Biomarkers, 2008; 13(7–8): 637–657

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Biosensors for biomarkers in medical diagnostics

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Abstract

At present, most biomarker testing is taking place at centralised dedicated laboratories using large, automated analysers, increasing waiting time and costs. Smaller, faster and cheaper devices are highly desired for replacing these time-consuming laboratory analyses and for making analytical results available at the patient's bedside (point-of-care diagnostics). Innovative biosensor-based strategies could allow biomarkers to be tested reliably in a decentralised setting, although several challenges and limitations remain, which need to be improved, in the design and application of biosensors for the appropriate interpretation of the identified and quantified biomarkers. The development of biosensors is probably one of the most promising ways to solve some of the problems concerning the increasing need to develop highly sensitive, fast and economic methods of analysis in medical diagnostics. In this review, some consideration will be given to biosensors and their application in medical diagnostics, taking into account several crucial features.

Keywords: *aptamers, biosensors, cancer, cardiovascular disease, hormones, point-of-care*

(Received 1 July 2008; accepted 4 November 2008)

Introduction

Since stricter requirements regarding human health have led to a rising number of clinical tests, there is an increasing need to develop highly sensitive, fast and economic methods of analysis. The elaboration of biosensors is probably one of the most promising ways to solve some of the problems concerning sensitive, fast and cheap measurements.

The definition of a biosensor has recently been selected by IUPAC (Thevenot et al. 2001), but a more 'modern-time appropriate' definition has been chosen by Newman et al. (2004). They referred to a biosensor as: 'a compact analytical device incorporating a biological or biologically-derived sensing element either integrated within or intimately associated with a physicochemical transducer'.

The earliest biosensors were catalytic systems that integrated especially enzymes with transducers that convert the biological response into an electronic signal. The next generation of biosensors, affinity biosensors, took advantage of different biological elements, such as antibodies, receptors (natural or synthetic), or nucleic

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ISSN 1354-750X print/ISSN 1366-5804 online © 2008 Informa UK Ltd.
DOI: 10.1080/13547500802645905

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acids. In all of these interactions, the binding between the target analyte and the immobilised biomolecule on the transduction element is governed by an affinity interaction, such as the antigen–antibody (Ag–Ab), the DNA–DNA or the protein–nucleic acid binding. The specificity of the biosensor system is given by the immobilised molecule. The transducers used in biosensors are electrochemical, optical, thermometric, piezoelectric and magnetic. A general classification of biosensors is given in Figure 1, where the possible biorecognition elements and transducers are listed.

The research in the field of biosensors was initiated by Clark, whose study on the oxygen electrode was published in 1956 (Clark 1956). Since then, a huge number of biosensors have appeared in the literature and a great number with an application in medical diagnostics. Actually, >80% of the commercial devices based on biosensors are used in this domain (Dzyadevych et al. 2008), starting with the first commercial apparatus for glucose determination produced by Yellow Spring Instruments (YSI Incorporated 1975).

Apart from the huge space occupied in the market and in the literature by the glucose biosensor (Table I, (Newman et al. 2004)), recently reviewed by Wang (2008), and other enzyme-based catalytic biosensors (Singh et al. 2008, Arya et al. 2008), many examples related to the analysis of clinical relevant analytes by immunosensors have been reported in the last 20 years, when this approach first started (Lin & Ju 2005). More recently, in the last decade, DNA-based sensing has appeared for real applications in clinical diagnostics to detect the presence of pathogenic species responsible of infections, to identify genetic polymorphisms and to detect point mutations (Dell'Atti et al. 2006).

This paper reviews the recent (2006–08) literature on biosensors for medical diagnostics: the review is structured by focusing on biomarkers for different problems

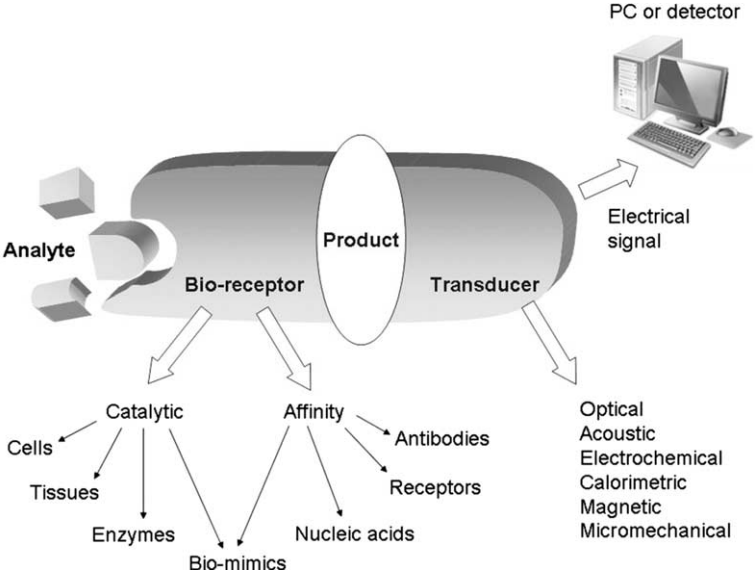

Figure 1. **Schematic representation of a biosensor.** The different biorecognition elements and transducers are depicted in the figure.

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Table I. Market-leading biosensor companies and target/sector involved.

