease, disorder or condition, severity of the disease, disorder or condition, the age of the subject, the concentration of the composition, the activity of the composition, and/or a combination thereof. It will also be appreciated that the effective dosage of the composition used for the treatment or prevention may increase or decrease over the course of a particular treatment or prevention regime. Changes in dosage may result and become apparent by standard diagnostic assays known in the art. In some instances, chronic administration may be required. For example, the compositions are administered to the subject in an amount and for a duration sufficient to treat the subject.

[00119] The above disclosure generally describes the present application. A more complete understanding can be obtained by reference to the following specific examples. These examples are described solely for the purpose of illustration and are not intended to limit the scope of the disclosure. Changes in form and substitution of equivalents are contemplated as circumstances might suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

[00120] The following non-limiting examples are illustrative of the present disclosure:

EXAMPLES

Materials and methods:

Bacteria

[00121] A field isolate of *E. coli* from a turkey with septicemia, was used as the challenge strain. This *E. coli* was serogroup O2, nonhemolytic, serum-resistant, produced aerobactin, a K1 capsule and Type 1 pili. Aliquots of bacteria were stored at -70 °C in 50% brain–heart infusion broth (BHI; Difco, Detroit, MI) supplemented with 25% (w/v) glycerol (VWR Scientific, Inc.,

Montreal, Quebec). Bacteria for use as the challenge were cultured on sheep blood agar plates for 24 hours at 37 °C. One colony was added to 100 ml of Luria broth in a 250ml Erlenmeyer flask. The culture was grown at 37 °C for 16 - 18 hours with shaking at 150 rpm. Stationary phase culture contained
5 approximately 1×10^9 colony forming units (CFUs) of bacteria per milliliter. The cultures were further diluted in sterile saline to the concentration of bacteria required for challenge (1×10^5 or 1×10^4 CFU/ bird). Viable bacterial counts were determined by plating serial dilutions of the diluted culture on sheep blood agar plates, incubating for 24 hours at 37 °C then counting the number
10 of colonies.

Animal model

[00122] This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Day-old broiler chicks or
15 hatching eggs were obtained from a local hatchery or broiler breeder operation in Saskatchewan, Canada. Eggs were incubated at the Animal Care Unit at the Western College of Veterinary Medicine, University of Saskatchewan. Groups of chicks were allocated randomly into animal isolation rooms at the Animal Care Unit, University of Saskatchewan. Water
20 and commercial broiler ration were provided *ad libitum*. Each room was ventilated with filtered, non-recirculated air at a rate of 10–12 changes/hour. Air pressure differentials and strict sanitation were maintained in this isolation facility. The subjects received either 1×10^5 or 1×10^4 CFUs of stationary -
phase *E. coli* in a 0.25 ml volume, inoculated by subcutaneous injection in the
25 neck of neonatal chicks and resulted in *E. coli* septicemia. In this model, *E. coli* septicemia with airsacculitis, pericarditis, or perihepatitis develops in 60%–90% of birds that are not protected by treatment intervention. Two doses of *E. coli* were given to groups of birds to simulate field conditions since
30 all birds in a commercial poultry barn will not be exposed to a constant dose of *E. coli*. Birds were evaluated three times daily at the critical stage then

twice thereafter for 7 days post challenge. Birds were observed for clinical signs and each individual was assigned a daily clinical score: 0 = normal; 0.5 = slightly abnormal appearance, slow to move; 1 = depressed, reluctant to move; 1.5 = reluctant to move, may take a drink and peck some; 2 = unable to stand or reach food or water; and 3 = found dead. Birds that received a clinical score of 2 were euthanatized by cervical dislocation. Chicks that were found dead or euthanatized were necropsied immediately. On day-7 post *E. coli* challenge, the remaining birds were euthanatized by cervical dislocation. Bacterial swabs were taken from the air sacs and cultured on sheep blood agar using a typical method of inoculation and streaking on four quadrants of the plate of medium. A semi quantitative estimate of *E. coli* isolation was conducted on sheep blood agar. Growth on these plates was recorded on a scale from 0 to 4+, where 0 = no growth; 1+ = growth of bacteria on the area 1; 2+ = growth of the bacteria on areas 1 and 2; 3+ = growth of bacteria on areas 1, 2, and 3; and 4+ = growth of bacteria on areas 1, 2, 3, and 4.

Synthetic CpG ODN and CpG ODN formulations.

