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(54) Title: METHOD FOR DETECTING BINDING AND CHEMICAL REACTIONS IN AN AQUEOUS SAMPLE

(57) Abstract: The present invention relates to a method to detect binding and reactions of substances in an aqueous sample, wherein the binding or reaction changes the spectroscopic properties of water, and the binding or reaction is detected by observing or measuring any change in the spectroscopic properties of water.

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Method for detecting binding and chemical reactions in an aqueous sample

DESCRIPTION

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Field of the Invention

The present invention generally relates to a method to detect binding between substances or chemical reactions in an aqueous sample, by observing or measuring a change in the spectroscopic properties of the water. It is based on the fact that water's vibrational properties, like vibrational frequencies, absorption and scattering cross sections, are different when it is bound to molecules or ions than when surrounded by other water molecules, i.e. as in "bulk water". Thus, monitoring changes in water's spectroscopic properties enables us to detect macromolecular interactions, conformational changes, binding, chemical reactions etc. Examples of vibrational spectroscopy include infrared spectroscopy in the near-infrared, mid-infrared and far-infrared spectral range, terahertz spectroscopy, Raman spectroscopy, photoacoustic spectroscopy, vibrational circular dichroism, and non-linear techniques like sum frequency generation spectroscopy.

25 Background of the Invention

A great challenge in the pharmaceutical field, as well as the fields of organic chemistry, physical chemistry, analytical chemistry, biotechnology, the life sciences and surface science, is the monitoring of different interactions between molecules and the reactivity of molecules. A non-exhaustive list includes the binding of a small molecule to a macromolecule, for instance in ligand-receptor interactions, the binding of two macromolecules, the interaction of ions, molecules or macromolecules with membranes, conformational changes in a macromolecule such as an enzyme or other protein, measurement of chemical reactivity, for instance enzymatic activity etc.

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The ability to accurately monitor such processes is of key importance to all Life Sciences.

There is a wide array of quantitative and qualitative analytical methods available within the field of chemistry, that may or may not be suitable for such purposes (see Mendelsohn and Brent Science 18 (1999) 1948-1950 (protein-protein interactions); Zimmermann et al. Targets 1 (2002) 66-73; Haake et al. Fresenius J. Anal. Chem. 366 (2000) 576-585 (surface plasmon resonance); Barth & Zscherp FEBS Lett. 477 (2000) 151-156 (infrared spectroscopy); Deng & Callender Meth. Enz. 308 (1999) 176-201 (Raman spectroscopy); Hovius et al. TIPS 21 (2000) 266-273; Kakehi et al. Anal. Biochem. 297 (2001) 111-116 (fluorescence), Legrain & Selig FEBS Lett. 480 (2000) 32-36 (protein-protein interactions); Hernández & Robinson J. Biol. Chem. 276 (2001) 46685-46688 (mass spectrometry); Meyer & Peters Ang. Chem. Int. Ed. 42 (2003) 864-890 (NMR)); for an overview of some of the most common techniques). However, very few of these methods are generally applicable to different systems without modification or specific assay development, or they may have other drawbacks such as high cost, inconvenient and/or time consuming analysis and/or preparation, unphysiological conditions, destruction of the sample to be analyzed etc. In drug development, this often requires a unique binding assay to be developed for each new drug target, which is costly and time consuming. The lack of universal methods and other drawbacks with existing techniques spur the continued development of new methods for monitoring molecular interactions.

Vibrational spectroscopy

Vibrational spectroscopy, including e.g. infrared spectroscopy (in the near-infrared, mid-infrared and far-infrared spectral range), terahertz spectroscopy, Raman spectroscopy, photoacoustic spectroscopy, vibrational circular dichroism, and non-linear techniques like sum frequency generation spectroscopy, is one of the classical methods for structure de-

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termination of small molecules. This standing is due to its sensitivity to the chemical composition and architecture of molecules. The high information content in a vibrational spectrum carries over also to biological systems. This makes
5 vibrational spectroscopy a valuable tool for the investigation of protein and DNA structure, of the molecular mechanism of protein reactions and of protein folding, unfolding and misfolding. The wealth of information in the vibrational spectrum can be exploited even for complex systems. A striking
10 example is the possibility to identify bacterial strains from the infrared spectrum and to differentiate and classify microorganisms (Naumann in: Infrared and Raman Spectroscopy of Biological Materials, eds. Gremlich & Yan, Marcel Dekker Inc., New York, 2001, 323-377).

