**Title**: REMOTE OPTICAL SWITCH FOR LOCALIZED CONTROL OF GENE INTERFERENCE

**Abstract**: The disclosure provides a remote control switch of gene interference in living cells by using Oligonucleotides on a Nanoplasmonic Carrier-based Optical Switch (ONCOS) and a near infrared (NIR) laser transmitter. It is possible activate a variety of different nanoscale receivers by tuning optical transmission frequency, which is selectively determined by matching the resonant transmission frequency with the gold nanoplasmonic particle (GNP) carriers.
Remote Optical Switch for Localized Control of Gene Interference

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. §119 from Provisional Application Serial No. 61/076,805, filed June 30, 2008, the disclosure of which is incorporated herein by reference.

TECHNICAL FIELD

[0002] The disclosure provides compositions and methods for modulating gene expression. More particularly, the disclosure provides oligonucleotides conjugated to nanostructures for gene modulation.

BACKGROUND

[0003] There are currently no methods or compositions for modulating gene expression on a per cell level.

SUMMARY

[0004] The disclosure provides a remote optical switch of gene interference with having spatial and temporal control in living cells. Nanoparticle optical switches carry gene interfering oligonucleotides into cells. These nanoparticle optical switches can, for example, be activated to thermally release an interfering oligonucleotide using light by converting optical energy to thermal heat at the surface of the nanoparticle. Various wavelengths of light can be used depending upon the type (e.g., compositions, geometry etc.) of the nanoparticle. For example, nanoparticles can be tuned such that optical activation can be achieved at a specific near infrared wavelength with a longer penetration depth and where cellular photo-damage is minimized. High spatial resolution of gene interference is possible from the activation of a single nanoparticle optical switch. High temporal resolution is possible by remote control of the optical source. The disclosure provides a new paradigm for
systems biology, cancer biology, and molecular cell biology
where gene interference can be controlled precisely in space
and in time.

[0005] The disclosure provides a composition comprising a
nanostructure that absorbs energy at a near infrared
wavelength; and a double stranded oligonucleotide comprising a
sense strand and an antisense strand, wherein the sense strand
is conjugated to the nanostructure and the antisense strand is
hybridized to the sense strand. In a further embodiment, the
nanostructure is selected to produce a photothermal energy
upon contact with a near infrared wavelength that is equal to
or higher than the $T_m$ of the double stranded oligonucleotide.
In one embodiment, the sense oligonucleotide is conjugated to
the nanostructure via a thiol linkage. In another embodiment,
the antisense strand is complementary to a sense strand of a
target gene. In yet another embodiment, the nanostructure
comprises a geometry selected from a bowl, a crescent, a rod,
a square, a hollow sphere, a solid sphere and a triangle. In
yet a further embodiment, the nanostructure comprises a noble
metal (e.g., gold). In one embodiment, the composition further
comprises a pharmaceutically acceptable carrier. In yet
another embodiment, the sense, antisense or both the sense and
antisense oligonucleotides comprise at least one modified base
or modified sugar backbone of an oligonucleotide. The at
least one modified base or modified sugar backbone of an
oligonucleotide renders may be useful for improving nuclease
resistance.

[0006] The disclosure also provides a composition
comprising a nanostructure that absorbs energy at a near
infrared wavelength; a single stranded fixed strand
oligonucleotide, or a chemically modified derivative of a
single stranded oligonucleotide analog which is resistant to
nucleases, wherein the single stranded oligonucleotide is
conjugated to the surface of the nanostructure; a second
single stranded therapeutic oligonucleotide, or a chemically
modified derivative of a single stranded oligonucleotide
analog which resistant to nucleases, wherein the therapeutic
stranded hybridizes to the fixed strand, wherein the nanostructure is covalently bound to the fixed strand and wherein the fixed strand is bound to the therapeutic strand by non-covalent Watson-Crick base-pairing interactions, or Hoogstein base-pairing interactions. In one embodiment, the fixed strand is covalently bound to the nanostructure via a thiol linkage. In another embodiment, the therapeutic strand comprises an oligonucleotide, or is a chemically modified derivative of an oligonucleotide analog, comprising an antisense sequence which is complementary to the sense strand of a target gene or target mRNA. In yet a further embodiment, the therapeutic strand is an oligonucleotide, or is a chemically modified derivative of an oligonucleotide analog, comprising an RNA interference agent having a sequence which, through its interaction with endogenous cellular enzymes, macromolecules, and/or biomolecular structures, results in the suppression of the biosynthesis of the protein product of a target gene, through a down-regulation effect upon the messenger RNA (mRNA) corresponding to the target gene. In one embodiment, the nanostructure comprises a geometry selected from a bowl, a crescent, a rod, a square, a hollow sphere, a solid sphere and a triangle. In another embodiment, the nanostructure comprises a noble metal (e.g., gold). The composition may further comprise a pharmaceutically acceptable carrier. In yet another embodiment, an additional organic molecule and/or molecular chain is conjugated to the surface of the nanostructure to aid in the beneficial biocompatibility and to aid in the beneficial absorption, distribution, metabolism, and elimination (ADME) behavior of the composite nanostructure in a subject.

[0007] The disclosure also provides a method of modulating gene expression or gene product production in a cell comprising contact a cell with any one of the compositions described above and herein and contacting the cell with a wavelength that causes excitation of the nanostructure for a sufficient time to cause denaturation of double stranded oligonucleotide. In one embodiment, the cell is in vivo. In
another embodiment, the cell is in vitro. In yet a further embodiment, the cell comprises a phenotype of a disease or disorder caused by overexpression of a gene.

[0008] The disclosure also provides a method of modulating gene expression or gene product production in a cell comprising heating of a composite nanostructure, the composite nanostructure comprising (i) a nanostructure, conjugated to (ii) a fixed strand oligonucleotide, wherein the fixed strand oligonucleotide is bonded to (iii) a complementary therapeutic strand oligonucleotide to form a duplex. In one embodiment, the therapeutic strand is an antisense strand. In a further embodiment, the heating is by near infrared irradiation so as to result in the thermal dissociation or thermal denaturation of the duplex to release the therapeutic strand from the nanostructure complex. In another embodiment, the duplex comprises a therapeutic RNAi molecule. In one embodiment, the duplex is selectively denatured in a tissue. In yet another embodiment, the tissue is a disease tissue. In a further embodiment, the tissue is in vivo. The method can be used to treat a subject comprising a disease or disorder associated with overexpression of a gene. In yet another embodiment, the sense, antisense or both the sense and antisense oligonucleotides comprise at least one modified base or modified sugar backbone of an oligonucleotide. The at least one modified base or modified sugar backbone of an oligonucleotide renders may be useful for improving nuclease resistance. In one embodiment, the nanostructure upon contact with a near infrared wavelength generates heat equal to or higher than the $T_m$ of a double stranded oligonucleotide comprising the fixed and therapeutic strand oligonucleotides.

[0009] The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

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Figure 1A-B depicts an embodiment of the disclosure comprising gene release by ONCOS activation. (a) Thiol-modified sense oligonucleotides are attached to gold nanoparticle (GNP) carriers. Antisense oligonucleotides are then hybridized to the sense oligonucleotides. NIR illumination photothermally heats the gold carriers, causing the double-stranded oligonucleotides to denature at their melting temperature and the antisense oligonucleotides to be released from the carriers. (b) ONCOS-activated gene interference: Conjugated gold nanoparticles (GNPs) are internalized within cells. The figure illustrates transcription of DNA into mRNA, followed by NIR activation to photothermally heat GNP carriers and release antisense oligonucleotides within the cells, the binding of the antisense oligonucleotides to mRNA, the digestion of the heteroduplex mRNA by RNase H enzyme, thus silencing the gene of interest at the translational step and ultimately protein translation is halted. Since old protein is continually degraded and no new protein is being synthesized, a decrease in the overall protein expression occurs.

Figure 2A-G shows experimental characterization of ONCOS activation. (a) Tunable rod-shaped GNP carriers based on different aspect ratios. Each GNP solution corresponds to a maximum absorbance (left to right): (i) 652 nm, (ii) 676 nm, (iii) 694 nm, (iv) 715 nm, (v) 785 nm. (b) Scanning electron microscopy image showing the GNPs’ aspect ratio (length/diameter) is 3.5. The aspect ratio is tuned so maximum light absorbance is at a wavelength in the NIR range of interest (785 nm). (c) Axisymmetric Femlab simulation demonstrating localized heat distribution at the GNP’s surface at steady-state. (d) For experimental characterization of temperature on the nanoparticle, melting profiles for three different lengths of oligonucleotides (15 bp, 25 bp, and 50 bp) with known melting temperatures (50°C, 63°C, and 70°C respectively) are collected. Antisense oligonucleotides are FAM-labeled. Temperature is correlated to input energy by analyzing their melting profiles. (e) Absorption spectrum of

[0012] Figure 3A-B shows in vitro demonstration of gene release by ONCOS activation. Concept of localized activation of oligonucleotide release from immobilized nanoparticles. Fluorescence is from the TAMRA-labeled antisense strand of the double-stranded oligonucleotides attached to immobilized nanoparticles on glass. (a) Concept and fluorescent images before ONCOS activation. (b) Concept and fluorescence images after localized oligonucleotide release.

[0013] Figure 4A-C shows visualization of intracellular ONCOS activation. Concept of localized activation of oligonucleotide release inside BT474 breast carcinoma cells. Antisense oligonucleotides are FAM-labeled. (a) Concept and fluorescence images before ONCOS activation. Inset shows surface plot of fluorescence intensity of a cell (located on the left-hand side) before ONCOS activation. (b) Concept and brightfield image of localized NIR activation. (c) Concept and fluorescent images after localized release of oligonucleotides inside BT474 breast carcinoma cells. Inset shows surface plot of fluorescence intensity of a cell (located on the left-hand side) after ONCOS activation.

[0014] Figure 5A-D shows ERBB2 gene interference by ONCOS. (a) Concept, flow cytometric results, and representative image of indirect immunofluorescent staining of ERBB2 after 48 hrs in control cells which have been treated with conjugated GNP carriers but have not been exposed to NIR light. (b) Concept, flow cytometric results, and representative image of indirect immunofluorescent staining of ERBB2 after 48 hours in control cells which do not contain conjugated GNP carriers but have
been exposed to light. (c) Concept, flow cytometric results, and representative image of indirect immunofluorescent staining ERBB2 in ONCOS-activated BT474 breast carcinoma cells. (d) Table summarizing flow cytometric results by % of cells whose protein levels have dropped below threshold, average fluorescence intensity μ (a.u.), and coefficient of variation CV (a.u.).

[0015] Figure 6A-C shows ERBB2 gene interference by ONCOS. (a) Calcein AM, a dye which converts from nonfluorescent cell-permeant calcein AM into fluorescent calcein by intracellular esterase enzymes in living cells, is used as a measure of cell viability. Flow cytometry is used to analyze the samples 2 days and 5 days after activation (b) Representative DIC image of BT474 cells after ONCOS activation. (c) Representative fluorescence image of live/dead cells after ONCOS activation. Live cells are stained with Calcein AM. Dead cells are stained with Ethidium homodimer.

