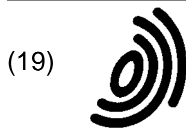
(19) 

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(54) **Method of calculating association and dissociation constants using a polymer chip for identifying ionic polymers**

Verfahren zum Kalkulieren von Assoziations- und Dissoziationskonstanten mit einem Polymerchip zum Auffinden von ionischen Polymeren

Méthode pour calculer des constantes d'association et de dissociation en utilisant une puce de polymères pour l'identification des polymères ioniques

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(73) Proprietor: **HITACHI SOFTWARE ENGINEERING CO., LTD.**
Yokohama-shi, Kanagawa 230-0045 (JP)

(72) Inventors:
- **Nishimura, Yoshifumi,**
  Yokohama City University
  Yokohama-shi,
  Kanagawa 230-0045 (JP)
- **Murai, Hiroshi,**
  Hitachi Software Eng. Co. Ltd.
  Tokyo 140-0002 (JP)
- **Nakao, Motonao,**
  Hitachi Software Eng. Co. Ltd.
  Tokyo 140-0002 (JP)
- **Morita, Toshiki,**
  Hitachi Software Eng. Co. Ltd.
  Tokyo 140-0002 (JP)

(74) Representative: **Liesegang, Roland**
FORRESTER & BOEHMERT
Pettenkoferstrasse 20-22
80336 München (DE)

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Description

BACKGROUND OF THE INVENTION

1. Technical Field

[0001] The present invention relates to a method of calculating the association constant and the dissociation constant between a probe and a protein, a method of searching for the functionality of proteins using the calculation method, and a method of identifying proteins using the calculation method. In particular, the invention relates to a method of searching for the functionality of proteins by calculating the DNA-binding property of the proteins, and a method of identifying the proteins.

2. Background Art

[0002] It is expected that searching for the functionality of DNA and proteins or identifying them would provide tools for the development of drugs or for medical examinations. DNA-binding proteins, in particular, are considered to be among the important substances controlling or adjusting the expression of gene functions, the important substances controlling or adjusting the expression of gene functions, and clarification of their DNA binding properties is an important task.

[0003] A DNA is a polymer formed by a sequence of four kinds of bases, and two DNAs can specifically bind to one another through their sequences. Because the nucleic acids of both such DNAs are negatively charged, hybridization is carried out in a highly ionic solvent. Conventional biochips take advantage of this fact to detect a sample DNA by immobilizing a probe DNA on a substrate.

[0004] However, a DNA-binding protein, for example, cannot be identified by a conventional biochip. This is because a protein as a sample may be specifically adsorbed on a probe DNA on a biochip by its complementary DNA portion, and the protein may also be nonspecifically adsorbed on the probe DNA even though this is not supposed to happen, in light of the DNA. Because of such specific and nonspecific adsorptions that apparently occur on the probe DNA, it has been virtually impossible to identify sample proteins using a conventional biochip.

[0005] Thus, there is a need to develop a chip that can specifically bind to ionic polymers such as proteins and/or DNA, and a technique of accurately identifying ionic polymers using this chip.

[0006] There is also an expectation that investigating the interactions between ionic polymers in a living organism would contribute to the development of new drugs and medical examinations. Particularly, attention is being focused on association constant (KA) and dissociation constant (KD) as important indicators of the interactions between ionic polymers. The association constant (KA) and the dissociation constant (KD) are reciprocal to each other:

$$KA = 1/KD$$

[0007] Conventionally, the association constant and the dissociation constant between ionic polymers are calculated based on a binding curve or dissociation curve obtained by monitoring the adsorption of a substance using an apparatus for measuring the strength of bonding between ionic polymers, such as a surface plasmon resonance measuring apparatus (see JP Patent Publication (Kohyo) No. 7-507865, for example).

[0008] In an example of the surface plasmon resonance measuring apparatus, a ligand is immobilized on a sensor surface, and a sample containing a substance that acts on the ligand is added via a microchannel system. Minute mass changes due to the association or dissociation of molecules on the sensor surface are then monitored in real time in terms of changes in a surface plasmon resonance signal. This technique is effective in studying the interaction between biomolecules or the correlation between structure and function. Surface plasmon resonance is being used in a wide variety of areas including basic research, protein engineering, and the screening of new drugs. Specific examples where surface plasmon resonance is utilized include research into the mechanism of AIDS or cancer development, immunologic response, signal transduction, the ways in which receptors and ligands bind to one another, and the mechanism of gene expression control (see Chong L et al. A human telomeric protein. Science 1995 Dec 8; 270 (5242): 1663-7, for example).

[0009] However, in order to determine the dissociation constant between a DNA binding protein and a number of double-stranded DNAs, it is necessary to fabricate a special sensor chip for each double-stranded DNA to be measured. Normally, it takes about 4 to 6 hours to fabricate a sensor chip, including the time for preprocessing. Thus, it has been virtually impossible to calculate the dissociation constant in a number of double-stranded DNA samples in a short time.

[0010] WO 02/18648 discloses a method of analysis of binding interactions, wherein a protein is bound to a nucleic acid chip. Fluorescence values are normalized versus the highest fluorescence intensity on a spot for the particular measurement. Selective washing steps to increase the specificity of binding are not disclosed.
US 6,355,428 discloses a method for determining the relative binding affinity between DNA immobilized on a chip and DNA-binding proteins, wherein the second ionic polymer is labeled. Again, selective washing steps for increasing the specificity are not disclosed.

[0011] Conversely, the method using an ionic-polymer identifying chip makes it possible to determine the presence or absence of binding between double-stranded DNA and DNA-binding proteins or the relative association strengths for a large number of proteins simultaneously. This method, however, is not capable of calculat-

ing the association and dissociation constants, which are absolute values.

