Introduction to Fluorescence Sensing

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Springer

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ISBN 978-1-4020-9002-8 e-ISBN 978-1-4020-9003-5

Library of Congress Control Number: 2008936100

Printed on acid-free paper

springer.com

Preface

The field of molecular sensing is immense. It is nearly the whole world of natural and synthetic compounds that have to be analyzed in a variety of conditions and for a variety of purposes. In the human body, we need to detect and quantify virtually all the genes (genomics) and the products of these genes (proteomics). In our surrounding there is a need to analyze a huge number of compounds including millions of newly synthesized products. Among them, we have to select potentially useful compounds (e.g., drugs) and discriminate those that are inefficient and harmful. No less important is to control agricultural production and food processing. There is also a practical necessity to provide control in industrial product technologies, especially in those that produce pollution. Permanent monitoring is needed to maintain the safety of our environment. Protection from harmful microbes, clinical diagnostics and control of patient treatment are the key issues of modern medicine. New problems and challenges may appear with the advancement of human society in the XXI century. We have to be ready to meet them.

Modern society needs the solution of these problems on the highest possible scientific and technological level. The science of intermolecular interactions is traditionally a part of physical chemistry and molecular physics. Now it becomes a strongly requested background for modern sensing technologies. The most specific and efficient sensors are found in the biological world and the sensors based on biomolecular recognition (biosensors) have acquired a strong impulse for development and application. A strong move is observed for improving them by endowing new features or even by making fully synthetic analogs of them. Modern electronics and optics make their own advance in providing the most efficient means for supplying the sensors with the input and output signals and now becomes oriented at satisfying the needs of not only researchers but a broad community of users.

This book is focused on one sensing technology, which is based on fluorescence. This is not only because of limited space or limited expertise of the present author. Indeed, fluorescence techniques are the most sensitive; their sensitivity reaches the absolute limit of single molecules. They offer very high spatial resolution; that with overcoming the light diffraction means the limit has reached molecular scale. They are also the fastest; their response develops on the scale of fluorescence lifetime and can be as short as 10^{-8}–10^{-10} s. However, their greatest advantage is versatility. Fluorescence sensing can be provided in solid, liquid and gaseous media and at all

kinds of interfaces between these phases. It is because the fluorescence reporter and the detecting instrument are connected via light emission that fluorescence detection can be made non-invasive and equally well suited for remote industrial control and for sensing different targets within the living cells. All these features explain their high popularity.

The fascinating field of fluorescence sensing needs new brains. Therefore, this book is primarily addressed to students and young scientists. Together with a basic knowledge they will obtain an overview of different ideas in research and technology and will be guided in their own creative activity. Providing a link between the basic sciences needed to understand sensor performance and the frontiers of research, where new ideas are explored and new products developed, this book will make a strong link between research and education. For the active researcher it will also be a source of useful information in nearly all areas where fluorescence sensing is used.

Thus, this book is organized with the aim to satisfy both curious student and busy researchers. After a short introduction, a comparative analysis of basic principles used in fluorescence sensing will be made. We then provide a formal description of binding equilibrium and binding kinetics that are the background to sensing technologies. The focus will be on techniques of obtaining information from fluorescent reporters and on analysis of their structures and properties. The design of various types of recognition units will be reviewed, including those selected from large libraries. A deeper understanding of the basic mechanism of signal transduction in fluorescence sensing will be our focus, with special attention paid to the new possibilities provided by support structures, scaffolds and integrated units that expand the range of sensor applications. Non-conventional generation and transformation of response signals will also be described. Fluorescence sensing is realized with optical instrumentation, so these devices are overviewed, including microarrays, microfluidics and flow cytometry. Detection of different targets from physical, chemical and biological worlds is discussed, with many examples presented. We will also address the analytical means of detecting different targets inside the living cells based on modern microscopy. Finally, the frontiers of modern research are overviewed with the prospects for fluorescence sensing behind the horizon. Each chapter is terminated by the section “Sensing and thinking”, in which, after a short summary, a series of questions and exercises is suggested for the reader.

Enjoy your reading.