[0016] Other embodiments of the invention will be apparent from detailed description, examples and the appended claims.

DETAILED DESCRIPTION

[0017] As used herein and in the appended claims, the singular forms "a," "and," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nanoparticle" includes a plurality of such particles and reference to "the oligonucleotide" includes reference to one or more oligonucleotides and so forth.

[0018] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although any methods and reagents similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, the exemplary methods and materials are now described.

[0019] Also, the use of "or" means "and/or" unless stated otherwise. Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting.
[0020] It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of."

[0021] All publications mentioned herein are incorporated herein by reference in full for the purpose of describing and disclosing the methodologies, which are described in the publications, which might be used in connection with the description herein. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior disclosure.

[0022] The disclosure provides nanostructure-oligonucleotide composites and nanostructure compositions useful for modulating and regulating gene expression and protein production. The disclosure provides a nanoscale biophotonic transmitter and receiver system, ONCOS, which can modulate (e.g., inhibit or interfere) with gene expression in a single cell, cells or tissues for applications requiring nanometer-scale targeted release and precise temporal control of release.

[0023] Using the methods and compositions of the disclosure it is possible to control the temporal and localized delivery of therapeutics or diagnostic agents for treatment of various diseases, genetic studies, systems biology, and molecular cell biology-related studies. Using the devices, compositions and methods described herein gene interference can be programmed at a desired phase of a cell cycle or a certain moment after external growth factor stimulation, making it possible to study dynamic changes in genetic response that would normally be hidden by ensemble averaging of bulk population response.

[0024] The disclosure provides technology that allows for the perturbation of single gene expression and the study of
this single effect on an entire system of genes and proteins, thus having far-reaching implications in biotechnology, systems biology, cancer therapy and gene therapy.

[0025] As described herein, a better, more precise control of gene interference in living cells is useful for studying cellular signaling pathways, quantitative cell biology, systems biology, and molecular cell biology. In order to advance these dynamic cellular studies, nanoscale intracellular transmitter and receiver systems are required for the remote manipulation of biological systems. Control of the spatial and temporal resolution of optical activation, as well as the selective coupling of optical transmission frequency to different nanoscale transmitters is provided. The disclosure provides a remote control switch of gene interference in living cells by using Oligonucleotides on a Nanoplasmonic Carrier-based Optical Switch (ONCOS) and a near infrared (NIR) laser transmitter.

[0026] Using the methods of the disclosure it is possible to activate a variety of different nanoscale receivers by tuning optical transmission frequency, which is selectively determined by matching the resonant transmission frequency with the metal nanoplasmonic structure (e.g., a gold nanoplasmonic particle (GNP)) carriers. This is accomplished by tuning the plasmon resonant wavelength based on the nanoparticles size, composition and geometry (Fig. 2A, E). For example, the nanoparticles can be tuned as nanoscale receivers such that optical activation can be achieved at a specific NIR wavelength with a longer penetration depth and where cellular photo-damage is minimized.

[0027] Optically-active nanoparticle receivers are conjugated with an oligonucleotide (e.g., a single stranded oligonucleotide) (referred to herein as the “fixed strand”). In one embodiment, fixed strand oligonucleotide is in a sense orientation relative to an expressed gene. The oligonucleotide can form a duplex or double stranded molecule comprising a sense and antisense strand. In one embodiment, the antisense strand is not conjugated to the nanoparticle,
but rather forms base pairs with a complementary oligonucleotide that is conjugated to the nanoparticle to form a duplex. The complementary oligonucleotides (e.g., a sense and antisense strand, dsRNA, dsDNA etc.) form nanostructure carrier-receivers. These devices (i.e., nanostructure complexes) can then be introduced into cells, a tissue or a subject. At desired times and at specific intracellular locations, the therapeutic oligonucleotides are released from their carriers via photothermal heating of the nanostructure carrier-receivers with a remote optical excitation. The photothermal heating causes a localized increase in the area surrounding the nanoparticle (e.g., within a few nanometers to 10 or 100 of nanometers) sufficient to denature the double stranded oligonucleotide (e.g., sense/antisense duplex), thereby releasing the therapeutic oligonucleotide from the carrier-nanostructure. In another embodiment, the duplex oligonucleotide comprises an RNAi that upon delivery undergoes processing within a cell. This method accomplishes gene interference or expression inhibition with nanometer-scale spatial resolution (less than 100 nm resolution) and with minimal temperature disturbance to the surrounding cellular environment, since the heat transfer from the surface of nanoparticle exponentially decays within a few nanometers, as previously shown using nanocrescents and nanoshells.

[0028] Any of a various number of metallic, metal alloy or layered metallic materials can be used in the methods and compositions of the disclosure.

[0029] Metals, alloys and materials useful for the formation of a nanostructure of the disclosure can be obtained based upon a functional layer or thermal bias layer. The material is selected from the group of noble metal and transition metal including, but not limited to, Ag, Au, Cu, Al, Fe, Co, Ni, Ru, Rh, Pd, and Pt. A further surface functional layer can be added or formed in combination with the noble or transition metal core material. Such functional layers can include, but are not limited to, Ag oxide, Au oxide, SiO₂, Al₂O₃, Si₃N₄, Ta₂O₅, TiO₂, ZnO, ZrO₂, HfO₂, Y₂O₃, Ti
oxide, antimony oxide, and other oxides; Ag doped with chlorine or chloride, Au doped chlorine or chloride, Ethylene and Chlorotrifluoroethylene (ECTFE), Poly(ethylene-co-butyl acrylate-co-carbon monoxide) (PEBA), Poly(allylamine hydrochloride) (PAH), Polystyrene sulfonate (PSS), Polytetrafluoroethylene (PTFE), Polyvinyl alcohol (PVA), Polyvinyl chloride (PVC), Polyvinylidene fluoride (PVDF), Polyvinylprorolidone (PVP), and other polymers; stacked multiple layers at least two layers including above listed metal layers and non-metal layers, and the like. A typical material is a metal such as Au, Ag, Ti, Ni, Cr, Pt, Ru, Ni--Cr alloy, NiCrN, Pt--Rh alloy, Cu--Au--Co alloy, Ir--Rh alloy or/and W--Re alloy. The material used should be biocompatible.

[0030] The geometry or structure of the nanomaterial can incorporate the functional capabilities of nanotip, nanosphere, and nanoring geometries. Other geometries can include spherical, circular, triangle, quasi-triangle, square, rectangular, hexagonal, oval, elliptical, rectangular with semi-circles or triangles and the like. The nanostructures of the materials and geometries ideally have an absorbance or excitation wavelength in the near infrared range. Selection of suitable materials and geometries are known in the art. Excitation at longer wavelengths provides deeper penetration into tissue with minimal photothermal damage, and excitation of the nanostructure does not cause fluorescence of other biomolecules.

[0031] Various nanostructure geometries are capable near-infrared (NIR) excitation. For example, nanopins, crescents, bowls, hollow spheres and the like (see, e.g., International Application Publ. No. WO/2006/099494, the disclosure of which is incorporated herein) have a higher local field-enhancement factor in the near-infrared wavelength region due to the simultaneous incorporation of SERS hot spots including sharp nanotip and nanoring geometries, leading to the strong hybrid resonance modes from nanocavity resonance modes and tip-tip intercoupling modes. The structures of the disclosure have a
much stronger field emitting or "antenna" effect than those obtained from nanotips and nanorings. The excited "hotspot" of such structure have been demonstrated to have an enhancement factor larger than $10^{10}$.

[0032] One of skill in the art will recognize that the size, shape, and thickness or where multi-layers are present layer thickness can all be individually controlled by modifying the size of a sacrificial nanostructure template, the deposition angle, the deposited layer thickness, and the material of each layer. Since the plasmon-resonance wavelength of the metallic nanostructures is dependent on these parameters, the optical properties of the nanostructure are tunable during fabrication.

[0033] In one embodiment, the nanostructure comprises a spherical or semi-spherical structure commonly produced in the art. In another embodiment, the nanostructure has the shape of a crescent moon when viewed cross-sectionally, such nanostructures, called "nanocrescent SERS probes" and "nanobowls" herein, may also include two or more layers of different metals.

[0034] For example, asymmetric, hollow metallic nanocrescent SERS probes feature a large surface area (for better molecular adsorptions) and a long edge length for the maximized total integration of multiple surface-enhanced Raman scattering (SERS) spectroscopy tips when compared with conventional spherical and/or solid-core nanoshells. Owing to its hollowness, the inner and outer surfaces can be modified with different materials for a wide variety of optical characteristics. Moreover, the sharp edge of the nanocrescent SERS probes results in even higher degree of field enhancement.

[0035] The metallic composition of composite nanostructures of the disclosure are biocompatible, and thus can be bio-functionalized.

[0036] The term "functionalized" is meant to include structures with two or more layers of different metals, structures with functional groups attached thereto, and the
like. For example, to form a linkage to an oligonucleotide, to prolong or target analyte interaction with a noble metal nanostructure, a binding agent/targeting domain can be used to promote interaction of a nanostructure with a desired target. An alkanethiol, such as 1-decanethiol, can be used to form the capture layer on the noble metal (Blanco Gomis et al., J. Anal. Chim. Acta 436:173 [2001]; Yang et al., Anal. Chem. 34:1326 [1995]). Other exemplary capture molecules include longer-chained alkanethiols, cyclohexyl mercaptan, glucosamine, boronic acid and mercapto carboxylic acids (e.g., 11-mercaptopoundecanoic acid).

[0037] Attached functional groups are used to attach thiolated oligonucleotides. The disclosure demonstrates that a thiol-group containing oligonucleotide molecule, can be attached to the surface of the nanostructure through Au sulfide bonds.

[0038] Oligonucleotides contemplated for attachment to a nanoparticle include those which modulate expression of a gene product expressed from a target gene. Accordingly, antisense oligonucleotides which hybridize to a target polynucleotide and inhibit expression, translation, siRNA oligonucleotides which hybridize to a target polynucleotide and initiate an RNAse activity (for example RNAse H), triple helix forming oligonucleotides which hybridize to double-stranded polynucleotides and inhibit transcription, and ribozymes which hybridize to a target polynucleotide and inhibit translation can be used in the methods, devices and compositions of the disclosure.

[0039] Each nanostructure utilized in the methods, devices and compositions described herein has a plurality of oligonucleotides conjugated to the nanostructure. As a result, each nanostructure-oligonucleotide device has the ability to deliver a plurality of therapeutic (e.g., antisense) oligonucleotides. For example, if a specific mRNA is targeted, a single nanostructure has the ability to deliver multiple copies of an inhibitory/therapeutic oligonucleotide.
[0040] In one embodiment, methods are provided wherein the
nanostructure is functionalized with identical
oligonucleotides, i.e., each oligonucleotide has the same
length and the same sequence. In another embodiment, the
nanostructure is functionalized with two or more
oligonucleotides which are not identical, i.e., at least one
of the attached oligonucleotides differ from at least one
other attached oligonucleotide in that it has a different
length and/or a different sequence. In another embodiment,
wherein different oligonucleotides are attached to the
nanostructure, these different oligonucleotides can bind to
the same single target gene or mRNA but at different
locations, or bind to different target genes or mRNA which
encode different gene products Accordingly, in various
aspects, a single functionalized nanostructure may be used as
a method to inhibit expression of more than one gene product.
The therapeutic oligonucleotides can thus target specific
genes or mRNAs, whether at one or more than one specific
region in the target gene or mRNA, or over the entire length
of the target gene or mRNA as the need may be to effect a
desired level of inhibition of gene product expression.