[0012] Thus, there is a need to develop a technique capable of calculating the association and dissociation constants of many kinds of ionic polymers with double-stranded DNA in a simple and quick manner.

SUMMARY OF THE INVENTION

[0013] The invention was made upon the realization that the calculation of rough values of association and dissociation constants of many kinds of double-stranded DNA samples, which used to take a long time, can be made in a short time by using a specific protein identifying chip. The aforementioned need is solved by a method according to claim 1.

[0014] There is disclosed a method of calculating an association constant and a dissociation constant comprising this method not forming part of the invention :

a first step of preparing a polymer chip for identifying an ionic polymer, the polymer chip comprising a substrate on which a first ionic polymer is immobilized, wherein a second ionic polymer is complementarily bound to thefirst ionic polymer to form a probe;
a second step of causing a third ionic polymer as a sample to specifically bind to the probe, and measuring the relative association strength between the probe comprising the first and second ionic polymers and the third ionic polymer;
a third step of calculating an accurate association constant and dissociation constant between the probe comprising the first and second ionic polymers and the third ionic polymer, using a method of measuring the association strength between ionic polymers; and
a fourth step of calculating the association and dissociation constants between the probe in which the first and second ionic polymers are complementarily bound and the third ionic polymer, using a correlation between the relative association strength between ionic polymers obtained in the second step and the accurate association and dissociation constants between ionic polymers obtained in the third step.

[0015] There is also disclosed a method of calculating an association constant and a dissociation constant comprising, this method not forming part of the invention:

a first step of preparing a polymer chip for identifying ionic polymers, the chip comprising a substrate on which a first ionic polymer is immobilized, wherein a second ionic polymer that is labeled is caused to complementarily bind to the first ionic polymer in an ionic solvent to form a probe which is then dried;
a second step wherein a third ionic polymer as a sample is caused to be adsorbed on the probe, the chip is then washed with an ionic solvent to remove the third ionic polymer that is nonspecifically bound, the chip is further washed with a non-ionic solvent to remove the second, labeled ionic polymer except where the third ionic polymer is specifically bound, and the remaining amount of the label is read;
a third step of calculating an accurate association and dissociation constant between the probe formed by the first and second ionic polymers and the third ionic polymer using a method of measuring the association strength between ionic polymers, and
a fourth step of preparing an analytical curve based on a correlation between the relative association strength between ionic polymers obtained in the second step and the accurate association and dissociation constants between ionic polymers obtained in the third step, in order to calculate the association contestant and dissociation constant between the probe in which the first and second ionic polymers are complementarily bound and the third ionic polymer.

[0016] Preferably, the first to third ionic polymers may be selected from the group consisting of proteins, polyamino acids, DNA, RNA, and synthetic polymers.

[0017] In one aspect, the invention provides a method of calculating association and dissociation constants between a probe and a protein, comprising:

a first step of preparing a DNA chip for identifying proteins, the DNA chip comprising a substrate on which a first DNA is immobilized, wherein a second DNA which is labeled is complementarily bound to the first DNA in an ionic solvent to form a probe having a double-stranded DNA, which probe is then dried;
a second step where a protein as a sample is caused to adsorb on the probe, the DNA chip is then washed with an ionic solvent to remove proteins that are nonspecifically bound, and the DNA chip is then washed with a non-ionic solvent to remove the second, labeled DNA except where proteins are specifically bound, and the amount of remaining label is read;
a third step of calculating accurate association and dissociation constants between the probe having a double-stranded DNA consisting of the first and second DNA and the protein, using a method of measuring the association strenght between a double-stranded DNA and a protein, such as surface plasmon resonance methods and gel shift assays
a fourth step of preparing an analytical curve based on a correlation between the relative association strength between the double-stranded DNA and the protein obtained in the second step and the accurate association and dissociation constants between the double-stranded DNA and the protein obtained in the third step, in order to calculate the association and dissociation constants between the probe having the double-stranded DNA in which the first and

second DNA are complementarily bounded and the protein.

[0018] Examples of the method of measuring the association strength between a double-stranded DNA and a protein include a surface plasmon resonance method and a gel shift assay method. Particularly, the surface plasmon resonance method is superior in accuracy and operability, and is suitable for the method of calculating the association and dissociation constants according to the invention.

[0019] The invention also provides a method of searching for the functionality of proteins using the method of calculating association and dissociation constants as described above, wherein the binding property between the probe in which the first and second DNA are complementarily bound and the protein is evaluated.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020]

Fig. 1 schematically shows a protein-identifying DNA chip as an example of the polymer chip for identifying ionic polymers according to the invention.

Fig. 2 shows a chart illustrating the method of binding a protein using the protein-identifying DNA chip of Fig. 1.

Fig. 3 shows the principle of measurement using a surface plasmon measuring apparatus.

Fig. 4 shows an example of the biochip.

Fig. 5 shows an image after DNA hybridization.

Fig. 6 is an image after addition of TRF protein and washing with pure water.

Fig. 7 shows a table showing the results of standardization of a quantized image that has been read, and the dissociation constants measured by the surface plasmon measuring apparatus.

Fig. 8 is a graph (analytical curve) indicating a correlation between the dissociation constants measured by the surface plasmon measuring apparatus and the ratios of fluorescent intensities on the biochip.

DESCRIPTION OF THE INVENTION

[0021] The material and shape of the substrate used in the present invention are not particularly limited, and the materials and shapes typically used for the substrates for biochips can be used. Preferably, the material is selected from the group consisting of glass, silica gel, polystyrene, polypropylene, and membrane. The shape is preferably either plate-like or bead-like.

[0022] The marker to be provided to the second DNA is selected from those typically used in the field of biochips. Particularly, a fluorescent substance is preferable from the viewpoint of optical processes in a read step.

