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(71) **Applicant**: **AURASENSE THERAPEUTICS, LLC** [US/US]; 8045 Lamon Avenue, Suite 410, Skokie, IL 60077-5318 (US).

(72) **Inventors**: **RADOVIC-MORENO, Aleksandar, Filip**; 1055 Crabapple Drive, State College, PA 16801 (US). **GOEL, Askriti**; 8045 Lamon Avenue Suite 410, Skokie, IL 60077-5318 (US). **RISCHE, Clayton**; 8045 Lamon Avenue Suite 410, Skokie, IL 60077-5318 (US). **MADER, Christopher, C.**; 8045 Lamon Avenue, Suite 410, Skokie, IL 60077-5318 (US). **KANG, Richard**; 3312 Central Street, Evanston, IL 60201 (US). **NALLAGATLA, Subbarao**; 8045 Lamon Avenue, Suite 410, Skokie, IL 60077-5318 (US). **GRYAZNOV, Sergei**; 138 W. Bellevue Avenue, San Mateo, CA 94402 (US). **PATEL, Pinal**; 8045 Lamon Avenue, Suite 410, Skokie, IL 60077-5318 (US). **DANIEL, Weston**; 1513 Hinman Avenue, Apt. 2n, Evanston, IL 60201 (US). **GILJOHANN, David, A.**; 7659 N. Bosworth #c1, Chicago, IL 60626 (US).

(74) **Agent**: **LOCKHART, Helen C.**; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210-2206 (US).

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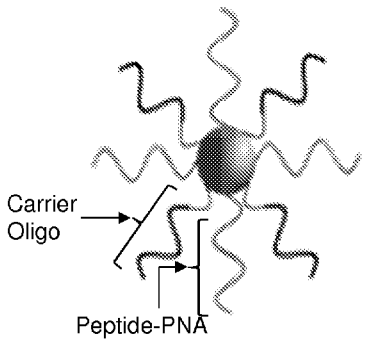
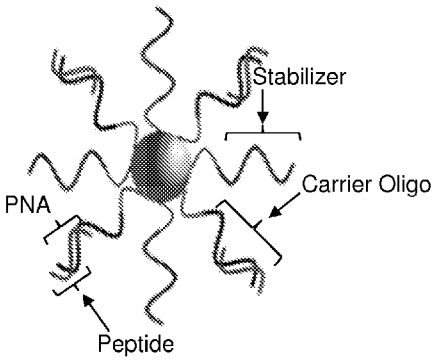


Peptide-PNA: KFF-acpP
Carrier Oligo: *acpP*-18-PO

Figure 1A



Peptide-PNA: KFF-acpP
Carrier Oligo: compl-acpP
Stabilizer: PEG

Figure 1B

(57) **Abstract**: Spherical nanoparticles for the treatment of bacteria are provided. The nanoparticles may be self-assembling particles having a core decorated by oligonucleotides and therapeutic pharmacophores. The nanoparticles are useful for prophylactic and therapeutic applications as well as research and diagnostic indications.

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SPHERICAL NANOPARTICLES AS ANTIBACTERIAL AGENTS

Related Applications

This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application Serial No. 62/019,557, entitled "SPHERICAL NANOPARTICLES AS ANTIBACTERIAL AGENTS" filed on July 1, 2014, which is herein incorporated by reference in its entirety.

Background of the Invention

Antibiotics are agents that have the ability to either eliminate or inhibit the growth of microorganisms. Antibiotic development began as early as the 1920s and is still evolving due to rapid appearance of resistant strains. Resistance to antibiotics can be due to either spontaneous or induced gene mutations or acquisition of resistant genes from another bacterial species by horizontal gene transfer. Antibiotic resistant genes are, however, not limited to bacterial genomes. They can be transferred horizontally from one organism to another and even across species. Emergence of antibiotic resistance has been prevalent since only a few years after antibiotic development began. Once any antibiotic was widely used, resistant strains that were capable of inactivating the drug became prevalent. As a result, with evolution of antibiotics and their widespread use, resistance to antibiotics also became more common.

Antibiotic resistance can be explained by the following mechanisms- (1) inactivation of the antibiotic, (2) development of a resistant metabolic pathway, (3) alteration of the receptor for the antibiotic drug or/and (4) decrease in the amount of antibiotic that is able to reach the receptor by altering drug uptake. For example, tetracycline resistance, fosfomycin resistance, aminoglycoside resistance are all developed due to poor drug uptake. In addition, drug efflux, a mechanism that cycles toxins out of bacterial cells, often contributes to antibiotic resistance.

Currently, most antibiotics being developed are either natural products from the bacteria with antibiotic activity or synthetic antimicrobial agents. With increased appearance of antibiotic resistance mechanisms, new ways to treat bacterial infections are desirable.

Summary of the Invention

The invention in an aspect is a nanostructure which includes a core, one or more carrier oligonucleotides positioned on the exterior of the core, and a therapeutic pharmacophore coupled to the carrier oligonucleotides. In one embodiment the carrier oligonucleotides form an oligonucleotide shell on the exterior of the core and wherein the therapeutic pharmacophore is hybridized to the carrier oligonucleotides.

In some embodiments the oligonucleotide shell is comprised of carrier oligonucleotides and at least one other molecule. In other embodiments the oligonucleotide shell is comprised entirely of oligonucleotides.

In some embodiments the oligonucleotides are comprised of single-stranded or double-stranded DNA oligonucleotides. In other embodiments the oligonucleotides are comprised of single-stranded or double-stranded RNA oligonucleotides. In other embodiments the oligonucleotides are comprised of chimeric RNA-DNA oligonucleotides. In another embodiment the carrier oligonucleotides are comprised of RNA-DNA or DNA-RNA oligonucleotide heteroduplexes. In another embodiment the oligonucleotides are comprised of combinations of single-stranded or double-stranded DNA, RNA, or chimeric RNA-DNA oligonucleotides.

In another embodiment the oligonucleotides have structurally and nucleotide sequence identical oligonucleotides. In some embodiments the oligonucleotides have at least two structurally and nucleotide sequence different oligonucleotides.

In other embodiments the oligonucleotides have 2-10 different nucleotide sequences.

In an embodiment at least one of the carrier oligonucleotides is a modified oligonucleotide. In some embodiments at least 50% of the carrier oligonucleotides are modified oligonucleotides. In yet other embodiments all of the carrier oligonucleotides are modified oligonucleotides. In some embodiments the oligonucleotides have at least one phosphorothioate linkage. In other embodiments the oligonucleotides do not have a phosphorothioate linkage.

In another embodiment the nanostructure comprises a liposomal core having a lipid bilayer.

In some embodiments at least one oligonucleotide has its 5'- terminus exposed to the outside surface of the nanostructure. In other embodiments all of the oligonucleotides have their 5'- terminus exposed to the outside surface of the nanostructure. In other embodiments at least one carrier oligonucleotide has its 3'- terminus exposed to the outside surface of the nanostructure. All of the carrier oligonucleotides have their 3'- terminus exposed to the outside surface of the nanostructure in other embodiments.

In another embodiment the oligonucleotides are directly linked to the core. In some embodiments the oligonucleotides are indirectly linked to the core through a linker. In other embodiments the oligonucleotides are indirectly linked to the core through more than one linker. The carrier oligonucleotides may also be directly linked to the core. In some embodiments at least one carrier oligonucleotide is positioned laterally on the surface of the nanostructure. In other embodiments all of the carrier oligonucleotides are positioned laterally on the surface of the nanostructure.

In other embodiments the oligonucleotides (including carrier oligonucleotides and therapeutic pharmacophores) are reversibly or irreversibly coupled to the core. In some embodiments, the carrier oligonucleotides are irreversibly coupled to the core. In yet other embodiments the therapeutic pharmacophore is reversibly coupled to the core or carrier oligonucleotide.

The linker in some embodiments is a chemical structure containing one or more thiol groups, including various chain length alkane thiols, cyclic dithiol, lipoic acid, PEG-thiol, and other thiol group containing linkers. Optionally the carrier oligonucleotides are linked to a liposomal core and the linker is one or more of the following linkers: tocopherols, sphingolipids such as sphingosine, sphingosine phosphate, methylated sphingosines and sphinganines, ceramides, ceramide phosphates, 1-0 acyl ceramides, dihydroceramides, 2-hydroxy ceramides, sphingomyelin, glycosylated sphingolipids, sulfatides, gangliosides, phosphosphingolipids, and phytosphingosines of various lengths and saturation states and their derivatives, phospholipids such as phosphatidylcholines, lysophosphatidylcholines, phosphatidic acids, lysophosphatidic acids, cyclic LPA, phosphatidylethanolamines, lysophosphatidylethanolamines, phosphatidylglycerols, lysophosphatidylglycerols, phosphatidylserines, lysophosphatidylserines, phosphatidylinositols, inositol phosphates,

LPI, cardiolipins, lysocardiolipins, bis(monoacylglycero) phosphates, (diacylglycero) phosphates, ether lipids, diphytanyl ether lipids, and plasmalogens of various lengths, saturation states, and their derivatives, sterols such as cholesterol, desmosterol, stigmasterol, lanosterol, lathosterol, diosgenin, sitosterol, zymosterol, zymostenol, 14-demethyl-lanosterol, cholesterol sulfate, DHEA, DHEA sulfate, 14-demethyl-14-dehydrlanosterol, sitostanol, campesterol, ether anionic lipids, ether cationic lipids, lanthanide chelating lipids, A-ring substituted oxysterols, B-ring substituted oxysterols, D-ring substituted oxysterols, side-chain substituted oxysterols, double substituted oxysterols, cholestanoic acid derivatives, fluorinated sterols, fluorescent sterols, sulfonated sterols, phosphorylated sterols, and polyunsaturated sterols of different lengths, saturation states and derivatives thereof.

In another embodiment the oligonucleotides comprise 2-1,000 oligonucleotides.

The core in some embodiments is a solid or hollow core and may be inert, paramagnetic or supramagnetic. In some embodiments the solid core is comprised of noble metals, including gold and silver, transition metals including iron and cobalt, metal oxides including silica, polymers or combinations thereof. In other embodiments the core is a polymeric core and wherein the polymeric core is comprised of amphiphilic block copolymers, hydrophobic polymers including polystyrene, poly(lactic acid), poly(lactic co-glycolic acid), poly(glycolic acid), poly(caprolactone) and other biocompatible polymers.

In another embodiment the liposomal core is comprised of one type of lipid. In some embodiments the liposomal core is comprised of 2-10 different lipids.

The liposomal core in some embodiments is comprised of one or more lipids selected from: sphingolipids such as sphingosine, sphingosine phosphate, methylated sphingosines and sphinganines, ceramides, ceramide phosphates, 1-0 acyl ceramides, dihydroceramides, 2-hydroxy ceramides, sphingomyelin, glycosylated sphingolipids, sulfatides, gangliosides, phosphosphingolipids, and phytosphingosines of various lengths and saturation states and their derivatives, phospholipids such as phosphatidylcholines, lysophosphatidylcholines, phosphatidic acids, lysophosphatidic acids, cyclic LPA, phosphatidylethanolamines, lysophosphatidylethanolamines, phosphatidylglycerols, lysophosphatidylglycerols, phosphatidylserines, lysophosphatidylserines, phosphatidylinositols, inositol phosphates,

LPI, cardiolipins, lysocardiolipins, bis(monoacylglycero) phosphates, (diacylglycero) phosphates, ether lipids, diphytanyl ether lipids, and plasmalogens of various lengths, saturation states, and their derivatives, sterols such as cholesterol, desmosterol, stigmasterol, lanosterol, lathosterol, diosgenin, sitosterol, zymosterol, zymostenol, 14-demethyl-lanosterol, cholesterol sulfate, DHEA, DHEA sulfate, 14-demethyl-14-dehydrlanosterol, sitostanol, campesterol, ether anionic lipids, ether cationic lipids, lanthanide chelating lipids, A-ring substituted oxysterols, B-ring substituted oxysterols, D-ring substituted oxysterols, side-chain substituted oxysterols, double substituted oxysterols, cholestanoic acid derivatives, fluorinated sterols, fluorescent sterols, sulfonated sterols, phosphorylated sterols, and polyunsaturated sterols of different lengths, saturation states, and derivatives thereof.

The therapeutic pharmacophore in some embodiments is an antisense oligonucleotide. The therapeutic pharmacophore may be a peptide nucleic acid (PNA). Optionally an antibacterial cell penetrating peptide conjugated to the antisense oligonucleotide or PNA.

In another embodiment the nanostructure further comprises an active agent. In some embodiments the active agent is mixed together with the nanostructure. In other embodiments the active agent is linked directly to the oligonucleotide shell and/or the therapeutic pharmacophore. In some embodiments the active agent is linked indirectly to the oligonucleotide shell and/or the therapeutic pharmacophore through a linker. In other embodiments the active agent is linked directly to the core. In yet another embodiment the active agent is linked indirectly to the core through a linker. In another embodiment an active agent -oligonucleotide conjugate is linked to the core through oligonucleotide hybridization. In some embodiments the active agent is associated with the core by being embedded within the core, optionally the liposomal core. In some embodiments the active agent is encapsulated within the liposomal core in an inner aqueous layer. In other embodiments the active agent is attached non-covalently to the oligonucleotide of the oligonucleotide shell.

In some embodiments the nanostructure is a self-assembling nanostructure.

In other embodiments the therapeutic pharmacophore is an RNA oligonucleotide, which is optionally an siRNA.

The nanostructure may include an antibacterial agent conjugated to the antisense oligonucleotide.

In some embodiments the therapeutic pharmacophore is an oligonucleotide having at least a region that is antisense to acyl carrier protein P (acpP). In other embodiments the therapeutic pharmacophore is an oligonucleotide is coupled to a peptide. The peptide may be a cell penetrating peptide and is conjugated to the oligonucleotide through a glycol linkage. Optionally the cell penetrating peptide has the following sequence: KFFKFFKFFK (SEQ ID NO: 1).

In some embodiments a PEG is incorporated into the nanostructure. The PEG may be 1,000-40,000 Daltons.

A method for treating a subject having a bacterial infection is provided The method involves administering to a subject a nanostructure as described and claimed herein, in an effective amount to treat the bacterial infection. In one embodiment the bacterial infection is selected from the group consisting of *Escherichia coli, Acinetobacter baumannii, Helicobacter pyloris*, *Borelia burgdorferi*, *Legionella pneumophilia*, *Mycobacteria sps* (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansaii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic species.), *Streptococcus pneumoniae*, pathogenic *Campylobacter sp.*, *Enterococcus sp.*, *Haemophilus influenzae*, *Bacillus antracis*, *corynebacterium diphtheriae*, *corynebacterium sp.*, *Erysipelothrix rhusiopathiae*, *Clostridium perfringers*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides sp.*, *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidium*, *Treponema pertenue*, *Leptospira*, *Rickettsia,* and *Actinomyces israelli.*

In some embodiments the nanostructure is delivered by a route selected from the group consisting of oral, nasal, sublingual, intravenous, subcutaneous, mucosal, respiratory, direct injection, enema, and dermally.

In other aspects the invention is a method for treating a surface by applying to a surface a nanostructure as described or claimed herein, in an effective amount to disrupt a bacterial infection on the surface. In one embodiment the surface is a surgical device or implant.

An article is provided in other aspects of the invention. The article is a device having a coating of a nanoparticle as described or claimed herein. In an embodiment the device is a surgical device or implant. In another embodiment the nanoparticle partially coats the device or fully coats the device. In yet other embodiments the nanoparticle coats the device by being embedded in the surface of the device.

In another aspect the composition for use in the treatment of disease comprises the nanostructure and embodiments thereof.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. The details of one or more embodiments of the invention are set forth in the accompanying Detailed Description, Examples, Claims, and Figures. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

Brief Description of the Drawings

The accompanying drawings are not intended to be drawn to scale. In the drawings, each identical or nearly identical component that is illustrated in various figures is represented by a like numeral. For purposes of clarity, not every component may be labeled in every drawing. In the drawings:

Figures 1A-1B: General structure of the spherical nucleic acids (SNAs) described herein. (A) General structure of a co-loading model, wherein peptide-peptide nucleic acids (PNAs) (therapeutic pharmacophores) and carrier oligonucleotides (oligos) are both

conjugated directly to the gold core. (B) General structure of peptide-PNA loaded on the gold core via hybridization to a complementary carrier oligo (hybridization model).

Figures 2A-2B: Antibacterial efficacy of Peptide-peptide nucleic acid (PNA) spherical nucleic acid (SNA) construct (hybridization model) with KFF as the cell penetrating peptide (CPP) and PNA oligomer targeted towards the *acpP* gene. The negative control is a SNA construct with similar structure, with KFF as the CPP but scrambled PNA oligomer, not targeted towards any *Escherichia coli* gene. The targeted SNA constructs Peptide-PNA:Oligo 1:1 (closed circles) and Peptide-PNA:Oligo 2:1 (closed squares), control SNA construct (CTL Peptide-PNA:Oligo; 1:1; closed up triangles) and free-peptide-PNA (Peptide-PNA Only; closed down triangles) were added to *Escherichia coli K-12* (A) and *Escherichia coli DH5a* (B). The data are expressed in terms of estimated peptide-PNA concentration, assuming there is 100% hybridization of peptide PNA added to the complementary oligo.

Figures 3A-3B: Antibacterial efficacy of Peptide-PNA SNA construct (hybridization model) with KFF as the CPP and PNA oligomer targeted towards the *acpP* gene. Bacteria were treated with the peptide-PNA SNA constructs Peptide-PNA:Oligo 1:1, Peptide-PNA:Oligo 2:1, negative control (CTL Peptide-PNA:Oligo 1:1), free peptide-PNA (Peptide-PNA Only), or left untreated, and the number of colony forming units (CFU)/mL was counted. The CFU/mL count was plotted as a comparison to the untreated cells to show the growth inhibition for treated and untreated *Escherichia coli K-12* (A) and *Escherichia coli DH5a* (B).

Figures 4A-4B: Antibacterial efficacy of Peptide- PNA SNAs (hybridization model) with KFF as the cell penetrating peptide and PNA oligomers targeted towards the *acpP* gene. The control is peptide-PNAs hybridized to the oligos (Peptide-PNA:Oligo Free; arrow point to closed circles), not functionalized on the gold core, to establish the role of gold core as a carrier of the peptide-PNA into the bacterial cell. (A) A bacterial turbidity assay was conducted and the data are expressed in terms of oligo concentration as Peptide-PNA:Oligo 1:1 (arrow points to closed circles), Peptide-PNA:Oligo 2:1 (closed squares), Peptide-PNA:Oligo 3:1 (closed up triangles), and Peptide-PNA:Oligo 4:1 (closed down triangles), based on the oligo loading that was identified using a fluorescence assay. (B) The results of

turbidity assay were confirmed by the colony forming unit (CFU)/mL viability assay and the data is presented.

Figures 5A-5B: Time-dependent antimicrobial efficacy of nanostructures having a liposomal core functionalized with oligonucleotides. The oligonucleotides are hybridized to peptide-peptide nucleic acids (pPNAs) via Watson-Crick hybridization in either duplex or triplex conformations. Each pPNA sequence is antisense to the acyl carrier protein in *A. baumannii*. The nanostructures were administered to the bacteria and the percentage population as related to untreated cells at t = 4 hours (A) or at t = 7 hours (B) were measured, with the liposomal pPNAs (Liposome+pPNA; open diamonds) compared to free pPNAs (pPNA only; open squares).

Figure 6: Comparison of nanostructures having a liposomal core -SNAs to free peptide-peptide nucleic acids (pPNAs) and gold-core spherical nucleic acids functionalized with oligonucleotides. The oligonucleotides are hybridized to pPNAs via Watson-Crick hybridization in either duplex or triplex conformations. Each pPNA sequence is antisense to the acyl carrier protein in *A. baumannii* or *E. coli*. The nanostructures having a liposomal core were administered to the bacteria on a 96-well plate, using a serial dilution, single-time dosage approach. Results indicate the percentage population as related to untreated cells after incubation overnight, with the liposomal pPNAs (LSNA-pPNAs; closed diamonds) compared to free pPNAs (closed squares).

Figures 7A-7B: Antibacterial efficacy and general structure of the SNAs evaluated herein. (A) Activity and structure of the co-loading model, wherein peptide-PNAs and stabilizing oligonucleotides (oligos) are both conjugated directly to the gold core via thiol linkage. Antibacterial efficacy of Peptide-PNA SNAs (co-loading model) with KFF as the cell penetrating peptide and PNA oligomers targeted towards the acpP gene. The Target Oligo & Peptide-PNA (closed diamonds), control construct CTL oligo & PeptidePNA (closed triangles), and PeptidePNA Only (closed squares) were added to bacteria. The data are expressed in terms of estimated peptide-PNA concentration, assuming there is 100% hybridization of peptide PNA added to the complementary oligo. (B) Activity and structure of peptide-PNA loaded on the gold core via hybridization to a complementary oligo (hybridization model). The structure also contains proposed PEG molecule to increase the

stability and circulation time of the SNA construct. Antibacterial efficacy of Peptide-PNA SNAs (hybridization model) with KFF as the cell penetrating peptide and PNA oligomers targeted towards the acpP gene. The data are expressed in terms of oligo concentration as Peptide-PNA:Oligo 1:1 (arrow points to closed circles), Peptide-PNA:Oligo 2:1 (closed squares), Peptide-PNA:Oligo 3:1 (closed up triangles), and Peptide-PNA:Oligo 4:1 (closed down triangles).

Figures 8A-8B: SNAs without carrier oligonucleotide and peptide nucleic acid do not show antibacterial efficacy. SNAs were prepared to target either the acyl carrier protein gene (acpP) using the indicated chemistries and targets: T1=TTCTTCGATAGTGCTCAT (SEQ ID NO: 2), T2=CATACTCTTAAATTTCCT (SEQ ID NO: 3), T3=TAGTGCTCATACTCTT (SEQ ID NO: 4). PO=all phosphodiester backbone, 2'OMe=2'OMe modifications to all bases, G/PS=Gap-mer with 18 total bases, from 5' to 3' containing six 2'OMe modified bases followed by six 2'deoxy nucleobases followed by six 2'OMe modified bases (all linked by phosphorothioate backbone). (A) Increase in optical density at 900 nm (OD900) following overnight incubation with an inoculum of $1x10^4$ bacteria per well at SNA = 100 nM (approximate oligonucleotide concentration = 10 uM). PBS = 0.7676. (B) CFU/mL measurement obtained via plating the cells treated as indicated overnight with SNAs at 100 nM (approximate oligonucleotide concentration = 10 uM) from part A.

Figure 9: General structure of silver spherical nucleic acid (SNA) design and interaction described herein. Using a silver nanoparticle core, a basic SNA is formulated with the adsorption of 3'-thiol-oligonucleotide and methoxyl poly(ethylene glycol) thiol. SNAs are known to adhere to or transfect across various types of cell membranes, which serve as the driving force behind a silver-derived SNA. The unique properties of Ag-SNAs enable the use of these particles as potent antimicrobials.

Figure 10:. TEM, DLS, and zeta-potential characterization of silver particles. (A) The transmission electron microscopy (TEM) size analysis of silver nanoparticles (Ag-NPs) yielded a mean diameter of 25.8 ± 4.8 nm. Functionalized bacteria-targeting silver-core spherical nucleic acid (BT-Ag-SNA) based constructs (B) possess a mean diameter of 19.7 ±4.05 nm. (C) The dynamic light scattering (DLS) number size distributions display the

peak sizes of the Ag-NPs and BT-Ag-SNAs. There is an upward shift of about 5-10 nm between Ag-NPs and BT-Ag-SNAs. (D) The difference in zeta-potential indicates the presence of negatively charged oligonucleotides on the surface of BT-Ag-SNAs.

Figure 11: Growth curves for treated bacteria. (A) *A. baumannii* AYE treated with PS-BT-Ag-SNAs demonstrates an minimum inhibitory concentration (MIC) value of 0.3125 nM. The corresponding PO-BT-Ag-SNAs and Ag-NPs displayed MICs of 0.625 and 5 nM, respectively. *A. baumannii* UNT086-1 (B) demonstrated MICs of 0.625, 1.25, and 5 nM when comparing PS-BT-Ag-SNAs, PO-BT-Ag-SNAs, and Ag-NPs, respectively. (C) MRSA showed a similar reaction to the particles, with MICs of 2.5, 5, and 10 nM for PS-BT-Ag-SNAs, PO-BT-Ag-SNAs, and Ag-NPs, respectively. Unconjugated oligonucleotide did not display activity against any bacteria tested. (D) The time-dependent kill curve of BT-Ag-SNAs and Ag-NPs shows that the SNAs were able to completely eliminate bacteria within 7 hours, whereas Ag-NPs were only able to reduce the bacterial population at the same Ag concentration.

Figure 12: Interactions of BT-Ag-SNAs with bacteria. Fluorescence microscopy images of untreated (A) and fluorescent-BT-Ag-SNA treated (B) *A. baumannii* AYE demonstrate the association of particles with cells in a bacterial population. TEM images of untreated (C) and BT-Ag-SNA-treated (D) *A. baumannii* AYE cells display the accumulation of BT-Ag-SNAs on the bacterial membrane.

Figure 13: Ampicillin acts synergistically with BT-Ag-SNAs and Ag-NPs. Graphs were obtained by comparing the fractional inhibitory concentrations of ampicillin with Ag-SNAs, where each point represents data that deviated from an additive relationship between the drugs (the dotted line). Both methicillin-resistant *Staphylococcus aureus* (MRSA) and *A. baumannii* UNT086-1 were challenged with a combination of ampicillin and BT-Ag-SNAs. Both strains of bacteria are resistant to ampicillin or more generally, β-lactam antibiotics. (A) In MRSA, the best results were seen when BT-Ag-SNAs and ampicillin were dosed at 0.78 nM and 0.49 μg/mL, respectively. (B) The clinical baumannii strain, UNT086-1, displayed similar results. Only 0.25 nM of BT-Ag-SNAs and 31.2 μg/mL of ampicillin were used at the lowest MIC with respect to ampicillin. In both tested bacterial strains, these

numbers represent 4-fold and-128 fold decreases in the respective dosage of BT-Ag-SNAs and ampicillin.