15 Further advantages of vibrational spectroscopy are a large application range from small soluble molecules to large macromolecules like DNA or proteins, a high time resolution, short measuring times, the low amount of sample required
20 (typically 10 - 100 µg) and the relatively low costs of instrumentation. The molecules of interest are observed directly. Contrary to fluorescence spectroscopy, no labelling that might be difficult to achieve or disturb the biological system under investigation is required. In addition, the
25 molecules can be studied in aqueous solution under near physiological conditions in contrast to X-ray crystallography which requires time-consuming crystallization, and to surface plasmon resonance which can detect interactions between molecules only when one of them is attached to a solid substrate.

30 A drawback of using vibrational spectroscopy for structural analysis in an aqueous medium is the strong absorption of water, which interferes with the vibrational data for the molecules of interest. In infrared spectroscopy, for example, the
35 absorption near 1645 cm⁻¹ overlaps with the important amide I band of proteins and some amino acid side chain bands. Similar problems are encountered throughout the infrared spectral region.

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The obstacles imposed by the strong water absorption have so far prevented the wide-spread use of infrared spectroscopy to study the binding of substances to macromolecules directly, although specialised techniques have been applied (Barth & Zscherp FEBS Lett. 477 (2000) 151-156). Both, small path-length and high concentrations make the use of standard mixing devices in the mid-infrared range difficult (though not impossible) and the high water absorption decreases the signal to noise ratio in regions of water absorption.

Summary of the Invention

The present invention generally relates to a method to detect binding between substances or chemical reactivity in an aqueous sample, by observing or measuring a change in the spectroscopic properties of water. Most surprisingly, the applicant has discovered that the strong absorption of water, previously considered a drawback of using vibrational spectroscopy, can be used in itself to detect molecular interactions in aqueous media indirectly.

In a first aspect, the present invention therefore relates to a method to detect binding of at least one substance A to at least one other substance B in an aqueous sample, wherein the binding changes the spectroscopic properties of water, characterized in that the binding is detected by observing or measuring any change in the spectroscopic properties of water.

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In a second aspect, the present invention relates to a method to detect a chemical reaction or measure its reaction rate, wherein the reaction changes the spectroscopic properties of water, characterized in that the reaction is detected by observing or measuring any change in the spectroscopic properties of water.

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In one embodiment of the invention, the change in the properties of water is measured using infrared, terahertz, Raman, sum frequency generation, or photoacoustic spectroscopy, or vibrational circular dichroism.

5

In other embodiments of the invention, substances A and B are biomolecules such as proteins, nucleic acids or parts of a biomembrane. More specifically, substances A and B may be therapeutic targets, candidate drugs, herbicide targets or herbicide candidates.

10

In yet other embodiments of the invention, many substances are checked for binding or reaction simultaneously, so that the at least one substance A and/or B is a mixture of substances A1, A2... Ai and/or B1, B2... Bj. Alternatively, or in addition thereto, the method may also comprise a mixing device for mixing substances A and B, and/or high throughput screening, for instance on a microtiter plate.

15

In yet other embodiments of the invention, a catalyst, like an enzyme, catalyzes a reaction from at least one reactant to at least one product.

20

25 **Detailed Description of the Invention**

Water is an essential participant in many molecular interactions, in particular in living organisms. Its vibrational properties depend on the hydrogen bonding pattern. Therefore they are different for water around hydrophobic solutes, around ions and in bulk water (Worley & Klotz 1966, J. Chem. Phys. 2868-71, Sharp et al. 2001, J. Chem. Phys. 114, 1791-6).

30

Hydrophobic, polar and ionic groups on the surface of biomolecules such as proteins affect the structure of close-by water molecules. Freda et al. (J. Phys. Chem. B 105 (2001) 12714) show that the absorption of water is different around

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hydrophobic molecules. In their Fig. 3 they demonstrate this difference directly in a difference spectrum (the absorption of water in the presence of hydrophobic molecules minus the absorption of bulk water) which reveals a downshift of the OH stretching band position (in cm^{-1}) due to the presence of hydrophobic molecules. Fischer et al. (Phys. Chem. Chem. Phys. 3 (2001) 4228) show that anions and cations affect the water spectrum, in their Fig. 2 for the OH stretching region and in their Fig. 3 for the band of the bending vibration of water. These effects can also be observed in the near-infrared spectral region (Worley & Klotz J. Chem. Phys. 45 (1966) 2868 Fig. 3). That the water spectrum of a protein at low hydration level is indeed different from that of bulk water has been demonstrated by (Grdadolnik & Maréchal Biopolymers 62 (2001) 54, Fig. 1). When a dry protein film is hydrated, the water absorption at low hydration level has its maximum at 3500 cm^{-1} (panel A of their Fig. 3) whereas liquid water has its maximum at 3404 cm^{-1} at 25°C (Venyaminov & Prendergast Anal. Biochem. 248 (1997) 234). Even single water molecules within proteins have been detected and characterized (Maeda Biochemistry (Moscow) 66 (2001) 1555; Kandori Biochim. Biophys. Acta 1460 (2000) 177). They show characteristic absorption spectra depending on their interactions with the protein.

