

METHOD FOR DETECTING DNA-BINDING PROTEINS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims benefit of, and priority from, U.S. provisional patent application No. 61/526,913 filed on August 24, 2011, the contents of which are hereby incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The invention relates to methods of detecting binding of a DNA-binding protein to a double-stranded DNA recognition sequence.

BACKGROUND OF THE INVENTION

[0003] DNA-binding proteins are important biomolecules for life; interactions of DNA-binding proteins with sequence-specific DNA regulate the expression of specific genes during specific cellular events. Such interactions between DNA-binding proteins and their target DNA recognition sequences are essential for many crucial cellular processes such as transcription, replication and recombination of genes. DNA binding proteins may be involved in regulation of various cellular mechanisms including control of cell cycle, cell death, cell response to various external signalling events and cell metabolism.

[0004] The biological functions that are regulated by DNA-binding proteins underlie the importance of these molecules (e.g. transcription factors) as biomarkers or targets for disease diagnosis and drug development. Thus, it is desirable to develop simple and robust methods for identifying DNA-binding proteins and to monitor their DNA-binding activities. Preferably, such identification methods would be amenable to high-throughput screening techniques.

[0005] To date, methods to detect DNA-binding proteins with sequence specificity, such as fluorescent titration method, gel shift assay, DNA foot-printing assays

etc. are complicated, labor-intensive and time-consuming. The use of radioactive or fluorescent labels and specialized instrument/facilities in most of these techniques imposes limitations on cost, safety, usability and sensitivity for practical use, and is not adaptable to high-throughput formats as often needed in biomedical research.

[0006] Metal nanoparticles (mNP) have unique optical properties which arise from their ability to support a localized surface plasmon resonance (LSPR), resulting in characteristic color for a solution containing the nanoparticles, which color can change depending on changes to the nanoparticles. Bioassays using nanoparticles have been developed for a wide range of analytes, including DNA, metal ions, small molecules, *etc.*

[0007] One straightforward method for mNP-based colorimetric assays involves functionalizing two sets of mNPs separately, each with probe and target biomolecules, and then directly detecting aggregation of the differently functionalized mNPs as a result of direct recognition between probe and target. However, this strategy is less than ideal for the design of protein-related assays, since conjugation may change the conformation of a protein and affect the specific biomolecular interactions in which the protein is involved.

[0008] Many metal nanoparticles (mNP)-based colorimetric assays for DNA binding proteins are designed based on a crosslinking mechanism, in which two sets of mNP-conjugates are linked/assembled (in the presence of analyte) through permanent interparticle bond formation. This on-particle biorecognition process is slow and can take up to 12 hours to observe a color change. In addition, such analyte-induced aggregation mechanisms tend to produce false positive results caused by destabilizing effects of unrelated molecules that may be present in the reaction buffer, causing aggregation of the mNPs.

[0009] In contrast to a crosslinking mechanism, non-crosslinking mechanisms use unmodified mNPs that involve no inter-particle linkage and may provided relatively fast colorimetric response (within minutes). The stability of the mNPs is achieved via the controlled loss and/or gain of stabilization forces on the particle surface. For example, the addition of a salt to the reaction buffer may neutralize surface charges on the mNPs,

causing the mNPs to aggregate. However, this type of assay is not suitable for use in protein sensing due to the largely available non-specific binding of proteins to the bare mNPs surface.

[0010] Other strategies involve the use of particle aggregates to detect a protein analyte that dissociates the aggregates into dispersed particles. A typical example is the endonuclease (DNase I) sensor for the detection of enzymatic cleavage activity and inhibition. This technology involves intensive inter-particle hybridization and requires careful monitoring of melting (dissociation) behavior of DNA. In addition, due to inaccessibility of DNA embedded inside the particle aggregates, this assay is limited in application, and is not useful for proteins that are large in size or which do not possess an enzymatic cleavage function.

SUMMARY OF THE INVENTION

[0011] The invention relates to a metal nanoparticle-based protein-DNA binding assay that is based on an elegant design of double-stranded DNA-metal nanoparticle (dsDNA-mNP) conjugates. The assay involves two sets of dsDNA-mNP conjugates, each carrying a half site segment of a functional dsDNA recognition sequence for a DNA-binding protein of interest. These dsDNA half sites are designed to each contain a complementary single-stranded overhang in which the overhang contains a small number of nucleotides, allowing for the complementary single-stranded regions to anneal under suitable buffer conditions. Thus, the dsDNA half sites have a tendency to anneal to form a transient full-length dsDNA sequence (with a nick on each strand), which presents a full-length target recognition sequence for protein binding. This annealing tendency also acts as a driving force to induce particle aggregation in the presence of ionic conditions, for example an appropriate concentration of salt ions, in the absence of the DNA-binding protein.

[0012] The detection methods of the invention for detection of specific protein-DNA binding are based on the observation that the mixture of these two sets of dsDNA-mNPs with complementary overhangs experiences a particle aggregation under annealing conditions in the presence of ionic species, for example under appropriate salt conditions

that allow for annealing of the DNA sticky ends and that also allow for neutralization of charges that occur at the surface of the nanoparticles. Particle aggregation is modulated by the presence of the DNA-binding protein that is able to bind to the transiently formed full-length target recognition sequence; the binding of the protein to the DNA site is thought to act as a steric spacer between adjacent nanoparticles, separating the particles to a greater extent than compared to when the particles are aggregated. This difference in aggregation states is indicated by a color change, which is readily detectable, for example by colorimetric methods.

[0013] This “light off” sensing strategy using the analyte DNA-binding protein as a stabilizer to prevent particle aggregation may avoid false positive results which are often seen in “light on” assays in which the analyte binding results in particle aggregation.

[0014] Other known protein detection methods using metal nanoparticles may have long incubation periods, may provide false positive results, may involve multiple tedious steps or stringent assay conditions, or may have low specificity. The method as described herein has been designed to allow for fast, simple and specific detection methods. This method allows for combination of the specificity found in cross-linking approaches and the rapidity of noncross-linking methods into a single assay.

[0015] Furthermore, modulation of aggregation of the metal nanoparticles by the DNA-binding protein helps to avoid false positive signals that arise in other methods as a result of spontaneous aggregation.