[0023] In cases where the probe on which the labeled second DNA is complementarily bound to the first DNA and the protein as a sample have the same charge, a fourth, sample polymer with an opposite ionic property should preferably be added so that they can be firmly immobilized, followed by a step of washing them with a nonionic solvent.

[0024] For the first and second DNA , the number of base sequences in the DNA should preferably be between 4 to 13 for each polymer. When the number of base sequences is in this range, the complementary binding of the DNA comprising the first and second DNA is made stronger, and further the specific binding between the DNA chip comprising these polymers and the DNA-binding proteins as samples based on their chemical and physical structures is made stronger. As a result, the search for and identification of the functionality of the sample proteins can be made more easily and accurately.

[0025] The nonionic solvent used in the invention is not particularly limited. For example, it is a mixture of one or more kinds of substances selected from the group consisting of pure water, alcohol, acetone, and hexane. The ionic solvent is not particularly limited either. It may be an aqueous solution of sodium chloride or potassium chloride.

[0026] Biopolymers such as DNA and proteins (polyamino acids and polypeptides) have a charge, which is negative in the case of DNA due to the phosphate group and either positive or negative in the case of proteins depending on the kind of amino acids. The double strand of DNA normally has a negative ion and is therefore repulsive, so that it does not hybridize in pure water. However, it can hybridize by hydrogen bonding in a system where a positive ion exists, as an Na^+ ion chelates with the phosphor group of DNA, for example. Proteins with a positive charge bind with DNA having a negative charge at the charge portion.

[0027] By washing the biochip with a solvent with varied ionic strengths in which the biopolymers are bonded via their charges, the polymers with the same charge are dissociated when the ionic strength is low, whereas the polymers with opposite charges strongly bind to one another. When the ionic strength is high, even polymers with the same charge can bind to one another, while polymers with different charges lose their association strength and are dissociated.

[0028] By thus changing the ionic strength, it becomes possible to identify various polymers, and to search the functionality of those polymers.

[0029] The DNA-binding proteins as used herein refer to proteins that have affinity for DNA and that specifically or nonspecifically bind to base sequences. Examples of such proteins include, mainly, 1) double-stranded DNA-binding proteins that control gene expression by causing changes in DNA structure, 2) single-stranded DNA-binding proteins that are indispensable for the process of duplication, recombination, or repair of DNA, 3) proteins involved with the maintenance of higher-order

structures of chromosomes, 4) ATP-dependent Dnase, and 5) topoisomerases forming the DNA superhelix structure. Many of 1) are transcription factors, for which several structural motifs are known, such as helix-turn-helix that was found in the structures of lambda phage Cro protein and cAMP receptor proteins, zinc finger in which cysteine and histidine chelate a zinc ion, and leucine zipper in which two molecules of proteins comprising an alpha helix with leucine disposed on one side which are combined like a zipper. The proteins 2) are called SSB (single-stranded DNA-binding proteins), and they are found in various life forms ranging from bacteriophage to higher forms of life. The proteins 3) are represented by the histone proteins that exist in the chromosomes of eukaryotes, and they form a nucleosome structure. It is known that similar HU proteins bind to form a nucleosome-like structure in bacterium, too. The proteins 4) include helicase that promote DNA replication (DnaB proteins, for example), recombination (RecA and RecBC proteins), and the unwinding of double-stranded DNA. Further, in the present invention, not only the above-mentioned proteins that inherently have DNA binding property but also proteins to which a DNA binding property has been given by physical or chemical treatments may be used.

[0030] Thus, in the present invention, the above-mentioned various DNA-binding proteins can be used. The invention includes cases using not only one kind of DNA-binding proteins but two or more kinds of DNA-binding proteins.

[0031] DNA-binding proteins characteristically have a positive charge at the DNA binding domain and bind to DNA or RNA with negative charges either specifically or nonspecifically. The double-stranded DNA to which these DNA-binding proteins have bound has increasing association strength with decreasing ionic strength.

[0032] Fig. 1 shows a protein identifying DNA chip as an example of the polymer chip for identifying ionic polymers according to the invention. A first DNA 2 having a base sequence GCTA is immobilized on a substrate 1. As a second DNA 3 labeled with a fluorescent substance and having a base sequence CGAT is added to an ionic solvent in the presence of the first DNA 2, they complimentarily bind to each other and form a double strand. Similarly, as a second DNA 6 labeled with the same fluorescent substance and having a base sequence GGTT is added to another DNA 5 having a base sequence CCAA, they complimentarily bind to each other and form a double strand. As a protein 4 that has not been identified is added to the DNA chip, the protein 4 binds to the double strand with the complementary binding of DNA 2 and DNA 3. However, the protein 4 does not bind to the double strand with the complementary biding of DNA 5 and DNA 6. Such phenomena can be measured with a fluorescence intensity meter and then analyzed in order to identify the protein.

[0033] Fig. 2 shows a chart illustrating the process of identifying proteins using the protein identifying DNA chip shown in Fig. 1. First, a DNA chip is prepared, which comprises a substrate 1 on which DNA 2 and DNA 5 are immobilized (a). Then, DNA 3 and DNA 6 that are labeled with a fluorescent substance are caused to complimentarily bind on the DNA chip in the presence of an ionic solvent (b). After removing the ionic solvent, unidentified proteins 4 are added. The proteins 4 specifically bind to the double strands of DNAs 2 and 3 as they should, and they also nonspecifically bind to the double strands of DNAs 5 and 6 although they should not (c). Thus, the DNA chip is washed with an ionic solvent to remove the nonspecifically bound proteins (d). The DNA chip is further washed with a nonionic solvent to remove DNA 6 that is labeled with fluorescent substance (e). The remaining fluorescence is then read, and the protein is identified based on the result of reading (f).