[0041] In yet another embodiment, the oligonucleotides are
designed with knowledge of the target sequence. Methods of
making oligonucleotides of a predetermined sequence are well-
known (see, e.g., Sambrook et al., Molecular Cloning: A
Laboratory Manual (2nd ed. 1989) and F. Eckstein (ed.)
Oligonucleotides and Analogues, 1st Ed. (Oxford University
Press, New York, 1991)). Solid-phase synthesis methods can be
used for both oligoribonucleotides and
oligodeoxyribonucleotides (the well-known methods of
synthesizing DNA are also useful for synthesizing RNA).
Oligoribonucleotides and oligodeoxyribonucleotides can also be
prepared enzymatically. Synthesized oligonucleotides can have
modified nucleotides that increase stability and/or have
reduced degradation from nucleases.

[0042] In yet another embodiment, oligonucleotides are
selected from a library (see, e.g., U.S. Patent Application
Publication 20050214782). Such libraries can be screened for biologically active therapeutic oligonucleotides, using the methods, devices and compositions described herein.

[0043] The term "oligonucleotides" as used herein refers to DNA or RNA of a length of about three to several hundred bases in length, but typically about 5-200, about 5-100, about 5-50, about 5-40, about 5-35, about 5-30, about 5-25, about 5-20, or about 5-15 nucleotides in length. An oligonucleotide includes modified forms as discussed herein as well as those otherwise known in the art which are used to regulate gene expression. An oligonucleotide can include naturally-occurring nucleotides as well as modified nucleotides or combinations thereof that can be polymerized into a molecule that functions in a biological organism. Methods are also contemplated wherein the oligonucleotide is about 5 to about 90 nucleotides in length, about 5 to about 80 nucleotides in length, about 5 to about 70 nucleotides in length, about 5 to about 60 nucleotides in length, about 5 to about 50 nucleotides in length about 5 to about 45 nucleotides in length, about 5 to about 40 nucleotides in length, about 5 to about 35 nucleotides in length, about 5 to about 30 nucleotides in length, about 5 to about 25 nucleotides in length, about 5 to about 20 nucleotides in length, about 5 to about 15 nucleotides in length, about 5 to about 10 nucleotides in length, and all oligonucleotides intermediate in length of the sizes specifically disclosed to the extent that the oligonucleotide is able to achieve the desired result. Accordingly, oligonucleotides of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, and 100 nucleotides in length are contemplated.

[0044] "Hybridization" means an interaction between two strands of nucleic acids by hydrogen bonds in accordance with
the rules of Watson-Crick DNA complementarity, Hoogstein binding, or other sequence-specific binding known in the art. Hybridization can be performed under different stringency conditions known in the art.

[0045] In various embodiments, the methods include use of an oligonucleotide which is 100% complementary to the target gene or mRNA. In other embodiments, the oligonucleotide is at least 70%, 80%, 85%, 90%, 95%, 98%, or 99% complementary to the target gene or mRNA over the length of the oligonucleotide and the oligonucleotide is able to achieve the desired degree of inhibition of a target gene product expression.

[0046] It is understood in the art that the sequence of an antisense strand need not be 100% complementary to that of its target gene or mRNA to be specifically hybridize to the gene or mRNA. It will also be understood that an oligonucleotide may hybridize over one or more segments such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure or hairpin structure). Percent complementarity of an antisense strand with a region of a target gene or mRNA can be determined using routine publicly available algorithms (e.g., BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., J. Mol. Biol., 1990, 215, 403-410; Zhang and Madden, Genome Res., 1997, 7, 649-656)).

[0047] In other embodiments, the devices, methods and compositions include oligonucleotides which are DNA oligonucleotides, RNA oligonucleotides, or combinations of the two types. Modified forms of oligonucleotides are also contemplated which include those having at least one modified internucleotide linkage or modified nucleobase. In one embodiment, the oligonucleotide is all or in part a peptide nucleic acid. Other modified internucleoside linkages include at least one phosphorothioate linkage. Still other modified oligonucleotides include those comprising one or more universal bases. "Universal base" refers to molecules capable of substituting for binding to any one of A, C, G, T and U in nucleic acids by forming hydrogen bonds without significant
structure destabilization. The oligonucleotide incorporated with the universal base analogues is able to function as a probe in hybridization, as a primer in PCR and DNA sequencing. Examples of universal bases include but are not limited to 5' nitroindole-2'-deoxyriboside, 3-nitropyrrrole, inosine and hypoxanthine.

[0048] Oligonucleotides may also include base modifications or substitutions. As used herein, "unmodified" or "natural" bases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified bases include other synthetic and natural bases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiouracil, 5-halouracil and cytosine, 5-propynyl uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-aminoadenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified bases include tricyclic pyrimidines such as phenoxazine cytidine (1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzox-azin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified bases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Certain of these bases are useful for increasing the binding affinity and include 5-
substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 degree. C. and are, in certain aspects combined with 2'-O-methoxyethyl sugar modifications. See, U.S. Pat. No. 3,687,808; U.S. Pat. Nos. 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; 5,750,692 and 5,681,941, the disclosures of which are incorporated herein by reference.

[A0049] A "modified base" or other similar term refers to a composition which can pair with a natural base (e.g., adenine, guanine, cytosine, uracil, and/or thymine) and/or can pair with a non-naturally occurring base. In certain aspects, the modified base provides a Tm differential of 15, 12, 10, 8, 6, 4, or 2 °C or less.

[A0050] Oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. Modified oligonucleotide backbones containing a phosphorus atom include, for example, chiral phosphorothioates, phosphorothioates, phosphorodithioates, aminoalkylphosphotriesters, phosphotriesters, methyl and alkyl phosphonates (e.g., chiral phosphonates, 5'-alkylene phosphonates, 3'-alkylene phosphonates), phosphinites, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage.

[A0051] Modified oligonucleotide backbones that do not include a phosphorus atom can be used in the methods, device and compositions of the disclosure. For example, an
oligonucleotide backbone lacking a phosphorus atom include those having backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages; siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

[0052] In another embodiment, oligonucleotide mimetics wherein both one or more sugar and/or one or more internucleotide linkage of the nucleotide units are replaced with "non-naturally occurring" groups. The bases of the oligonucleotide are maintained for hybridization with the target polynucleotide. In one embodiment, a peptide nucleic acid (PNA) can be used. In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone.

[0053] In yet another embodiment, oligonucleotides are provided with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and including -CH₂-NH-O-CH₂-, -CH₂-N(CH₃)O-CH₂-, -CH₂-O-N(CH₃)-CH₂-, -CH₂-N(CH₃)-N(CH₃)-CH₂- and -O-N(CH₃)-CH₂-CH₂-. Also contemplated are oligonucleotides with morpholino backbone structures.

[0054] Modified oligonucleotides may also contain one or more substituted or modified sugar moieties. A sugar backbone may comprise any naturally occurring sugar. Examples of naturally occurring sugars include, but are not limited to, ribose, deoxyribose, and/or 2-deoxyribose. Sugar units of a backbone may be modified such that the modified sugar backbone is resistant to cleavage. The sugars of a backbone may be modified by methods known in the art, for example, to achieve resistance to nuclease cleavage. Examples of modified sugars
include, but are not limited to, 2'-O-alkyl riboses, such as 2'-O-methyl ribose, and 2'-O-allyl ribose. The sugar units may be joined by phosphate linkers. Typical sugar units of the invention may be linked to each other by 3'-5', 3'-3', or 5'-5' linkages. Additionally, 2'-5' linkages are also possible if the 2' OH is not otherwise modified.

[0055] A non-sugar backbone may comprise any non-sugar molecule to which bases may be attached. Non-sugar backbones are known in the art. Examples include, but are not limited to, morpholino and peptide nucleic acids (PNAs). A morpholino backbone is made up of morpholino rings (tetrahydro-1,4-oxazine) and may be joined by non-ionic phosphorodiamidate groups. Modified morpholinos known in the art may be used in the present invention.

[0056] PNAs result when bases are attached to an amino acid backbone by molecular linkages. Examples of such linkages include, but are not limited to, methylene carbonyl, ethylene carbonyl, and ethyl linkages. The amino acids may be any amino acid, natural or non-natural, modified or unmodified, and are preferably alpha amino acids. The amino acids may be identical or different from one another. One non-limiting example of a suitable amino acid includes an amino alkyl-amino acid, such as (2-aminoethyl)-amino acid.

[0057] Examples of PNAs include, but are not limited to, N-(2-aminoethyl)-glycine, cyclohexyl PNA, retro-inverso, phosphene, propynyl, and aminoproline-PNA. PNAs may be chemically synthesized by methods known in the art. Examples include, but are not limited to, modified Fmoc and/or tBoc peptide synthesis protocols.

[0058] An oligonucleotide of the disclosure may comprise one of the following at the 2' position: OH; P; O, S, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted 1-10 carbon alkyl or 2-10 carbon alkenyl and alkynyl. Other side groups may be linked to the nucleotide base to improve pharmacokinetic properties or pharmacodynamic properties of an oligonucleotide.
[0059] In another embodiment, a modified oligonucleotide can comprise Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring, thereby forming a bicyclic sugar moiety.

[0060] Another modification of the oligonucleotides contemplated involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. These moieties or conjugates can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups contemplated include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligonucleotides, and groups that enhance the pharmacokinetic properties of oligonucleotides. Typical conjugate groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhoda-mines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties include groups that improve uptake, enhance resistance to degradation, and/or strengthen sequence-specific hybridization with the target nucleic acid. Groups that enhance the pharmacokinetic properties include groups that improve uptake, distribution, metabolism or excretion of the compounds of the present invention. Also contemplated are groups that enhance binding or association of the oligonucleotide or a targeting agent to its target (either the target polynucleotide of target of the targeting agent) by bringing either or both into proximity of the target through association or interaction with the actin/myosin intracellular framework, the early to late endosome framework, the translational to endoplasmic reticulum to golgi network pathway, and the like).

[0061] Still other conjugate moieties include proteins, peptides, and peptide mimetics. In one aspect, members from this group of moieties are selected based on their binding specificity to a ligand expressed in or on a target cell type
or a target organ. Alternatively, moieties of this type include a receptor for a ligand on a target cell (instead of the ligand itself), and in still other aspects, both a receptor and its ligand are contemplated in those instances wherein a target cell expresses both the receptor and the ligand. In other aspects, members from this group are selected based on their biological activity, including for example enzymatic activity, agonist properties, antagonist properties, multimerization capacity (including homo-multimers and hetero-multimers). With regard to proteins, conjugate moieties contemplated include full length protein and fragments thereof which retain the desired property of the full length proteins. Fusion proteins, including fusion proteins wherein one fusion component is a fragment or a mimic, are also contemplated. This group also includes antibodies along with fragments and derivatives thereof, including but not limited to Fab’ fragments, F(ab) fragments, Fv fragments, Fc fragments, one or more complementarity determining regions (CDR) fragments, individual heavy chains, individual light chain, dimeric heavy and light chains (as opposed to heterotetrameric heavy and light chains found in an intact antibody, single chain antibodies (scAb), humanized antibodies (as well as antibodies modified in the manner of humanized antibodies but with the resulting antibody more closely resembling an antibody in a non-human species), chelating recombinant antibodies (CRABs), bispecific antibodies and multispecific antibodies, and other antibody derivative or fragments known in the art.