[00123] The sequence of CpG ODN used was 5'-TCGTCGTTGTCGTTTTGTCGTT-3' (SEQ ID NO:1). ODNs were produced with a phosphorothioate backbone. This synthetic CpG-ODN is available through a commercial source, for example Eurofins MWG Operon, Huntsville, Alabama, USA. CpG-ODN formulations were prepared either with SWNTs or lipid surfactant composites (LSC) at the School of Pharmacy, University of Waterloo, Canada. Synthetic CpG-ODN was diluted in sterile, pyrogen-free saline or formulation substrates and administered in a 100 µl volume by either the *in ovo* route into the amniotic cavity through the air cell of the egg using a 20 gauge, 1 inch needle,. The volume of the injection and the length of the needle were selected to simulate the *in ovo* technology used in the poultry industry.

CNT formulations

[00124] SWNT#1 CpG-ODN was prepared using 0.1 mg/mL HiPco SWNTs (Unidym, Inc., Sunnyvale, CA) and 0.5 mg/mL CpG-ODN aqueous solution (quantum satis with sterile water).

- 5 [00125] SWNT#2 CpG-ODN contained 0.1 mg/mL Puretubes SWNTs (NanoIntegris, Inc., Menlo Park, CA; supplied as a powder) and 0.5 mg/mL CpG-ODN aqueous solution (quantum satis with sterile water).

- [00126] Both of the above CNT formulations were prepared by weighing out the required amount of the SWNT powder, adding the required amount of the CpG-ODN aqueous solution to the SWNT powder in a glass vial, and then bath sonicating the mixture for 5 h at temperatures not exceeding 40 °C. Final doses contained 10 µg SWNT and 50 µg CpG-ODN per 100 µl.

Lipid-surfactant formulations

- [00127] LSC-CpG-ODN formulations (LSC#1) were made up of two phases: an aqueous phase (AP; 1.1 mg/mL gemini surfactant 12-7N-12 [synthesized in house according to Wettig et al. 2007 ³²], 0.5mg/ml CpG-ODN, quantum satis with sterile water [Fisher Scientific, Toronto, ON, Canada]) and a lipid phase (LP; 2% w/w Phospholipon 90H [Nattermann Phospholipids GmbH, Koln, Germany], 0.2% w/w cholesterol [Croda, Vaughan, ON, Canada], 4% w/w propylene glycol [Spectrum Chemicals, Gardena, CA]). In a glass vial, the required amount of CpG-ODN solution was added to the gemini surfactant powder and topped up with the sterile water (AP). In a second glass vial, all of the LP ingredients were weighed out and melted in a 70 °C water bath to a clear liquid. The premixed, prewarmed (to 40 °C) AP was added to the LP and incorporated by intermittent vortexing/heating until a homogenous formulation formed. This final mixture was then sonicated in a cup-horn Sonicator 4000 (Misonix, Qsonica, LLC., Newtown, CT) on amplitude 11 for 30 min.

[00128] Gemini surfactant-CpG-ODN complexes were prepared with 0.125% (w/v) gemini surfactant 12-3-12 (synthesized in house according to Donkuru et al. 2012³³) at 0.5 mg/mL CpG-ODN. The mixture was bath sonicated for 3 h at temperatures not exceeding 40 °C.

5 **Experimental design**

Delivery of CpG ODN formulations by the in-ovo route

[00129] The objective of this experiment was to study whether CpG-ODN formulated with SWNTs or LSC enhanced the immunoprotective effects in neonatal birds. Day 18 old embryonated eggs were injected with 50 µg of
 10 CpG-ODN formulated with SWNT (SWNT#1 CpG-ODN), LSC-CpG-ODN (LSC#1), unformulated CpG-ODN or saline. Each of four groups of embryonating eggs received 100 µl of formulation or saline by the *in ovo* route in the amniotic cavity through the air cell using 21 gauge needles with 1 inch length. Forty birds were randomly allocated into each of four groups. Groups
 15 of birds were inoculated on day 1 of age or 24 h following post-hatch with 1×10^5 or 1×10^4 CFU of stationary phase *E. coli* by the subcutaneous route in the neck. Half of each group (n=20) were inoculated with 1×10^5 CFU of *E. coli* and the remaining of the group (n=20) was inoculated with 1×10^4 CFU of *E. coli*. Birds were examined daily for 7 days for clinical signs following *E. coli*
 20 challenge. Bacterial isolations from air sacs were conducted in all dead and euthanized birds during the clinical evaluation period, and at the termination of the experiment.

