

Biomolecule-Based Metal Nanoclusters as Biosensors

Technical Field

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The present invention generally relates to a method for determining the ability of a ligand to bind a target molecule by forming metal nanoclusters (MNCs) with the target molecules under denaturing conditions and assaying
10 for a signal emitted by the MNCs. The present invention also relates to use of the method for drug screening.

Background

Serum albumin is the most abundant blood plasma
15 protein produced in the liver. Serum albumin serves to regulate blood volume by maintaining the colloid osmotic pressure of the blood compartment. It also serves as a carrier protein for molecules of low solubility such as steroids, fatty acids and thyroid hormones, and some drug
20 molecules such as warfarin and phenytoin. Competition between drugs for albumin binding sites may lead to drug-drug interaction, which in turn may affect drug potency. It is therefore important to determine drug binding sites and their binding affinity to albumin proteins in order to
25 better understand drug pharmacokinetics.

Both Bovine (BSA) and human serum albumin (HSA) are variants of albumin that are commonly used in biophysical and biochemical studies. Both proteins share highly similar homology (88%) in their sequences. HSA constitutes about
30 50% of human plasma proteins and is often used to replace lost fluids and restore blood volume in trauma, burns and surgery patients. BSA and HSA are predominantly helical proteins, comprising three similar domains (I-III), each of

which can be further classified into two subdomains (A and B). The primary drug binding sites of albumin are located in two distinct hydrophobic pockets of subdomains IIA and IIIA, which are known as Sudlow's site I and site II
5 respectively. Site I preferably binds to large heterocyclic and negatively charged molecules (e.g. warfarin) while site II binds to small aromatic compounds with a distal negatively charged carboxylic acid (e.g. ibuprofen).

Binding interactions between proteins (such as
10 albumin) and drugs may be studied using a variety of methods. Protein-based metal nanoclusters, such as protein-based gold nanoclusters (AuNCs) have remarkable fluorescent properties that render them particularly useful in drug screening and as biosensors. The preparation of protein-
15 based gold nanoclusters typically involves encapsulation of gold ions by protein molecules, followed by reduction of the encapsulated gold ions. The reduction is typically facilitated by use of a strong reducing agent (such as NaBH_4), or by mildly reducing amino acids (such as tyrosine
20 residues) in the protein that has been activated by alkaline conditions in the solution. However, a disadvantage of this method of preparation is the harsh conditions involved in the process, which typically lead to undesirable loss of protein bioactivity. Furthermore,
25 preparation of fluorescent AuNCs using native proteins at ambient temperatures suffers the disadvantage of long reaction time, typically more than 10 hours.

Other biophysical methods available to study protein-drug binding interactions suffer from various
30 disadvantages, such as high complexity, involving tedious procedures, and having low sensitivity. For example, surface plasmon resonance (SPR), which is the "gold standard" method for studying protein-ligand interaction, is costly, and requires covalent immobilization of proteins

on the chip surface, which may complicate the binding scheme. In addition, the general solubility of a ligand in dimethyl sulfoxide (DMSO) requires a "solvent correction" step due to the bulk response signal from DMSO. Being a
5 heterogenous detection system, deviations in binding constants may be observed as compared to those obtained from homogeneous detection systems.

X-ray crystallography and nuclear magnetic resonance (NMR) are also widely used to obtain structural information
10 on protein-drug interactions. However, not all proteins can form diffractable crystals, thereby limiting the information that can be gained from X-ray crystallography. Similarly, use of NMR is restricted to only small proteins (<50 kDa), requires skilled personnel to perform the
15 experiments, and can be expensive as it requires ^{15}N isotope labeling.

Another method that may be used for studying protein-drug binding interaction is circular dichroism (CD). In general, CD is widely used for routine studies of protein
20 secondary structure and conformational changes. Upon binding, a ligand induces a conformational change in the protein, thus allowing one to detect the protein-ligand interaction. However, the secondary structures are not always ideal in real protein structure, which complicates
25 data interpretation. In addition, CD suffers the disadvantage of having lower sensitivity. Woo et al.¹ has demonstrated that this technique could not detect subtle changes in protein structures when compared to a metal nanoparticle-based colorimetric assay.

30 Yet another method that may be used for studying protein-drug binding interaction is intrinsic tryptophan fluorescence measurement. Protein fluorescence is dominated by the tryptophan (Trp) residues due to their high extinction coefficient values. Upon binding, a ligand

generally causes a detectable change in the local environment of the Trp residues. The associated change in protein fluorescence emission can be used to detect protein-ligand interaction. This technique has the
5 disadvantage of limited sensitivity, particularly when a ligand binding results in fluorescence quenching. Furthermore, this method cannot be used for Trp-free proteins. For example, HSA contains only a single Trp residue (Trp214) in domain IIA, which results in low
10 sensitivity when detecting relative binding affinity among different drug ligands.

Therefore, there is a need to provide a method for determining protein-drug binding interaction that overcomes, or at least ameliorates, one or more of the
15 disadvantages described above.

There is a need to provide a method that is less time consuming, more accurate and sensitive, simple, and cost-effective for determining protein-drug binding interactions.

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Summary

According to a first aspect, there is provided a method for detecting binding of a ligand to a target
25 molecule comprising:

contacting a sample comprising the ligand and the target molecule with a metal nanocluster precursor under a denaturing condition, the precursor being capable of forming a metal nanocluster under a reducing condition to
30 produce a detectable signal;

wherein detection of a signal that substantially differs from the signal of a control in the absence of the ligand indicates that the ligand binds to the target molecule.

In one embodiment, the reducing condition is applied while the denaturing condition is present.

In one embodiment, the denaturing condition is selected from the group consisting of a chemical denaturant and a physical denaturant. In one embodiment, the physical denaturant is heat. Advantageously, application of the denaturing condition to the method significantly reduces the time required for obtaining a detectable signal on which determination of whether the ligand binds to the target molecule can be based.

In one embodiment, the method is conducted in a homogenous system. Advantageously, being in homogeneity enhances the accuracy in determining the binding of the ligand to the target molecule, and simplifies the detection method.

According to a second aspect, there is provided a method for determining the strength of binding of a ligand to a target molecule, comprising:

- (a) detecting binding of the ligand to the target molecule according to the method of the first aspect at varying concentrations of the ligand; and
- (b) determining the dissociation constant (K_D) of the binding from a correlation between the concentration of the ligand and the detectable signal.