[0034] Fig. 3 shows the principle of measurement carried out by a surface plasmon resonance measuring apparatus available from Biacore. A gold thin film is formed at the bottom of a prism to a thickness of 50 nm, and the interface is irradiated with polarized light of wavelength 760 nm. This produces an energy wave called an evanescent wave on the thin film. Because this evanescent wave is utilized for the resonance of a free electron wave or a plasmon wave on the gold thin film, a loss of energy is observed at a certain angle of the reflected light. When the intensity of the reflected light is measured with a photodiode array, an optical valley is recognized, as indicated by I. This optical phenomena is the surface plasmon resonance, in which the loss angle varies depending on changes in the concentration of the solvent on the gold film surface.

[0035] For example, in the case where a solution containing a DNA binding protein is added after the double-stranded DNA chip has been immobilized on the thin film, the mass of the surface of the double-stranded DNA chip acting as a sensor increases due to the specific binding reaction on the surface. As a result, the angle at which the optical valley occurs shifts from I to II. This shift is displayed after converting it into a chronological binding curve called a "sensor gram."

[0036] Hereafter, a specific method for calculating the association constant and dissociation constant of proteins will be described. First, a synthesized oligo DNA is spotted on a substrate, and a complementary oligo DNA with a labeled terminal is hybridized, thus manufacturing a double-stranded DNA chip. A substance of which the association constant is to be measured is added onto the double-stranded DNA chip as a sample. When the DNA chip is washed with pure water, the complementary DNA is dissociated if the sample has not bound to the double-stranded DNA. Finally, by reading the fluorescence on the chip, it can be determined to what degree the sample to be measured has bound to the double-stranded DNA. On the other hand, the values of dissociation constants KD for three or more kinds of samples on the chip are determined using surface plasmon measuring equipment, and an analytical curve is made. As a corre-

lation can be observed between the fluorescent intensity ratio on the chip and the sample dissociation constant KD values with respect to binding to the double-stranded DNA, the dissociation constant KD values can be determined from the fluorescence intensity ratio using the analytical curve.

Example

[0037] In the following example, the protein of TRF1 that has already been identified was used as a sample. TRF1 is introduced in the above-mentioned Non-Patent Document 1 (Chong L et al. A human telomeric protein. Science 1995 Dec 8; 270(5242): 1663-7).

[0038] A biochip was fabricated as follows.

[0039] Four kinds of probe DNA to be immobilized on a chip were provided, and their respective 5' terminals were biotinylated. They were then spotted on six locations of an avidin-coated slide glass for each kind of probe using SPBIO (available from Hitachi Software Engineering Co., Ltd.). In the present example, four kinds of probe DNA were used, so that 24 spots were made. The sequences of the chip-immobilized probe DNA were 5'-GTTAGGGTTAGGG-3, 5'-GTTAAGGTCAGGG-3, 5'-GTTAAGGTTAGGG-3, and 5'-GTTAGGGCTAGGG-3. In the following, they will be referred to as an AGTT probe, an AATC probe, an AATT probe, and an AGCT probe. The concentration of the spotted probe DNA was adjusted to 1 mM with pure water. An example of the thus spotted biochip is shown in Fig. 4.

[0040] A DNA sample with a sequence 3'-CAAYYCCRRTCCC-5' (Y=C or T, R=A or G) was synthesized as the DNA sample for hybridization that contains the complementary strands for all of the four kinds. The 5'-terminal was labeled with a fluorescent pigment Cy5.

[0041] Then, a 5xSSC solution was prepared containing the sample DNA to a concentration of 100 nM, and 10 μl of the solution was added onto the prepared biochip to effect hybridization for 30 minutes in a humid environment so as to avoid drying.

[0042] After hybridization, the chip was washed with 1xSSC, dried, and then read using a fluorescent scanner CRBIO2 (available from Hitachi Software Engineering Co., Ltd.).

[0043] An example of an image that has been read is shown in Fig. 5. In Fig. 5, darkened spots indicate the locations where the Cy5-labeled sample exists. Thus, the formation of a double-stranded DNA can be confirmed.

[0044] Thereafter, a TRF1 protein sample solution prepared with 5 mM of KPB (phosphoric acid) and 30 mM of NaCl to a TRF1 protein concentration of 110 μM was added onto the hybridized biochip, and hybridization was effected for 10 minutes.

[0045] Then, the chip was washed with 0.2xSSC at 40 °C for 3 minutes to remove the nonspecific adsorption of proteins. The chip was further washed with pure water at 40 °C to facilitate the dissociation of the Cy5-labeled sample DNA. Finally, the chip was read using CRBIO2. Fig. 6 shows an image that has been read after processing in Fig. 5.

[0046] The double-stranded DNA hybridized with TRF1 does not dissociate even in pure water. Thus, it can be concluded that the difference in fluorescent intensity among the four kinds of probes shown in Fig. 6 corresponds to the difference in association strength between the TRF1 protein and the double-stranded DNA chip.

[0047] The fluorescent intensities at the spot positions in the image of Fig. 6 were quantified and a mean of six spots was obtained (T). The fluorescent intensities at the spots positions in the image of Fig. 5 were quantified and a mean of six spots was obtained (H). Thus, a standardized fluorescent intensity ratio (T/H) can be obtained. Fig. 7 shows a table of the fluorescent intensity ratios for the individual probe sequences.

[0048] An accurate dissociation constant between each of the four kinds of double-stranded DNA and TRF 1 was examined using a surface plasmon resonance measuring apparatus Biacore (R) X available from Biacore. The details of the method is as follows.

