METHODS FOR DETERMINING SITE SPECIFIC BINDING OF AT LEAST ONE LIGAND TO A COMPOSITE NUCLEIC ACID, AND KITS THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of Singapore provisional application No.201308043-7, filed 29 October 2013, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates to the field of molecular biology, biochemistry and nanotechnology. More particularly, the invention relates to methods for determining site-specific ligand binding to nucleic acids composites comprising metal nanoparticles.

BACKGROUND OF THE INVENTION

[0003] In molecular biology and genetics, the regulation of gene expression largely relies on the sequence-selective recognition of nucleic acids by repressor and/or activator ligands, such as proteins. For example, one such type of repressor and/or activator proteins are transcription factors (TF), that may perform their function alone or in combination with other proteins in a complex, by promoting (as an activator), or blocking (as a repressor) the recruitment of RNA polymerase (the enzyme that performs the transcription of genetic information from DNA to RNA) to specific genes and binding sequences(Nikolov et al., Proc. Natl. Acad. Sci. U. S. A. 1997, 94, 15-22).

[0004] Often, for gene transcription to occur, a number of TFs must bind to DNA regulatory sequences synergistically. To understand the complex gene regulation mechanisms for a

specific gene regulation, it is critical to reveal the molecular details of the co-localization of multiple proteins.

[0005] Of the techniques commonly utilized to establish nucleic acid binding properties of polypeptides, most of them are technically challenging, require the knowledge of specialized biochemical procedures and expensive equipment, and are time and labor intensive. Footprinting and Electrophoretic mobility shift assay (EMSA), for example, can define a protein's binding selectivity but they involve biohazard labeling and arduous assays. Surface plasmon resonance (SPR) spectroscopy can quantitatively determine protein-DNA binding affinity, sequence selectivity, and binding stoichiometry. But SPR experiments require expensive equipment and extensive optimization of surface chemistry for DNA or protein immobilization. The Fluorescent Intercalator Displacement (FID) assay is nondestructive, technically non-demanding, and amenable for high-throughput assay. However, this method is insensitive for differentiation of subtle affinity difference.

[0006] For studying multiple polypeptide binding to nucleic acids, the above techniques have severe limitations as shown in Figure 2 disclosed herein. In particular, EMSA requires a full set of DNA with selective mutation of each sites in order to determine the preferable protein binding (Petz et al., Mol Endo 2000, 14, 972-985. SPR measurement does not provide protein identity due to the principle of measuring the refractive index. The refractive index change is insensitive to mass change distance within the 200 nm evanescent wave field. The study of multiple protein binding require specific antibody in a successive step (Neo et al., Anal Chem 2009, 81, 3344-3349). Not only this will add additional assay steps, the study of complex protein binding is subject to the availability of specific antibodies for the proteins of

interest. Finally, based on experiments with FID detection of high affinity sequence specific interactions (ERα-ERE), low affinity binding (ERα-½ ERE and Sp1-CG boxes), and nonspecific interaction (Sp1-random DNA), this method can only differentiate high affinity binding relative to low affinity ones. It cannot differentiate low affinity binding and nonspecific binding (i.e. between ERα-½ ERE, Sp1-CG boxes, and Sp1-random DNA). This shows that the traditional FID has limited sensitivity, and is thus incapable of studying site specific protein binding.

[0007] A deeper insight of the ligand binding involved, the delineation of the sequences to which they bind, the interaction between ligands and the subsequent impact on gene transcription action and deactivation holds significant promise in therapeutic medicine.

[0008] As such, there is a need to provide methods that can further elucidate ligand-nucleic acid binding, and particular site specific polypeptide binding, which may at least partially ameliorate one or more of the disadvantages described above with regards to the use of any bulky expensive equipment or consumable items and hazardous labeling.

SUMMARY OF THE INVENTION

[0009] In one aspect, the present invention provides a method for determining site specific binding of at least one ligand to a composite nucleic acid, comprising: providing a composite nucleic acid comprising a metal nanoparticle attached to a nucleic acid having a predetermined length; saturating said composite nucleic acid with an intercalating dye; measuring an initial fluorescence intensity (F_0) of said saturated composite nucleic acid; contacting said saturated composite nucleic acid with said ligand; measuring a further fluorescence

intensity (F_1) of said contacted saturated composite nucleic acid and determining a relative change in fluorescence intensity (ΔF), between F_0 and F_1; and correlating the relative change in fluorescence intensity with the predetermined length of the nucleic acid to determine a distance (R) of the binding of the ligand from the metal nanoparticle.

[0010] In a second aspect, the present invention provides a kit for detecting site specific binding of at least one ligand to a nucleic acid, comprising: a nucleic acid composite comprising a nanoparticle attached to said nucleic acid, and an intercalator dye.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0012] **Figure 1.** Shows a schematic illustration of (A) even InD emission in dsDNA and (B&C) uneven emission in dsDNA conjugated to AuNPs of a smaller (B) or (C) a larger size. According to the function, the AuNPs in B and C are termed "quenching profiler" and "enhancement profiler", respectively. The initial dye intensity in A is denoted as F_0. The resulting intensity in (B) and (C) are denoted as F'_0 and F''_0, respectively. Upon addition of protein, InD will be displaced, as shown representatively by (A). The resulting intensity is denoted as F_1, F'_1 and F''_1, respectively.

[0013] **Figure 2.** Shows a table of limitations of existing techniques for studying multiple protein-composite DNA interactions.

[0014] **Figure 3.** Shows a schematic diagram of the DNA-conjugated AuNPs, showing the total length and the distance of each site to AuNPs of sequences with different spacers between

½ ERE and Sp1 sites. Spacer 4, 8, 12, and 18 are selected to show the distance calculation.

[0015] **Figure 4.** Shows (A) UV-vis spectra of 20 nm AuNP, 100 nm AuNP, and 80 nm AgNP and their conjugates with the 52 bp DNA. (B) TO's emission spectrum when intercalated in dsDNA52-AuNPs20nm. TO's fluorescent intensity increases as the ratio of TO:DNA is incrementally varied from 1:1 to 30:1 molar ratio. The dashed line in (B) (at the top) represents 23:1 of TO:DNA ratio and the dotted line (most bottom) was free TO (without DNA) at the highest concentration used in this study. The AuNPs used to conjugate with the DNA is 20 nm. The DNA is the longest strand of 52 bp. The formed construct is denoted as TO/dsDNA52bp-AuNPs20nm.

[0016] **Figure 5.** Shows (A) Fluorescence curve from TO intercalated into 38 bp DNA, 38 bp DNA-AuNP20nm, and 38 bp DNA-AuNP100nm. (B). resulting intensity (at 535 nm) of saturate amount of TO intercalated to various length DNA in the absence of AuNPs (circle), presence of 20 nm AuNPs (diamond) and 100nm AuNPs (triangle).

[0017] **Figure 6.** Shows relative fluorescence intensity of TO intercalated to DNA-AuNPs20nm of 38, 44, and 52 bp DNA (F'_0), with TO in respective DNA without AuNPs (F_0) as reference.

[0018] **Figure 7.** Shows schematic illustration of the two TO/dsDNA-AuNPs20nm setting used to identify preferential binding site of Sp1 at two Sp1 motifs, with the DNA conjugated to AuNPs at the (A) ½ ERE site and (B) Sp1/P site. The bp number refers to the distance of the Sp1 sites to AuNPs (4bp spacer between ½ ERE and Sp1/D). (C) The relative decrease in fluorescent intensity (at 535 nm) induced by first molar and second molar of Sp1 binding for dsDNA/TO without AuNP and for dsDNA-AuNPs/TO composites in "A setting" and "B setting".

[0019] **Figure 8.** Shows (A) TO fluorescent intensity decrease (at 535 nm) for ½ ERE-Sp1 DNA (spacer 4-18 bp) conjugated at

5′ end to 20nm AuNP, caused by 1 st molar (left bars) and 2nd molar (right bars) Sp1 binding. (B) Decrease in intensity at 535 nm upon binding of 1st molar Sp1 (square) and 2nd molar Sp1 (triangle). The dashed line is the calculated fluorescence contribution for each InD fitted to data in A to deduce the position of protein binding. The relative standard deviation between three repeated experiments are ~5%.

[0020] **Figure 9.** Shows the detection of preferential Sp1 binding to distal Sp1 site using (A) 100 nm AuNPs and (B) 80 nm AgNPs assisted TO displacement. Blue and green bars are the fluorescent intensity drop corresponding to the binding of 1st Sp1. Red and orange bars are the 2nd molar Sp1 binding. (C) TO intensity decrease (at 535 nm) relative to non-enhanced TO intensity drop measured with control setting without AuNPs, as a function of total length of the composite DNA, i.e. the distance of the Sp1 sites to mNP. The relative standard deviation between three repeated experiments are ~5%.

[0021] **Figure 10.** Shows a bar diagram of the decrease in fluorescent intensity of TO induced by Sp1 and ERα binding. The DNA are the composite sequence with 8 bp spacer. The DNA is conjugated to 20 nm AuNPs via either 5′ end or 3′ end.

[0022] **Figure 11.** Shows a bar diagram of the decrease in fluorescent intensity of TO induced by ERα binding in the (A) 5′end TO/dsDNA-AuNPs and (B) 3′ end TO/dsDNA-AuNPs rulers. The relative standard deviation between three repeated experiments are <7%.

[0023] **Figure 12.** Shows TO fluorescent intensity drop (at 535 nm) induced by ERα binding without Sp1 (A-C) and with Sp1 (A′-C′), measured by conventional FID (A ad A′), and (B and B′) AuNP100nm-assited FID and (C and C′) AgNP80nm-assited FID. The solid line is the average value decrease in fluorescent intensity and red dotted lines are the three standard

deviations. The relative standard deviation between three repeated experiments are <7%.

Definitions

[0024] The following words and terms used herein shall have the meaning indicated:

[0025] The term "ligand" is interchangeable with "nucleic acid binders" and both refer to any molecule, compound or substance that is capable of binding to a nucleic acid. In particular, the "ligand" may be a polypeptide, protein, small molecule, antibody or organic compound.

[0026] The term "nanoparticle" refers to a particle that covers a range between about 1 nm and about 2000 nm. Nanoparticles may or may not exhibit size-related properties that differ significantly from those observed in fine particles or bulk materials. The nanoparticle may have different shapes, including but not limited to spheres, discs, rods, fibers, cups, boxes, pyramids, tetrahedron, octahedron, decahedron, tetrapods, multipods, stars, and the like.

[0027] The term "conjugated" or "coupled" or "attached" in the context of the mNPs and nucleic acid refers to the forming of a bond between the metal surface of the mNP and the nucleic acid. This bond may refer to a chemical bond formed between a nucleic acid molecule and the surface of a nanoparticle.

[0028] The term "nucleic acid" refers to a molecule made from one or more nucleotides as is well known. The nucleic acid molecule may be single stranded (ss) or double stranded (ds). Nucleic acid molecules may include, but are not limited to, deoxyribonucleotide (DNA), ribonucleic acid (RNA), small interfering RNA (siRNA), peptide nucleic acid (PNA), locked nucleic acid (LNA) and the like.

[0029] The term "binding site" refers to a region of a nucleotide sequence to which a ligand binds in a specific or non-specific manner. The binding site may be a "partial" or "fragment" of the binding site, whereby a one or more nucleotides are absent from the "full" binding site and the function as a binding site is maintained.

[0030] The term "polypeptide" refers to a large molecule comprising one or more chains of amino acids. The polypeptide may be negatively charged or positively charged. The polypeptide may have a vast array of functions, including but not limited to, catalysis, gene regulation, responding to stimuli and the like.

[0031] The term "enhance" in reference to fluorescence for the purposes of this disclosure, refers to any process that increases the fluorescence intensity of a given substance. Enhancement may be a result of, but not limited to, excited state reactions, energy transfer, electron transfer, complex formation, colloidal quenching and the like. Enhancement may be static or dynamic. The term "enhanceable" should be construed accordingly.

[0032] The term "quench" in reference to fluorescence for the purposes of this disclosure, refers to any process that decreases the fluorescence intensity of a given substance. Quenching may be a result of, but not limited to, excited state reactions, energy transfer, electron transfer, complex formation, colloidal quenching and the like. Quenching may be static or dynamic. The term "quenchable" should be construed accordingly.

[0033] The term "plasmonic" in the context of the present invention and metal nanoparticles refers to the exploitation of surface plasmons, which are produced from the interaction of light with metal-dielectric materials. Under specific conditions, the incident light couples with the surface

plasmons to create self-sustaining, propagating electromagnetic waves known as surface plasmon polaritons (SPPs). Once launched, the SPPs ripple along the metal-dielectric interface and do not stray from this narrow path.

DETAILED DESCRIPTION

[0034] The present invention discloses a method for site-specific determination of ligand binding to a composite nucleic acid that may comprise multiple binding sites, by exploiting metal nanoparticle-assisted fluorescent intercalator displacement (mNPs-FID) principle, as further elaborated herein.

[0035] The fluorescent intercalator displacement assay (FID) is a well-known technique for detecting nucleic acid binders such as proteins, on a single nucleic acid binding site. In conventional FID, the addition of binding ligands, such as proteins and other nucleic acid binders, produces a drop in fluorescence emission due to the displacement of the InD from the nucleic acid.

[0036] In the present invention, mNPs are attached to one end of the nucleic acid to perform plasmonic mediated FID. In the presence of the mNPs, the magnitude of fluorescent intensity decrease is determined not only by the affinity of the nucleic acid binders to the nucleic acid, but also largely by the binding position of nucleic acid binders relative to the mNPs. In particular, when the binding affinity difference is minor (undetectable by conventional FID), the present invention is able to measure which binding sites the specific nucleic acid binders bind to.

[0037] The combined use of mNPs with a conventional FID assay is termed as a "plasmonic-fluorescent ruler", due to the underline rationale that mNPs can modulate the proximal

fluorophores in a distance dependent manner. In particular, the mNPs act as a 'profiler' that gives each InD its own contribution to the overall fluorescence intensity, according to their distance to the mNPs, thus allowing differentiation among InDs.

[0038] The concept of the InD fluorescence intensity in the present invention and "plasmonic-fluorescent ruler" is illustrated in Figure 1.

[0039] Advantageously, the modulation of the InD's fluorescence intensity in a distance dependent manner makes it possible to determine site specific ligand binding on a nucleic acid without involving a full set of control nucleic acids with selective site mutations. Therefore, ligand binding to a particular nucleic acid sequence can be approximated, by back-calculating the distance, based on the decrease in fluorescence intensity.

[0040] Accordingly, in a first embodiment the present invention refers to a method for determining site specific binding of at least one ligand to a composite nucleic acid, comprising: a) providing a composite nucleic acid comprising a metal nanoparticle attached to a nucleic acid having a predetermined length; b) saturating said composite nucleic acid with an intercalating dye; c) measuring an initial fluorescence intensity (F_0) of said saturated composite nucleic acid; d) contacting said saturated composite nucleic acid with said ligand; e) measuring a further fluorescence intensity (F_1) of said contacted saturated composite nucleic acid and determining the relative change in fluorescence intensity (ΔF), between F_0 and F_1; and f) correlating the relative change in fluorescence intensity with the predetermined length of the nucleic acid to determine a distance (R) of the binding of the ligand from the metal nanoparticle.

[0041] In one embodiment, the ligand may be any molecule, substance or compound that is capable of binding to a nucleic acid and displacing an Intercalating dye molecule. In particular, the ligand may be selected from proteins, polypeptides, antibodies, small molecules or organic compounds.

[0042] In one embodiment, the ligand may be a polypeptide. In accordance with aspects of the invention the polypeptide may be referred to as a binding protein. The terms "polypeptide" and "binding protein" may be used interchangeably and may refer to any polypeptide/protein that binds to a nucleic acid. Examples of polypeptides or binding proteins include, without limitation, transcription factors, including activators or suppressors of transcription, proteins involved in replication, or enzymes. In one embodiment, the polypeptide is a transcription factor. In another embodiment, the transcription factor may be any one of FoxA1, AP-2γ, ERα, ERβ or SP1.

[0043] The polypeptide may bind to the nucleic acid in a sequence-specific manner, thus recognizing and binding to a specific sequence of nucleotides found within the nucleic acid. The specific nucleic acid sequence to which the protein binds is referred to herein as a binding site, a sequence motif or a specific binding sequence. These terms may be used interchangeably. The binding proteins bind to the nucleic acid in a sequence-specific manner, and thus have a measurable binding affinity for target consensus sequences and may bind to sequences that have slightly altered sequences with a different (e.g., either greater or lesser) affinity. As such, in one embodiment the nucleic acid comprises at least one binding site for the polypeptide. In one embodiment, the binding site may be a full or partial binding site.

[0044] In another embodiment, the nucleic acid comprises at least one, two, three, four or more binding sites. In one embodiment, the nucleic acid comprises multiple binding sites separated from one another by spacer sequence regions. The spacer sequences may comprise a sequence of at least 4 bp, at least 5 bp, at least 6 bp, at least 7bp, at least 8 bp, at least 9 bp, at least 10 bp, at least 11 bp, at least 12 bp, at least 13 bp, at least 14bp, at least 15 bp, at least 16 bp, at least 17 bp, at least 18 bp. In another embodiment, the spacer sequence is 4 bp, 6 bp, 8 bp, 10 bp, 12 bp, 14 bp, 16 bp or 18 bp.

[0045] In another embodiment the nucleic acid is DNA or RNA, preferably a double stranded DNA (dsDNA). The double stranded nucleic acid molecule may further comprise any one of a loop, tetraloop, stem-loop, hairpin loop, junction, bulge, pseudoknot or internal loop.

[0046] In one embodiment, the nucleic acid is a predetermined length of base pairs, whereby the precise length may depend on factors such as the type of nanoparticles, such as gold or silver NP, the length of the target recognition sequence, the size of the binding protein, and/or the manner in which the binding protein binds to the target recognition sequence. A skilled person can readily test a suitable length of nucleic acid required for any given nanoparticle, target recognition sequence and binding protein, using routine laboratory methods, and as illustrated in the Examples provided herein.