[0016] Thus, in one aspect, the present invention provides a method for detecting binding of a DNA-binding protein to a target recognition sequence. The method comprises mixing in a reaction buffer a first set of metal nanoparticles, a second set of metal nanoparticles and a DNA-binding protein to form a mixture, and detecting the aggregation state of the mixture of metal nanoparticles. The first set of metal nanoparticles has a first double-stranded DNA molecule conjugated thereto, the first double-stranded DNA molecule having a first single-stranded overhang at one end, the second set of metal nanoparticles having a second double-stranded DNA molecule

conjugated thereto. The second double-stranded DNA molecule has a second single-stranded overhang at one end, the second single-stranded overhang being complementary to the first single-stranded overhang such that annealing of the first and the second single-stranded overhang results in formation of the target recognition sequence that specifically binds the DNA-binding protein. The mixing thereby results in annealing of the first single-stranded overhang with the second single-stranded overhang. The reaction buffer comprises an ionic species in a concentration sufficient to result in aggregation of the metal nanoparticles upon annealing of the first and second single-stranded overhang.

[0017] The first set of metal nanoparticles and the second set of metal nanoparticles may comprise a noble metal or a noble metal alloy, including for example gold or silver, or an alloy thereof.

[0018] The first and second double-stranded DNA molecules may each be conjugated to the first and second set of metal nanoparticles respectively by a thioether linkage.

[0019] The ionic species may comprise KCl, NaCl, CaCl₂ or phosphate buffered saline.

[0020] The first single-stranded overhang and the second-single stranded overhang may each be from 2 to 8 nucleotides in length.

[0021] The DNA-binding protein may be a transcription factor, a protein involved in replication, or an enzyme.

[0022] Detecting may comprise one or more of microscopy techniques, dynamic light scattering techniques, visual observation of color, UV-vis absorption techniques, and localized surface plasmon resonance techniques.

[0023] The method may further comprise pre-incubating the DNA-binding protein with a free double-stranded DNA molecule that comprises pre-incubating a competitor target recognition sequence prior to the mixing.

[0024] Other aspects and features of the present invention will become apparent to those of ordinary skill in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] The tables and figures, which illustrate, by way of example only, embodiments of the present invention, are as follows.

[0026] **Figure 1.** Schematic illustration of an embodiment of the detection method of the invention, using ER α and vit ERE as a model system. (SEQ ID NO: 1)

[0027] **Figure 2.** TEM images of A) v1 AuNPs, and B) complementary mixture of v1-v2 AuNPs, each in a 25mM KCl-containing protein binding buffer. C) UV-vis spectra of v1-AuNPs alone (curve a) and complementary v1-v2 AuNP mixture at 1:1 ratio (curve b).

[0028] **Figure 3.** UV-vis spectra (left) and TEM images (right) of non-complementary v1-s1 AuNPs mixture in the 25 mM KCl-containing protein binding buffer.

[0029] **Figure 4.** Color photographs of A) v1_{xbp}-AuNPs and B) (Left) complementary v1_{xbp}-v2_{xbp} AuNP mixture in protein binding buffer. B) (Right) Aggregate size of particles (measured as effective hydrodynamic diameter by DLS) formed in the complementary mixture of AuNPs. C) UV-vis spectra of the complementary v1_{xbp}-v2_{xbp} AuNPs.

[0030] **Figure 5.** (A) UV-vis spectra and (B) kinetic of LSPR peak ($\lambda_{\max}$) shift of the complementary mixture of v1-v2 AuNPs (1:1 ratio) in the 25 mM KCl-containing protein binding buffer with ER α (curve a, solid sphere) and without ER α (curve b, hollow sphere). UV-vis spectra, color photographs (inset of A) and DLS data (inset of B) of the AuNPs.

[0031] **Figure 6.** TEM images of the complementary mixture of (A) v1-v2 AuNPs and (B) s1-s2 AuNPs in the presence of ER α .

[0032] **Figure 7.** UV-vis spectra of the complementary mixture of v1-v2 particles conjugates for (A) AuNPs and (B) AgNPs with b) 200 nM, c) 100 nM, d) 50 nM, e) 25 nM and f) 0 nM of ER α in 50 mM KCl-containing protein binding buffer. UV-vis spectra of the stable v1-mNPs conjugates under the same buffer conditions (curve a) is shown as a reference.

[0033] **Figure 8.** Schematic illustration of competition assay for DNA-binding specificity study.

[0034] **Figure 9.** A) UV-Vis spectrum showing the sequence specific competition assay. B) Aggregation degree (measured as A₆₅₀/A₅₂₆) and calculated deviation percentage ($[(X-Y)/Y]*100\%$; X and Y denote samples with and without competitors, respectively).

[0035] **Table 1.** Lists the sequences of various oligonucleotides used in an exemplified embodiment of the method of the invention.

[0036] **Table 2.** Lists the sequences of various oligonucleotides used in an exemplified embodiment of the method of the invention.

DETAILED DESCRIPTION

[0037] The methods as described herein relate to a strategy for measuring sequence-specific interactions between DNA-binding proteins and their target recognition sequences within double-stranded DNA. The methods use a novel design of double-stranded DNA segments having “sticky ends” (i.e. single-stranded overhang regions) that can anneal to form an intact target recognition sequence, each segment being conjugated to a metal nanoparticle. The use of metal nanoparticles allows for colorimetric detection of protein binding to the target recognition sequence due to the surface plasmon resonant properties of the nanoparticles.

[0038] The methods as described herein relate to the discovery that the DNA binding protein acts to stabilise self-assembled constructs of complementary dsDNA-metal nanoparticles (dsDNA-mNP) in solution under conditions where the

complementary ends of the double-stranded DNA segments anneal and under ionic conditions where such annealing would otherwise promote aggregation of the metal nanoparticles to which the dsDNA segments are conjugated.