Company	Target/sector
Abbott (Molecular Diagnostics and Diabetes Care)	Glucose
Affymetrix	Pharmaceutical and general bioscience research
Applied Biosystems and HTS Biosystems	Affinity chip
ARKRAY, Inc.	Glucose
Bayer Diagnostics/Kyoto Daiichi /Menarini	Glucose
Becton Dickinson	Glucose
Biacore	Affinity sensors for pharmaceutical and general bioscience research
Biosite	Medical
Eppendorf	Glucose and lactate
LifeScan	Glucose
Molecular Devices	Diagnostics, pharmaceutical and defence
Nanogen	Diagnostics
Nova Biomedical	Glucose, urea, lactate and creatinine
Roche Diagnostics	Glucose
YSI	Glucose and lactate

(cancer, cardiac diseases, hormones). A separate section is centred on DNA biosensors, which can represent a valid alternative to the well-accepted immunosensors.

Despite the impressive number of publications on biosensors in the diagnostics field, the commercialisation of this technology is feasible only minimally in the near future, besides the blood glucose and lactate biosensors and a few others (Table II) (http://www.biotechblog.org/entry/srinakharinwirot-universitys-technology-detects-tb-within-60-seconds). This can partially be due to the need to prove the stability and reliability of the biosensors: validation by well-established procedures must be conducted with the analysis of real samples to prove the effectiveness of biosensors for the technology to be transferred to the laboratory diagnostics market (Loung et al. 2008).

For this reason and to make a selection among the huge number of works published in the last 3 years, the authors centre their attention on those papers that report a real application of the developed biosensor with the analysis of real samples and/or a comparison with a reference method.

Review of the literature related to biosensors for biomarkers

Biosensors for cancer biomarkers

Cancer is the second most common cause of mortality and morbidity in western countries and most other countries, with >1.4 million cases in the US this year, with a similar incidence across the EU, with almost 1.5 million cases. The diagnosis and treatment of cancer represents a major area of unmet need across Europe and all other areas of the world (www.cancerworld.org). There is, however, a strong connection between early detection and positive patient outcome: early detection is the best hope

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Table II. New biosensors that have appeared in the literature for several biomarkers.

Target/biomarker	Disease	Biorecognition element	Transduction	Ref.
α-fetoprotein (AFP)	Cancer	Antibody Competitive assay	Electrochemical (array)	Wilson & Nie 2006
		Antibody Sandwich assay	Electrochemical	Wu et al. 2007a
BRCA1 gene	Breast cancer	DNA	Electrochemical	Castaneda et al. 2007
Cancer antigen 125 (CA 125)	Ovarian cancer	Antibody Competitive assay	Electrochemical (array)	Wilson & Nie 2006
Cancer antigen 15-3 (CA 15-3)	Breast cancer	Antibody Competitive assay	Electrochemical (array)	Wilson & Nie 2006
Cancer antigen 19-9 (CA 19-9)	Gastrointestinal tract carcinoma	Antibody Competitive assay	Electrochemical (array)	Wilson & Nie 2006
Carcinoembryonic antigen (CEA)	Cancer	Antibody Competitive assay	Electrochemical (array)	Wilson & Nie 2006
		Antibody Direct assay	Electrochemical	Wu et al. 2006
		Antibody Competitive assay	Electrochemical	He et al. 2008
Cardiac troponin T (cTnT)	Acute myocardial infarction	Antibody Direct assay	Optical (SPR)	Fireman et al. 2007
Cardiac troponin I (cTnI)	Acute myocardial infarction	Antibody Sandwich assay	Electrochemical	Ko et al. 2007
		Antibody Direct assay	Optical (FRET)	Cody Stringer et al. 2007
Cortisol	General physical stress	Antibody Competitive assay	Optical (SPR)	Stevens et al. 2008
C-reactive protein (CRP)	Inflammation Cardiovascular diseases	Antibody Sandwich assay	Magnetic	Meyer et al. 2007
Epidermal growth factor receptor 2 (HER-2)	Breast cancer	Antibody Direct assay	Optical (SPR)	Martin et al. 2008
Ferritin	Anaemia Cancer	Antibody Competitive assay	Electrochemical (array)	Wilson & Nie 2006
Hepatitis B virus	Hepatitis	DNA	Electrochemical	Ding et al. 2008
		PNA	Acoustic (QCM)	Yao et al. 2008
Human chorionic gonadotrophin (hCG)	Cancer	Antibody Competitive assay	Electrochemical (array)	Wilson & Nie 2006
		Antibody Direct assay	Electrochemical	Chai et al. 2008, Tan et al. 2007
IL-6	Various (Cancer)	Antibody Direct assay	Acoustic (SAW)	Krishnamoorthy et al. 2008
IL-8	Oral cancer	Antibody Sandwich assay	Optical (Fluorescence)	Tan et al. 2008
BCR/ABL gene	Chronic myelogenous leukaemia	DNA	Electrochemical	Chen et al. 2008
L1 viral region	Human papilloma virus	DNA	Magnetoresistive	Xu et al. 2008
		DNA	Acoustic (QCM)	Dell'Atti et al. 2007

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Table II (*Continued*)

Target/biomarker	Disease	Biorecognition element	Transduction	Ref.
Platelet-derived growth factor (PDGF)	Atherosclerosis, fibrosis, malignant diseases	Aptamer	Electrochemical	Lai et al. 2007
Progesterone		Antibody Competitive assay	Optical (SPR)	Yuan et al. 2007
Prostate-specific antigen (PSA)	Prostate cancer	Antibody Sandwich assay	Optical (SPR)	Cao et al. 2006
		Antibody Sandwich assay	Electrochemical	Yu et al. 2006
Thrombin	Cardiovascular diseases	Aptamer Sandwich assay	Electrochemical	Centi et al. 2007, Zheng et al. 2007

in all cancers to improve patient survival and disease prognosis, and may lead to cancer prevention.