[0047] In another embodiment, the nucleic acid may have a sequence of at least 5 bp, at least 6bp, at least 7bp, at least 8 bp, or may be in the range of any one of about 5 base pairs (bp) to about 100 base pairs (bp), about 5 bp to about 10 bp, about 5 bp to about 15 bp, about 5 bp to about 20 bp, about 5 bp to about 25 bp, about 5 bp to about 50 bp, about 5 bp to about 75 bp, about 10 bp to about 15 bp, about 10 bp to

about 20 bp, about 10 bp to about 25 bp, about 10 bp to about 50 bp, about 10 bp to about 75 bp, about 10 bp to about 100 bp, about 15 bp to about 20 bp, about 15 bp to about 25 bp, about 15 bp to about 50 bp, about 15 bp to about 75 bp, about 15 bp to about 100 bp, about 20 bp to about 25 bp, about 20 bp to about 50 bp, about 20 bp to about 75 bp, about 20 bp to about 100 bp, about 25 bp to about 50 bp, about 25 bp to about 75 bp, about 25 bp to about 100 bp, about 50 bp to about 75 bp, about 50 bp to about 100 bp or about 75 bp to about 100 bp.

[0048] The metal nanoparticles provided herein are conjugated to the nucleic acid to form a nucleic acid composite. The nucleic acid may be conjugated/ attached to the nanoparticle by any method used to conjugate/attach an organic molecule such as DNA to a metal. For example, free thiol groups react with metal, forming a bond between the metal surface and the sulphur atom. Thus, the nucleic acid may be synthesized with an end group containing a free thiol on one end, such as the 5' end, of one strand of the nucleic acid, using standard laboratory techniques. The nucleic acid with a free thiol may be conjugated to the metal nanoparticle for use in any of the methods provided herein, thereby forming a thioether linkage between the metal nanoparticle and the DNA molecule.

[0049] In one embodiment, the metal nanoparticles provided herein may be any one of an alkali metal, alkaline earth metal, transition metal, post-transition metal, lanthanide metal, actinide metal or any alloy thereof. In one embodiment, the metal nanoparticle may be a noble metal nanoparticle. The noble metal may be any one of ruthenium (Ru), rhodium (Rh), palladium (Pd), silver (Ag), osmium (Os), iridium (Ir), platinum (Pt), gold (Au) or any alloy thereof. In one embodiment, the metal nanoparticle is a noble metal nanoparticle. In another embodiment, the noble metal

nanoparticle is a gold (Au) NP (AuNP) or a silver (Ag) NP (AgNP).

[0050] Advantageously, the mNP may have unique optical and electronic properties, whereby the interaction of mNPs with light is strongly dictated by their environment, size and physical dimensions. Oscillating electric fields of a light ray propagating near a colloidal nanoparticle may interact with free electrons, causing a concerted oscillation of electron charge that is in resonance with the frequency of light. These resonant oscillations, known as plasmons, may depend on the size of the mNP. For examples, the surface plasmon resonance phenomena of small monodisperse (~30 nm) AuNPs, may cause an absorption of light in the blue-green portion of the spectrum (~450 nm) while red light (~700 nm) to be reflected. As particle size increases, the wavelength of surface plasmon resonance related absorption shifts to longer wavelengths. Red light is then absorbed and blue light is reflected. The surface plasmon resonance may be tuned by varying the size or shape of the mNP, leasing to particles with tailored optical properties for different applications.

[0051] As illustrated in **Figure 1**, in a conventional FID assay without mNPs (Figure 1A), the change in the fluorescence intensity due to protein binding are roughly uniformly distributed among all of the dyes being displaced from the nucleic acid, for example if there was 5 intercalated dyes displaced, the contribution of each dye would be 20%.

[0052] In the constructed mNP coupled FID of the present invention, the initially observed fluorescent intensity of the InD inserted to the nucleic acid composite is a weighted average from each InD. Figure 1A shows an even emission in dsDNA without mNPs, and Figure 1(B & C) shows the uneven emission in dsDNA with mNPs conjugates of smaller mNPs (5-80 nm) or larger mNPs (> 80 nm). In (B) and (C), the overall (or

apparent/resulting) InD intensity is either lower (quenched) or higher (enhanced) relative to that without AuNPs as in (A).

[0053] Accordingly, when smaller mNPs are present as a quenching profiler (Figure 1B), the InD located nearer to the mNP surface has a lower weighting factor or less contribution to the overall intensity, since it is quenched much stronger by surface Plasmon resonance of the mNP, and *vice versa* for the one further away. On the other hand, when larger mNPs are used as an enhancement profiler (Figure 1C), the InD located nearer to the mNP surface has a higher weighting factor or more contribution to the overall intensity, because the enhancement effect is stronger as it closer to mNPs. By using the example with a "quenching profiler" (Figure 1B) as illustration, the denoted weighting factor of the 5 InDs (A, B, C, D, and E, with A being the closest to mNP's surface and E as the furthest entity) of a, b, c, d and e; the relative weighting factor of (in fraction) each InD is roughly $a < b < c < d < e$, and the summation $(a + b + c + d + e)$ is equal to one.

[0054] As such, the material and size of the mNPs, may impact upon the overall initial fluorescence emission of the InD in the nucleic acid composite as being either lower (quenched by a smaller mNPs) or higher (enhanced by a larger mNPs) in comparison to nucleic acid composites without a mNP profiler. The latter will provide higher sensitivity for ligand binding near to the particle surface.

[0055] Accordingly, in one embodiment the mNPs are either smaller or larger mNPs. In another embodiment, the mNPs may have a size in the range of about 5 nm to about 1000nm, about 5 nm to about 500 nm, about 5 nm to about 300nm, about 5 nm to about 200nm, about 5 nm to about 10 nm, about 5 nm to about 15 nm, about 5 nm to about 50 nm, about 5 nm to about 70 nm, about 5 nm to about 80 nm, about 5 nm to about 100 nm, about 5

nm to about 150 nm, about 10 nm to about 15 nm, about 10 nm to about 50 nm, about 10 nm to about 70 nm, about 10 nm to about 80 nm, about 10 nm to about 100 nm, about 10 nm to about 150 nm, about 10 nm to about 200 nm, about 15 nm to about 50 nm, about 15 nm to about 70 nm, about 15 nm to about 80 nm, about 15 nm to about 100 nm, about 15 nm to about 150 nm, about 15 nm to about 200 nm, about 50 nm to about 70 nm, about 50 nm to about 80 nm, about 50 nm to about 100 nm, about 50 nm to about 150 nm, about 50 nm to about 200 nm, about 70 nm to about 80 nm, about 70 nm to about 100 nm, about 70 nm to about 150 nm, about 70 nm to about 200 nm, about 80 nm to about 100 nm, about 80 nm to about 150 nm, about 80 nm to about 200 nm, about 100 nm to about 150 nm, about 100 nm to about 200 nm or about 150 nm to about 200 nm.

[0056] In yet another embodiment, the mNPs may have a size that is less than 80 nm (smaller mNP) or greater than about 80 nm (larger mNP). In one embodiment, the mNPs may have a size that is less than 80 nm and said fluorescent intensity of said intercalating dye decreases proximal to said nanoparticle. In another embodiment, the mNPs may have a size that is greater than 80 nm and said fluorescent intensity of said intercalating dye increases proximal to said nanoparticle.

[0057] In one embodiment, the mNP may have different shapes selected from any one of spheres, discs, rods, fibers, cups, boxes, pyramids, tetrahedron, octahedron, decahedron, tetrapods, multipods, or stars.

[0058] Advantageously, the present invention and underlying principle of mNPs assisted FID, is particularly useful for constructing the ruler with composite nucleic acid up to tens to hundreds of base pairs in length, due to the long range fluorescent quenching and enhancement effect (up to tens of nm) of the smaller or larger mNPs.

[0059] As described herein, the present invention utilizes mNP assisted FID with intercalator dyes (InD). DNA intercalator dyes (InD) may be aromatic cations with planar structures that enter between stacked base pairs in the DNA duplex to form dsDNA/InD composites. In one embodiment, the intercalater dye is selected from propidium iodide, oxazole yellow, TO-PRO, ethidium bromide or thiazole orange.

[0060] The relative weighting factor of each InD is highly dependent on the length of the nucleic acid length attached to the mNP, the excitation/emission spectrum and quantum yield of the InD, and the surface Plasmon resonance/absorption spectra of mNP used in the method, all of which can be readily selected and adapted by those skilled in the art based upon the desired use.

[0061] The ratio of the InD to the nucleic acid impacts upon the sensitivity of the present invention. In this regard, as well known in the art when using conventional FID assays, the ratio of InD to the nucleic acid is dependent upon the chemical structure of the InD. Those skilled in the art can readily determine the optimal ratio (i.e. reaching saturation) to use by applying conventional techniques in the art, as illustrated in Example 1 described herein. In particular, in one embodiment where TO is the InD, the ratio is 3 TO molecules to 7 bp in length.

[0062] In one embodiment, the InD emits fluorescence in the range of any one of about 200 nm to about 800 nm, about 200 nm to about 300 nm, about 200 nm to about 400 nm, about 200 nm to about 500 nm, about 200 nm to about 600 nm, about 200 nm to about 700 nm, about 300 nm to about 400 nm, about 300 nm to about 500 nm, about 300 nm to about 600 nm, about 300 nm to about 700 nm, about 400 nm to about 500 nm, about 400 nm to about 600 nm, about 400 nm to about 700 nm, about 500 nm to about 600 nm, about 500 nm to about 700 nm or about 600 nm to

about 700 nm. In one embodiment, the InD may emit fluorescence in the range of about 350 nm to about 700 nm, 450 nm to about 700 nm, 500 nm to about 700 nm, 520 nm to about 600 nm, 530 nm to about 580 nm.

[0063] The fluorescence intensity of the InD may be measured by well known techniques in the art and those readily utilized in the convention FID assay. In one embodiment, the fluorescence intensity may be measured by a fluorescence spectrometer or a microplate reader, as is well known in the art.

[0064] In one embodiment, the InD and emission wavelength may be selected to overlap with the UV-vis absorption of the selected mNPs. In particular, the InD fluorescence emission and plasmon peak emission of the mNPs may be within the same range of about 350 nm to about 700 nm, 450 nm to about 700 nm, 500 nm to about 700 nm, 520 nm to about 600 nm, 530 nm to about 580 nm. It will be appreciated that any selectable InD can be used in the present invention.

[0065] An InD once saturated into a nucleic acid, such as dsDNA, will yield a much higher fluorescence emission relative to its free form in solution. As such, contacting the saturated nucleic acid composite with the ligand results in a decrease of fluorescence due to the displacement of the InD from said composited. The decrease of fluorescence is a function and determinant of the relative change in fluorescence intensity, whereby both the decrease and relative change in fluorescence intensity may be expressed as a percentage ranging from 0.01% to 99.99% or any value inbetween.

[0066] In one embodiment, the relative change in fluorescence intensity (ΔF) is calculated from the equation:

- $\Delta F = (F_1 - F_0)/F_0$.

[0067] The F_1 is the measured fluorescent intensity after addition of the ligand (to displace InD) and F_0 the initial intensity of InD saturated nucleic acid composite before displacement.

[0068] The relative change in fluorescence intensity in comparison to the base pair length of the nucleic acid can be used for determining the positioning of an unknown binding site according to a ligand binding induced intensity decrease. The decrease magnitude in fluorescent intensity when a ligand binds to the saturated nucleic acid composite can be compared against a calibration curve to directly determine a specific base pair number away from the mNP's surface (distance (R)) where the InD has been displaced by the ligand. As such, in one embodiment the binding site is determined based on comparing the relative intensity decrease on a calibration curve. The calibration curve may be obtained from a theoretical calculation based on the FRET theory and equation that is well known in the art.

[0069] In particular, using the equation of $Q= R_o^6/ (R^6 + R_o^6)$, where R_o is the Förster distance, i.e. the distance where the emission intensity is 50% quenched and is constant, the extent of quenching Q and remaining fluorescence intensity I (I = 1-Q) may be calculated for each distance R. R^6 and R_0^6 relate to the distance (R) and Forster distance (R_o) in the 6th power law wherein the FRET equation calculates the energy transfer based on dipole-dipole coupling mechanism. For single dipole, the distance factor is D^3, hence for dipole coupling, the distance factor is D^6.

[0070] As such, in one embodiment the calibration curve is obtained by calculating Q for each distance R and plotting the results on a graph to form said calibration curve. This

principle is further illustrated in the working examples with respect to Figure 8B and the like.

[0071] In one embodiment, the distance (R) is equivalent to the ligand binding site as measured in terms of distance from mNP's surface used as the reference point. In one embodiment, the distance (R) is calculated based on a value of 0.34 nm per nucleotide base, wherein 0.34 nm is the distance between successive nucleotides, as is well known in the art (Chatterjee, S.; Lee, J. B.; Valappil, N. V.; Luo, D.; Menon, V. M. *Biomed. Opt. Express.* 2011, *2*, 1727-1733).

[0072] Therefore, advantageously the present invention enables the ligand binding to the nucleic acid composite to be approximated, by back-calculating the distance (R), based on the decrease in relative fluorescence intensity.

[0073] In one embodiment of the methods described herein, it may be desired to determine the preferential binding site of the at least one ligand, wherein the at least one ligand may bind to more than one binding site in the nucleic acid composite.

[0074] In this regard, another embodiment of the method described herein, the step of contacting said saturated composite nucleic acid with said at least one ligand may further comprise contacting said saturated composite nucleic acid with a first ligand in a 1st molar amount and measuring a first further fluorescence intensity (F'_1) of said contacted saturated composite nucleic acid; contacting said saturated composite nucleic acid with a second ligand in a 2nd molar amount and measuring a second further fluorescence intensity (F'_2) of said contacted saturated composite nucleic acid. The relative change in fluorescence intensity (ΔF), between F_0, F'_1, and F'_2 may then be determined as described herein, whereby the relative change in fluorescence intensity determines the preferential binding site of the ligand.

[0075] In one embodiment, the first and second ligands are the same. In particular, in an embodiment where the ligand is a polypeptide, the "same" polypeptide refers to a polypeptide with an identical amino acid sequence or a polypeptide variant thereof, that maintains the ability to bind to the same binding site in the nucleic acid composite. As such, the same polypeptide (2nd polypeptide) may be one that comprises an amino acid sequence which differs from the reference amino acid sequence (1st polypeptide) by one or more mutations, substitutions, insertions or deletions. Accordingly, in one embodiment the first and second polypeptide may have 100% sequence identity with one another, or may be polypeptide variants with at least 70%, at least 80%, at least 85%, at least 90%, at least 95% sequence identity and have maintained the ability to bind to the same binding site of the nucleic acid composite.

[0076] In another embodiment, the first and second ligands may be contacted with the nucleic acid composite at suitable molar ratios relative to the nucleic acid concentration. Those skilled in the art can readily determine the ligand concentration that is sufficient to bind to the nucleic acid composite. In one embodiment, the molar ratio may be 1:1 (1 molar ligand) to 4:1 (4 mol ligand) according to the binding stoichiometry. In another embodiment, the concentration of the first and second ligand is always the same concentration, wherein the 1st and 2nd molar amounts are the same.

[0077] In one embodiment, the first and second ligands are the same and the metal nanoparticle is a smaller mNP that is less than 80nm in size. In the event that the 1st molar amount of the first ligand measures a higher decrease in fluorescence intensity than the 2nd molar amount of the second ligand, the replaced InD by the first ligand had higher contribution to overall intensity, this being indicative of the binding site

being located further away from the mNP due to the weakly quenched InD. In the event that the 1st molar amount of the first ligand measures a lower drop in fluorescence intensity than the 2nd molar amount of the second ligand, the replaced InD by the first ligand had lower contribution to overall intensity, this being indicative of the binding site being located closer to the mNP due to the heavily quenched InD.

[0078] In one embodiment, the first and second ligand are the same and the metal nanoparticle may be a larger mNP that is greater than 80nm in size. In the event that the 1st molar amount of the first ligand measures a higher decrease in fluorescence intensity than the 2nd molar amount of the second ligand, the replaced InD by the first ligand had higher contribution to overall intensity, this being indicative of the binding site being located closer (proximal) to the mNP due to the higher enhancement of InD. In the event that the 1st molar amount of the first ligand measures a lower drop in fluorescence intensity than the 2nd molar amount of the second ligand, the replaced InD by the first ligand had lower contribution to overall intensity, this being indicative of the binding site being further away (distal) to the mNP due to the lower enhancement of the InD.

[0079] As described herein, when using smaller or larger mNP the contribution of InD at a proximal distance to the mNP varies and thus the change in fluorescence intensity also varies, as exemplified in Example IV disclosed herein with respect to smaller mNPs. Thus, in applying this principle the methods described herein may determine the identity of ligand based upon the magnitude of the decrease in fluorescence intensity as described herein, and correlating the same with one or more binding sites of the nucleic acid composite.

[0080] In this regard, another embodiment of the method described herein and the step of contacting said saturated

composite nucleic acid with said at least one ligand may further comprise contacting said saturated composite nucleic acid with a first ligand and measuring a first further fluorescence intensity (F'_1) of said contacted saturated composite nucleic acid; and contacting said saturated composite nucleic acid with a second ligand and measuring a second further fluorescence intensity (F'_2) of said contacted saturated composite nucleic acid. The relative change in fluorescence intensity (ΔF), between F_0, F'_1, and F'_2 may then be determined, whereby the relative change in fluorescence intensity with the predetermined length of the nucleic acid determines the identity of the first and/or second ligand and a distance (R) of the binding of the first and/or second ligands from the metal nanoparticle.

[0081] In one embodiment, the first and second ligands may be the same or different. In the embodiment where the ligand is a polypeptide, the term "same" is as defined above. A "different" polypeptide refers to one that is distinct in either its function or activity when bound to the nucleic acid composite, or distinct in the binding site it binds to, and may refer to a polypeptide and protein that belongs to a different protein family and functional characterization from a reference polypeptide. Accordingly, in one embodiment the first and second polypeptide may be different from one another.

[0082] In yet another embodiment, the first and second ligand are contacted with the composite nucleic acid simultaneously, sequentially or separately.