According to a third aspect, there is provided a method for determining the site of binding of a ligand with an unknown binding site on a target molecule, comprising:

- (a) detecting binding of a ligand with a known binding site on the target molecule according to the method of the first aspect;
- (b) adding the ligand with the unknown binding site to the sample; and

- (c) comparing the detectable signal obtained in (a) to the detectable signal obtained in (b).

In one embodiment of the third aspect, a decrease in detectable signal indicates that the ligand with the unknown binding site binds to the same site on the target molecule as the ligand with the known binding site, and an increase in detectable signal indicates that the ligand with the unknown binding site binds to a different site on the target molecule as the ligand with the known binding site.

According to a fourth aspect, there is provided a method for detecting binding of a ligand to a target molecule comprising:

- (a) applying a denaturing condition to a pre-incubated sample comprising the ligand and the target molecule to form a template for the synthesis of fluorescent metal nanoclusters, and
- (b) contacting the pre-incubated sample with a metal nanocluster precursor under reducing conditions to form metal nanoclusters, the metal nanoclusters producing a detectable fluorescence signal upon binding to the target molecule;

wherein detection of a signal that substantially differs from the signal of a control in the absence of the ligand indicates that the ligand binds to the target molecule.

According to a fifth aspect, there is provided a method for comparing the strength of binding of a plurality of ligands to a target molecule, comprising:

- (a) detecting the binding of each of the plurality of ligands to the target molecule according to the method of the first aspect or the fourth aspect; and
- (b) comparing the extent to which each detectable signal obtained from step (a) for each of the

plurality of ligands differs from the detectable signal of the control.

Definitions

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The following are some definitions that may be helpful in understanding the description of the present invention. These are intended as general definitions and should in no way limit the scope of the present invention to those terms alone, but are put forth for a better understanding of the following description.

The terms "polypeptide" and "protein" are used interchangeably and refer to any polymer of amino acids (dipeptide or greater) linked through peptide bonds or modified peptide bonds, whether produced naturally or synthetically. The polypeptides may comprise non-peptidic components, such as carbohydrate groups.

The word "substantially" does not exclude "completely" e.g. a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

Unless the context requires otherwise or specifically stated to the contrary, integers, steps, or elements of the invention recited herein as singular integers, steps or elements clearly encompass both singular and plural forms of the recited integers, steps or elements.

As used in this application, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "an agent" includes a plurality of agents, including mixtures thereof.

Unless specified otherwise, the terms "comprising" and "comprise", and grammatical variants thereof, are intended

to represent "open" or "inclusive" language such that they include recited elements but also permit inclusion of additional, unrecited elements.

As used herein, the term "about", in the context of
5 concentrations of components of the formulations, typically means +/- 5% of the stated value, more typically +/- 4% of the stated value, more typically +/- 3% of the stated value, more typically, +/- 2% of the stated value, even more typically +/- 1% of the stated value, and even more
10 typically +/- 0.5% of the stated value.

Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible
15 limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as
20 from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of
25 the range.

Certain embodiments may also be described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the disclosure. This includes the generic
30 description of the embodiments 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.

Disclosure of Optional Embodiments

Disclosed herein is a method for detecting binding of a ligand to a target molecule comprising:

5 contacting a sample comprising the ligand and the target molecule with a metal nanocluster precursor under a denaturing condition, the precursor being capable of forming a metal nanocluster under a reducing condition to produce a detectable signal;
10 wherein detection of a signal that substantially differs from the signal of a control in the absence of the ligand indicates that the ligand binds to the target molecule.

 The binding may be any interaction between two or more molecules, for example between a ligand and a target
15 molecule.

 The ligand may be any agent (e.g. a molecule or other chemical entity) that is capable of binding to, or interacting or otherwise associating with, a target molecule. The ligand may bind the target molecule when the
20 target molecule is in its native conformation, or when it is partially or totally unfolded or denatured. The ligand is not limited to an agent that binds a recognized functional region of the target molecule, but can also be an agent that binds any surface or internal sequences or
25 conformational domains of the target molecule. The ligand may be a molecule affecting physiological function, such as a drug.

 In one embodiment, the ligand is selected from the group consisting of a small-molecular weight compound, and
30 a metal ion.

 A small-molecular weight compound typically refers to a compound, for example an organic compound, with a molecular weight of less than about 1,000 daltons, less

than about 750 daltons, less than about 600 daltons, or less than about 500 daltons.

In one embodiment, the small-molecular weight compound is selected from the group consisting of ibuprofen, warfarin, phenytoin, sulfanilamide, sulfamethazine, sulfonated azo dyes, metal-oxide clusters, thiosemicarbazone, heteroaromatic compounds, halogenated salicyl derivatives, polycyclic aromatic ketones, and non-redox active compounds.

10 In one embodiment, the target molecule is serum albumin and the small-molecular weight compound is selected from the group consisting of ibuprofen, warfarin, phenytoin, sulfanilamide, and sulfamethazine.

In one embodiment, the target molecule is a transcription factor and the small-molecular weight compound is selected from the group consisting of sulfonated azo dyes, metal-oxide clusters, thiosemicarbazone, heteroaromatic compounds, halogenated salicyl derivatives, polycyclic aromatic ketones, and non-redox active compounds. The non-redox active compounds may be selected from the National Cancer Institute (NCI) compound library.

A nucleic acid ligand may be, for example an aptamer.

The target molecule may be any molecule that is capable of being bound by the ligand, and that has one or more binding sites for the ligand. The term "binding site" refers to a region of a molecule to which another molecule can bind. The target molecule may be a biomolecule, which may be any compound isolated from a living organism, as well as analogs (including engineered and/or synthetic analogs), derivatives, mutants or variants and/or biologically active fragments of the same. The biomolecule can also be an engineered or synthetic analog of a compound isolated from a living cell that is

structurally different from the compound but retains a biological activity characteristic of that compound. Exemplary biomolecules include, but are not limited to, peptides, proteins, nucleic acids (e.g. polynucleotides, oligonucleotides), carbohydrates, lipids, or complexes thereof. The term also includes portions of peptides, proteins, and other biomolecules which are capable of acquiring secondary, tertiary, or quaternary structure through folding, coiling or twisting. The target molecule may be substituted with substituents including, but not limited to, cofactors, coenzymes, prosthetic groups, lipids, oligosaccharides, or phosphate groups.

In one embodiment, the target molecule is selected from the group consisting of a protein, a DNA, a RNA and a peptide.