Alexander P. Demchenko

Contents

Introduction

The simplest common definition of a sensor is that "a sensor is something that senses", i.e., receives information and transforms it into a form compatible with our perception, knowledge and understanding. Our body is full of sensors that respond to light, heat, taste, etc. With the development of civilization they became insufficient for the orientation of personality, community or the whole society in new conditions. More and more we need objective knowledge on what is happening inside and outside of our body and what are benefits and threats to the whole society. There is a necessity to know what compounds are useful and what are harmful, how safe and healthful is our environment and to monitor them continuously. Different industrial processes, including that of production of agricultural goods, food processing and storage need to be controlled as well. The human genome is a very useful piece of knowledge only when we can analyze gene expression and find its relation with individuality, age and disease. We need to know the distribution inside living cells of many compounds, including enzymes and their regulators and also substrates and products of these reactions. This information may need to be obtained throughout the whole cell life cycle including its division, differentiation, aging and apoptosis. All that can be accomplished only with the help of man-made sensors. They will be the subject of the present book.

The man-made sensors are often called 'chemical sensors' and those of them that involve biology-related compounds and/or biospecific target binding – 'biosensors'. According to the definition approved by IUPAC, 'a chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal'. Thus the sensor can be regarded as both a designed molecule and a miniaturized analytical device that delivers real-time and online information on the presence of specific compounds in complex samples (Thevenot et al. 1999). In a narrow sense, the sensor is a molecule or assembled supra-molecular unit (or nanoparticle), which is able to selectively bind the target molecule (or supra-molecular structure, living cell) and provide information about this binding. In a broader sense it should include control and processing electronics, interconnecting networks, software and other elements needed to make the signal not only recordable but understandable.

Molecular, supra-molecular and cellular mechanisms of acquisition of primary information on the presence and amount of target compounds, particles and cells and of reporting about that in the form of fluorescence signal will be of primary concern in this book. The relevant analytic devices will also be discussed in due course but in a much lesser extent. Our view is that the immense world of potential target compounds is incomparably larger than the variations of instrumental design based on modern electronics and optics. In principle, each member of this world needs its own sensor. It is a great challenge to create them.

Because of this broad range of potential (Cooper 2003a) applications, sensing techniques are attracting an increasing interest of researchers. A number of excellent reviews have been published in the field of chemical sensors, biosensors and nanosensors. By addressing a number of publications one can make a comparative analysis of different sensing strategies: electrochemical (Palecek et al. 2002; Warsinke et al. 2000); microcantilever (Carrascosa et al. 2006); optical (Baird et al. 2002; Baird and Myszka 2001) including surface plasmon resonance (SPR) (Baird et al. 2002; Homola 2003) and microrefractometric-microreflectometric techniques (Gauglitz 2005). Regarding fluorescence sensing techniques one can find important information in the books of Lakowicz and Valeur (Lakowicz 1999, 2007; Valeur 2002) and reviews (de Silva et al. 1997, 2001; Geddes and Lakowicz 2005). In some reviews the applications in particular areas are outlined: food safety (Patel 2002), clinical applications and environment monitoring (Andreescu and Sadik 2004, 2005; Nakamura and Karube 2003), detection of biological warfare agents (Gooding 2006), pharmacology and toxicology (Cooper 2003a). Particular recognition units were highlighted from antibodies (Luppa et al. 2001) and aptamers (O'Sullivan 2002; Tombelli et al. 2005) to functional nanoparticles (Apostolidis et al. 2004) and to whole living cells (Pancrazio et al. 1999). Sensing technologies have started to be used not only in cells but also on the level of whole human bodies (Wilson and Ammam 2007). It is difficult to become oriented in this broad and permanently increasing mass of information. Therefore, a systematization of obtained results and their critical evaluation are badly needed.

The general problem in any sensor technology can be formulated as follows. We have the target molecule, particle or cell dispersed in a medium that may contain many similar molecules, particles or cells. We have to provide a sensor that has to be incorporated into this medium or exposed to contact with it. The presence of a target should be revealed by its selective binding to the sensor. This binding should be detected and, if necessary, quantified in target concentration. This requires some transduction mechanism that connects the binding (molecular event) and its detection by the instrument, on the scale of our vision and understanding.