[0039] Without being limited to any particular theory, it appears that under annealing conditions that include ionic species in the annealing buffer, such as salt ions, and in the absence of DNA binding protein, base-pairing of the complementary overhang regions together with neutralization of surface charges on the nanoparticles by the ionic species in solution synergistically drive interactions between the metal nanoparticles to which the dsDNA segments are conjugated, as depicted in Figure 1. The addition of the DNA binding protein to the system appears to result in binding of the protein to the full-length recognition sequence that is formed by annealing of complementary dsDNA segments, the protein thus acting as a steric spacer between adjacent nanoparticles, thereby reducing the tendency of the particles to aggregate. This steric spacing appears to space the nanoparticles sufficiently apart to influence the resonance response of the particles, resulting in a color change as compared to the aggregated state. Thus, due to the surface plasmon resonant properties of the nanoparticles, solutions containing the nanoparticles will reflect different colours when the nanoparticles are in an aggregated state as compared to when the nanoparticles are sterically spaced by the stabilizing effect of the DNA binding protein.

[0040] Thus, there is provided a method of detecting a DNA-binding protein in a sample, including quantification or assessing the sequence specificity of the DNA-binding protein for a particular target recognition sequence.

[0041] The DNA-binding protein may be any DNA-binding protein that binds to double-stranded DNA in a sequence-specific manner, thus recognizing and binding to a specific sequence of nucleotides found within a double-stranded DNA molecule, termed a target recognition sequence. Many DNA-binding proteins and their particular consensus target recognition sequences are known. The DNA-binding protein may be, for example, a transcription factor, including an activator or a suppressor of transcription, a protein involved in replication, or an enzyme. The DNA-binding protein binds dsDNA in a

sequence-specific manner, and thus has a measurable binding affinity for its target consensus sequence, and may bind to sequences that have slightly altered sequences with a different (i.e. either greater or lesser) affinity.

[0042] The method involves mixing two sets of metal nanoparticles together in a reaction buffer. Each set of metal nanoparticles has a double-stranded DNA (dsDNA) molecule conjugated to the surface of the nanoparticles, in which the first dsDNA molecule conjugated to the first set of metal nanoparticles comprises a portion of a target recognition sequence for the DNA-binding protein. The first dsDNA will also have an overhang of single-stranded nucleotides at one end, the end not conjugated to the metal nanoparticles.

[0043] Similarly, the second dsDNA molecule conjugated to the second set of metal nanoparticles also comprises a portion of a target recognition sequence for the DNA-binding protein, and also has an overhang of single-stranded nucleotides at one end of the DNA molecule, the end not conjugated to the metal nanoparticles.

[0044] Thus, although each DNA molecule conjugated to a set of metal nanoparticles is described as “double-stranded”, it will be appreciated that the DNA molecules are substantially double-stranded with a short single-stranded overhang region at one end, the end not conjugated to the nanoparticle.

[0045] The sequences of the first and second dsDNA molecules are designed so that the single-stranded overhang of the first dsDNA molecule is complementary to and capable of annealing to the single-stranded overhang of the second dsDNA molecule, in order to form, at least transiently, one longer, entirely double-stranded DNA molecule. Further, the sequences of the first and second dsDNA molecules are designed so that when annealed, the longer formed DNA molecule contains an intact target recognition sequence that is available to be bound by any of the DNA-binding protein that may be present in a sample.

[0046] The single-stranded overhang portion may be from 2 nucleotides in length up to 8 nucleotides in length, and thus may be 2, 3, 4, 5, 6, 7 or 8, nucleotides in length.

The overhang may be a 3' overhang, meaning that the single-stranded overhang portion falls at the 3' end of the longer of the two strands that make up the dsDNA molecule (i.e. the "top strand" has the overhang). Alternatively, the overhang may be a 5' overhang, meaning that the single-stranded overhang portion falls at the 5' end of the longer of the two strands that make up the dsDNA molecule (i.e. the "bottom strand" has the overhang).

[0047] The dsDNA molecules are designed such that the total length of the DNA molecule that is transiently formed by annealing of the two overhangs provides sufficient space for the DNA-binding protein to fit between adjacent, aggregated metal nanoparticles, while binding to the transiently formed target recognition site. For example, the full-length DNA molecule, once assembled, which will contain the assembled target recognition sequence, may be from 10 to 100 nucleotides. The precise length will depend on factors such as the nanoparticles chosen, the length of the target recognition sequence, the size of the DNA-binding protein and the manner in which the DNA-binding protein binds to the target recognition sequence. A skilled person can readily test a suitable length of DNA required for any given nanoparticle, target recognition sequence and DNA-binding protein, using routine laboratory methods, and as set out in the Examples provided herein.

[0048] As will be appreciated, metal nanoparticles have unique optical properties arising from their ability to support a localized surface plasmon resonance (LSPR). Free electrons in the metal nanoparticle interact with the oscillating electric fields of light, resulting in an oscillating electron charge in the nanoparticles that is in resonance with a visible light frequency. The size, shape and material of the metal nanoparticle will influence the LSPR. As well, interparticle distance will influence the LSPR and thus the color appearance of the solution containing the nanoparticles.

[0049] Thus, the metal nanoparticles used in the method may be any metal nanoparticles that possess LSPR properties, including metal nanoparticles that comprise a noble metal, for example, silver, and gold, or an alloy of such a metal. In some embodiments, the metal nanoparticles comprise gold, silver, a gold alloy or a silver alloy.

As will be appreciated, nanoparticles are available with a variety of different nanostructures, and include for example gold nanorods, silver nanoplates, gold nanoplates.

[0050] The metal nanoparticles used in each of the two sets of nanoparticles may be the same nanoparticles prior to conjugation of any DNA molecule. Alternatively, different metal nanoparticles may be used for each of the two sets of nanoparticles. That is, the metal nanoparticles used in the first set may have a different size, may comprise a different metal, or may have a different nanostructure than the nanoparticles used in the second set.

[0051] The metal nanoparticles have dimensions in the nanometer range, for example in the range of 5 to 100 nm. The size of the metal nanoparticles chosen may influence the length of the DNA molecules. That is, with increasing dimension of nanoparticles, the total length of the annealed full-length DNA containing the assembled target recognition sequence may need to increase, in order to provide sufficient space for the DNA-binding protein to fit between adjacent nanoparticles linked by annealed dsDNA molecules. A shorter length of the total assembled DNA once annealed may result in the nanoparticles sterically hindering the DNA-binding protein, preventing access to the assembled full-length target recognition sequence. Thus, typically, the larger the dimensions of the nanoparticles, the longer the total length of the assembled DNA may need to be. A skilled person can readily test a suitable length of DNA required for any given nanoparticle, using routine laboratory methods, and as set out in the Examples provided herein.