Figure 14: Toxicity of BT-Ag-SNAs with HFK cells. To establish an in vitro toxicity index between the effective treatment of bacteria and toxicity to host cells, human foreskin keratinocytes were treated with various concentrations of BT-Ag-SNAs and Ag-NPs.

Figure 15: Topical Infection of *A. baumannii* UNT086-1 treated with BT-Ag-SNAs. BT-Ag-SNAs displayed a decrease in CFUs after treatment, with a significant log10 reduction of 1.51 ($p < 0.05$) compared to the untreated control. Ag-NPs and unconjugated oligonucleotide did not demonstrate significant activity, with minor log10 reductions of 0.63 and 0.58.

Figure 16: Growth curve analysis of *P. aeruginosa* and *E. coli* in vitro. A comparison of Ag-SNAs and Ag-NPs with unconjugated phosphorothioate (PS) oligonucleotide reinforces the increased efficacy of the Ag-SNA, particularly against Gram-negative species. When challenging *P. aeruginosa* in (A), the MIC of Ag-SNAs is 0.3125 nM, which is 8-fold lower than the MIC of Ag-NPs. Similarly, in (B) the MIC of Ag-SNAs was 0.16525 nM, a 16-fold reduction from Ag-NPs. In (C), an ATP detection cell viability assay was used to verify the results of (B).

Figure 17: Vancomycin and ciprofloxacin synergy with Ag-SNAs and Ag-NPs. *Acinetobacter baumannii* UNT086-1 (MIC = 1 nM, Ag-SNAs) was challenged using the checkerboard method for synergy. (A) Vancomycin displayed an MIC of 15.6 μg/mL, and demonstrated significant synergy at 32- and 62-fold reductions of Ag-SNAs and vancomycin, respectively. (B) Ciprofloxacin's MIC value was 30 μg/mL, and displayed a respective 2- and 120-fold reduction in Ag-SNA and ciprofloxacin concentration at the point of greatest synergy.

Figure 18: BT-Ag-SNA and silver ion growth curve analysis of *A. baumannii* UNT086-1. *A. baumannii* UNT086-1 were treated with silver ions (Ag^+; closed triangles) or silver spherical nucleic acids (Ag-SNAs; closed squares) and the data is represented as percent of untreated (% UNT) cells as a function of increasing concentration of Ag atoms

(nM). This comparison was made on an atomic basis, assuming that the entire outer layer of silver atoms on each SNA was free to interact with bacteria.

Detailed Description of the Invention

Methods and products for delivery of therapeutic molecules in a multivalent fashion are provided herein. The methods and products may be used for, for example, antibacterial applications, prevention or treatment of infectious disease, modification or elimination of natural and artificial bacterial flora, disruption or elimination of biofilms, and modification of industrial bacterial cultures.

Nanostructures having a core and one or more carrier oligonucleotides and therapeutic pharmacophores positioned on the exterior of the core are provided herein. This novel class of nanostructures has unexpectedly high anti-microbial activity. These nanostructures are supra-molecular assemblies, which are spherical nucleic acids (sometimes referred to as SNAs), and can deliver combinations of anti-microbial agents in a highly spatiotemporally controlled manner to cells (Examples of the SNA structures are shown in Figure 1). A distinctive feature of these nanostructures is the incorporation of at least two distinct sets of nucleic acids on the exterior of the core.

Incorporation of the dual oligonucleotides in the SNA construct confers unique properties on the structure including but not limited to preferable chemical properties, enhanced bioavailablity, enhanced bacterial targeting, enhanced drug product efficacy, enhanced in vivo pharmacodynamics and pharmacokinetic properties. Importantly, these advantages as they relate to antibiotic development cannot be achieved by delivering either of the separate components individually or in simultaneous combination but not physically associated. Assembly of all of the components into a single structure is critical to obtaining the desired enhanced properties and effects.

The properties of the structures described herein represent a substantial improvement over the existing art. Currently, common methods used in the clinic as antibiotic treatments are small molecule compounds that block or impair essential life processes in the microorganism such as penicillin derivatives, cephalosporins, quinolones, and sulfanomides. However, rapid development of antibiotic resistance by bacteria have resulted in the clinical

expansion of multi-drug resistant (MDR) strains of many pathogens which requires the development of new antibiotic treatments. In addition to continued development of small molecule antibiotics which has significant limitations known to those skilled in the art, more recently, gene regulatory compounds such as antisense DNA have been explored as a way to overcome MDR strains of many pathogens. In order to overcome the propensity of nucleic acid degrading enzymes secreted by many pathogens into their immediate environment, strategies toward heavily modifying the antisense DNA strands including, 2'O-methyl or 2' O-methoxy modification of the ribose ring, replacement of the natural phosphodiester backbone linkages with phosphorothioate or phosphoroamidate linkages, or more exotic modifications including the use of bridged nucleic acids, locked nucleic acids or peptide nucleic acids have been required. However, each of these approaches has limitations known to those skilled in the art.

Achieving specific delivery of a therapeutic pharmacophore to achieve bactericidal effects is a significant challenge. The prior art typically relies heavily on significant chemical modification of nucleic acids, which results in relatively poor efficacy requiring very large dosages and high incidences of side effects and toxicity. In contrast, the methods of the invention achieve antibiotic effects that are enhanced relative to the use of single agents alone.

The nanostructure of the invention includes a core. The core may be a solid or a hollow core, such as a liposomal core. A solid core is a spherical shaped material that does not have a hollow center. The term spherical as used herein refers to a general shape and does not imply or is not limited to a perfect sphere or round shape. It may include imperfections.

Solid cores can be constructed from a wide variety of materials known to those skilled in the art including but not limited to: noble metals (gold, silver), transition metals (iron, cobalt) and metal oxides (silica). In addition, these cores may be inert, paramagnetic, or supramagentic. These solid cores can be constructed from either pure compositions of described materials, or in combinations of mixtures of any number of materials, or in layered compositions of materials. In addition, solid cores can be composed of a polymeric core such as amphiphilic block copolymers, hydrophobic polymers such as polystyrene,

poly(lactic acid), poly(lactic co-glycolic acid), poly(glycolic acid), poly(caprolactone) and other biocompatible polymers known to those skilled in the art.

The core may alternatively be a hollow core, which has at least some space in the center region of a shell material. Hollow cores include liposomal cores. A liposomal core as used herein refers to a centrally located core compartment formed by a component of the lipids or phospholipids that form a lipid bilayer. "Liposomes" are artificial, self closed vesicular structure of various sizes and structures, where one or several membranes encapsulate an aqueous core. Most typically liposome membranes are formed from lipid bilayers membranes, where the hydrophilic head groups are oriented towards the aqueous environment and the lipid chains are embedded in the lipophilic core. Liposomes can be formed as well from other amphiphilic monomeric and polymeric molecules, such as polymers, like block copolymers, or polypeptides. Unilamellar vesicles are liposomes defined by a single membrane enclosing an aqueous space. In contrast, oligo- or multilamellar vesicles are built up of several membranes. Typically, the membranes are roughly 4 nm thick and are composed of amphiphilic lipids, such as phospholipids, of natural or synthetic origin. Optionally, the membrane properties can be modified by the incorporation of other lipids such as sterols or cholic acid derivatives.

The lipid bilayer is composed of two layers of lipid molecules. Each lipid molecule in a layer is oriented substantially parallel to adjacent lipid bilayers, and two layers that form a bilayer have the polar ends of their molecules exposed to the aqueous phase and the non-polar ends adjacent to each other. The central aqueous region of the liposomal core may be empty or filled fully or partially with water, an aqueous emulsion, oligonucleotides, or other therapeutic or diagnostic agent such as an antimicrobial agent.

"Lipid" refers to its conventional sense as a generic term encompassing fats, lipids, alcohol-ether-soluble constituents of protoplasm, which are insoluble in water. Lipids usually consist of a hydrophilic and a hydrophobic moiety. In water lipids can self organize to form bilayers membranes, where the hydrophilic moieties (head groups) are oriented towards the aqueous phase, and the lipophilic moieties (acyl chains) are embedded in the bilayers core. Lipids can comprise as well two hydrophilic moieties (bola amphiphiles). In that case, membranes may be formed from a single lipid layer, and not a bilayer. Typical

examples for lipids in the current context are fats, fatty oils, essential oils, waxes, steroid, sterols, phospholipids, glycolipids, sulpholipids, aminolipids, chromolipids, and fatty acids. The term encompasses both naturally occurring and synthetic lipids. Preferred lipids in connection with the present invention are: steroids and sterol, particularly cholesterol, phospholipids, including phosphatidyl, phosphatidylcholines and phosphatidylethanolamines and sphingomyelins. Where there are fatty acids, they could be about 12-24 carbon chains in length, containing up to 6 double bonds. The fatty acids are linked to the backbone, which may be derived from glycerol. The fatty acids within one lipid can be different (asymmetric), or there may be only 1 fatty acid chain present, e.g. lysolecithins. Mixed formulations are also possible, particularly when the non-cationic lipids are derived from natural sources, such as lecithins (phosphatidylcholines) purified from egg yolk, bovine heart, brain, liver or soybean.

The liposomal core can be constructed from one or more lipids known to those in the art including but not limited to: sphingolipids such as sphingosine, sphingosine phosphate, methylated sphingosines and sphinganines, ceramides, ceramide phosphates, 1-0 acyl ceramides, dihydroceramides, 2-hydroxy ceramides, sphingomyelin, glycosylated sphingolipids, sulfatides, gangliosides, phosphosphingolipids, and phytosphingosines of various lengths and saturation states and their derivatives, phospholipids such as phosphatidylcholines, lysophosphatidylcholines, phosphatidic acids, lysophosphatidic acids, cyclic LPA, phosphatidylethanolamines, lysophosphatidylethanolamines, phosphatidylglycerols, lysophosphatidylglycerols, phosphatidylserines, lysophosphatidylserines, phosphatidylinositols, inositol phosphates, LPI, cardiolipins, lysocardiolipins, bis(monoacylglycero) phosphates, (diacylglycero) phosphates, ether lipids, diphytanyl ether lipids, and plasmalogens of various lengths, saturation states, and their derivatives, sterols such as cholesterol, desmosterol, stigmasterol, lanosterol, lathosterol, diosgenin, sitosterol, zymosterol, zymostenol, 14-demethyl-lanosterol, cholesterol sulfate, DHEA, DHEA sulfate, 14-demethyl-14-dehydrlanosterol, sitostanol, campesterol, ether anionic lipids, ether cationic lipids, lanthanide chelating lipids, A-ring substituted oxysterols, B-ring substituted oxysterols, D-ring substituted oxysterols, side-chain substituted oxysterols, double substituted oxysterols, cholestanoic acid derivatives,

fluorinated sterols, fluorescent sterols, sulfonated sterols, phosphorylated sterols, and polyunsaturated sterols of different lengths, saturation states, and their derivatives.

The oligonucleotides (including carrier oligonucleotides and therapeutic pharmacophores) are positioned on the exterior of the core. An oligonucleotide that is positioned on the core is typically referred to as coupled to the core. Coupled may be direct or indirect. One or more therapeutic pharmacophores may also be coupled to the carrier oligonucleotide. While the carrier may be reversibly or irreversibly coupled to the core, preferably the therapeutic pharmacophore is reversibly coupled to the core or carrier oligonucleotide. Reversibly coupled compounds are associated with one another using a susceptible linkage. A susceptible linkage is one which is susceptible to separation under physiological conditions. For instance Watson crick base pairing is a susceptible linkage. Cleavable linkers are also susceptible linkages. It is describable for the therapeutic pharmacophore to become uncoupled from the carrier oligonucleotide in a cell or body. In some instances the therapeutic pharmacophore is hybridized to the carrier oligonucleotides.

At least two oligonucleotides are on the exterior, a carrier and a therapeutic pharmacophore oligonucleotide (referred to herein interchangeably with "therapeutic oligonucleotide") . In some embodiments at least 25, 50, 75, 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1,000 oligonucleotides (carrier oligonucleotide and/or therapeutic pharmacophores) or any range combination thereof are on the exterior of the core. In some embodiments, 1-1000, 10-500, 50-250, or 50-300 oligonucleotides are present on the surface.

An oligonucleotide, as used herein, refers to any nucleic acid containing molecule. The nucleic acid may be DNA, RNA, PNA, LNA, ENA or combinations or modifications thereof. It may also be single, double or triple stranded. The carrier oligonucleotide and/or therapeutic pharmacophore may be a wide variety of molecules including but not limited to: single-stranded deoxyribonucleotides, ribonucleotides, and other single-stranded oligonucleotides incorporating one or a multiplicity of modifications known to those skilled in the art, double-stranded deoxyribonucleotides, ribonucleotides, and other double-stranded oligonucleotides incorporating one or a multiplicity of modifications known to those skilled in the art, as well as peptide nucleic acids (PNAs).

A carrier oligonucleotide is any oligonucleotide. It may or may not be a therapeutic pharmacophore. However, the carrier oligonucleotide is different from the therapeutic pharmacophore on any given nanostructure. It may be different in nucleotide sequence, structure, modification, etc. The carrier may have a therapeutic or diagnostic functionality or it may be non-functional. In some instance it is complementary fully or partially or with mismatches with the therapeutic pharmacophore.

A "therapeutic pharmacophore" or "therapeutic oligonucleotide" as used herein refers to a nucleic acid or partial nucleic acid having some therapeutic or diagnostic activity. The pharmacophore may be, for instance, an inhibitory nucleic acid or molecule involved in gene knockdown or an activation oligonucleotide involved in upregulating gene expression.

It is particularly desirable according to the invention for a therapeutic pharmacophore to have an anti-microbial therapeutic effect. Thus, the therapeutic pharmacophore may be a nucleic acid or other molecule that knocks down the expression of a bacterial mRNA and/or protein. The therapeutic pharmacophores of the invention may target essential genes of bacteria in order to reduce or ameliorate a bacterial infection. Essential genes that regulate or control bacterial growth, proliferation, virulence and synthesis of important living-dependent substances include but are not limited to *fbpA/ fbpB/fbpC* and *glnA1* in *Mycobacterium tuberculosis*, *gyrA/ompA* in *Klebsiella pneumonia*, *inhA* in *Mycobacterium smegmatis*, *oxyR/ahpC* in *Mycobacterium avium*, *NPT/EhErd2* in *Entamoeba histolytica*, *gtfB* in *Streptococcus mutans*, *fmhB/ gyrA/hmrB* and *fabI* in *Staphylococcus aureus*, *23S rRNA*, 16S rRNA plus *lacZ/bla,* and RNAse P in *Escherichia coli,* and *acpP* in *Burkholderia cepacia*, *Escherichia coli* as well as *Salmonella enterica serovar Typhimurium*, the *marRAB* operon in *Escherichia coli, aac(6')-Ib*, *act* in *Escherichia coli*, *vanA* in *Enterococcus faecalis*, *cmeA* in *Campylobacter jejun*, *mecA* in *Staphylococcus aureus*, *metS/murB* in *Bacillus anthracis* and *oprM* in *Pseudomonas aeruginossa.*

Therapeutic pharmacophores include but are not limited to antisense nucleic acids (single or double stranded), RNAi oligonucleotides, ribozymes, peptides, DNAzymes, peptide nucleic acids (PNAs), triple helix forming oligonucleotides, and aptamers and modified form(s) thereof directed to sequences in gene(s), RNA transcripts, or proteins.

Antisense nucleic acids include modified or unmodified RNA, DNA, or mixed polymer nucleic acids, and primarily function by specifically binding to matching sequences resulting in modulation of peptide synthesis. Antisense nucleic acid binds to target RNA by Watson Crick base-pairing and blocks gene expression by preventing ribosomal translation of the bound sequences either by steric blocking or by activating RNase H enzyme. Antisense molecules may also alter protein synthesis by interfering with RNA processing or transport from the nucleus into the cytoplasm.

As used herein, the term "antisense nucleic acid" or "antisense oligonucleotide" describes a nucleic acid that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of that mRNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence.

Examples of antisense oligonucleotides useful according to the invention are set forth in the Table below.

Table: List of Applicable Antisense Sequences and their Gene Targets

SEQ ID NO:	Antisense Sequence (5' to 3')	Gene Target	Organism	Reference
5	CTTCGATAGTG	acpP	*E. coli*	doi:10.1093/jac/dkp392
6	CTCATACTCT	acpP	*E. coli*	doi:10.1038/86753
7	TTTGCTCCAT	rpoD	*E. coli*	doi:10.1016/j.biomaterials.2011.09.075
8	CGGTCAGGGTAAC	Gyrase A	*E. coli*	doi:10.1073/pnas.1112561108
9	GACGTCGTCGGGCGTCTT	glnA1	*M. tuberculosis*	doi:10.1073/pnas.97.1.418
10	CATGCCGGACCCGTTGTCGCC	glnA1	*M. tuberculosis*	doi:10.1073/pnas.97.1.418
11	CCACAGCGACTGATGACAGTGCAT	glnA1	*M. tuberculosis*	doi:10.1073/pnas.97.1.418
12	TTTCTCGTCA	rpoD	*MRSA*	doi:10.1371/journal.pone.0029886

13	TTCTCGTCAG	rpoD	*MRSA*	doi:10.1371/journal.pone.0029886
14	GTTTCTCGTCAG	rpoD	*MRSA*	doi:10.1371/journal.pone.0029886
15	ATTCTCCTCAT	acpP	*A. baumannii*	doi:10.1093/infdis/jit460
16	GTGGCGTTTGA	acpP	*A. baumannii*	doi:10.1093/infdis/jit460
17	TCAAATGAGGC	ftsZ	*A. baumannii*	doi:10.1093/infdis/jit460
18	TAGACATACCA	rpsJ	*A. baumannii*	doi:10.1093/infdis/jit460
19	TACCAGTAAAC	rpsJ	*A. baumannii*	doi:10.1093/infdis/jit460

A broad range of RNAi-based modalities could be employed to inhibit expression of a gene in a cell, such as siRNA-based oligonucleotides and/or altered siRNA-based oligonucleotides. Altered siRNA based oligonucleotides are those modified to alter potency, target affinity, safety profile and/or stability, for example, to render them resistant or partially resistant to intracellular degradation. Modifications, such as phosphorothioates, for example, can be made to oligonucleotides to increase resistance to nuclease degradation, binding affinity and/or uptake. In addition, modification of siRNAs at the 2'-sugar position and phosphodiester linkage confers improved serum stability without loss of efficacy. RNAi therapies may be desirable in knocking down gene expression in a eukaryotic cell that is exposed to a bacteria in order to remove an inhibitor of bacterial resistance.

Ribozymes have also been proposed as a means of both inhibiting gene expression of a mutant gene and of correcting the mutant by targeted trans-splicing. Ribozyme activity may be augmented by the use of, for example, non-specific nucleic acid binding proteins or facilitator oligonucleotides. Multitarget ribozymes (connected or shotgun) have been suggested as a means of improving efficiency of ribozymes for gene suppression. Triple helix approaches have also been investigated for sequence-specific gene suppression. Triple helix forming oligonucleotides have been found in some cases to bind in a sequence-specific manner. Similarly, peptide nucleic acids have been shown to inhibit gene expression (Hanvey et al., Antisense Res. Dev. 1(4):307-17, 1991; Knudsen and Nielson Nucleic Acids Res. 24(3):494-500, 1996; Taylor et al., Arch. Surg. 132(11):1177-83, 1997). Minor-groove binding polyamides can bind in a sequence-specific manner to DNA targets and hence may represent useful small molecules for suppression at the DNA level. The diverse array of suppression strategies that can be employed includes the use of DNA and/or RNA aptamers that can be selected to target a protein of interest.

In some embodiments the therapeutic pharmacophore is 100% identical to the nucleic acid target and/or to portions of the carrier oligonucleotide. In other embodiments it is at least 99%, 95%, 90%, 85%, 80%, 75%, 70%, or 50% identical to the nucleic acid target and/or to portions of the carrier oligonucleotide. The term "percent identical" refers to sequence identity between two nucleotide sequences. Percent identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. Expression as a percentage of identity refers to a function of the number of identical amino acids or nucleic acids at positions shared by the compared sequences. Various alignment algorithms and/or programs may be used, including FASTA, BLAST, or ENTREZ-FASTA and BLAST are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default settings. ENTREZ is available through the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.

Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, Calif., USA. Preferably, an alignment program that permits gaps in the sequence is utilized to align the sequences. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See Meth. Mol. Biol. 70: 173-187 (1997). Also, the GAP program using the Needleman and Wunsch alignment method can be utilized to align sequences. An alternative search strategy uses MPSRCH software, which runs on a MASPAR computer. MPSRCH uses a Smith-Waterman algorithm to score sequences on a massively parallel computer. This approach improves ability to pick up distantly related matches, and is especially tolerant of small gaps and nucleotide sequence errors. Nucleic acid-encoded amino acid sequences can be used to search both protein and DNA databases.

An therapeutic pharmacophore may be designed to have partial or complete complementarity with one or more target genes. Depending on the particular target gene, the nature of the therapeutic pharmacophore and the level of expression of therapeutic pharmacophore (e.g. depending on copy number, promoter strength) the procedure may provide partial or complete loss of function for the target gene. Quantitation of gene

expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

"Inhibition of gene expression" refers to the absence or observable decrease in the level of protein and/or mRNA product from a target gene. "Specificity" refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell.

In some instances the therapeutic pharmacophore is a peptide nucleic acid (PNA). PNA oligomers have greater binding strength and specificity in the formation of a PNA/DNA duplex or PNA/DNA/PNA triplex as compared to a DNA/DNA duplex. PNAs also have increased stability to nucleases and proteases over a wide pH range, which makes them resistant to enzymatic degradation.

Despite the beneficial effects of PNA oligomers as antimicrobial agents, there have been indications that in vivo dosages of these molecules tend to be insufficient for a prolonged drug effect. This is likely due to the host's ability to clear small molecules and toxins from the blood stream, resulting in a decrease of the effective time for each dose in the body. This may also be due to the lower solubility of PNAs in aqueous solution, enzymatic degradation, microbial uptake, the ability of PNAs to penetrate and dissipate throughout the host tissues, or a combination of these factors. The nanostructures provided herein overcome these issues, thereby extending the lifetime of these types of biomolecules, resulting in the stabilization and/or enhancement of the effects of PNAs.

Additionally a cell penetrating peptide (CPP) may be coupled to the therapeutic pharmacophore. In some instances the CPP is covalently coupled to a PNA. CPP is believed to improve the bacterial cytosolic delivery of the therapeutic molecule. Coupling CPP to a therapeutic pharmacophore enhances stability and enables the penetration of the bacterial cell wall and membrane.

An exemplary structure including a PNA was synthesized (as described in more detail in the Examples). In the structure carrier phosphodiester oligonucleotides having the following sequence: 5'- AAAAAAAAAAGAGTATGAGAA -3' (SEQ ID NO: 20); acpP) were 5' modified with thiol and incorporated onto 13 nm diameter gold nanoparticles (AuNP). A thiol-modified polyethylene glycol (PEG) of 20 kDa average molecular weight was also incorporated into the nanostructure. Lastly, a peptide-peptide nucleic acid (peptide-PNA or pPNA) conjugate containing a PNA active drug product coupled to a cell penetrating peptide was Watson-Crick hybridized to the carrier oligonucleotide. The PNA sequence is complementary to the carrier oligonucleotide and antisense to the acyl carrier protein P (acpP) gene in E. coli and is coupled via a glycol linkage to the KFFKFFKFFK (SEQ ID NO: 1) cell penetrating peptide (5'-3' or N-C: KFFKFFKFFK (SEQ ID NO: 1)-O-CTCATACTCT (SEQ ID NO: 6)) (Figure 1). The PNA was selected on the basis of its ability to downregulate gene expression of the acpP gene in E. coli which is classified as an essential gene for bacterial growth. The cell penetrating peptide was selected based on its ability to facilitate PNA function from a screen of peptides.

In some instances the carrier oligonucleotides and optionally the therapeutic oligonucleotides form an oligonucleotide shell. An oligonucleotide shell is formed when at least 50% of the available surface area of the exterior surface of the core includes an oligonucleotide. In some embodiments at least 60%, 70%, 80%, 90%, 95%, 96%, 97% 98% or 99% of the available surface area of the exterior surface of the core includes an oligonucleotide.

The oligonucleotides of the oligonucleotide shell or therapeutic pharmacophores may be oriented in a variety of directions. In some embodiments the oligonucleotides are oriented radially outwards. The orientation of these oligonucleotides can be either 5' distal/3' terminal in relation to the core, or 3' distal/5'terminal in relation to the core, or

laterally oriented around the core. In one embodiment one or a multiplicity of different oligonucleotides are present on the same surface of a single SNA. In all cases, at least 1 oligonucleotide is present on the surface but up to 10,000 can be present.

The oligonucleotides (carrier oligonucleotide and/or therapeutic pharmacophores) may be linked to the core or to one another and/or to other molecules such an active agents either directly or indirectly through a linker. The oligonucleotides may be conjugated to a linker via the 5' end or the 3' end, e.g. [Sequence, 5'-3']-Linker or Linker-[Sequence, 5'-3']. Some or all of the oligonucleotides of the nanostructure may be linked to one another either directly or indirectly through a covalent or non-covalent linkage. The linkage of one oligonucleotide to another oligonucleotide may be in addition to or alternatively to the linkage of that oligonucleotide to liposomal core. One or more of the oligonucleotides may also be linked to other molecules such as an anti-microbial. The oligonucleotides may be linked to the anti-microbial of the core either directly or indirectly through a covalent or non-covalent linkage.

The oligonucleotide shell formed of at least carrier oligonucleotides may be anchored to the surface of the core through one or multiple of linker molecules, including but not limited to: any chemical structure containing one or multiple thiols, such as the various chain length alkane thiols, cyclic dithiol, lipoic acid, or other thiol linkers known to those skilled in the art.