[0062] Modified oligonucleotides can increase resistance to nuclease degradation, increase cellular uptake, increased stability and/or increased binding affinity for the target gene or mRNA. In other embodiments, the modification serves as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide-mediated inhibition of gene expression. The cleavage of
RNA:RNA hybrids can, in like fashion, be accomplished through the actions of endoribonucleases, such as RNAseL which cleaves both cellular and viral RNA. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

[0063] Oligonucleotides, particularly the fixed strand or sense strand can be conjugated to a nanostructure through any number of techniques known in the art. Regardless of the method by which the oligonucleotide is attached to the nanostructure, attachment can be accomplished through a 5' linkage, a 3' linkage, some type of internal linkage, or any combination of these attachments.

[0064] In one embodiment, the nanostructure, the fixed oligonucleotide or both are functionalized in order to attach the oligonucleotide to the nanostructure. Methods to functionalize nanostructures and oligonucleotides are known in the art. For instance, oligonucleotides functionalized with alkanethiols at their 3'-termini or 5'-termini readily attach to gold nanostructures (see, e.g., Whitesides, Proceedings of the Robert A. Welch Foundation 39th Conference On Chemical Research Nanophase Chemistry, Houston, Tex., pp. 109-121, 1995; and Mucic et al., Chem. Commun., pp. 555-557, 1996). The alkanethiol method can also be used to attach oligonucleotides to other metal, semiconductor and magnetic colloids and to the other types of nanostructures described herein. Other functional groups for attaching oligonucleotides to solid surfaces include phosphorothioate groups (see, for example, U.S. Pat. No. 5,472,881 for the binding of oligonucleotide-phosphorothioates to gold surfaces), substituted alkylsiloxanes (see, for example, Burwell, Chemical Technology, 4:370-377, 1974; and Matteucci and Caruthers, J. Am. Chem. Soc., 103: 3185-3191, 1981) for binding of oligonucleotides to silica and glass surfaces, and Grabar et al., Anal. Chem., 57:735-743, for binding of aminoalkylsiloxanes and for similar binding of mercaptoalkylsiloxanes). Oligonucleotides with a 5'

Cyclic disulfides may be used to functionalize oligonucleotides in certain and have 5 or 6 atoms in their rings, including two sulfur atoms. Suitable cyclic disulfides are available commercially or are synthesized by known procedures. When cyclic disulfides are used, oligonucleotides are attached to a nanostructure through one or more linkers. The linker can comprise a hydrocarbon moiety attached to a cyclic disulfide. Suitable hydrocarbons are available commercially, and are attached to the cyclic disulfides.

Method are provided wherein the oligonucleotide is bound to the nanostructure at a surface density of at least about 0.3 pmol/cm² to 50 pmol/cm² or more.

In some embodiments, functionalized nanostructure are conjugated to oligonucleotides through a spacer. A spacer includes, for example, a polymer, an oligonucleotide, a
peptide, an oligosaccharide, a carbohydrate, a lipid, an ethylglycol, or combinations thereof. The spacer serves to extend the distance between the nanostructure surface and the fixed oligonucleotide or therapeutic oligonucleotide (indirectly).

[0068] The target gene or mRNA may be present in a eukaryotic cell or prokaryotic cell. An mRNA target typically includes an mRNA encoding a gene product, wherein the ONCOS device, composition and method (upon activation) inhibits translation of the gene product. Where the target is a gene, transcription of the gene product is inhibited. For example, a target region of a target gene or mRNA can include the 5' untranslated region (5'UTR), the portion of an mRNA in the 5' direction from the translation initiation codon, including nucleotides between the 5' cap site and the translation initiation codon of an mRNA (or corresponding nucleotides on the gene), and the 3' untranslated region (3'UTR), the portion of an mRNA in the 3' direction from the translation termination codon, including nucleotides between the translation termination codon and 3' end of an mRNA (or corresponding nucleotides on the gene).

[0069] An inhibiting effective amount of an ONCOS construct of the disclosure refers to that amount wherein expression of the target gene product is inhibited by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% compared to gene product expression in the absence of an ONCOS construct.

[0070] The ONCOS construct comprising an antisense oligonucleotide can be used to downregulate or silence the translation or transcription (i.e., expression) of a gene of interest. Genes of interest include, but are not limited to,
genes associated with viral infection and survival, genes associated with metabolic diseases and disorders (e.g., liver diseases and disorders), genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0071] Genes associated with viral infection and survival include those expressed by a virus in order to bind, enter, and replicate in a cell. Of particular interest are viral sequences associated with chronic viral diseases. Viral sequences of particular interest include sequences of Hepatitis viruses (Hamasaki et al., FEBS Lett., 543:51 (2003); Yokota et al., EMBO Rep., 4:602 (2003); Schlomai et al., Hepatology, 37:764 (2003); Wilson et al., Proc. Natl. Acad. Sci., 100:2783 (2003); Kapadia et al., Proc. Natl. Acad. Sci., 100:2014 (2003); and FIELDS VIROLOGY (Knipe et al., eds. 2001)); Human Immunodeficiency Virus (HIV) (Banerjea et al., Mol. Ther., 8:62 (2003); Song et al., J. Virol., 77:7174 (2003); Stephenson, JAAM, 289:1494 (2003); Qin et al, Proc. Natl. Acad. Sci., 100:183 (2003)); Herpes viruses (Jia et al., J. Virol., 77:3301 (2003)); and Human Papilloma Viruses (HPV) (Hall et al, J. Virol., 77:6066 (2003); Jiang et al., Oncogene, 21:6041 (2002)). Exemplary hepatitis viral nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences involved in transcription and translation (e.g., En1, En2, X, P) and nucleic acid sequences encoding structural proteins (e.g., core proteins including C and C-related proteins, capsid and envelope proteins including S, M, and/or L proteins, or fragments thereof) (see, e.g., FIELDS VIROLOGY, 2001, supra). Exemplary Hepatitis C nucleic acid sequences that can be silenced include, but are not limited to, serine proteases (e.g., NS3/NS4), helicases (e.g. NS3), polymerases (e.g., NS5B), and envelope proteins (e.g., E1, E2, and p7). Hepatitis A nucleic acid sequences are set forth in, e.g., Genbank Accession No. NC001489; Hepatitis B nucleic acid sequences are set forth in,
e.g., Genbank Accession No. NC003977; Hepatitis C nucleic acid sequences are set forth in, e.g., Genbank Accession No. NC004102; Hepatitis D nucleic acid sequence are set forth in, e.g., Genbank Accession No. NC001653; Hepatitis E nucleic acid sequences are set forth in, e.g., Genbank Accession No. NC001434; and Hepatitis G nucleic acid sequences are set forth in, e.g., Genbank Accession No. NC001710. Silencing of sequences that encode genes associated with viral infection and survival can conveniently be used in combination with the administration of conventional agents used to treat the viral condition.

[0072] Genes associated with metabolic diseases and disorders (e.g., disorders in which the liver is the target and liver diseases and disorders) include, for example, genes expressed in dyslipidemia (e.g., liver X receptors such as LXRα and LXRβ (Genbank Accession No. NM007121), farnesoid X receptors (FXR) (Genbank Accession No. NM005123), sterol-regulatory element binding protein (SREBP), Site-1 protease (SIP), 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG coenzyme-A reductase), Apolipoprotein (ApoB), and Apolipoprotein (ApoE); and diabetes (e.g., Glucose 6-phosphatase) (see, e.g., Forman et al., Cell, 81:687 (1995); Seol et al., Mol. Endocrinol., 9:72 (1995), Zavacki et al., PNAS USA, 94:7909 (1997); Sakai et al., Cell, 85:1037-1046 (1996); Duncan et al., J. Biol. Chem., 272:12778-12785 (1997); Willy et al., Genes Dev., 9:1033-1045 (1995); Lehmann et al., J. Biol. Chem., 272:3137-3140 (1997); Janowski et al., Nature, 383:728-731 (1996); and Peet et al., Cell, 93:693-704 (1998)). One of skill in the art will appreciate that genes associated with metabolic diseases and disorders (e.g., diseases and disorders in which the liver is a target and liver diseases and disorders) include genes that are expressed in the liver itself as well as and genes expressed in other organs and tissues. Silencing of sequences that encode genes associated with metabolic diseases and disorders can conveniently be used in combination with the administration of conventional agents used to treat the disease or disorder.
Examples of gene sequences associated with tumorigenesis and cell transformation include translocation sequences such as MLL fusion genes, BCR-ABL (Wilda et al., Oncogene, 21:5716 (2002); Scherr et al., Blood, 101:1566 (2003)), TEL-AML1, EWS-FLI1, TLS-FUS, PAX3-FKHR, BCL-2, AML1-ETO, and AML1-MTG8 (Heidenreich et al., Blood, 101:3157 (2003)); overexpressed sequences such as multidrug resistance genes (Nieth et al., FEBS Lett., 545:144 (2003); Wu et al., Cancer Res., 63:1515 (2003)), cyclins (Li et al., Cancer Res., 63:3593 (2003); Zou et al., Genes Dev., 16:2923 (2002)), beta-Catenin (Verma et al., Clin Cancer Res., 9:1291 (2003)), telomerase genes (Kosciolek et al., Mol Cancer Ther., 2:209 (2003)), c-MYC, N-MYC, BCL-2, ERBB1, and ERBB2 (Nagy et al. Exp. Cell Res., 285:39 (2003)); and mutated sequences such as RAS (reviewed in Tuschl and Borkhardt, Mol. Interventions, 2:158 (2002)). Silencing of sequences that encode DNA repair enzymes find use in combination with the administration of chemotherapeutic agents (Collis et al., Cancer Res., 63:1550 (2003)). Genes encoding proteins associated with tumor migration are also target sequences of interest, for example, integrins, selectins, and metalloproteinases. The foregoing examples are not exclusive. Any whole or partial gene sequence that facilitates or promotes tumorigenesis or cell transformation, tumor growth, or tumor migration can be included as a template sequence.

Angiogenic genes are able to promote the formation of new vessels. Of particular interest is Vascular Endothelial Growth Factor (VEGF) (Reich et al., Mol. Vis., 9:210 (2003)) or VEGFr. siRNA sequences that target VEGFr are set forth in, e.g., GB 2396864; U.S. Patent Publication No. 20040142895; and CA2456444.