[0052] As indicated above, each of the two sets of nanoparticles has either the first dsDNA molecule or the second dsDNA molecule conjugated to the surface of the nanoparticles. The dsDNA may be conjugated by any method used to conjugate 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 dsDNA may be synthesized with an end group containing a free thiol on one end (e.g. the 5' end) of one strand of dsDNA molecule, using standard laboratory techniques. The DNA

molecule with a free thiol may thus be conjugated to the metal nanoparticle for use in the method, thereby forming a thioether linkage between the metal nanoparticle and the DNA molecule.

[0053] The two sets of metal nanoparticles, each with either the first dsDNA molecule or the second dsDNA molecule conjugated to the surface, are mixed together in a reaction buffer and under conditions designed to provide appropriate conditions to allow for the complementary overhang regions of the dsDNA molecules to anneal. Annealing conditions for short DNA sequences are well known, including appropriate pH, temperature and ionic strength conditions.

[0054] The reaction buffer is also designed to provide appropriate conditions (e.g. pH, ionic concentration and non-denaturing conditions) to allow for the binding of the DNA-binding protein to the formed target recognition sequence.

[0055] The reaction buffer contains an ionic species, for example a salt. The ionic species is selected to assist with the annealing conditions for the complementary overhang regions of the DNA molecules. In addition, the ionic species is selected for its ability to neutralise at least partially the charge present on the surface of the metal nanoparticles, resulting in aggregation of the nanoparticles upon annealing.

[0056] Thus, the reaction buffer comprises an effective amount of an ionic species sufficient to drive annealing of the overhang regions and concomitant aggregation of the nanoparticles. The ionic species may be, for example, any inorganic or organic salt. As will be appreciated, the precise identity and concentration of the ionic species selected for inclusion in the reaction buffer will depend on the nature of the nanoparticle used and the design of the DNA molecules and the complementary overhang regions. For example, different types of nanoparticles (e.g. gold versus silver) may have different lower limits for the concentration of ionic species in order to still aggregate upon annealing of the complementary overhang regions. Similarly, the same type of nanoparticle (e.g. gold) may have different lower limits for the concentration of ionic species, depending on the particular ionic species used and other buffer components (e.g. KCl versus PBS). In some embodiments, the ionic species used comprises KCl, NaCl, or

CaCl₂ at a final concentration of 25 mM or greater in the reaction buffer.

[0057] It is this combination of annealing and charge neutralization that results in aggregation of the metal nanoparticles in the reaction buffer in the absence of DNA-binding protein. Metal nanoparticles conjugated with only one of the first or second nucleotide will not aggregate in solution, even in the presence of the ionic species in the solution. Annealing of the complementary overhang regions between the two sets of nanoparticles in the absence of sufficient concentration of ionic species may still result in aggregation of the nanoparticles, but any such aggregation will occur at a measurably slower rate than with concomitant charge neutralization.

[0058] As stated above, aggregation of metal nanoparticles influences the resonant frequencies of the nanoparticles, and thus results in a shift in the colorimetric properties of the nanoparticles. Thus, the aggregated state of the nanoparticles can be readily detected using one or more visualization methods, including colorimetric or uv/vis detection methods. For example, the aggregation state of the nanoparticles can be observed using microscopic examination or dynamic light scattering to determine whether the nanoparticles are clumped together or are evenly dispersed in the reaction buffer. More rapid detection may be achieved by observation of the color of the reaction buffer by visual inspection, measurement of uv-vis absorbance, measurement of localized surface plasmon resonance.

[0059] The aggregation of nanoparticles is controlled by the presence of any DNA-binding protein in the sample that is added to the reaction buffer.

[0060] Sample that may contain the DNA-binding protein may be added to the reaction buffer prior to, simultaneously with, or subsequently to the mixing together of the two sets of metal nanoparticles. That is, the DNA-binding protein will not inhibit the annealing and aggregated if present during the mixing of the two sets of metal nanoparticles. As well, the DNA-binding protein is capable of binding to the full-length target recognition sequence if added after the metal nanoparticles have aggregated.

[0061] That is, when the mixture of the two sets of nanoparticles also contains a

DNA-binding protein that is capable of recognizing and binding to the full-length target recognition sequence formed by annealing of the two dsDNA molecules, DNA-binding protein will bind to full-length target recognition sequences formed between nanoparticles, modulating the aggregation state. As indicated above, the DNA-binding protein may act as a steric spacer, resulting in a reduction of aggregation between nanoparticles, thus influencing the interparticle distance and as a result affecting the resonance parameters of the nanoparticles.

[0062] Thus, provided there is a detectable amount of DNA-binding protein present (i.e. the amount of protein is above the lower detection limit of the assay), the presence of the DNA-binding protein will have a detectable effect on the aggregation state of the nanoparticles. That is, the nanoparticles will appear less aggregated or even monodispersed in the reaction as compared to the aggregation state in the absence of DNA-binding protein, when observed using for example microscopy techniques or dynamic light scattering techniques. Similarly, colorimetric measurement of the reaction buffer will also be affected by the presence of DNA-binding protein, and a change in color or a shift in UV-vis absorption or localized surface plasmon resonance, as compared to in the absence of DNA-binding protein, can be detected.

[0063] The method therefore includes detecting the aggregation state of the metal nanoparticles in order to determine whether any DNA-binding protein is present in the sample (and optionally how much), as described above, using visual, colorimetric or other methods to determine the interparticle distance (i.e. the state of aggregation or dispersion) of the nanoparticles in the reaction buffer. As indicated above, the results of such detection are compared to the aggregated state of nanoparticles in the reaction buffer in the absence of DNA-binding protein.

[0064] The binding of the DNA-binding protein to the target recognition is a specific binding event, meaning that the binding is reversible, measurable and saturatable (also referred to as “specific binding” or as one of the DNA-binding protein or the DNA target recognition sequence “specifically binding” to the other). Thus, the binding of the DNA-binding protein to the target recognition sequence will have a specific affinity

constant that can be measured. Thus, it may be possible using the method to determine the concentration of DNA-binding protein in a sample using titration methods, in order to develop a binding curve. Similarly, it may be possible to measure the affinity binding constant between the DNA-binding protein and the target recognition sequence if the titration is performed using known concentrations of DNA-binding protein and nanoparticles.