In an embodiment containing a liposomal core, the oligonucleotide shell may be anchored to the surface of the liposomal core through conjugation to one or a multiplicity of linker molecules including but not limited to: tocopherols, sphingolipids such as sphingosine, sphingosine phosphate, methylated sphingosines and sphinganines, ceramides, ceramide phosphates, 1-0 acyl ceramides, dihydroceramides, 2-hydroxy ceramides, sphingomyelin, glycosylated sphingolipids, sulfatides, gangliosides, phosphosphingolipids, and phytosphingosines of various lengths and saturation states and their derivatives, phospholipids such as phosphatidylcholines, lysophosphatidylcholines, phosphatidic acids, lysophosphatidic acids, cyclic LPA, phosphatidylethanolamines, lysophosphatidylethanolamines, phosphatidylglycerols, lysophosphatidylglycerols, phosphatidylserines, lysophosphatidylserines, phosphatidylinositols, inositol phosphates,

LPI, cardiolipins, lysocardiolipins, bis(monoacylglycero) phosphates, (diacylglycero) phosphates, ether lipids, diphytanyl ether lipids, and plasmalogens of various lengths, saturation states, and their derivatives, sterols such as cholesterol, desmosterol, stigmasterol, lanosterol, lathosterol, diosgenin, sitosterol, zymosterol, zymostenol, 14-demethyl-lanosterol, cholesterol sulfate, DHEA, DHEA sulfate, 14-demethyl-14-dehydrlanosterol, sitostanol, campesterol, ether anionic lipids, ether cationic lipids, lanthanide chelating lipids, A-ring substituted oxysterols, B-ring substituted oxysterols, D-ring substituted oxysterols, side-chain substituted oxysterols, double substituted oxysterols, cholestanoic acid derivatives, fluorinated sterols, fluorescent sterols, sulfonated sterols, phosphorylated sterols, and polyunsaturated sterols of different lengths, saturation states, and their derivatives.

The carrier oligonucleotide is positioned on the exterior of the core. It may be associated with the core by being embedded within the core (liposomal core) or it may be attached or linked, either indirectly (i.e. non-covalently or covalently through other molecules such a linkers) or directly (i.e. covalently).

The terms "oligonucleotide" and "nucleic acid" are used interchangeably to mean multiple nucleotides (i.e., molecules comprising a sugar (e.g., ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g., cytosine (C), thymidine (T) or uracil (U)) or a substituted purine (e.g., adenine (A) or guanine (G)). Thus, the term embraces both DNA and RNA oligonucleotides. The terms shall also include oligonucleosides (i.e., a oligonucleotide minus the phosphate) and any other organic base containing polymer. Oligonucleotides can be obtained from existing nucleic acid sources (e.g., genomic or cDNA), but are preferably synthetic (e.g., produced by nucleic acid synthesis).

The oligonucleotides may be single stranded or double stranded. A double stranded oligonucleotide is also referred to herein as a duplex. Double-stranded oligonucleotides of the invention can comprise two separate complementary nucleic acid strands.

As used herein, "duplex" includes a double-stranded nucleic acid molecule(s) in which complementary sequences or partially complementary sequences are hydrogen bonded to each other. The complementary sequences can include a sense strand and an

antisense strand. A double-stranded oligonucleotide can be double-stranded over its entire length, meaning it has no overhanging single-stranded sequences and is thus blunt-ended. In other embodiments, the two strands of the double-stranded oligonucleotide can have different lengths producing one or more single-stranded overhangs. A double-stranded oligonucleotide of the invention can contain mismatches and/or loops or bulges. In some embodiments, it is double-stranded over at least about 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% of the length of the oligonucleotide. In some embodiments, the double-stranded oligonucleotide of the invention contains at least or up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 mismatches.

Oligonucleotides associated with the invention can be modified such as at the sugar moiety, the phosphodiester linkage, and/or the base. As used herein, "sugar moieties" includes natural, unmodified sugars, including pentose, ribose and deoxyribose, modified sugars and sugar analogs. Modifications of sugar moieties can include replacement of a hydroxyl group with a halogen, a heteroatom, or an aliphatic group, and can include functionalization of the hydroxyl group as, for example, an ether, amine or thiol.

Modification of sugar moieties can include 2'-O-methyl nucleotides, which are referred to as "methylated." In some instances, oligonucleotides associated with the invention may only contain modified or unmodified sugar moieties, while in other instances, oligonucleotides contain some sugar moieties that are modified and some that are not.

In some instances, modified nucleomonomers include sugar- or backbone-modified ribonucleotides. Modified ribonucleotides can contain a non-naturally occurring base such as uridines or cytidines modified at the 5'-position, *e.g.*, 5'-(2-amino)propyl uridine and 5'-bromo uridine; adenosines and guanosines modified at the 8-position, *e.g.*, 8-bromo guanosine; deaza nucleotides, *e.g.*, 7-deaza-adenosine; and N-alkylated nucleotides, *e.g.*, N6-methyl adenosine. Also, sugar-modified ribonucleotides can have the 2'-OH group replaced by an H, alkoxy (or OR), R or alkyl, halogen, SH, SR, amino (such as NH_2, NHR, NR_2,), or CN group, wherein R is lower alkyl, alkenyl, or alkynyl. In some embodiments, modified ribonucleotides can have the phosphodiester group connecting to adjacent ribonucleotides replaced by a modified group, such as a phosphorothioate group.

In some aspects, 2′-O-methyl modifications can be beneficial for reducing undesirable cellular stress responses, such as the interferon response to double-stranded nucleic acids. Modified sugars can include D-ribose, 2′-O-alkyl (including 2′-O-methyl and 2′-O-ethyl), *i.e.*, 2′-alkoxy, 2′-amino, 2′-S-alkyl, 2′-halo (including 2′-fluoro), 2′-methoxyethoxy, 2′-allyloxy ($-OCH_2CH=CH_2$), 2′-propargyl, 2′-propyl, ethynyl, ethenyl, propenyl, and cyano and the like. The sugar moiety can also be a hexose.

The term "alkyl" includes saturated aliphatic groups, including straight-chain alkyl groups (*e.g.*, methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, *etc.*), branched-chain alkyl groups (isopropyl, tert-butyl, isobutyl, *etc.*), cycloalkyl (alicyclic) groups (cyclopropyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl), alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. In some embodiments, a straight chain or branched chain alkyl has 6 or fewer carbon atoms in its backbone (*e.g.*, C_1-C_6 for straight chain, C_3-C_6 for branched chain), and more preferably 4 or fewer. Likewise, preferred cycloalkyls have from 3-8 carbon atoms in their ring structure, and more preferably have 5 or 6 carbons in the ring structure. The term C_1-C_6 includes alkyl groups containing 1 to 6 carbon atoms.

Unless otherwise specified, the term alkyl includes both "unsubstituted alkyls" and "substituted alkyls," the latter of which refers to alkyl moieties having independently selected substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. The term "alkenyl" includes unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double bond. Unless otherwise specified, the term alkenyl includes both "unsubstituted alkenyls" and "substituted alkenyls," the latter of which refers to alkenyl moieties having independently selected substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone.

The term "base" includes the known purine and pyrimidine heterocyclic bases, deazapurines, and analogs (including heterocyclic substituted analogs, *e.g.*, aminoethyoxy phenoxazine), derivatives (*e.g.*, 1-alkyl-, 1-alkenyl-, heteroaromatic- and 1-alkynyl derivatives) and tautomers thereof. Examples of purines include adenine, guanine, inosine, diaminopurine, and xanthine and analogs (*e.g.*, 8-oxo-N^6-methyladenine or 7-diazaxanthine)

and derivatives thereof. Pyrimidines include, for example, thymine, uracil, and cytosine, and their analogs (*e.g.*, 5-methylcytosine, 5-methyluracil, 5-(1-propynyl)uracil, 5-(1-propynyl)cytosine and 4,4-ethanocytosine). Other examples of suitable bases include non-purinyl and non-pyrimidinyl bases such as 2-aminopyridine and triazines.

The term "nucleoside" includes bases which are covalently attached to a sugar moiety, preferably ribose or deoxyribose. Examples of preferred nucleosides include ribonucleosides and deoxyribonucleosides. Nucleosides also include bases linked to amino acids or amino acid analogs which may comprise free carboxyl groups, free amino groups, or protecting groups. Suitable protecting groups are well known in the art (see P. G. M. Wuts and T. W. Greene, "Protective Groups in Organic Synthesis", 2nd Ed., Wiley-Interscience, New York, 1999).

As used herein, the term "linkage" used in the context of an internucleotide linkage includes a naturally occurring, unmodified phosphodiester moiety (-O-(PO^{2-})-O-) that covalently couples adjacent nucleomonomers. As used herein, the term "substitute linkage" or "modified linkage" or modified internucleotide linkage" includes any analog or derivative of the native phosphodiester group that covalently couples adjacent nucleomonomers. Substitute linkages include phosphodiester analogs, *e.g.*, phosphorothioate, phosphorodithioate, and P-ethyoxyphosphodiester, P-ethoxyphosphodiester, P-alkyloxyphosphotriester, methylphosphonate, and nonphosphorus containing linkages, *e.g.*, acetals and amides. Such substitute linkages are known in the art (*e.g.*, Bjergarde *et al.* 1991. Nucleic Acids Res. 19:5843; Caruthers *et al.* 1991. Nucleosides Nucleotides. 10:47). In certain embodiments, non-hydrolizable linkages are preferred, such as phosphorothioate linkages.

In some aspects, oligonucleotides of the invention comprise 3′ and 5′ termini (except for circular oligonucleotides). The 3′ and 5′ termini of a oligonucleotide can be substantially protected from nucleases, for example, by modifying the 3′ or 5′ linkages (*e.g.*, U.S. Pat. No. 5,849,902 and WO 98/13526). Oligonucleotides can be made resistant by the inclusion of a "blocking group." The term "blocking group" as used herein refers to substituents (*e.g.*, other than OH groups) that can be attached to oligonucleotides or nucleomonomers, either as protecting groups or coupling groups for synthesis (*e.g.*, FITC, propyl (CH_2-CH_2-CH_3),

glycol (-O-CH_2-CH_2-O-) phosphate (PO_3^{2-}), hydrogen phosphonate, or phosphoramidite). "Blocking groups" also include "end blocking groups" or "exonuclease blocking groups" which protect the 5′ and 3′ termini of the oligonucleotide, including modified nucleotides and non-nucleotide exonuclease resistant structures.

Exemplary end-blocking groups include cap structures (*e.g.*, a 7-methylguanosine cap), inverted nucleomonomers, *e.g.*, with 3′-3′ or 5′-5′ end inversions (see, *e.g.*, Ortiagao *et al.* 1992. *Antisense Res. Dev.* 2:129), methylphosphonate, phosphoramidite, non-nucleotide groups (*e.g.*, non-nucleotide linkers, amino linkers, conjugates) and the like. The 3′ terminal nucleomonomer can comprise a modified sugar moiety. The 3′ terminal nucleomonomer comprises a 3′-O that can optionally be substituted by a blocking group that prevents 3′-exonuclease degradation of the oligonucleotide. For example, the 3′-hydroxyl can be esterified to a nucleotide through a 3′→3′ internucleotide linkage. For example, the alkyloxy radical can be methoxy, ethoxy, or isopropoxy, and preferably, ethoxy. Optionally, the 3′→3′linked nucleotide at the 3′ terminus can be linked by a substitute linkage. To reduce nuclease degradation, the 5′ most 3′→5′ linkage can be a modified linkage, *e.g.*, a phosphorothioate or a P-alkyloxyphosphotriester linkage. Preferably, the two 5′ most 3′→5′ linkages are modified linkages. Optionally, the 5′ terminal hydroxy moiety can be esterified with a phosphorus containing moiety, *e.g.*, phosphate, phosphorothioate, or P-ethoxyphosphate.

In some aspects, oligonucleotides can be chimeric RNA-DNA oligonucleotides which include both DNA and RNA or DNA-RNA or RNA-DNA duplexes.

The oligonucleotides (including carrier oligonucleotides and/or therapeutic pharmacophores) are preferably in the range of 2 to 1000, 2-500, 2-100, 5-500, 5-100, 10-500, 10-100, 10-50, 15-500, 15-100, 15-50, 20-500, 20-100, 20-50, or 20-40 bases in length. However, nucleic acids of other sizes are useful.

In some embodiments the oligonucleotides have a modified backbone such as a phosphorothioate (PS) backbone. In other embodiments the oligonucleotides have a phosphodiester (PO) backbone. In yet other embodiments oligonucleotides have a mixed or chimeric PO and PS backbone.

In some embodiments, PEG of different sizes is incorporated into the structure to alter the in vivo properties including but not limited to sizes from 1,000 Da to 40,000 Da.

The nanostructure may also include an active agent. An active agent as used herein is a molecule capable of providing some therapeutic or diagnostic advantage to a cell or subject. Active agents include, for instance, anti-microbial agents.

Active agents can be attached to the structures by the externally-facing oligonucleotides through covalent or non-covalent, e.g. Watson/Crick hybridization. Alternatively or additionally the active agents may be incorporated into a liposomal bilayer via conjugation to a hydrophobic moiety. In yet another embodiment, active agent may be incorporated inside the inner aqueous layer of the liposome.

In one embodiment, active agent is conjugated to the liposomal nanostructure via interactions with the oligonucleotide shell. In some instances the active agent – oligonucleotide conjugate is linked to the core through oligonucleotide hybridization. In other words the oligonucleotide is hybridized to a complementary or partially complementary oligonucleotide to form a duplex or partial duplex. One or both of the oligonucleotides of the duplex is linked directly to the core and the active agent which is external facing (on the outside of the lipid bilayer) or which is internal (in the inner aqueous layer) and not directly linked to the core is linked to one or both of the oligonucleotides in the duplex. In another embodiment, active agent is conjugated to the liposomal nanostructure via direct interactions with the core. The active agent can be anchored to the surface of the liposomal core through conjugation to one or a multiplicity of linker molecules including but not limited to: tocopherols, sphingolipids such as sphingosine, sphingosine phosphate, methylated sphingosines and sphinganines, ceramides, ceramide phosphates, 1-0 acyl ceramides, dihydroceramides, 2-hydroxy ceramides, sphingomyelin, glycosylated sphingolipids, sulfatides, gangliosides, phosphosphingolipids, and phytosphingosines of various lengths and saturation states and their derivatives, phospholipids such as phosphatidylcholines, lysophosphatidylcholines, phosphatidic acids, lysophosphatidic acids, cyclic LPA, phosphatidylethanolamines, lysophosphatidylethanolamines, phosphatidylglycerols, lysophosphatidylglycerols, phosphatidylserines, lysophosphatidylserines, phosphatidylinositols, inositol phosphates,

LPI, cardiolipins, lysocardiolipins, bis(monoacylglycero) phosphates, (diacylglycero) phosphates, ether lipids, diphytanyl ether lipids, and plasmalogens of various lengths, saturation states, and their derivatives, sterols such as cholesterol, desmosterol, stigmasterol, lanosterol, lathosterol, diosgenin, sitosterol, zymosterol, zymostenol, 14-demethyl-lanosterol, cholesterol sulfate, DHEA, DHEA sulfate, 14-demethyl-14-dehydrlanosterol, sitostanol, campesterol, ether anionic lipids, ether cationic lipids, lanthanide chelating lipids, A-ring substituted oxysterols, B-ring substituted oxysterols, D-ring substituted oxysterols, side-chain substituted oxysterols, double substituted oxysterols, cholestanoic acid derivatives, fluorinated sterols, fluorescent sterols, sulfonated sterols, phosphorylated sterols, and polyunsaturated sterols of different lengths, saturation states, and their derivatives.

The invention also encompasses the use of the nanostructures for the treatment of a subject having an infectious disease. A "subject having an infectious disease" is a subject that has had contact with a microorganism. Thus the microorganism has invaded the subjects body. The word "invade" as used herein refers to contact by the microorganism with the external surface of the subject, e.g., skin or mucosal membranes and/or refers to the penetration of the external surface of the subject by the microorganism.

An "infectious disease" as used herein, refers to a disorder arising from the invasion of a host, superficially, locally, or systemically, by an infectious microorganism. Infectious microorganisms include bacteria, viruses, and fungi. Bacteria are unicellular organisms which multiply asexually by binary fission. They are classified and named based on their morphology, staining reactions, nutrition and metabolic requirements, antigenic structure, chemical composition, and genetic homology. Bacteria can be classified into three groups based on their morphological forms, spherical (coccus), straight-rod (bacillus) and curved or spiral rod (vibrio, campylobacter, spirillum, and spirochaete). Bacteria are also more commonly characterized based on their staining reactions into two classes of organisms, gram-positive and gram-negative. Gram refers to the method of staining which is commonly performed in microbiology labs. Gram-positive organisms retain the stain following the staining procedure and appear a deep violet color. Gram-negative organisms do not retain the stain but take up the counter-stain and thus appear pink.

Bacteria have two main structural components, a rigid cell wall and protoplast (material enclosed by the cell wall). The protoplast includes cytoplasm and genetic material. Surrounding the protoplast is the cytoplasmic membrane which includes some of the cell respiratory enzymes and is responsible for the permeability of bacteria and transport of many small molecular weight substances. The cell wall surrounding the cytoplasmic membrane and protoplast is composed of mucopeptides which include complex polymers of sugars cross-linked by peptide chains of amino acids. The wall is also composed of polysaccharides and teichoic acids.

Infectious bacteria include, but are not limited to, gram negative and gram positive bacteria. Gram positive bacteria include, but are not limited to *Pasteurella* species, *Staphylococci* species, and *Streptococcus* species. Gram negative bacteria include, but are not limited to, *Escherichia coli*, *Pseudomonas* species, and *Salmonella* species. Specific examples of infectious bacteria include but are not limited to: *Helicobacter pyloris*, *Borelia burgdorferi*, *Legionella pneumophilia*, *Mycobacteria sps* (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansaii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic species.), *Streptococcus pneumoniae*, pathogenic *Campylobacter sp.*, *Enterococcus sp.*, *Haemophilus influenzae*, *Bacillus antracis*, *corynebacterium diphtheriae*, *corynebacterium sp.*, *Erysipelothrix rhusiopathiae*, *Clostridium perfringers*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides sp.*, *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidium*, *Treponema pertenue*, *Leptospira*, *Rickettsia,* and *Actinomyces israelli*.

The methods of the invention involve nanostructures that optionally further include an anti-microbial agent for the treatment or prevention of infectious disease. An anti-microbial agent, as used herein, refers to a naturally-occurring or synthetic compound which is capable of killing or inhibiting infectious microorganisms. The type of anti-microbial agent useful according to the invention will depend upon the type of microorganism with which the subject is infected or at risk of becoming infected. One type of anti-microbial

agent is an antibacterial agent. Antibacterial agents kill or inhibit the growth or function of bacteria. A large class of antibacterial agents is antibiotics.

Antibiotics, which are effective for killing or inhibiting a wide range of bacteria, are referred to as broad spectrum antibiotics. Other types of antibiotics are predominantly effective against the bacteria of the class gram-positive or gram-negative. These types of antibiotics are referred to as narrow spectrum antibiotics. Other antibiotics which are effective against a single organism or disease and not against other types of bacteria, are referred to as limited spectrum antibiotics.

Antibacterial agents are sometimes classified based on their primary mode of action. In general, antibacterial agents are cell wall synthesis inhibitors, cell membrane inhibitors, protein synthesis inhibitors, nucleic acid synthesis or functional inhibitors, and competitive inhibitors. Cell wall synthesis inhibitors inhibit a step in the process of cell wall synthesis, and in general in the synthesis of bacterial peptidoglycan. Cell wall synthesis inhibitors include β-lactam antibiotics, natural penicillins, semi-synthetic penicillins, ampicillin, clavulanic acid, cephalolsporins, and bacitracin.

The β-lactams are antibiotics containing a four-membered β-lactam ring which inhibits the last step of peptidoglycan synthesis. β-lactam antibiotics can be synthesized or natural. The natural antibiotics are generally produced by two groups of fungi, *penicillium* and *cephalosporium* molds. The β-lactam antibiotics produced by *penicillium* are the natural penicillins, such as penicillin G or penicillin V. These are produced by fermentation of *penicillium chrysogenum*. The natural penicillins have a narrow spectrum of activity and are generally effective against *streptococcus*, *gonococcus*, and *staphylococcus*. Other types of natural penicillins, which are also effective against gram-positive bacteria, include penicillins F, X, K, and O.

Semi-synthetic penicillins are generally modifications of the molecule 6-aminopenicillanic acid produced by a mold. The 6-aminopenicillanic acid can be modified by addition of side chains which produce penicillins having broader spectrums of activity than natural penicillins or various other advantageous properties. Some types of semi-synthetic penicillins have broad spectrums against gram-positive and gram-negative bacteria, but are inactivated by penicillinase. These semi-synthetic penicillins include

ampicillin, carbenicillin, oxacillin, azlocillin, mezlocillin, and piperacillin. Other types of semi-synthetic penicillins have narrower activities against gram-positive bacteria, but have developed properties such that they are not inactivated by penicillinase. These include, for instance, methicillin, dicloxacillin, and nafcillin. Some of the broad spectrum semi-synthetic penicillins can be used in combination with β-lactamase inhibitors, such as clavulamic acids and sulbactam. The β-lactamase inhibitors do not have anti-microbial action but they function to inhibit penicillinase, thus protecting the semi-synthetic penicillin from degradation.

Another type of β-lactam antibiotic is the cephalolsporins. Cephalolsporins are produced by *cephalolsporium* molds, and have a similar mode of action to penicillin. They are sensitive to degradation by bacterial β-lactamases, and thus, are not always effective alone. Cephalolsporins, however, are resistant to penicillinase. They are effective against a variety of gram-positive and gram-negative bacteria. Cephalolsporins include, but are not limited to, cephalothin, cephapirin, cephalexin, cefamandole, cefaclor, cefazolin, cefuroxine, cefoxitin, cefotaxime, cefsulodin, cefetamet, cefixime, ceftriaxone, cefoperazone, ceftazidine, and moxalactam.

Bacitracin is another class of antibiotics which inhibit cell wall synthesis. These antibiotics, produced by *bacillus* species, prevent cell wall growth by inhibiting the release of muropeptide subunits or peptidoglycan from the molecule that delivers the subunit to the outside of the membrane. Although bacitracin is effective against gram-positive bacteria, its use is limited in general to topical administration because of its high toxicity. Since lower effective doses of bacitracen can be used when the compound is administered with the nanostructures of the invention, this compound can be used systemically and the toxicity reduced.

Carbapenems are another broad spectrum β-lactam antibiotic, which is capable of inhibiting cell wall synthesis. Examples of carbapenems include, but are not limited to, imipenems. Monobactems are also broad spectrum β-lactam antibiotics, and include, euztreonam. An antibiotic produced by *streptomyces*, vancomycin, is also effective against gram-positive bacteria by inhibiting cell membrane synthesis.

Another class of anti-bacterial agents is the anti-bacterial agents that are cell membrane inhibitors. These compounds disorganize the structure or inhibit the function of bacterial membranes. Alteration of the cytoplasmic membrane of bacteria results in leakage of cellular materials from the cell. Compounds that inhibit or interfere with the cell membrane cause death of the cell because the integrity of the cytoplasmic and outer membranes is vital to bacteria. One problem with anti-bacterial agents that are cell membrane inhibitors is that they can produce effects in eucaryotic cells as well as bacteria because of the similarities in phospholipids in bacterial and eukaryotic membranes. Thus these compounds are rarely specific enough to permit these compounds to be used systemically and prevent the use of high doses for local administration.

One clinically useful anti-bacterial agent that is a cell membrane inhibitor is Polymyxin, produced by Bacillus polymyxis. Polymyxins interfere with membrane function by binding to membrane phospholipids. Polymyxin is effective mainly against Gram-negative bacteria and is generally used in severe *Pseudomonas* infections or *Pseudomonas* infections that are resistant to less toxic antibiotics. It is also used in some limited instances topically. It's limited use is due to the severe side effects associated with systemic administration, such as damage to the kidney and other organs.

Other cell membrane inhibitors include Amphotericin B and Nystatin produced by the bacterium *Streptomyces* which are also anti-fungal agents, used predominantly in the treatment of systemic fungal infections and *Candida* yeast infections respectively. Imidazoles, produced by the bacterium *Streptomyces*, are another class of antibiotic that is a cell membrane inhibitor. Imidazoles are used as bacterial agents as well as anti-fungal agents, e.g., used for treatment of yeast infections, dermatophytic infections, and systemic fungal infections. Imidazoles include but are not limited to clotrimazole, miconazole, ketoconazole, itraconazole, and fluconazole.

Many anti-bacterial agents are protein synthesis inhibitors. These compounds prevent bacteria from synthesizing structural proteins and enzymes and thus cause inhibition of bacterial cell growth or function or cell death. In general these compounds interfere with the processes of transcription or translation. Anti-bacterial agents that block transcription include but are not limited to Rifampins, produced by the bacterium *Streptomyces* and

Ethambutol, a synthetic chemical. Rifampins, which inhibit the enzyme RNA polymerase, have a broad spectrum activity and are effective against gram-positive and gram-negative bacteria as well as *Mycobacterium tuberculosis.* Ethambutol is effective against *Mycobacterium tuberculosis.*

Anti-bacterial agents which block translation interfere with bacterial ribosomes to prevent mRNA from being translated into proteins. In general this class of compounds includes but is not limited to tetracyclines, chloramphenicol, the macrolides (e.g. erythromycin) and the aminoglycosides (e.g. streptomycin).

Some of these compounds bind irreversibly to the 30s ribosomal subunit and cause a misreading of the mRNA, e.g., the aminoglycosides. The aminoglycosides are a class of antibiotics which are produced by the bacterium *Streptomyces,* such as, for instance streptomycin, kanamycin, tobramycin, amikacin, and gentamicin. Aminoglycosides have been used against a wide variety of bacterial infections caused by Gram-positive and Gram-negative bacteria. Streptomycin has been used extensively as a primary drug in the treatment of *tuberculosis.* Gentamicin is used against many strains of Gram-positive and Gram-negative bacteria, including *Pseudomonas infections*, especially in combination with Tobramycin. Kanamycin is used against many Gram-positive bacteria, including penicillin-resistant *staphylococci.* One side effect of aminoglycosides that has limited their use clinically is that at dosages which are essential for efficacy, prolonged use has been shown to impair kidney function and cause damage to the auditory nerves leading to deafness.