Anti-angiogenic genes are able to inhibit neovascularization. These genes are particularly useful for treating those cancers in which angiogenesis plays a role in the pathological development of the disease. Examples of anti-angiogenic genes include, but are not limited to, endostatin (see, e.g., U.S. Pat. No. 6,174,861), angiostatin (see, e.g.,
U.S. Pat. No. 5,639,725), and VEGF-R2 (see, e.g., Decaussin et al., J. Pathol., 188: 369-377 (1999)).

[0076] Immunomodulator genes are genes that modulate one or more immune responses. Examples of immunomodulator genes include, without limitation, cytokines such as growth factors (e.g., TGF-α, TGF-β, EGF, FGF, IGF, NGF, PDGF, CGF, GM-CSF, SCF, etc.), interleukins (e.g., IL-2, IL-4, IL-12 (Hill et al., J. Immunol., 171:691 (2003)), IL-15, IL-18, IL-20, etc.), interferons (e.g., IFN-α, IFN-β, IFN-γ, and the like) and TNF. Fas and Fas Ligand genes are also immunomodulator target sequences of interest (Song et al, Nat. Med., 9:347 (2003)). Genes encoding secondary signaling molecules in hematopoietic and lymphoid cells are also included in the present invention, for example, Tec family kinases such as Bruton's tyrosine kinase (Btk) (Reinonen et al, FEBS Lett., 527:274 (2002)).

[0077] Cell receptor ligands include ligands that are able to bind to cell surface receptors (e.g., insulin receptor, EPO receptor, G-protein coupled receptors, receptors with tyrosine kinase activity, cytokine receptors, growth factor receptors, etc.), to modulate (e.g., inhibit, activate, etc.) the physiological pathway that the receptor is involved in (e.g., glucose level modulation, blood cell development, mitogenesis, etc.). Examples of cell receptor ligands include, but are not limited to, cytokines, growth factors, interleukins, interferons, erythropoietin (EPO), insulin, glucagon, G-protein coupled receptor ligands, etc. Templates coding for an expansion of trinucleotide repeats (e.g., CAG repeats) find use in silencing pathogenic sequences in neurodegenerative disorders caused by the expansion of trinucleotide repeats, such as spinobulbar muscular atrophy and Huntington's Disease (Caplen et al., Hum. Mol. Genet., 11:175 (2002)).

[0078] In addition to its utility in silencing the expression of any of the above-described genes for therapeutic purposes, the ONCOS technology described herein is also useful in research and development applications as well as diagnostic, prophylactic, prognostic, clinical, and other healthcare applications. As a non-limiting example, the
therapeutic or diagnostic oligonucleotide in the ONCOS construct can be used in target validation studies directed at testing whether the gene of interest has the potential to be a therapeutic target.

[0079] The disclosure provides a non-limiting example of the process using breast carcinoma cells and optically activating the release of antisense oligonucleotides at a specific time and blocking the translation of the ERBB2 (also known as HER-2, neu, and EGFR-2) mRNA, with precise position, minimal toxic effects and high post treatment cell viability. The methods and compositions of the disclosure have applicability to any number of oligonucleotides useful for inhibiting expression and to any number of cell or tissue types. Thus, the disclosure has far-reaching implications in biotechnology, systems biology, and gene therapy.

[0080] In one embodiment, ONCOS uses near infra-red (NIR)-absorbing gold nanoparticles (GNPs) that are conjugated with the sense strand of a double-stranded oligonucleotide. The double-stranded complex is formed by a thiol-modified sense oligonucleotide attached to the GNP’s surface (Fig. 1A) and an antisense (e.g., siRNA) oligonucleotide hybridized to the sense strand.

[0081] In addition to a conjugated oligonucleotide, a targeting ligand can be conjugated to the nanostructure (e.g., a receptor bound to the surface of a nanostructure that interacts reversibly or irreversibly with a specific analyte). Alternatively or in addition an uptake moiety can be linked to the nanoparticle (e.g., a TAT moiety, see, for example, International Patent Publ. No. WO/2007/095152). Examples of targeting ligands include antigen-antibody pairs, receptor-ligand pairs, and carbohydrates and their binding partners. Binding ligands to a wide variety of analytes are known or can be readily identified using known techniques. As will be appreciated by those in the art, any two molecules that will associate, may be used, either as the analyte or the functional group (e.g., targeting/binding ligand). Suitable analyte/binding ligand pairs include, but are not limited to,
antibodies/antigens, receptors/ligand, proteins/nucleic acids; nucleic acids/nucleic acids, enzymes/substrates and/or inhibitors, carbohydrates (including glycoproteins and glycolipids) /lectins, carbohydrates and other binding partners, proteins/proteins; and protein/small molecules. In one embodiment, the binding ligands are portions (e.g., the extracellular portions) of cell surface receptors.

[0082] Alternatively, a self-assembled monolayer (SAM) can be formed on the nanostructure surface to concentrate the analyte of interest near the surface of the nanostructure. Exemplary SAMs include, but are not limited to, 4-aminothiophenol, L-cystein, 3-mercaptopropionicacid, 11-mercaptopoundecanoic acid, 1-hexanethiol, 1-octanethiol, 1-DT, 1-hexadecanethiol, poly-DL-lysine, 3-mercaptop-1-propanesulfonic acid, benzenethiol, and cyclohexylmercaptan. Typically the SAM is comprised of straight chain alkanethiols.

[0083] In some embodiments, polyethylene glycol (PEG) is immobilized on nanostructure surfaces to prevent nonspecific interactions and prolong circulation times. In some embodiments, silica sensor surfaces not coated with silver are PEGylated with silane terminated monomethoxy PEG and silver coated nanoparticle surfaces are coated with oligoethylene glycol terminated alkanethiols.

[0084] In yet another aspect biotin-avidin functionalization can be used. Biotin and avidin are capable of interacting with one another to form a binding complex.

[0085] In order to accomplish selective optical control of gene release, specific size and geometry of nanoplasmic carriers (i.e. GNPs) are used in the design of ONCOS. GNPs were selected because of their stable and nontoxic properties in biological applications. For example, the rod-shaped GNPs have an aspect ratio (length/diameter) of 3.5, as shown in a scanning electron microscopy image in Fig. 2B. Such geometry allows highly efficient photothermal conversion due to the matched resonant frequency, making it possible to activate gene release with minimized optical exposure time and low optical power. Other geometries can be used having similar
properties. The nanostructures can be excited at a wavelength of between about 800-1300 nm. In one aspect, the nanostructure (e.g., the composition and/or geometry) used in the methods, compositions and devices of the disclosure is selected for the generation of a photothermal energy upon contact with an electromagnetic radiation sufficient to increase the temperature of the milieu surrounding the oligonucleotide to above the $T_m$ (i.e., sufficient to denature the double stranded oligonucleotide). GNPs are one type of nanostructure useful in the disclosure. GNPs were selected because of the maximum light absorbance at a wavelength in the NIR range of interest (e.g., about 785 nm). Since cells are essentially transparent in this wavelength regime (e.g. about 800-1300 nm), cell damage can be minimized while the cell is exposed to the light source. Furthermore, due to the GNPs’ large surface area, each GNP is able to carry hundreds of oligonucleotides, making it is possible to interfere with gene expression from the activation of a single GNP. Using the surface density of single-stranded DNA ($9.0 \times 10^{12}$ ssDNA molecules/cm$^2$) and the dimensions of the GNP (radius=7.5 nm, height=50 nm), it is estimated that each GNP will release approximately 250 molecules of oligonucleotides upon activation.

[0086] When light is absorbed by a nanostructure of the disclosure (e.g., a GNP) at a specific NIR wavelength, the resulting excess energy is dissipated in the conversion of optical energy to heat, otherwise known as the photothermal effect. When the temperature on the nanostructure (e.g., GNP) reaches the melting temperature of the oligonucleotides, the double-stranded oligonucleotides denature. The sense oligonucleotides remain attached to the nanostructure (e.g., GNP) through thiol bonds while the antisense oligonucleotides are released. The heat transfer from the surface of nanostructure to the surrounding cellular environment is highly localized and decays exponentially within a few nanometers (Fig. 2c).

[0087] This optical switch can be used to interfere with gene expression and protein expression in living cells. At the
beginning of protein production, mRNA is transcribed from DNA in the nucleus and exported out to the cytoplasm (Fig. 1B, part 1). The mRNA is then translated into the corresponding amino acids. This primary sequence of amino acids then folds into its final protein structure and is transported to the proper location in the cell.

[0088] The nanostructures conjugated to the oligonucleotides are internalized within living cells. When NIR light is used to activate this optical switch, the antisense oligonucleotides linked to a sense strand which in turn is linked to the nanostructure are released and bind to a portion of a complementary mRNA target (Fig. 1B, part 2). Once an mRNA heteroduplex is formed, which is recognized and degraded by RNase H enzymes within the cell, thereby inhibiting mRNA translation and thus, interfering with the expression of that gene product.

[0089] RNA Interference ("RNAi") or RNA silencing is an evolutionarily conserved process by which specific mRNAs can be targeted for enzymatic degradation. To initiate the RNAi pathway, a double-stranded RNA (dsRNA) is introduced into a cell. The dsRNA is converted into multiple siRNA duplexes of 21-23 bp length ("siRNAs") by the enzymatic activity of dsRNA-specific endonucleases ("Dicer") of the RNase III family. The siRNAs are subsequently recognized by RNA-induced silencing complexes ("RISC") that promote the unwinding of the siRNA through an ATP-dependent process. The unwound antisense strand of the siRNA guides the activated RISC to the targeted mRNA comprising a sequence complementary to the siRNA antisense strand. The targeted mRNA and the antisense strand form an A-form helix, and the major groove of the A-form helix is recognized by the activated RISC. The mRNA is cleaved by activated RISC at a single site defined by the binding site of the 5'-end of the siRNA strand. The activated RISC is recycled to catalyze another cleavage event.

[0090] In one embodiment, the disclosure provides double-stranded ribonucleic acid (dsRNA) molecule, wherein a fixed strand (in some embodiments, the sense strand) is conjugated
to a nanostructure. In this embodiment, the thermal activation of the nanostructure causes a release and processing of the dsRNA to form siRNA molecules capable of silencing expression of a target gene or inhibiting mRNA translation. In one embodiment, the dsRNA comprises and RNAl molecule.

[0091] In another embodiment, the duplex comprises a fixed strand and a "non-fixed strand" (e.g., a strand complementary and bound to the fixed strand) wherein the duplex comprises e a dsDNA or dsRNA or a chimeric duplex of DNA and RNA that can be thermally activated to release the non-fixed strand at NIR wavelengths for inhibiting the expression of a target gene in a cell. In one embodiment, the double stranded nucleic acid comprises an antisense strand comprising a region of complementarity which is complementary to at least a part of an mRNA formed in the expression of the target gene, and wherein the region of complementarity is less than 30 nucleotides in length and wherein the sense strand of the dsRNA is conjugated to the nanostructure.