[0083] As exemplified in Example 5 disclosed herein, the binding of a first polypeptide may improve the binding of a second polypeptide to a binding site, in comparison to when only one polypeptide is bound to a binding site. Without being bound by theory the aforementioned cooperative binding and

improvement may be attributed to protein-protein contact and interaction. Accordingly, in another aspect of the methods described herein, it may be desired to determine if two or more ligands cooperatively bind to the nucleic acid composite, and also determine the optimum separation distance between binding sites for a cooperative binding effect.

[0084] In this regard, another embodiment of the method described herein and step of contacting said saturated composite nucleic acid with said at least one ligand may further comprise contacting said saturated composite nucleic acid with a first ligand and measuring a first further fluorescence intensity (F'_1) of said contacted saturated composite nucleic acid; and contacting said saturated composite nucleic acid with the first ligand and one or more further ligand and measuring a second further fluorescence intensity (F'_2) of said contacted saturated composite nucleic acid, wherein the contacting steps may be carried out separately. The relative change in fluorescence intensity (ΔF), between F_0, F'_1, and F'_2 may then be determined as described herein, whereby the relative change in fluorescence intensity determines the cooperative binding of the first and further one or more ligands.

[0085] In another embodiment of the above method, the optimal separation distance between the bound first and second ligands for a cooperative binding effect may be determined. In particular, the above method and series of steps may be repeated once or more for nucleic acid composites comprising a spacer between the binding sites of at least 4 bp, at least 5 bp, at least 6bp, at least 7bp, at least 8 bp, at least 9 bp, at least 10 bp, at least 11 bp, at least 12 bp, at least 13 bp, at least 14bp, at least 15 bp, at least 16 bp, at least 17 bp, at least 18 bp. In another embodiment, the spacer sequence is 4 bp, 6 bp, 8 bp, 10 bp, 12 bp, 14 bp, 16 bp or 18 bp.

[0086] In a second aspect of the present invention, the methods described herein may be used in an assay kit. In particular, the present invention provides a kit for detecting site specific binding of at least one ligand to a nucleic acid, comprising: a nucleic acid composite comprising a nanoparticle attached to said nucleic acid at the 5′ end or 3′ end, and an intercalator dye bound to said nucleic acid.

[0087] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0088] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0089] Other embodiments are within the following claims and non- limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

Example 1:

Materials and Methods

[0090] Nucleic Acid Sequence: A series of ½ ERE/Sp1 composite DNA derived from the +571 ERE/Sp1 composite site in promoter A of the human PR gene (Petz et al., J. Steroid Biochem. Mol. Biol. 2004, 88, 113-122) is used to demonstrate the idea and concept of the present invention.

[0091] The composite DNA elements containing a half-site estrogen response element (½ ERE, TGACC) upstream of two adjacent Sp1 sites (CCGCCC), i. e. proximal "Sp1/P" and distal "Sp1/D". The distance between the ½ ERE and the Sp1/D is 4-18 bp. The purpose of having different number of bps between ½ ERE and Sp1/D is to illustrate the optimal separation distance between ½ ERE and Sp1 motifs for favorable "cooperation" of ERα and Sp1 to bind to the DNA.

[0092] **Table 1.** Composite DNA sequences containing ½ ERE motif (pink) and Sp1 motifs (blue) with varied spacer length of 4-18 bp (black).

[0093]

Spacer between ½ ERE & Sp1/D	Sequence	Total length (nm) (bp)

4 bps	**AuNPs** - 5′-TAG GAG **CTG ACC** AGC GCC GCC CTC CCC CGC CCC CGA CC-3′ **Identity** ½ ERE spacer Sp1/D Sp1/P	13.8 nm (38bp)
6 bps	**AuNPs**-5′-TAG GAG **CTG ACC** AGAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	14.4nm (40bp)
8 bps	**AuNPs**-5′-TAG GAG **CTG ACC** AGATAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	15.1 nm (42bp)
10 bps	**AuNPs**-5′-TAG GAG **CTG ACC** AGATATAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	15.8 nm (44bp)
12 bps	**AuNPs**-5′-TAG GAG **CTG ACC** AGATATATAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	16.5 nm (46bp)
14 bps	**AuNPs**-5′-TAG GAG **CTG ACC** AGATATATATAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	17.2 nm (48bp)
16 bps	**AuNPs**-5′-TAG GAG **CTG ACC** AGATATATATATAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	17.8 nm (50bp)
18 bps	**AuNPs**-5′-TAG GAG **CTG ACC** AGATATATATATATAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	18.5 nm (52bp)

[0094] The length of DNA of the selective spacer and the distance of each site to the AuNPs for a different spacer is shown in **Figure 3**. The calculation is based on 0.34 nm per base pair (Murphy et al., Biophysical Journal, 2004, **86**, 2530-2537) and with the 0.83 nm C6 linker taken into consideration, assuming the C-C carbon bond length in the alkane chain.

[0095] Metal Nanoparticle: AuNPs (20 nm and 100nm) and AgNPs (80 nm) were used to demonstrate the "quenching ruler" and "enhancement ruler" concepts.

[0096] Conjugation of dsDNA to mNPs was performed using the conventional thiol-AuNP conjugation chemistry. One single strand of the dsDNA is labeled with a thiol group at 5′ end (separated by a linker containing 6 Carbon atoms) as prepared by Sigma Life Science. The thiolated single stranded DNA (ssDNA, i.e. the sense strand) was activated with tris(2-carboxyethyl)phosphine) (TCEP, 10 molar excess) and stirred for 10 min at room temperature. The final solution was centrifuged, with a Sigma-Aldrich microcon centrifugal filter device, YM-3 (NMWCO 3 kDa), to remove TCEP before conjugation to AuNPs. Conjugation of activated thiolated DNA to AuNP was done as described by Zhang et al (X. Zhang, M. R. Servos, J. Liu, *J. Am. Chem. Soc.* **2012**, *134*, 7266). Activated ssDNA was mixed with AuNPs at desired ratio (100:1) and incubated for 5

min. Then, pH of solution was lowered to 3 and salt concentration was increased to 30 mM by adding HCl and NaCl, respectively. After 20 min, NaOH was added to return the pH to neutral range. The ssDNA-AuNPs conjugates were then annealed with its complimentary DNA strands at 90 °C for 5 min and left to cool down to room temperature (RT).

[0097] Intercalator Dye(InD): **Thiazole orange (TO)** was chosen as the InD to constitute the "dsDNA-AuNPs/InD" plasmonic-fluorescent ruler with the composite ½ ERE/Sp1 DNA. TO was chosen because it emits at 535 nm, near to the wavelength of the AuNPs' plasmon peak (520-580 nm from 20 to 100 nm), thus more pronounced quenching (or enhancement) by AuNPs and therefore better sensitivity can be expected. In addition, TO intercalation in DNA shows a less sequence dependency and a high fluorescence emission upon intercalation to the dsDNA.

Optimal Intercalating Dye amount that can saturate the dsDNA

[0098] TO was added to the longest dsDNA, i.e. 52 bp $dsDNA_{52bp}$-AuNPs conjugate, with increasing TO:DNA ratio from 1:1 to 30:1. The mixture of TO and $dsDNA_{52bp}$-AuNPs was incubated for 15 min to allow TO intercalation, and the fluorescence intensity was measured by using a fluorescence spectrometer or a microplate reader. Optimal TO amount was determined from the TO:DNA ratio that would not result in further increase of fluorescence intensity.

Metal Nanoparticle Size-Effect on the Intercalating Dye Emission.

[0099] TO, 38 bp up to 52 bp dsDNA were conjugated to 20 nm AuNPs, 100 nm AuNPs, and 80 nm AgNPs. TO was added to the $dsDNA_{36-52bp}$ $-AuNPs_{20nm,100nm}$ or $dsDNA_{36-52bp}$ $-AgNPs_{80nm}$ conjugates at TO:DNA molar ratio of 23:1 and incubated for 15 min to allow

TO intercalation. The fluorescence intensity of intercalated TO in the dsDNA-AuNPs or dsDNA-AgNPs conjugates was measured by using a fluorescence spectrometer or a microplate reader.

Determining the DNA Length Dependency of TO Emission.

[00100] 38 bp, 46 bp, and 52 bp dsDNA were conjugated to 20 nm AuNPs. TO was added to the $dsDNA_{38,46,52bp}$ -$AuNPs_{20nm}$ conjugates at TO:DNA molar ratio of 23:1 and incubated for 15 min to allow TO intercalation. The fluorescence intensity of intercalated TO in the dsDNA-AuNPs conjugates was measured by using a fluorescence spectrometer or a microplate reader.

Results

OptimalIntercalating Dye amount that can saturate the dsDNA.

[00101] As shown in Figure 4A the UV-vis spectra of 20 nm, 100 nm AuNPs, and 80nm AgNPs and their conjugates with the 52bp dsDNA. Figure 4B shows the TO emission intensity in the 52 bp DNA conjugated with 20 nm AuNPs (i.e. $dsDNA_{52bp}$-$AuNPs_{20nm}$), at increasing molar ratio of TO:DNA-AuNPs from 1:1 to 30:1. Saturation was achieved at 23:1 and further addition of TO would not result in further increase in fluorescence intensity (dashed line in Figure 4B). Since the DNA consists of 52 bps, then the ratio was approximately 3 TO molecules to 7 bps.

[00102] This ratio obtained for the longest DNA sequence (52 bp) would be applied to the shorter DNAs as well.

Metal Nanoparticle Size-Effect on the Intercalating Dye Emission.

[00103] Using dsDNA/TO without AuNPs conjugated as reference (control), it was found that 20 nm AuNPs quenches the overall fluorescence of the intercalated TO, whereas 100 nm AuNPs and 80 nm AgNPs enhance the overall emission intensity.

[00104] Spanning from 38 up to 52 bps of the dsDNA, the fluorescent intensities increased linearly with the number of DNA's bps, which suggested that higher number of TOs were intercalated for longer DNA and *vice versa*. If the points were fitted with a linear line, the R^2 of the lines were approximately 0.98. R^2 refers to R-squared value of the linear regression of the curve. It indicates how fit the regression model with the curve is. The value ranges 0 (no fit) to 1 (perfect fit), with typical value of > 0.95 implies a good fit.

[00105] From the series of dsDNA/TO without AuNPs (circles in Figure 5), the line intercepted the y-axis at 304, while DNA-AuNPs$_{20nm}$/TO and DNA-AuNPs$_{100nm}$/TO series cut the y-axis at -14313 and +7804, respectively. The small positive value (304) supports that the overall intensity is linearly proportional to the amount of TOs for TO/DNA series and intensity should go to 0 as the DNA used becomes shorter and shorter (approaching 0) whereby no TO can be intercalated.

[00106] On the contrary, the large negative value (-14313) of DNA-AuNPs$_{20nm}$/TO series signified fluorescent intensity was only measureable for DNA consisted of at least 8 bps (extrapolating the line to intercept at the x-axis). Thereby it is critical to ensure the DNA's bp used must consist of at least 8 bp. On the other hand, even for hypothetical 1 bp, DNA-AuNPs$_{100nm}$/TO can still produce fluorescence.

[00107] This type of assembly can be a method of choice if the DNA consists of a few nucleotides, where AuNP$_{20nm}$ completely suppresses the intercalating dyes fluorescence.

Determining the DNA Length Dependency of TO Emission.

[00108] The TO fluorescence intensity in their DNA-AuNPs conjugates were 48%, 60%, and 64%, respectively (**Figure 6**).

[00109] This demonstrates that as the length of DNA increases, quenching of intercalated TO distant away from AuNP becomes weaker. Therefore, the overall fluorescence intensity is progressively restored with the increasing number of bp's and approaching that without AuNPs, yet the fluorescence intensity of dsDNA-AuNPs$_{20nm}$ will always be lower because the TO at close proximity to AuNP$_{20nm}$ is in a quenched state.

Example 2:

Site Specific Detection of "proximal" and "distal" Protein Binding Sites using a "quenching profiler" ruler.

[00110] As seen in **Table 1**, the composite DNA has two Sp1 sites (Sp1/D and Sp1/P). Previous EMSA and *in vitro* DNase I footprinting assays have shown that Sp1 protein binds to the two Sp1 sites in a "additive manner", either it binds to the 10-bp proximal Sp1 site (Sp1/P) first, and then the distal site (Sp1/D) (Petz and Nardulli, Mol. Endocrinology 2000, 14, 972-985).

[00111] In this example, the capability of current dsDNA-AuNPs/InD assembly to report the preferential binding of Sp1 is demonstrated. AuNPs of 20 nm (as a quenching profiler) and a composite DNA sequence with 4 bps spacer (between the ½ ERE and Sp1/P) are used to illustrate the concept.

Materials and Methods

[00112] To eliminate the potential steric limitation imposed to any motif, two sets of dsDNA$_{38bp}$-AuNPs conjugates were prepared, with the conjugation at either the 5′ end or 3′ end of the sense strand (**Figure 7A and 7B**), respectively. Sp1 was added incrementally from one to a few molar ratios relative to

the DNA concentration every 1 molar ratio. The 38 bp dsDNA contained 4 bp spacers.

[00113] The relative intensity drop caused by 1st and 2nd molar Sp1 were recorded **(Figure 7C)**. The 1st molar refers to the addition of Sp1 in 1:1 molar ratio relative to DNA concentration. The 2nd molar refers to 2:1 molar ratio of Sp1 relative to DNA concentration. The relative intensity was determined and recorded as described herein.

[00114] Experiments were carried out and extended to higher number of bp spacing between ½ ERE and the Sp1 motif (6 bp – 18 bp) using the 5′ end conjugation to further confirm the trend and strengthen the above findings **(Figure 8A)**.

[00115] The 1st molar refers to the addition of Sp1 in 1:1 molar ratio relative to DNA concentration. The 2nd molar refers to 2:1 molar ratio of Sp1 relative to DNA concentration. In Figure 8A, the Y axis is not the "relative intensity" but the decrease in fluorescence intensity, which can be calculated and recorded as described herein as F_1-F_0.

Results

[00116] For the first two molar Sp1 added, the fluorescence intensity decreased gradually and stayed constant above 2 molar ratios. This confirms the "additive" binding behavior of Sp1 to Sp1/P and Sp1/D (Petz and Nardulli, Mol. Endocrinology 2000, 14, 972-985).

[00117] As shown by the relative intensity drop for the **"A"** assembly (**center cluster in Figure 7C**), introduction of the 1st molar Sp1 gave a higher decrease in intensity than the 2nd molar of Sp1, which meant the TO displaced by the 1st molar Sp1 had higher contribution to overall intensity. This being attributed to the weakly quenched TO dyes in Sp1/P site that are located further away from AuNP (26 bp away).

[00118] On the **"B"** assembly, Sp1/P is positioned closer to AuNPs (6 bp away). As expected a lower drop in intensity was observed for the 1st molar of Sp1 protein than the 2nd molar (**right cluster in Figure 7C**), which meant the 1st molar Sp1 bound at Sp1/P site and displaced the heavily quenched TO dyes in there.

[00119] Using conventional FID using DNA without conjugated AuNPs, the addition of 1st and 2nd molar of Sp1 gave similar change in intensity drop (**left cluster in Figure 7C**). As such, the above supports that conventional FID is incapable of detecting preferential protein binding at specific site.

[00120] Similar to the results of 4 bp spacer, a higher intensity decrease was always observed for the 1st molar Sp1, corresponded to binding to the Sp1/D at 26-40 bps away from the AuNP's surface, relative to when 2nd molar Sp1 bound to the proximal site (16-30 bps away from AuNPs), which displaced TO dyes that have smaller contribution to the overall intensity.

[00121] Interestingly, the longer the DNA, the higher is the quenching for each addition of Sp1. This was anticipated as the longer the spacing between ½ ERE and Sp1 motif, the further away Sp1 motifs is from the AuNP. Hence, the smaller TO quenching and its higher role in overall fluorescence intensity. This also explains why the magnitude of difference between 1st and 2nd molar Sp1 becomes lesser with the increase of overall DNA length (**Figure 8A**).

[00122] The decrease in fluorescence intensity caused by 1st and 2nd molar Sp1 measured for all sequences of different length (4-18 bp spacer) were then fitted to the TO fluorescence contribution calculated by FRET equation (**Figure 8B**). The calculated Förster distance (R_o), i.e. the distance where the emission intensity is 50% quenched, was 7.01 nm, taking into account the thiolated-C6 chain (0.83 nm) sat at

the head of each DNA sequences, which was used for conjugating DNA to AuNPs.

[00123] The purpose of figure 8B is to show the correlation between the decrease in fluorescence intensity and the distance of the binding site from AuNP's surface (R). The correlation is derived from the equations:

- $I = 1-Q$; and
- $Q = R_o^6/ (R^6 + R_o^6)$.

[00124] Specifically, the extent of quenching of TO at a certain distance from the AuNP is calculated with $Q = R_o^6/ (R^6 + R_o^6)$, where R is the distance (determined by the number of bp) from AuNP's surface, and thus TO fluorescence remaining is $I = 1-Q$. Starting from R in the x axis of Figure 8B, R correlates to Q (the extent of TO quenching) based on equation $Q = R_o^6/ (R^6 + R_o^6)$. Q correlates to I (remaining fluorescence intensity of TO) based on the equation $I = 1-Q$. For example, if the distance (R) of the binding site from the mNP surface is 11 nm, where $R_o = 7.01$ nm, it can be calculated that $Q = R_o^6/ (R^6 + R_o^6) = 0.063$. $I = 1-Q = 0.937$ as shown in the Y axis of Figure 8B.

[00125] The above equations are a FRET quenching correlation with distance (R). These equations are used in combination with the equation $\Delta F = (F_1 - F_0)/F_0$ in which I (of $I = 1-Q$) is calculated from the decrease in fluorescence as $\Delta F/ F_0$ or $(F_0-F_1)/ F_0$ based on ΔF to establish the correlation of R to the decrease in fluorescence intensity in Figure 8A.

[00126] The relative change in fluorescence intensity is calculated from $\Delta F = (F_1 - F_0)/F_0$. The F_1 is the measured fluorescent intensity after addition of Sp1 (to displace TO) and F_0 the initial intensity of saturated TO before displacement. The calculated value was fitted to the curve

from theoretical calculation (**Figure 8B**). The close correlation of the experimental results with the theoretical fitting curve suggests that the quenching of TO by $AuNP_{20nm}$ obeyed the conventional FRET theory. FRET is widely employed to determine distance at molecular scale level owing to the sensitivity of energy transfer at this scale.