The sensor and biosensor technologies used for performing this task are based on different physical principles. They develop in parallel, competing with and enriching each other. Some transduction principles, as those used in surface plasmon resonance (SPR) sensors, acoustic sensors, microcantilevers and microcalorimeters, can be applied to any molecular interaction because they are based on the changes in mass or in heat, which are general features of complex formation (Cooper 2003b). However, these approaches generally require sophisticated instrumentation, restricting

their use to research purposes. In contrast, electrochemical sensors that are based on redox reactions at electrodes (Palecek et al. 2002) are very simple since they allow a direct conversion of a signal on target binding into an electrical signal. But they are still not always applicable because the sensing mechanism is not general enough. For instance, in biosensing they are mostly based on enzymatic activity generating a detectable product and are therefore restricted to the monitoring of the substrate(s) or effector(s) of a particular enzyme. Therefore, there is a need for generic sensing strategies that can be applied to the detection of any target, rely on low cost and easy-to-use instrumentation and are suitable for on-the-spot or field analysis. In addition, as required in some applications, the response should be very fast and the spatial resolution high enough to allow obtaining microscope images of target distribution and reading from sensors assembled in microarrays containing thousands of spots. This method exists, it is fluorescence.

Thus, what distinguishes fluorescence from all other methods suggested for reporting about sensor-target interaction? Primarily it is its *ultra-high sensitivity* (Lakowicz 1999, 2007; Valeur 2002). This feature is especially needed if the analyte exists in trace amounts. High sensitivity may allow avoiding time-consuming and costly enrichment steps. Meanwhile, one has to distinguish the absolute sensitivity, which is the sensitivity of detecting the fluorescent dye (or particle) from sensitivity in response to target binding. With proper dye selection and proper experimental conditions, the absolute sensitivity may reach the limit of single molecules. This is sufficient and very attractive for many applications, particularly for those in which the dyes are used as labels and the primary response from them is not required. High sensitivity is necessary to achieve to provide the necessary dynamic range of variation of the recorded fluorescence parameters in detecting the sensor-target interaction. This is a much harder task, which we will discuss in detail.

The second distinguishing feature of fluorescence is the *high speed* of response. This response can be as fast as 10^{-8}–10^{-10} s and is limited by fluorescence lifetime and the speed of the photophysical or photochemical event that provides the response. Usually, such high speed is not needed but sometimes it is essential. For instance, probing the rate of action potential propagation in excitable cells needs submicrosecond time resolution. The speed of sensor response is not commonly limited by fluorescence reporting. It is limited by other factors, such as the rate of target – sensor mutual diffusion and the establishment of the dynamic equilibrium between bound and unbound target.

The *very high spatial resolution* that can be achieved with fluorescence is important. It allows detecting cellular images and operating with dense multi-analyte sensor arrays. This resolution in common microscopy is limited to about 500 nm (in visible light). The limit is due to the effect of the diffraction of light when the dimensions become similar or shorter than the wavelength. Even this limit can be overcome in special conditions.

The *non-destructive* and *non-invasive* character of fluorescence sensing may be beneficial primarily for many biological and medical applications. In fluorescence sensing the reporter dye and the detecting instrument are located at a certain distance and connected via the propagation of light. This is why fluorescence detection

is equally well suited to the remote control of chemical reactions in industry and to sensing different targets within living cells.

The greatest advantage of fluorescence reporting is its *versatility*, coming from the basic event of the fluorescence response. It is essentially a photophysical event coupled to a molecular event of sensor-target interaction. That is why it can be achieved in any environment: in solid, liquid and gaseous media and at all kinds of interfaces between them. The basic mechanism of response remains always the same. It does not impose any limit on the formation of any supra-molecular structures, incorporation of reporter into any nano-composite, attachment to solid support, etc. This allows not only creating smart molecular sensing devices: their attachment to the surfaces in heterogeneous assays or integration into nanoparticles endows new functional possibilities. Due to these facts, homogeneous assays in liquid media develop into nanosensor technologies in which, in addition to fluorescence, different self-assembling, magnetic and optical properties can be explored. In microfluidic devices the detection volume can be reduced to nanoliters. Heterogeneous assays develop into multi-analyte microarrays (sensor chips), which allow the simultaneous detection of several hundreds and thousands of analytes. Two-photon and confocal microscopies allow one to obtain 3-dimensional images, which allows localizing target compounds in space.