[0092] The dsRNA comprises two RNA strands that are sufficiently complementary to hybridize to form a duplex structure. One strand of the dsRNA (the antisense strand) comprises a region of complementarity that is substantially complementary, and typically fully complementary, to a target sequence, derived from the sequence of an mRNA formed during the expression of the target gene, the other strand (the sense strand) comprises a region which is complementary to the antisense strand, such that the two strands hybridize and form a duplex structure when combined under suitable conditions. The duplex structure is between about 15 and 30 (e.g., between about 18 and 25), typically between about 19 and 24 (e.g., between 21 and 23) base pairs in length. Similarly, the region of complementarity to the target sequence is between 15 and 30 (e.g., between about 18 and 25), typically between about 19 and 24 (e.g., between 21 and 23) base pairs in length. The dsRNA of the disclosure may further comprise one or more single-stranded nucleotide overhang(s). The dsRNA can
be synthesized by standard methods known in the art, e.g., by use of an automated DNA synthesizer, such as are commercially available from, for example, Biosearch, Applied Biosystems, Inc. The dsRNA can comprise a ribonucleotide backbone or a modified backbone.

[0093] The compositions of the disclosure are useful for modifying expression of a gene in a cell, tissue or subject. For example, to modulate expression of a target gene in a cell (e.g., for research purposes or therapeutic purposes), a nanostructure such as a GNP comprising a double-stranded oligonucleotide is delivered to a cell. The nanostructure is then contacted with a wavelength of energy that causes a thermal change (i.e., an increase) in the nanostructure or local environment for a sufficient period of time to cause denaturation of the double-stranded oligonucleotide. Upon denaturation the fixed strand oligonucleotide (in some embodiments the sense strand) remains conjugated to the nanostructure while the therapeutic strand become biological active either as an siRNA or an antisense molecule. In one embodiment, the therapeutic strand oligonucleotide is an antisense strand that binds to the mRNA comprising the target sequence or the target gene. In another embodiment, a nanostructure comprising a double-stranded oligonucleotide is delivered to a tissue, either locally or systemically (e.g., comprising a targeting ligand specific for a cell-type or tissue). In yet another embodiment, a nanostructure comprising a double-stranded oligonucleotide is delivered to a subject.

[0094] The methods of the disclosure are useful for treating diseases or disorders wherein aberrant (e.g., increased) gene expression is present. For example, the methods and compositions of the disclosure are useful for treating or studying cell proliferative disorder such as cancer, inflammatory disorder and autoimmune disorders to name a few.

[0095] A nanostructure comprising a conjugated oligonucleotide can be formulated in pharmaceutically
acceptable carrier. Pharmaceutically acceptable carriers useful for administration to a cell, tissue or subject are well known in the art and include, for example, aqueous solutions such as water or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters. A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of the conjugate. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the physico-chemical characteristics of the therapeutic agent and on the route of administration of the composition, which can be, for example, orally or parenterally such as intravenously, and by injection, intubation, or other such method known in the art. The pharmaceutical composition also can contain a second (or more) compound(s) such as a diagnostic reagent, nutritional substance, toxin, or therapeutic agent, for example, a cancer chemotherapeutic agent and/or vitamin(s).

[0096] In some embodiments, the invention provides kits and systems for modulating gene expression in a sample or subject.

[0097] Excitation of the nanostructures of the disclosure can be performed by contacting the nanostructure with appropriate electromagnetic radiation (e.g., an excitation wavelength). Wavelengths in the visible spectrum comprise light radiation that contains wavelengths from approximately 360 nm to approximately 800 nm. Ultraviolet radiation comprises wavelengths less than that of visible light, but greater than that of X-rays, and the term "infrared spectrum" refers to radiation with wavelengths of greater 800 nm.
Typically, the desired wavelength can be provided through standard laser and electromagnetic radiation techniques.

[0098] The following examples are intended to illustrate but not limit the disclosure. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

**EXAMPLES**

[0099] The disclosure demonstrates the efficacy of ONCOS for the remote control of photothermal gene release. GNPs are conjugated with double-stranded oligonucleotides and immobilized onto a glass surface. The exemplary experiments are based on permanently-attached nanoparticles. The antisense oligonucleotides are labeled with fluorescent dye to visually monitor their release using fluorescence microscopy. To demonstrate wavelength specificity, a laser at the peak optical absorption (785 nm) is compared to a laser outside the peak optical absorption (658 nm) for photothermal release. The entire viewing window is illuminated using each laser, which is positioned above the sample, and an inverted microscope operating in epi-fluorescence mode is used to visualize ONCOS gene release. Photothermal release is monitored by observing the fluorescence intensity of an area away from the conjugated GNPs. Initially, the normalized fluorescence intensity of this area is set to 0. When the temperature on the nanoparticle reaches the melting temperature of the double-stranded oligonucleotides, the antisense oligonucleotides are released and the fluorescence intensity in the observed area increases. Fig. 2f shows that the photothermal efficiency is two and a half times greater at the peak optical absorption wavelength.

[00100] To minimize laser exposure to cells, the minimal power necessary to achieve photothermal release was used. Conjugated GNPs, with fluorescently-labeled antisense strands, are immobilized onto a glass surface and a 785 nm laser is used to illuminate the GNPs at various power densities: 2
mW/cm², 19 mW/cm², and 46 mW/cm². Fig. 2g illustrates that the photothermal efficiency rises with increasing power densities.

[00101] The temperature on the surface of the GNPs was also analyzed. If three different oligonucleotides of known length (15 bp, 25 bp, and 50 bp) with known melting temperatures (50°C, 63°C, and 70°C respectively) are photothermally melted, the temperature at the surface of the nanostructure could be correlated as a function of input energy by visualizing their melting profiles. When the temperature on the GNPs reaches the melting temperature of the oligonucleotides for each length, the fluorescent intensity decreases sharply, indicating that the antisense oligonucleotides have been released into solution. Using each profile, the energy required to reach each melting temperature is approximated by \( E = \alpha P t \), where \( E \) is the energy in J/cm², \( \alpha \) is the absorptivity of gold, \( P \) is the power of the laser in W/cm², and \( t \) is the time in seconds (shown in inset of Fig. 2d). Experimental characterization shows that melting temperatures as low as 50 °C requires 2 mJ/cm² of energy and temperatures as high as 70 °C requires 5 mJ/cm² of energy. This demonstrates that low temperature activation can be achieved with ONCOS.

[00102] In addition to experimental analysis, FEMLab™ software is used to simulate an axisymmetric model of the heat distribution of a single GNP at steady-state (Fig. 2c). Fig. 2c shows the temperature distribution as a function of distance from the surface of the GNP. From the surface of the GNP outward, the temperature profile begins at the melting temperature of the oligonucleotides and falls off exponentially to 37°C within 100 nm from the surface of the GNP. This demonstrates that the heat generated using ONCOS is highly localized to the surface of the GNP and should not propagate significantly intracellularly. Thus, cell viability will remain uncompromised after ONCOS activation.

[00103] As a qualitative test of ONCOS’ high spatial resolution, optically-controlled gene release was performed both outside and inside of biological cells. Here, a NIR laser is focused to a 5 μm diameter spot size using an objective
lens located above the sample. Using this focused NIR laser, certain immobilized GNP s (with fluorescently-labeled antisense oligonucleotides) are illuminated while other neighboring GNP s are not (Fig. 3). When the temperature on the GNP s reaches the melting temperature, the antisense oligonucleotides are released into solution and the fluorescence intensity on the illuminated GNP s vanish (Fig. 3b).

[00104] Having established that oligonucleotides can be released in a highly-localized manner, the localized release of oligonucleotides within BT474 breast carcinoma cells was investigated. GNP s with fluorescently-labeled antisense strands are internalized within a population of cells, as shown in the fluorescence image in Fig. 4a. Control experiments were carried out to verify that cells lacking the fluorescently-labeled antisense strands did not show fluorescence. To show localized photothermal gene release, a focused NIR laser is used to illuminate a specific location within a cell, as shown in the brightfield image in Fig. 4b. When the temperature reaches the melting temperature, the double-stranded oligonucleotides denature and the labeled antisense strands diffuse into the surrounding cytosol.

[00105] To ensure that oligonucleotides are well-dispersed in the cells after ONCOS activation, the illumination time was chosen such that it was longer than the estimated diffusion time of the oligonucleotides. During illumination, no oligonucleotide back-binding of released oligonucleotides back onto the GNP s occurs due to the elevated temperature at the surface of the GNP s. The diffusion time is approximately 30 s, assuming the diffusion length is approximated by the diameter of the cell (10^{-5} m) and the diffusion coefficient is 3 \times 10^{12} m^2/s) for a 100 bp oligonucleotide. Therefore, the cells were illuminated for 2 minutes at 2 mW/cm^2 to allow enough time for oligonucleotides to be well-dispersed in the cytoplasm. A surface plot of the fluorescence intensity is shown as an inset in Fig. 4a and c to clearly demonstrate the decrease in intensity at the illuminated location while the surrounding areas remain unaffected. At the area of laser activation, the
surface plot shows that the maximum peak fluorescence intensity decreases and broadens out at the activated area. Control experiments were carried out to confirm that change in fluorescence is not due to the movement of the GNPs themselves during activation.

[00106] After validating steps essential in using ONCOS, the methods and compositions were used in breast carcinoma cells to block the mRNA translation of ERBB2, an oncoprotein commonly overexpressed in 20-30% of breast cancers. General transfection methods of bringing DNA into cells, such as viral vectors or cationic liposome-mediated transfection, lack the spatial and temporal control of gene interference. Nevertheless, as a conventional measure of gene interference, a multi-component lipid-based transfection reagent was used to initially determine the optimal modulation of ERBB2 expression in BT474 breast carcinoma cells by antisense oligonucleotides. ERBB2 receptors are stained with fluorescently-labeled antibodies against ERBB2. Flow cytometry results show that there is no significant change in expression 24 hours after transfection. However, for 9.0% of transfected cells, the ERBB2 levels dropped below threshold after 48 hours.

[00107] After determining the experimental parameters for inhibiting ERBB2 expression using conventional transfection methods, conjugated GNPs are internalized within cultured BT474 breast carcinoma cells. It has been suggested that there are between 168 and 336 ERBB2 molecules per cell. Since it was estimated that each GNP releases approximately 250 molecules of oligonucleotides, a difference in expression was expected since there should be enough released oligonucleotides to bind with ERBB2 mRNA. To initiate photothermal gene release, a NIR laser is used to illuminate an entire population of cells. The cells are then allowed to culture for 48 hours. Two control experiments are conducted to insure that ONCOS activation is the reason for gene interference: (1) cells which have internalized conjugated GNPs but have not been exposed to NIR illumination (Fig. 5a) and (2) cells which do not contain GNPs but are exposed to NIR illumination (Fig. 5b). If ONCOS is in
fact inhibiting ERBB2 expression, both control samples should express ERBB2 while ONCOS-activated cells should not (Fig. 5c). To quantitatively analyze ERBB2 inhibition, ONCOS-activated cells and both control samples are stained with PE-labeled antibodies against ERBB2 and their fluorescence is analyzed by flow cytometry. The flow cytometric results (mean μ, coefficient of variation CV, % cells whose protein levels which have dropped below threshold) for the ONCOS-activated sample and both control samples are summarized in a table in Fig. 5d. Fig. 5 shows that for 13.2% of the cells treated with ONCOS, the protein levels dropped below threshold. It is especially noteworthy to point out that the average ERBB2 protein level of the entire cell population decreased by 55% after ONCOS activation. In Fig. 5, the average fluorescence intensity of the control sample (contains no GNPs but illuminated with light) is 178.6 a.u. and the average fluorescence intensity of the ONCOS-activated sample is 79.5 a.u. The average fluorescence intensity of the control sample which contains GNPs but is not illuminated with light is 199.4 a.u. To qualitatively visualize ERBB2 inhibition, the cells are fixed and immunostained with fluorescently labeled antibodies against the ERBB2 (Fig. 5c, d). The decrease in fluorescence in the ONCOS-activated cells versus the control sample indicates that ONCOS has effectively interfered with ERBB2 gene expression.