At present, the most important cancer diagnostic indicators are morphological and histological characteristics of tumours or single biomarkers, such as prostate-specific antigen (PSA). Several examples of biosensors for the detection of cancer biomarkers can be found in the literature and further attention will be given to the detection of PSA in the next subsection, as this antigen is a biomarker strictly associated with one kind of cancer, prostate cancer. Among other cancer biomarkers that have been considered as possible targets for a single-analyte biosensor, α-fetoprotein (AFP) (Maeng et al. 2008), carcinoembryonic antigen (CEA) (Wu et al. 2006, He et al. 2008), epidermal growth factor receptor-2 (HER-2) (Martin et al. 2006) and interleukin-8 (IL-8) and interleukin-6 (IL-6) (Krishnamoorthy et al. 2008, Tan et al. 2008) have recently been taken into account.

No single oncogene or tumour suppressor, however, has been discovered to be universally altered in all adult cancers and, besides genome-related changes, other complex molecular alterations, such as protein over/underexpression, can result during the course of tumorigenesis (Soper et al. 2006). Considering these statements, a plethora of molecular biomarkers can be analysed for tumour classification, for diagnosis and for monitoring treatment and disease recurrence.

In this regard, two works are described here that report the development of biosensors with an array format for the simultaneous detection of different tumour markers (Wilson & Nie 2006, Wu et al. 2007a). These can be considered of particular significance because most markers are not specific to a particular tumour and the use of panels of tumour markers can increase their diagnostic value (Wu et al. 2007b). Both biosensors are immunosensors with an electrochemical detection. The first one (Wilson & Nie 2006) was constructed for the detection of seven tumour markers (AFP, ferritin, β-human chorionic gonadotropin (β-hCG), CEA, cancer antigen 125 (CA 125), cancer antigen 15-3 (CA 15-3) and cancer antigen 19-9 (CA 19-9)) by a competitive immunoassay conducted on an array of IrOx electrodes. The detection was accomplished by using in the competitive assay a secondary anti-IgG antibody labelled with alkaline phosphatase (AP): the enzyme substrate hydroquinone diphosphate (HQDP) was added at the end of the assay and the oxidation current was registered simultaneously for all the electrodes after the application of a potential of 320 mV. The sensor had good precision and accuracy and was comparable with the

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corresponding single analyte ELISAs with a detection limit of <2 ng/ml for all the markers. Serum control samples were used to validate the sensor, which demonstrated a good correlation with clinical analysers (within 8% agreement).

The second multi-analyte biosensor (Wu et al. 2007a) was created for the simultaneous detection of AFP, β-hCG, CEA and CA 125 by using a screen-printed carbon electrode to capture the specific horseradish peroxidase-labelled antibodies in a competitive assay format. The detection was achieved by monitoring the mediator-catalysed enzymatic response to hydrogen peroxide. Clinical serum samples were analysed with good inter-assay and intra-assay precision, and good correlation coefficients with a commercial electrochemiluminescence analyser (Elecsys 010, Roche).

Biosensors for the detection of PSA. Prostate cancer has become an important health issue because it is, on a global scale, the third most common cancer in men (Jemal et al. 2006). Prostate-specific antigen has been identified as a biomarker to screen prostate cancer patients and it has been shown that PSA is the most reliable tumour marker to detect prostate cancer at the early stage and to monitor the recurrence of the disease after treatment (Stephan et al. 2006). The PSA is found in serum, either free or in a complex with various protease inhibitors, and a total PSA level of 10 ng/ml or higher is a highly probable indicator for prostate cancer (Benson et al. 1992). Anyway, a PSA measurement above cutoff value of 4 ng/ml is generally regarded as positive and might indicate the need for a biopsy (Stenman et al. 1999).

At present, most PSA testing takes place at centralised dedicated laboratories using large, automated analysers, increasing waiting time and costs (Healy et al. 2007, Lin et al. 2008). Faster and cheaper devices are highly desired to replace these time-consuming laboratory analyses.

Innovative biosensor strategies could represent alternative strategies for reliable cancer testing: several biosensors for PSA detection have been presented in the last few years based on different transduction techniques, from electrochemical (Meyerhoff 1999, Fernandez-sanchez 2004, Sarkar 2002) to piezoelectric (Wu et al. 2001, Wee et al. 2005) and optical (Besselink et al. 2005, Huang et al. 2005) methods.

More recently, surface plasmon resonance (SPR) based detection of different PSA isoforms down to 1 ng/ml has been reported (Cao et al. 2006). The detection of a complex of PSA with α1-antichymotrypsin (PSA-AC) (Stenman et al. 1991) in both buffer and serum was demonstrated by using an alkanethiolated-modified surface for the attachment of specific antibodies. The detection limit was improved further with a sandwich format down to 10.2 and 18.1 ng/ml in buffer and in serum, respectively.

Lower detection limits have recently been reached by several published electrochemical biosensors using carbon nanotubes (Yu et al. 2006, Okuno et al. 2006) and nanoparticles (Lin et al. 2008, Choi et al. 2008) for signal amplification. In particular, an immunochromatographic electrochemical biosensor coupled to nanoparticles (Lin et al. 2008), with a detection limit of 0.02 ng/ml, was tested also in serum samples. This biosensor was validated with a human serum sample and a commercial ELISA PSA kit: the results of the biosensor were consistent with those of ELISA, with recoveries for spiked samples of 105 – 111%.

Particular attention should be given to an electrochemical immunosensor based on carbon nanotubes (Yu et al. 2006), which reached a detection limit for PSA of 4 pg/ml and was also tested in several human serum and tissue samples. The application of

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biosensors to the analysis of tissue samples is particularly challenging because very low volumes are available and sensitivity should be very high. In this case, PSA was detected with high sensitivity by using only 10 μl of serum or tissue lysates containing ~1000 cells.