As mentioned above, the absorption of water often gives rise to "noise" when studying macromolecules in an aqueous solute with infrared spectroscopy. However, the applicant has most surprisingly discovered that the cause of this "noise", the absorption of water, can be a tell tale sign of molecular interactions and reactions in itself. The underlying idea is to detect these events by a change in water properties like absorption, scattering, vibrational circular dichroism, photoacoustic signal, or by a change in water mediated non-linear interaction of photons.

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For example, macromolecules are surrounded by water with different structure and properties as compared to bulk water (see Cameron et al. 1997, Cell Biol. Int. 21, 99-113; Smith et al. 2002, J. Mol. Liq. 101, 27-33). The water shell
5 gives rise to a particular vibrational signature, since for example vibrational frequencies depend on the degree of hydrogen bonding. This hydrogen bonding is different as compared to bulk water because the macromolecular surface is generally composed of hydrophobic and ionic groups not found
10 in bulk water. Binding of a substance to a macromolecule partially alters this water shell and transfers some of the bound water from the macromolecular surface to bulk water where these water molecules have different vibrational properties. This change in water's vibrational properties can be
15 detected and thus gives evidence for the binding process.

Likewise, the water shells around the reactants of a given chemical reaction and the products of this reaction are different, in particular when the number of charged species
20 changes. Therefore the vibrational properties of the hydration shells of reactants are different from those of the products which can be detected and gives evidence for the chemical reaction and enables measurement of the reaction velocity.

25 Thus, the present invention proposes to detect the binding and/or reactivity of molecules in aqueous media by monitoring bound and/or bulk water using vibrational spectroscopy. The invention therefore generally relates to a method to detect
30 binding or reaction of at least one substance A to at least one other substance B in an aqueous sample, wherein the binding or the reaction changes the spectroscopic properties of water, characterized in that the binding or the reaction is detected by observing or measuring any change in the spectro-
35 scopic properties of water.

As a person skilled in the art will appreciate, the method according to the invention comprises a wide variety of prac-

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tical approaches. The at least one substance A and/or B may be a principally pure substance in aqueous solution, or a mixture of substances. For instance, substance A may be a mixture of *i* binding candidates A1, A2... Ai, suspected of
5 binding to either of *j* binding targets B1, B2... Bj. In such a case, a change in the spectroscopic properties of the water would indicate binding between one or several of substances A and B, and additional trials may be necessary to pinpoint the binding pair(s).

10

The method according to the invention is not dependent on any particular detection means for observing or measuring the change in spectroscopic properties. Any known means in the art is suitable, for instance infrared, Raman, sum frequency
15 generation, photoacoustic, or terahertz spectroscopy, or vibrational circular dichroism.

The inventive method may be practically incorporated into any conceivable device or array of devices, and may for instance
20 comprise a mixing device for mixing substances A and B, a microtiter plate for high throughput scanning of several substances, a thermal light source, a laser, or a light-emitting diode, a multi-array detector etc.

25 According to one embodiment of the invention, the method is performed wherein:

- a) substance B is supplied as an aqueous solution to a mixing device;
 - b) substance A is supplied as a separate aqueous solution to
30 the mixing device;
 - c) the two aqueous solutions are mixed to form a mixed solution; and
 - d) the spectroscopic properties of water in the mixed solution in c) are compared to the properties of water in a)
35 and/or b), to detect any changes in said properties.
- The sequence of a) and b) maybe reversed or may happen at the same time.

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According to another embodiment of the invention, the method is performed wherein:

- a) substance A is supplied as an aqueous solution to a mixing device;
 - 5 b) a catalyst or enzyme is supplied as a separate aqueous solution to the mixing device;
 - c) the two aqueous solutions are mixed to form a mixed solution; and
 - d) the spectroscopic properties of water in the mixed solution in c) is compared to the properties of water in a) and/or b), to detect any changes in said properties.
- 10 The sequence of a) and b) maybe reversed or may happen at the same time.

- 15 Especially the method according to the invention could be performed wherein substance A is the substrate of a catalyst or of an enzyme and substance B its product.