In one embodiment, the target molecule is a protein. The protein may be selected from the group consisting of serum albumin, a transcription factor, and a protein drug target. The serum albumin may be selected from the group consisting of bovine serum albumin (BSA) and human serum albumin (HSA). The protein drug target may be any protein that forms a target for a drug.

A sample comprising the ligand and target molecule may be in fluid form, which facilitates contact with the metal nanocluster precursor to form a metal nanocluster.

In one embodiment, the ligand is a small-molecular weight compound and the target molecule is a protein. In one embodiment, the ligand is ibuprofen and the target molecule is a serum albumin (e.g. HSA or BSA). In one embodiment, the ligand is warfarin and the target molecule is a serum albumin (e.g. HSA or BSA). In one embodiment, the ligand is phenytoin and the target molecule is a serum albumin (e.g. HSA or BSA). In one embodiment, the ligand is

sulfanilamide and the target molecule is a serum albumin (e.g. HSA or BSA).

The denaturing condition may be applied to the sample comprising the ligand and the target molecule by any
5 suitable means of bringing the sample into contact with a denaturing agent. Without being bound by theory, it is believed that the denaturing condition allows the protein to unfold and interact with the metal nanocluster precursor for forming protein-based metal nanoclusters.

10 A denaturing condition may be any condition or agent that, when exposed to a target molecule, causes, affects or influences the degradation or denaturation of the target molecule. Where the target molecule is a protein, the denaturation condition may cause a biochemical change in
15 the protein such that a property or activity of the protein is diminished or eliminated. The biochemical change can be a loss or partial loss of the tertiary or secondary structure of the protein, i.e. full or partial unfolding of the protein. For example, the protein may be about 10%,
20 about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80% or about 90% partially unfolded. The protein may also be 100% unfolded (i.e. fully unfolded). The property or activity can be completely abolished or can be reduced by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%,
25 90%, 95% or more in the presence of the denaturing condition than in its absence. Where the target molecule is a nucleic acid, the denaturing condition may cause disruption of hydrogen-bonded base pairs between two regions of partial or complete complementary nucleotide
30 sequences. This includes the disruption of hybridization between two nucleic acid molecules (intermolecular), and the disruption of hybridization between two complementary regions in one nucleic acid molecule (intramolecular secondary structure). The denaturation may be reversible or

irreversible, and may lead to the formation of oligomers or aggregates.

A denaturing condition can be an external stress or physical condition (e.g. temperature, time of storage, 5 absence of a stabilizer, agitation), or can be an exposure to a denaturing agent. For example, the denaturing condition can be heat, acid or a chemical denaturant. Exemplary denaturing conditions include, but are not limited to, the presence of elevated temperature (e.g. 10 heat), urea, a strong acid or base, a concentrated inorganic salt, an organic solvent (e.g. alcohol or chloroform), high or low pH (extremes of pH), excipients that can be denaturing (e.g. phenolic preservatives or detergent), and low or substantially no stabilizing agent 15 that otherwise is required for stability of the target molecule.

In one embodiment, the denaturing condition is selected from the group consisting of a chemical denaturant and a physical denaturant. The chemical denaturant may be 20 selected from a group consisting of urea, guanidine hydrochloride, and salt. The concentration of urea may be selected from a group consisting of about 1.6M, about 3.2M, about 4.8M and about 6.4M. The concentration of urea may be at least about 0.5M, at least about 1M, at least about 25 1.5M, at least about 2M, at least about 2.5M, at least about 3M, at least about 3.5M, at least about 4M, at least about 4.5M, at least about 5M, at least about 6M, at least about 7M, or at least about 8M.

In one embodiment, the physical denaturant is heat. 30 The heat may be applied at a temperature that is above the temperature at which the target molecule denatures. Generally, the temperature may be temperatures that are greater than room temperature or ambient temperature. This may be at least, greater than, or about 30°C, such as 30°C

to 42°C, and generally 32°C to 37°C or 35°C to 37°C, inclusive. Room or ambient temperature typically refers to a range from about or at 18°C to about or at 32°C. Those of skill in the art will appreciate that room or ambient temperature varies by location and prevailing conditions. For example, the heat may be applied at a temperature of at least about 30°C, at least about 40°C, at least about 50°C, at least about 60°C, at least about 70°C, at least about 80°C, or more. In one embodiment, the temperature is about 60°C.

Advantageously, the denaturation accelerates the formation of the target molecule metal nanoclusters, and reduces the time taken to detect ligand binding to a target molecule from hours to minutes.

The denaturing condition may be applied after incubation of the ligand and the target molecule for a predetermined period of time. Without being bound by theory, it is believed that the binding of the ligand to the target molecule stabilizes the target molecule against denaturation by the denaturing condition. As used herein, "predetermined period of time" refers to a time that is established or decided in advance. For example, the predetermined period of time can be a time chosen in advance that is associated with the desired duration of incubation of the ligand with the target molecule to enable binding of the ligand to the target molecule. A predetermined period of time can be minutes, hours, days, months or years, but typically a predetermined period of time is in minutes or hours.

In one embodiment, the method is conducted in a homogenous system. Advantageously, being in a homogeneous system allows better interaction between the ligand and the target molecule. The incubation of the ligand with the target molecule prior to denaturation of the target

molecule ensures that the target molecule retains its bioactivity during interaction with the ligand. The incubation may be for at least about 30 min, at least about 45 min, at least about 60 min, at least about 75 min, at least about 90 min, or more. In one embodiment, the incubation time is about 60 min.

The denatured sample comprising the ligand and target molecule may be contacted with a metal nanocluster precursor by any mode of bringing together the denatured sample with the metal nanocluster precursor, under reducing conditions, such that they can interact to form the metal nanoclusters. Broadly, the term refers to placing the denatured sample comprising the ligand and target molecule in solution with the metal nanocluster precursor so that the metal nanocluster precursor can interact with the target molecule to produce a detectable signal, upon application of a reducing condition. Less broadly, contacting refers to the mixing, stirring, turning, swirling, shaking or vibrating of a solution of the denatured sample and the metal nanocluster precursor. Mixing can be accomplished, for example, by repeated uptake and discharge through a pipette tip, either manually or using an automated pipetting device.

Advantageously, the contacting of the sample comprising the ligand and the target molecule with the metal nanocluster precursor is also conducted in a homogeneous system which allows better interaction between the sample and the metal nanocluster precursor in forming a metal nanocluster. It is envisaged that the metal nanocluster is formed and encapsulated in the ligand-bound target molecule under reducing conditions as defined herein, to produce a detectable signal that is reflective of the binding and binding strength of the ligand to the target molecule.