[0049] Two hundred μl of each of the four kinds of chip-immobilized probes (with biotinylated 5'-terminals) was prepared to a concentration of 30 μM. On the other hand, 20 μl of a hybridized DNA sample that is not Cy5-labled was prepared to a concentration of 30 μM. The hybridization DNA sample was then mixed with each of the chip-immobilized probes and then denatured at 95°C for 10 minutes. The temperature was then slowly returned to room temperature, thereby preparing four kinds of double-stranded DNA probes.

[0050] The above-described double-stranded DNA probes were then immobilized on an avidin-coated chip using the surface plasmon resonance measuring apparatus. The avidin coat was Sensor Chip SA available from Biacore. The immobilization buffer employed 10 mM HEPES pH7.4 + 3M EDTA + 150 mM NaCl. The flow volume during immobilization was 5 μl/min.

[0051] The TRF1 protein was caused to flow on the immobilized chip in five different concentrations of 10 nM, 25 nM, 50 nM, 75 nM, and 100 nM. The TRF 1 binding amount was monitored using the surface plasmon measuring apparatus to prepare an association curve and a dissociation curve, based on which the dissociation constant (KD value) was calculated. The coupling buffer employed 10 mM HEFES pH 7.4 + 3M EDTA + 50 mM NaCl, and the flow volume was 20 μl/min. The accurate dissociation constants of the four kinds of probe are shown in the table of Fig. 7.

[0052] A correlation can be observed between the accurate dissociation constants (KD) thus measured and the fluorescent intensity ratios (T/H) obtained from the chip. Accordingly, an analytical curve was prepared by measuring the dissociation constants of at least three kinds of double-stranded DNA. Fig. 8 shows an analytical curve prepared based on the probes AGTT, AATC, and

AATT. In Fig. 8, the vertical axis indicates the dissociation constant (KD value), while the horizontal axis indicates the fluorescent intensity ratio (T/H).

[0053] If the dissociation constants of other kinds of double-stranded DNA are to be determined, the fluorescent intensity can read from the relevant spot on the biochip and compared with the analytical curve in order to calculate a rough dissociation constant. For example, the fluorescent intensity of the AGCT probe is 0.437, as shown in Fig. 7, so that it can be expected that the dissociation constant lies roughly at the latter half of 10^{-8}, based on the analytical curve of Fig. 8. When measured using the surface plasmon measuring apparatus, the accurate dissociation constant (KD value) was 8.17×10^{-8}, as shown in Fig. 7. Thus, it can be seen that estimates can be made using the analytical curve shown in Fig. 8.

[0054] Thus, in accordance with the invention, in a mutation on a double-stranded DNA to which an ionic polymer such as protein binds, the association constant (KA value) and the dissociation constant (KD value), which are indicators of the structure and function of the mutation, can be comprehensively and effectively estimated by a simple method. Particularly, the invention makes it possible to calculate rough association and dissociation constants in a number of mutants in which single base substitution such as SNP has occurred in an easy and quick manner. Thus, the invention can provide tools for drug development and/or medical examinations.

Claims

1. A method of calculating association and dissociation constants between a probe and a protein, comprising:

    a first step of preparing a DNA chip for identifying proteins, the DNA chip comprising a substrate on which a first DNA is immobilized, wherein a second DNA which is labeled is complementarily bound to the first DNA in an ionic solvent to form a probe having a double-stranded DNA, which probe is then dried;
    a second step where a protein as a sample is caused to adsorb on the probe, a third step of calculating accurate association and dissociation constants between the probe having a double-stranded DNA consisting of the first and second DNA and the protein, using a method of measuring the association strength between a double-stranded DNA and a protein, such as surface plasmon resonance methods and gel shift assays, and
    a fourth step of preparing an analytical curve based on a correlation between the relative association strength between the double-stranded DNA and the protein obtained in the second step and the accurate association and dissociation constants between the double-stranded DNA and the protein obtained in the third step, in order to calculate the association and dissociation constants between the probe having the double-stranded DNA in which the first and second DNA are complementarily bound and the protein,

    characterized in that,
    in the second step, after a protein as a sample is caused to adsorb on the probe, the DNA chip is then washed with an ionic solvent to remove proteins that are nonspecifically bound, and the DNA chip is then washed with a non-ionic solvent to remove the second labeled DNA except where proteins are specifically bound, and the amount of remaining label is read.

2. The method of calculating association and dissociation constants according to claim 1, wherein the method of measuring the association strength between a double-stranded DNA and a protein is a surface plasmon method or a gel shift assay method.

3. A method of searching for the functionality of proteins using the method of calculating association and dissociation constants according to any one of claims I to 2, wherein the binding property between the probe in which the first and second DNA are complementarily bound and the protein is evaluated.

4. A method of identifying proteins using the method of calculating association and dissociation constants according to any one of claims 1 to 2,wherein the binding property between the probe in which the first and second DNA are complementarily bound and the protein is evaluated.