In any sensing technology the sensor should switch between two distinguishable states – free and with bound target. There are two possibilities for reporting about a binding event and for providing a signal to distinguish these states and both of them can be realized in fluorescence sensing. First is *indirect*: to label one of these states and then to provide a quantitative measure for labeling that will be connected with the quantity of bound target. This needs additional reagents and special treatments to separate the bound and unbound label and that is why we call this approach indirect. The reporter in this case should provide a stable and bright fluorescence response and additional manipulation with the sample makes this response informative. The other possibility is *direct*: the sensor reports immediately and without any treatments on the primary act of sensor-target interaction. This requires a different property from the fluorescence reporter: to change the parameters of its emission to the very act of target binding. It is the variation of this parameter that can be calibrated in target concentration. Both possibilities show their merits and weaknesses and both of them allow broad possibilities for technical solutions.

The last decade has seen tremendous progress in the development of molecular binders – recognition units of molecular sensors, nanosensors and sensing devices. Each of them should exhibit a high affinity to a target analyte and a high level of discrimination against the species of a similar structure. This can be achieved in many ways: by using complementarity in DNA and RNA sequences, by applying monoclonal antibodies and their recombinant fragments, natural protein receptors and their analogs, combinatorial peptide and polynucleotide libraries, compounds forming inclusion complexes, imprinted polymers, etc. Imagine that one succeeded to select or to design the whole range of necessary binders. In order to benefit from that and to make efficient sensors an efficient mechanism of transduction of this effect of binding into a detectable signal should be applied. The response has to be

developed based on available fluorescent dyes and a preferable fluorescence parameter to be recorded. Synthetic chemistry offers tremendous numbers of fluorescent organic dyes (fluorophores) plus many types of nanoparticles and nanocomposites, whereas optical detection methods are much more limited. They involve measuring only several parameters, such as fluorescence excitation and emission spectra and also relative intensities, anisotropies (or polarizations) and lifetimes at particular wavelengths. The optimal choice among these possibilities means the optimal strategy in fluorescence sensing technology.

Particular attention should be given to the coupling of sensing elements with fluorescent reporter dyes and to the methods for producing efficient fluorescence response. Fluorescence reporter units are commonly referred to as "dyes". Indeed, in most cases they are organic dyes that contain extended π-electronic systems with excitation and emission in the convenient visible range of spectrum. In addition, coordinated transition metal ions can be used since they produce a luminescence emission with extended lifetimes. (In this and similar cases it should be more correct to use the term 'luminescence sensing' but it is still not in common use.) Some semiconductor nanoparticles known as quantum dots can generate a narrow-band emission and this property can also be used in sensing. The other possibility that is very attractive for intracellular studies is related to green fluorescent protein (GFP) and its analogs. In this case, the fluorescent moiety appears as a result of a folding of the polypeptide chain and a reaction between proximate amino acid side groups.

One cannot predict the long-run future developments of sensor technologies. But what is sure, they are rapidly becoming a part of everyday life. Thus, for helping diabetes patients the color-changing glucose sensor molecules are already incorporated into plastic eye contact lenses (Badugu et al. 2003) and there has been a reported development of 'an ingestible one-use nanotechnology biosensor' that can be swallowed like a vitamin to report in fluorescent light about the pathological changes in human tissues (Kfouri et al. 2008). So, what will happen next?

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Chapter 1
Basic Principles

The boom in sensor technologies is a response to a strong demand in society. As a result, almost every physical principle and technique that can detect interactions between molecules, particles and interfaces has been suggested and tested for application in sensing. In this Chapter we will provide a short survey of these techniques and try to determine the role of those of them that are based on fluorescence detection. In recent years, intensive research and development led to the establishment of several important strategies for sensor operation. Some of them are of a rather general nature and some are specific for fluorescence techniques. An overview is given below.