[00127] The relative intensity drop vs. DNA length can be used as a calibration curve for determining the unknown binding site according to a protein binding induced intensity drop. The decrease magnitude in fluorescent intensity when a protein binds to DNA can be either substituted back into the FRET equation or it can be compared against the calibration curve directly to determine the distance (R). The value of R corresponds directly to a specific base pair number away from the mNP's surface where dye has been replaced. In other words, the distance calculated is equivalent to the protein binding site as measured in terms of distance from mNP's surface used as the reference point.

Example 3:

Site specific detection of polypeptide binding to "proximal" and "distal" binding sites using a "enhancement profiler" ruler.

[00128] In previous Example 2 using a "quenching profiler", the detection of protein binding, especially to the DNA motif located close to the surface AuNP would be challenging, due to the original fluorescent intensity of the InD being already very weak due to the strong quenching.

[00129] In order to circumvent this limitation, the construct in Example 2 is modified by replacing the smaller AuNPs with a larger gold or silver NP (i.e. 100 nm AuNP and 80

nm AgNP). These larger particles exhibit greater light scattering contribution than the absorption, which eventually enhance the fluorescence of the proximal fluorophores (the intercalated dyes in this case). The enhancement effect is also distanced dependent, i.e. becoming depleted with the increase of the distanced.

Materials and Methods

[00130] To test the ability of the larger particle "enhancement profiler" coupled with FID for detecting preferential Sp1 binding, two molars of Sp1 were added one after another. The experiments were repeated for all DNA spacers.

[00131] dsDNA (containing 4 bp - 18 bp spacer) were conjugated to 100 nm AuNPs and 80 nm AgNPs.

[00132] TO was added to the dsDNA -$AuNPs_{100nm}$ conjugates and dsDNA -$AgNPs_{80nm}$ conjugates at TO:DNA molar ratio of 23:1 and incubated for 15 min to allow TO intercalation. The fluorescence intensity of intercalated TO in the dsDNA -$AuNPs_{100nm}$ conjugates and dsDNA -$AgNPs_{80nm}$ conjugates was measured by using a fluorescence spectrometer or a microplate reader. A first molar of Sp1 was added in the equal molar ratio of Sp1 to DNA. After 15 min incubation, the fluorescence intensity of TO after addition of the first molar of Sp1 was measured. Second molar of Sp1 was added to make the final molar ratio of Sp1:DNA to 2:1. After 15 min incubation, the fluorescence intensity of TO after Sp1 second molar addition was measured.

Results

[00133] Binding of first molar of Sp1 to the Sp1/P site that is further away from mNPs surface produced a lower change in fluorescent intensity than second molar Sp1 that binds to the

closer SP1/D site for all tested DNA (**Figure 9A & 9B**).This is because the TO dyes located further apart from mNPs have lower apparent intensity or lower contribution to overall intensity (due to the lower enhancement effect) than the dyes close to mNPs. The opposite trend from the current Example III relative to previous Example II (higher intensity drop by the 1st molar Sp1) is due to the opposing nature of the role of mNP, being as a "quenching profiler" and "enhancement profiler", respectively.

[00134] Figure 8C is a summary of TO intensity decrease (at 535 nm) relative to non-enhanced TO intensity drop measured with control setting without AuNPs ($\Delta F/F_0$), as a function of total length of the composite DNA, or the distance of the Sp1 sites to mNP. The dashed line is the calculated fluorescence contribution for each InD fitted to data in A and B to deduce the position of protein binding.

[00135] The enhancement effect is larger for shorter DNA, due to the same reason as discussed above. The comparison between AuNPs and AgNPs shows that $AgNP_{80nm}$ has stronger enhancement effect than $AuNP_{100nm}$.

[00136] It must be acknowledged that for larger mNPs there is not a universal formulation available to calculate the distance based on fluorescence enhancement as FRET for quenching. In order to circumvent this limitation, a controlled distance study was carried out to compare the fluorescence intensity of FAM-labeled DNA in free solution and on $AuNP_{100nm}$ or $AgNP_{80nm}$, by using dsDNA as rigid rod spacer. The enhancement as function of distance is well fitted with exponential curve. The same curve was then further used to calculate the distance of which TOs have already been displaced, as given by the drop in intensity. From the fitting curve, it gave quite precise location of the motifs from $AuNP_{100nm}$ /$AgNP_{80nm}$ surface (Figure 9C).

Example 4:

Identification of ERα or Sp1 protein binding to composite DNA with a ruler of a "quenching profiler"

[00137] In Figure 7A, the ½ ERE is located very close to the AuNP (10 bp away), whereas the Sp1 sites are further away. In this example, it is demonstrated that with a 20 nm AuNPs profiler, it can be detected whether the added protein is ERα (that binds to the ½ ERE) or Sp1 that binds to the Sp1 sites by the differential TO intensity drops. This is based on the fact that the contribution of TO at proximal distance is much smaller and thus the change in fluorescence intensity when ERα bound to ½ ERE is much smaller.

Methods and Materials

[00138] A dsDNA composite sequence with 8 bp spacer that is not conjugated, a dsDNA composite sequence with 4-18 bp spacer conjugated to 20 nm AuNPs via 5′ end, and a dsDNA composite sequence with 4-18 bp spacer conjugated to 20 nm AuNPs via 3′ end, were used in this example.

[00139] TO was added to the unconjugated dsDNA, dsDNA conjugated to 20 nm AuNPs via 5′ end, and dsDNA conjugated to 20 nm AuNPs via 3′ end at TO:DNA molar ratio of 23:1 and incubated for 15 min to allow TO intercalation. The initial fluorescence intensity was measured by using a fluorescence spectrometer or a microplate reader. Sp1 was added in the equal molar ratio of Sp1 to DNA. After 15 min incubation, the fluorescence intensity of TO after addition of Sp1 was measured. Then, ERα was added in the equal molar ratio of ERα

to DNA. After 15 min incubation, the fluorescence intensity of TO after ERα addition was measured.

Results

[00140] As illustrated in **Figure 10**, for the 5′ end conjugation (center panel), there is clear demarcation upon ERα binding and Sp1 binding to composite DNA (8 bp spacer). The magnitude of TO intensity decrease is much greater for Sp1 binding than ERα binding. For the 3′ end conjugation (right panel, Figure 9), the result is reversed.

[00141] In the control experiment with the conventional FID without the AuNPs (left panel), Sp1 binding to the composite DNA didn't generate significant difference compared to ERα binding over the tested protein concentration. This indicates that the affinity of the ERα to the ½ ERE and the affinity of Sp1 to Sp1 sites are not different sustainably.

[00142] Due to the insignificant affinity difference, the intensity drop value in the traditional FID couldn't conclude binder's identity. In contrast the AuNPs-assisted FID (current invention) can generate significant differentiation for the protein binding due to the relative distance of the binding motifs to the AuNPs.

[00143] In conclusion with the plasmonic-fluorescent ruler assay, the identity of the protein binding of different identity but similar affinity can be determined.

[00144] To further verify the function of the profiler AuNPs for determining distance dependency of protein binding, ERα binding to 5′ end conjugates for the entire series of composite DNA (4-18 bp spacer) was tested. Since the ½ ERE site was located at the same distance from the surface of AuNP for the entire series of DNA, the degree of quenching is pretty uniform upon ERα binding to the ½ ERE site (~800 a.u.; see **Figure 11A**). Conversely, when AuNPs is conjugated via the

3′ end, ½ ERE is located approximately 27 bp, 31 bp, and 35 bp (equivalent to 10.0, 11.4, and 12.7 nm) from the surface of AuNP for the 4 bp, 8bp and 12 bp DNA (**Figure 11B**).

[00145] Thus, the magnitude of intensity drop due to ERα binding is dependent on the ½ ERE distance from AuNP's surface and also much greater than the respective 5′ end conjugates, mainly owing to the greater distance of ½ ERE from AuNP's surface in 3′ end conjugate.

Example 5

Detection of spacer dependent cooperative ERα and Sp1 binding using a ruler with a "enhancement profiler"

[00146] Sp1 protein is known to be involved in regulating progesterone receptor gene expression, especially at these sites presence of Sp1 sites next to half-ERE (typically 10-17 bp away). It is reported that binding of Sp1 protein to the Sp1 site next to half-ERE could increase the amount of ERα protein recruited compared to ERα alone to half-ERE, through additional protein-protein contact. The enhancement effect or cooperative Sp1-ERα binding should be spacer dependent. Unfortunately, bio-informatic simulation failed to give an exact model, or weakly conclusive of the cooperative effect.

[00147] In this example, plasmonic-fluorescent rulers with an "enhancement profiler" are used to study the binding of one protein (ERα) in the presence of another (Sp1), or the cooperative action.

Method and Materials

[00148] TO/dsDNA-$AuNP_{100nm}$ and TO/dsDNA-$AgNP_{80nm}$ rulers are used for demonstration of the concept, by measuring ERα

binding to Sp1 saturated DNA (Figure 12A'-C'). The binding of ERα without Sp1 is used as control (Figure 12A-C).

[00149] An unconjugated dsDNA composite sequence with 4-18 bp spacer, dsDNA composite sequence with 4-18 bp spacer conjugated to 100 nm AuNPs via 5' end, and dsDNA composite sequence with 4-18 bp spacer conjugated to 80 nm AgNPs via 3' end are used in this experiemtnt.

[00150] TO was added to the conjugated and unconjugated dsDNA at a TO:DNA molar ratio of 23:1 and incubated for 15 min to allow TO intercalation. The initial fluorescence intensity was measured by using a fluorescence spectrometer or a microplate reader. To prepare Sp1 saturated dsDNA (for both conjugated and unconjugated dsDNA), Sp1 was added in the molar ratio of Sp1:DNA at 2:1. After 15 min incubation, the fluorescence intensity of TO after Sp1 saturation was measured. Then, ERα was added in the equal molar ratio of ERα to DNA. After 15 min incubation, the fluorescence intensity of TO after ERα addition to Sp1 saturated dsDNA was measured. For control experiment, ERα was added directly to dsDNA in the equal molar ratio of ERα to DNA without prior addition of Sp1.

Results

[00151] The binding of ERα without Sp1 shows similar decrease in emission intensity across the DNA series. This is expected because ERα affinity to ½ ERE is uniform for all DNA bp tested in the absence of Sp1 protein. However, when the DNA is first saturated by Sp1, the intensity drop caused by the subsequent ERα are found different across the tested spacer length with the rulers (Figure 11B' and C').

[00152] At the spacer >10bp, ERα induced intensity drop is significantly larger than the average measured for the short spacers. As Petz reported (Mol Endo 2000), at the shorter

spacers (< 8 bp), owing to the close proximity between ½ ERE and Sp1 motifs and the size of Sp1 protein, it is possible that steric repulsion can hinder ERα binding to ½ ERE motif efficiently. The present invention and ruler results confirm this point and further shows that a spacer >10 bp is more favorable for cooperative effect to occur to enhance ERα binding (Figure 11B′ and C′).

[00153] Using conventional FID assay (Figure 11A′), no such effect is detectable (the signals are significantly higher than the average from the short spacer cases). This is because the limited sensitivity of the FID in differentiation of subtle affinity difference. However, with the "enhancement profiler" rulers, such subtle difference become detectable, because the TO at the ½ ERE site is close to the mNPs and their emission is amplified. The displacement of those dyes generated larger signal.

[00154] In other words, the "mNPs profiler" acts like a magnifying glass. The finding of favorable cooperative interactions between ERα-Sp1 at spacer of 10-18 bp is consistent with the data using Biacore T200.

Applications

[00155] The advantages of the present invention are evident. Advantageously, mNPs and intercalator dyes are used to construct a method, for determining site specific ligand binding, where the nucleic acid to be studied is covalently attached to mNPs. Specifically, through proper selection of InD, metal nanoparticles (mNPs), and proper detection of optimal TO amount (Example I), it is demonstrated that mNPs coupled FID or the "plasmonic-fluorescent ruler" is able to serve the following purposes:

[00156] Detecting the binding of ligands of the same identity to a nucleic acid composite at specific binding site via a "quenching profiler" (Example II) or a "enhancement profiler" (Example III).

[00157] Detecting the binding of ligands of different identity to a nucleic acid composite at a specific site (Example IV).

[00158] Determining cooperative binding between multiple ligands and optimum separation distance for cooperative effect (Example V).

Claims

what we claim is:

1. A method for determining site specific binding of at least one ligand to a composite nucleic acid, comprising:

a) providing a composite nucleic acid comprising a metal nanoparticle attached to a nucleic acid having a predetermined length;

b) saturating said composite nucleic acid with an intercalating dye;

c) measuring an initial fluorescence intensity (F_0) of said saturated composite nucleic acid;

d) contacting said saturated composite nucleic acid with said ligand;

e) measuring a further fluorescence intensity (F_1) of said contacted saturated composite nucleic acid and determining the relative change in fluorescence intensity (ΔF), between F_0 and F_1; and

f) correlating the relative change in fluorescence intensity with the predetermined length of the nucleic acid to determine a distance (R) of the binding of the ligand from the metal nanoparticle.

2. The method of claim 1, wherein the ligand is a polypeptide.

3. The method of claim 2, wherein the polypeptide is a transcription factor.

4. The method of any one of claims 1-3, wherein the nucleic acid is DNA or RNA.

5. The method of any of claims 1-4, wherein the nucleic acid comprises at least one binding site for said at least one ligand.

6. The method of any of claims 5, wherein the binding site is a full or partial binding site.

7. The method of any of claims 1-6, wherein the nucleic acid is a double stranded DNA comprising at least 8 base pair.

8. The method of any of claims 1-7, wherein the metal nanoparticle is selected from the group consisting of gold, silver, palladium, ruthenium, rhodium, osmium, iridium and platinum.

9. The method of any of claims 1-8, wherein the size of the metal nanoparticle is from 5nm to 2000nm.

10. The method of any of claims 1-9, wherein the intercalating dye is ethidium bromide or thiazole orange.

11. The method of any of claims 1-10, wherein said metal nanoparticle is less than 80 nm, and said fluorescent intensity of said intercalating dye decreases proximal to said nanoparticle.

12. The method of any of claims 1-10, wherein said metal nanoparticle is greater than 80 nm and said fluorescent intensity of said intercalating dye increases proximal to said nanoparticle.

13. The method of any of claims 1-12, wherein the relative change in fluorescence intensity is a percentage decrease in intensity.

14. The method of any of claims 1-13, wherein the relative change in fluorescence intensity (ΔF) is calculated by

$$\Delta F = (F_1 - F_0)/F_0.$$

15. The method of any of claims 1-14, wherein the distance (R) is a specific nucleotide base number relative to the nanoparticle.

16. The method of claim 15, wherein the distance between each nucleotide base is 0.34 nm.

17. The method of any one of claims 1-16, wherein the binding site is determined based on the relative intensity decrease on a calibration curve.

18. The method of any of claims 1-17, further comprising:

contacting said saturated composite nucleic acid with a first ligand in a 1st molar amount and measuring a first further fluorescence intensity (F'_1) of said contacted saturated composite nucleic acid;

contacting said saturated composite nucleic acid with a second ligand in a 2nd molar amount and measuring a second further fluorescence intensity (F'_2) of said contacted saturated composite nucleic acid;

determining the relative change in fluorescence intensity (ΔF), between F_0, F'_1, and F'_2;

correlating the relative change in fluorescence intensity with the preferential binding site of the ligand,

wherein the nucleic acid comprises at least one binding site for said first and second ligand.

19. The method of any of claims 1-17, further comprising:

contacting said saturated composite nucleic acid with a first ligand and measuring a first further fluorescence intensity (F'_1) of said contacted saturated composite nucleic acid;

contacting said saturated composite nucleic acid with a second ligand and measuring a second further fluorescence intensity (F'_2) of said contacted saturated composite nucleic acid;

determining the relative change in fluorescence intensity (ΔF), between F_0, F'_1, and F'_2;

correlating the relative change in fluorescence intensity to determine the identity of the first and/or second ligand and a distance (R) of the binding of the first and/or second ligands from the metal nanoparticle.

20. The method of claim 18 or 19, wherein the first and second ligands may be the same or different.

21. The method of any of claims 18-20, wherein the first and second ligand are contacted with the composite nucleic acid separately and sequentially.

22. The method of any of claims 1-17, further comprising:

contacting said saturated composite nucleic acid with a first ligand and measuring a first further fluorescence intensity (F'_1) of said contacted saturated composite nucleic acid;

contacting said saturated composite nucleic acid with the first ligand and one or more further ligands and measuring a second further fluorescence intensity (F'_2) of said contacted saturated composite nucleic acid;

determining the relative change in fluorescence intensity (ΔF), between F_0, F'_1, and F'_2;

correlating the relative change in fluorescence intensity to determine the cooperative binding of the first and further one or more ligands.

23. The method of claim 22, wherein the optimal separation distance between the bound ligands for a cooperative binding effect is determined.

24. The method of claim 23, wherein the method is repeated once or more for a nucleic acid composite comprising a spacer sequence of about 4 bp to 18 bp.

25. A kit for detecting site specific binding of at least one ligand to a nucleic acid, comprising: a nucleic acid composite comprising a nanoparticle attached to an end of said nucleic acid, and an intercalator dye.

26. The kit of claim 25, wherein the nucleic acid comprises at least one binding site for said at least one ligand.

Abstract

METHODS FOR DETERMINING SITE SPECIFIC BINDING OF AT LEAST ONE LIGAND TO A COMPOSITE NUCLEIC ACID, AND KITS THEREOF

[00159] The present invention relates to methods for determining site-specific ligand binding to nucleic acid composites comprising metal nanoparticles. In this regard, the present invention exploits a metal nanoparticle-assisted fluorescent intercalator displacement (mNPs-FID) principle, whereby the InD's fluorescence intensity is modulated by mNPs in a distance dependent manner making it possible to determine site specific ligand binding on a nucleic acid.