Patentansprüche

1. Verfahren zum Berechnen von Assoziations- und Dissoziationskonstanten zwischen einer Sonde und einem Protein, umfassend:

    einen ersten Schritt der Herstellung eines DNA-Chip zum Identifizieren von Proteinen, wobei der DNA-Chip ein Substrat umfaßt, auf dem eine erste DNA immobilisiert ist, wobei eine zweite DNA, die markiert ist, an die erste DNA in einem ionischen Lösungsmittel komplementär gebunden ist, um eine Sonde mit einer doppelsträngigen DNA zu bilden, wobei die Sonde dann getrocknet wird;
    einen zweiten Schritt, bei dem ein Protein als eine Probe dazu gebracht wird, auf der Sonde zu adsorbieren,
    einen dritten Schritt der Berechnung von genau-

en Assoziations- und Dissoziationskonstanten zwischen der Sonde mit einer doppelsträngigen DNA, bestehend aus der ersten und zweiten DNA, und dem Protein, unter Verwendung eines Verfahrens zum Messen der Assoziationsstärke zwischen einer doppelsträngigen DNA und einem Protein, wie etwa Oberflächenplasmonresonanzverfahren und Gel-Shift-Assays, und
einen vierten Schritt der Herstellung einer analytischen Kurve, basierend auf einer Korrelation zwischen der relativen Assoziationsstärke zwischen der doppelsträngigen DNA und dem Protein, erhalten in dem zweiten Schritt, und den genauen Assoziations- und Dissoziationskonstanten zwischen der doppelsträngigen DNA und dem Protein, erhalten in dem dritten Schritt, um die Assoziations- und Dissoziationskonstanten zwischen der Sonde mit der doppelsträngigen DNA, in der die erste und zweite DNA komplementär gebunden sind, und dem Protein zu berechnen,

dadurch gekennzeichnet,
daß in dem zweiten Schritt, nachdem ein Protein als eine Probe dazu gebracht wird, auf der Sonde zu adsorbieren, der DNA-Chip dann mit einem ionischen Lösungsmittel gewaschen wird, um Proteine zu entfemen, die nicht-spezifisch gebunden sind, und daß der DNA-Chip dann mit einem nicht-ionischen Lösungsmittel gewaschen wird, um die zweite markierte DNA zu entfemen, außer da, wo Proteine spezifisch gebunden sind, und daß die Menge an verbleibender Markierung abgelesen wird.

2. Verfahren zum Berechnen von Assoziations- und Dissoziationskonstanten nach Anspruch 1, wobei das Verfahren zum Messen der Assoziationsstärke zwischen einer doppelsträngigen DNA und einem Protein ein Oberflächenplasmonverfahren oder ein Gel-Shift-Assay-Verfahren ist.

3. Verfahren zum Untersuchen der Funktionalität von Proteinen unter Verwendung des Verfahrens zum Berechnen von Assoziations- und Dissoziationskonstanten nach einem der Ansprüche 1-2, wobei die Bindungseigenschaft zwischen der Sonde, in der die erste und zweite DNA komplementär gebunden sind, und dem Protein bewertet wird.

4. Verfahren zum Identifizieren von Proteinen unter Verwendung des Verfahrens zum Berechnen von Assoziations- und Dissoziationskonstanten nach einem der Ansprüche 1-2, wobei die Bindungseigenschaft zwischen der Sonde, in der die erste und zweite DNA komplementär gebunden sind, und dem Protein bewertet wird.

Revendications

1. Procédé de calcul de constantes d'association et de dissociation entre une sonde et une protéine comprenant :

une première étape de préparation d'une puce ADN pour identifier des protéines, la puce ADN comprenant un substrat sur lequel un premier ADN est immobilisé, un second ADN étiqueté étant lié de façon complémentaire au premier ADN dans un solvant ionique pour former une sonde ayant un ADN à double hélice, cette sonde étant ensuite séchée;
une deuxième étape dans laquelle une protéine en tant qu'échantillon est amenée à s'adsorber sur la sonde ;
une troisième étape de calcul de constantes précises d'association et de dissociation entre la sonde ayant un ADN à double hélice consistant en les premier et second ADN et la protéine, en utilisant un procédé de mesure de la force d'association entre un ADN en double hélice et une protéine, tel que des procédés de résonance plasmon en surface et des essais de décalage de gel ; et
une quatrième étape de préparation d'une courbe analytique basée sur une corrélation entre la force d'association relative entre l'ADN en double hélice et la protéine obtenue dans la seconde étape et les constantes précises d'association et de dissociation entre l'ADN en double hélice et la protéine obtenue lors de la troisième étape, pour calculer les constantes d'association et de dissociation entre la sonde ayant l'ADN en double hélice dans laquelle les premier et second ADN sont liés de façon complémentaire et la protéine ;

caractérisé en ce que, lors de la deuxième étape, après qu'une protéine échantillon soit amenée à s'adsorber sur la sonde, la puce ADN est lavée par un solvant ionique pour enlever les protéines qui ne sont pas spécifiquement liées, et la puce ADN est ensuite lavée par un solvant non ionique pour enlever le second ADN étiqueté sauf là où les protéines sont spécifiquement liées, et la quantité d'étiquette restante est lue.

2. Procédé de calcul de constantes d'association et de dissociation selon la revendication 1, dans lequel le procédé de mesure de la force d'association entre l'ADN en double hélice et une protéine est un procédé de plasmon de surface ou un procédé d'essai de décalage de gel.

3. Procédé de recherche de la fonctionnalité de protéines en utilisant le procédé de calcul de constantes

d'association et de dissociation selon l'une des revendications 1 ou 2, dans lequel la propriété de liaison entre la sonde dans laquelle les premier et second ADN sont liés de façon complémentaire, et la protéine est évaluée.

4. Procédé d'identification de protéines en utilisant le procédé de calcul de constantes d'association et de dissociation selon l'une des revendications 1 ou 2, dans lequel la propriété de liaison entre la sonde, dans laquelle les premier et second ADN sont liés de façon complémentaire, et la protéine est évaluée.