1.1 Overview of Strategies in Molecular Sensing

No interaction – no information. This principle is clearly seen in the background of all sensing technologies. In every interaction, we have at least two partners. One is, of course, the object that has to be detected. It is commonly called the *target* or *analyte*. It can be an object of any size and complexity, starting from protons, small molecules and ions up to large particles and living cells. The other partner, designed or selected for target detection, is the *sensor*.

The sensor has two functions. The first is to provide interaction with the target in a highly selective way, recognizing it from other objects of similar structure and properties that can be present in the probed system. Addressing the demand of target detection, it can also be the object of any size and complexity. The structure responsible for that is called *recognition unit* or *receptor*.

The other function is to 'visualize' this interaction, to report about it by providing a signal that can be analyzed and counted. The structure responsible for the generation of this signal is called a *reporter*. The transformation of the signal about a binding event into a response of a reporter is called transduction and if additional elements of the structure are needed for that, they are named *transducers*. Sensors in a broad sense also involve instrumentation, which is important as an interface between the micro-world of molecules and the macro-world in which we live.

A.P. Demchenko, *Introduction to Fluorescence Sensing*,

In the micro-world the elementary events in sensing occur and we have to analyze the results and make our decisions in the macro-world.

1.1.1 Basic Definitions: Sensors and Assays, Homogeneous and Heterogeneous

Assay is a broader term than sensing, since it may involve different manipulations with the tested system that may include different chemical and biochemical reactions. *Sensing* is always a more direct procedure, which is based on an interaction with the target of a particular sensor unit and on a detectable response to this interaction. The latter can be a molecule, a particle or a solid surface in which additional reactions are not needed or can be used only for the amplification of a primary effect of this interaction. Both assays and sensors can operate in heterogeneous or homogeneous formats.

Heterogeneous formats for assays and sensors are those that require the separation of the sensor-analyte complex for subsequent detection by any analytical method, including fluorescence. Most conveniently, this can be done in a *heterogeneous system*, in which the sensor elements are immobilized on a solid surface. In this case, after incubation in the tested medium, the unreacted species that are present in the system can be removed simply by washing and, if necessary, supplied with additional reagents for the visualization or generation of a response signal (Fig. 1.1).

In a solution, this type of assay is also possible but it requires separation by chromatography, electrophoresis, etc. As we will see below, the employment of unbound and specifically bound species of this assay principle in sensor technologies allows us to achieve the broadest dynamic range of quantitative determination of the target. Because of the implied washing step, the result is less sensitive to interference from non-specifically bound components of the test system. However, the assay is limited to high-affinity binding, since only in this case will the target-sensor complexes not be destroyed during manipulations of the sample. All additional operations, such as separation and washing, are time-consuming, which does not allow one to obtain immediate results.

Homogeneous assays are those that provide the necessary signal upon target binding in the test medium without any separation or washing (Fig. 1.2). Therefore, they are often called ‘mix-and-read’ assays. Many sensor technologies operate according to this principle. They use different physical mechanisms of response to primary sensor-target binding. Such sensors are often called *direct* sensors (Altschuh et al. 2006). If such a response is provided, then there is no need for separation, reagent addition or washing. Therefore, such sensors can also be called *reagent-independent* sensors. We will see below that there is no general simple and straightforward way to provide such direct responses. Nevertheless, there are many possibilities, especially in the cases of high target binding affinity.

A strong advantage of homogeneous assay formats is the possibility of the quantitative determination of analytes of relatively low affinities, in which a dynamic concentration-dependent equilibrium is established between a free and bound target.