Another type of translation inhibitor anti-bacterial agent is the tetracyclines. The tetracyclines bind reversibly to the 30s ribosomal subunit and interfere with the binding of charged tRNA to the bacterial ribosome. The tetracyclines are a class of antibiotics, produced by the bacterium *Streptomyces,* that are broad-spectrum and are effective against a variety of gram-positive and gram-negative bacteria. Examples of tetracyclines include tetracycline, minocycline, doxycycline, and chlortetracycline. They are important for the treatment of many types of bacteria but are particularly important in the treatment of Lyme disease.

Anti-bacterial agents such as the macrolides bind reversibly to the 50s ribosomal subunit and inhibits elongation of the protein by peptidyl transferase or prevents the release

of uncharged tRNA from the bacterial ribosome or both. The macrolides contain large lactone rings linked through glycoside bonds with amino sugars. These compounds include erythromycin, roxithromycin, clarithromycin, oleandomycin, and azithromycin. Erythromycin is active against most Gram-positive bacteria, *Neisseria*, *Legionella* and *Haemophilus*, but not against the *Enterobacteriaceae*. Lincomycin and clindamycin, which block peptide bond formation during protein synthesis, are used against gram-positive bacteria.

Another type of translation inhibitor is chloramphenicol. Chloramphenicol binds the 70S ribosome inhibiting the bacterial enzyme peptidyl transferase thereby preventing the growth of the polypeptide chain during protein synthesis. Chloramphenicol can be prepared from Streptomyces or produced entirely by chemical synthesis. One serious side effect associated with chloramphenicol is aplastic anemia. Aplastic anemia develops at doses of chloramphenicol which are effective for treating bacteria in a small proportion (1/50,000) of patients. Chloramphenicol which was once a highly prescribed antibiotic is now seldom uses as a result of the deaths from anemia. Because of its effectiveness it is still used in life-threatening situations (e.g. typhoid fever). By combining chloramphenicol with the nanostructures of the invention these compounds can again be used as anti-bacterial agents because nanostructures allow a lower dose of the chloramphenicol to be used, a dose which may avoid side effects.

Some anti-bacterial agents disrupt nucleic acid synthesis or function, e.g., bind to DNA or RNA so that their messages cannot be read. These include but are not limited to quinolones and co-trimoxazole, both synthetic chemicals and rifamycins, a natural or semi-synthetic chemical. The quinolones block bacterial DNA replication by inhibiting the DNA gyrase, the enzyme needed by bacteria to produce their circular DNA. They are broad spectrum and examples include norfloxacin, ciprofloxacin, enoxacin, nalidixic acid and temafloxacin. Nalidixic acid is a bactericidal agent that binds to the DNA gyrase enzyme (topoisomerase) which is essential for DNA replication and allows supercoils to be relaxed and reformed, inhibiting DNA gyrase activity. The main use of nalidixic acid is in treatment of lower urinary tract infections (UTI) because it is effective against several types of Gram-negative bacteria such as *E. coli*, *Enterobacter aerogenes*, *K. pneumoniae* and *Proteus*

species which are common causes of UTI. Co-trimoxazole is a combination of sulfamethoxazole and trimethoprim, which blocks the bacterial synthesis of folic acid needed to make DNA nucleotides. Rifampicin is a derivative of rifamycin that is active against Gram-positive bacteria (including *Mycobacterium tuberculosis* and meningitis caused by *Neisseria meningitidis*) and some Gram-negative bacteria. Rifampicin binds to the beta subunit of the polymerase and blocks the addition of the first nucleotide which is necessary to activate the polymerase, thereby blocking mRNA synthesis.

Another class of anti-bacterial agents is compounds that function as competitive inhibitors of bacterial enzymes. The competitive inhibitors are mostly all structurally similar to a bacterial growth factor and compete for binding but do not perform the metabolic function in the cell. These compounds include sulfonamides and chemically modified forms of sulfanilamide which have even higher and broader antibacterial activity. The sulfonamides (e.g. gantrisin and trimethoprim) are useful for the treatment of *Streptococcus pneumoniae*, beta-hemolytic *streptococci* and *E. coli*, and have been used in the treatment of uncomplicated UTI caused by E. coli, and in the treatment of meningococcal meningitis.

A major problem associated with anti-microbial drug resistance is that the particular anti-microbial agent is then useless in the treatment of the infection by the microorganism. As this resistance develops, additional therapies need to be identified or the infection, which was once manageable will become serious and untreatable.

The nanostructures of the invention are useful for the prevention of anti-microbial resistance. When the nanostructures are administered optionally with an anti-microbial agent resistant strains may be prevented from developing. The nanostructures may be administered before, at the same time as, or after the anti-microbial agent as long as it is within a time period that is sufficient to effectively treat the disease or prevent the drug resistance. Preferably, the anti-microbial is incorporated as part of the nanostructure.

The nanostructures of the invention may also be coated with or administered in conjunction with an anti-microbial agent. An anti-microbial agent, as used herein, refers to a naturally-occurring or synthetic compound which is capable of killing or inhibiting infectious microorganisms. The type of anti-microbial agent useful according to the

invention will depend upon the type of microorganism with which the subject is infected or at risk of becoming infected.

The nanostructure can be combined with other therapeutic agents. The nanostructure and/or other therapeutic agent may be administered simultaneously or sequentially. When the other therapeutic agents are administered simultaneously they can be administered in the same or separate formulations, but are administered at the same time. The other therapeutic agents are administered sequentially with one another and with the nanostructure anti-microbial agent, when the administration of the other therapeutic agents and the nanostructure and anti-microbial agent is temporally separated. The separation in time between the administration of these compounds may be a matter of minutes or it may be longer.

The term "effective amount" of an nanostructure refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of an nanostructure for treating or preventing infectious disease is that amount necessary to prevent the infection with the microorganism if the subject is not yet infected or is that amount necessary to prevent an increase in infected cells or microorganisms present in the subject or that amount necessary to decrease the amount of the infection that would otherwise occur in the absence of the nanostructure. Combined with the teachings provided herein, by choosing among the various active compounds and weighing factors such as potency, relative bioavailability, patient body weight, severity of adverse side-effects and preferred mode of administration, an effective prophylactic or therapeutic treatment regimen can be planned which does not cause substantial toxicity and yet is entirely effective to treat the particular subject. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular nanostructure being administered the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular nanostructure without necessitating undue experimentation.

Subject doses of the compounds described herein typically range from about 0.1 μg to 10,000 mg, more typically from about 1 μg/day to 8000 mg, and most typically from about 10 μg to 100 μg. Stated in terms of subject body weight, typical dosages range from

about 0.1 μg to 20 mg/kg/day, more typically from about 1 to 10 mg/kg/day, and most typically from about 1 to 5 mg/kg/day.

In some instances, the nanostructure is administered with a sub-therapeutic dosage of an anti-microbial agent. When the two classes of drugs are used together, they may be administered in sub-therapeutic doses in order to produce a desirable therapeutic result. A "sub-therapeutic dose" as used herein refers to a dosage which is less than that dosage which would produce a therapeutic result in the subject. Thus, the sub-therapeutic dose of an anti-microbial agent is one which would not produce the desired therapeutic result in the subject in the absence of the nanostructure. Therapeutic doses of anti-microbial agent are well known in the field of medicine for the treatment of infectious disease. These dosages have been extensively described in references such as Remington's Pharmaceutical Sciences, 18th ed., 1990; as well as many other medical references relied upon by the medical profession as guidance for the treatment of infectious disease.

The nanostructures of the invention may be delivered to a subject *in vivo or ex vivo* for therapeutic and/or diagnostic use or may be used *in vitro, ex vivo* or *in vivo* for research purposes. The nanostructures may be administered alone or in any appropriate pharmaceutical carrier, such as a liquid, for example saline, or a powder, for administration *in vivo*. They can also be co-delivered with larger carrier particles or within administration devices. The nanostructures may be formulated or unformulated. The formulations of the invention can be administered in pharmaceutically acceptable solutions, which may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, adjuvants, and optionally other therapeutic ingredients. In some embodiments, nanostructures are mixed with a substance such as a lotion (for example, aquaphor) and are administered to the skin of a subject, whereby the nanostructures are delivered through the skin of the subject. The nanostructures may also be sterile.

In other embodiments of the invention, the nanostructure is administered on a routine schedule. A "routine schedule" as used herein, refers to a predetermined designated period of time. The routine schedule may encompass periods of time which are identical or which differ in length, as long as the schedule is predetermined. For instance, the routine schedule

may involve administration of the nanostructure on a daily basis, every two days, every three days, every four days, every five days, every six days, a weekly basis, a monthly basis or any set number of days or weeks there-between, every two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, twelve months, etc. Alternatively, the predetermined routine schedule may involve administration of the nanostructure on a daily basis for the first week, followed by a monthly basis for several months, and then every three months after that. Any particular combination would be covered by the routine schedule as long as it is determined ahead of time that the appropriate schedule involves administration on a certain day.

For use in therapy, an effective amount of the nanostructures can be administered to a subject by any mode that delivers the nanostructures to the desired cell. Administering pharmaceutical compositions may be accomplished by any means known to the skilled artisan. Routes of administration include but are not limited to oral, parenteral, intramuscular, intravenous, subcutaneous, mucosal, intranasal, sublingual, intratracheal, inhalation, ocular, vaginal, dermal, rectal, and by direct injection.

A subject shall mean a human or vertebrate animal including but not limited to a dog, cat, horse, cow, pig, sheep, goat, turkey, chicken, primate, e.g., monkey, and fish (aquaculture species), e.g. salmon. Thus, the invention can also be used to treat infections in non-human subjects.

As used herein, the term treat, treated, or treating when used with respect to an disorder such as an infectious disease refers to a prophylactic treatment which increases the resistance of a subject to development of the disease (e.g., to infection with a bacteria) or, in other words, decreases the likelihood that the subject will develop the disease (e.g., become infected with the bacteria) as well as a treatment after the subject has developed the disease in order to fight the disease (e.g., reduce or eliminate the bacteria) or prevent the disease from becoming worse.

In another aspect, the present invention is directed to a kit including one or more of the compositions previously discussed. A "kit," as used herein, typically defines a package or an assembly including one or more of the compositions of the invention, and/or other compositions associated with the invention, for example, as previously described. Each of

the compositions of the kit, if present, may be provided in liquid form (e.g., in solution), or in solid form (e.g., a dried powder). In certain cases, some of the compositions may be constitutable or otherwise processable (e.g., to an active form), for example, by the addition of a suitable solvent or other species, which may or may not be provided with the kit. Examples of other compositions that may be associated with the invention include, but are not limited to, solvents, surfactants, diluents, salts, buffers, emulsifiers, chelating agents, fillers, antioxidants, binding agents, bulking agents, preservatives, drying agents, antimicrobials, needles, syringes, packaging materials, tubes, bottles, flasks, beakers, dishes, frits, filters, rings, clamps, wraps, patches, containers, tapes, adhesives, and the like, for example, for using, administering, modifying, assembling, storing, packaging, preparing, mixing, diluting, and/or preserving the compositions components for a particular use, for example, to a sample and/or a subject.

In some embodiments, a kit associated with the invention includes one or more components of the nanostructure. For instance the kit may include liposomes for forming a liposome core or a metal for forming a solid core, and or carrier or therapeutic oligonucleotides for the exterior of the nanostructure. A kit can also include one or more anti-microbials and or other therapeutic agents.

A kit of the invention may, in some cases, include instructions in any form that are provided in connection with the compositions of the invention in such a manner that one of ordinary skill in the art would recognize that the instructions are to be associated with the compositions of the invention. For instance, the instructions may include instructions for the use, modification, mixing, diluting, preserving, administering, assembly, storage, packaging, and/or preparation of the compositions and/or other compositions associated with the kit. In some cases, the instructions may also include instructions for the use of the compositions, for example, for a particular use, e.g., to a sample. The instructions may be provided in any form recognizable by one of ordinary skill in the art as a suitable vehicle for containing such instructions, for example, written or published, verbal, audible (e.g., telephonic), digital, optical, visual (e.g., videotape, DVD, etc.) or electronic communications (including Internet or web-based communications), provided in any manner.

Examples

In order that the invention described herein may be more fully understood, the following examples are set forth. The examples described in this application are offered to illustrate the compounds, pharmaceutical compositions, and methods provided herein and are not to be construed in any way as limiting their scope.

Example 1. AuNP SNA Synthesis

Gold nanoparticles were synthesized by initially dissolving gold chloride trihydrate and trisodium citrate separately into nanopure water. The gold chloride trihydrate solution was then added to a larger volume of nanopure water while stirring the mixture using a stirrer plate. After waiting for this solution to reflux rapidly, trisodium citrate solution was added to the reaction mixture. Upon addition of this solution, the reaction mixture changed color from clear to black and then red. Color change is an indication of a successful reaction. The solution was allowed to stir and reflux for 30 minutes with heat before it was allowed to cool down. After the solution cools down, the gold nanoparticles were filtered using a 0.45 μm filter paper and a UV-vis reading was taken to verify the size of the nanoparticles to be 13 nm.

For functionalization of these gold nanoparticles with oligonucleotides and peptide PNA, a surfactant (10% Tween-20) was added at 0.01% volume/volume to the desired volume of gold nanoparticles. The next step was adding phosphate buffer (pH 7.4), which was followed by addition of carrier phosphodiester oligonucleotides of sequence 5'-AAAAAAAAAAGAGTATGAGAA -3' ((SEQ ID NO: 20); acpP) that were 5' modified with thiol. The oligonucleotides were added up to 500 fold excess of the gold nanoparticle amount. This was followed by addition of 300 mM salt and overnight incubation at room temperature to allow for conjugation of oligonucleotides to the gold surface. The following day, particles were washed and peptide-PNA was added in varying ratios to the number of oligonucleotide strands present on the gold core during the formulation process. The PNA sequence was complementary to the carrier oligonucleotide and antisense to the acyl carrier protein P (acpP) gene in E. coli and is coupled via a glycol linkage to the KFFKFFKFFK

(SEQ ID NO: 1) cell penetrating peptide (5'-3' or N-C: KFFKFFKFFK(SEQ ID NO: 1)-O-CTCATACTCT(SEQ ID NO: 6)). The number of oligonucleotide strands per gold nanoparticle was measured using a characterization method developed previously, wherein a fluorescent dye was used to bind to the oligonucleotide bases, dye was excited and a measurement of emission predicted the oligonucleotide concentration. After addition of varying amounts of peptide PNA, the functionalized gold nanoparticles were allowed to incubate overnight at room temperature again. The following day, functionalized SNAs were washed with water after which they were ready to treat bacteria.

PEG of different molecular weights may be incorporated into the structure, PEG of average molecular weight ranging from 1000 Da to 40,000 Da were added to the gold nanoparticles at varying concentrations after addition of the surfactant. The gold nanoparticles were then allowed to incubate at room temperature for 2 hours before addition of phosphate buffer (pH 7.4), carrier oligonucleotide and peptide-PNA as described above.

Example 2. Liposome SNA Synthesis

Liposomes were synthesized using 1,2-Dioleoyl-sn-glycero-3-Phosphatidylcholine (DOPC) via an extrusion process which utilized filtration to obtain a relatively monodisperse population of particles. DOPC was first suspended in dichloromethane (DCM) as a solvent. The DCM was then evaporated under a nitrogen line while rotating the glass vial containing the mixture. This allowed for the deposition of a thin DOPC film on the walls of the vial. The container was then lyophilized overnight to remove any remaining moisture or solvent. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and sodium chloride buffer was used to resuspend the dried DOPC. The contents were then subjected to sonication and vortexing until the DOPC was completely resuspended in the buffer solution. This solution was then freeze-fractured using liquid nitrogen via shell freezing method multiple times, allowing for room-temperature thaws between freezing. After freeze-fracture, the particles were then subjected to extrusion and filtration through a 100 nm filter followed by progressively smaller filters up to, but not limited to, 20 nm in size. Liposomes were stored at 4 °C following extrusion and filtration, until addition and functionalization with oligonucleotides and peptide-PNAs.

Carrier phosphodiester oligonucleotides of the sequence 5'-AAAAAAAAAAATGAGGAGAAT -3' (SEQ ID NO: 21) were 5' modified with thiol and coupled to a reactive 1,2-Dimyristoyl-sn-Glycero-3-Phosphoethanolamine-polyethylene glycol-maleimide (DMPE-PEG-Mal) molecule. The 5' thiol and maleimide form a stable bond which then allows for the functionalization of the liposome with oligonucleotide via the incorporation of hydrophobic phospholipid, DMPE, into the liposomal structure. A peptide-peptide nucleic acid (peptide-PNA or pPNA) of the composition (5'-3' or N-C: KFFKFFKFFK(SEQ ID NO: 1)-O-ATTCTCCTCAT(SEQ ID NO: 15)) was Watson-Crick hybridized to this coupled oligonucleotide via duplex or triplex, and the combined hybrid was then added to liposomes in aqueous suspension. The cell penetrating peptide KFFKFFKFFK (SEQ ID NO: 1) was selected based on its ability to facilitate PNA function from a screen of peptides. In this embodiment reduced to practice, the PNA sequence is antisense to the carrier oligonucleotide and antisense to the acyl carrier protein P (acpP) gene in A. baumannii, which was classified as an essential gene for bacterial growth. The PNA sequence was selected based on its ability to downregulate expression of the acpP gene in the bacteria A. baumannii.

The PEG at 5,000 Da in molecular weight was added. It should be noted that hybridization of the pPNAs to the oligonucleotide may be conducted before or after functionalization of the liposome.

Example 3. In Vitro Testing

It was demonstrated herein that the nanostructure of the invention was a more effective antibiotic agent against Escherica coli than the independent components alone. The activity of peptide-PNAs functionalized on the gold core was explored (peptide-PNA SNAs, Figure 1). The peptide-PNA was either (Figure 1A) co-surface functionalized using a thiol group at the end of the peptide-PNA (co-loading model) or (Figure 1B) hybridized to a complementary oligo for the peptide-PNA without a thiol group at the end (hybridization model).

The co-loading model involves peptide-PNAs (therapeutic pharmacophores) and stabilizing oligonucleotides (carrier oligonucleotides) both conjugated directly to the gold

core. In this particular case, the 13nm gold core was functionalized with stabilizing oligos with phosphodiester linkages (PO) and densely functionalized with peptide-PNAs that had a thiol group at the end for direct conjugation with the gold core that targeted the *acpP* gene (see Table 1 for sequences). In the hybridization model peptide-PNA was loaded on the gold core via hybridization to a complementary carrier oligo. A two-step process was used to build these constructs. First, the 13nm gold core was functionalized with an oligo that was complementary to the PNA portion of the peptide-PNA. Based on the loading of the oligo, peptide-PNA was added in different fold excess of the carrier oligo loading to achieve maximum loading density and 100% hybridization of the peptide-PNA. Addition of a PEG molecule was also proposed to increase the stability and circulation time of the SNA construct.

Table 1: Sequences of oligonucleotides and peptides used in these experiments.

Name	Type	Sequence (5'-3' or N-C)
Peptide-PNA	CPP+AS	KFFKFFKFFK(SEQ ID NO: 1)-O-CTCATACTCT(SEQ ID NO: 6)
CTL Peptide-PNA	CPP+AS	KFFKFFKFFK(SEQ ID NO: 1)-O-ATTCTCCTCAT(SEQ ID NO: 15)
Complementary oligo	AS	AAAAAAAAAAGAGTATGAGAA (SEQ ID NO: 20)
CTL complementary oligo	AS	AAAAAAAAAAATGAGGAGAAT (SEQ ID NO: 21)

Notes: AS = antisense oligo, CPP = cell penetrating peptide. The Peptide-PNA sequences used for co-loading are modified with a -SH (thiol) group at the 3' end/C terminal. The complementary oligo was modified with one spacer 18 molecule and a 5' thiol group to conjugate to the gold core.

During preliminary experiments, the hybridized model SNAs appeared to be more stable compared to the other co-surface loaded orientation under the conditions tested. It is possible that the slightly positive charge of the peptide-PNA reduces the nanoparticle's colloidal stability by reducing the electrostatic charge density. As a result, the latter SNA construct was chosen for further experimentation to evaluate antibacterial activity.

The antibacterial efficacy of the peptide-PNA SNAs was assessed using the dose-response behavior in preventing the growth of Escherichia coli. The results of testing

antibacterial efficacy of Peptide-PNA SNA construct (hybridization model) with KFF as the CPP and PNA oligomer targeted towards the *acpP* gene are shown in Figure 2. Different SNA formulations have different fold excess of peptide-PNA added during synthesis, for example, a 1:1 and 2:1 ratio of peptide-PNA to oligo, to ensure maximum loading of peptide-PNA by formation of PNA/DNA duplex. The negative control was a SNA construct with similar structure, with KFF as the CPP but scrambled PNA oligomer, not targeted towards any *Escherichia coli* gene. The targeted SNA constructs, control SNA construct and free-peptide-PNA were treated to *Escherichia coli K-12* (A) and *Escherichia coli DH5a* (B). The data are expressed in terms of estimated peptide-PNA concentration, assuming there was 100% hybridization of peptide PNA added to the complementary oligo. The results show in this case that Peptide-PNAs were able to achieve complete growth inhibition of bacteria at higher concentrations of Peptide-PNA.

We also sought to explore the ability to target different strains of the same pathogen. The peptide-PNA SNA construct has increased antimicrobial activity at higher concentrations which were significantly below the concentration at which the free peptide-PNA demonstrates comparable activity. Complete growth inhibition was also achieved at highest SNA concentration for the construct with peptide-PNA to carrier oligonucleotide loading ratio of 2:1.

To further increase our understanding of the mechanism of action of these constructs, as well as confirming their activity through orthogonal means, a colony forming unit (CFU) assay was performed. Figure 3 shows the data for antibacterial efficacy of Peptide-PNA SNA construct (hybridization model) with KFF as the CPP and PNA oligomer targeted towards the *acpP* gene. The bacteria treated with highest concentrations of peptide-PNA SNA construct, negative control and free peptide-PNA (Figure 2) as well as the untreated bacteria were diluted to various dilutions and plated on Tryptic Soy Broth (TSB) agar plates. The TSB agar plates incubated overnight at 37C and the number of colony forming units (CFU) were counted manually the following day. The resulting CFU count was plotted as a comparison to the untreated cells to show the growth inhibition. The CFU assay was performed for treated and untreated *Escherichia coli K-12* (A) and *Escherichia coli DH5a* (B). The data are expressed in terms of colony forming units counted per volume of the

bacterial cells. The results of this assay confirm increased antibacterial activity of the targeted peptide-PNA SNA construct as compared to the negative control and the free peptide-PNA.

The data show (Figure 2-3) that (1) peptide-PNA SNA constructs are effective as antibacterial constructs, (2) loading of peptide-PNA appeared to clearly have an improved impact on the antibacterial efficacy of the construct and (3) both the bacterial turbidity assay and the colony forming unit assay show similar outcomes. These data confirm the activity of the proposed construct and validate the results. It was also clear from these results that the effect was mediated by activity stemming from interactions with the targeted mRNA, since the control SNA construct has decreased activity as compared to the proposed targeted SNA construct.

Once the structure was optimized for stability, the SNA were functionalized with increasing density of peptide-PNA hybridized to a complementary oligo (see Table 1 for sequences) and their antimicrobial activity was assessed by treating Escherichia coli. Figure 4 demonstrates the antibacterial efficacy of Peptide-PNA SNAs (hybridization model) with KFF as the cell penetrating peptide and PNA oligomers targeted towards the *acpP* gene. Different SNA formulations have different fold excess of peptide-PNA added during synthesis (1:1, 2:1, 3:1 and 4:1 ratio of peptide-PNA to oligo) to ensure maximum loading of peptide-PNA by formation of PNA/DNA duplex. The control is peptide-PNAs hybridized to the oligos, not functionalized on the gold core, to establish the role of gold core as a carrier of the peptide-PNA into the bacterial cell. The targeted SNA constructs and control construct were tested against *Escherichia coli* K-12. (A) The bacterial turbidity assay data are expressed in terms of oligo concentration, based on the oligo loading that was identified using a fluorescence assay. The results show that Peptide-PNAs were able to achieve complete growth inhibition of bacteria at higher concentrations of Peptide-PNA for lower peptide-PNA densities. But in case of higher peptide-PNA density, complete growth inhibition was observed at even lower SNA concentrations. (B) Results of turbidity assay were confirmed by the colony forming unit (CFU) viability assay. The bacteria treated with highest concentrations of peptide-PNA SNA construct and the untreated bacteria were diluted to various dilutions and plated on Tryptic Soy Broth (TSB) agar plates. The TSB

agar plates incubated overnight at 37C and the number of colony forming units (CFU) were counted manually the following day. The resulting CFU count was plotted as a comparison to the untreated cells to show the growth inhibition. The results confirm the increased antibacterial effect of peptide-PNA SNA constructs with higher density of peptide-PNA. The data are expressed in terms of colony forming units counted per volume of the bacterial cells.

The peptide-PNA SNA constructs lead to complete growth inhibition at concentrations which were significantly below the concentration at which the free peptide-PNA demonstrates comparable activity. Complete growth inhibition was also achieved at all SNA concentrations for the construct with peptide-PNA to carrier oligonucleotide loading of 4:1. The liposomal SNAs were further shown to be active against the pathogenic organism Acinetobacter baumannii as shown in Figure 5.

In Figure 5 time-dependent antimicrobial efficacy of liposomes functionalized with oligonucleotides were examined. The oligonucleotides were hybridized to peptide-peptide nucleic acids (pPNAs) via Watson-Crick hybridization in either duplex or triplex conformations. Each pPNA sequence is antisense to the acyl carrier protein in *A. baumannii*. The oligonucleotides were anchored into the surface of the liposomes via a conjugation to a strand of polyethylene glycol that was conjugated to the phospholipid 2-Dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE). The liposomes were administered to the bacteria on a 96-well plate, using a serial dilution, single-time dosage approach. (A) Results indicate the percentage population as related to untreated cells at t = 4 hours, with the liposomal pPNAs compared to free pPNAs. (B) represents the data at t = 7 hours. Due to the single dosage approach, the bacterial population that was not eradicated with the first dose was able to replenish itself. Higher dosages indicate a prolonged therapeutic effect, with populations remaining relatively low.