Definitions

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As used herein, the term "bulk water" generally refers to water molecules in an aqueous medium that are substantially surrounded by other water molecules or that are not associated with the substances of interest, such as macromolecules and ligands of which binding is studied or reactants and products of a reaction. It will be appreciated that bulk water can still contain non-water molecules, for example comprising an aqueous buffer or ion solution. Specifically, "bulk water" has spectroscopic properties that are distinct from "bound water", as defined below. Substances, like ions, polar or hydrophobic molecules, may be added to "bulk water" in order to enhance the difference between the spectroscopic properties of "bulk water" and "bound water".

- 35 As used herein, the term "bound water" generally refers to water molecules in the hydration layer(s) around the substances of interest, such as macromolecules and ligands of which binding is studied or reactants and products of a reac-

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tion. Generally, "bound water" is in rapid exchange with "bulk water". It will be appreciated that "bound water molecules" may hydrate a non-water molecule in several layers, and that each bound water molecule need not be associated directly with the hydrated molecule. Specifically, "bound water" has spectroscopic properties that are distinct from those of "bulk water".

As used herein, the term "water" generally refers to water consisting of any oxygen or hydrogen isotope or of a mixture of isotopes.

As used herein, the term "spectroscopic property", such as in the phrase "spectroscopic properties of water", means any property that can be measured using any known spectroscopic technique, for instance absorption, scattering, vibrational circular dichroism, photoacoustic signal, the non-linear interaction of photons etc.

As used herein, the term "vibrational spectroscopy" comprises methods that detect properties of vibrations or properties that are modulated by vibrations, like absorption and scattering cross section. By way of non-limiting examples, vibrational spectroscopy comprises infrared, Raman, terahertz, sum frequency generation and photoacoustic spectroscopy as well as vibrational circular dichroism.

As used herein, the term "near infrared spectral range" refers to the spectral range of 700 to 2500 nanometer; the term "mid infrared spectral range" refers to the spectral range of 2.5 to 50 micrometer and the term "far infrared spectral range" refers to the spectral range of 50 to 100 micrometer.

Use and Practice of the Invention

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The present invention may generally be used to monitor molecular interactions and reactions in aqueous media. Specifically, the invention provides an easy means to determine

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whether one molecule or substance binds to another or not, and whether at least one molecule or substance reacts or not, as such a binding or reaction would register in the vibrational signature of the bound water. Quantitative measurements are also possible, as the degree of change in vibrational signature would correlate to strength of binding or amount of bound molecules, i.e. allowing several binding candidates to be compared, or to amount of reacted molecules for example.

10

As such, the invention has particular application in the fields of pharmaceutical development, or in the development of herbicides. The present invention will be especially useful in the field of proteomics, i.e. in monitoring protein-protein interactions. Nucleic acid interactions, for instance DNA-RNA interactions or DNA-protein interactions, and the measurement of enzymatic activity are also applicable fields for the invention.

20

To further clarify the invention, several non-exhaustive examples of inhibitor binding to a protein are discussed below, merely as an illustration of how the inventive concept may be used in practice. These examples are also applicable to the measurement of chemical reactivity or enzymatic activity. The examples below should not in any way be construed as limiting the scope of the invention as presented in the claims.

25

As explained above, inhibitor binding to a protein may for instance be observed by a change in water absorption. The absorption spectra of water in the presence of protein, in the presence of inhibitor and in the presence of the inhibitor-protein complex can be recorded separately - either one by one in conventional cuvettes or with more sophisticated approaches.

30

(i) Binding can be observed in a time-resolved way with mixing devices if the time for binding is slower than the mixing time.

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(ii) Mixing devices can be used that allow the recording of spectra in the presence of inhibitor and protein separately before binding and of the complex after binding. For example, the device can be constructed such that the flow of inhibitor and the flow of protein combine and mix by diffusion. Water absorption can then be detected at different distances from the point of combining the two flows. Close to that point, inhibitor and protein will still be largely separated, allowing the detection of the combined absorption of inhibitor and protein before complex formation. Distant from the point of combining the flows, diffusion has mixed inhibitor and protein and the complex has formed. A measurement here measures the water absorption in the presence of the complex.

(iii) Alternatively, absorption of the separated inhibitor and the separated protein can be measured in the two channels leading to the mixing chamber, and the absorption of the complex in the channel that leads away from the mixing chamber. A variation of this method is the separation of inhibitor and protein by an optically transparent wall or membrane in the cuvette, which is removed to initiate binding.

(iv) The inhibitor can be added to the protein via a semi-permeable membrane.

(v) Binding can be blocked initially by chemical modification of inhibitor or protein which is then relieved to initiate binding. An example is the use of caged compounds or caged proteins (Dynamic Studies in Biology. Phototriggers, Photoswitches and Caged Biomolecules, eds. Goeldner & Givens, Wiley-VCH, Weinheim, 2005). In this case the blockage is removed by photocleavage.