[00130] The second part of the objective was to study the immunoprotective effect of CpG-ODN formulated with two other delivery
 25 systems: SWNT (SWNT#2 CpG-ODN) and gemini surfactant-CpG-ODN. *In ovo* delivery of formulations, *E. coli* challenge experiments following clinical evaluation, mortality and bacterial isolations were performed as stated above. In this trial the group sizes were SWNT#2-CpG-ODN: n = 33; gemini surfactant-CpG-ODN: n = 40; CpG-ODN: n = 26; and Saline n = 40.

Safety of CpG-ODN formulated with CNT or LSC

[00131] The third objective of these experiments was to study the safety and toxicity of these CpG-ODN formulations in neonatal broiler chickens until 9 days post-hatch. This experiment was conducted in parallel to the above experiments. Four groups of birds (n=18) were injected with SWNT#1-CpG-ODN, LSC-CpG-ODN, CpG-ODN or saline by the *in ovo* route as described above, but were not challenged with *E. coli*. Birds were examined daily for any clinical signs and mortality. Tissue sections were collected from the gastrointestinal tract (crop, esophagus, proventriculus, gizzard, duodenum, jejunum, ileum, ceca), kidneys, lungs, trachea and lymphoid organs (spleen, bursa, thymus) at 19, 20 and 21 days of incubation and at 3, 6 and 9 days post-hatch for histopathology. At each time point, three embryonated eggs or birds were randomly selected and euthanized for sample collection. Tissue sections were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5µm thickness and stained with hematoxylin and eosin (H&E).

Statistical analysis

[00132] Survival and other data were analyzed with the use of Prism (Prism 5.0, GraphPad Software Inc., San Diego, CA) and Statistix7 (Analytical Software, Tallahassee, FL) with a significance level of $P < 0.05$. The survival patterns and median survival times were compared using the log-rank test and chi-square statistics. The relative risk of mortality for control subjects was calculated using proportional hazards regression. The clinical score for each bird was summed over the 8-day observation period and the significance of differences among groups was tested with the use of the Kruskal-Wallis nonparametric analysis of variance. The homogeneity of distribution of bacterial scores was tested using chi-square statistics.

Results:

Delivery of CpG ODN formulations by the in-ovo route

[00133] The unchallenged viability control birds experienced no mortality, and the groups of birds that received CpG-ODN either alone or in any formulation experienced about half the relative risk of mortality as the
5 birds that received saline (0.48; $p=0.0020$ in the first trial and 0.51; $p=0.0044$ in the second trial).

[00134] Birds in the first trial that received either CpG-ODN formulated with SWNT#1 or LSC#1 (> 75% survival) experienced one-third (0.32; $p<0.001$) the relative risk of mortality following a lethal dose of *E. coli*
10 compared to CpG-ODN alone and saline control groups (30% survival) (Fig. 1). The cumulative clinical score was significantly lower in birds who received CpG-ODN formulated with SWNT#1 or LSC#1 compared to birds who received either CpG-ODN alone or saline ($p<0.05$) (Figure 2). Moreover, birds who received CpG-ODN formulated with either SWNT#1 or LSC#1 had
15 a significantly greater proportion of lower bacterial loads compared to birds that received CpG-ODN alone or saline ($p<0.005$) (Figure 3 and Table 1).

[00135] The eggs that were set and treated did not all hatch in one day; hence some group sizes were reduced. SWNT#2-CpG $n=33$; gemini surfactant-CpG-ODN $n=40$; unformulated CpG $n=26$; Saline $n=40$). The
20 second challenge, birds which received CpG-ODN formulated with SWNT#2, gemini surfactant-CpG-ODN – experienced significantly lower risk of mortality (0.57; $p=0.0149$) compared to the birds who received CpG-ODN alone or saline (Fig. 4) The cumulative clinical score was significantly different among groups ($p=0.016$) and there were 2 sets of groups between which the CCS
25 was significantly different ($p<0.05$) as shown in Figure 5. Additionally, birds who received various treatments had significantly different bacterial loads (overall Chi-square=30.94; $p=0.002$), and birds that received CpG-ODN alone or saline had the higher levels of bacteria (Figure 6).

Safety of CpG-ODN formulated with CNT or LSC

[00136] No mortality or any clinical signs were detected in any of the unchallenged birds during the experiment. Histopathological lesions were not noted in any of the organs at any of the time points examined in the groups of birds who received SWNT#1 CpG-ODN, LSC # 1 CpG-ODN, CpG-ODN or saline.