(no suitable figure)

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METHODS FOR DETERMINING SITE SPECIFIC BINDING OF AT LEAST ONE LIGAND TO A COMPOSITE NUCLEIC ACID, AND KITS THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of Singapore provisional application No.201308043-7, filed 29 October 2013, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates to the field of molecular biology, biochemistry and nanotechnology. More particularly, the invention relates to methods for determining site-specific ligand binding to nucleic acids composites comprising metal nanoparticles.

BACKGROUND OF THE INVENTION

[0003] In molecular biology and genetics, the regulation of gene expression largely relies on the sequence-selective recognition of nucleic acids by repressor and/or activator ligands, such as proteins. For example, one such type of repressor and/or activator proteins are transcription factors (TF), that may perform their function alone or in combination with other proteins in a complex, by promoting (as an activator), or blocking (as a repressor) the recruitment of RNA polymerase (the enzyme that performs the transcription of genetic information from DNA to RNA) to specific genes and binding sequences(Nikolov et al., Proc. Natl. Acad. Sci. U. S. A. 1997, 94, 15-22).

[0004] Often, for gene transcription to occur, a number of TFs must bind to DNA regulatory sequences synergistically. To understand the complex gene regulation mechanisms for a

specific gene regulation, it is critical to reveal the molecular details of the co-localization of multiple proteins.

[0005] Of the techniques commonly utilized to establish nucleic acid binding properties of polypeptides, most of them are technically challenging, require the knowledge of specialized biochemical procedures and expensive equipment, and are time and labor intensive. Footprinting and Electrophoretic mobility shift assay (EMSA), for example, can define a protein's binding selectivity but they involve biohazard labeling and arduous assays. Surface plasmon resonance (SPR) spectroscopy can quantitatively determine protein-DNA binding affinity, sequence selectivity, and binding stoichiometry. But SPR experiments require expensive equipment and extensive optimization of surface chemistry for DNA or protein immobilization. The Fluorescent Intercalator Displacement (FID) assay is nondestructive, technically non-demanding, and amenable for high-throughput assay. However, this method is insensitive for differentiation of subtle affinity difference.

[0006] For studying multiple polypeptide binding to nucleic acids, the above techniques have severe limitations as shown in Figure 2 disclosed herein. In particular, EMSA requires a full set of DNA with selective mutation of each sites in order to determine the preferable protein binding (Petz et al., Mol Endo 2000, 14, 972-985. SPR measurement does not provide protein identity due to the principle of measuring the refractive index. The refractive index change is insensitive to mass change distance within the 200 nm evanescent wave field. The study of multiple protein binding require specific antibody in a successive step (Neo et al., Anal Chem 2009, 81, 3344-3349). Not only this will add additional assay steps, the study of complex protein binding is subject to the availability of specific antibodies for the proteins of

interest. Finally, based on experiments with FID detection of high affinity sequence specific interactions (ERα-ERE), low affinity binding (ERα-½ ERE and Sp1-CG boxes), and nonspecific interaction (Sp1-random DNA), this method can only differentiate high affinity binding relative to low affinity ones. It cannot differentiate low affinity binding and nonspecific binding (i.e. between ERα-½ ERE, Sp1-CG boxes, and Sp1-random DNA). This shows that the traditional FID has limited sensitivity, and is thus incapable of studying site specific protein binding.

[0007] A deeper insight of the ligand binding involved, the delineation of the sequences to which they bind, the interaction between ligands and the subsequent impact on gene transcription action and deactivation holds significant promise in therapeutic medicine.

[0008] As such, there is a need to provide methods that can further elucidate ligand-nucleic acid binding, and particular site specific polypeptide binding, which may at least partially ameliorate one or more of the disadvantages described above with regards to the use of any bulky expensive equipment or consumable items and hazardous labeling.

SUMMARY OF THE INVENTION

[0009] In one aspect, the present invention provides a method for determining site specific binding of at least one ligand to a composite nucleic acid, comprising: providing a composite nucleic acid comprising a metal nanoparticle attached to a nucleic acid having a predetermined length; saturating said composite nucleic acid with an intercalating dye; measuring an initial fluorescence intensity (F_0) of said saturated composite nucleic acid; contacting said saturated composite nucleic acid with said ligand; measuring a further fluorescence

intensity (F_1) of said contacted saturated composite nucleic acid and determining a relative change in fluorescence intensity (ΔF), between F_0 and F_1; and correlating the relative change in fluorescence intensity with the predetermined length of the nucleic acid to determine a distance (R) of the binding of the ligand from the metal nanoparticle.

[0010] In a second aspect, the present invention provides a kit for detecting site specific binding of at least one ligand to a nucleic acid, comprising: a nucleic acid composite comprising a nanoparticle attached to said nucleic acid, and an intercalator dye.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0012] **Figure 1.** Shows a schematic illustration of (A) even InD emission in dsDNA and (B&C) uneven emission in dsDNA conjugated to AuNPs of a smaller (B) or (C) a larger size. According to the function, the AuNPs in B and C are termed "quenching profiler" and "enhancement profiler", respectively. The initial dye intensity in A is denoted as F_0. The resulting intensity in (B) and (C) are denoted as F'_0 and F''_0, respectively. Upon addition of protein, InD will be displaced, as shown representatively by (A). The resulting intensity is denoted as F_1, F'_1 and F''_1, respectively.

[0013] **Figure 2.** Shows a table of limitations of existing techniques for studying multiple protein-composite DNA interactions.

[0014] **Figure 3.** Shows a schematic diagram of the DNA-conjugated AuNPs, showing the total length and the distance of each site to AuNPs of sequences with different spacers between

½ ERE and Sp1 sites. Spacer 4, 8, 12, and 18 are selected to show the distance calculation.

[0015] **Figure 4.** Shows (A) UV-vis spectra of 20 nm AuNP, 100 nm AuNP, and 80 nm AgNP and their conjugates with the 52 bp DNA. (B) TO's emission spectrum when intercalated in dsDNA52-AuNPs20nm. TO's fluorescent intensity increases as the ratio of TO:DNA is incrementally varied from 1:1 to 30:1 molar ratio. The dashed line in (B) (at the top) represents 23:1 of TO:DNA ratio and the dotted line (most bottom) was free TO (without DNA) at the highest concentration used in this study. The AuNPs used to conjugate with the DNA is 20 nm. The DNA is the longest strand of 52 bp. The formed construct is denoted as TO/dsDNA52bp-AuNPs20nm.

[0016] **Figure 5.** Shows (A) Fluorescence curve from TO intercalated into 38 bp DNA, 38 bp DNA-AuNP20nm, and 38 bp DNA-AuNP100nm. (B). resulting intensity (at 535 nm) of saturate amount of TO intercalated to various length DNA in the absence of AuNPs (circle), presence of 20 nm AuNPs (diamond) and 100nm AuNPs (triangle).

[0017] **Figure 6.** Shows relative fluorescence intensity of TO intercalated to DNA-AuNPs20nm of 38, 44, and 52 bp DNA (F'_0), with TO in respective DNA without AuNPs (F_0) as reference.

[0018] **Figure 7.** Shows schematic illustration of the two TO/dsDNA-AuNPs20nm setting used to identify preferential binding site of Sp1 at two Sp1 motifs, with the DNA conjugated to AuNPs at the (A) ½ ERE site and (B) Sp1/P site. The bp number refers to the distance of the Sp1 sites to AuNPs (4bp spacer between ½ ERE and Sp1/D). (C) The relative decrease in fluorescent intensity (at 535 nm) induced by first molar and second molar of Sp1 binding for dsDNA/TO without AuNP and for dsDNA-AuNPs/TO composites in "A setting" and "B setting".

[0019] **Figure 8.** Shows (A) TO fluorescent intensity decrease (at 535 nm) for ½ ERE-Sp1 DNA (spacer 4-18 bp) conjugated at

5′ end to 20nm AuNP, caused by 1 st molar (left bars) and 2nd molar (right bars) Sp1 binding. (B) Decrease in intensity at 535 nm upon binding of 1st molar Sp1 (square) and 2nd molar Sp1 (triangle). The dashed line is the calculated fluorescence contribution for each InD fitted to data in A to deduce the position of protein binding. The relative standard deviation between three repeated experiments are ~5%.

[0020] **Figure 9.** Shows the detection of preferential Sp1 binding to distal Sp1 site using (A) 100 nm AuNPs and (B) 80 nm AgNPs assisted TO displacement. Blue and green bars are the fluorescent intensity drop corresponding to the binding of 1st Sp1. Red and orange bars are the 2nd molar Sp1 binding. (C) TO intensity decrease (at 535 nm) relative to non-enhanced TO intensity drop measured with control setting without AuNPs, as a function of total length of the composite DNA, i.e. the distance of the Sp1 sites to mNP. The relative standard deviation between three repeated experiments are ~5%.

[0021] **Figure 10.** Shows a bar diagram of the decrease in fluorescent intensity of TO induced by Sp1 and ERα binding. The DNA are the composite sequence with 8 bp spacer. The DNA is conjugated to 20 nm AuNPs via either 5′ end or 3′ end.

[0022] **Figure 11.** Shows a bar diagram of the decrease in fluorescent intensity of TO induced by ERα binding in the (A) 5′end TO/dsDNA-AuNPs and (B) 3′ end TO/dsDNA-AuNPs rulers. The relative standard deviation between three repeated experiments are <7%.

[0023] **Figure 12.** Shows TO fluorescent intensity drop (at 535 nm) induced by ERα binding without Sp1 (A-C) and with Sp1 (A′-C′), measured by conventional FID (A ad A′), and (B and B′) AuNP100nm-assited FID and (C and C′) AgNP80nm-assited FID. The solid line is the average value decrease in fluorescent intensity and red dotted lines are the three standard

deviations. The relative standard deviation between three repeated experiments are <7%.

Definitions

[0024] The following words and terms used herein shall have the meaning indicated:

[0025] The term "ligand" is interchangeable with "nucleic acid binders" and both refer to any molecule, compound or substance that is capable of binding to a nucleic acid. In particular, the "ligand" may be a polypeptide, protein, small molecule, antibody or organic compound.

[0026] The term "nanoparticle" refers to a particle that covers a range between about 1 nm and about 2000 nm. Nanoparticles may or may not exhibit size-related properties that differ significantly from those observed in fine particles or bulk materials. The nanoparticle may have different shapes, including but not limited to spheres, discs, rods, fibers, cups, boxes, pyramids, tetrahedron, octahedron, decahedron, tetrapods, multipods, stars, and the like.

[0027] The term "conjugated" or "coupled" or "attached" in the context of the mNPs and nucleic acid refers to the forming of a bond between the metal surface of the mNP and the nucleic acid. This bond may refer to a chemical bond formed between a nucleic acid molecule and the surface of a nanoparticle.

[0028] The term "nucleic acid" refers to a molecule made from one or more nucleotides as is well known. The nucleic acid molecule may be single stranded (ss) or double stranded (ds). Nucleic acid molecules may include, but are not limited to, deoxyribonucleotide (DNA), ribonucleic acid (RNA), small interfering RNA (siRNA), peptide nucleic acid (PNA), locked nucleic acid (LNA) and the like.

[0029] The term "binding site" refers to a region of a nucleotide sequence to which a ligand binds in a specific or non-specific manner. The binding site may be a "partial" or "fragment" of the binding site, whereby a one or more nucleotides are absent from the "full" binding site and the function as a binding site is maintained.

[0030] The term "polypeptide" refers to a large molecule comprising one or more chains of amino acids. The polypeptide may be negatively charged or positively charged. The polypeptide may have a vast array of functions, including but not limited to, catalysis, gene regulation, responding to stimuli and the like.

[0031] The term "enhance" in reference to fluorescence for the purposes of this disclosure, refers to any process that increases the fluorescence intensity of a given substance. Enhancement may be a result of, but not limited to, excited state reactions, energy transfer, electron transfer, complex formation, colloidal quenching and the like. Enhancement may be static or dynamic. The term "enhanceable" should be construed accordingly.

[0032] The term "quench" in reference to fluorescence for the purposes of this disclosure, refers to any process that decreases the fluorescence intensity of a given substance. Quenching may be a result of, but not limited to, excited state reactions, energy transfer, electron transfer, complex formation, colloidal quenching and the like. Quenching may be static or dynamic. The term "quenchable" should be construed accordingly.

[0033] The term "plasmonic" in the context of the present invention and metal nanoparticles refers to the exploitation of surface plasmons, which are produced from the interaction of light with metal-dielectric materials. Under specific conditions, the incident light couples with the surface

plasmons to create self-sustaining, propagating electromagnetic waves known as surface plasmon polaritons (SPPs). Once launched, the SPPs ripple along the metal-dielectric interface and do not stray from this narrow path.

DETAILED DESCRIPTION

[0034] The present invention discloses a method for site-specific determination of ligand binding to a composite nucleic acid that may comprise multiple binding sites, by exploiting metal nanoparticle-assisted fluorescent intercalator displacement (mNPs-FID) principle, as further elaborated herein.

[0035] The fluorescent intercalator displacement assay (FID) is a well-known technique for detecting nucleic acid binders such as proteins, on a single nucleic acid binding site. In conventional FID, the addition of binding ligands, such as proteins and other nucleic acid binders, produces a drop in fluorescence emission due to the displacement of the InD from the nucleic acid.

[0036] In the present invention, mNPs are attached to one end of the nucleic acid to perform plasmonic mediated FID. In the presence of the mNPs, the magnitude of fluorescent intensity decrease is determined not only by the affinity of the nucleic acid binders to the nucleic acid, but also largely by the binding position of nucleic acid binders relative to the mNPs. In particular, when the binding affinity difference is minor (undetectable by conventional FID), the present invention is able to measure which binding sites the specific nucleic acid binders bind to.

[0037] The combined use of mNPs with a conventional FID assay is termed as a "plasmonic-fluorescent ruler", due to the underline rationale that mNPs can modulate the proximal

fluorophores in a distance dependent manner. In particular, the mNPs act as a 'profiler' that gives each InD its own contribution to the overall fluorescence intensity, according to their distance to the mNPs, thus allowing differentiation among InDs.

[0038] The concept of the InD fluorescence intensity in the present invention and "plasmonic-fluorescent ruler" is illustrated in Figure 1.

[0039] Advantageously, the modulation of the InD's fluorescence intensity in a distance dependent manner makes it possible to determine site specific ligand binding on a nucleic acid without involving a full set of control nucleic acids with selective site mutations. Therefore, ligand binding to a particular nucleic acid sequence can be approximated, by back-calculating the distance, based on the decrease in fluorescence intensity.

[0040] Accordingly, in a first embodiment the present invention refers to a method for determining site specific binding of at least one ligand to a composite nucleic acid, comprising: a) providing a composite nucleic acid comprising a metal nanoparticle attached to a nucleic acid having a predetermined length; b) saturating said composite nucleic acid with an intercalating dye; c) measuring an initial fluorescence intensity (F_0) of said saturated composite nucleic acid; d) contacting said saturated composite nucleic acid with said ligand; e) measuring a further fluorescence intensity (F_1) of said contacted saturated composite nucleic acid and determining the relative change in fluorescence intensity (ΔF), between F_0 and F_1; and f) correlating the relative change in fluorescence intensity with the predetermined length of the nucleic acid to determine a distance (R) of the binding of the ligand from the metal nanoparticle.

[0041] In one embodiment, the ligand may be any molecule, substance or compound that is capable of binding to a nucleic acid and displacing an Intercalating dye molecule. In particular, the ligand may be selected from proteins, polypeptides, antibodies, small molecules or organic compounds.

[0042] In one embodiment, the ligand may be a polypeptide. In accordance with aspects of the invention the polypeptide may be referred to as a binding protein. The terms "polypeptide" and "binding protein" may be used interchangeably and may refer to any polypeptide/protein that binds to a nucleic acid. Examples of polypeptides or binding proteins include, without limitation, transcription factors, including activators or suppressors of transcription, proteins involved in replication, or enzymes. In one embodiment, the polypeptide is a transcription factor. In another embodiment, the transcription factor may be any one of FoxA1, AP-2γ, ERα, ERβ or SP1.

[0043] The polypeptide may bind to the nucleic acid in a sequence-specific manner, thus recognizing and binding to a specific sequence of nucleotides found within the nucleic acid. The specific nucleic acid sequence to which the protein binds is referred to herein as a binding site, a sequence motif or a specific binding sequence. These terms may be used interchangeably. The binding proteins bind to the nucleic acid in a sequence-specific manner, and thus have a measurable binding affinity for target consensus sequences and may bind to sequences that have slightly altered sequences with a different (e.g., either greater or lesser) affinity. As such, in one embodiment the nucleic acid comprises at least one binding site for the polypeptide. In one embodiment, the binding site may be a full or partial binding site.

[0044] In another embodiment, the nucleic acid comprises at least one, two, three, four or more binding sites. In one embodiment, the nucleic acid comprises multiple binding sites separated from one another by spacer sequence regions. The spacer sequences may comprise a sequence of at least 4 bp, at least 5 bp, at least 6 bp, at least 7bp, at least 8 bp, at least 9 bp, at least 10 bp, at least 11 bp, at least 12 bp, at least 13 bp, at least 14bp, at least 15 bp, at least 16 bp, at least 17 bp, at least 18 bp. In another embodiment, the spacer sequence is 4 bp, 6 bp, 8 bp, 10 bp, 12 bp, 14 bp, 16 bp or 18 bp.

[0045] In another embodiment the nucleic acid is DNA or RNA, preferably a double stranded DNA (dsDNA). The double stranded nucleic acid molecule may further comprise any one of a loop, tetraloop, stem-loop, hairpin loop, junction, bulge, pseudoknot or internal loop.

[0046] In one embodiment, the nucleic acid is a predetermined length of base pairs, whereby the precise length may depend on factors such as the type of nanoparticles, such as gold or silver NP, the length of the target recognition sequence, the size of the binding protein, and/or the manner in which the binding protein binds to the target recognition sequence. A skilled person can readily test a suitable length of nucleic acid required for any given nanoparticle, target recognition sequence and binding protein, using routine laboratory methods, and as illustrated in the Examples provided herein.