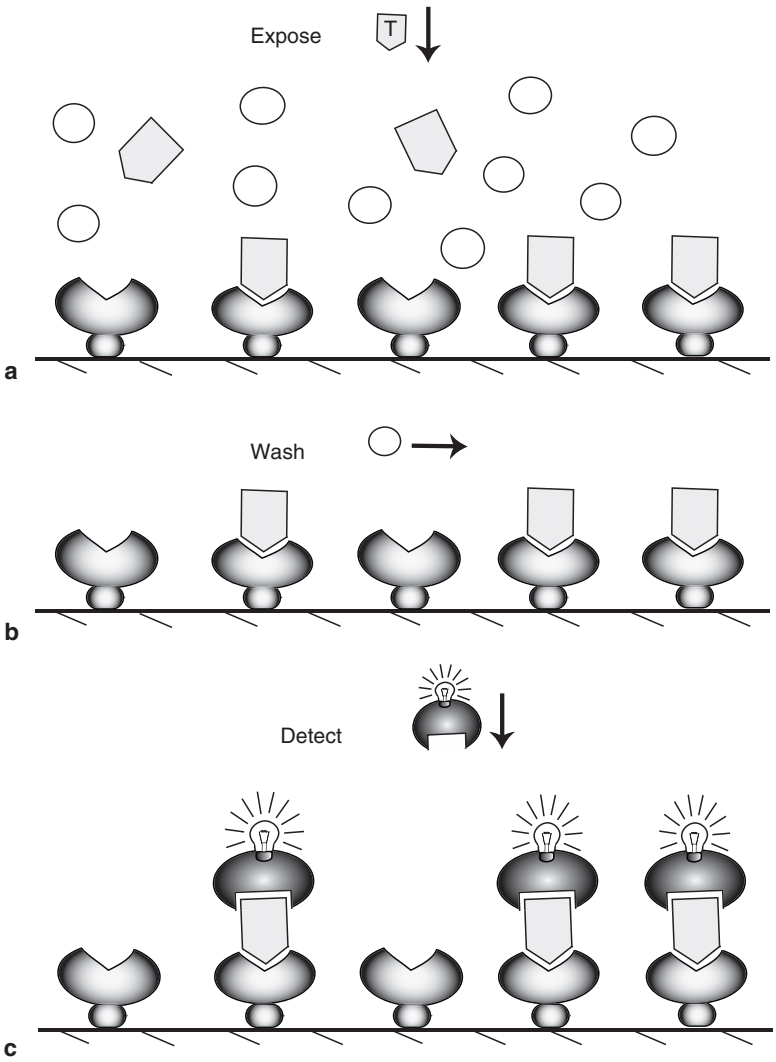

Fig. 1.1 An example of an heterogeneous assay, commonly consisting in three steps. **(a)** The plate with immobilized receptor molecules is exposed to the probed sample. The targets are strongly and specifically bound, whereas contaminating compounds remain in solution. **(b)** The plate is washed. It contains bound targets and all unbound components of the mixture are removed. **(c)** The plate is exposed to a solution of molecules or particles that are able to recognize the target and bind to it at a different site (indicator). The indicator contains a fluorescent label. Here and in other illustrations below, geometrical fitting indicates specific binding (recognition)

In this case, the *dynamic range* of the assay (the target concentration range, in which the variation of reporter signal is detected) is much narrower but the range of possible applications is dramatically increased. Direct sensors are especially desirable for different practical applications, due to the possibility of obtaining the results on-line.

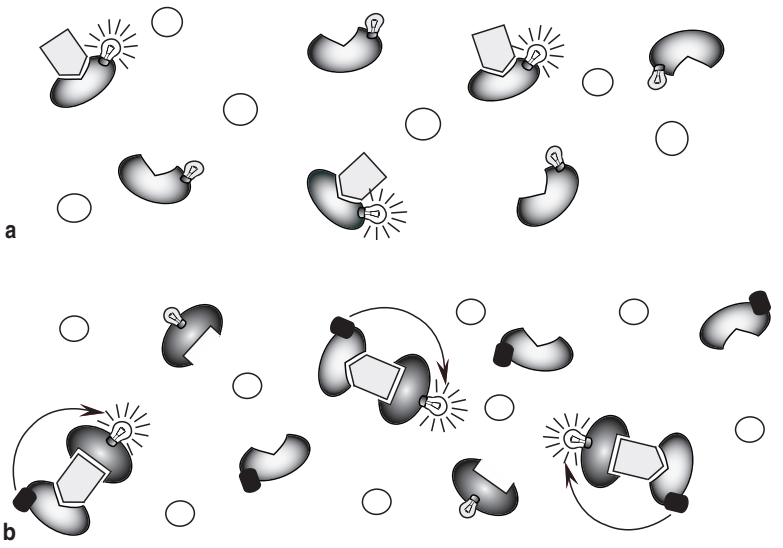

Fig. 1.2 Two examples of an homogeneous assay. **(a)** Sensor molecules comprises receptor and reporter groups that change their fluorescence on target binding. **(b)** Two different sensor molecules possessing different reporters bind the target at two different binding sites. The transduction of the reporter signal is generated on interaction between the reporter units. This is possible only in the case when they bind the same target and thus appear in close proximity. In both cases, the assay may occur without the sensor immobilization and without the separation of the complex from unreacted components