Discussion

[00137] Protective immunotherapeutic treatments have been a topic of great interest as a method to fight infectious disease because of their ability to stimulate the host's immune system and prepare the host to fight pathogenic microbes. In the food-animal industry, disease prevention by prophylactic measures or by immune stimulation is preferred compared to the use of therapeutic agents which may lead to concern about residues in edible products. This has become especially important due to a number of food safety and human health issues. The use of antibiotics in the animal industry has been related to the emergence of resistant strains of bacteria and the possibility of drug residues in meat products. For these reasons, novel immunostimulants may serve as alternative, desirable ways to prevent disease. CpG-ODN has been used as an immuno stimulant in many species, including chickens^{8-11, 26}. Previously, the present inventors have demonstrated that CpG-ODN is an effective immunostimulant in chickens and appeared to have its effectiveness for 6 days¹¹ following administration. Moreover, the present inventors were able to increase effectiveness of CpG-ODN by formulating with polyphosphazenes²⁴. In the present study, the present inventors demonstrated a significant health promoting effect of CpG-ODN formulated with CNT or LSC. CpG-ODN formulations significantly increase survival, reduce clinical signs and decrease the bacterial load in birds challenged with a lethal dose of *E. coli*.

[00138] *E. coli* or *Salmonella* septicemia is common in the neonatal period of broiler chickens and causes a significant mortality and production

loss¹². Furthermore, if broiler flocks experience mortality greater than 2% in the first week of life, the overall performance of the flock becomes very poor due to chronic infections, poor weight gain and uneven birds in the flock ²⁷. It is essential to have good health during the first week of broiler chicken growth as it will provide better performance and profitability¹. Currently in the broiler chicken industry, broiler chickens reach 2 kilograms body weight by 30-35 days post-hatch ²⁸. Thus, *in ovo* delivery of CpG-ODN formulations either with CNT or lipid formulations will provide effective immunostimulation and likely be a better alternative to antibiotics. Likewise, *in ovo* delivery of vaccines is in common place in the broiler chicken industry. Hence, delivery of CpG-ODN formulations are industry feasible. The present inventors have previously demonstrated that *in ovo* delivery of unformulated CpG-ODN are very effective against *E.coli* infection¹¹ or Salmonella¹² septicemia in neonatal broilers and here have demonstrated a significant synergy or potentiation of CpG-ODN with CNT or lipid delivery systems as an immunostimulant in chicken embryos. Moreover, CpG-ODN formulations significantly reduce the bacterial load in birds infected with *E. coli* becoming a significant consideration if CpG-ODNs are able to reduce bacterial load in birds in field. The animal model of *E. coli* cannot be used to validate this possibility since the animal model causes very high (between 60-80%) mortality following challenge. In the animal model of *E. coli*, 50% of birds were infected with 1×10^4 cfu of *E. coli* and the remaining 50% with 1×10^5 cfu a higher dose of *E. coli* to simulate field challenge since all the birds in the field are not exposed at a constant dose of *E. coli*.

[00139] Broiler chicken embryos are a well suited model to study toxicity of CNT. In addition, all organs can be studied for histopathology to identify any morphological changes. The present inventors did not see embryo mortality, decreased hatchability, clinical signs or any evidence of alteration of morphology of any internal organs that were studied during day-18 of incubation until day-9 post-hatch. Moreover, lipid based delivery systems

have proven to be safe and effective for delivering CpG ODN in many species^{22, 31}.

[00140] In conclusion, CpG-ODN formulated with CNT or lipid surfactants was observed to synergistically enhance the immunoprotective efficiency against *E. coli* infections in neonatal broiler chickens. CpG-ODN formulations with CNT did not have adverse reactions in chicken embryos or neonatal broiler chickens. This is the first demonstration of *in ovo* delivery of CpG-ODN formulated with CNT or lipid-surfactant delivery systems against a lethal bacterial infection in broiler chickens.

[00141] While the present disclosure has been described with reference to what are presently considered to be the examples, it is to be understood that the disclosure is not limited to the disclosed examples. To the contrary, the disclosure is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

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Table 1: Bacterial score per each bird according to bacterial growth on blood agar.

Bacterial swabs were taken from air sacs.