The biosensor was composed of 20–30-nm-long terminally carboxylated single-wallet carbon nanotubes (SWNTs) self-assembled (Chattopadhyay et al. 2001) on Nafion-iron oxide decorated conductive surfaces. Primary antibodies for PSA were attached to the SWNT forest and used to capture the target protein. Secondary antibodies labelled with horseradish peroxidase (HRP) were then used for the electrochemical detection (Figure 2). The high sensitivity was achieved by using these secondary antibodies linked to multiwallet carbon nanotubes (CNT) at high HRP/antibody ratio. PSA in human serum was measured with a ±5% accuracy compared with a referee ELISA method. Moreover, prostate tissue lysates were tested and the results demonstrated that differences in PSA concentration could be detected among different samples that cannot be distinguished by the immunohistochemical staining reference method (Gannot et al. 2005).

Biosensors for detection of hormones

Sex steroids are thought to participate in the regulation of immune response and may play a part in the modulation of some inflammatory and autoimmune disorders. Among these hormones, the measurement of progesterone is important in women. Progesterone, a C21 steroid secreted by the corpus luteum, promotes the development of the endometrial lining. Reference concentration ranges of progesterone in plasma are from 0.1 to 28 ng/ml in women.

Immunoassays are commonly used for the determination of progesterone in serum or saliva. In particular, analysis laboratories use commercially available radioimmunoassay (RIA) kits, which are able to detect progesterone with sufficient precision in the clinically relevant concentration range (Reinsberg et al. 1997). Given the inherent problems, different non-radioactive methods have been developed for measuring progesterone (Kakabakos & Khosravi 1992, Choi et al. 1997, Hong & Choi 2002).

Among the high number of immunoassay techniques, ELISA combined with a colorimetric measurement is the most widely used for measuring hormone concentrations (Basu et al. 2006). The use of immunosensors is another interesting alternative approach. Both electrochemical and optical biosensors have been reported, using screen-printing technology coupled to cyclic voltammetry (Xu et al. 2005) and chronoamperometry (Hart et al. 1997). For optical sensing, SPR has been used (Gillis et al. 2002, Yuan et al. 2007).

Screen-printed electrodes have been used in the immunosensor development for progesterone detection as solid phase for a competitive immunoassay (Minunni et al. 2007). The EC_{50} value (the analyte concentration necessary to displace 50% of the enzyme label) was calculated as 2 ng/ml, whereas the limit of detection (LOD), calculated by evaluation of the mean of the blank solution (containing the tracer only) response minus two times the standard deviation, was estimated as 32 pg/ml.

Among other hormones, human chorionic gonadotropin (hCG) has also been considered as a target for biosensors. Human chorionic gonadotropin is an important diagnostic marker of pregnancy and one of the most important carbohydrate tumour markers. Several immunoassay kits or strategies have been presented, including some

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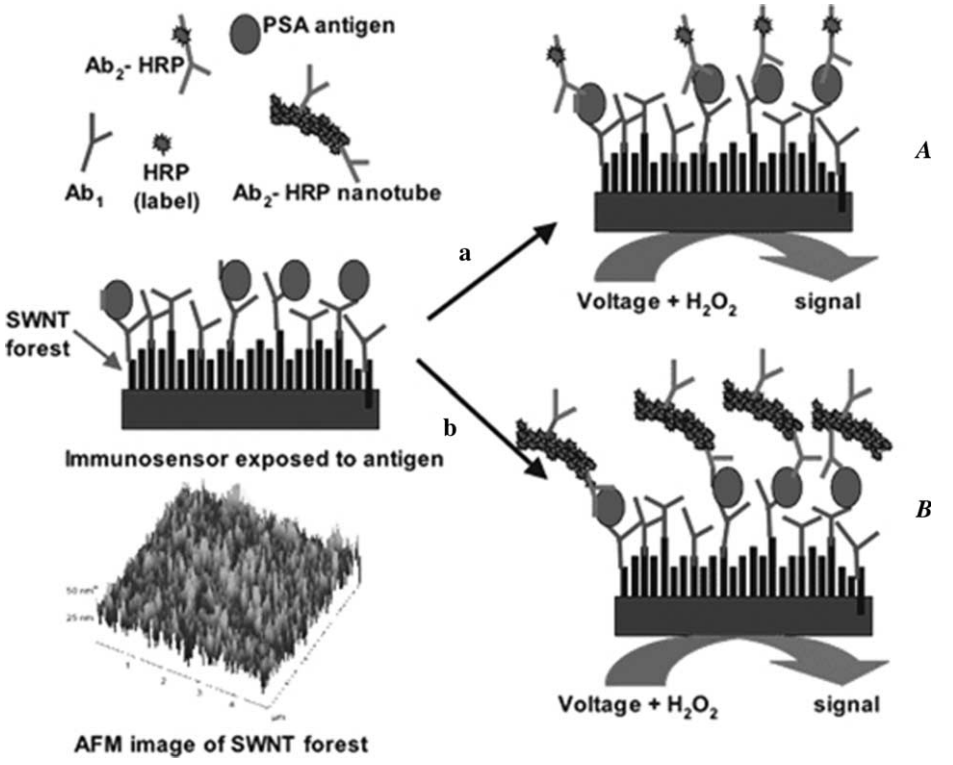

Figure 2. Illustration of detection principles of single-wallet carbon nanotube (SWNT) immunosensors. Bottom left: a tapping mode atomic force microscope image of a SWNT forest. Above this on the left is a cartoon of a SWNT immunosensor that has been equilibrated with an antigen, along with the biomaterials used for fabrication (horseradish peroxidase (HRP) is the enzyme label). (A) The immunosensor after treating with a conventional HRP-secondary antibody providing one label per binding event. (B) The immunosensor after treating with HRP-CNT (carbon nanotube) secondary antibody to obtain amplification by providing numerous enzyme labels per binding event.Adapted with permission from Fernandez-sanchez et al. 2004.AFM, α-fetoprotein; PSA, Prostate-specific antigen.

electrochemical immunosensors (Lim & Matsunaga 2001). More recently, other electrochemical immunosensors have been presented based on the use of gold nanoparticles (Chai et al. 2008) and of ormosil sol-gel membranes (Tan et al. 2007). The assays, for their analytical performances, fabrication reproducibility and operational stability, have been presented as promising tool for clinical diagnostics.