In one embodiment, the metal nanocluster precursor is in contact with a reducing agent. Contact with a reducing agent may be necessary to trigger the formation of the metal nanoclusters.

5 The reducing agent may be an "external" reducing agent that is added to the sample comprising the ligand and the target molecule, and the metal nanocluster precursor. For example, the reducing condition may comprise a reducing agent selected from a group consisting of sodium
10 borohydride (NaBH_4) and carbon monoxide (CO). The reducing agent may be in liquid form or gaseous form, for example gaseous carbon monoxide.

 Alternatively, an agent may be added which provides suitable conditions for reduction to take place. For
15 example, where the target molecule is a protein, sodium hydroxide (NaOH) may be added, which provides an alkaline condition that triggers or activates the reducing capability of the amino acids present in the protein. In this instance, the protein acts as the reducing agent to
20 reduce the metal nanocluster precursor. Hence, the reducing agent may be an "internal" reducing agent. In one embodiment, the reducing condition comprises a reducing amino acid of the protein as reducing agent. A reducing amino acid may be a tyrosine.

25 In one embodiment, the reducing condition is applied while the denaturing condition is present.

 In another embodiment, the reducing condition is applied after the denaturing condition.

 In one embodiment, the metal nanocluster precursor may
30 be selected from the group consisting of gold, silver, copper, palladium, platinum, ruthenium, and combinations thereof.

 The metal nanocluster precursor may be a noble metal nanocluster precursor.

The metal nanocluster precursor may be provided in salt form, for example HAuCl_4 , AgNO_3 , and the like.

In one embodiment, the metal nanocluster precursor is gold.

5 A plurality of the reduced metal nanocluster precursors may form a metal nanocluster.

In one embodiment, the metal nanocluster is in pure form. For example, the metal nanocluster may be a gold nanocluster, or it may be a silver nanocluster, or it may
10 be a copper nanocluster etc.

In another embodiment, the metal nanocluster is in the form of a metal core shell. In one embodiment, the metal core shell is a noble metal core shell. In one embodiment, the noble metal core shell comprises gold and silver. For
15 example, the noble metal core shell may comprise a silver core and a gold shell. Alternatively, the noble metal core shell may comprise a gold core and a silver shell.

In yet another embodiment, the metal nanocluster is an alloy. In one embodiment, the alloy is a noble metal alloy.

20 In one embodiment, the metal nanocluster is a gold nanocluster (i.e. aurium or Au) formed from gold nanocluster precursors. In one embodiment, the gold nanocluster precursor is a gold ion having a valency selected from the group consisting of +1, +2, and +3. In
25 one embodiment, the gold nanocluster precursor is HAuCl_4 . Upon application of the reducing condition, the gold ion may be reduced to a gold atom (i.e. having a valency of 0).

In one embodiment, the metal nanocluster has a size of less than about 2 nm, less than about 1 nm, or less than
30 about 0.5 nm. In one embodiment, the metal nanocluster is less than about 2 nm.

A detectable signal is produced after contacting the denatured sample with the metal nanocluster precursors for a predetermined period of time and upon exposure to a

reducing condition. The detectable signal may be any signal that is capable of being detected, for example perceived, sensed or measured, either by direct observation (e.g. visually) or instrumentally. The detectable signal
5 may be qualitative or quantitative. Detectable signals include but are not limited to fluorescent signals, visually detectable signals, luminescent signals, phosphorescence, electromagnetic waves, electrical signals, electrochemical signals, chemical signals
10 (chemiluminiscence), magnetic fields, and radiological signals.

In one embodiment, the detectable signal is a signal selected from the group consisting of fluorescence, visually detectable signal, and luminescence. For example,
15 when a complex forms between the target molecule and a metal nanocluster such that the metal nanocluster is encapsulated within a template formed by the target molecule, a change in the color of the sample may be observed visually. Hence, advantageously, the detectable
20 signal may be signal that can be easily detected visually without the need for any analytical instruments.

In one embodiment, the detectable signal is a fluorescence signal. For example, the fluorescence signal may be fluorescence intensity, fluorescence anisotropy,
25 fluorescence polarization or Förster resonance energy transfer (FRET). In one embodiment, the fluorescence signal is fluorescence intensity.

By comparing the detectable signal obtained from the sample against the detectable signal from a control, it is
30 possible to determine if a ligand binds to a target molecule. For example, where the detectable signal of the sample substantially differs from the detectable detection signal of the control, it indicates that the ligand binds

to the target molecule. A control may be one where the ligand is absent.

In one embodiment, a fluorescence intensity of a sample that is substantially lower than the fluorescence intensity of the control indicates that the ligand binds to the target molecule. The extent of the difference between the fluorescence intensity of the sample and the fluorescence intensity of the control can be correlated to the strength of the binding between the ligand and the target molecule. For example, a higher extent of the difference indicates a stronger binding of the ligand to the target molecule. Hence, different ligands having different binding strengths to a target molecule may produce detectable signals of different intensities. Accordingly, by comparing the fluorescent intensity difference among samples tested under the same reaction conditions, it is possible to determine the relative binding strength of different ligands to a target molecule.

In one embodiment, there is provided a method for comparing the strength of binding of a plurality of ligands to a target molecule, comprising:

- (a) detecting the binding of each of the plurality of ligands to the target molecule according to the method as disclosed above; and
- (b) comparing the extent to which each detectable signal obtained from step (a) for each of the plurality of ligands differs from the detectable signal of the control.

In one embodiment, the extent of the difference between the detectable signal from the control correlates to the strength of binding of the ligand to the target molecule.

The fluorescence intensity of the sample is substantially different from that of a control when the

fluorescence intensity of the sample is at least about 5%,
at least about 10%, at least about 20%, at least about 30%,
at least about 40%, at least about 50%, at least about 60%,
at least about 70%, at least about 80%, at least about 90%
5 or about 100% lower than the fluorescence intensity of the
control.

In another embodiment, a fluorescence intensity of a
sample that is substantially the same as the control
indicates that the ligand does not bind to the target
10 molecule.

In one embodiment, there is provided a method for
detecting binding of a ligand to a target molecule
consisting essentially of:

15 contacting a sample comprising the ligand and the
target molecule with a metal nanocluster
precursor under a denaturing condition, the
precursor being capable of forming a metal
nanocluster under a reducing condition to produce
a detectable signal;

20 wherein detection of a signal that substantially
differs from the signal of a control in the absence of
the ligand indicates that the ligand binds to the
target molecule.