In Figure 6 comparison of liposomal-SNAs to free peptide-peptide nucleic acids (pPNAs) and gold-core spherical nucleic acids functionalized with oligonucleotides were made. The oligonucleotides were hybridized to pPNAs via Watson-Crick hybridization in either duplex or triplex conformations. Each pPNA sequence was antisense to the acyl carrier protein in *A. baumannii* or *E. coli*. The oligonucleotides were anchored into the

surface of the liposomes via a conjugation to a strand of polyethylene glycol that is conjugated to the phospholipid 2-Dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE). The liposomes were administered to the bacteria on a 96-well plate, using a serial dilution, single-time dosage approach. (A) Results indicate the percentage population as related to untreated cells after incubation overnight, with the liposomal pPNAs compared to free pPNAs.

Antibacterial efficacy for the general structures of the SNAs shown in Figure 7 was tested. The structure of the co-loading model where peptide-PNAs and stabilizing oligonucleotides are both conjugated directly to the gold core via thiol linkage is shown in Figure 7A. The antibacterial efficacy of Peptide-PNA SNAs (co-loading model with a non-cleavable linker) with KFF as the cell penetrating peptide and PNA oligomers targeted towards the *acpP* gene is provided in the graph of Figure 7A. The stabilizing oligos have phosphodiester linkages (PO) and are targeted/non-targeted oligos towards the *acpP* gene (see Table 1 for sequences). The control is free peptide-PNA not conjugated to the gold core, to establish the role of the SNA contrsuct as a carrier of the peptide-PNA into the bacterial cell. The targeted and control SNA constructs were tested against *Escherichia coli* K-12. The bacterial turbidity assay data are expressed in terms of Peptide PNA concentration, based on the Peptide PNA loaded during functionalization. The results show that free Peptide-PNAs are able to achieve complete growth inhibition of bacteria but co-loaded SNA constructs having non-cleavable linkers do not achieve much growth inhibition at different SNA concentrations.

The structure of peptide-PNA loaded on the gold core via hybridization to a complementary oligo (hybridization model) is shown in Figure 7B. The structure also contains PEG molecules to increase the stability and circulation time of the SNA construct. Antibacterial efficacy of Peptide-PNA SNAs (hybridization model) with KFF as the cell penetrating peptide and PNA oligomers targeted towards the *acpP* gene is shown in the graph of Figure 7B. Different SNA formulations have different fold excess of peptide-PNA added during synthesis (1:1, 2:1, 3:1 and 4:1 ratio of peptide-PNA to oligo) to ensure maximum loading of peptide-PNA by formation of PNA/DNA duplex. The control is peptide-PNAs hybridized to the oligos, not functionalized on the gold core, to establish the

role of gold core as a carrier of the peptide-PNA into the bacterial cell. The targeted SNA constructs and control construct were tested against *Escherichia coli* K-12. The bacterial turbidity assay data are expressed in terms of oligo concentration, based on the oligo loading that was identified using a fluorescence assay. The results show that Peptide-PNAs are able to achieve complete growth inhibition of bacteria only at higher concentrations of Peptide-PNA for lower peptide-PNA densities. But in the case of higher peptide-PNA density, complete growth inhibition is observed at even lower SNA concentrations.

A study was conducted to compare the antibacterial efficacy of SNAs of the invention with SNAs lacking a carrier oligonucleotide and peptide nucleic acid. The data is shown in Figure 8. SNAs were prepared to target either the acyl carrier protein gene (acpP) using the indicated chemistries and targets: T1=TTCTTCGATAGTGCTCAT (SEQ ID NO : 2), T2=CATACTCTTAAATTTCCT (SEQ ID NO: 3), T3=TAGTGCTCATACTCTT (SEQ ID NO: 4). PO=all phosphodiester backbone, 2'OMe=2'OMe modifications to all bases, G/PS=Gap-mer with 18 total bases, from 5' to 3' containing six 2'OMe modified bases followed by six 2'deoxy nucleobases followed by six 2'OMe modified bases (all linked by phosphorothioate backbone). These SNAs were prepared as known in the art, see for example Giljohann D et al. Nano Letters 2007 7:3818-3821, and/or Patel P et al. Proc. Natl. Acad. Sci. USA. 2008 105:17222-17226. with oligonucleotides arranged around a gold core without incorporating the specific chemical changes that are shown to be important in this application.

An increase in optical density at 900 nm following overnight incubation with an inoculum of $1x10^4$ bacteria per well at SNA = 100 nM (approximate oligonucleotide concentration = 10 uM, PBS = 0.7676) was observed in Figure 8A. CFU/mL measurement obtained via plating the cells treated as indicated overnight with SNAs at 100 nM (approximate oligonucleotide concentration = 10 uM) from part A is shown in Figure 8B. The data show that independent of sequence and chemistry, SNAs lacking the carrier oligonucleotide and also lacking a PNA have significantly lower antibacterial efficacy than SNAs which utilize a carrier oligonucleotide. Using the SNAs of the invention it is possible to gain greater than 16X increase in potency.

Example 4. Design and Synthesis of BT-Ag-SNAs

One of the challenges in using silver-based antimicrobials, and silver nanoparticles in particular, is unwanted toxicity toward host cells.[20] Therefore, in order to increase the therapeutic index, pharmacokinetic properties and stability of the Ag-NPs, a common strategy has been to coat or functionalize Ag-NPs with various materials, such as chitosan.[21] While chitosan-coated silver nanoparticles demonstrate reduced toxicity, chitosan, as a positively-charged polymer, is subject to biofouling and low colloidal stability *in vivo*.[22]

Surprisingly a novel stable, negatively-charged formulation of colloidal silver that can resist protein biofouling has been discovered according to the invention. To increase the biocompatibility of these Ag-SNAs and reduce protein biofouling *in vivo*, we co-functionalized poly(ethylene glycol) (PEG) strands on the colloidal silver. As depicted in Figure 9, Ag-SNAs were synthesized through a simple stepwise addition of thiol-PEG followed by a large fold-excess of thiol-oligonucleotides, (either with natural phosphodiester (PO), or modified phosphorothioate (PS) internucleoside linkages), to Ag-NPs. Pre-treating the oligonucleotide to be loaded onto SNA with dithiothreitol (DTT) allowed us to increase the binding availability of oligonucleotides to Ag-NPs. Notably, this method produced greater yields of Ag-SNAs while using less oligonucleotide as compared with prior work.[13] BT-Ag-SNAs used in the experiments contained approximately 45 oligonucleotide strands per particle. In addition, our synthesis allows for the formulation of Ag-SNAs with variable oligonucleotide backbone chemistries while potentially improving particle stability.

Example 5. Particle Characterization

To verify the successful functionalization of Ag-NPs, we evaluated particle size and charge potential prior to *in vitro* and *in vivo* experimentation**.** Transmission electron microscopy (TEM) size analysis was used to determine the mean and distribution of size present in the Ag-SNAs and Ag-NPs used in these experiments (Figure 10A, B). Ag-NPs were not stabilized with capping agents and were suspended in H_2O. Using ImageJ software to evaluate particle diameter in the TEM images, the size of the Ag-NPs was found to be 25.8 ± 4.8 nm. The functionalized Ag-SNAs were measured to have a mean diameter of 19.7 ± 4.1 nm. In addition, our DLS analysis (Figure 10C) yielded diameter mean averages of 26.26 ± 3.49 nm (Ag-SNAs) and 21.94 ± 0.54 nm (Ag-NPs). Zeta potential measurements (Figure 10D) indicated the presence of negatively charged oligonucleotides on Ag-SNAs,

with a shift in potential from -2.04 mV to -16 mV after particle functionalization with oligonucleotides.

Example 6. BT-Ag-SNAs Antibacterial Activity *In Vitro*

Cultures of *A. baumannii* AYE, *A. baumannii* UNT086-1, and MRSA were each challenged with variations of BT-Ag-SNAs, Ag-NPs, and unconjugated oligonucleotides. Two groups of BT-Ag-SNAs were utilized: particles with phosphorothioate (PS) oligonucleotides and particles with phosphodiester (PO) oligonucleotides. The comparison of PS and PO allowed us to observe any potential influence of oligonucleotide backbone chemistry on particle efficacy. Figure 11A-C shows the growth curves of *A. baumannii* AYE, *A. baumannii* UNT086-1, and MRSA as detected via optical density using a 96-well plate reader at 900 nm. While all *A. baumannii*-targeted PS Ag-SNAs exhibited a 2-fold increase in efficacy over PO Ag-SNAs, both SNA groups had significantly lower MICs as compared to bare Ag-NPs. The differences in efficacy may be due to the enhanced stability and nuclease resistance of PS oligonucleotides or due to enhanced protein binding.[25] Interestingly, PS and PO Ag-SNAs displayed similar activity against MRSA. It is essential to note that neither PS nor PO unconjugated oligonucleotide displayed any activity when used alone against the bacterial cells. Our data, in conjunction with the low amount of oligonucleotide delivered at MIC, indicates that the SNA construct, and not the oligonucleotide strands alone, is largely responsible for the antimicrobial efficacy.

Figure 11D displays a time-dependent CFU kill curve of *A. baumannii* UNT086-1 challenged with BT-Ag-SNAs, Ag-NPs, and a gentamicin control. BT-Ag-SNAs effectively eliminated the bacteria after 5 hours of incubation, with no detectable growth via CFU by the 7-hour mark. In contrast, Ag-NPs did not manage to eliminate the bacteria by the ending time point of the experiment and were slower to treat the infection than the Ag-SNAs. Overall, a marked gap in efficacy existed between BT-Ag-SNAs and Ag-NPs, particularly in the case of the DR Gram-negative bacteria. This is a clear indication that the SNA construct is essential to improving the functionality of silver nanoparticles as an antimicrobial.

Cultures of P. aeruginosa and E.coli K-12 were each challenged with variations of BT-Ag-SNAs, Ag-NPs, and unconjugated oligonucleotides. Figure 16A-B shows the growth curves of P. aeruginosa and E.coli K-12 as detected via optical density using a 96-well plate

reader at 900 nm. While P. aeruginosa -targeted Ag-SNAs exhibited an 8-fold increase in efficacy over Ag-NPs, E.coli K-12-targeted Ag-SNAs exhibited a 16-fold increase in efficacy over Ag-NPs. PS unconjugated oligonucleotide failed to display any activity when used alone against the bacterial cells. Additionally, an Adenosine triphosphate (ATP) assay was also performed to verify the results of the optical density assay. Figure 16C indicates the results of the ATP assay, where in E.coli K-12-targeted Ag-SNAs again exhibit a 16-fold increase in efficacy over Ag-NPs. Observing similar data from different anti-bacterial assays reiterates and validates the results observed. Overall, a significant difference in efficacy existed between BT-Ag-SNAs and Ag-NPs, particularly in the case of the DR Gram-negative bacteria. In addition to the results observed earlier, this set of data verifies the broad spectrum activity of Ag-SNAs by treating a variety of bacterial strains with greatly improved efficacy as compared to Ag-NPs.

Example 7. BT-Ag-SNAs Target and Interact with Bacterial Cell Membranes

Next, we sought to understand how the BT-Ag-SNAs interacted with bacteria through microscopy techniques. To track the association of BT-Ag-SNAs with bacterial cell populations, fluorescence microscopy was utilized to look at the association of particles and cells. Ag-SNAs functionalized with 5'-Cy5 fluorescent oligonucleotides (ex/em = 650/670 nm) were used to treat bacteria *in vitro*. In Figure 12A, untreated *A. baumannii* AYE cells are shown. In Figure 12B, treated cells demonstrated a strong fluorescence signal that colocalized with the bacteria, suggesting that the BT-Ag-SNAs target the bacterial membrane. We used transmission electron microscopy (TEM) to determine whether the BT-Ag-SNAs penetrated the cytosol or if the particles were purely membrane-associated. Figure 12C, D feature images of untreated cells and BT-Ag-SNA-treated *A. baumannii* AYE, respectively. The images suggest BT-Ag-SNAs associated with bacterial cell membranes. This is likely to damaging to cell integrity, which is consistent with current understanding of silver's antimicrobial properties.[26] Antimicrobial effects of BT-Ag-SNAs were potentially a result of the attached negatively charged ions and particle-specific mechanisms observed from when silver nanoparticles were in close contact with microbes.[27]

Example 8. BT-Ag-SNAs Synergize with Ampicillin

Antimicrobial synergy is an area of particular interest for the potentiation of antibiotics with the use of silver. It has been indicated that silver nanoparticles functionalized with ampicillin attack Gram-negative bacteria by way of metal depletion, and interfere with DNA unwinding in Gram-positive species.[28] In a comparison of the synergistic effects of BT-Ag-SNAs or Ag-NPs with ampicillin against *A. baumannii* UNT086-1 and MRSA, data indicate Ag-SNAs to be superior in effect to bare silver particles. Ampicillin, to which both bacteria displayed resistance, had MIC values of 11.45 μM and 178.9 nM for *A. baumannii* UNT086-1 and MRSA, respectively. Ag-SNAs displayed MIC values of 1 nM and 3.125 nM for *A. baumannii* UNT086-1 and MRSA, respectively. The fractional inhibitory concentration (FIC) values of MRSA and *A. baumannii* UNT086-1 challenged by both groups of silver particles are shown in the graphs of Figure 13A, B. When treated in combination with one another, the effective MIC values of both Ag-SNA and ampicillin decrease drastically. Both bacterial groups tested demonstrated a significant 4-fold and 128-fold reduction of effective Ag-SNA and ampicillin MIC values, respectively. We also assessed synergy with vancomycin (Figure 17A) and ciprofloxacin (Figure 17B), showing 4-fold enhancement of both respective antibiotics. Synergy between β-lactam antibiotics and silver was observed. The data demonstrate the enhanced synergistic effects of BT-Ag-SNAs as compared to conventional colloidal silver in conjunction with antibiotics.

Example 9. BT-Ag-SNAs Strongly Synergize with Vancomycin and Ciprofloxacin.

BT-Ag-SNAs have been shown to display synergy with ampicillin against A. baumannii UNT086-1 and MRSA. To observe if this synergy is limited to ampicillin or if it can be translated to different antibiotics, A. baumannii UNT086-1 was treated with BT-Ag-SNAs and vancomycin or ciprofloxacin. The data indicated Ag-SNAs to be superior in effect to bare silver particles. Vancomycin and Ciprofloxacin, to which A. baumannii UNT086-1 displayed resistance, had MIC values of 15.6 μg/mL and 30μg/mL respectively. Ag-SNAs displayed MIC values of 1 nM for A. baumannii UNT086-1. The fractional inhibitory concentration (FIC) values of A. baumannii UNT086-1 challenged by both groups of silver particles are shown in the graphs of Figure 17A, B. When treated in conjunction with one another, the effective MIC values of both Ag-SNA and each antibiotic tested,

decreases drastically. Vancomycin demonstrated a significant 32-fold and 62-fold reduction of effective Ag-SNA and vancomycin concentration, respectively. Similarly, treating A. baumannii UNT086-1 synergistically with Ag-SNAs and ciprofloxacin demonstrates a 2-fold and a striking 120-fold reduction of effective Ag-SNA and ciprofloxacin concentration, respectively. Our data indicates the enhanced synergistic effects of BT-Ag-SNAs as compared to conventional colloidal silver in conjunction with numerous antibiotics.

Example 10. Reduced Toxicity of BT-Ag-SNAs as Compared to Ag-NPs in Human Foreskin Keratinocytes (HFKs)

Concerns over non-specific host toxicity associated with silver[20,30] were evaluated based on cell viability in the presence of silver. We executed an alamarBlue assay to establish a relative toxicity index for these particles *in vitro*. The graph in Figure 14 outlines the difference in toxicity between the BT-Ag-SNAs and the Ag-NPs. The data presents a 14-fold toxicity index between the MIC of *A. baumannii* AYE and the conservative estimates of HFK viability loss (estimated to be 4.375 nM, BT-Ag-SNAs). This index is in line with other compounds in the context of topical delivery. For example, polymyxin antibiotics, while known to display some adverse nephrotoxic side-effects when delivered intravenously, are often used topically with little concern over toxicity. In contrast, the index for intravenous doses have been observed at less than 2.5-fold (in the case of colistin).[31] Resistance to topical toxicity was factored into the *in vivo* model outlined in the next section, and cause for the use of 5 nM of BT-Ag-SNAs in the dose regimen. BT-Ag-SNAs demonstrate a decrease in host cell toxicity, which is critical in any *in vivo* applications of the particles. Our results again demonstrate the key influence of the conjugated PEG strands and oligonucleotides and their multi-faceted impact on both bacterial toxicity and host viability.

Example 11. *In Vivo* Topical Application of Ag-SNAs

Once we determined *in vitro* toxicity, our next step was to investigate the use of BT-Ag-SNAs *in vivo*. The treatment of an *A. baumannii* UNT086-1 infection in a topical wound was explored. In Figure 15, infections demonstrated a significant response to BT-Ag-SNA treatment with a $\log_{10}$ reduction of 1.51 when compared to the untreated controls. Comparisons with Ag-NPs ($\log_{10}$ reduction = 0.63) and unconjugated oligonucleotide ($\log_{10}$

reduction = 0.58) further emphasize the improved efficacy of BT-Ag-SNAs. Improved healing is likely a result of the antimicrobial properties of silver, as well as an anti-inflammatory effect and fibrogenic cytokine modulation. These results demonstrate that antibacterial activity of these Ag-SNAs extends beyond *in vitro* examination and is transferable to *in vivo* examinations, specifically for a clinically relevant species of DR Gram-negative bacteria.

BT-Ag-SNAs represent a new strategy in antibiotics with applicability against DR Gram-positive and DR Gram-negative organisms. Our data suggest that the BT-Ag-SNA constructs with oligonucleotides on the surfaces of these particles offer means for Ag-NPs to better interact with the surfaces of bacterial cells, while improving efficacy and decreasing the toxic effects for the host. The BT-Ag-SNA also represents the possibility of low-dose silver to be used synergistically with existing antibiotics, as ampicillin worked synergistically in conjunction with BT-Ag-SNAs. BT-Ag-SNAs exhibit a functional, stable, and broad spectrum approach to antimicrobials. Our data suggests that the polyvalent nature of the Ag-SNA combined with the potency of silver nanoparticles brings closer to the possibility of a safe and powerful silver-based antibiotic. These specialized nanoparticles differentiate themselves through their antimicrobial efficacy, potent antibiotic synergy, and improved control over unwanted toxicity as compared to Ag nanoparticles.

Example 12. Enhanced bacterial toxicity of Ag-SNAs relative to silver ions.

A comparison of silver ions was made with Ag-SNAs in *A. baumannii* UNT086-1, and the results indicated that SNAs possessed greater efficacy (Figure 18). This comparison was made on an atomic basis, assuming that the entire outer layer of silver atoms on each SNA was free to interact with bacteria. We made this assumption on the basis that no significant color change occurred in the SNAs during the experiment (color change would indicate a significant change in particle size and concentration).Together, these results demonstrate that the SNA construct can be used to couple therapeutics and that this action generates new and unexpected properties to the conjugate structure.

In the claims articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered

satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention includes embodiments in which more than one or all of the group members are present in, employed in or otherwise relevant to a given product or process.

Furthermore, the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, and descriptive terms from one or more of the listed claims is introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Where elements are presented as lists, e.g., in Markush group format, each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It should it be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements and/or features, certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements and/or features. For purposes of simplicity, those embodiments have not been specifically set forth in haec verba herein. It is also noted that the terms "comprising" and "containing" are intended to be open and permits the inclusion of additional elements or steps. Where ranges are given, endpoints are included. Furthermore, unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or sub–range within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

This application refers to various issued patents, published patent applications, journal articles, and other publications, all of which are incorporated herein by reference. If there is a conflict between any of the incorporated references and the instant specification, the specification shall control. In addition, any particular embodiment of the present invention that falls within the prior art may be explicitly excluded from any one or more of the claims. Because such embodiments are deemed to be known to one of ordinary skill in

the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the invention can be excluded from any claim, for any reason, whether or not related to the existence of prior art.

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation many equivalents to the specific embodiments described herein. The scope of the present embodiments described herein is not intended to be limited to the above Description, but rather is as set forth in the appended claims. Those of ordinary skill in the art will appreciate that various changes and modifications to this description may be made without departing from the spirit or scope of the present invention, as defined in the following claims.

CLAIMS

We claim:

1. A nanostructure, comprising

a core, one or more carrier oligonucleotides positioned on the exterior of the core, and a therapeutic pharmacophore coupled to the carrier oligonucleotides.

2. The nanostructure of claim 1, wherein the carrier oligonucleotides form an oligonucleotide shell on the exterior of the core and wherein the therapeutic pharmacophore is hybridized to the carrier oligonucleotides.

3. The nanostructure of claim 2, wherein the oligonucleotide shell comprises 2-1,000 oligonucleotides.

4. The nanostructure of any one of claims 1-3, wherein the carrier oligonucleotides are comprised of single-stranded or double-stranded DNA oligonucleotides.

5. The nanostructure of any one of claims 1-3, wherein the carrier oligonucleotides are comprised of single-stranded or double-stranded RNA oligonucleotides.

6. The nanostructure of any one of claims 1-3, wherein the carrier oligonucleotides are comprised of chimeric RNA-DNA oligonucleotides.

7. The nanostructure of any one of claims 1-3, wherein the carrier oligonucleotides are comprised of RNA-DNA or DNA-RNA oligonucleotide heteroduplexes.

8. The nanostructure of any one of claims 1-3, wherein the carrier oligonucleotides are comprised of combinations of single-stranded or double-stranded DNA, RNA, or chimeric RNA-DNA oligonucleotides.

9. The nanostructure of any one of claims 1-8, wherein the carrier oligonucleotides of the oligonucleotide shell are all structurally and nucleotide sequence identical oligonucleotides.

10. The nanostructure of any one of claims 1-8, wherein the carrier oligonucleotides of the oligonucleotide shell have at least two structurally and nucleotide sequence different oligonucleotides.

11. The nanostructure of any one of claims 1-8, wherein the carrier oligonucleotides of the oligonucleotide shell have 2-10 different nucleotide sequences.

12. The nanostructure of any one of claims 1-11, wherein at least one of the carrier oligonucleotides is a modified oligonucleotide.

13. The nanostructure of any one of claims 1-11, wherein at least 50% of the carrier oligonucleotides are modified oligonucleotides.

14. The nanostructure of any one of claims 1-11, wherein all of the carrier oligonucleotides are modified oligonucleotides.

15. The nanostructure of any one of claims 1-11, wherein the carrier oligonucleotides have at least one internucleoside phosphorothioate linkage.

16. The nanostructure of any one of claims 1-11, wherein the carrier oligonucleotides do not have an internucleoside phosphorothioate linkage.

17. The nanostructure of any one of claims 1-16, wherein at least one carrier oligonucleotide has its 5'- terminus exposed to the outside surface of the nanostructure.

18. The nanostructure of any one of claims 1-16, wherein all of the carrier oligonucleotides have their 5'- terminus exposed to the outside surface of the nanostructure.

19. The nanostructure of any one of claims 1-16, wherein at least one carrier oligonucleotide has its 3'- terminus exposed to the outside surface of the nanostructure.

20. The nanostructure of any one of claims 1-16, wherein all of the carrier oligonucleotides have their 3'- terminus exposed to the outside surface of the nanostructure.

21. The nanostructure of any one of claims 1-16, wherein the carrier oligonucleotides are directly linked to the core.

22. The nanostructure of any one of claims 1-16, wherein at least one carrier oligonucleotide is positioned laterally on the surface of the nanostructure.

23. The nanostructure of any one of claims 1-16, wherein all of the carrier oligonucleotides are positioned laterally on the surface of the nanostructure.

24. The nanostructure of any one of claims 1-23, wherein the carrier oligonucleotides are indirectly linked to the core through a linker.

25. The nanostructure of any one of claims 1-23, wherein the carrier oligonucleotides are indirectly linked to the core through more than one linker.

26. The nanostructure of any one of claims 1-23, wherein the carrier oligonucleotides are directly linked to the core.

27. The nanostructure of any one of claims 24 or 25, wherein the linker is a chemical structure containing one or more thiol groups, including various chain length alkane thiols, cyclic dithiol, lipoic acid, PEG-thiol, and other thiol group containing linkers.

28. The nanostructure of any one of claims 24 or 25, wherein the carrier oligonucleotides are linked to a liposomal core and the linker is one or more of the following linkers: tocopherols, sphingolipids such as sphingosine, sphingosine phosphate, methylated sphingosines and sphinganines, ceramides, ceramide phosphates, 1-0 acyl ceramides, dihydroceramides, 2-hydroxy ceramides, sphingomyelin, glycosylated sphingolipids, sulfatides, gangliosides, phosphosphingolipids, and phytosphingosines of various lengths and saturation states and their derivatives, phospholipids such as phosphatidylcholines, lysophosphatidylcholines, phosphatidic acids, lysophosphatidic acids, cyclic LPA, phosphatidylethanolamines, lysophosphatidylethanolamines, phosphatidylglycerols, lysophosphatidylglycerols, phosphatidylserines, lysophosphatidylserines, phosphatidylinositols, inositol phosphates, LPI, cardiolipins, lysocardiolipins, bis(monoacylglycero) phosphates, (diacylglycero) phosphates, ether lipids, diphytanyl ether lipids, and plasmalogens of various lengths, saturation states, and their derivatives, sterols such as cholesterol, desmosterol, stigmasterol, lanosterol, lathosterol, diosgenin, sitosterol, zymosterol, zymostenol, 14-demethyl-lanosterol, cholesterol sulfate, DHEA, DHEA sulfate, 14-demethyl-14-dehydrlanosterol, sitostanol, campesterol, ether anionic lipids, ether cationic lipids, lanthanide chelating lipids, A-ring substituted oxysterols, B-ring substituted oxysterols, D-ring substituted oxysterols, side-chain substituted oxysterols, double substituted oxysterols, cholestanoic acid derivatives, fluorinated sterols, fluorescent sterols, sulfonated sterols, phosphorylated sterols, and polyunsaturated sterols of different lengths, saturation states and derivatives thereof.