A very recent paper has appeared that is focused on the determination of another hormone, cortisol, by an SPR immunosensor (Stevens et al. 2008). Cortisol is a steroid hormone required for metabolic activities and cardiovascular function. It is considered to be an indicator of stress or disease state of a patient with normal serum levels between 20 and 140 ng/ml. Cortisol can also be found in saliva in a concentration range 1–8 ng/ml for healthy subjects: the most important advantage of detecting cortisol in saliva and not in blood/serum is the good correlation between salivary cortisol and levels of 'free' cortisol in serum, which is more biologically active than cortisol bound to transport proteins or albumin. The system that has been presented recently is based on a competition immunoassay with a six-channel portable SPR biosensor using cortisol-specific monoclonal antibody. In addition, an in-line

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filter composed of a hollow fibre membrane (20,000 molecular mass cutoff) served to separate small molecules from large molecular mass saliva components such as mucins. The detection limit of the biosensor was of 0.36 ng/ml in buffer and 1 ng/ml in saliva.

Biosensors for cardiovascular diseases

Laboratory tests are an important part in diagnosing heart infarction (World Health Organization 1979), and fast and cost-effective diagnostics is needed. At present cardiac troponin I or T (cTnI/T), myoglobin and natriuretic peptide (ANP), particularly of the B type (BNP), are determined by different immunoassay methods, such as ELISA (Katus et al. 1989), radioimmunoassay (Cummins et al. 1987) and immunochromatographic tests (Penttila et al. 1999). The first two are the most used but they are time-consuming because they require several steps; the last one is a qualitative test. As during the heart infarction the troponin T (TnT) is immediately released to the bloodstream, a biosensor able to monitor this biomarker in a short time ($<$ 10 min) could improve patient care by allowing a definite diagnosis of myocardial infarction in real time. In this regard, several biosensors for the detection of TnT and troponin I (TnI) have been published in the last few years (Fireman Dutra et al. 2007, Ko et al. 2007, Cody Stringer et al. 2008) based on electrochemical and optical transduction.

A very good sensitivity and specificity were reached by an optical biosensor coupled to quantum dots (Cody Stringer et al. 2008). The biosensor was based on fluorescence resonance energy transfer (FRET), a signal transduction method that occurs between two fluorescent molecules, a donor and an acceptor. In this regard, protein A was modified with carboxy-functionalised quantum dots, used here as donors, whereas an anti-TnI antibody was modified with a fluorescent dye, the acceptor. The two modified molecules were then incubated and, because protein A bound to the antibody, the donor and the acceptor were brought within an energy transfer distance. As the antigen TnI bound to the antibody, a conformational change occurred in the antibody molecule, altering the distance between the donor and the acceptor, resulting in a change of the energy transfer that could be detected by the fluorescence detector. The biosensor achieved a detection limit of 32 nM of TnI in buffer and of 55 nM TnI in human plasma. The response time of the biosensor was determined to be <1 min, which was much lower then the analysis time required by other TnI diagnostic assays.

Moreover, an SPR immunosensor has recently been presented (Fireman Dutra et al. 2007) for the detection of TnT based on the use of immobilised monoclonal antibodies specific for TnT. The biosensor presented a linear response range for TnT between 0.05 and 4.5 ng/ml with a good reproducibility (CV $=$ 4.4%). The amount of TnT was measured in human serum samples and the results were compared with a reference method (Roche Elecsys 2010 immunoassay analyser based on electrochemiluminescence immunoassay (ECLIA)). The measurements with the SPR biosensor showed good agreement with the ECLIA method at 95% confidence level.

Among other biomarkers, recently, the importance of inflammatory markers in the early detection of cardiovascular diseases has been shown (Rackley 2004). In particular, C-reactive protein (CRP), which is used for conventional inflammation diagnosis, can also serve as a diagnostic marker for low-grade inflammation for risk

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estimation of cardiovascular events (Ridker et al. 1998). Normal blood serum concentrations of humans range from 1 to 5 mg/l and protein levels >5 mg/l are an indication of inflammatory processes (Black et al. 2004). In routine clinical analysis CRP levels are determined by ELISA (Dominici et al. 2004) with detection limits down to 0.2 mg/l.

New approaches in medical CRP diagnosis for cardiovascular disease require rapid quantification in native matrices, such as saliva and urine (Christodoulides et al. 2005), which are not yet accessible for a CRP determination. A new magnetic biosensor was presented for the determination of CRP in human serum, saliva and urine (Meyer et al. 2007). Two CRP antibodies (clones C2 and C6) were used: C2 was used as a capturing antibody and it was immobilised onto polyethylene-sintered filters in ABICAP® plastic columns. Clone C6 served as a secondary antibody and it was biotinylated and attached to streptavidin-coated magnetic beads. This antibody–magnetic complex interacted with the captured CRP on the primary antibody, and it could be quantified by a magnetic reader. A very low detection limit (0.025 mg/l) was achieved with this system, which was also tested in CRP-spiked samples of serum, saliva and urine. Calibration curves were constructed for CRP in these matrices with good results.