The disclosed method may be used for determining the
25 strength of binding of a ligand to a target molecule, as
well as for determining the site of binding of a ligand
with an unknown binding site on a target molecule.

Hence, also disclosed herein is a method for
determining the strength of binding of a ligand to a target
30 molecule, comprising:

- (a) detecting binding of the ligand to the target
molecule according to the method as disclosed
above at varying concentrations of the ligand;
and

- (b) determining the dissociation constant (K_D) of the binding from a correlation between the concentration of the ligand and the detectable signal.

5 A plot of the detectable signal versus the varying concentrations of the ligand may be fitted with a kinetic equation, such as for example the Michaelis-Menten equation: $Y = R_{\max} * C / (K_D + C)$ where $R_{\max}$ indicates the maximum response signal, C is the concentration of the ligand and
10 K_D is the binding is the binding constant.

Further disclosed herein is a method for determining the site of binding of a ligand with an unknown binding site on a target molecule, comprising:

- (a) detecting binding of a ligand with a known
15 binding site on the target molecule according to the method as disclosed above;
- (b) adding the ligand with the unknown binding site to the sample; and
- (c) comparing the detectable signal obtained in (a)
20 to the detectable signal obtained in (b).

In one embodiment, a decrease in detectable signal indicates that the ligand with the unknown binding site binds to the same site on the target molecule as the ligand with the known binding site, and an increase in detectable
25 signal indicates that the ligand with the unknown binding site binds to a different site on the target molecule as the ligand with the known binding site. In one embodiment of this method, the detectable signal is a fluorescent signal.

30 Also disclosed herein is a method for detecting binding of a ligand to a target molecule comprising:

- (a) applying a denaturing condition to a pre-incubated sample comprising the ligand and the target molecule to

form a template for the synthesis of fluorescent metal nanoclusters, and

(b) contacting the pre-incubated sample with metal nanocluster precursor under reducing conditions to form metal nanoclusters, the metal nanoclusters producing a detectable fluorescence signal upon binding to the target molecule;

wherein detection of a signal that substantially differs from the signal of a control in the absence of the ligand indicates that the ligand binds to the target molecule. In one embodiment of this method, the detectable signal is a fluorescent signal.

Brief Description of Drawings

The accompanying drawings illustrate a disclosed embodiment and serve to explain the principles of the disclosed embodiment. It is to be understood, however, that the drawings are designed for purposes of illustration only, and not as a definition of the limits of the invention.

Figure 1 shows schematically the protein-directed AuNCs formation with and without ligand loading, under conditions of urea denaturation or heat treatment of the protein. The bottom photograph shows the HSA assisted-formation of AuNCs with urea or heat treatment in the presence of a drug ligand (low fluorescent intensity, left tube) and in the absence of a drug ligand (high fluorescent intensity, right tube).

Figure 2 shows the emission spectra of BSA-AuNCs prepared with (a) heat-treatment and (b) urea of different concentrations, recorded at 10 min and 1 hr respectively upon adding NaOH for reduction.

Figure 3 shows (a) the fluorescence evolution of BSA-AuNCs prepared in the absence and presence of ibuprofen, and treated with different concentrations of urea; and (b) a comparison of the fluorescence intensity of BSA-AuNCs in the absence and presence of ibuprofen, recorded 3 hr after addition of NaOH.

Figure 4 shows (a) emission spectra of HSA-AuNCs prepared in the presence of different ligands. A control experiment was carried out for the formation of HSA-AuNCs without ligands (black curve). Both spectra and pictures (see inset of Figure 3b) were taken 10 min after addition of NaOH; and (b) the normalized emission intensity of HSA-ligand-AuNCs against HSA-AuNCs sample without ligand (control).

Figure 5 shows (a) the results of tryptophan fluorescence quenching of HSA with increasing concentrations of ibuprofen; and (b) a plot of $\log[(F_0 - F)/(F - F_{\text{sat}})]$ against $\log[\text{ibuprofen}]$ to obtain the K_D value.

Figure 6 shows the dose response plots for the HSA binding ligands of different binding affinities: (a) warfarin and (b) ibuprofen, obtained by synthesizing HSA-ligand-AuNCs at increasing amounts of the ligand. The data were fitted to a single site binding model using Origin. The reported K_D based on homogenous NMR system is shown in the inset table for comparison.

Figure 7 shows the fluorescence spectra of (a) HSA-warfarin-AuNCs (site I probe) and (b) HSA-ibuprofen-AuNCs (site II probe) titrated with sulfamethazine (smz) of different concentrations; and (c) the overlaid structures of HSA-ibuprofen crystal (PDB: 2BXG; red) and HSA-warfarin crystal (PDB: 2BXP; blue). The tyrosine residues are shown as licorice models, with Tyr411 (orange; domain IIIA) and Tyr150 (cyan) involved in hydrogen bonding with ibuprofen and warfarin respectively, are shown as van der Waals

models. Cys34 was shown to illustrate the distance of domain I.

Figure 8 shows the dose response plots for the HSA-ibuprofen based on Biacore SPR.

5 **Figure 9** shows the CD spectra of HSA-AuNCs prepared at room temperature and through heat-treatment in presence of different ligands.

Examples

10 Non-limiting examples of the method of the invention and a comparative example will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of
15 the invention.

Example I: Serum Albumin Based-AuNCs Formation Under Different Denaturing Conditions

20 The inventors herein reports a novel method for screening serum albumin drugs by exploiting accelerated AuNCs formation directed by ligand-stabilized protein under urea denaturation or heat treatment conditions, as shown schematically in Figure 1. As can be seen in Figure 1, the
25 HSA assisted-formation of AuNCs with urea or heat treatment in the presence of a drug ligand resulted in low fluorescent intensity (left tube), while the formation of AuNCs with urea or heat treatment in the absence of a drug ligand resulted in high fluorescent intensity (right tube).

30 A denaturant was used not only to accelerate the reaction, but also to screen the ligand-induced protein stabilization. Two types of denaturants were tested for this purpose: urea (3.2 M and 6.4M) and heat-treatment. Urea is known to interact with peptide backbone and cause

solvation of hydrophobic core, whereas heat treatment is known to disrupt the hydrogen bonding and hydrophobic interactions of proteins. Both urea and heat treatments lead to protein unfolding. To compare the two denaturing conditions, BSA-AuNCs were used.