Bacterial score	SWNT #1- CpG ODN	LSC # 1-CpG ODN	CpG ODN	Saline
0	20	17	5	5
+1	8	10	7	7
+2	5	3	8	7
+3	4	6	15	14
+4	3	4	5	7

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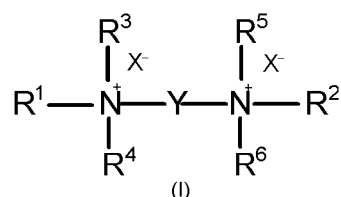
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CLAIMS:

1. A composition comprising a CpG oligonucleotide complexed with a dicationic gemini surfactant and a pharmaceutically acceptable carrier; wherein (i) the dicationic gemini surfactant has the configuration *m-s-m*, wherein *m* is the number of alkyl carbon atoms in the lipophilic tail and *s* is the number of carbon atoms in the spacer; or (ii) wherein the dicationic gemini surfactant comprises an –alkylene–N(CH₃)–alkylene– group having an *m-sN-m* configuration, wherein *m* is the number of alkyl carbon atoms in the lipophilic tail and *s* is the number of atoms in the main chain of the spacer, wherein *m* is from 3-40 and *s* is from 2-12.
2. The composition of claim 1, wherein the CpG oligonucleotide complexed with the dicationic gemini surfactant is further formulated with a lipid phase comprising a phospholipid to form a liposome.
3. The composition of claim 2, wherein the lipid phase comprises about 2 wt% of phospholipid, about 0.2 wt% cholesterol and about 4 wt% propylene glycol.
4. The composition of any one of claims 1 to 3, wherein the dicationic gemini surfactant has the configuration 12-3-12 or 12-7N-12.
5. The composition of any one of claims 1 to 3, wherein the dicationic gemini surfactant has a structure of Formula (I):



wherein,

each of R¹ and R² is the same or different and is independently selected from C₃₋₄₀alkyl and C₃₋₄₀alkenyl;

each of R³, R⁴, R⁵ and R⁶ is the same or different and is independently selected from C₁₋₃alkyl;

Y is selected C₂₋₁₂alkylene, C₃₋₁₂alkenylene and -C₁₋₆alkylene-NR⁷-C₁₋₆alkylene-, wherein R⁷ is H, C₁₋₄alkyl, C₂₋₄alkenyl or C₂₋₄alkynyl; and each X⁻ is the same or different and is a pharmaceutically acceptable counteranion.

6. The composition of claim 5, wherein R¹ and R² are both -(CH₂)₁₁CH₃; each of R³, R⁴, R⁵ and R⁶ is -CH₃; Y is -(CH₂)₃- and each X⁻ is the same and is Br⁻.
7. The composition of claim 5, wherein R¹ and R² are both -(CH₂)₁₁CH₃; each of R³, R⁴, R⁵ and R⁶ is -CH₃; Y is -(CH₂)₃-N(CH₃)-(CH₂)₃- and each X⁻ is the same and is Br⁻.
8. The composition of any one of claims 1 to 7, wherein the CpG oligonucleotide is unmethylated.
9. The composition of any one of claims 1 to 8, wherein the CpG oligonucleotide has a phosphorothioate backbone or a chimeric backbone.
10. The composition of any one of claims 1 to 9, wherein the CpG oligonucleotide is 8-40 nucleotides in length and contains at least one CG motif.
11. The composition of any one of claims 1 to 10, wherein the CpG oligonucleotides is of the formula: 5'-N₁n₁n₂CGn₃n₄N₂-3' (SEQ ID NO:7), wherein n₁, n₂, n₃ and n₄ are nucleotides and N₁ and N₂ are nucleic acid sequences composed of 0-25 nucleotides each.
12. The composition of any one of claims 1 to 10, wherein the CpG oligonucleotide is of the formula: 5'-TCN₁Tn₁n₂CGn₃n₄-3' (SEQ ID NO:8), wherein n₁, n₂, n₃ and n₄ are nucleotides and N₁ is a nucleic acid sequence composed of 0-25 nucleotides each.
13. The composition of claim 11 or 12, wherein n₁n₂ are selected from GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG; and n₃n₄ are selected from TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA and CpA.