Nucleic acid-based biosensors

A nucleic acid biosensor is defined as an analytical device incorporating an oligonucleotide, even a modified one, with a known sequence of bases, or a complex structure of nucleic acid (such as DNA from calf thymus) either integrated within or intimately associated with a signal transducer (Palchetti & Mascini 2008). Nucleic acid biosensors can be used to detect DNA/RNA fragments or either biological or chemical species. Most nucleic acid biosensors are based on the highly specific hybridisation of complementary strands of DNA or RNA molecules; this kind of biosensor is also called a genosensor. The probe, immobilised onto the transducer surface, acts as the biorecognition molecule and recognises the target DNA, whereas the transducer is the component that converts the biorecognition event into a measurable signal. Assembly of numerous (up to a few thousand) DNA biosensors onto the same detection platform results in DNA microarrays (or DNA chips), devices that are increasingly used for large-scale transcriptional profiling and single-nucleotide polymorphism (SNP) discovery.

In nucleic acid biosensors, the detection of the hybridisation event has been carried out through different detection technologies, from label-free methods, such as piezoelectric and SPR transduction, to other methods often requiring labels, such as electrochemical techniques. Several reviews have recently appeared in the literature (Lucarelli et al. 2008, Wang 2006) that elucidate all the critical aspects related to the transduction step. A possible scheme of a genosensor is shown in Figure 3 for the case of an SPR transduction.

As the specificity of the hybridisation reaction is essentially dependent on the biorecognition properties of the capture oligonucleotide, design of the capture probe is undoubtedly the most important preanalytical step. The probes can be linear oligonucleotides or structured (hairpin) oligonucleotides, which are being used with increasing frequency.

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The design of linear probes takes advantage of much commercially available software that can design capture oligonucleotides within the hypervariable or highly conserved regions of different genomes after their assembly and alignment. Candidate sequences, usually 18 – 25 nucleotides in length, are finally tested for theoretical melting temperature (Tm), hairpins and dimer formation and for homologies using a Basic Local Alignment Search Tool (BLAST) search (Lucarelli et al. 2008).

The experimental variables affecting the hybridisation event at the transducer–solution interface are referred to as stringency and they generally include hybridisation and posthybridisation-washing buffer composition and reaction temperature. When dealing with complex sets of probes the basic requirement for a functional system is the ability of all the different probes to hybridise their target sequences with high affinity and specificity under the same stringency conditions.

In addition, some probes, variable for chemical composition and conformational arrangement, have been used to assemble DNA biosensors. Peptide nucleic acids (PNAs) are DNA mimics in which the nucleobases are attached to a neutral *N*-(2-aminoethyl)-glycine pseudopeptide backbone. If compared with the conventional oligonucleotide probes, PNAs have appeared particularly interesting for the development of electrochemical genosensing, the main reason being the drastically different electrical characteristics of their molecular backbone.

Limiting the discussion to applications of this kind of biosensor to the field objects of this review, several DNA-based biosensors have been developed recently for the detection of virus-related sequences, such as hepatitis B virus (Ding et al. 2008, Yao et al. 2008) or papilloma virus (Xu et al. 2008, Dell'Atti et al. 2007), or for the recognition of disease-related sequences, such as leukaemia (Chen et al. 2008) or breast cancer (Castaneda et al. 2007). Several other papers could be listed related to this argument, but the opinion of the authors is that weight should be given to those

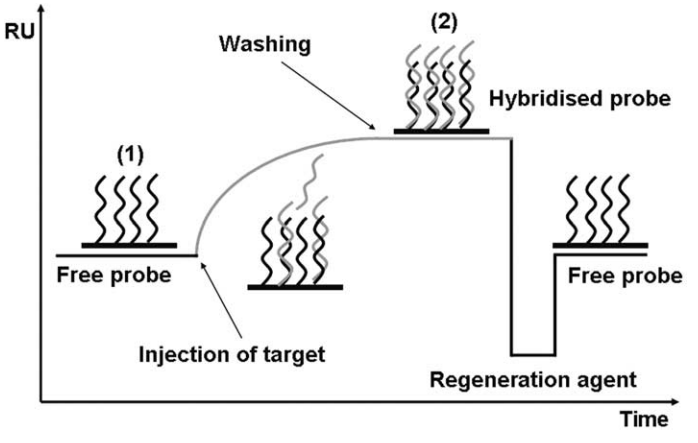

Figure 3. Schematic representation of a surface plasmon resonance (SPR) sensorgram recorded during a hybridisation cycle. The sensorgram reports the SPR signal in resonance units (RU) versus time. A baseline can be recorded with only the probe immobilised onto the SPR chip (1). The analyte (the complementary oligonucleotide) is then added and the signal is recorded. After the interaction the chip is washed with buffer and the signal is again recorded (2). The analytical datum usually considered for quantitative analysis is the signal difference between step (2) and step (1) when the sensor is in contact with the same running buffer. A regeneration step can then be performed to dissociate the hybrid and again have the immobilised probe available for a new hybridisation cycle.

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works in which real samples have actually been tested (Arora et al. 2007), not to papers where only a 'model' sequence has been considered using more simple synthetic oligonucleotides.

New frontiers in nucleic acid biosensors: aptamer-based biosensors for diagnostics

The need for analytical systems capable of rapid multi-analyte measurements of complex samples is experienced in the medical field where multi-parameter diagnostic systems are increasingly required in order to detect all the well known and the more recently known biomarkers for different diseases. When the detection system requires a biomolecular recognition event, antibody-based detection methodologies are still considered to be the standard assays in clinical analysis. These assays are well established and they have been demonstrated to reach the desired sensitivity and selectivity. However, the use of antibodies in multi-analyte detection methods and in the analysis of very complex samples could encounter some limitations, mainly deriving from the nature and synthesis of these protein receptors. To circumvent some of these drawbacks, other recognition molecules are being explored as alternatives.