However, the non-separation nature of these assays sets some major limitations to their performance. They can be very prone to interference from non-specific (non-target) binding. Moreover, the researcher is always restricted in finding a mechanism of response that is far from being universal.

It is a matter of terminology but some authors prefer to call heterogeneous assays the 'assays' while homogeneous assays are the 'sensors'. This is because in homogeneous assays it is much easier to develop sensors that will not need any manipulation in the course of or after measurement and that are applicable for the continuous monitoring of target concentration. According to the International Union of Pure and Applied Chemistry (IUPAC) nomenclature recommendations, a *biosensor* (that can be extended to any type of sensor) is defined as a self-contained analytical device, which is capable of providing quantitative or semi-quantitative analytical information using a biological recognition element either integrated within or intimately associated with a physicochemical transducer (Thevenot et al. 2001). It is clearly affirmed that "*a biosensor should be clearly distinguished from a bioanalytical system, which requires additional processing steps, such as the addition of reagents*". Meantime, this definition is not supported by many researchers and other definitions exist (Kellner et al. 2004). For instance, some authors suggest that sensors are the devices in which the response is produced in a chemical reaction with the analyte.

The distinction between 'sensors' and 'assays' is still not very clear. Moreover, the same molecules with a selective target-binding function can serve in both assay and sensor techniques. Therefore, it is important how the reporter signal is provided.

An example is the antibody, which is the most frequently used molecule with the function of biological recognition (Fig. 1.3). For participating in bioassays, this molecule can be labeled at the periphery, at any site outside its target-recognition site. This is enough to provide a response in heterogeneous format to the immobilized