14. The composition of any one of claims 11 to 13, wherein n_1n_2 are GpA or GpT and n_3n_4 are TpT.
15. The composition of any one of claims 11 to 14, wherein n_1n_2 are purines and n_3 or n_4 or both are pyrimidines.
16. The composition of any one of claims 11 to 14, wherein n_1n_2 is GpA and n_3 or n_4 or both are pyrimidines.
17. The composition of any one of claims 1 to 9, wherein the CpG oligonucleotide has a nucleotide sequence selected from TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO:1), TCGCGTGCCTTTTTCGTTTTGACGTT (SEQ ID NO:2), TCGTCGTTTTCGTTTTTTCGTT (SEQ ID NO:3), and ggGGGACGATCGTCggggG (SEQ ID NO:4); wherein, for SEQ ID NO:4, g represents a nucleotide linked together by a phosphorothioate linkage, and G represents a nucleotide linked together by a phosphodiester linkage.
18. The composition of any one of claims 1 to 9, wherein the CpG oligonucleotide has the nucleotide sequence 5'-TCGTCGTTGTCGTTTTGTCGTT - 3' (SEQ ID NO:1).
19. A use of composition of any one of claims 10 to 18 for promoting innate immunity *in ovo* in an avian subject; wherein the CpG oligonucleotide is unmethylated and the CpG oligonucleotide has a phosphorothioate backbone.
20. A use of composition of any one of claims 10 to 18 in the manufacture of a medicament for promoting innate immunity *in ovo* in an avian subject; wherein the CpG oligonucleotide is unmethylated and the CpG oligonucleotide has a phosphorothioate backbone.
21. A composition of any one of claims 10 to 18 for use in promoting innate immunity *in ovo* in an avian subject; wherein the CpG oligonucleotide is unmethylated and the CpG oligonucleotide has a phosphorothioate backbone.

22. The use of claim 19 or 20 or the composition for use of claim 21, for treating or preventing a bacterial, protozoan or viral disease or infection.
23. The use or composition for use of claim 22, wherein the bacteria is *E. coli* or *Salmonella*.
24. The use or composition for use of claim 22, wherein the protozoan disease or infection is *Eimeria*.
25. The use or composition for use of claim 22, wherein the viral disease or infection is infectious bursal disease or infectious bronchitis.
26. The use or composition for use of any one of claims 19 to 25, wherein the use *in ovo* is 2 to 3 days prior to hatching.
27. The use or composition for use of any one of claims 19 to 26, wherein the avian subject is a turkey or chicken.