BSA and HSA are known to unfold irreversibly and start to form a gel upon heating beyond 60°C. Hence, an optimum heating process at 60°C was used in all experiments unless otherwise stated. As shown in Figure 2(a), the preparation time for BSA-AuNCs was reduced significantly from (>10 hr) at room temperature to only 30 min at 60°C. The acceleration was due to the structural flexibility of the protein and the fast kinetic at 60°C, which enhanced the reduction of the gold ion. Similarly, as the urea concentration increased, the emission intensity increased accordingly (Figure 2(b)), indicating the unfolded protein facilitates the formation of AuNCs. However, using urea as a denaturant required a longer reaction time (>1 hr) to achieve reasonable emission intensity.

The same synthesis condition with heat-treatment was applied for HSA-AuNCs. Similar intensity and reaction time were obtained since the two proteins are structurally very similar.

Example II: Adopting Urea-treated AuNCs Formation Conditions for BSA-Drug Screening

It is known that ligand binding typically enhances protein stability. In this example, the adverse effect of ligand binding on AuNC formation under urea treatment was exploited for screening BSA binders. To test the ligand stabilization on BSA against urea denaturation, BSA (200 µL, 112 mM) was pre-incubated with ibuprofen (1 µL in DMSO, 450 mM) for 1 hr at room temperature. 2x excess of drug

ligand was used to ensure the binding sites were saturated. Urea (200 μ L, 10M or 20M) was then added into the mixture under vigorous stirring at 37°C. HAuCl₄ (200 μ L, 15mM) was then added followed by NaOH solution (20 μ L, 1.5 M) for
5 reduction reaction. The solution turned brown after 1 hr, indicating the formation of AuNCs. The reaction essentially took >6 hours to complete. In the case where a strong binder (ibuprofen) was used to stabilize BSA against urea-induced unfolding, the AuNCs formation process was retarded
10 to a certain extent, leading to their emission with lower intensity (lesser amount of AuNCs were formed), as compared to the sample without ligand. The differences between the sample with a ligand and the sample without a ligand could be observed clearly after 3 hr of reduction reaction
15 (Figure 3(a)). Owing to its long reaction time, heat-treatment is preferred for drug screening, as shown in Example III.

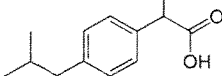
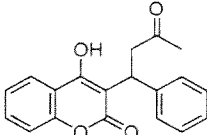
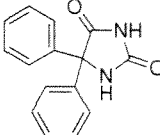
20 Example III: Adopting Heat-Treated AuNCs Formation Conditions for HSA-Drug Screening

The interaction of proteins with small ligands is known to increase thermostability of protein due to the coupling of binding with unfolding equilibrium.² As a
25 proof-of-concept experiment, HSA (200 μ L, 112 mM) was pre-incubated with ibuprofen (1 μ L in DMSO, 450 mM) for 1 hr at room temperature. The incubated solution was then heated up to 60°C for 10 min, and aqueous HAuCl₄ solution (400 μ L, 7.75 mM) and NaOH solution (20 μ L, 1.5 M) were added for
30 the reduction reaction. The colorless protein solution turned from light yellow to dark brown after 10 min, indicating the formation of AuNCs within the protein template. Similar to Example I, the strong binder (i.e. ibuprofen) had stabilized HSA against thermal-induced

unfolding, resulting in AuNCs with lower emission intensity, as compared to the sample without a ligand. Importantly, the difference between the two samples was more significant and observable after only 10 min of reaction time.

Based on this optimized condition, a total of four ligands with different binding affinity and drug binding sites on the HSA were tested (Table 1). Ibuprofen is known to bind to Sudlow's site II, whereas the other ligands are known to bind to Sudlow's site I with different affinity. Based on NMR study, it has been reported that ibuprofen has a stronger binding affinity (K_D) than warfarin, followed by phenytoin. It should be noted that the absolute K_D obtained from different techniques should not be compared directly due to the different sensitivity of each method. To confirm the ligand binding strength, Biacore Surface Plasmon resonance (SPR) experiments⁴ were performed to test ligand binding to HSA using sulfanilamide as a negative control ligand.

Table 1. HSA binding ligand of different binding sites and binding affinity tested in this study

Ligand	Structure	MW	Site Selectivity	Reported K_D (μ M)
Ibuprofen (ibp)		206	II	0.5 ± 1.0^3
Warfarin (war)		308	I	4.0 ± 2.8^3
Phenytoin (phe)		252	I	131.6 ± 12.5^3
Sulfanilamide (sul)		172	I	NA ⁴

³ Shortridge, M. D. (2010) *Nuclear Magnetic Resonance Affinity Screening Methods for Functional Annotation of Proteins and Drug Discovery*. Thesis, University of Nebraska.
⁴ GE Healthcare Biacore training notes. *Working with Small Molecules*.

As seen in Figure 4(a), the sample without the ligand shows the highest intensity amongst all, followed closely by the sample with sulfanilamide, indicating that the interaction between HSA and this ligand is weak. Figure 4(b) shows the normalized emission intensity of HSA-ligand-AuNCs obtained from multiple experiments. The intensity trend was reproducible and followed the trend of sulfanilamide > phenytoin > warfarin > ibuprofen, which is inversely proportional to the reported protein-drug binding strength (see reported binding constant (K_D) obtained using other methods in Table 1). The result implies the feasibility and sensitivity of the assay reported here to screen HSA-drug binding using a simple and reliable method.

Despite the K_D for some HSA binding ligands having been reported, different methods and conditions may yield different values. Hence, to further confirm the reliability of the results using the disclosed method reported here, the HSA-ligand binding constant was measured using the traditional tryptophan fluorescence quenching titration method, in which the detection signal relies solely on the weak fluorescence of a single tryptophan (Trp) residue (Trp214) in domain IIA of HSA protein. Briefly, HSA was titrated by successive addition of the drug ligand and its Trp emission fluorescence was recorded over time. As the ligand concentration increased, the fluorescence intensity gradually decreased, indicating ligand-binding induced conformational change. By plotting $\log [(F_0 - F)/(F - F_{sat})]$ against $\log[\text{ligand}]$, K_D and binding sites (n) can be obtained as the intercept and the slope (see Figure 5). The results for each HSA-ligand interaction are summarized in Table 2. The trend obtained is in accordance with our

screening results using HSA-ligand-AuNCs, but the K_D value for the ligands obtained using the Trp titration method are very close to each other. This indicates lower sensitivity of the fluorescence measurement based on intrinsic tryptophan fluorescence, which may be attributed to the presence of only a single Trp residue (Trp214) on HSA. This further demonstrates the high sensitivity of the disclosed method reported here for protein-ligand binding which resulted in large differences in fluorescent intensity for screening ligands of different binding strength, independent of the Trp content of the protein.