FIG.1

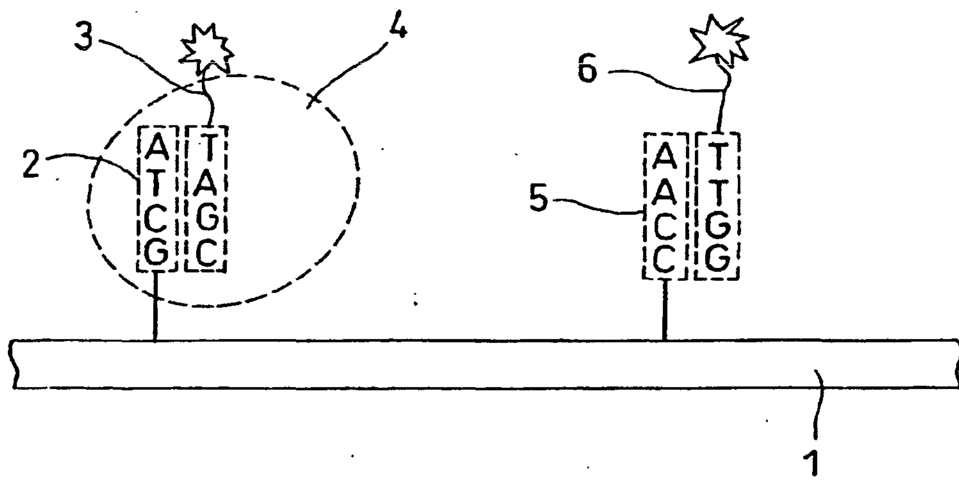
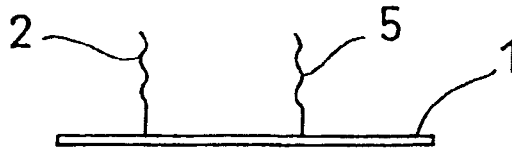
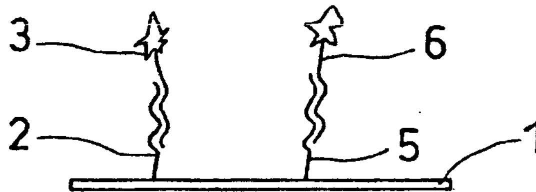