The awareness that nucleic acids can assume stable secondary structures and that they can be easily synthesised and functionalised has led to the idea of selecting new nucleic acid ligands called aptamers. Aptamers are artificial single-stranded DNA or RNA ligands that can be generated against amino acids, drugs, proteins and other molecules (Tombelli et al. 2005). Their name derives form the Latin word 'aptus', which means 'to fit'. They are generated, exploiting combinatorial chemistry technology, from very large random sequence oligonucleotide libraries, through an iterative process of absorption, recovery and reamplification called SELEX (systematic evolution of ligands by exponential enrichment) (Tuerk & Gold 1990, Ellington & Szostak 1990).

Several reviews on aptamers have appeared in the literature, even very recently (Famulok et al. 2007, Mairal et al. 2007), and the appealing features of their use in bioanalysis have been thoroughly reported (Ng et al. 2006). With respect to their application, aptamers were selected in the past mainly for their use as therapeutic agents; for the first time, an aptamer has recently been approved by the US Food and Drug Administration for the clinical treatment of age-related ocular vascular disease (Cole et al. 2007). In addition to the therapeutic field, aptamers were then used in several analytical methodologies, such as mass spectrometry (Cole et al. 2007) or biosensors (Tombelli et al. 2005). These aptamers-based methods have been used mainly in the clinical area for the development of diagnostic assays, despite the fact that the analytical application of aptamers in this field is still under investigation and the scientific community still needs further research to demonstrate the advancements brought by this new kind of ligand.

The important characteristics for the success of analytical and diagnostic assays based on aptamers are the affinity and the specificity of the aptamer that provides molecular recognition. The selected aptamers can bind to their targets with affinity ranging from the micromolar to the nanomolar level and they can discriminate between closely related targets. This is due to the adaptive recognition: aptamers, unstructured in solution, fold on associating with their molecular targets into molecular architectures in which the ligand becomes an intrinsic part of the nucleic acid structure (Hermann & Patel 2000, Aptamers in Bioanalysis 2008).

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This feature represents an almost unique mechanism, which has been exploited in the design of new electrochemical sensors (Willner & Zayats 2007). In this approach the interaction of a labelled aptamer with its target can modulate the distance of the electroactive labels from the sensor electrode altering the redox current. In this regard, a growth factor aptamer-based detection method has appeared in the literature centred on the use of a platelet-derived growth factor (PDGF) DNA aptamer (Lai et al. 2007). This approach has been presented as being well suited for point-of-care diagnostics, owing to the high sensitivity and selectivity. The assay was based on the use of the PDGF aptamer modified with methylthioninium chloride (MB) immobilised onto a gold electrode and exploits the capability of the aptamer to fold in its characteristic structure when in contact with the target molecule (Figure 4).

In the unfolded structure, hence in the absence of PDGF, the aptamer has only one of the three characteristic stems and MB, fixed at the aptamer end, is far from the electrode surface. In the presence of PDGF the aptamer adopts the three stems structure and the distance between MB and the electrode decreases, improving the electron-transfer activity, with an increase of current. Exceptional sensitivity was presented for this method with a detection limit of 50 pM for PDGF, with the sensitivity examined in buffer and 50% diluted serum.

Most of the reported aptamer-based biosensors, sensors or assays make use of the thrombin-binding aptamer. This DNA aptamer (15-mer, 5′-GGTTG GTGTGGTTGG-3′) was the first one selected *in vitro* specific for a protein without nucleic acid-binding properties (Bock et al. 1992). It has been investigated extensively and its G-quartet structure has been established (Smirnov & Shafer 2004). Moreover, Tasset et al., in 1997, reported about the selection of a new thrombin-binding aptamer, a 29-nucleotide single-stranded DNA with a Kd of 0.5 nM for the protein (Tasset et al. 1997). This new aptamer binds to the heparin-binding exosite of thrombin, whereas the previously selected 15-mer aptamer was known to bind the fibrinogen-recognition exosite.

Thrombin (factor IIa) is the last enzyme protease involved in the coagulation cascade and it converts fibrinogen to insoluble fibrin, which forms the fibrin gel either in physiological conditions or of a pathological thrombus (Holland et al. 2000).

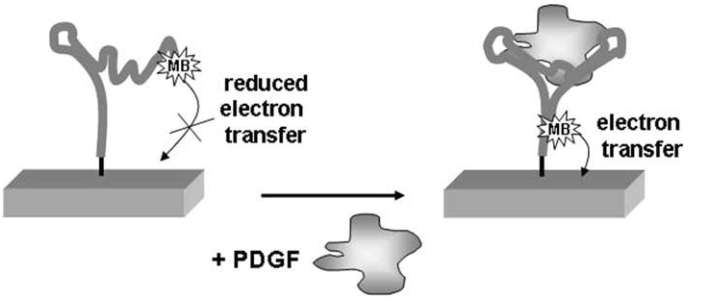

Figure 4. An approach based on the interaction of a labelled aptamer with its target modulating the distance of the electroactive labels from the sensor electrode and altering the redox current. The platelet-derived growth factor (PDGF) aptamer is immobilised onto the biosensor transducer (electrode). In the absence of target (PDGF) the aptamer is partially unfolded keeping the electroactive label (methylthioninium chloride; MB) far from the electrode. Upon target binding the interaction of the label is more efficient because the aptamer forms a stable structure holding the label close to the electrode surface, leading to an increased recorded current.

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Therefore, thrombin plays a central role in several cardiovascular diseases and it is thought to regulate many processes in inflammation and tissue repair at the vessel wall.