Table 2. Summary of K_D measured from Trp fluorescence quenching titration

Ligand	K_D (μ M)
Ibuprofen (ibu)	0.0743
Warfarin (war)	0.2207
Phenytoin (phe)	0.2357
Sulfanilamide (sul)	0.2597

Example IV: HSA-AuNCs for Binding Constant (K_D) Measurement in Homogeneous System

Driven by the consistent trend obtained, we further modified our protocol for K_D measurement. By synthesizing the nanoclusters in the presence of increased ligand amounts, the K_D can be evaluated by fitting the fluorescence plot with the Michaelis-Menten equation: $Y = R_{\max} * C / (K_D + C)$, where $R_{\max}$ indicates the maximum response signal, C is the concentration of the ligand and K_D is the binding constant. As proof-of-concept, warfarin and ibuprofen were chosen as drugs for site I and II, respectively. As shown in Figure 6, the increased amount of ligand rendered the has more stable against thermal denaturation, resulting in a decrease of the fluorescence signal due to the limited amount of AuNCs that could be

formed within the protein scaffold at short reaction time (i.e. 30 min). The calculated binding constant for warfarin and ibuprofen are 10.12 and 0.74 μM , respectively, comparable to those measured from homogenous-phased NMR study, i.e. 4.0 and 0.5 μM for warfarin and ibuprofen, respectively.

Example V: Competitive Assay using HSA-Ligand-AuNCs to Identify Drug Binding Sites

As aforementioned, HSA contains two primary drug binding sites. Identification of drug location on a target protein is of paramount importance. Herein, the brightly fluorescent AuNC system was adopted to study the effect of competitive binding based on the emission changes of the as-synthesized HSA-ligand-AuNCs probes (i.e. site I probe: HSA-warfarin-AuNCs and site II probe: HSA-ibuprofen-AuNCs) to determine the binding site of an unknown ligand.

In this study, the competitive binding assay was conducted using sulfamethazine which is known bind to site I with increasing concentrations of 10, 20, 30, 40, 50 and 60 mM. Figure 7(a) shows that upon adding sulfamethazine to the HSA-warfarin-AuNCs, the fluorescent intensity of the site I probe decreased with increasing concentration of the titrated ligand, sulfamethazine. Conversely, those titrated with the site II probes yielded further increments in the fluorescent intensity of the HSA-ibuprofen-AuNCs with increasing sulfamethazine concentrations (Figure 7(b)). While the mechanism behind the observation remains unclear, the results reported here imply that the binding of sulfamethazine to site I affects the emission of pre-bound warfarin and ibuprofen in the HSA-AuNCs. A possible explanation for such detectable change is the distance between the two domains may have decreased due to

structural change(s) in the HSA at alkaline pH, resulting in a significant effect on a neighboring site even when a ligand binds to a different region. This is supported by a Förster resonance energy transfer (FRET) study done by Shaw et al. to characterize the inter-domain distance of IB and IIIA.⁵ The result showed that at the aged form, the distance between the domains had decreased from 43.3 to 26.9 Å. To illustrate the distance of binding sites and domains IB, IIA (site I) and IIIA (site II), the crystal structures of HSA-ibuprofen and HSA-warfarin are shown in Figure 5(c).

Comparative Example 1

As a cross-validation test, the K_D of HSA-ibuprofen was measured using SPR (Figure 8). The value obtained using SPR (i.e. $23.21 \pm 9.01 \mu\text{M}$) differs from the values obtained using the disclosed method ($0.74 \pm 0.10 \mu\text{M}$) and by NMR ($0.5 \pm 1.0 \mu\text{M}$) (see Figure 6(b)). This may be due to the heterogeneous detection system of SPR that led to the deviation in binding constant as compared to those that were obtained from the homogeneous detection systems like NMR and the disclosed method.

Comparative Example 2

Circular dichroism (CD) results of AuNCs prepared with different HSA-ligand solutions showed non-distinguishable CD spectra (Figure 9). In contrast, the results obtained using the disclosed method clearly demonstrated the differences in fluorescence amongst AuNCs prepared with different HSA-ligand solutions (see Figure 4(a)). This shows the limited sensitivity of CD to detect subtle structural changes compared to the disclosed method.

Applications

In summary, the disclosed method is useful for rapid
5 detection of binding of a ligand to a target molecule by
formation of metal nanoclusters under denaturing
conditions. The disclosed method enables direct fluorescent
detection using a simple one-pot synthesis of metal
nanoclusters within a protein template. The disclosed
10 method is "label free" in that no isotope labelling is
necessary. The disclosed method therefore provides a simple
and cost-effective drug screening method without the need
for sophisticated instrument or crystal structure
information required.

15 The denaturing condition (urea or heat-treatment) had
shortened the reaction time of AuNCs formation presumably
due to the extensive interaction between the partially
unfolded protein and gold ions, resulting in faster
reduction of gold ions to form nanoclusters. Since ligand
20 binding occurred prior to the nanoclusters formation, there
is no concern of how well the protein retains the
bioactivity upon forming metal nanocluster. While urea
treatment required >3 hr to detect the ligand induced
stabilization effect, heat-treatment had successfully
25 reduced the reaction time to minutes scale (Examples II and
III above). Hence, the disclosed method enables fast
detection (<30 mins) of any ligand (such as drug) binding
to a target molecule (such as protein).

The disclosed method also enables sensitive
30 qualitative (visual) and quantitative measurements of
binding constant, and determination of ligand binding
sites. By preparing HSA-AuNCs in the presence of different
ligands under heat-treatment, a reverse relationship
between fluorescence intensity of AuNCs and HSA-drug

binding strength was observed (Example III above). This interesting finding can be harnessed for high-throughput drug screening, and provides further insights on the AuNCs formation.

5 In Example IV, the use of the disclosed method to determine the binding constant of drug molecules in homogenous solution was demonstrated. Through a simple competitive titration process, the disclosed method can also be used to identify the drug binding sites on HSA
10 protein for different small ligands (Example V above). The novelty and unique features of disclosed method as compared to conventional methods for protein-drug screening are summarized in Table 3 below.