[0065] As well, the specific binding affinity of the DNA-binding protein for various different target recognition sequences may be assessed using the method. That is, the method may be designed as a competitive assay, for example between a consensus target recognition site formed by annealing of the dsDNA molecules conjugated to each of the two sets of nanoparticles and a competitor altered target recognition sequence that differs from the consensus sequence.

[0066] A molar excess amount of the competitor DNA is mixed with the DNA-binding protein prior to addition of the DNA-binding protein to the reaction buffer. The competitor DNA comprises a free (i.e. unconjugated to a metal nanoparticle) double-stranded DNA molecule that contains the competitor target recognition site. If the competitor DNA is a strong competitor, the DNA-binding protein will bind predominantly to the free competitor DNA and will have a lesser or minimal effect in preventing aggregation of the two sets of metal nanoparticles upon annealing in the presence of an ionic species. If the competitor DNA is a weak competitor, more of the DNA-binding protein will be available to bind to the annealed target recognition sequence formed between nanoparticles, thus modulating the aggregation of the metal nanoparticles. In this way, a high throughput screening assay can be designed to test the binding affinity of the DNA-binding protein to a variety of different DNA sequences, without the need to synthesize a large number of different sets of metal nanoparticles conjugated with complementary DNA molecules.

[0067] As will be appreciated, the design of this method as described herein is general, and the method may be used to detect, quantify or test the specific binding affinity of any DNA-binding protein that binds to double-stranded DNA in a sequence-

specific manner. The sequences of the dsDNA molecules having complementary overhang regions can be readily synthesized to adapt the method for the DNA-binding protein of interest.

[0068] In contrast to other known assay methods that detect analyte-induced particle aggregation (“light on” assays), this method as described herein is a protein stabilization approach (“light-off” assay) and thus may reduce false positive results which are often caused by unrelated particle de-stabilizing effects.

[0069] In addition, the synergistic combination of annealing and charge neutralization at the nanoparticle surface to induce aggregation of the metal nanoparticles, combined with the steric spacing affect of the DNA-binding protein to reduce aggregation, allows for a rapid detection method that may not require long incubation periods.

[0070] In addition to the above methods, kits or commercial packages are provided for performing the method. The kit or commercial package comprises two sets of metal nanoparticles as described herein. Briefly, as described, each set of metal nanoparticles has a double-stranded DNA molecule conjugated to the surface of the nanoparticles. The double-stranded DNA molecule conjugated to each set of metal nanoparticles comprises a portion of a target recognition sequence and also comprises a short single-stranded overhang region that is complementary to the overhang region of the double-stranded DNA molecule conjugated to the other set of metal nanoparticles, such that when the complementary overhang regions anneal together, the two double-stranded DNA molecules form a full-length target recognition sequence that is available to be bound in the assay by a DNA-binding protein.

[0071] The kit or commercial package may further include a suitable reaction buffer as described herein.

[0072] The present methods are further exemplified by way of the following non-limiting examples.

EXAMPLES

[0073] The described detection assay was demonstrated using estrogen receptor α as a model DNA-binding protein together with double-stranded DNA segments that form an estrogen response element conjugated to gold or silver NPs as the sensing platform. UV-vis spectroscopy, TEM, and dynamic light scattering (DLS) measurements were conducted to characterize the particle aggregation/dispersion mechanism.

[0074] EXAMPLE 1

[0075] Design of dsDNA Conjugated AuNPs with Segmented DNA Elements

[0076] Estrogen receptor α (ER α), which binds specifically with its dsDNA response element from vitellogenin A2 gene (vit ERE) containing the core binding sequence of 5'-GGTCAnnnTGACC-3' (n: spacer nucleotide), is used as a model in this study. Figure 1 shows the basic principle underlying this assay.

[0077] **Figure 1.** Schematic illustration of sensing principle. Each of two sets of gold nanoparticles (AuNPs) is modified with a half ERE segment (v1 and v2) containing sticky ends of 3 bases in length. These particles, when mixed together at 1:1 molar ratio, have a tendency to aggregate through Watson-Crick base-pairing force (middle). Addition of KCl neutralizes the charge repulsion between DNA-AuNPs and promotes base pairing between the complementary half ERE segments, resulting in rapid particle aggregation and solution color change from red to purple (left). In the presence of ER α , binding of the protein to the transient full ERE sequence between AuNPs exerts steric force to stabilize the AuNPs, preventing the extensive aggregation effect; thus the solution color remains or returns to red (right).

[0078] **Table 1** sets out nucleotide sequences used in the ERE half sites and the scrambled half sites used as indicated in these Examples. The consensus vit dsERE (Table 1) was split into two half ERE segments (denoted as v1 and v2 respectively), each with a three base complementary sticky end. The half segments were conjugated onto AuNPs to form v1-AuNPs and v2-AuNPs conjugates, respectively. These as-prepared (unmixed) AuNPs conjugates (in 0.1 M PBS) were red in color and showed no traces of

aggregation even in 25 mM KCl buffers (see TEM image in Figure 2A). Noticeable particle aggregation was observed (Figure 2B) when two sets of complementary v1-AuNPs and v2-AuNPs conjugates were mixed at 1:1 ratio under same buffer conditions. The LSPR peak of the AuNPs conjugates was shifted to a longer wavelength with band broadening (from curve a to b, Figure 2C), accompanied by a visible solution color change from red to purple (insets of Figure 2C).

[0079] **Figure 2.** TEM images of A) v1 AuNPs and B) complementary mixture of v1-v2 AuNPs, in the 25mM KCl-containing protein binding buffer (0.1 M PBS, 25 mM KCl, 0.1 mM EDTA, 0.2 mM DTT and 1% of glycerol). TEM samples were prepared by dispensing the dsDNA-AuNPs onto a copper grid after 20 min of reaction with salt buffer. C) UV-vis spectra of v1-AuNPs alone (curve a) and complementary v1-v2 AuNP mixture at 1:1 ratio (curve b) were taken at 1 min upon addition of salt buffer.