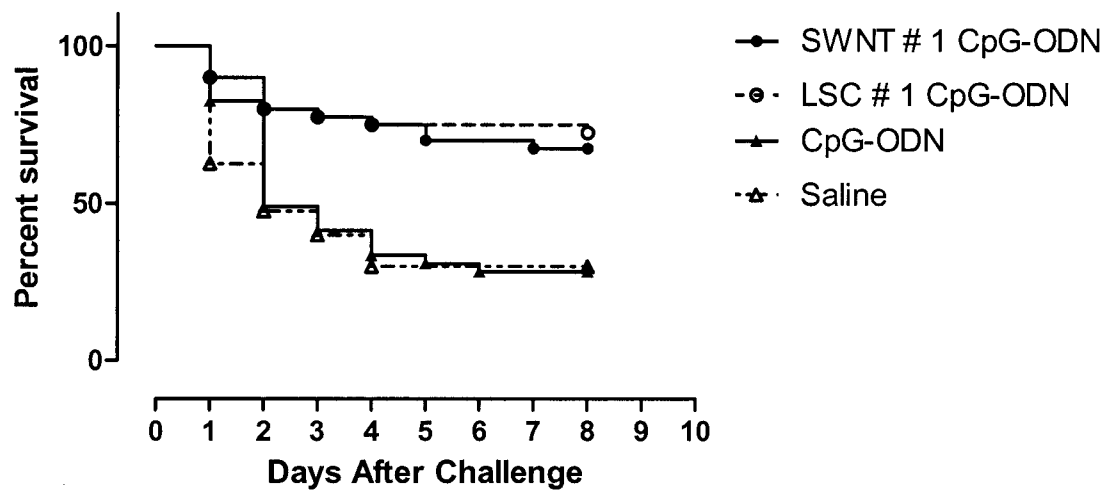


Figure 1

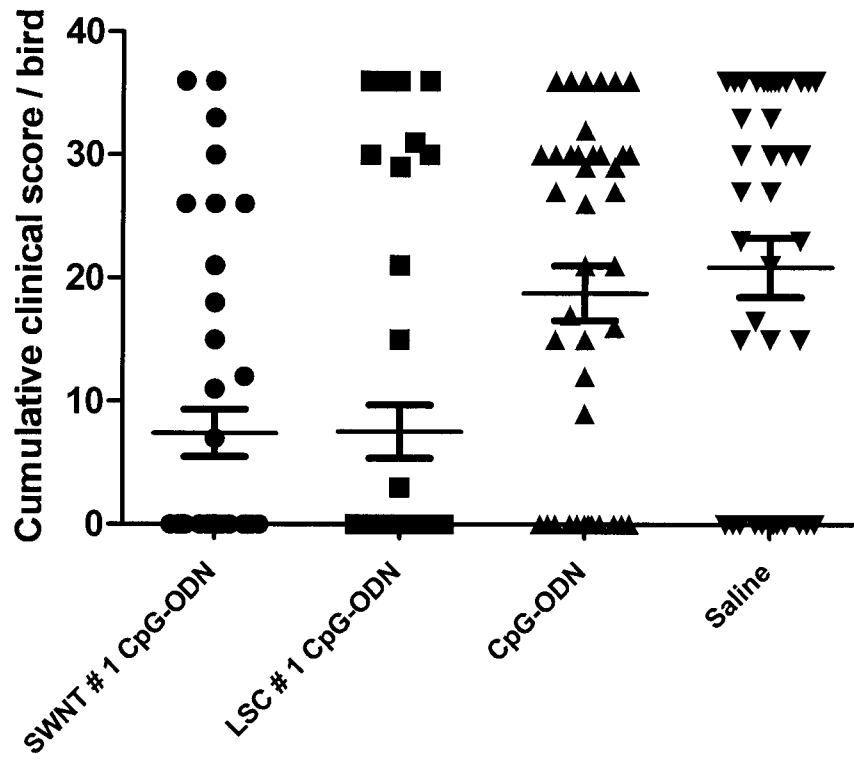


Figure 2

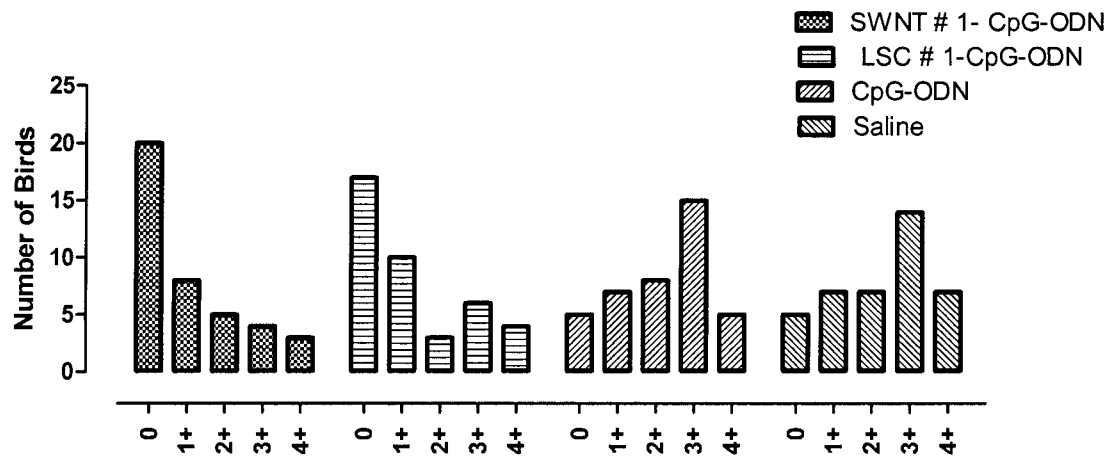


Figure 3