15 **Table 3.** Summary of advantages of disclosed method over conventional methods of protein-drug screening

Conventional methods	Problems associated with conventional methods	Solutions provided by disclosed method
Surface Plasmon Resonance	• tedious covalent protein immobilization on sensor chip or solid substrate • solvent correction step required • expensive instrument & sensor chip required	• protein-drug binding in homogenous solution • simple one step synthesis • bright fluorescent detection (possible without instrument)
X-ray crystallography	• limited crystallography information • requires longer time to obtain the crystal	• crystal structure information of the protein not needed • fast result
Nuclear magnetic resonance	• restricted to application for small proteins (< 50 kDa) • requires ¹⁵N isotope labeling	• no restriction on protein size • no labeling required • simple and cost-effective

	• tedious and expensive	
Circular dichrorism	• non-distinguishable secondary structural change of HSA with ligands of different binding affinity (Figure 8) • lower sensitivity	• sensitive fluorescent detection method • provides additional information in terms of binding constant and drug binding sites
Trp fluorescence measurement	• not applicable for Trp-free protein • limited sensitivity, determined by the number of Trp residues and their positions (Table 2)	• no restriction on Trp-containing protein • sensitive fluorescent detection method

In addition to drug screening and drug discovery, disclosed methods can therefore also be used to determine drug binding sites and their binding affinity to a target protein in order to better understand drug pharmacokinetics. For example, the disclosed method can be used to study protein stability and ligand-associated protein stabilization (i.e. protein structural and genomics studies).

It will be apparent that various other modifications and adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims.

References:

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15

20

CLAIMS

1. A method for detecting binding of a ligand to a target molecule comprising:

5 contacting a sample comprising the ligand and the target molecule with a metal nanocluster precursor under a denaturing condition, the precursor being capable of forming a metal nanocluster under a reducing condition to produce
10 a detectable signal;

wherein detection of a signal that substantially differs from the signal of a control in the absence of the ligand indicates that the ligand binds to the target molecule.

15

2. The method according to claim 1, wherein the denaturing condition is applied after incubation of the ligand and the target molecule for a predetermined period of time.

20

3. The method according to claim 1 or 2, wherein the reducing condition is applied while the denaturing condition is present.

25

4. The method according to claim 1 or 2, wherein the reducing condition is applied after the denaturing condition.

30

5. The method according to any one of the preceding claims, wherein the reducing condition comprises a reducing agent selected from a group consisting of sodium borohydride (NaBH_4) and carbon monoxide (CO).

35

6. The method according to any one of the preceding claims, wherein the target molecule is selected from

the group consisting of a protein, a DNA, a RNA, and a peptide.

7. The method according to claim 6, wherein the target molecule is a protein.

8. The method according to claim 7, wherein the protein is selected from the group consisting of serum albumin, transcription factor, and a protein drug target.

9. The method according to claim 8, wherein the serum albumin is selected from the group consisting of bovine serum albumin (BSA) and human serum albumin (HSA).

10. The method according to any one of claims 7 to 9, wherein the reducing condition comprises a reducing amino acid of the protein as reducing agent.

11. The method according to any one of the preceding claims, wherein the denaturing condition is selected from the group consisting of a chemical denaturant and physical denaturant.

12. The method according to claim 11, wherein the chemical denaturant is selected from the group consisting of urea, guanidine hydrochloride, and salt.

13. The method according to claim 11, wherein the physical denaturant is heat.

14. The method according to claim 13, wherein the heat is applied at a temperature that is above the temperature at which the target molecule denatures.

15. The method according to any one of the preceding claims, wherein the ligand is selected from the group consisting of a small-molecular weight compound, and a metal ion.
16. The method according to claim 15, wherein the small-molecular weight compound is selected from the group consisting of ibuprofen, warfarin, phenytoin, sulfanilamide, sulfamethazine, sulfonated azo dyes, metal-oxide clusters, thiosemicarbazone, heteroaromatic compounds, halogenated salicyl derivatives, polycyclic aromatic ketones, and non-redox active compounds.
17. The method according to any one of the preceding claims, wherein the metal nanocluster precursor is selected from the group consisting of gold, silver, copper, palladium, platinum, ruthenium, and combinations thereof.
18. The method according to any one of the preceding claims, wherein the method is conducted in a homogenous system.
19. The method according to any one of the preceding claims, wherein the detectable signal is a signal selected from the group consisting of fluorescence, visually detectable signal, and luminescence.
20. The method according to claim 19, wherein the detectable signal is a fluorescence signal.

21. The method according to claim 20, wherein the fluorescence signal is fluorescence intensity.
22. A method for determining the strength of binding of a ligand to a target molecule, comprising:
- (a) detecting binding of the ligand to the target molecule according to the method of any one of claims 1 to 21 at varying concentrations of the ligand; and
 - (b) determining the dissociation constant (K_D) of the binding from a correlation between the concentration of the ligand and the detectable signal.
23. A method for determining the site of binding of a ligand with an unknown binding site on a target molecule, comprising:
- (a) detecting binding of a ligand with a known binding site on the target molecule according to the method of any one of claims 1 to 21;
 - (b) adding the ligand with the unknown binding site to the sample; and
 - (c) comparing the detectable signal obtained in (a) to the detectable signal obtained in (b).
24. The method according to claim 23, wherein a decrease in detectable signal indicates that the ligand with the unknown binding site binds to the same site on the target molecule as the ligand with the known binding site, and wherein an increase in detectable signal indicates that the ligand with the unknown binding site binds to a different site on the target molecule as the ligand with the known binding site.

25. A method for detecting binding of a ligand to a target molecule comprising:

- 5 (a) applying a denaturing condition to a pre-incubated sample comprising the ligand and the target molecule to form a template for the synthesis of fluorescent metal nanoclusters, and
- (b) contacting the pre-incubated sample with a metal nanocluster precursor under reducing conditions
- 10 to form metal nanoclusters, the metal nanoclusters producing a detectable fluorescence signal;

wherein detection of a signal that substantially differs from the signal of a control in the absence of the ligand

15 indicates that the ligand binds to the target molecule.

26. A method for comparing the strength of binding of a plurality of ligands to a target molecule, comprising:

- 20 (a) detecting the binding of each of the plurality of ligands to the target molecule according to the method of any one of claims 1 to 21 or 26; and
- (b) comparing the extent to which each detectable signal obtained from step (a) for each of the plurality of ligands differs from the detectable
- 25 signal of the control.