[0080] It was found that the complementary mixture of v1-AuNPs and v2-AuNPs also aggregated in buffer conditions without KCl after exposure to 0.1 M PBS for 20 min (data not shown). This observation indicates that the complementary sticky ends of the v1- and v2-sequences introduce base-pairing forces to facilitate nanoparticle aggregation, which is assisted by the presence of ions in solution.

[0081] To test this hypothesis, a control experiment was conducted using a mixture of dsDNA-AuNPs conjugates that carry non-complementary overhang ends, *i.e.*, v1-AuNPs with s1-AuNPs (s1 is a half site of a non ER α binding DNA, in which the ERE site is scrambled. s1 has a non-complementary overhang end with v1 sequence. The sequences of the two half sites of scrambled DNA, *i.e.*, s1 and s2, are shown in Table 1). The UV-vis spectrum of the v1-AuNPs and s1-AuNPs mixture (1:1 ratio) measured after 20 min of incubation in the 25 mM KCl-containing protein binding buffer overlapped with the stable v1-AuNPs conjugates alone under the same buffer condition (Figure 3).

[0082] **Figure 3.** UV-vis spectra (left) and TEM images (right) of non-complementary v1-s1 AuNPs mixture in the 25 mM KCl-containing protein binding buffer (0.1 M PBS, 25 mM KCl, 0.1 mM EDTA, 0.2 mM DTT and 1% of glycerol). The spectra and TEM images were taken after 20 min of incubation.

[0083] Based on the above-described results and discussion above, it is inferred that the hybridization tendency of the complementary nucleotides brings the particles into close proximity with each other, and the aggregation is driven at least in part by the presence of salt ions in the solution. As the melting temperature of the complementary nucleotides (-CAG-) used in this study is only 10°C, it is likely that the three DNA base pairs are not strong enough to stabilize the 34bp full length vit ERE-AuNP structure for a significant period of time at room temperature. Furthermore, when the two particles are in close enough proximity, the charge screening effects by salt ions is amplified, leading to an observed extensive particle aggregation that increased as time elapsed.

[0084] EXAMPLE 2

[0085] Length effect of dsDNA-AuNPs Conjugates and Stability Test

[0086] To further understand the condition for the complementary conjugates to form aggregates, two shorter (15 and 25 bp) and one longer (45 bp) length of EREs were used to investigate the effect of the transient DNA length on aggregate formation.

[0087] Each of the EREs was split into two half segments with a complementary sticky end (see sequences in Table 2, and were conjugated onto AuNPs to form v1_xbp-AuNPs and v2_xbp-AuNPs (x=15, 25, 34, and 45), respectively. Dynamic light scattering (DLS) was used to measure the hydrodynamic diameter (D_h) of the particles. DLS results as shown in Figure 4 revealed that large aggregates were formed only in the complementary mixture of AuNPs conjugates modified with the longer ERE segments of 34 bp ($D_h = 488.6 \pm 36.8$ nm) and 45 bp ($D_h = 548.4 \pm 56.2$ nm). For the complementary particle mixtures conjugated with shorter segments of EREs (15 and 25 bp), the D_h (32.4 ± 0.2 nm) remain similar to the v1-AuNPs alone (34.2 ± 0.2 nm). The longer dsDNA-capping structures appear to promote base pairing between the sticky end sequences that are farther away from the particle interfaces, and thus lead to faster aggregation. The corresponding color change (from red to purple) for the 34 and 45 bp of DNA-AuNPs mixtures (left panel of Figure 4B) and the LSPR spectra shift of these particles mixture (Figures 2C and 4C) correlate well with the DLS results, showing that the large aggregates have large hydrodynamic sizes, red shift spectra and darkened solution color.

[0088] **Figure 4.** Length effects of dsDNA on AuNPs aggregate formation. Color photograph of A) v1_{xbp}-AuNPs and B) (Left) complementary v1_{xbp}-v2_{xbp} AuNPs mixture, in protein binding buffer containing 0.1 M PBS, 25 mM KCl, 0.1 mM EDTA, 0.2 mM DTT and 1% of glycerol. (Right) Aggregates size of particles (measured as effective hydrodynamic diameter by DLS) formed in the complementary mixture of AuNPs conjugates modified with different length of dsDNA ($x = 15, 25, 34$ and 45bp). DLS data and color photograph of the AuNPs were taken at 10 min upon addition of salt buffer. C) UV-vis spectra of the complementary v1_{xbp}-v2_{xbp} AuNPs taken at 1 min upon mixing in the 25 mM KCl-containing protein binding buffer.

[0089] **EXAMPLE 3**

[0090] **Protein Sensing using Segmented dsDNA-AuNPs Conjugates**

[0091] Using the 34bp consensus ERE sequence as an example, the instability of the complementary mixture in KCl-containing PBS buffer was used to detect a sequence-specific protein binding event.

[0092] Only a small shift in peak position ($\lambda_{\text{max}} = 530 \text{ nm}$) was observed in the complementary mixture of AuNPs conjugates when ERE-binding protein (i.e., ER α) was added (Figure 5A). The time course of the particle aggregation (shown as a function of peak wavelength shift over time in Figure 5B) for the mixture of v1-v2 AuNPs with (+) and without (-) ER α further confirms the distinctive stabilization effect of ER α throughout the tested period of 20 min. The same protein binding experiment was conducted for the AuNPs modified with the complementary half site of 45bp ERE (v1_{45bp}-v2_{45bp} AuNPs). A similar stabilization effect was also observed in the presence of ER α (data not shown). It appears that the specific binding of ER α to its recognizable vit ERE sequence (formed through initial base-pairing between the complementary v1 and v2 half sites) may be responsible for the retainable stability of particle mixture, which would otherwise aggregate in the presence of the salt-containing buffer. The formation of v1-AuNPs/ER α /v2-AuNPs complex stabilizes the transient full ERE and inserts a sufficient steric barrier between the particles to prevent them from further aggregation.