FIG2a



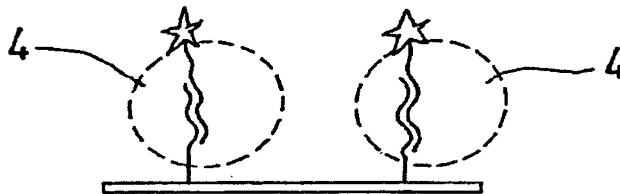
IONIC SOLVENT

FIG2b



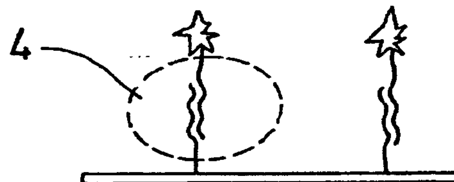
ADD PROTEIN

FIG2c



WASH WITH IONIC SOLVENT

FIG2d



WASH WITH NON-IONIC SOLVENT

FIG2e

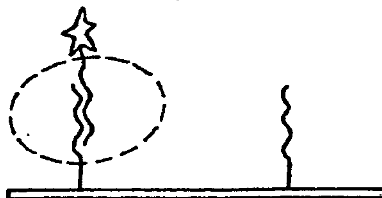


FIG2f

READ FLUORESCENCE

FIG3

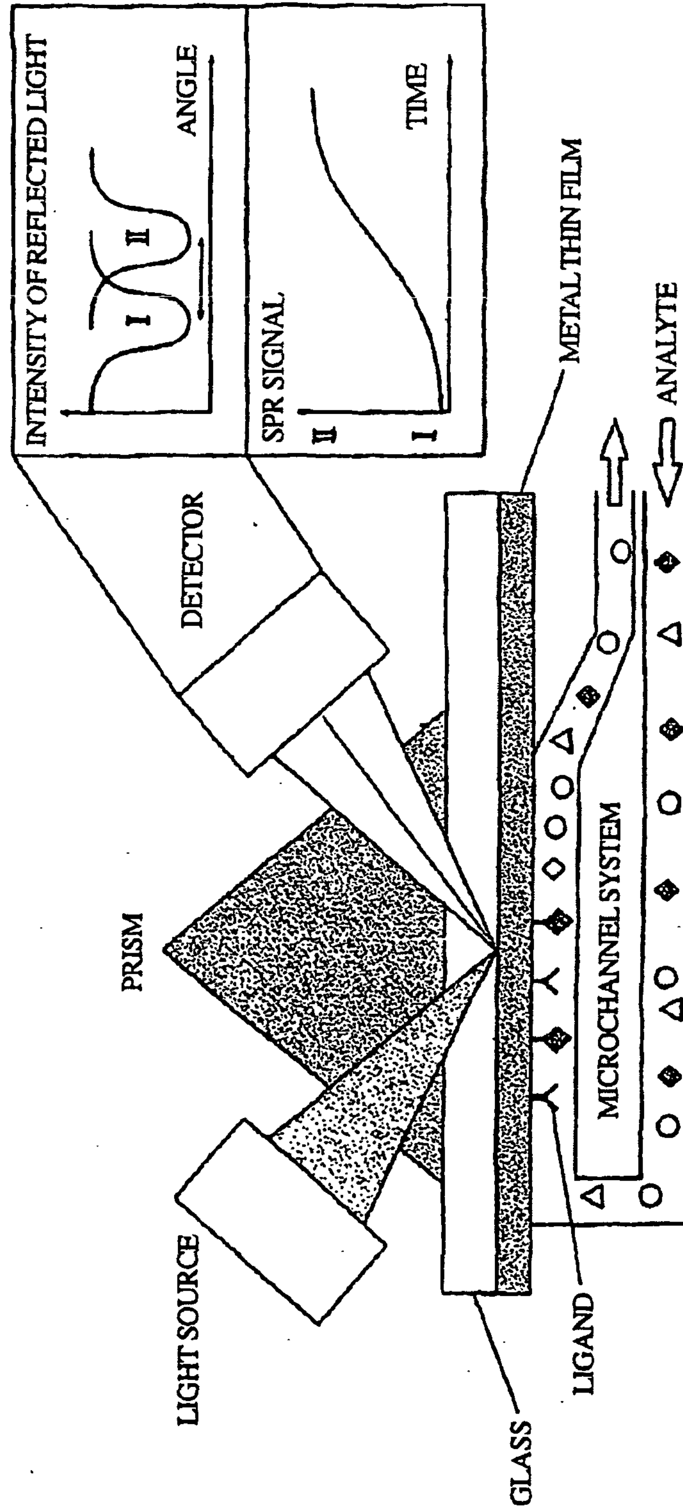


FIG4

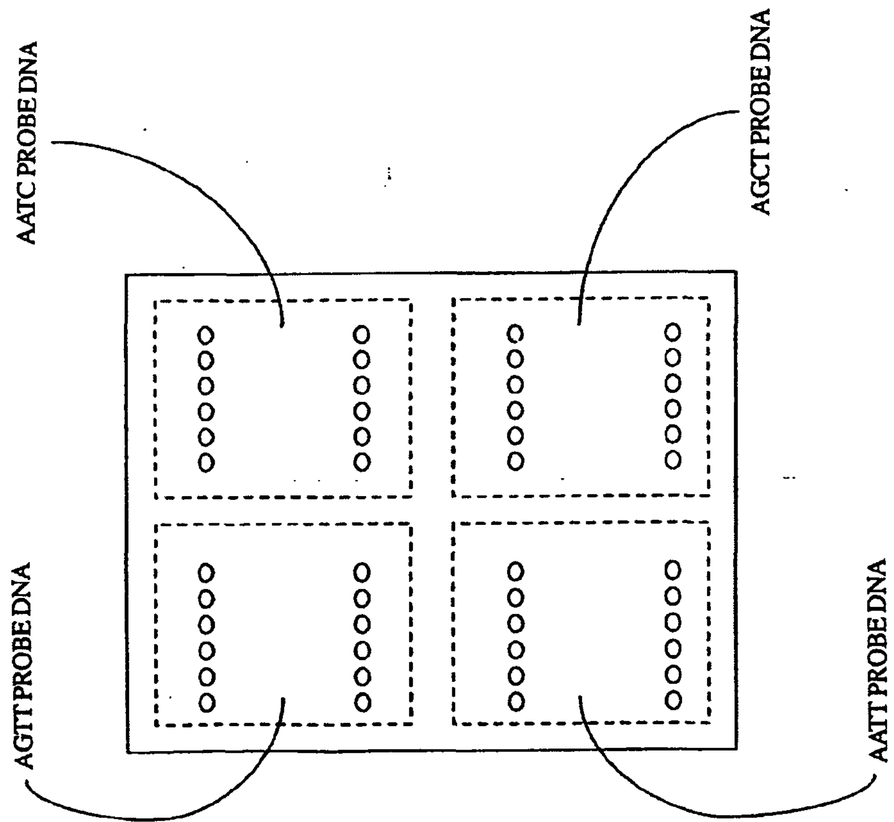


FIG.5

BEFORE WASHING WITH PURE WATER

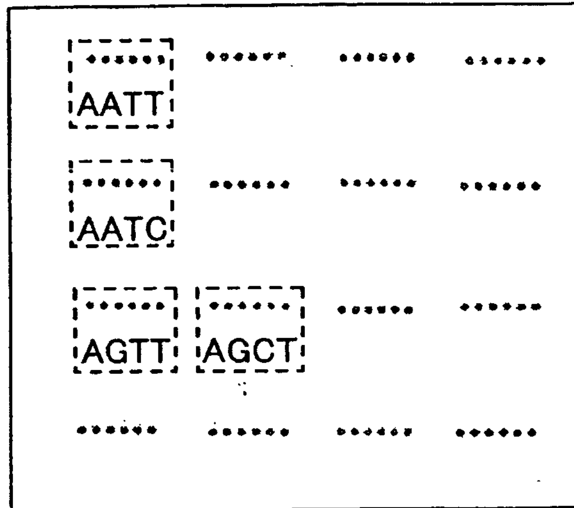


FIG.6

AFTER WASHING WITH PURE WATER

1 mM TRF2 PROTEIN ADDED
x0.2 SSC WASHING
WASHING WITH PURE WATER

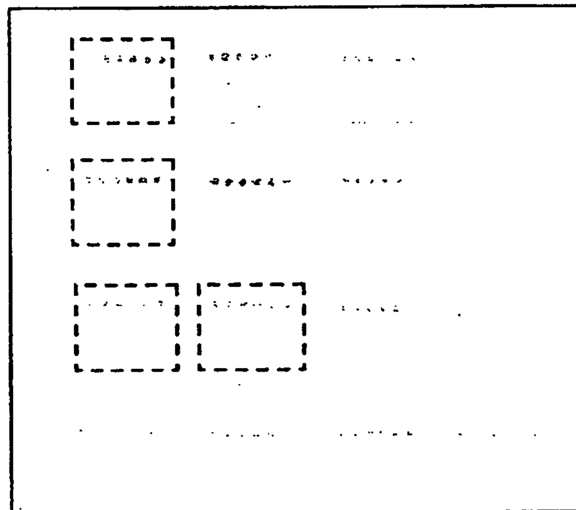


FIG7

PROBE	CHIP (T/H RATIO)	BIACORE (K_D VALUE)
AGTT	0.451	7.77×10^{-8}
AATC	0.369	4.81×10^{-7}
AATT	0.232	2.50×10^{-6}
AGCT	0.437	8.17×10^{-8}

FIG8