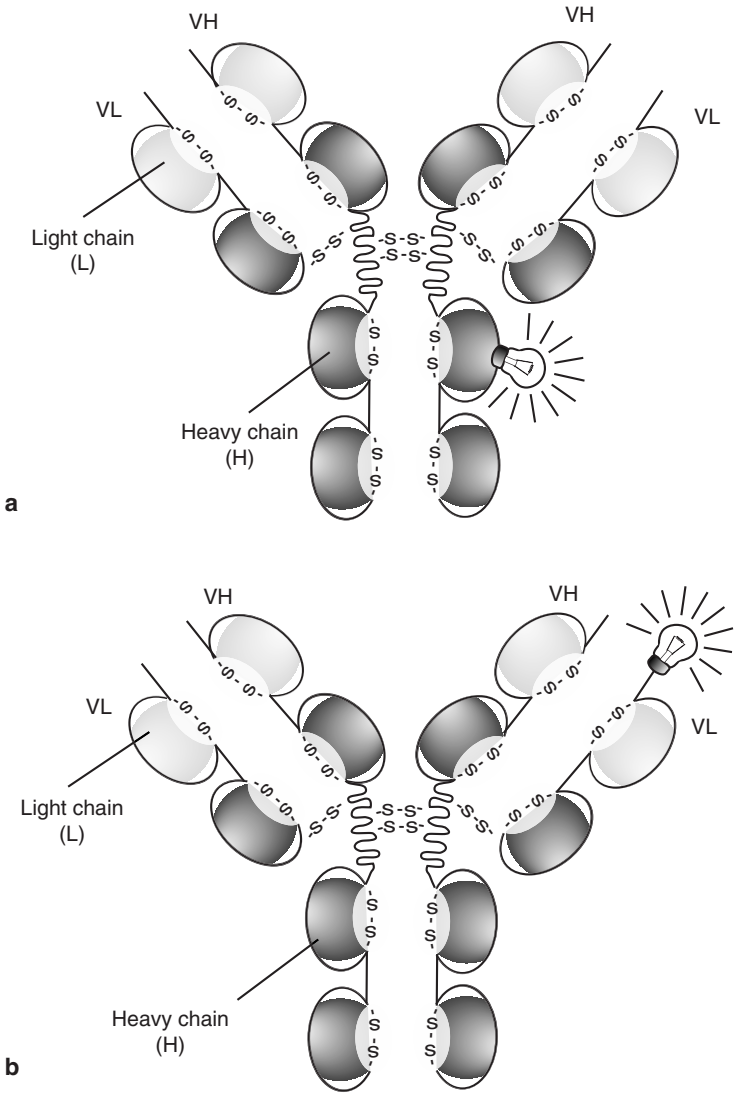

Fig. 1.3 The labeling of antibodies with fluorescence dyes for use in bioassays and biosensors. The IgG antibody is shown. It is a protein molecule composed of two light (L) and two heavy (H) chains forming domains, cross-linked by disulfide (-S-S-) bonds. The VL and VH domains form the target antigen binding site. The site of labeling determines the range of the applications of the antibodies. **(a)** The fluorescent dye is bound at the periphery of the antibody molecule and serves only for the purpose of labeling. Its response is insensitive to antigen binding. **(b)** The reporter dye is located close to the antigen binding site so that the interaction with antigen changes its fluorescence parameters providing the reporting signal

target. In contrast, for its participation in sensing in homogeneous format, stringent additional requirements should be satisfied. The label should directly or indirectly sense the interaction with the target and provide a detectable reporting signal. One of the solutions to achieving this is to locate the label at the target-binding site.

1.1.2 Principles of Sensor Operation

Different physical principles can be applied in sensor technologies to generate a measurable signal in response to sensor-target interaction (Fig. 1.4). The primary event of target binding by *receptor* (recognition element of the sensor) should generate some response, which is provided by a *reporter element*. These functions are different but they must be strongly coupled. This response (optical, heat, mass, etc.) is transformed into a measurable electrical signal.

The sensors can be classified according to three basic elements of their operation.

1. *The recognition element*. It can be as small as a group of atoms chelating a metal cation and as big as large protein molecule, DNA, membrane and even the living cell.
2. *The reporter element and the principle of reporting*. Optical (light absorption, reflection, fluorescence), electrochemical (amperometric, conductometric), mass, heat and acoustic effects can be used for this purpose.

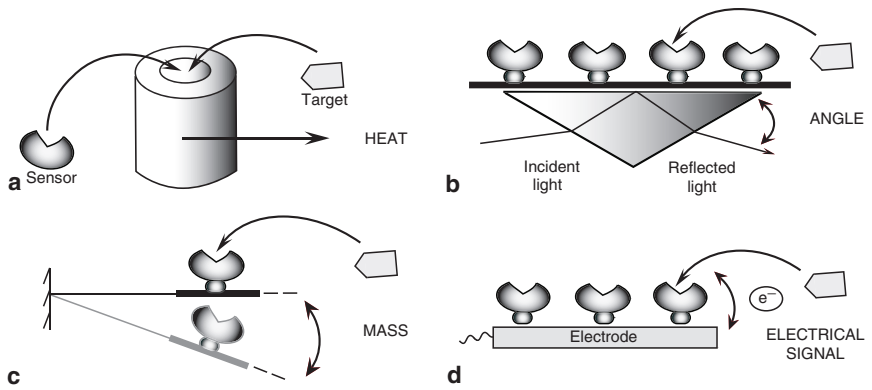

Fig. 1.4 Schematics illustrating the operation principles of sensing based on several alternative technologies. **(a)** Calorimetry. When target and receptor are mixed in a calorimeter, they produce heat effects. **(b)** Surface plasmon resonance. The layer of receptors is formed on the surface of a thin layer of gold or silver. The target binding causes the change of the refractive index close to this surface that modulates the angular dependence of the reflected light beam. **(c)** Micro-cantilever technique. The target binding results in the mechanical signal that is detected as the change of mass. (d) Electrochemistry. The target binding usually results in an electron transfer between the electroactive compound and the transducer (electrode) or in a change of the existing electrochemical signal