[00108] In order for ONCOS to be used as a spatially- and temporally-controlled gene interference method, viability after ONCOS activation is desirable. Calcein AM, a dye which converts from nonfluorescent cell-permeant calcein AM into fluorescent calcein by intracellular esterase enzymes in living cells, is used as a measure of cell viability. Flow cytometry is used to analyze the samples 2 days and 5 days after activation. As a control for the Calcein AM dye, cells are intentionally killed using sonication and stained with calcein AM. Two days after activation, cell samples show between 96-99% viability. Five days after activation, cell samples show between 93-97% viability (Fig. 6a). There is only
2-3% variation in the viability between the control sample which contains GNPs but has not been exposed to light, the control sample which does not contain GNPs but has been exposed to light, and the ONCOS-activated sample. A representative DIC image and fluorescent image (live cells stained with Calcein AM, dead cells stained with Ethidium homodimer) of the ONCOS-activated sample is shown in Fig. 6b and c and confirms the efficacy of ONCOS.

[00109] The mechanism by which GNPs enter cells deserves discussion. Conjugated GNPs enter the cytosol by the cells’ internalization mechanism. Since GNPs are encapsulated within endosomes upon internalization, the localized heat generated by ONCOS not only releases oligonucleotides from their carriers but can also denature the endosomes, allowing antisense oligonucleotides to enter the cytosol and bind to mRNA. Charge neutralization of an oligonucleotide can also promote uptake.

[00110] As the flow cytometric results for conventional transfection and ONCOS-activated gene interference (Fig. 5) indicate, the delivery of oligonucleotides into the cell with high efficiency remains challenging. It is however conceivable that this limitation can be overcome. For example, magnetic field driving of GNPs containing magnetic cores may be used to penetrate cell membranes with high efficiency. Once inside the cells, ONCOS can be activated with high efficiency. ONCOS still has several key advantages over conventional methods to inhibit protein translation. Whereas various methods can interfere with gene expression within a population of cells, precise control of single cell’s gene expression is not feasible. ONCOS can be activated to release oligonucleotides within a single cell, enabling the precise regulation of stochastic gene expression to better understand and control cellular signaling pathways. Furthermore, ONCOS provides a temporally-controlled optical switch to precisely regulate a single cell’s gene expression and study its effect on an entire system. Finally, unlike other nanoparticle-based gene interference methods, ONCOS can achieve nanometer-scale
spatial resolution while maintaining high cell viability due
to the low temperature mechanism of release.

[00111] Preparation of oligonucleotide-conjugated GNPs. Rod-
shaped GNPs with aspect ratio of 3.5 (Fig. 2 b) are
synthesized using a previously reported seed-mediated growth
method. As shown in Fig. 2 a and e, these GNPs are selected
because of the maximum light absorbance at a wavelength in the
NIR range of interest (785 nm). Phosphorothioate
oligonucleotides sequences directed against the 5’ region of
the ERBB2 mRNA molecule are purchased from Integrated DNA
Technologies (Coralville, IA). The sense sequence is 3’-
GTGAGCACCATGGAG-5’-SH (SEQ ID NO:1) and the antisense sequence
is 3’ - CTCCATGGTGCTCAC-5’ (SEQ ID NO:2). Unless otherwise
specified, all sequences are purchased from Integrated DNA
Technologies and all sequences are not labeled with dye. To
conjugate sense oligonucleotides with GNPs, 100 µl of 100 µM
thiolated, sense oligonucleotides is incubated with 2000 µl of
GNPs (1x10^9 particles/ml) and 40 µl of phosphate-buffered
saline (PBS) on a rocker for 24 hours. The sense
oligonucleotides attach to the GNPs through the thiol (-SH)
group on the 5’ end. To hybridize antisense oligonucleotides
to the sense oligonucleotides, 100 µl of 100 µM antisense
oligonucleotides is added to the conjugated GNPs solution.
This mixture is then heated for 2 minutes at 80°C and then
heated for 15 minutes at 65°C. The mixture is finally
incubated at room temperature on a rocker for 24 hours to
ensure maximum hybridization. For use with cell culture, the
conjugated GNPs are concentrated to 1x10^14 particles/µL by
centrifugation at 5000 rpm for 30 minutes, removal of the
supernatant, and sonication for 1 minute to resuspend the
GNPs.

[00112] Surface immobilization of conjugated GNPs. TAMRA-
modified (559 nm excitation/583 nm emission), phosphorothioate
antisense oligonucleotides (TAMRA - 3’ - CTCCATGGTGCTCAC-5’
(SEQ ID NO:2) are used to visually monitor the release of
antisense oligonucleotides from the gold GNPs. Conjugated GNPs
are electrostatically attached to the glass slide. The
nanoparticle solution is firstly dispensed onto the glass slide. Immediately after drying on the slide, the nanoparticles were re-immersed in buffer solutions. A few times of washing steps were also performed to remove free standing nanoparticles. When re-immersed in solution, the conjugated GNPs remain attached to the glass surface.

**[00113]** Characterizations of laser power, wavelength specificity, and temperature. A 785 nm laser (model APM50/1557, Power Technologies, Little Rock, AR) is positioned above the sample. The beam of the laser is broadened to a spot size of 5mm in order to illuminate the entire viewing window by using a concave lens (25mm focal length, Edmund Industrial Optics) located above the sample. An inverted microscope operating in epi-fluorescence mode is used to visualize ONCOS gene release.

**[00114]** To characterize laser power, the power intensity is measured using an optical power meter (model 1830-C, Newport Corp, Irvine, CA). Immobilized GNPs with TAMRA-labeled antisense oligonucleotides are illuminated at three different power intensities: 2 mW/cm², 19 mW/cm², and 46 mW/cm² for 30 minutes. At 10 minute intervals, the fluorescence intensity of an area away from the conjugated GNPs is captured using a color CCD camera (Qfire, Olympus America, Inc.).

**[00115]** To characterize wavelength specificity, a 658 nm laser (model LM658-65C, Newport Corporation, Irvine, CA), outside the peak optical absorption, is also used in addition to the 785 nm laser. Immobilized GNPs with TAMRA-labeled antisense oligonucleotides are illuminated at the same power density of 46 mW/cm² for 30 minutes using each laser. At 10 minute intervals, the fluorescence intensity of an area away from the conjugated GNPs is captured using a color CCD camera (Qfire, Olympus America, Inc.). Matlab™ software is used to analyze the fluorescence intensity by integrating the signal over the area of the image. Various areas located at the same radial distance from the immobilized GNPs are measured to obtain statistical information (mean, standard deviation).
[00116] To characterize the temperature on the GNPs, three different lengths of oligonucleotides (15 bp, 25 bp, and 50 bp) with known melting temperatures (50°C, 63°C, and 70°C respectively) are purchased: (15 bp sense) 5'-GTGAGCACCATGGAG-3' SH (SEQ ID NO:3), (15 bp antisense) 5'-CTCCATGGTGCTCAC-3' FAM (SEQ ID NO:4), (25 bp sense) 5'-AACCTCCTCAGACCTCAACCTCAGACCT-3' SH (SEQ ID NO:5), (25 bp antisense) 5'-AGGTTCGAGGAGGTGTTCCGAGGAGGTT-3' FAM (SEQ ID NO:6), (50 bp sense) 5'-AACCTCCTCGAACC TCTCAGACCTCAACCTCAGACCTCAACCTCAGACCT-3' SH (SEQ ID NO:7), and (50 bp antisense) 5'-AGGTTCGAGGAGGTGTTCCGAGGAGGTTAGGTTCC GAGGAGGTTTCGAGGAGGTT-3' FAM (SEQ ID NO:8). Here, all the antisense strands are labeled with FAM dye (495 nm excitation/520 nm emission) on the 3' end to visually monitor the release of antisense oligonucleotides from the GNPs. To characterize the temperature, immobilized GNPs with different lengths of oligonucleotides are illuminated with 2 mW/cm² for 15 minutes. The fluorescence intensity of the conjugated GNPs is captured at 3 frames/sec using a color CCD camera (Qfire, Olympus America, Inc.).

[00117] **Cell preparation.** The human breast carcinoma line BT474 was purchased from the American Type Culture Collection (Rockville, MD). Cells are cultured in DMEM media supplemented with 10% heat-inactivated fetal bovine serum and are maintained in a 37°C incubator with 5% CO₂ humidified air. Cells are initially seeded in either 6-well plate at a cell density of 400,000 cells/well (9.6 cm²) or 96-well plates at 20,000 cells/well. The cells are allowed to plate for 24 hours prior to treatment with conjugated GNPs.

[00118] **Experimental setup for localized activation of photothermal gene release.** To show highly localized activation of photothermal gene release, 785 nm laser is positioned above a sample using a micro-manipulated xyz stage (MP-285, Sutter Instrument Company). The laser is then focused to a 5 μm diameter spot size using a 4X objective lens located above the sample. An inverted microscope operating in epi-fluorescence mode is used to visualize ONCOS gene release. Conjugated GNPs
are illuminated using the focused laser at 2 mW/cm² for 2 minutes without illuminating other neighboring GNFs. Images were captured using a color CCD camera (Qfire, Olympus America, Inc.) before and after activation.

[00119] For highly localized intracellular activation, 10 μL of conjugated GNFs (1x10¹¹ particles/μL) are internalized inside BT474 breast carcinoma cells (which are plated on glass coverslips in the 6-well plates) for 8 hours. The antisense strand is labeled with FAM fluorescent dye. After 8 hours, the coverslips containing cells are removed from the 6-well plate and are washed three times with 1X PBS. The coverslip containing cells is then placed on a microscope slide. Cured PDMS is used to form a well around the coverslip on the microscope slide. This well is then filled with media. Conjugated GNFs are illuminated using the focused laser at 2 mW/cm² for 2 minutes without illuminating other neighboring GNFs within a cell. Images were captured using a highly sensitive monochrome CCD camera (Cascade 512B, Photometrics) before and after activation.

[00120] Experimental setup for conventional transfection of antisense oligonucleotides. Antisense oligonucleotides that are complementary to ERBB2 mRNA (FAM - 3’-CTCCATGGTGCTCAC-5’ (SEQ ID NO:2)) are transfected into BT474 breast carcinoma cells using Fugene 6 (Roche Diagnostics) transfection reagent. Briefly, 10 μl of Fugene is mixed with 90 μl of Optimem media (Gibco) and incubated for 5 minutes at room temperature. Then 0.5 μg of antisense oligonucleotides is added to the Fugene/Optimem mixture and allowed to incubate for 20 minutes at room temperature. Finally, this solution is added to one wells of the 6-well plate containing BT474 cells and media. After 24 hours and 48 hours, the samples are prepared for flow cytometry analysis.