29. The nanostructure of any one of claims 1-27, wherein the core is a solid or hollow core.

30. The nanostructure of any one of claims 1-27, wherein the core is inert, paramagnetic or supramagnetic.

31. The nanostructure of any one of claims 1-27, wherein the core is a solid core.

32. The nanostructure of claim 31, wherein the solid core is comprised of noble metals, including gold and silver, transition metals including iron and cobalt, metal oxides including silica, polymers or combinations thereof.

33. The nanostructure of claim 31, wherein the core is a polymeric core and wherein the polymeric core is comprised of amphiphilic block copolymers, hydrophobic polymers including polystyrene, poly(lactic acid), poly(lactic co-glycolic acid), poly(glycolic acid), poly(caprolactone) and other biocompatible polymers.

34. The nanostructure of any one of claims 1-27, wherein the core is a liposomal core.

35. The nanostructure of claim 34, wherein the liposomal core is comprised of one or more lipids selected from: sphingolipids such as sphingosine, sphingosine phosphate, methylated sphingosines and sphinganines, ceramides, ceramide phosphates, 1-0 acyl ceramides, dihydroceramides, 2-hydroxy ceramides, sphingomyelin, glycosylated sphingolipids, sulfatides, gangliosides, phosphosphingolipids, and phytosphingosines of various lengths and saturation states and their derivatives, phospholipids such as phosphatidylcholines, lysophosphatidylcholines, phosphatidic acids, lysophosphatidic acids, cyclic LPA, phosphatidylethanolamines, lysophosphatidylethanolamines, phosphatidylglycerols, lysophosphatidylglycerols, phosphatidylserines, lysophosphatidylserines, phosphatidylinositols, inositol phosphates, LPI, cardiolipins, lysocardiolipins, bis(monoacylglycero) phosphates, (diacylglycero) phosphates, ether lipids, diphytanyl ether lipids, and plasmalogens of various lengths, saturation states, and their derivatives, sterols such as cholesterol, desmosterol, stigmasterol, lanosterol, lathosterol, diosgenin, sitosterol, zymosterol, zymostenol, 14-demethyl-lanosterol, cholesterol sulfate, DHEA, DHEA sulfate, 14-demethyl-14-dehydrlanosterol, sitostanol, campesterol, ether anionic lipids, ether cationic lipids, lanthanide chelating lipids, A-ring substituted oxysterols, B-ring substituted oxysterols, D-ring substituted oxysterols, side-chain substituted oxysterols, double substituted oxysterols, cholestanoic acid derivatives, fluorinated sterols, fluorescent sterols, sulfonated sterols, phosphorylated sterols, and polyunsaturated sterols of different lengths, saturation states, and derivatives thereof.

36. The nanostructure of any one of claims 34-35, wherein the liposomal core is comprised of one type of lipid.

37. The nanostructure of any one of claims 34-35, wherein the liposomal core is comprised of 2-10 different lipids.

38. The nanostructure of any one of claims 1-37, wherein the therapeutic pharmacophore is an antisense oligonucleotide.

39. The nanostructure of claim 38, wherein the therapeutic pharmacophore is a peptide nucleic acid (PNA).

40. The nanostructure of claim 38 or 39, further comprising an antibacterial cell penetrating peptide conjugated to the antisense oligonucleotide.

41. The nanostructure of claim 38 or 39, further comprising an antibacterial agent conjugated to the antisense oligonucleotide.

42. The nanostructure of any one of claims 1-37, wherein the therapeutic pharmacophore is an RNA oligonucleotide.

43. The nanostructure of any one of claims 1-37, wherein the therapeutic pharmacophore is an siRNA.

44. The nanostructure of any one of claims 1-43, further comprising an active agent.

45. The nanostructure of claim 44, wherein the active agent is mixed together with the nanostructure.

46. The nanostructure of claim 44, wherein the active agent is linked directly to the oligonucleotide shell.

47. The nanostructure of claim 44, wherein the active agent is linked indirectly to the oligonucleotide shell or the core through a linker.

48. The nanostructure of claim 44, wherein the active agent is linked directly to the core.

49. The nanostructure of claim 44, wherein an active agent –oligonucleotide conjugate is linked to the core through oligonucleotide hydrogen-bond based hybridization.

50. The nanostructure of claim 44, wherein the active agent is encapsulated within the core.

51. The nanostructure of claim 44, wherein the active agent is attached non-covalently to the carrier oligonucleotide of the oligonucleotide shell.

52. The nanostructure of any one of claims 44-51, wherein the active agent is an antibacterial agent.

53. The nanostructure of any one of claims 1-52, wherein the nanostructure is a self-assembling nanostructure.

54. The nanostructure of any one of claims 1-52, wherein the therapeutic pharmacophore is an oligonucleotide having at least a region that is antisense to acyl carrier protein P (acpP).

55. The nanostructure of any one of claims 1-52, wherein the therapeutic pharmacophore is an oligonucleotide that is coupled to a peptide.

56. The nanostructure of claim 55, wherein the peptide is a cell penetrating peptide and is conjugated to the oligonucleotide through a glycol linkage.

57. The nanostructure of claim 56, wherein the cell penetrating peptide has the following sequence: KFFKFFKFFK (SEQ ID NO: 1).

58. The nanostructure of any one of claims 1-57, further comprising a PEG incorporated into the nanostructure.

59. The nanostructure of claim 58, wherein the PEG is 1,000-40,000 Daltons.

60. A method for treating a subject having a bacterial infection, comprising administering to a subject a nanostructure of any one of claims 1-59, in an effective amount to treat the bacterial infection.

61. The method of claim 60, wherein the bacterial infection is selected from the group consisting of *Escherichia coli, Acinetobacter baumannii, Helicobacter pyloris, Borelia burgdorferi, Legionella pneumophilia, Mycobacteria sps* (e.g. *M. tuberculosis, M. avium, M. intracellulare, M. kansaii, M. gordonae*), *Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis, Streptococcus bovis, Streptococcus* (anaerobic species.), *Streptococcus pneumoniae*, pathogenic *Campylobacter sp., Enterococcus sp., Haemophilus influenzae, Bacillus antracis, corynebacterium diphtheriae, corynebacterium*

sp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringers*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides sp.*, *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidium*, *Treponema pertenue*, *Leptospira*, *Rickettsia,* and *Actinomyces israelli*.

62. The method of claim 60, wherein the nanostructure is delivered by a route selected from the group consisting of oral, nasal, sublingual, intravenous, subcutaneous, mucosal, respiratory, direct injection, enema, and dermally.

63. A composition for use in the treatment of disease, comprising the nanostructure of any one of claims 1-59.

64. A method for treating a surface, comprising

applying to a surface a nanostructure of any one of claims 1-59, in an effective amount to disrupt a bacterial infection on the surface.

65. The method of claim 64, wherein the surface is a surgical device or implant.

66. An article , comprising:

a device having a coating of a nanoparticle of any one of claims 1-59.

67. The article of claim 66, wherein the device is a surgical device or implant.

68. The article of claim 66 or 67, wherein the nanoparticle partially coats the device.

69. The article of claim 66 or 67, wherein the nanoparticle fully coats the device.

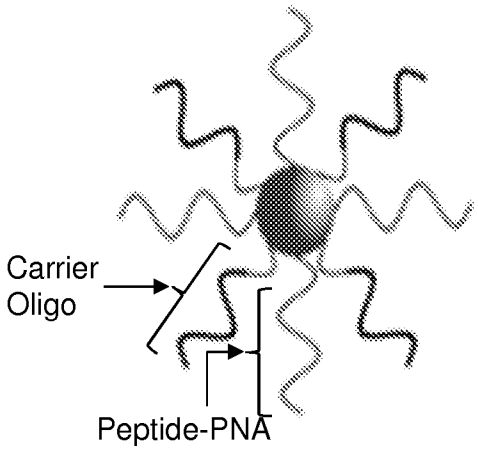
70. The article of claim 66 or 67, wherein the nanoparticle coats the device by being embedded in the surface of the device.

71. A nanostructure, comprising

a silver core, one or more carrier oligonucleotides positioned on the exterior of the core, and a PEG molecule coupled to the core or carrier oligonucleotides.

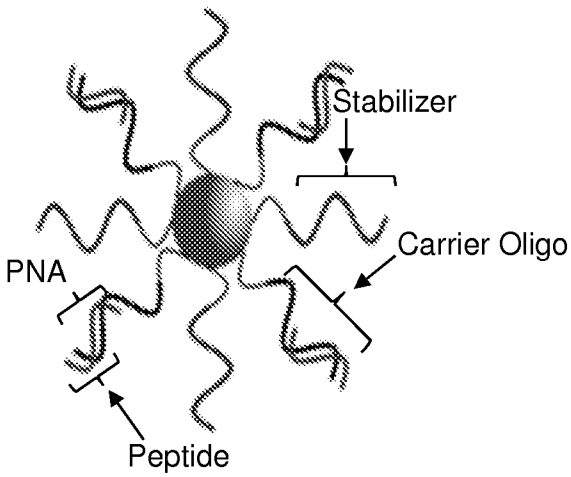
72. The nanostructure of claim 71, wherein the carrier oligonucleotides form an oligonucleotide shell on the exterior of the core and wherein the PEG molecule is hybridized to the carrier oligonucleotides.