[0047] In another embodiment, the nucleic acid may have a sequence of at least 5 bp, at least 6bp, at least 7bp, at least 8 bp, or may be in the range of any one of about 5 base pairs (bp) to about 100 base pairs (bp), about 5 bp to about 10 bp, about 5 bp to about 15 bp, about 5 bp to about 20 bp, about 5 bp to about 25 bp, about 5 bp to about 50 bp, about 5 bp to about 75 bp, about 10 bp to about 15 bp, about 10 bp to

about 20 bp, about 10 bp to about 25 bp, about 10 bp to about 50 bp, about 10 bp to about 75 bp, about 10 bp to about 100 bp, about 15 bp to about 20 bp, about 15 bp to about 25 bp, about 15 bp to about 50 bp, about 15 bp to about 75 bp, about 15 bp to about 100 bp, about 20 bp to about 25 bp, about 20 bp to about 50 bp, about 20 bp to about 75 bp, about 20 bp to about 100 bp, about 25 bp to about 50 bp, about 25 bp to about 75 bp, about 25 bp to about 100 bp, about 50 bp to about 75 bp, about 50 bp to about 100 bp or about 75 bp to about 100 bp.

[0048] The metal nanoparticles provided herein are conjugated to the nucleic acid to form a nucleic acid composite. The nucleic acid may be conjugated/ attached to the nanoparticle by any method used to conjugate/attach an organic molecule such as DNA to a metal. For example, free thiol groups react with metal, forming a bond between the metal surface and the sulphur atom. Thus, the nucleic acid may be synthesized with an end group containing a free thiol on one end, such as the 5' end, of one strand of the nucleic acid, using standard laboratory techniques. The nucleic acid with a free thiol may be conjugated to the metal nanoparticle for use in any of the methods provided herein, thereby forming a thioether linkage between the metal nanoparticle and the DNA molecule.

[0049] In one embodiment, the metal nanoparticles provided herein may be any one of an alkali metal, alkaline earth metal, transition metal, post-transition metal, lanthanide metal, actinide metal or any alloy thereof. In one embodiment, the metal nanoparticle may be a noble metal nanoparticle. The noble metal may be any one of ruthenium (Ru), rhodium (Rh), palladium (Pd), silver (Ag), osmium (Os), iridium (Ir), platinum (Pt), gold (Au) or any alloy thereof. In one embodiment, the metal nanoparticle is a noble metal nanoparticle. In another embodiment, the noble metal

nanoparticle is a gold (Au) NP (AuNP) or a silver (Ag) NP (AgNP).

[0050] Advantageously, the mNP may have unique optical and electronic properties, whereby the interaction of mNPs with light is strongly dictated by their environment, size and physical dimensions. Oscillating electric fields of a light ray propagating near a colloidal nanoparticle may interact with free electrons, causing a concerted oscillation of electron charge that is in resonance with the frequency of light. These resonant oscillations, known as plasmons, may depend on the size of the mNP. For examples, the surface plasmon resonance phenomena of small monodisperse (~30 nm) AuNPs, may cause an absorption of light in the blue-green portion of the spectrum (~450 nm) while red light (~700 nm) to be reflected. As particle size increases, the wavelength of surface plasmon resonance related absorption shifts to longer wavelengths. Red light is then absorbed and blue light is reflected. The surface plasmon resonance may be tuned by varying the size or shape of the mNP, leasing to particles with tailored optical properties for different applications.

[0051] As illustrated in **Figure 1**, in a conventional FID assay without mNPs (Figure 1A), the change in the fluorescence intensity due to protein binding are roughly uniformly distributed among all of the dyes being displaced from the nucleic acid, for example if there was 5 intercalated dyes displaced, the contribution of each dye would be 20%.

[0052] In the constructed mNP coupled FID of the present invention, the initially observed fluorescent intensity of the InD inserted to the nucleic acid composite is a weighted average from each InD. Figure 1A shows an even emission in dsDNA without mNPs, and Figure 1(B & C) shows the uneven emission in dsDNA with mNPs conjugates of smaller mNPs (5-80 nm) or larger mNPs (> 80 nm). In (B) and (C), the overall (or

apparent/resulting) InD intensity is either lower (quenched) or higher (enhanced) relative to that without AuNPs as in (A).

[0053] Accordingly, when smaller mNPs are present as a quenching profiler (Figure 1B), the InD located nearer to the mNP surface has a lower weighting factor or less contribution to the overall intensity, since it is quenched much stronger by surface Plasmon resonance of the mNP, and *vice versa* for the one further away. On the other hand, when larger mNPs are used as an enhancement profiler (Figure 1C), the InD located nearer to the mNP surface has a higher weighting factor or more contribution to the overall intensity, because the enhancement effect is stronger as it closer to mNPs. By using the example with a "quenching profiler" (Figure 1B) as illustration, the denoted weighting factor of the 5 InDs (A, B, C, D, and E, with A being the closest to mNP's surface and E as the furthest entity) of a, b, c, d and e; the relative weighting factor of (in fraction) each InD is roughly $a < b < c < d < e$, and the summation $(a + b + c + d + e)$ is equal to one.

[0054] As such, the material and size of the mNPs, may impact upon the overall initial fluorescence emission of the InD in the nucleic acid composite as being either lower (quenched by a smaller mNPs) or higher (enhanced by a larger mNPs) in comparison to nucleic acid composites without a mNP profiler. The latter will provide higher sensitivity for ligand binding near to the particle surface.

[0055] Accordingly, in one embodiment the mNPs are either smaller or larger mNPs. In another embodiment, the mNPs may have a size in the range of about 5 nm to about 1000nm, about 5 nm to about 500 nm, about 5 nm to about 300nm, about 5 nm to about 200nm, about 5 nm to about 10 nm, about 5 nm to about 15 nm, about 5 nm to about 50 nm, about 5 nm to about 70 nm, about 5 nm to about 80 nm, about 5 nm to about 100 nm, about 5

nm to about 150 nm, about 10 nm to about 15 nm, about 10 nm to about 50 nm, about 10 nm to about 70 nm, about 10 nm to about 80 nm, about 10 nm to about 100 nm, about 10 nm to about 150 nm, about 10 nm to about 200 nm, about 15 nm to about 50 nm, about 15 nm to about 70 nm, about 15 nm to about 80 nm, about 15 nm to about 100 nm, about 15 nm to about 150 nm, about 15 nm to about 200 nm, about 50 nm to about 70 nm, about 50 nm to about 80 nm, about 50 nm to about 100 nm, about 50 nm to about 150 nm, about 50 nm to about 200 nm, about 70 nm to about 80 nm, about 70 nm to about 100 nm, about 70 nm to about 150 nm, about 70 nm to about 200 nm, about 80 nm to about 100 nm, about 80 nm to about 150 nm, about 80 nm to about 200 nm, about 100 nm to about 150 nm, about 100 nm to about 200 nm or about 150 nm to about 200 nm.

[0056] In yet another embodiment, the mNPs may have a size that is less than 80 nm (smaller mNP) or greater than about 80 nm (larger mNP). In one embodiment, the mNPs may have a size that is less than 80 nm and said fluorescent intensity of said intercalating dye decreases proximal to said nanoparticle. In another embodiment, the mNPs may have a size that is greater than 80 nm and said fluorescent intensity of said intercalating dye increases proximal to said nanoparticle.

[0057] In one embodiment, the mNP may have different shapes selected from any one of spheres, discs, rods, fibers, cups, boxes, pyramids, tetrahedron, octahedron, decahedron, tetrapods, multipods, or stars.

[0058] Advantageously, the present invention and underlying principle of mNPs assisted FID, is particularly useful for constructing the ruler with composite nucleic acid up to tens to hundreds of base pairs in length, due to the long range fluorescent quenching and enhancement effect (up to tens of nm) of the smaller or larger mNPs.

[0059] As described herein, the present invention utilizes mNP assisted FID with intercalator dyes (InD). DNA intercalator dyes (InD) may be aromatic cations with planar structures that enter between stacked base pairs in the DNA duplex to form dsDNA/InD composites. In one embodiment, the intercalater dye is selected from propidium iodide, oxazole yellow, TO-PRO, ethidium bromide or thiazole orange.

[0060] The relative weighting factor of each InD is highly dependent on the length of the nucleic acid length attached to the mNP, the excitation/emission spectrum and quantum yield of the InD, and the surface Plasmon resonance/absorption spectra of mNP used in the method, all of which can be readily selected and adapted by those skilled in the art based upon the desired use.

[0061] The ratio of the InD to the nucleic acid impacts upon the sensitivity of the present invention. In this regard, as well known in the art when using conventional FID assays, the ratio of InD to the nucleic acid is dependent upon the chemical structure of the InD. Those skilled in the art can readily determine the optimal ratio (i.e. reaching saturation) to use by applying conventional techniques in the art, as illustrated in Example 1 described herein. In particular, in one embodiment where TO is the InD, the ratio is 3 TO molecules to 7 bp in length.

[0062] In one embodiment, the InD emits fluorescence in the range of any one of about 200 nm to about 800 nm, about 200 nm to about 300 nm, about 200 nm to about 400 nm, about 200 nm to about 500 nm, about 200 nm to about 600 nm, about 200 nm to about 700 nm, about 300 nm to about 400 nm, about 300 nm to about 500 nm, about 300 nm to about 600 nm, about 300 nm to about 700 nm, about 400 nm to about 500 nm, about 400 nm to about 600 nm, about 400 nm to about 700 nm, about 500 nm to about 600 nm, about 500 nm to about 700 nm or about 600 nm to

about 700 nm. In one embodiment, the InD may emit fluorescence in the range of about 350 nm to about 700 nm, 450 nm to about 700 nm, 500 nm to about 700 nm, 520 nm to about 600 nm, 530 nm to about 580 nm.

[0063] The fluorescence intensity of the InD may be measured by well known techniques in the art and those readily utilized in the convention FID assay. In one embodiment, the fluorescence intensity may be measured by a fluorescence spectrometer or a microplate reader, as is well known in the art.

[0064] In one embodiment, the InD and emission wavelength may be selected to overlap with the UV-vis absorption of the selected mNPs. In particular, the InD fluorescence emission and plasmon peak emission of the mNPs may be within the same range of about 350 nm to about 700 nm, 450 nm to about 700 nm, 500 nm to about 700 nm, 520 nm to about 600 nm, 530 nm to about 580 nm. It will be appreciated that any selectable InD can be used in the present invention.

[0065] An InD once saturated into a nucleic acid, such as dsDNA, will yield a much higher fluorescence emission relative to its free form in solution. As such, contacting the saturated nucleic acid composite with the ligand results in a decrease of fluorescence due to the displacement of the InD from said composited. The decrease of fluorescence is a function and determinant of the relative change in fluorescence intensity, whereby both the decrease and relative change in fluorescence intensity may be expressed as a percentage ranging from 0.01% to 99.99% or any value inbetween.

[0066] In one embodiment, the relative change in fluorescence intensity (ΔF) is calculated from the equation:

- $\Delta F = (F_1 - F_0)/F_0$.

[0067] The F_1 is the measured fluorescent intensity after addition of the ligand (to displace InD) and F_0 the initial intensity of InD saturated nucleic acid composite before displacement.

[0068] The relative change in fluorescence intensity in comparison to the base pair length of the nucleic acid can be used for determining the positioning of an unknown binding site according to a ligand binding induced intensity decrease. The decrease magnitude in fluorescent intensity when a ligand binds to the saturated nucleic acid composite can be compared against a calibration curve to directly determine a specific base pair number away from the mNP's surface (distance (R)) where the InD has been displaced by the ligand. As such, in one embodiment the binding site is determined based on comparing the relative intensity decrease on a calibration curve. The calibration curve may be obtained from a theoretical calculation based on the FRET theory and equation that is well known in the art.

[0069] In particular, using the equation of $Q= R_o^6/ (R^6 + R_o^6)$, where R_o is the Förster distance, i.e. the distance where the emission intensity is 50% quenched and is constant, the extent of quenching Q and remaining fluorescence intensity I (I = 1-Q) may be calculated for each distance R. R^6 and R_0^6 relate to the distance (R) and Forster distance (R_o) in the 6th power law wherein the FRET equation calculates the energy transfer based on dipole-dipole coupling mechanism. For single dipole, the distance factor is D^3, hence for dipole coupling, the distance factor is D^6.

[0070] As such, in one embodiment the calibration curve is obtained by calculating Q for each distance R and plotting the results on a graph to form said calibration curve. This

principle is further illustrated in the working examples with respect to Figure 8B and the like.

[0071] In one embodiment, the distance (R) is equivalent to the ligand binding site as measured in terms of distance from mNP's surface used as the reference point. In one embodiment, the distance (R) is calculated based on a value of 0.34 nm per nucleotide base, wherein 0.34 nm is the distance between successive nucleotides, as is well known in the art (Chatterjee, S.; Lee, J. B.; Valappil, N. V.; Luo, D.; Menon, V. M. *Biomed. Opt. Express.* 2011, *2*, 1727-1733).

[0072] Therefore, advantageously the present invention enables the ligand binding to the nucleic acid composite to be approximated, by back-calculating the distance (R), based on the decrease in relative fluorescence intensity.

[0073] In one embodiment of the methods described herein, it may be desired to determine the preferential binding site of the at least one ligand, wherein the at least one ligand may bind to more than one binding site in the nucleic acid composite.

[0074] In this regard, another embodiment of the method described herein, the step of contacting said saturated composite nucleic acid with said at least one ligand may further comprise contacting said saturated composite nucleic acid with a first ligand in a 1st molar amount and measuring a first further fluorescence intensity (F'_1) of said contacted saturated composite nucleic acid; contacting said saturated composite nucleic acid with a second ligand in a 2nd molar amount and measuring a second further fluorescence intensity (F'_2) of said contacted saturated composite nucleic acid. The relative change in fluorescence intensity (ΔF), between F_0, F'_1, and F'_2 may then be determined as described herein, whereby the relative change in fluorescence intensity determines the preferential binding site of the ligand.

[0075] In one embodiment, the first and second ligands are the same. In particular, in an embodiment where the ligand is a polypeptide, the "same" polypeptide refers to a polypeptide with an identical amino acid sequence or a polypeptide variant thereof, that maintains the ability to bind to the same binding site in the nucleic acid composite. As such, the same polypeptide (2nd polypeptide) may be one that comprises an amino acid sequence which differs from the reference amino acid sequence (1st polypeptide) by one or more mutations, substitutions, insertions or deletions. Accordingly, in one embodiment the first and second polypeptide may have 100% sequence identity with one another, or may be polypeptide variants with at least 70%, at least 80%, at least 85%, at least 90%, at least 95% sequence identity and have maintained the ability to bind to the same binding site of the nucleic acid composite.

[0076] In another embodiment, the first and second ligands may be contacted with the nucleic acid composite at suitable molar ratios relative to the nucleic acid concentration. Those skilled in the art can readily determine the ligand concentration that is sufficient to bind to the nucleic acid composite. In one embodiment, the molar ratio may be 1:1 (1 molar ligand) to 4:1 (4 mol ligand) according to the binding stoichiometry. In another embodiment, the concentration of the first and second ligand is always the same concentration, wherein the 1st and 2nd molar amounts are the same.

[0077] In one embodiment, the first and second ligands are the same and the metal nanoparticle is a smaller mNP that is less than 80nm in size. In the event that the 1st molar amount of the first ligand measures a higher decrease in fluorescence intensity than the 2nd molar amount of the second ligand, the replaced InD by the first ligand had higher contribution to overall intensity, this being indicative of the binding site

being located further away from the mNP due to the weakly quenched InD. In the event that the 1st molar amount of the first ligand measures a lower drop in fluorescence intensity than the 2nd molar amount of the second ligand, the replaced InD by the first ligand had lower contribution to overall intensity, this being indicative of the binding site being located closer to the mNP due to the heavily quenched InD.

[0078] In one embodiment, the first and second ligand are the same and the metal nanoparticle may be a larger mNP that is greater than 80nm in size. In the event that the 1st molar amount of the first ligand measures a higher decrease in fluorescence intensity than the 2nd molar amount of the second ligand, the replaced InD by the first ligand had higher contribution to overall intensity, this being indicative of the binding site being located closer (proximal) to the mNP due to the higher enhancement of InD. In the event that the 1st molar amount of the first ligand measures a lower drop in fluorescence intensity than the 2nd molar amount of the second ligand, the replaced InD by the first ligand had lower contribution to overall intensity, this being indicative of the binding site being further away (distal) to the mNP due to the lower enhancement of the InD.

[0079] As described herein, when using smaller or larger mNP the contribution of InD at a proximal distance to the mNP varies and thus the change in fluorescence intensity also varies, as exemplified in Example IV disclosed herein with respect to smaller mNPs. Thus, in applying this principle the methods described herein may determine the identity of ligand based upon the magnitude of the decrease in fluorescence intensity as described herein, and correlating the same with one or more binding sites of the nucleic acid composite.

[0080] In this regard, another embodiment of the method described herein and the step of contacting said saturated

composite nucleic acid with said at least one ligand may further comprise contacting said saturated composite nucleic acid with a first ligand and measuring a first further fluorescence intensity (F'_1) of said contacted saturated composite nucleic acid; and contacting said saturated composite nucleic acid with a second ligand and measuring a second further fluorescence intensity (F'_2) of said contacted saturated composite nucleic acid. The relative change in fluorescence intensity (ΔF), between F_0, F'_1, and F'_2 may then be determined, whereby the relative change in fluorescence intensity with the predetermined length of the nucleic acid determines the identity of the first and/or second ligand and a distance (R) of the binding of the first and/or second ligands from the metal nanoparticle.

[0081] In one embodiment, the first and second ligands may be the same or different. In the embodiment where the ligand is a polypeptide, the term "same" is as defined above. A "different" polypeptide refers to one that is distinct in either its function or activity when bound to the nucleic acid composite, or distinct in the binding site it binds to, and may refer to a polypeptide and protein that belongs to a different protein family and functional characterization from a reference polypeptide. Accordingly, in one embodiment the first and second polypeptide may be different from one another.

[0082] In yet another embodiment, the first and second ligand are contacted with the composite nucleic acid simultaneously, sequentially or separately.