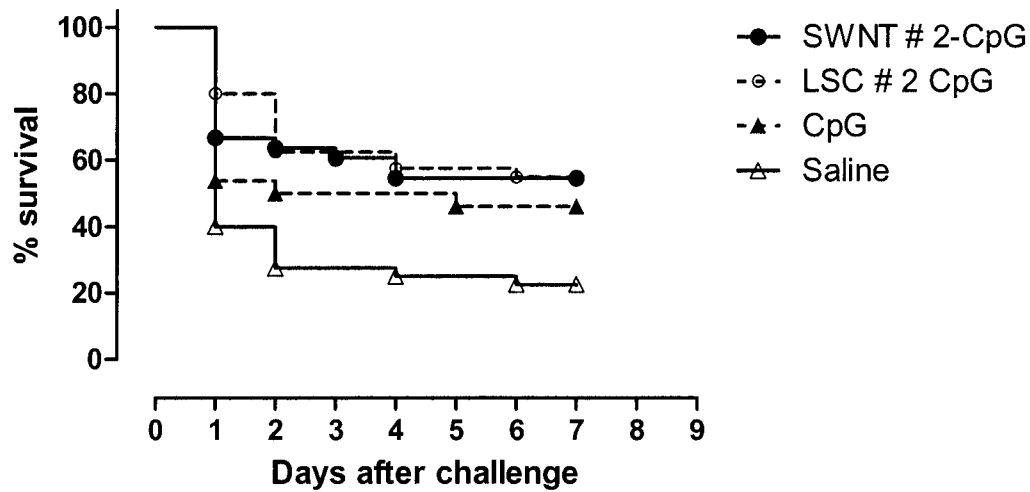


Figure 4

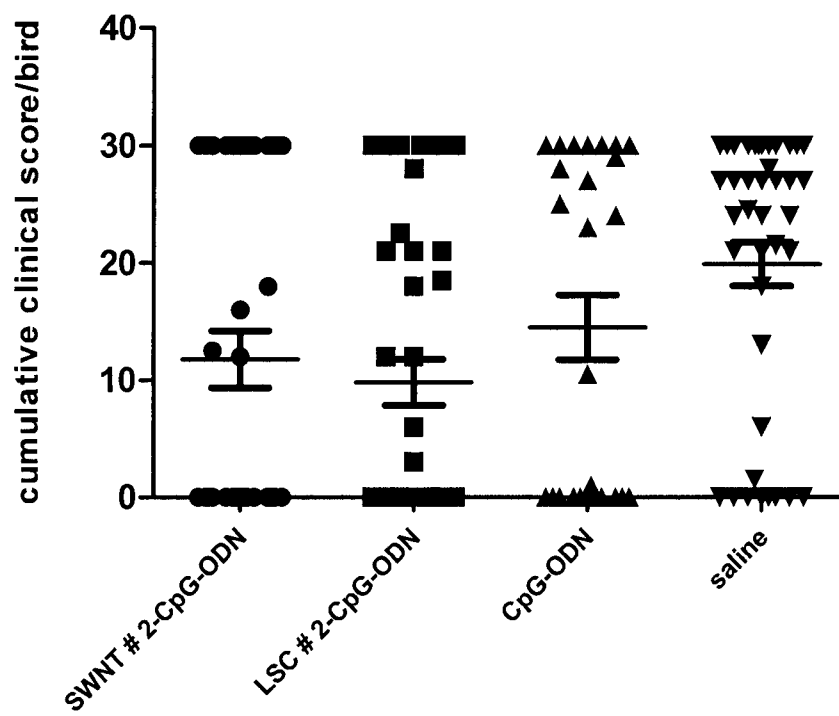


Figure 5

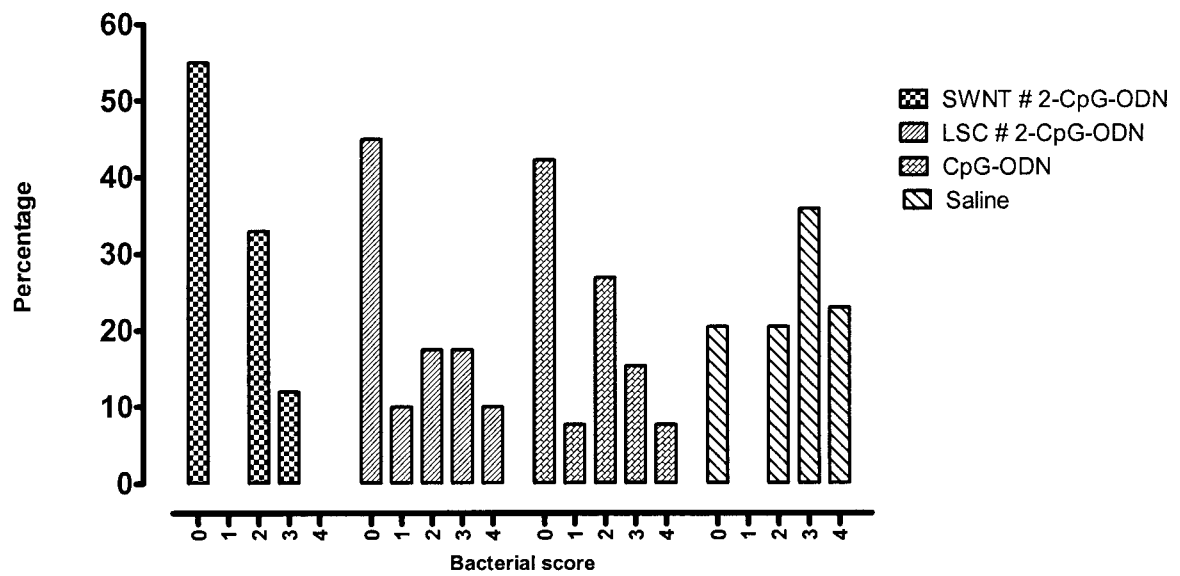


Figure 6