73. The nanostructure of claim 72, wherein the oligonucleotide shell comprises 2-1,000 oligonucleotides.



Peptide-PNA: KFF-acpP
Carrier Oligo: *acpP*-18-PO

Figure 1A



Peptide-PNA: KFF-acpP
Carrier Oligo: compl-acpP
Stabilizer: PEG

Figure 1B

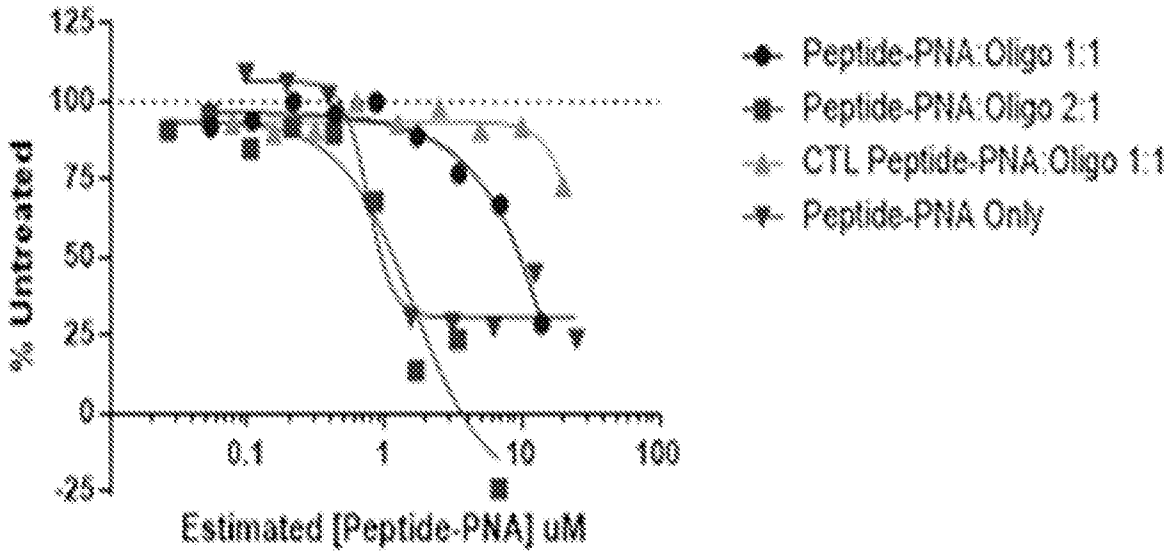


Figure 2A

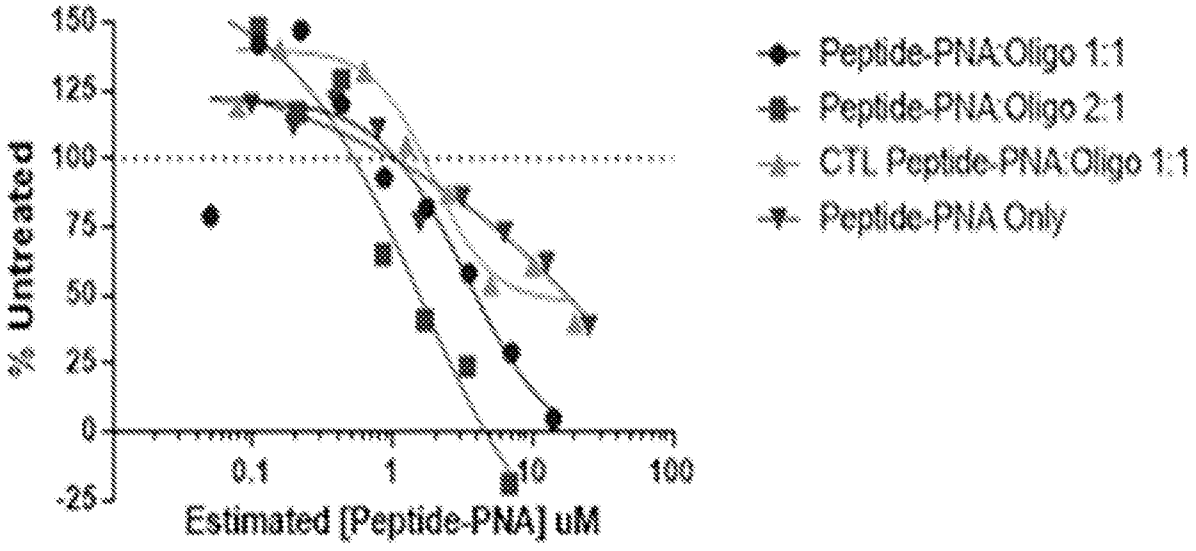


Figure 2B

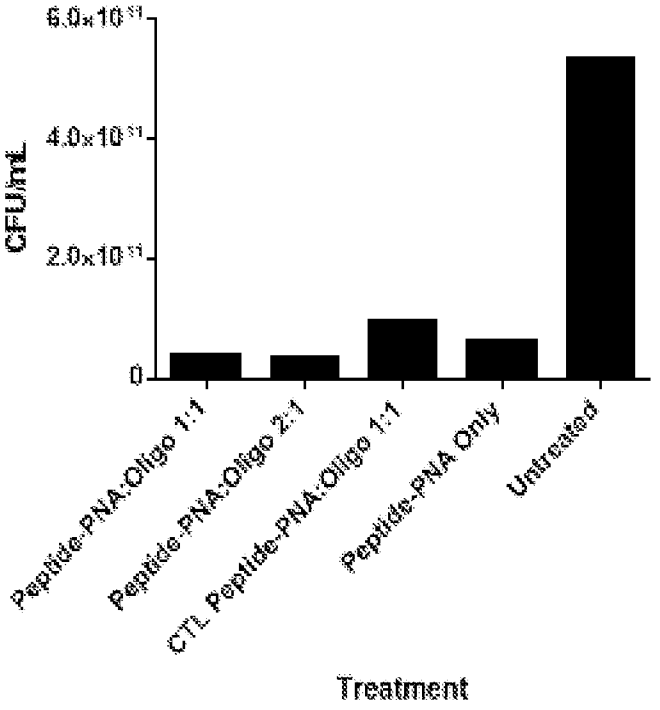


Figure 3A

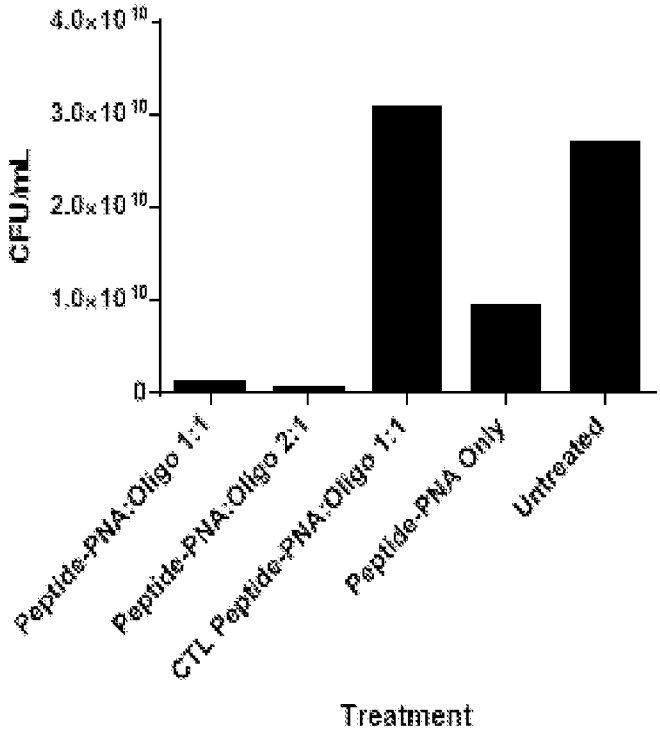


Figure 3B

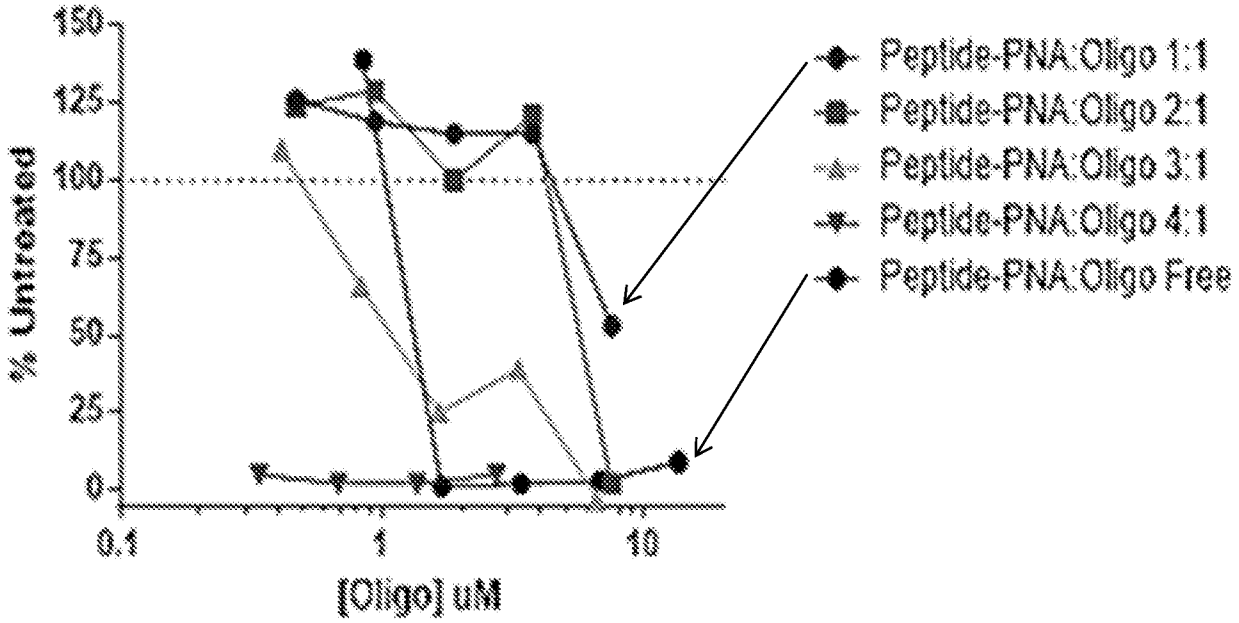


Figure 4A

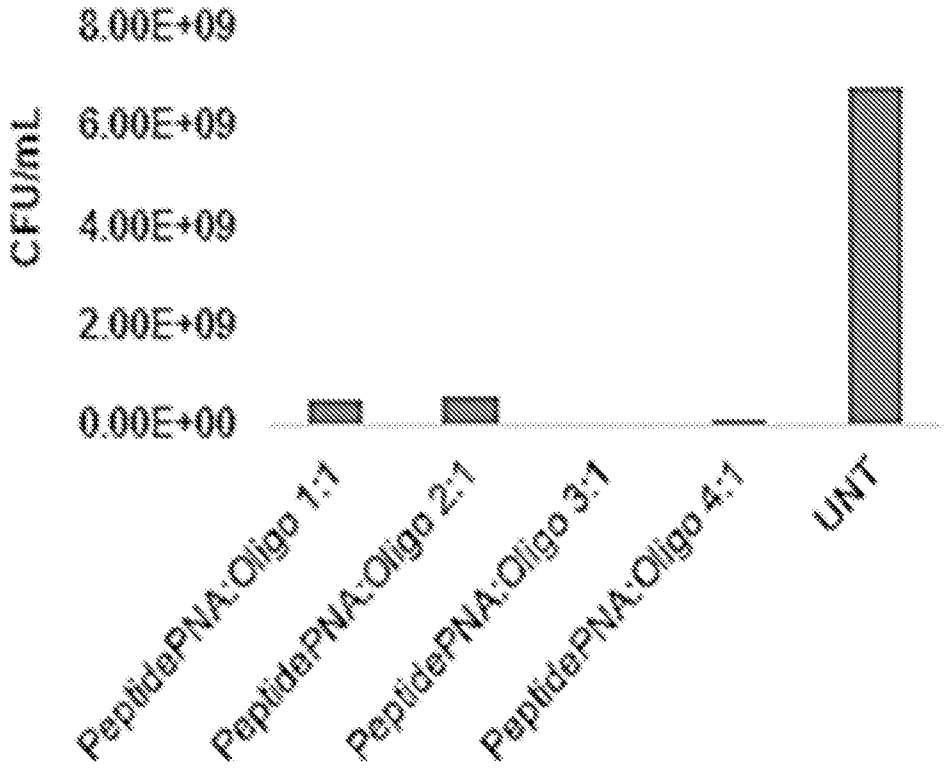


Figure 4B

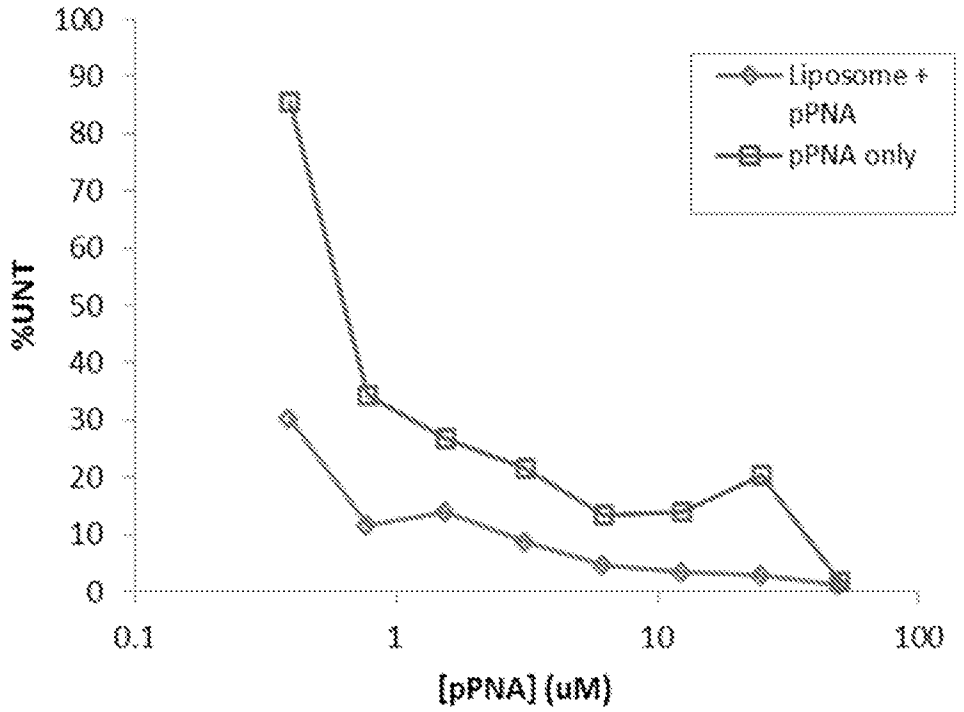


Figure 5A

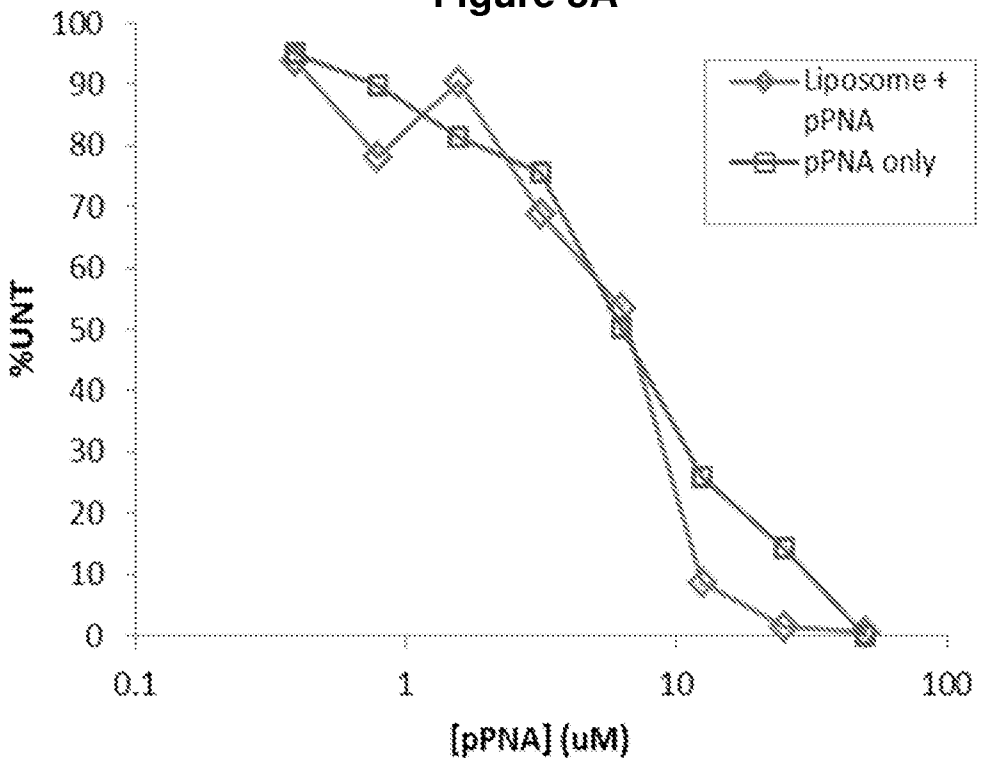


Figure 5B

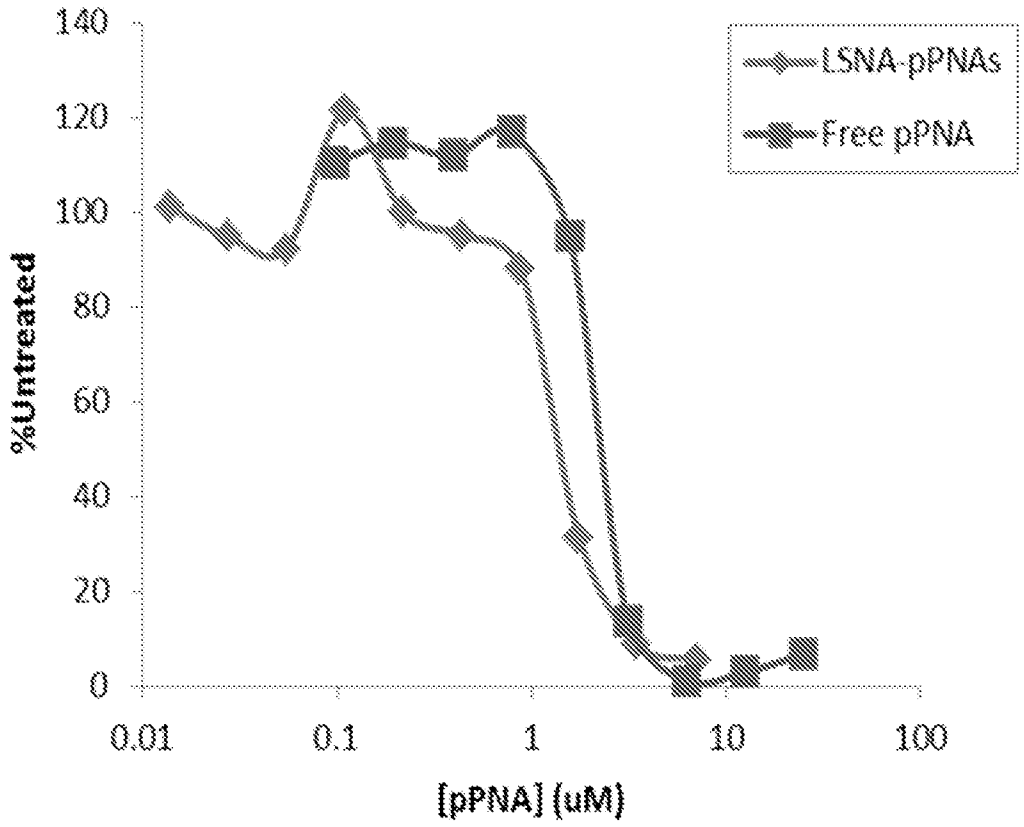
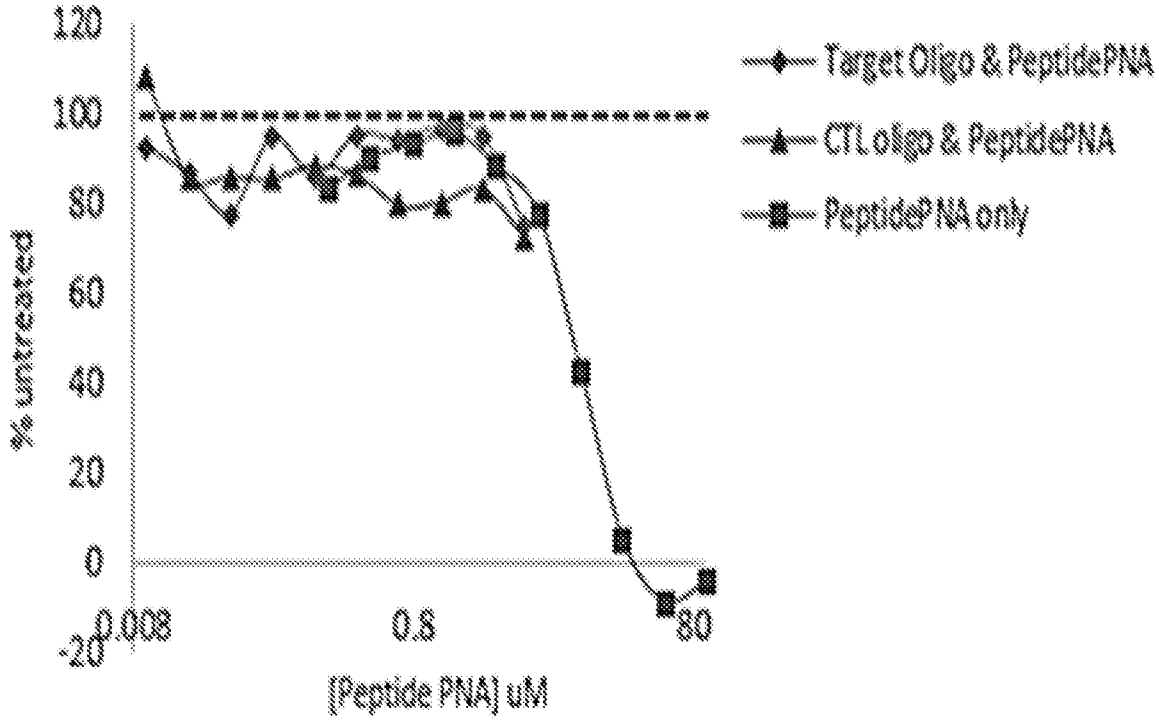
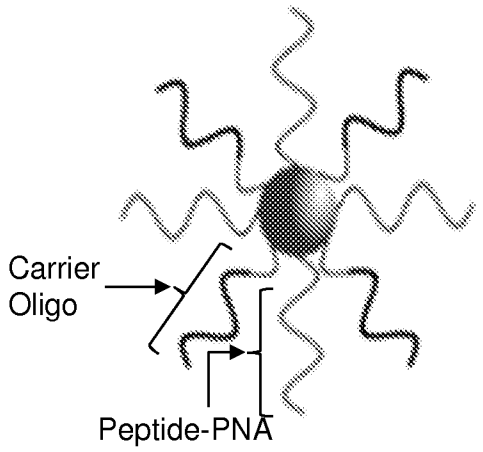

140
120
100
80
60
40
20
0
%Untreated
0.01
0.1
1
10
100
[pPNA] (uM)
LSNA-pPNAs
Free pPNA

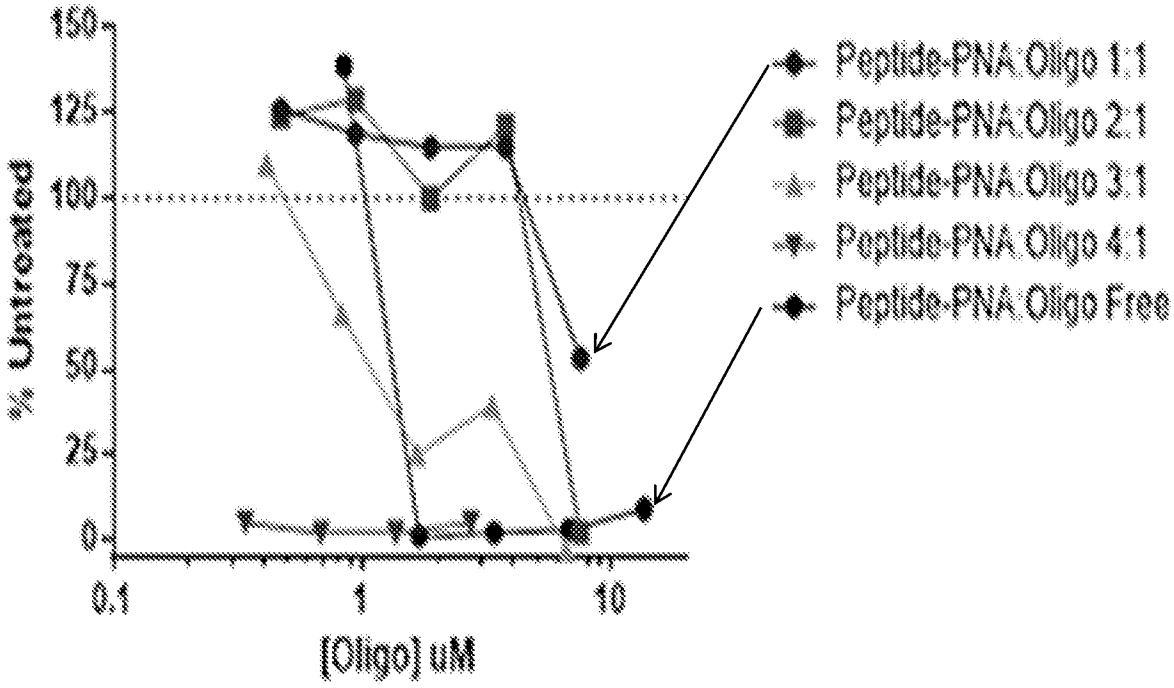
Figure 6

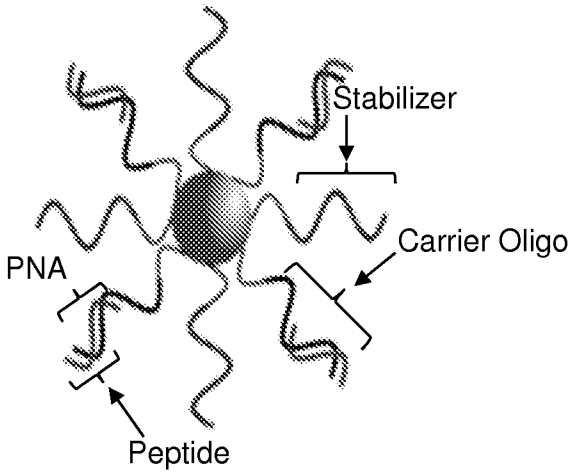




Peptide-PNA: KFF-acpP
Carrier Oligo: *acpP*-18-PO

Figure 7A





Peptide-PNA: KFF-acpP
Carrier Oligo: compl-acpP
Stabilizer: PEG

Figure 7B

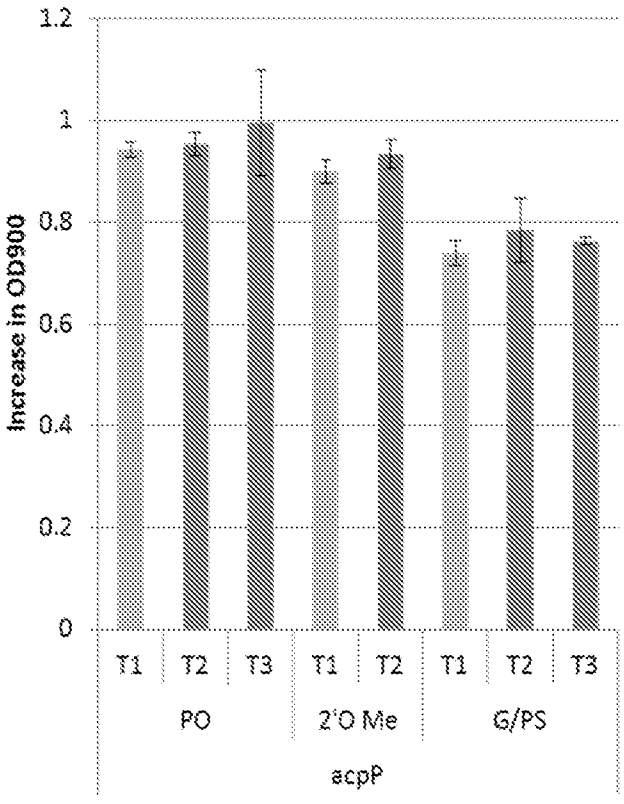


Figure 8A

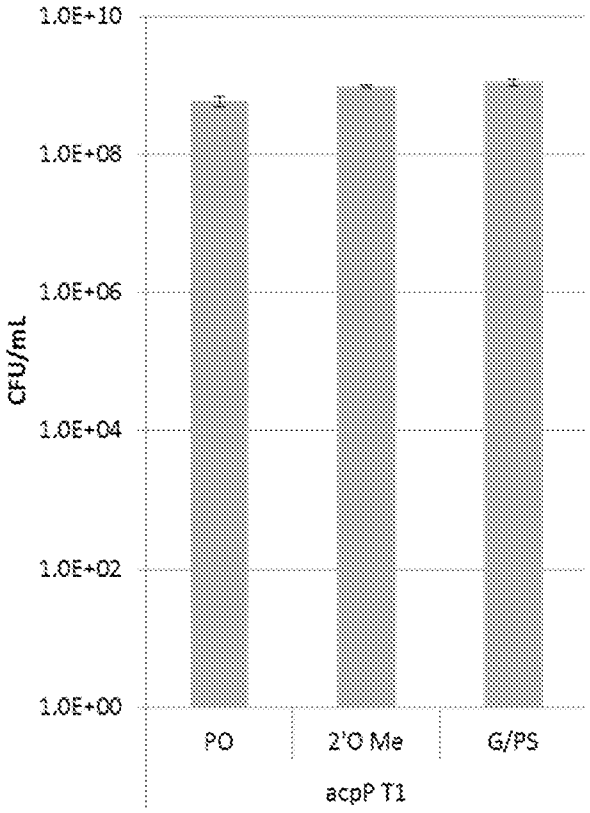


Figure 8B

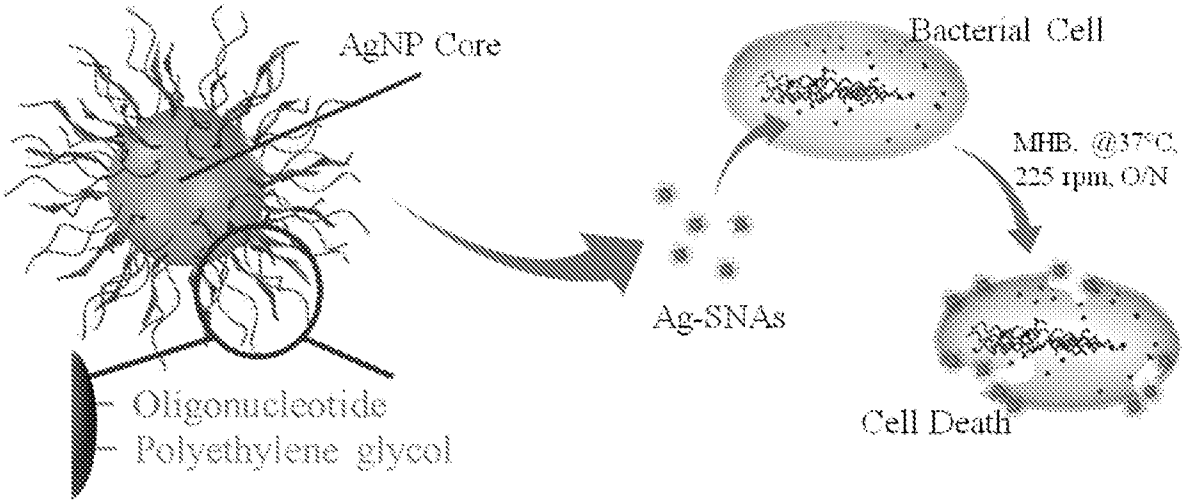
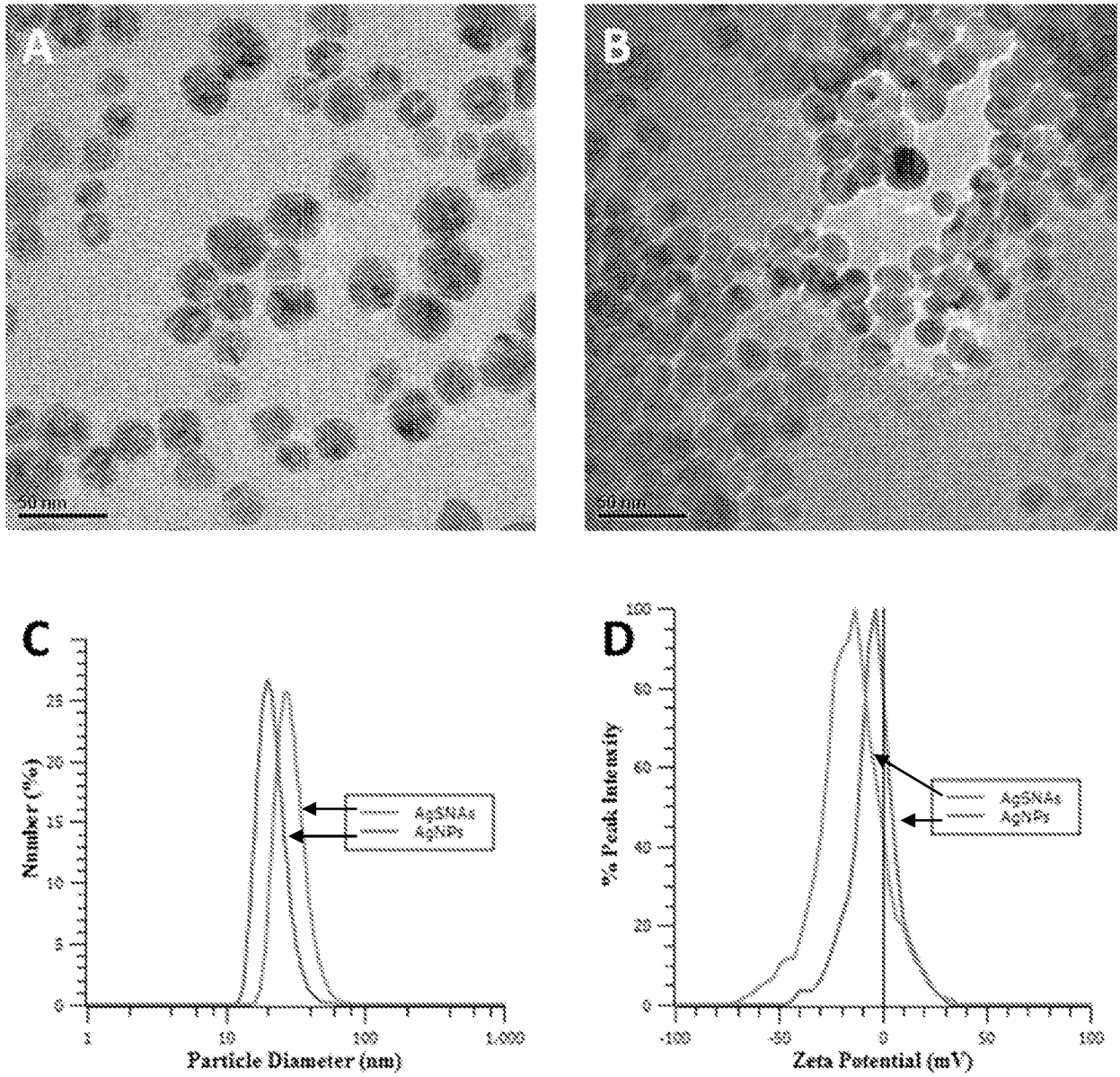
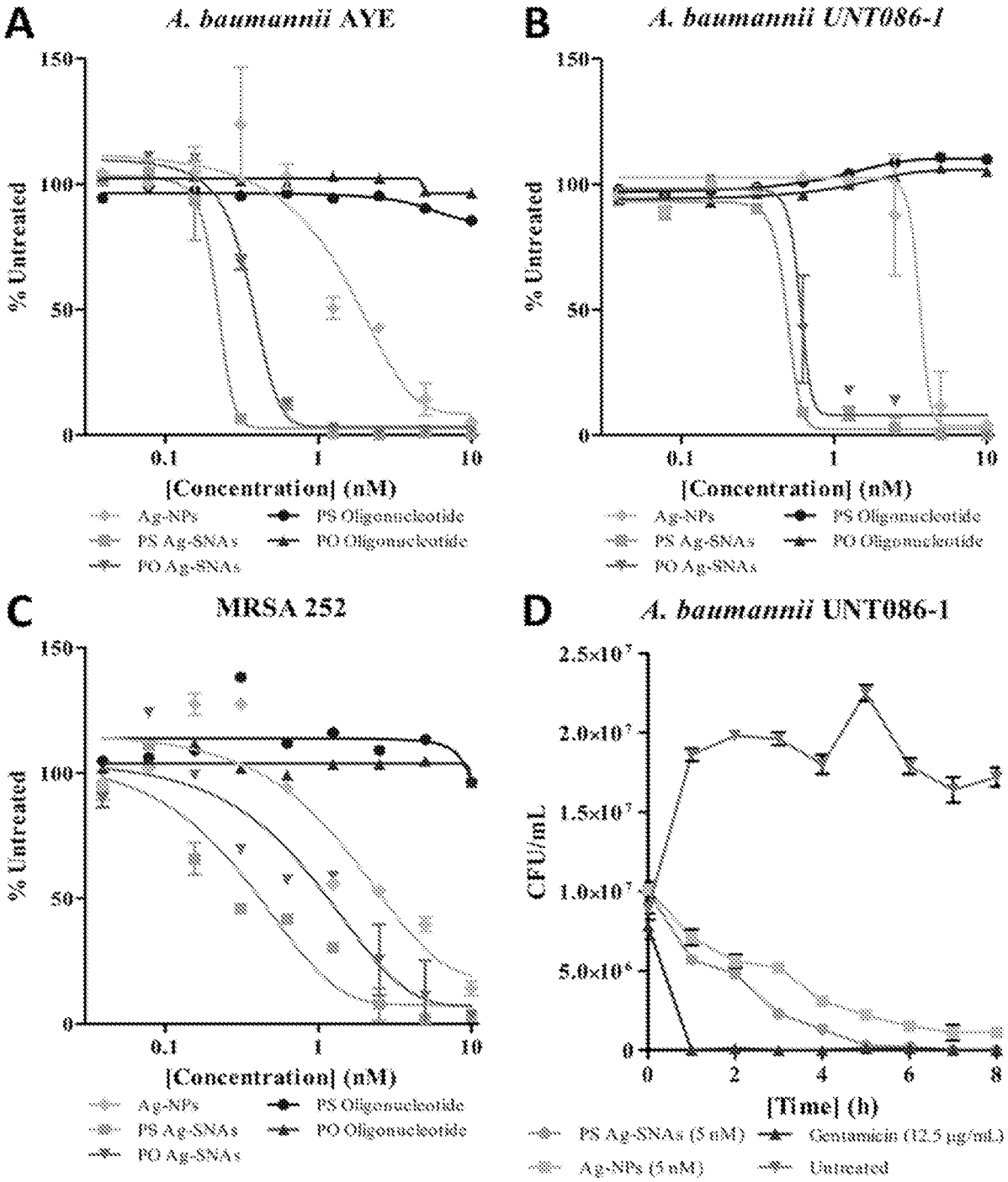

AgNP Core
Bacterial Cell
MHB, @37°C,
225 rpm, O/N
Ag-SNAs
Oligonucleotide
Polyethylene glycol
Cell Death