[0083] As exemplified in Example 5 disclosed herein, the binding of a first polypeptide may improve the binding of a second polypeptide to a binding site, in comparison to when only one polypeptide is bound to a binding site. Without being bound by theory the aforementioned cooperative binding and

improvement may be attributed to protein-protein contact and interaction. Accordingly, in another aspect of the methods described herein, it may be desired to determine if two or more ligands cooperatively bind to the nucleic acid composite, and also determine the optimum separation distance between binding sites for a cooperative binding effect.

[0084] In this regard, another embodiment of the method described herein and step of contacting said saturated composite nucleic acid with said at least one ligand may further comprise contacting said saturated composite nucleic acid with a first ligand and measuring a first further fluorescence intensity (F'_1) of said contacted saturated composite nucleic acid; and contacting said saturated composite nucleic acid with the first ligand and one or more further ligand and measuring a second further fluorescence intensity (F'_2) of said contacted saturated composite nucleic acid, wherein the contacting steps may be carried out separately. The relative change in fluorescence intensity (ΔF), between F_0, F'_1, and F'_2 may then be determined as described herein, whereby the relative change in fluorescence intensity determines the cooperative binding of the first and further one or more ligands.

[0085] In another embodiment of the above method, the optimal separation distance between the bound first and second ligands for a cooperative binding effect may be determined. In particular, the above method and series of steps may be repeated once or more for nucleic acid composites comprising a spacer between the binding sites of at least 4 bp, at least 5 bp, at least 6bp, at least 7bp, at least 8 bp, at least 9 bp, at least 10 bp, at least 11 bp, at least 12 bp, at least 13 bp, at least 14bp, at least 15 bp, at least 16 bp, at least 17 bp, at least 18 bp. In another embodiment, the spacer sequence is 4 bp, 6 bp, 8 bp, 10 bp, 12 bp, 14 bp, 16 bp or 18 bp.

[0086] In a second aspect of the present invention, the methods described herein may be used in an assay kit. In particular, the present invention provides a kit for detecting site specific binding of at least one ligand to a nucleic acid, comprising: a nucleic acid composite comprising a nanoparticle attached to said nucleic acid at the 5′ end or 3′ end, and an intercalator dye bound to said nucleic acid.

[0087] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0088] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0089] Other embodiments are within the following claims and non- limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

Example 1:

Materials and Methods

[0090] Nucleic Acid Sequence: A series of ½ ERE/Sp1 composite DNA derived from the +571 ERE/Sp1 composite site in promoter A of the human PR gene (Petz et al., J. Steroid Biochem. Mol. Biol. 2004, 88, 113-122) is used to demonstrate the idea and concept of the present invention.

[0091] The composite DNA elements containing a half-site estrogen response element (½ ERE, TGACC) upstream of two adjacent Sp1 sites (CCGCCC), i. e. proximal "Sp1/P" and distal "Sp1/D". The distance between the ½ ERE and the Sp1/D is 4-18 bp. The purpose of having different number of bps between ½ ERE and Sp1/D is to illustrate the optimal separation distance between ½ ERE and Sp1 motifs for favorable "cooperation" of ERα and Sp1 to bind to the DNA.

[0092] **Table 1.** Composite DNA sequences containing ½ ERE motif (pink) and Sp1 motifs (blue) with varied spacer length of 4-18 bp (black).

[0093]

Spacer between ½ ERE & Sp1/D	Sequence	Total length (nm) (bp)

4 bps	**AuNPs** - 5′-TAG GAG CTG ACC AGC GCC GCC CTC CCC CGC CCC CGA CC-3′ **Identity** [redacted] spacer Sp1/D Sp1/P	13.8 nm (38bp)
6 bps	**AuNPs**-5′-TAG GAG CTG ACC AGAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	14.4nm (40bp)
8 bps	**AuNPs**-5′-TAG GAG CTG ACC AGATAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	15.1 nm (42bp)
10 bps	**AuNPs**-5′-TAG GAG CTG ACC AGATATAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	15.8 nm (44bp)
12 bps	**AuNPs**-5′-TAG GAG CTG ACC AGATATATAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	16.5 nm (46bp)
14 bps	**AuNPs**-5′-TAG GAG CTG ACC AGATATATATAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	17.2 nm (48bp)
16 bps	**AuNPs**-5′-TAG GAG CTG ACC AGATATATATATAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	17.8 nm (50bp)
18 bps	**AuNPs**-5′-TAG GAG CTG ACC AGATATATATATATAGCGCC GCC CTC CCC CGC CCC CGA CC-3′	18.5 nm (52bp)

[0094] The length of DNA of the selective spacer and the distance of each site to the AuNPs for a different spacer is shown in **Figure 3**. The calculation is based on 0.34 nm per base pair (Murphy et al., Biophysical Journal, 2004, **86**, 2530-2537) and with the 0.83 nm C6 linker taken into consideration, assuming the C-C carbon bond length in the alkane chain.

[0095] Metal Nanoparticle: AuNPs (20 nm and 100nm) and AgNPs (80 nm) were used to demonstrate the "quenching ruler" and "enhancement ruler" concepts.

[0096] Conjugation of dsDNA to mNPs was performed using the conventional thiol-AuNP conjugation chemistry. One single strand of the dsDNA is labeled with a thiol group at 5′ end (separated by a linker containing 6 Carbon atoms) as prepared by Sigma Life Science. The thiolated single stranded DNA (ssDNA, i.e. the sense strand) was activated with tris(2-carboxyethyl)phosphine) (TCEP, 10 molar excess) and stirred for 10 min at room temperature. The final solution was centrifuged, with a Sigma-Aldrich microcon centrifugal filter device, YM-3 (NMWCO 3 kDa), to remove TCEP before conjugation to AuNPs. Conjugation of activated thiolated DNA to AuNP was done as described by Zhang et al (X. Zhang, M. R. Servos, J. Liu, *J. Am. Chem. Soc.* **2012**, *134*, 7266). Activated ssDNA was mixed with AuNPs at desired ratio (100:1) and incubated for 5

min. Then, pH of solution was lowered to 3 and salt concentration was increased to 30 mM by adding HCl and NaCl, respectively. After 20 min, NaOH was added to return the pH to neutral range. The ssDNA-AuNPs conjugates were then annealed with its complimentary DNA strands at 90 °C for 5 min and left to cool down to room temperature (RT).

[0097] Intercalator Dye(InD): **Thiazole orange (TO)** was chosen as the InD to constitute the "dsDNA-AuNPs/InD" plasmonic-fluorescent ruler with the composite ½ ERE/Sp1 DNA. TO was chosen because it emits at 535 nm, near to the wavelength of the AuNPs' plasmon peak (520-580 nm from 20 to 100 nm), thus more pronounced quenching (or enhancement) by AuNPs and therefore better sensitivity can be expected. In addition, TO intercalation in DNA shows a less sequence dependency and a high fluorescence emission upon intercalation to the dsDNA.

Optimal Intercalating Dye amount that can saturate the dsDNA

[0098] TO was added to the longest dsDNA, i.e. 52 bp $dsDNA_{52bp}$-AuNPs conjugate, with increasing TO:DNA ratio from 1:1 to 30:1. The mixture of TO and $dsDNA_{52bp}$-AuNPs was incubated for 15 min to allow TO intercalation, and the fluorescence intensity was measured by using a fluorescence spectrometer or a microplate reader. Optimal TO amount was determined from the TO:DNA ratio that would not result in further increase of fluorescence intensity.

Metal Nanoparticle Size-Effect on the Intercalating Dye Emission.

[0099] TO, 38 bp up to 52 bp dsDNA were conjugated to 20 nm AuNPs, 100 nm AuNPs, and 80 nm AgNPs. TO was added to the $dsDNA_{36\text{-}52bp}$ -$AuNPs_{20nm,100nm}$ or $dsDNA_{36\text{-}52bp}$ -$AgNPs_{80nm}$ conjugates at TO:DNA molar ratio of 23:1 and incubated for 15 min to allow

TO intercalation. The fluorescence intensity of intercalated TO in the dsDNA-AuNPs or dsDNA-AgNPs conjugates was measured by using a fluorescence spectrometer or a microplate reader.

Determining the DNA Length Dependency of TO Emission.

[00100] 38 bp, 46 bp, and 52 bp dsDNA were conjugated to 20 nm AuNPs. TO was added to the $dsDNA_{38,46,52bp}$ -$AuNPs_{20nm}$ conjugates at TO:DNA molar ratio of 23:1 and incubated for 15 min to allow TO intercalation. The fluorescence intensity of intercalated TO in the dsDNA-AuNPs conjugates was measured by using a fluorescence spectrometer or a microplate reader.

Results

OptimalIntercalating Dye amount that can saturate the dsDNA.

[00101] As shown in Figure 4A the UV-vis spectra of 20 nm, 100 nm AuNPs, and 80nm AgNPs and their conjugates with the 52bp dsDNA. Figure 4B shows the TO emission intensity in the 52 bp DNA conjugated with 20 nm AuNPs (i.e. $dsDNA_{52bp}$-$AuNPs_{20nm}$), at increasing molar ratio of TO:DNA-AuNPs from 1:1 to 30:1. Saturation was achieved at 23:1 and further addition of TO would not result in further increase in fluorescence intensity (dashed line in Figure 4B). Since the DNA consists of 52 bps, then the ratio was approximately 3 TO molecules to 7 bps.

[00102] This ratio obtained for the longest DNA sequence (52 bp) would be applied to the shorter DNAs as well.

Metal Nanoparticle Size-Effect on the Intercalating Dye Emission.

[00103] Using dsDNA/TO without AuNPs conjugated as reference (control), it was found that 20 nm AuNPs quenches the overall fluorescence of the intercalated TO, whereas 100 nm AuNPs and 80 nm AgNPs enhance the overall emission intensity.

[00104] Spanning from 38 up to 52 bps of the dsDNA, the fluorescent intensities increased linearly with the number of DNA's bps, which suggested that higher number of TOs were intercalated for longer DNA and *vice versa*. If the points were fitted with a linear line, the R^2 of the lines were approximately 0.98. R^2 refers to R-squared value of the linear regression of the curve. It indicates how fit the regression model with the curve is. The value ranges 0 (no fit) to 1 (perfect fit), with typical value of > 0.95 implies a good fit.

[00105] From the series of dsDNA/TO without AuNPs (circles in Figure 5), the line intercepted the y-axis at 304, while DNA-$AuNPs_{20nm}$/TO and DNA-$AuNPs_{100nm}$/TO series cut the y-axis at -14313 and +7804, respectively. The small positive value (304) supports that the overall intensity is linearly proportional to the amount of TOs for TO/DNA series and intensity should go to 0 as the DNA used becomes shorter and shorter (approaching 0) whereby no TO can be intercalated.

[00106] On the contrary, the large negative value (-14313) of DNA-$AuNPs_{20nm}$/TO series signified fluorescent intensity was only measureable for DNA consisted of at least 8 bps (extrapolating the line to intercept at the x-axis). Thereby it is critical to ensure the DNA's bp used must consist of at least 8 bp. On the other hand, even for hypothetical 1 bp, DNA-$AuNPs_{100nm}$/TO can still produce fluorescence.

[00107] This type of assembly can be a method of choice if the DNA consists of a few nucleotides, where $AuNP_{20nm}$ completely suppresses the intercalating dyes fluorescence.

Determining the DNA Length Dependency of TO Emission.

[00108] The TO fluorescence intensity in their DNA-AuNPs conjugates were 48%, 60%, and 64%, respectively (**Figure 6**).

[00109] This demonstrates that as the length of DNA increases, quenching of intercalated TO distant away from AuNP becomes weaker. Therefore, the overall fluorescence intensity is progressively restored with the increasing number of bp's and approaching that without AuNPs, yet the fluorescence intensity of dsDNA-AuNPs$_{20nm}$ will always be lower because the TO at close proximity to AuNP$_{20nm}$ is in a quenched state.

Example 2:

Site Specific Detection of "proximal" and "distal" Protein Binding Sites using a "quenching profiler" ruler.

[00110] As seen in **Table 1**, the composite DNA has two Sp1 sites (Sp1/D and Sp1/P). Previous EMSA and *in vitro* DNase I footprinting assays have shown that Sp1 protein binds to the two Sp1 sites in a "additive manner", either it binds to the 10-bp proximal Sp1 site (Sp1/P) first, and then the distal site (Sp1/D) (Petz and Nardulli, Mol. Endocrinology 2000, 14, 972-985).

[00111] In this example, the capability of current dsDNA-AuNPs/InD assembly to report the preferential binding of Sp1 is demonstrated. AuNPs of 20 nm (as a quenching profiler) and a composite DNA sequence with 4 bps spacer (between the ½ ERE and Sp1/P) are used to illustrate the concept.

Materials and Methods

[00112] To eliminate the potential steric limitation imposed to any motif, two sets of dsDNA$_{38bp}$-AuNPs conjugates were prepared, with the conjugation at either the 5′ end or 3′ end of the sense strand (**Figure 7A and 7B**), respectively. Sp1 was added incrementally from one to a few molar ratios relative to

the DNA concentration every 1 molar ratio. The 38 bp dsDNA contained 4 bp spacers.

[00113] The relative intensity drop caused by 1st and 2nd molar Sp1 were recorded (**Figure 7C**). The 1st molar refers to the addition of Sp1 in 1:1 molar ratio relative to DNA concentration. The 2nd molar refers to 2:1 molar ratio of Sp1 relative to DNA concentration. The relative intensity was determined and recorded as described herein.

[00114] Experiments were carried out and extended to higher number of bp spacing between ½ ERE and the Sp1 motif (6 bp - 18 bp) using the 5′ end conjugation to further confirm the trend and strengthen the above findings (**Figure 8A**).

[00115] The 1st molar refers to the addition of Sp1 in 1:1 molar ratio relative to DNA concentration. The 2nd molar refers to 2:1 molar ratio of Sp1 relative to DNA concentration. In Figure 8A, the Y axis is not the "relative intensity" but the decrease in fluorescence intensity, which can be calculated and recorded as described herein as F_1-F_0.

Results

[00116] For the first two molar Sp1 added, the fluorescence intensity decreased gradually and stayed constant above 2 molar ratios. This confirms the "additive" binding behavior of Sp1 to Sp1/P and Sp1/D (Petz and Nardulli, Mol. Endocrinology 2000, 14, 972-985).

[00117] As shown by the relative intensity drop for the **"A"** assembly (**center cluster in Figure 7C**), introduction of the 1st molar Sp1 gave a higher decrease in intensity than the 2nd molar of Sp1, which meant the TO displaced by the 1st molar Sp1 had higher contribution to overall intensity. This being attributed to the weakly quenched TO dyes in Sp1/P site that are located further away from AuNP (26 bp away).

[00118] On the **"B"** assembly, Sp1/P is positioned closer to AuNPs (6 bp away). As expected a lower drop in intensity was observed for the 1st molar of Sp1 protein than the 2nd molar (**right cluster in Figure 7C**), which meant the 1st molar Sp1 bound at Sp1/P site and displaced the heavily quenched TO dyes in there.

[00119] Using conventional FID using DNA without conjugated AuNPs, the addition of 1st and 2nd molar of Sp1 gave similar change in intensity drop (**left cluster in Figure 7C**). As such, the above supports that conventional FID is incapable of detecting preferential protein binding at specific site.

[00120] Similar to the results of 4 bp spacer, a higher intensity decrease was always observed for the 1st molar Sp1, corresponded to binding to the Sp1/D at 26-40 bps away from the AuNP's surface, relative to when 2nd molar Sp1 bound to the proximal site (16-30 bps away from AuNPs), which displaced TO dyes that have smaller contribution to the overall intensity.

[00121] Interestingly, the longer the DNA, the higher is the quenching for each addition of Sp1. This was anticipated as the longer the spacing between ½ ERE and Sp1 motif, the further away Sp1 motifs is from the AuNP. Hence, the smaller TO quenching and its higher role in overall fluorescence intensity. This also explains why the magnitude of difference between 1st and 2nd molar Sp1 becomes lesser with the increase of overall DNA length (**Figure 8A**).

[00122] The decrease in fluorescence intensity caused by 1st and 2nd molar Sp1 measured for all sequences of different length (4-18 bp spacer) were then fitted to the TO fluorescence contribution calculated by FRET equation (**Figure 8B**). The calculated Förster distance (R_o), i.e. the distance where the emission intensity is 50% quenched, was 7.01 nm, taking into account the thiolated-C6 chain (0.83 nm) sat at

the head of each DNA sequences, which was used for conjugating DNA to AuNPs.

[00123] The purpose of figure 8B is to show the correlation between the decrease in fluorescence intensity and the distance of the binding site from AuNP's surface (R). The correlation is derived from the equations:

- $I = 1-Q$; and
- $Q = R_o^6/ (R^6 + R_o^6)$.

[00124] Specifically, the extent of quenching of TO at a certain distance from the AuNP is calculated with $Q = R_o^6/ (R^6 + R_o^6)$, where R is the distance (determined by the number of bp) from AuNP's surface, and thus TO fluorescence remaining is $I = 1-Q$. Starting from R in the x axis of Figure 8B, R correlates to Q (the extent of TO quenching) based on equation $Q = R_o^6/ (R^6 + R_o^6)$. Q correlates to I (remaining fluorescence intensity of TO) based on the equation $I = 1-Q$. For example, if the distance (R) of the binding site from the mNP surface is 11 nm, where $R_o = 7.01$ nm, it can be calculated that $Q = R_o^6/ (R^6 + R_o^6) = 0.063$. $I = 1-Q = 0.937$ as shown in the Y axis of Figure 8B.

[00125] The above equations are a FRET quenching correlation with distance (R). These equations are used in combination with the equation $\Delta F = (F_1 - F_0)/F_0$ in which I (of $I = 1-Q$) is calculated from the decrease in fluorescence as $\Delta F/ F_0$ or $(F_0-F_1)/ F_0$ based on ΔF to establish the correlation of R to the decrease in fluorescence intensity in Figure 8A.

[00126] The relative change in fluorescence intensity is calculated from $\Delta F = (F_1 - F_0)/F_0$. The F_1 is the measured fluorescent intensity after addition of Sp1 (to displace TO) and F_0 the initial intensity of saturated TO before displacement. The calculated value was fitted to the curve

from theoretical calculation (**Figure 8B**). The close correlation of the experimental results with the theoretical fitting curve suggests that the quenching of TO by $AuNP_{20nm}$ obeyed the conventional FRET theory. FRET is widely employed to determine distance at molecular scale level owing to the sensitivity of energy transfer at this scale.