[0093] **Figure 5.** (A) UV-vis spectra and (B) kinetic of LSPR peak ($\lambda_{\max}$) shift of the complementary mixture of v1-v2 AuNPs (1:1 ratio) in the 25 mM KCl-containing protein binding buffer with ER α (curve a, solid sphere) and without ER α (curve b, hollow sphere). UV-vis spectra, color photographs (inset of A) and DLS data (inset of B) of the AuNPs mixture were taken at 20 min upon addition of salt buffer.

[0094] To verify that the particle stabilization is due to the sequence-specific binding of ER α -ERE, experiments using a non-ER α binding scrambled DNA sequence as control were conducted. Two of the scrambled DNA half site, i.e., s1 and s2 (see sequences in Table 1) were functionalized on two sets of AuNPs respectively. The complementary scrambled dsDNA-AuNPs were then mixed together (1:1 molar ratio) and tested for stability in the presence of 100 nM of ER α . TEM images in Figure 6 show that the complementary mixture of s1-s2 AuNPs (which can transiently anneal) underwent extensive aggregation, while the v1-v2 AuNP mixture shows no large aggregates formation but a few strands of short linear particle assemblies in the stable particle mixtures. These results show that the sequence-specific protein-DNA binding event is responsible for a higher stability of the complementary dsDNA-AuNPs mixtures using segmented protein binding sequences.

[0095] **Figure 6.** TEM images of the complementary mixture of (A) v1-v2 AuNPs and (B) s1-s2 AuNPs in the presence of ER α (100 nM, final concentration), taken 20 min upon addition of 20 mM KCl buffer.

[0096] **EXAMPLE 4**

[0097] **Detection Sensitivity of Protein-DNA Binding Assay**

[0098] In this experiment, the detection sensitivity of ER α binding to the complementary mixture of v1-v2 AuNPs was tested for a range of protein concentration (0 to 200 nM) in 50 mM KCl-containing buffer.

[0099] Figure 7A shows that ER α effectively retarded the aggregation of the complementary mixture of v1-v2 AuNPs. The degree of stabilization was increased with

increasing protein concentration. For example, only a slight shift in the UV-vis spectrum was observed for the v1-v2 AuNP mixture in the presence of 200 nM ER α (the highest concentration tested) (curve b) relative to a stable v1-AuNP conjugate (curve a) at $t = 1$ min. In such a short incubation time, ER α down to 50 nM can be detected by a noticeable stabilization effect. Both the improved ER α -ERE binding efficiency and the larger shift in LSPR peak (relative to a stable v1-AuNPs at $\lambda_{\max_v1} = 520$ nm) for the v1-v2 AuNPs mixture in the 50 mM KCl-containing buffer (*i.e.*, $\lambda_{\max_v1v2} = 545$ nm, curve f in Figure 7) are attributable to the effective detection of low concentration of ER α in a short incubation time. Indeed, when the comparison was made for the 1 min binding of 100 nM ER α in the 25 mM KCl- (Figure 2C) and 50 mM KCl-containing buffer solutions, it was found that the recovery of the peak wavelength (*i.e.*, $\Delta\lambda_{\max} = \lambda_{\max_v1v2} - \lambda_{\max_v1v2-ER\alpha}$) for the former and latter conditions are 5 and 15 nm, respectively. These results suggest that the less stable the v1-v2 AuNP mixture is in (high) salt solution, the faster the aggregation will occur and the more readily stabilization effects of ER α can be detected in short assay time. The protein binding-particle stabilization mechanism used in this assay design also reduces the risk of obtaining false positive results caused by unrelated particle de-stabilizing effects.

[00100] **Figure 7.** UV-vis spectra of the complementary mixture of v1-v2 particles conjugates for (A) AuNPs and (B) AgNPs with b) 200 nM, c) 100 nM, d) 50 nM, e) 25 nM and f) 0 nM of ER α in 50 mM KCl-containing protein binding buffer (0.1 M PBS, 50 mM KCl, 0.1 mM EDTA, 0.2 mM DTT and 1% of glycerol). UV-vis spectra of the stable v1-mNPs conjugates under the same buffer conditions (curve a) is shown as a reference. All the spectra were taken at 1min upon addition of salt buffer.

[00101] In this study, segmented DNA-conjugated silver nanoparticles (AgNPs) were also prepared and tested for ER α binding to confirm the sensing strategy and to demonstrate the utility of AgNPs in this instance. The dsDNA-AgNP stability test shows that the mixture of the complementary v1-v2 AgNP conjugates (no ER α added) aggregated drastically in the 50 mM KCl-containing protein binding buffer solution (curve f, Figure 7B). A noticeable dampening of the UV-vis spectra of AgNPs,

particularly the drop in absorbance from its original peak wavelength of 400 nm (curve a, Figure 7B) was observed, accompanied by an intense color change from yellow to pale brown. The binding of ER α (curve b-e, Figure 7B), on the other hand, retarded the particle aggregation effectively in an ER α concentration-dependent manner. The detection limit of ER α binding using the AgNP assembly platform was found to be 25 nM, which is lower than that obtained using the AuNP sensing probe under the same buffer conditions (50 mM KCl) and assay time ($t = 1$ min). Thus, AgNPs may, under certain conditions, provide a more sensitive platform for protein detection in these methods.

[00102] EXAMPLE 5

[00103] Competition Assay for DNA-binding Protein Sequence Selectivity Studies

[00104] The application of the controlled assembly of segmented DNA-AuNP conjugates for biosensing may be further developed into a competition assay format for fast screening of protein selectivity to various DNA sequence. In this assay, free ERE competitor dsDNA was added to compete with the transiently formed full-length EREs conjugated to the AuNPs for ER β binding (see schematic diagram in Figure 8).

[00105] In this study, excess unmodified DNA competitor (i.e., wtERE and scrDNA, are used as strong and weak competitors, respectively) was pre-incubated with the ER β at room temperature for 20 minutes to allow for complex formation (molar ratio of ER β :dsDNA is 1:4), followed by the addition to the complementary mixture of v1-v2-AuNPs in 56 mM KCl, with final ER β concentration of 360 nM. In the presence of strong competitor (wtERE), limited amount of ER β bound to the ERE anchored on the AuNPs surface, leading to extensive particle aggregation in salt solution. The opposite result was seen with the weak competitor that is unable to form a complex with ER β in the incubation buffer. The unbound ER β was thus available to bind with the dsERE-AuNP conjugates, leading to particle stabilization through protein binding event as observed in the direct binding assay (see Example 3).