Alternatively, complex formation can be revealed in a titration without recording the water spectra in the presence of the two separated interaction partners. A reference signal in the presence of one of the interaction partners (A) is measured first. Then the other interaction partner (B) is added. In the first additions of the titration, the water property will change because of complex formation. When all binding sites on A are saturated, further additions change the water

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property because of addition of partner B to the aqueous solution of the AB complex. In this type of experiment, the binding constant can be determined.

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CLAIMS

1. A method to detect binding or reaction of at least one substance A to at least one other substance B in an aqueous sample, wherein the binding or reaction changes the spectroscopic properties of water, **characterized in that** the binding or reaction is detected by observing or measuring any change in the spectroscopic properties of water.
2. The method according to claim 1, wherein the change in the properties of bound water is measured using infrared (in the near-infrared, mid-infrared and far-infrared spectral range), Raman, terahertz, sum frequency generation, or photoacoustic spectroscopy, or vibrational circular dichroism.
3. The method according to claim 1 or 2, wherein substance A and/or B is a biomolecule, such as a protein, nucleic acid or part of a biomembrane.
4. The method according to claim 1 or 2, wherein substance A is a substrate of a catalyst or of an enzyme, for example a therapeutic or herbicide target protein, and substance B its product.
5. A method according to any preceding claim, wherein substance B is a therapeutic target protein, such as a receptor or enzyme, and wherein substance A is a candidate drug, such as a ligand, agonist or antagonist, or a substrate.
6. The method according to claim 1, 2 or 3, wherein substance B is a herbicide target in a plant, and wherein substance A is a herbicide candidate.
7. A method according to any preceding claim, wherein the at least one substance A is a mixture of i substances A₁, A₂... A_i and/or the at least one substance B is a mixture of j substances B₁, B₂... B_j, so that a change in the spectroscopic

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properties of water may be observed if there is any binding or reaction between either of the substances A and B.

8. A method according to any preceding claim, wherein

- 5 a) substance B is supplied as an aqueous solution to a mixing device;
- b) substance A is supplied as a separate aqueous solution to the mixing device;
- 10 c) the two aqueous solutions are mixed to form a mixed solution; and
- d) the spectroscopic properties of water in the mixed solution in c) is compared to the properties of water in a) and/or b), to detect any changes in said properties.

15 9. A method according to claim 4, wherein

- a) substance A is supplied as an aqueous solution to a mixing device;
- b) a catalyst or enzyme is supplied as a separate aqueous solution to the mixing device;
- 20 c) the two aqueous solutions are mixed to form a mixed solution; and
- d) the spectroscopic properties of water in the mixed solution in c) is compared to the properties of water in a) and/or b), to detect any changes in said properties.

25

10. A method according to any preceding claim, wherein any binding or reaction between substances A and B is detected in a high throughput assay, so that several substances or mixtures of substances can be checked rapidly for binding or re-
- 30 action, for instance on a microtiter plate.

11. A method according to any preceding claim, wherein the spectroscopic properties of any water isotope is detected.

- 35 12. A method according to any preceding claim, wherein any of the known light sources are used, such as thermal sources, lasers and diodes.

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13. A method according to any preceding claim, wherein at least one substance is added to the aqueous solution in order to enhance the difference between the spectroscopic properties of "bulk water" and "bound water".

INTERNATIONAL SEARCH REPORT

International application No.

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A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 20060029941 A1 (KOO, T-W ET AL), 9 February 2006 (09.02.2006), the whole document --	1-13
A	LING-SHU WAN ET AL: "Diffusion and Structure of Water in Polymers Containing /V-Vinyl-2-pyrrolidone ", J. Phys. Chem. B February 8th 2007, volume 111, no 5, pages 922-928. See the whole document --	1-13
A	FREDA, M ET AL: "Infrared and dielectric spectroscopy study of the water perturbation induced by two small organic solutes", Journal of Molecular Structure, volumes 565-566 (2001), pages 153-157. See the whole document -- -----	1-13



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See patent family annex.

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Cited literature, if any, will be enclosed in paper form.

INTERNATIONAL SEARCH REPORT
Information on patent family members

26/01/2008

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PCT/SE2008/000221

US	20060029941	A1	09/02/2006	DE	112005001895	T	21/06/2007
				GB	0700616	D	00/00/0000
				GB	2433987	A	11/07/2007
				US	20060216742	A	28/09/2006
				US	20060216743	A	28/09/2006
				WO	2006137824	A	28/12/2006