[00127] The relative intensity drop vs. DNA length can be used as a calibration curve for determining the unknown binding site according to a protein binding induced intensity drop. The decrease magnitude in fluorescent intensity when a protein binds to DNA can be either substituted back into the FRET equation or it can be compared against the calibration curve directly to determine the distance (R). The value of R corresponds directly to a specific base pair number away from the mNP's surface where dye has been replaced. In other words, the distance calculated is equivalent to the protein binding site as measured in terms of distance from mNP's surface used as the reference point.

Example 3:

Site specific detection of polypeptide binding to "proximal" and "distal" binding sites using a "enhancement profiler" ruler.

[00128] In previous Example 2 using a "quenching profiler", the detection of protein binding, especially to the DNA motif located close to the surface AuNP would be challenging, due to the original fluorescent intensity of the InD being already very weak due to the strong quenching.

[00129] In order to circumvent this limitation, the construct in Example 2 is modified by replacing the smaller AuNPs with a larger gold or silver NP (i.e. 100 nm AuNP and 80

nm AgNP). These larger particles exhibit greater light scattering contribution than the absorption, which eventually enhance the fluorescence of the proximal fluorophores (the intercalated dyes in this case). The enhancement effect is also distanced dependent, i.e. becoming depleted with the increase of the distanced.

Materials and Methods

[00130] To test the ability of the larger particle "enhancement profiler" coupled with FID for detecting preferential Sp1 binding, two molars of Sp1 were added one after another. The experiments were repeated for all DNA spacers.

[00131] dsDNA (containing 4 bp - 18 bp spacer) were conjugated to 100 nm AuNPs and 80 nm AgNPs.

[00132] TO was added to the dsDNA -$AuNPs_{100nm}$ conjugates and dsDNA -$AgNPs_{80nm}$ conjugates at TO:DNA molar ratio of 23:1 and incubated for 15 min to allow TO intercalation. The fluorescence intensity of intercalated TO in the dsDNA -$AuNPs_{100nm}$ conjugates and dsDNA -$AgNPs_{80nm}$ conjugates was measured by using a fluorescence spectrometer or a microplate reader. A first molar of Sp1 was added in the equal molar ratio of Sp1 to DNA. After 15 min incubation, the fluorescence intensity of TO after addition of the first molar of Sp1 was measured. Second molar of Sp1 was added to make the final molar ratio of Sp1:DNA to 2:1. After 15 min incubation, the fluorescence intensity of TO after Sp1 second molar addition was measured.

Results

[00133] Binding of first molar of Sp1 to the Sp1/P site that is further away from mNPs surface produced a lower change in fluorescent intensity than second molar Sp1 that binds to the

closer SP1/D site for all tested DNA (**Figure 9A & 9B**).This is because the TO dyes located further apart from mNPs have lower apparent intensity or lower contribution to overall intensity (due to the lower enhancement effect) than the dyes close to mNPs. The opposite trend from the current Example III relative to previous Example II (higher intensity drop by the 1st molar Sp1) is due to the opposing nature of the role of mNP, being as a "quenching profiler" and "enhancement profiler", respectively.

[00134] Figure 8C is a summary of TO intensity decrease (at 535 nm) relative to non-enhanced TO intensity drop measured with control setting without AuNPs ($\Delta F/F_0$), as a function of total length of the composite DNA, or the distance of the Sp1 sites to mNP. The dashed line is the calculated fluorescence contribution for each InD fitted to data in A and B to deduce the position of protein binding.

[00135] The enhancement effect is larger for shorter DNA, due to the same reason as discussed above. The comparison between AuNPs and AgNPs shows that $AgNP_{80nm}$ has stronger enhancement effect than $AuNP_{100nm}$.

[00136] It must be acknowledged that for larger mNPs there is not a universal formulation available to calculate the distance based on fluorescence enhancement as FRET for quenching. In order to circumvent this limitation, a controlled distance study was carried out to compare the fluorescence intensity of FAM-labeled DNA in free solution and on $AuNP_{100nm}$ or $AgNP_{80nm}$, by using dsDNA as rigid rod spacer. The enhancement as function of distance is well fitted with exponential curve. The same curve was then further used to calculate the distance of which TOs have already been displaced, as given by the drop in intensity. From the fitting curve, it gave quite precise location of the motifs from $AuNP_{100nm}$ /$AgNP_{80nm}$ surface (Figure 9C).

Example 4:

Identification of ERα or Sp1 protein binding to composite DNA with a ruler of a "quenching profiler"

[00137] In Figure 7A, the ½ ERE is located very close to the AuNP (10 bp away), whereas the Sp1 sites are further away. In this example, it is demonstrated that with a 20 nm AuNPs profiler, it can be detected whether the added protein is ERα (that binds to the ½ ERE) or Sp1 that binds to the Sp1 sites by the differential TO intensity drops. This is based on the fact that the contribution of TO at proximal distance is much smaller and thus the change in fluorescence intensity when ERα bound to ½ ERE is much smaller.

Methods and Materials

[00138] A dsDNA composite sequence with 8 bp spacer that is not conjugated, a dsDNA composite sequence with 4-18 bp spacer conjugated to 20 nm AuNPs via 5′ end, and a dsDNA composite sequence with 4-18 bp spacer conjugated to 20 nm AuNPs via 3′ end, were used in this example.

[00139] TO was added to the unconjugated dsDNA, dsDNA conjugated to 20 nm AuNPs via 5′ end, and dsDNA conjugated to 20 nm AuNPs via 3′ end at TO:DNA molar ratio of 23:1 and incubated for 15 min to allow TO intercalation. The initial fluorescence intensity was measured by using a fluorescence spectrometer or a microplate reader. Sp1 was added in the equal molar ratio of Sp1 to DNA. After 15 min incubation, the fluorescence intensity of TO after addition of Sp1 was measured. Then, ERα was added in the equal molar ratio of ERα

to DNA. After 15 min incubation, the fluorescence intensity of TO after ERα addition was measured.

Results

[00140] As illustrated in **Figure 10**, for the 5′ end conjugation (center panel), there is clear demarcation upon ERα binding and Sp1 binding to composite DNA (8 bp spacer). The magnitude of TO intensity decrease is much greater for Sp1 binding than ERα binding. For the 3′ end conjugation (right panel, Figure 9), the result is reversed.

[00141] In the control experiment with the conventional FID without the AuNPs (left panel), Sp1 binding to the composite DNA didn't generate significant difference compared to ERα binding over the tested protein concentration. This indicates that the affinity of the ERα to the ½ ERE and the affinity of Sp1 to Sp1 sites are not different sustainably.

[00142] Due to the insignificant affinity difference, the intensity drop value in the traditional FID couldn't conclude binder's identity. In contrast the AuNPs-assisted FID (current invention) can generate significant differentiation for the protein binding due to the relative distance of the binding motifs to the AuNPs.

[00143] In conclusion with the plasmonic-fluorescent ruler assay, the identity of the protein binding of different identity but similar affinity can be determined.

[00144] To further verify the function of the profiler AuNPs for determining distance dependency of protein binding, ERα binding to 5′ end conjugates for the entire series of composite DNA (4-18 bp spacer) was tested. Since the ½ ERE site was located at the same distance from the surface of AuNP for the entire series of DNA, the degree of quenching is pretty uniform upon ERα binding to the ½ ERE site (~800 a.u.; see **Figure 11A**). Conversely, when AuNPs is conjugated via the

3′ end, ½ ERE is located approximately 27 bp, 31 bp, and 35 bp (equivalent to 10.0, 11.4, and 12.7 nm) from the surface of AuNP for the 4 bp, 8bp and 12 bp DNA (**Figure 11B**).

[00145] Thus, the magnitude of intensity drop due to ERα binding is dependent on the ½ ERE distance from AuNP's surface and also much greater than the respective 5′ end conjugates, mainly owing to the greater distance of ½ ERE from AuNP's surface in 3′ end conjugate.

Example 5

Detection of spacer dependent cooperative ERα and Sp1 binding using a ruler with a "enhancement profiler"

[00146] Sp1 protein is known to be involved in regulating progesterone receptor gene expression, especially at these sites presence of Sp1 sites next to half-ERE (typically 10-17 bp away). It is reported that binding of Sp1 protein to the Sp1 site next to half-ERE could increase the amount of ERα protein recruited compared to ERα alone to half-ERE, through additional protein-protein contact. The enhancement effect or cooperative Sp1-ERα binding should be spacer dependent. Unfortunately, bio-informatic simulation failed to give an exact model, or weakly conclusive of the cooperative effect.

[00147] In this example, plasmonic-fluorescent rulers with an "enhancement profiler" are used to study the binding of one protein (ERα) in the presence of another (Sp1), or the cooperative action.

Method and Materials

[00148] TO/dsDNA-$AuNP_{100nm}$ and TO/dsDNA-$AgNP_{80nm}$ rulers are used for demonstration of the concept, by measuring ERα

binding to Sp1 saturated DNA (Figure 12A'-C'). The binding of ERα without Sp1 is used as control (Figure 12A-C).

[00149] An unconjugated dsDNA composite sequence with 4-18 bp spacer, dsDNA composite sequence with 4-18 bp spacer conjugated to 100 nm AuNPs via 5' end, and dsDNA composite sequence with 4-18 bp spacer conjugated to 80 nm AgNPs via 3' end are used in this experiemtnt.

[00150] TO was added to the conjugated and unconjugated dsDNA at a TO:DNA molar ratio of 23:1 and incubated for 15 min to allow TO intercalation. The initial fluorescence intensity was measured by using a fluorescence spectrometer or a microplate reader. To prepare Sp1 saturated dsDNA (for both conjugated and unconjugated dsDNA), Sp1 was added in the molar ratio of Sp1:DNA at 2:1. After 15 min incubation, the fluorescence intensity of TO after Sp1 saturation was measured. Then, ERα was added in the equal molar ratio of ERα to DNA. After 15 min incubation, the fluorescence intensity of TO after ERα addition to Sp1 saturated dsDNA was measured. For control experiment, ERα was added directly to dsDNA in the equal molar ratio of ERα to DNA without prior addition of Sp1.

Results

[00151] The binding of ERα without Sp1 shows similar decrease in emission intensity across the DNA series. This is expected because ERα affinity to ½ ERE is uniform for all DNA bp tested in the absence of Sp1 protein. However, when the DNA is first saturated by Sp1, the intensity drop caused by the subsequent ERα are found different across the tested spacer length with the rulers (Figure 11B' and C').

[00152] At the spacer >10bp, ERα induced intensity drop is significantly larger than the average measured for the short spacers. As Petz reported (Mol Endo 2000), at the shorter

spacers (< 8 bp), owing to the close proximity between ½ ERE and Sp1 motifs and the size of Sp1 protein, it is possible that steric repulsion can hinder ERα binding to ½ ERE motif efficiently. The present invention and ruler results confirm this point and further shows that a spacer >10 bp is more favorable for cooperative effect to occur to enhance ERα binding (Figure 11B′ and C′).

[00153] Using conventional FID assay (Figure 11A′), no such effect is detectable (the signals are significantly higher than the average from the short spacer cases). This is because the limited sensitivity of the FID in differentiation of subtle affinity difference. However, with the "enhancement profiler" rulers, such subtle difference become detectable, because the TO at the ½ ERE site is close to the mNPs and their emission is amplified. The displacement of those dyes generated larger signal.

[00154] In other words, the "mNPs profiler" acts like a magnifying glass. The finding of favorable cooperative interactions between ERα-Sp1 at spacer of 10-18 bp is consistent with the data using Biacore T200.

Applications

[00155] The advantages of the present invention are evident. Advantageously, mNPs and intercalator dyes are used to construct a method, for determining site specific ligand binding, where the nucleic acid to be studied is covalently attached to mNPs. Specifically, through proper selection of InD, metal nanoparticles (mNPs), and proper detection of optimal TO amount (Example I), it is demonstrated that mNPs coupled FID or the "plasmonic-fluorescent ruler" is able to serve the following purposes:

[00156] Detecting the binding of ligands of the same identity to a nucleic acid composite at specific binding site via a "quenching profiler" (Example II) or a "enhancement profiler" (Example III).

[00157] Detecting the binding of ligands of different identity to a nucleic acid composite at a specific site (Example IV).

[00158] Determining cooperative binding between multiple ligands and optimum separation distance for cooperative effect (Example V).

Claims

what we claim is:

1. A method for determining site specific binding of at least one ligand to a composite nucleic acid, comprising:

a) providing a composite nucleic acid comprising a metal nanoparticle attached to a nucleic acid having a predetermined length;

b) saturating said composite nucleic acid with an intercalating dye;

c) measuring an initial fluorescence intensity (F_0) of said saturated composite nucleic acid;

d) contacting said saturated composite nucleic acid with said ligand;

e) measuring a further fluorescence intensity (F_1) of said contacted saturated composite nucleic acid and determining the relative change in fluorescence intensity (ΔF), between F_0 and F_1; and

f) correlating the relative change in fluorescence intensity with the predetermined length of the nucleic acid to determine a distance (R) of the binding of the ligand from the metal nanoparticle.

2. The method of claim 1, wherein the ligand is a polypeptide.

3. The method of claim 2, wherein the polypeptide is a transcription factor.

4. The method of any one of claims 1-3, wherein the nucleic acid is DNA or RNA.

5. The method of any of claims 1-4, wherein the nucleic acid comprises at least one binding site for said at least one ligand.

6. The method of any of claims 5, wherein the binding site is a full or partial binding site.

7. The method of any of claims 1-6, wherein the nucleic acid is a double stranded DNA comprising at least 8 base pair.

8. The method of any of claims 1-7, wherein the metal nanoparticle is selected from the group consisting of gold, silver, palladium, ruthenium, rhodium, osmium, iridium and platinum.

9. The method of any of claims 1-8, wherein the size of the metal nanoparticle is from 5nm to 2000nm.

10. The method of any of claims 1-9, wherein the intercalating dye is ethidium bromide or thiazole orange.

11. The method of any of claims 1-10, wherein said metal nanoparticle is less than 80 nm, and said fluorescent intensity of said intercalating dye decreases proximal to said nanoparticle.

12. The method of any of claims 1-10, wherein said metal nanoparticle is greater than 80 nm and said fluorescent intensity of said intercalating dye increases proximal to said nanoparticle.

13. The method of any of claims 1-12, wherein the relative change in fluorescence intensity is a percentage decrease in intensity.

14. The method of any of claims 1-13, wherein the relative change in fluorescence intensity (ΔF) is calculated by

$$\Delta F = (F_1 - F_0)/F_0.$$

15. The method of any of claims 1-14, wherein the distance (R) is a specific nucleotide base number relative to the nanoparticle.

16. The method of claim 15, wherein the distance between each nucleotide base is 0.34 nm.

17. The method of any one of claims 1-16, wherein the binding site is determined based on the relative intensity decrease on a calibration curve.

18. The method of any of claims 1-17, further comprising:

contacting said saturated composite nucleic acid with a first ligand in a 1st molar amount and measuring a first further fluorescence intensity (F'_1) of said contacted saturated composite nucleic acid;

contacting said saturated composite nucleic acid with a second ligand in a 2nd molar amount and measuring a second further fluorescence intensity (F'_2) of said contacted saturated composite nucleic acid;

determining the relative change in fluorescence intensity (ΔF), between F_0, F'_1, and F'_2;

correlating the relative change in fluorescence intensity with the preferential binding site of the ligand,

wherein the nucleic acid comprises at least one binding site for said first and second ligand.

19. The method of any of claims 1-17, further comprising:

contacting said saturated composite nucleic acid with a first ligand and measuring a first further fluorescence intensity (F'_1) of said contacted saturated composite nucleic acid;

contacting said saturated composite nucleic acid with a second ligand and measuring a second further fluorescence intensity (F'_2) of said contacted saturated composite nucleic acid;

determining the relative change in fluorescence intensity (ΔF), between F_0, F'_1, and F'_2;

correlating the relative change in fluorescence intensity to determine the identity of the first and/or second ligand and a distance (R) of the binding of the first and/or second ligands from the metal nanoparticle.

20. The method of claim 18 or 19, wherein the first and second ligands may be the same or different.

21. The method of any of claims 18-20, wherein the first and second ligand are contacted with the composite nucleic acid separately and sequentially.

22. The method of any of claims 1-17, further comprising:

contacting said saturated composite nucleic acid with a first ligand and measuring a first further fluorescence intensity (F'_1) of said contacted saturated composite nucleic acid;

contacting said saturated composite nucleic acid with the first ligand and one or more further ligands and measuring a second further fluorescence intensity (F'_2) of said contacted saturated composite nucleic acid;

determining the relative change in fluorescence intensity (ΔF), between F_0, F'_1, and F'_2;

correlating the relative change in fluorescence intensity to determine the cooperative binding of the first and further one or more ligands.

23. The method of claim 22, wherein the optimal separation distance between the bound ligands for a cooperative binding effect is determined.

24. The method of claim 23, wherein the method is repeated once or more for a nucleic acid composite comprising a spacer sequence of about 4 bp to 18 bp.

25. A kit for detecting site specific binding of at least one ligand to a nucleic acid, comprising: a nucleic acid composite comprising a nanoparticle attached to an end of said nucleic acid, and an intercalator dye.

26. The kit of claim 25, wherein the nucleic acid comprises at least one binding site for said at least one ligand.

Abstract

METHODS FOR DETERMINING SITE SPECIFIC BINDING OF AT LEAST ONE LIGAND TO A COMPOSITE NUCLEIC ACID, AND KITS THEREOF

[00159] The present invention relates to methods for determining site-specific ligand binding to nucleic acid composites comprising metal nanoparticles. In this regard, the present invention exploits a metal nanoparticle-assisted fluorescent intercalator displacement (mNPs-FID) principle, whereby the InD's fluorescence intensity is modulated by mNPs in a distance dependent manner making it possible to determine site specific ligand binding on a nucleic acid.

(no suitable figure)

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