[00106] **Figure 8.** Schematic illustration of competition assay for DNA-binding protein sequence selectivity study. Unmodified DNA of different sequences can be used as competitor DNA to compete with the DNA-conjugated AuNPs for sequence-specific protein binding. The presence of a strong competitor will cause the aggregation of segmented dsDNA-AuNPs conjugates due to the lost of binding molecules to stabilize the transient DNA structure against charge screening by salt ions.

[00107] The aggregation state of the AuNPs was quantified using UV-Vis absorption spectrum as indicated by the shift of LSPR peak at 526nm wavelength to a longer wavelength (e.g. 650 nm). The UV-Vis absorbance ratio at 650nm to that at 526nm, i.e., A_{650}/A_{526} is indicative of the particle aggregation degree where a higher ratio is directly related to a larger extent of aggregation. In this study, the deviation percentage (i.e., $[(X-Y)/Y]*100\%$) was calculated based on the difference in aggregation ratio of AuNP samples (measure as A_{650}/A_{526}) with competitors (denoted as X) relative to that without competitor (denoted as Y) at 5 minutes after mixing in the 56 mM KCl-containing protein binding buffer. A distinct difference in UV-Vis spectrum and aggregation degree is shown in Figure 9. A 9.0% of deviation was recorded for the scrambled DNA sequence (scrDNA). This result shows that the weak competitor, i.e., scrDNA, is unable to form complex with ER β efficiently, due to the sequence recognition of ER β to the consensus ERE (see Table 1). Ideally, there should be no uptake of ER β for complex formation with unmodified dsDNA, which eases the maximum stabilization effect. Here, the deviation of up to 9.0% observed reveals the non-specific binding of ER β to the unrecognizable dsDNA present. On the other hand, AuNP sample with the wild type ERE sequence as competitor recorded a deviation as high as 35.0% from the maximum stabilization exerted by the 360nM ER β to the system (without competitor) in 56 mM KCl solution. This result shows a high uptake of ER β to form a complex with the competitor wtERE sequence during the incubation step, causing a large percentage of ER β to be unavailable for stabilization of the DNA conjugated AuNPs in salt buffer.

[00108] **Figure 9.** A) UV-Vis spectrum showing the sequence specific competition assay. B) Aggregation degree (measured as A_{650}/A_{526}) and calculated deviation percentage ($[(X-Y)/Y]*100\%$; X and Y denote samples with and without competitors,

respectively) upon mixing in 56mM KCl-containing protein binding buffer for 5 min.

[00109] The ER β sequence specificity studies above demonstrates the potential of this transient DNA-linked AuNP assembly for high throughput screening of sequence selectivity of DNA-binding protein in a competition format. Tedious and time-consuming conjugation of AuNPs with each DNA sequence can be avoided via this competition assay, which allows fast and easy detection with the direct addition of unmodified DNA for sequence selectivity test.

[00110] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

[00111] As used in this specification and the appended claims, the singular forms “a”, “an” and “the” include plural reference unless the context clearly dictates otherwise. As used in this specification and the appended claims, the terms “comprise”, “comprising”, “comprises” and other forms of these terms are intended in the non-limiting inclusive sense, that is, to include particular recited elements or components without excluding any other element or component. As used in this specification and the appended claims, all ranges or lists as given are intended to convey any intermediate value or range or any sublist contained therein. Unless defined otherwise all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

[00112] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for detecting binding of a DNA-binding protein to a consensus target recognition sequence in the presence of a competitor target recognition sequence, the method comprising:

incubating the DNA-binding protein with a molar excess of a free double-stranded DNA molecule that comprises the competitor target recognition sequence that differs from the consensus target recognition sequence, to form a pre-incubated DNA-binding protein;

mixing in a reaction buffer a first set of metal nanoparticles, a second set of metal nanoparticles and the pre-incubated DNA-binding protein to form a test mixture,

said first set of metal nanoparticles having a first double-stranded DNA molecule conjugated thereto, said first double-stranded DNA molecule having a first single-stranded overhang at one end,

said second set of metal nanoparticles having a second double-stranded DNA molecule conjugated thereto, said second double-stranded DNA molecule having a second single-stranded overhang at one end, said second single-stranded overhang being complementary to said first single-stranded overhang such that annealing of said first and said second single-stranded overhang results in formation of the consensus target recognition sequence that specifically binds the DNA-binding protein,

thereby annealing said first single-stranded overhang with said second single-stranded overhang,

said reaction buffer comprising an ionic species in a concentration sufficient to result in aggregation of the metal nanoparticles upon annealing of the first and second single-stranded overhang;

detecting the aggregation state of the test mixture of metal nanoparticles; and

comparing the aggregation state of the test mixture with the aggregation state of a control mixture of metal nanoparticles formed by mixing in the reaction buffer the first set of metal nanoparticles, the second set of metal nanoparticles and the DNA-binding protein that has not been incubated with the free double-stranded DNA molecule.

2. The method of claim 1, wherein the first set of metal nanoparticles and the second set of metal nanoparticles comprise a noble metal or a noble metal alloy.
3. The method of claim 2, wherein the noble metal comprises gold or silver.
4. The method of claim 2, wherein the noble metal alloy comprises an alloy of gold or silver.
5. The method of any one of claims 1 to 4, wherein the first and second double-stranded DNA molecules are each conjugated to the first and second set of metal nanoparticles respectively by a thioether linkage.
6. The method of any one of claims 1 to 5, wherein the ionic species comprises KCl, NaCl, CaCl₂ or phosphate buffered saline.
7. The method of any one of claims 1 to 6, wherein the first single-stranded overhang and the second-single stranded overhang are each from 2 to 8 nucleotides in length.
8. The method of any one of claims 1 to 7, wherein the DNA-binding protein is a transcription factor, a protein involved in replication, or an enzyme.
9. The method of any one of claims 1 to 8, wherein said detecting comprises one or more of microscopy techniques, dynamic light scattering techniques, visual observation of color, UV-vis absorption techniques, and localized surface plasmon resonance techniques.