[00121] Experimental setup for ERBB2 interference using ONCOS. 10 μL of conjugated GNFs (1x10¹¹ particles/μL) to each well of the 96-well plate. The BT474 breast carcinoma cells are incubated for 24 hours at 37°C with the conjugated GNFs. After 24 hours, a 785 nm laser is positioned above a sample
and the spot size of the laser is broadened to 5 mm in diameter using a concave lens (25 mm focal length) to illuminate the entire cell sample. The laser is used to illuminate the cells at 2 mW/cm² for 2 minutes. The cells are then incubated at 37°C for 48 hours.

[00122] **Indirect immuno-fluorescence staining of ERBB2.**

Cells are washed three times with 1X PBS, fixed using 2% paraformaldehyde (15712-S, Electron Microscope Sciences, Hatfield, PA) for 10 minutes, and blocked with bovine serum albumin (15260-037, Invitrogen Corporation) for 30 minutes. The cells are then stained for ERBB2 using mouse anti-ERBB2 (OP15T, Calbiochem) primary antibody (1:100 dilution) for 90 minutes and anti-mouse IgG (F5262-1ML, Sigma-Aldrich) secondary antibody conjugated with (1:128 dilution) FITC dye (488 nm excitation/518 nm emission) for 30 minutes.

[00123] **Viability analysis.** Cells are seeded in 6-well plates as explained in the cell preparation method above. After 24 hours, cells are incubated with 10 µl of concentrated GNP (1x10¹⁵ particles/µL) in 2 ml of Optimem media per well for 24 hours. After 24 hours, the cells are washed with 1X PBS and placed back in media. The laser is used to illuminate cells at 2 mW/cm² for 2 minutes. The cells are then incubated at 37°C for 2 days and 5 days.

[00124] Two days and five days, the cells are prepared for analysis with flow cytometry. The cells are not fixed when they are analyzed. The cells are detached from the 6 well plates using 0.25% trypsin for 2 minutes, collected using centrifugation (1100 rpm, 8 minutes), and re-suspended in 1X PBS which contains 2 M Calcein AM. As a control, un-adhered dead cells are collected from the media in the 6 well plates and sonicated for 10 seconds. These dead cells are then collected using centrifugation (1100 rpm, 8 minutes), and re-suspended in 1X PBS which contains 2µM Calcein AM (Invitrogen) for 20 minutes. These samples are then immediately transferred to FACS tubes for flow cytometry analysis. Their fluorescence is analyzed by flow cytometry using the Coulter EPICS XL flow cytometer (Beckman Coulter Inc., Fullerton, CA) at an average
flow rate of 500 cells/second. Flow cytometry profiles were analyzed using WinMDI software.

[00125] To qualitatively view viability, cells are adhered onto glass-cover slips and prepared for ONCOS activation in the same way as explained the above paragraph. Five days after activation, the cells are washed in 1X PBS and incubated in 1X PBS containing 2μM Calcein AM and 4 μM Ethidium homodimer (Invitrogen). The cells are imaged using DIC and fluorescence microscopy.

[00126] Flow cytometry analysis of ERBB2. For flow cytometry analysis, duplicate samples are made in the 96-well plates so that the total cell number per sample is approximately 500,000 cells. After treatment, the cells are washed three times with 1X PBS, detached from the cell culture dish using 1m M EDTA for 10 minutes, collected using centrifugation (1100 rpm, 8 minutes), and re-suspended in PBS containing 2% fetal bovine serum (16000-036, Invitrogen Corporation) and 0.1% sodium azide. The suspended cells are then stained for ERBB2 using 10 μl of mouse anti-ERBB2 conjugated with PE dye (340552, BD Biosciences) for 60 minutes in the dark. Cells in the isotype control are stained with mouse IgG1 conjugated with phycoerythrin (PE) dye (340761, BD Biosciences) to ensure no non-specific antibody binding takes place. The suspended cells are finally fixed in 2% paraformaldehyde for 10 minutes and transferred to FACS tubes for flow cytometry analysis. Their fluorescence is analyzed by flow cytometry using the Coulter EPICS XL flow cytometer (Beckman Coulter Inc., Fullerton, CA) at an average flow rate of 500 cells/second. Flow cytometry profiles were analyzed using WinMDI software.

[00127] A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.
WHAT IS CLAIMED IS:

1. A composition comprising:
   a nanostructure that absorbs energy at a near infrared wavelength;
   a double stranded oligonucleotide comprising a sense strand and an antisense strand, wherein one strand of the double strand oligonucleotide is conjugated to the nanostructure.

2. The composition of claim 1, wherein the sense oligonucleotide is conjugated to the nanostructure via a thiol linkage.

3. The composition of claim 1 or 2, wherein the antisense strand is complementary to a sense strand of a target gene.

4. The composition of claim 1, wherein the nanostructure comprises a geometry selected from a bowl, a crescent, a rod, a square, a hollow sphere, a solid sphere and a triangle.

5. The composition of claim 1 or 4 wherein the nanostructure comprises a noble metal.

6. The composition of claim 5, wherein the noble metal is gold.

7. The compositions of claim 1, further comprising a pharmaceutically acceptable carrier.

8. The composition of claim 1, wherein the sense oligonucleotide comprises at least one modified base or modified sugar backbone of an oligonucleotide.

9. The composition of claim 8, wherein the at least one modified base or modified sugar renders the sense strand oligonucleotide nuclease resistant.
10. The composition of claim 1, wherein the antisense strand comprises at least one modified base or modified sugar backbone of an oligonucleotide.

11. The composition of claim 10, wherein the at least one modified base or modified sugar renders the antisense oligonucleotide nuclease resistant.

12. The composition of claim 1, wherein the nanostructure upon contact with a near infrared wavelength generates heat equal to or higher than the Tm of the double stranded oligonucleotide.

13. The composition of claim 12, wherein the generated heat denatures the double stranded oligonucleotide.

14. A composition comprising:
   a nanostructure that absorbs energy at a near infrared wavelength;
   an single stranded fixed strand oligonucleotide, or a chemically modified derivative of a single stranded oligonucleotide analog which is resistant to nucleases, wherein the single stranded oligonucleotide is conjugated to the surface of the nanostructure;
   a second single stranded therapeutic oligonucleotide, or a chemically modified derivative of a single stranded oligonucleotide analog which resistant to nucleases, wherein the therapeutic stranded hybridizes to the fixed strand, wherein the nanostructure is covalently bound to the fixed strand and wherein the fixed strand is bound to the therapeutic strand by non-covalent Watson-Crick base-pairing interactions, or Hoogstein base-pairing interactions.

15. The composition of claim 14, wherein the fixed strand is covalently bound to the nanostructure via a thiol linkage.
16. The composition of claim 14 or 15, wherein the therapeutic strand comprises an oligonucleotide, or is a chemically modified derivative of an oligonucleotide analog, comprising an antisense sequence which is complementary to the sense strand of a target gene or target mRNA.

17. The composition of claim 14 or 15, wherein the therapeutic strand is an oligonucleotide, or is a chemically modified derivative of an oligonucleotide analog, comprising an RNA interference agent having a sequence which, through its interaction with endogenous cellular enzymes, macromolecules, and/or biomolecular structures, results in the suppression of the biosynthesis of the protein product of a target gene, through a down-regulation effect upon the messenger RNA (mRNA) corresponding to the target gene.

18. The composition of claim 14, wherein the nanostructure comprises a geometry selected from a bowl, a crescent, a rod, a square, a hollow sphere, a solid sphere and a triangle.

19. The composition of claim 14 or 18, wherein the nanostructure comprises a noble metal.

20. The composition of claim 19, wherein the noble metal is gold.

21. The compositions of claim 14, further comprising a pharmaceutically acceptable carrier.

22. The composition of any one of claims 14, 18, 20 or 21, wherein additional organic molecules and/or molecular chains are conjugated to the surface of the nanostructure to aid in the beneficial bio-compatibility and to aid in the beneficial absorption, distribution, metabolism, and elimination (ADME) behavior of the composite nanostructure in a subject.
23. A method of modulating gene expression or gene product production in a cell comprising contact a cell with any one of the compositions of claims 1, 2, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 18, 20 or 21 and contacting the cell with a wavelength that causes excitation of the nanostructure for a sufficient time to cause denaturation of double stranded oligonucleotide.

24. The method of claim 23, wherein the cell is in vivo.

25. The method of claim 23, wherein the cell is in vitro.

26. The method of claim 25, wherein the cell comprises a phenotype of a disease or disorder caused by overexpression of a gene.

27. A method of modulating gene expression or gene product production in a cell comprising heating of a composite nanostructure, the composite nanostructure comprising:

- a nanostructure, conjugated to an oligonucleotide fixed strand oligonucleotide, wherein the fixed strand oligonucleotide is bonded to a complementary therapeutic strand oligonucleotide to form a duplex,

- by near infrared irradiation so as to result in the thermal dissociation or thermal denaturation of the duplex to release the therapeutic strand from the nanostructure.

28. The method of claim 27, wherein the duplex is selectively denatured in a tissue.

29. The method of claim 28, wherein the tissue is a disease tissue.

30. The method of claim 29, wherein the tissue is in vivo.
31. The method of claim 30, wherein the method is used to treat a subject comprising a disease or disorder associated with overexpression of a gene.

32. The method of claim 27, wherein the oligonucleotide fixed strand oligonucleotide comprises at least one modified base or modified sugar backbone of an oligonucleotide.

33. The method of claim 32, wherein the at least one modified base or modified sugar renders the fixed strand oligonucleotide strand nuclease resistant.

34. The method of claim 27, wherein the therapeutic strand oligonucleotide comprises at least one modified base or modified sugar backbone of an oligonucleotide.

35. The method of claim 34, wherein the at least one modified base or modified sugar renders the therapeutic strand oligonucleotide is nuclease resistant.

36. The method of claim 27, wherein the nanostructure upon contact with a near infrared wavelength generates heat equal to or higher than the Tm of the double stranded oligonucleotide.
FIGURE 2
FIGURE 3
a Control: unactivated GNPs & no NIR light

unactivated GNPs

coverslip

b Control: no GNPs exposed to NIR light

NIR light

coverslip

c ONCOS: activated GNPs by NIR light

NIR light

activated GNPs

coverslip

d

<table>
<thead>
<tr>
<th>Sample</th>
<th>% cells below threshold</th>
<th>Mean, ( \mu ) (a.u.)</th>
<th>CV (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control: unactivated GNPs &amp; no NIR light</td>
<td>2.4</td>
<td>199.4</td>
<td>13.72</td>
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<tr>
<td>Control: no GNPs exposed to NIR light</td>
<td>1.5</td>
<td>178.6</td>
<td>10.8</td>
</tr>
<tr>
<td>ONCOS: activated GNPs by NIR light</td>
<td>13.2</td>
<td>79.5</td>
<td>17.18</td>
</tr>
</tbody>
</table>

FIGURE 5
FIGURE 6