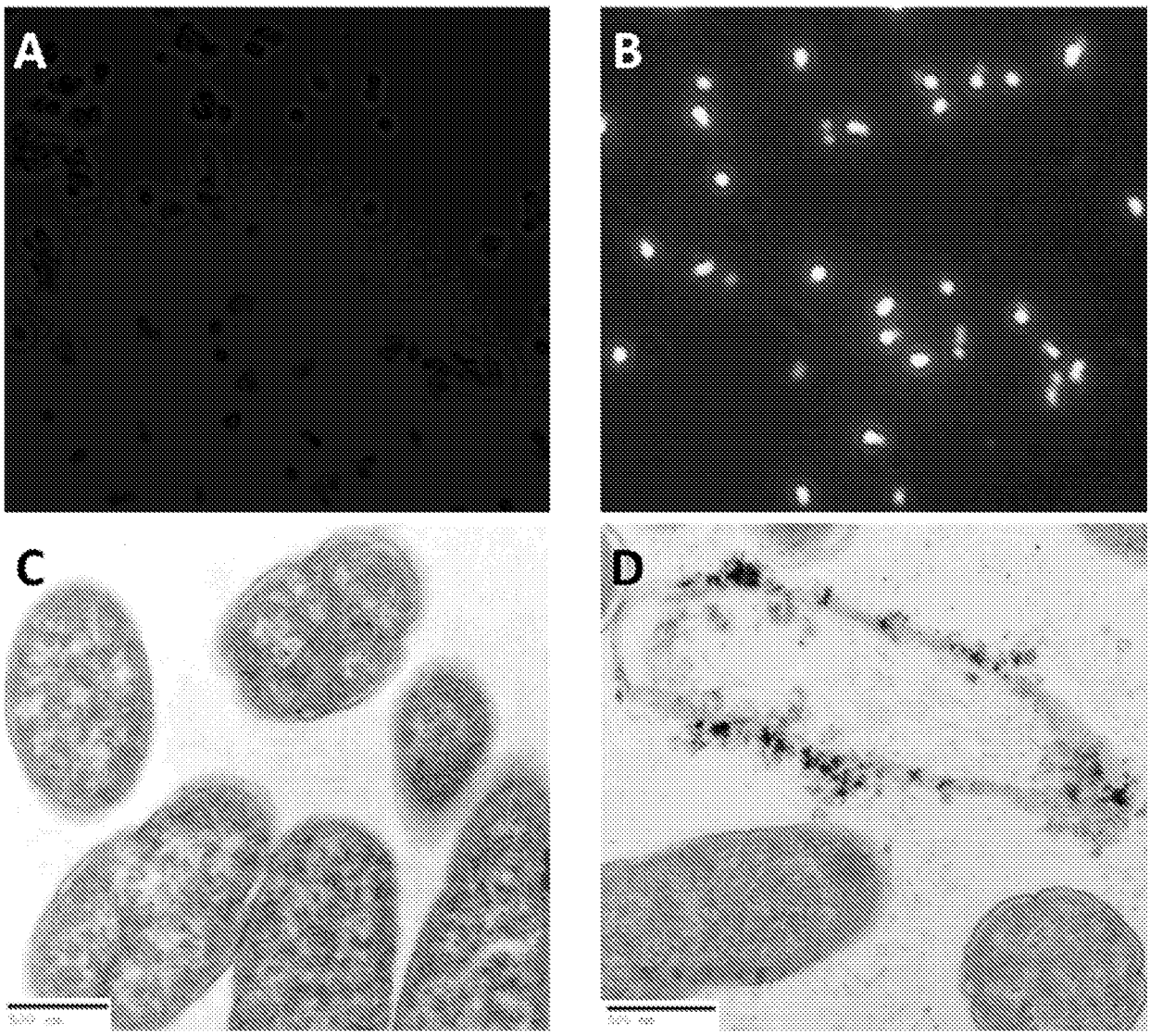
Figure 9



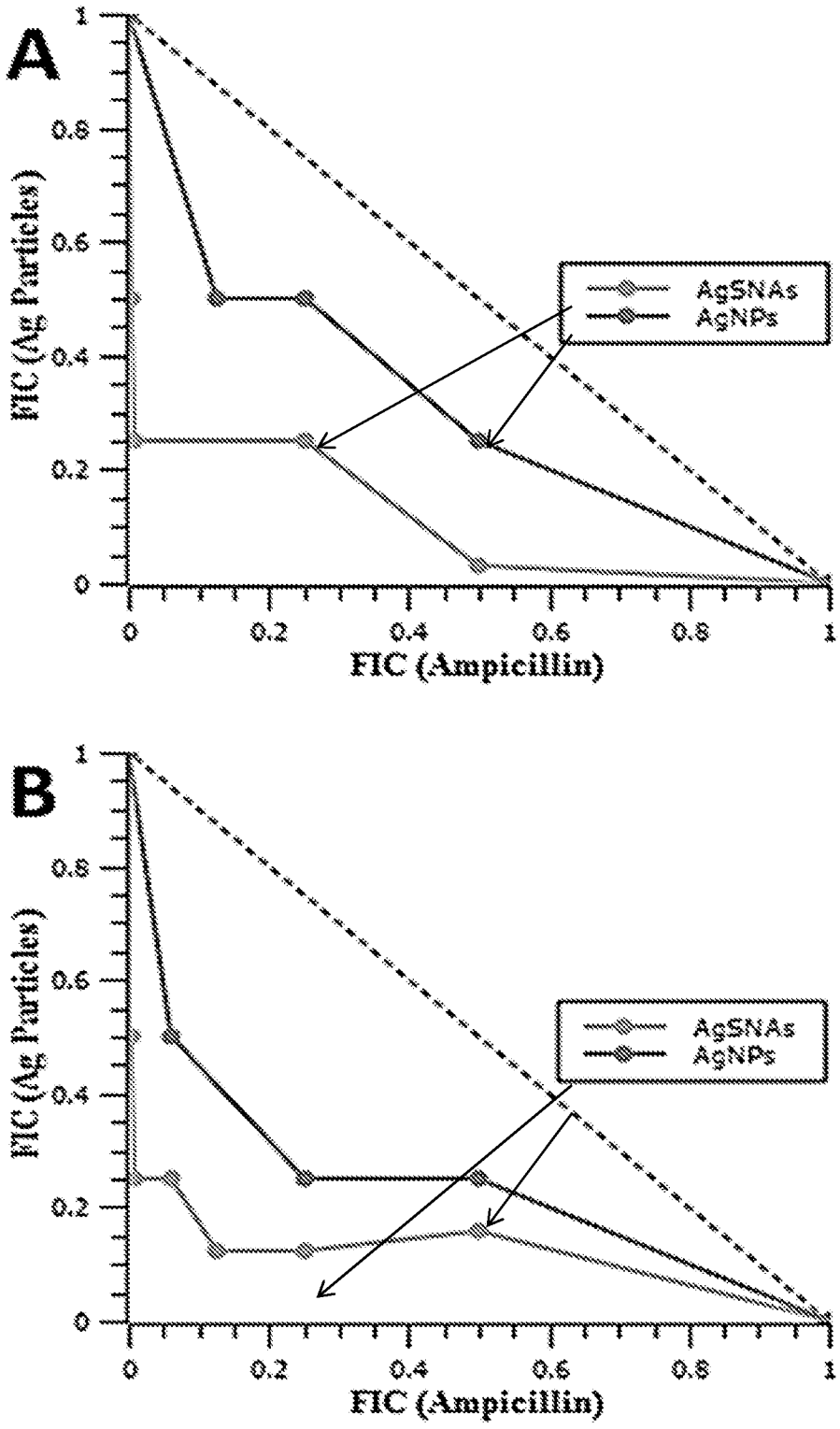
Figures 10A-10D



Figures 11A-11D



Figures 12A-12D



Figures 13A-13B

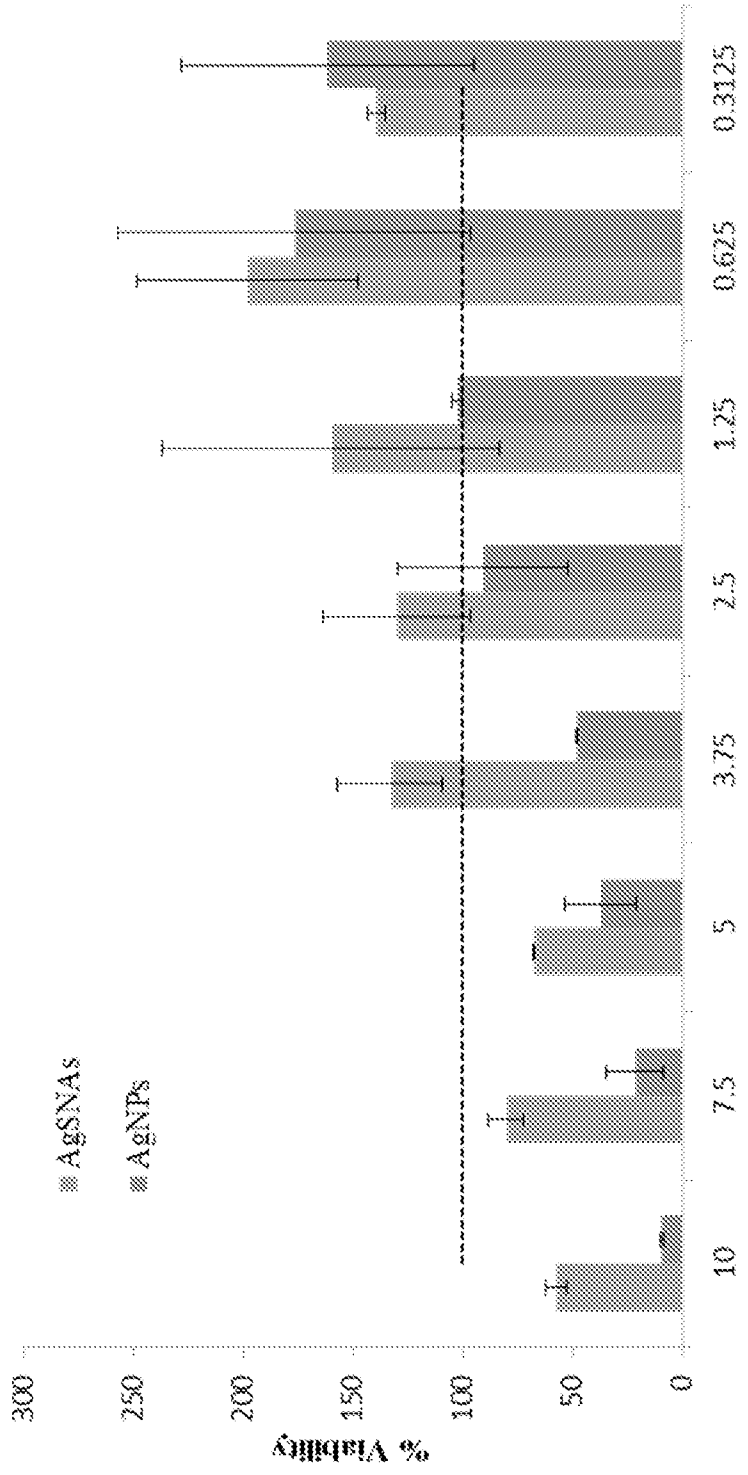

% Viability
300
250
200
150
100
50
0
AgSNAs
AgNPs
10
7.5
5
3.75
2.5
1.25
0.625
0.3125
[Ag Particles] (nM)

Figure 14

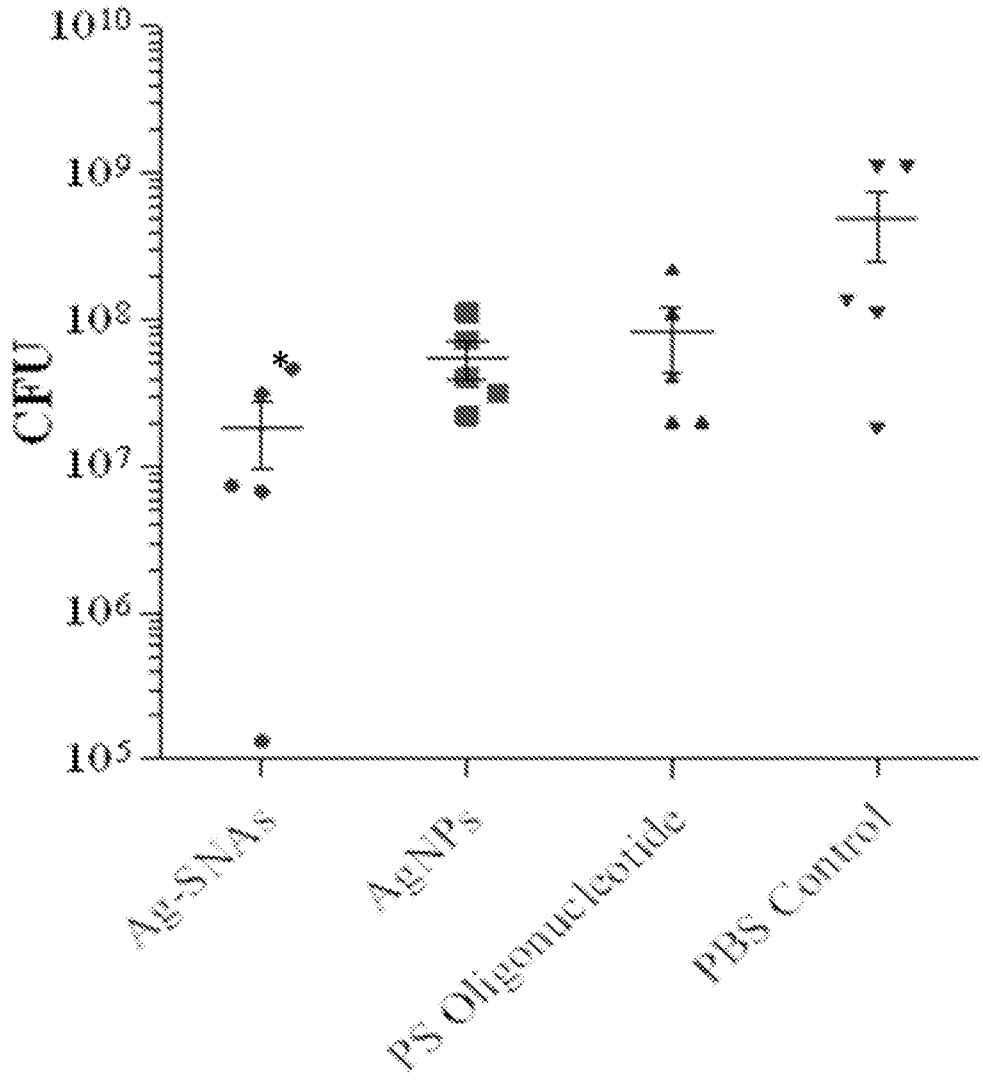

10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
CFU
*
Ag-SNAs
AgNPs
PS Oligonucleotide
PBS Control

Figure 15

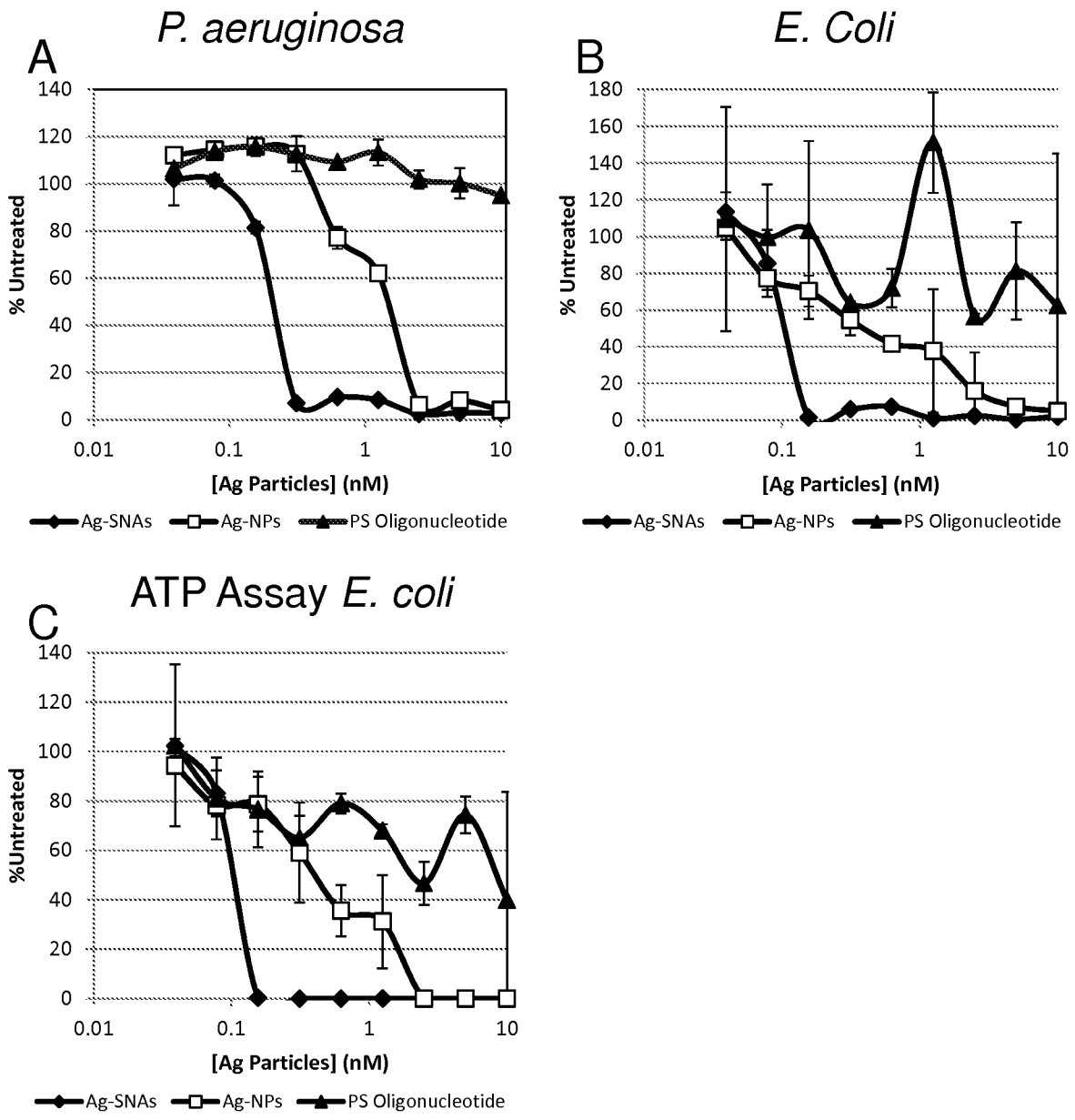
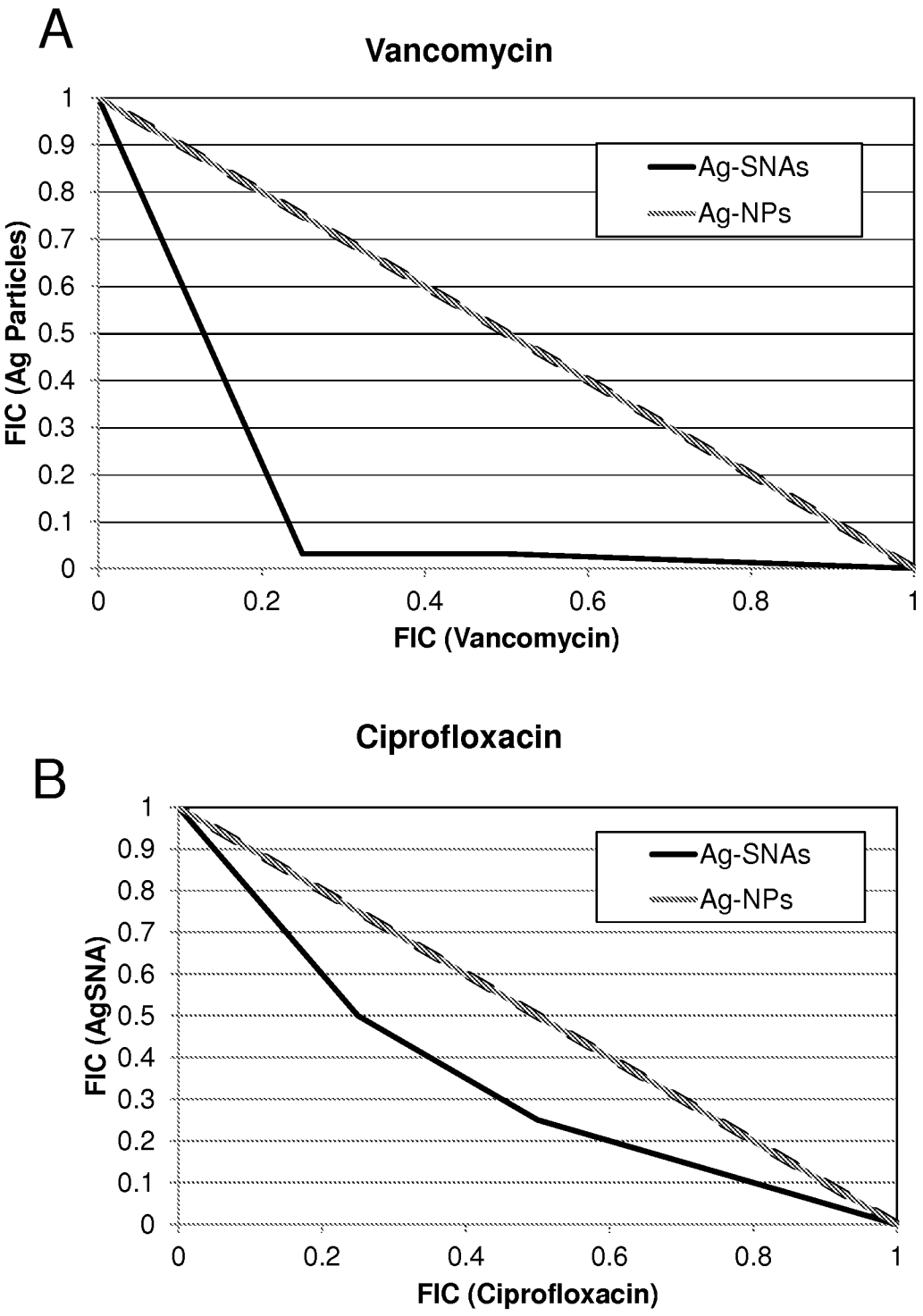


Figure 16A-16C



Figures 17A-17B

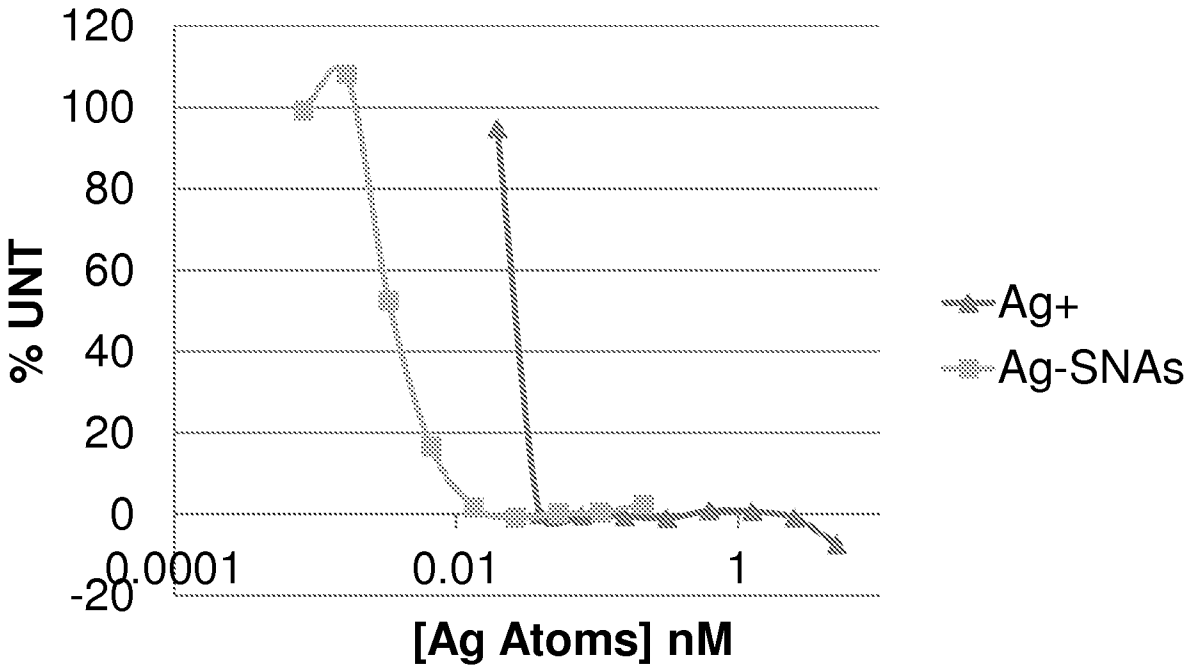


Figure 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/038771

A. CLASSIFICATION OF SUBJECT MATTER

A61K 9/14 (2006.01) A61K 31/7088 (2006.01) A61P 31/04 (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

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

Databases: WPIAP, EPODOC, MEDLINE, HCAPLUS, BIOSIS, EMBASE, ESPACENET, PATENTSCOPE. Keywords: Nanostructure, nanoparticle, nanocrystal, spherical nucleic acid, nanoscale construct, oligonucleotide, polynucleotide, nucleic acid, DNA, RNA, liposome, silver, gold, AUNP, AGSNA, pharmacophore, peptide nucleic acid, aptamer, antisense, PEG, polyethylene glycol, and similar terms, synonyms, and plurals (as appropriate).

Applicant and Inventor Name searches of the patent and non-patent literature were performed using Patentscope (http://www/wipo.int/patentscope/en/) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 2015	30 September 2015
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Amanda Lim AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61-2-6283 2157

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. PCT/US2015/038771
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/120420 A1 (NORTHWESTERN UNIVERSITY) 21 October 2010 Paragraphs [0040], [0052], [0062], [0065], [0084], [0085], [0088], [0090]-[0123], [0135], [0137], [0138], [0143], [0156], [0154], [0178], [0179], Figures; Examples; claims	1-73
X	CUTLER, J. I. et al., 'Spherical Nucleic Acids', 9 Jan 2012, Journal of the American Chemical Society, Vol. 134, No. 3, pages 1376-1391. Figure 2; page 1377, right-hand column; Figure 9, page 1386; page 1378, page 1380, right-hand column; pages 1384-1385	1-73
X	ZHENG, J. et al., 'A Spherical Nucleic Acid Platform Based on Self-Assembled DNA Biopolymer for High-Performance Cancer Therapy', 27 August 2013, ASC Nano, Vol. 7, No: 8, pages 6545-6554; published on-line 10 July 2013. Scheme 1; page 6547	1-73
X	WO 2011/113054 A2 (AURASENSE LLC) 15 September 2011 Abstract; Figure 1; claims; paragraphs [0010], [0045], [0053], [0054], [0056], [0061], [0081], [00106]-[00107], [00111], [00117], [00118], [00151], [00153]-[00156], [00159], [00171], [00191]	1-73
X	US 2014/0065425 A1 (UNIVERSITY OF MASSACHUSETTS) 06 March 2014 Claims 25, 30, 42-44; paragraphs [0018], [0044]	1-73
A	WO 2011/072133 A1 (WILLIAM MARSH RICE UNIVERSITY) 16 June 2011 Claims	1-73
A	WO 1998/004740 A1 (NORTHWESTERN UNIVERSITY) 05 February 1998 Pages 5, 6, 9, 38	1-73
A	GRIJALVO, S. et al., 'Oligonucleotide delivery: a patent review (2010 -2013), 2014, Expert Opinion on Therapeutic Patents, Vol. 24, No.7, pages 801-819, published on-line 05 May 2014. Whole document	1-73
P,X	BARNABY, S. N., et al., 'Therapeutic Applications of Spherical Nucleic Acids,' 21 April 2015, Cancer treatment and research, Vol. 166, pages 23-50. Whole document	1-9, 12, 15, 16, 21-24, 26, 27-34, 38-40, 42-53, 63 AND 71-73
P,X	RANDERIA, P. S., et al., 'What Controls the Hybridization Thermodynamics of Spherical Nucleic Acids?', 4 March 2015, Journal of the American Chemical Society, Vol. 137, No. 10, pages 3486-3489. Whole document	1-4, 9, 12, 15, 16, 21, 22, 23, 26, 27, 29-32, 38, 42, 43 AND 53
P,X	ROUGE, J. L., et al., 'Spherical nucleic acids as a divergent platform for synthesizing RNA-nanoparticle conjugates through enzymatic ligation', 21 August, 2014, ACS Nano, Vol. 8, No. 9, pages 8837-8843. Whole document	1-4, 8, 9, 12, 14, 15, 17, 18, 21-23, 26, 27, 29-32, 38, 42, 43 AND 63
	WO 2015/013673 A1 (AURASENSE THERAPEUTICS, LLC) 29 January 2015	

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/US2015/038771**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Abstract; claims; pages 2, 16-18, 24-26, 31; Table 1; Figure 1B; claims	1-5, 8, 9, 12, 14, 15-23, 26, 27, 29-32, 38, 42-45, 47-49, 51, 52 AND 60-67

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2015/038771**

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INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2015/038771**

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INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2015/038771**

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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
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WO 2015/013673 A1	29 January 2015	WO 2015013673 A1	29 Jan 2015

End of Annex

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