Many assays, mainly biosensors, based on the thrombin-binding aptamer for the detection of thrombin have been developed in the last few years (Radi et al. 2006, Zhang et al. 2006), but only a few of them have really been used as an analytical method for the detection of thrombin in real samples.

A sandwich assay was developed by using two selected aptamers binding thrombin in two different sites (Centi et al. 2007). The protein captured by the first aptamer was detected after the addition of the second biotinylated aptamer and of streptavidin labelled with alkaline phosphatase, and the detection of the product generated by the enzymatic reaction was achieved by differential pulse voltammetry (DPV). Good sensitivity and selectivity were demonstrated by the sensor with a detection limit of 0.45 nM in buffer and negligible signal generated by negative control proteins. The system was also demonstrated to recognise the target analyte in protein-rich media such as thrombin-spiked serum and plasma samples. Moreover, mimicking the physiological clogging event, thrombin was generated *in situ* by the conversion of its precursor prothrombin present in plasma and its concentration was measured at different incubation times. The results correlated well with thrombogram-mimicking software.

The best sensitivity for the detection of thrombin with a detection limit of 7.82 aM was reached by an electrochemical assay coupled to a new amplification strategy (Zheng et al. 2007). This ultrasensitive aptamer-based bioanalytical method is based on a sandwich format with the target protein captured by the first aptamer-functionalised magnetic nanoparticles. A second aptamer was attached to gold nanoparticles, which served for the electrochemical transduction (Figure 5). The innovative amplification strategy was achieved by forming network-like thiocyanuric acid/gold nanoparticles whose aggregation greatly enhanced the transduction of the aptamer-protein recognition event.

Discussion

Over the last decade we have witnessed a tremendous amount of activity in the area of biosensors. Biosensors for monitoring blood glucose at home have achieved prominence in the world diagnostics market and are now being joined by a diverse array of biosensors for detecting other analytes of clinical importance.

Now the problem has shifted to the miniaturisation of the devices, which means making a quantitative detection with $<1\mu l$ of whole blood, then with minor pain and the possible use of multiple sites for taking the sample, such as legs, forearms, and so on.

Some consideration regarding biosensors and their application in medical diagnostics can be done taking into account several crucial features.

Point-of-care testing

Point-of-care systems are viewed as integrated systems that can process clinical samples for several different types of biomarker in a variety of settings, such as clinical laboratories, ambulances, doctors' offices and the patient's home.

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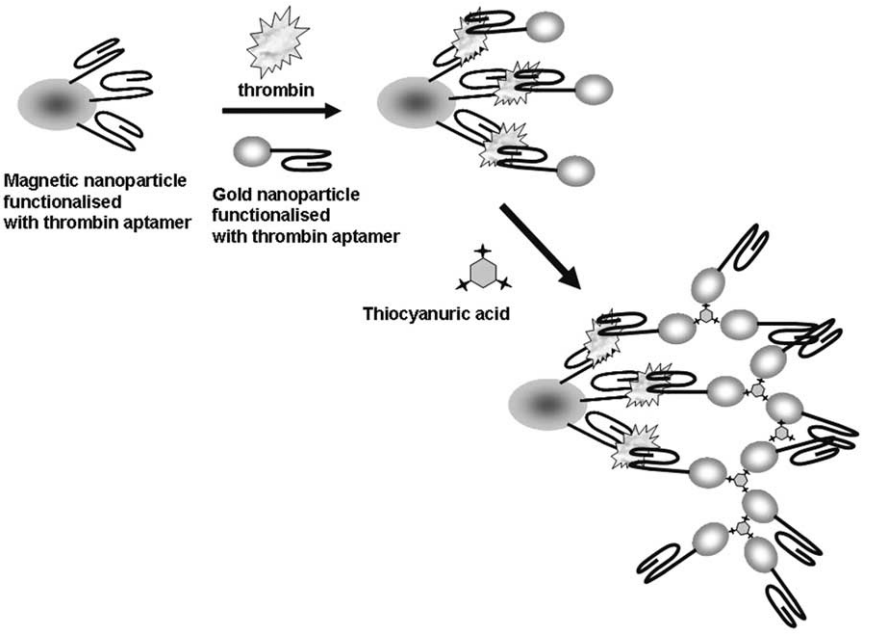

Figure 5. Schematic representation of a thrombin aptasensor. Electrochemical assay coupled to a new amplification strategy based on a sandwich format with the target protein captured by the first aptamer-functionalised magnetic nanoparticles. A second aptamer is attached to gold nanoparticles, which served for the electrochemical transduction. Signal amplification is achieved by forming network-like thiocyanuric acid/gold nanoparticles.

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Diagnostics at hospitals is based on either large-scale automated equipment or ELISA techniques based on bioassays, which are not suitable for bedside and emergency medicine. In the case of general practitioners (i.e., family doctors), diagnostics is limited to either non-quantitative sticks or sending samples to a central lab. At present, in the case of home care of patients, patients with diabetes do self-monitoring in a simple and cost-effective way.

Therefore, validated, intelligent, next-generation diagnostic devices and systems based on biosensor technology with new, radically enhanced detection capabilities and integrated sample-handling to address the most common diagnostic problems are greatly needed. These systems must reduce sample volumes and improve limit of detection, and make the point-of-care diagnostics fast, easy to handle and cost-effective. Interestingly, such devices could be assembled for specific biomarkers such as thrombin, C-reactive protein, troponin, and so on, limiting the hospital stay; this could bring an important cut in hospital expenses and would be very welcome in many industrial countries.

Disposable systems?

Some considerations are important in this regard: when developing a biosensor for clinical diagnostics, a relevant aspect is the simplification of operation, and test strips are at present still superior. Their mass production allowed the development of very cheap devices that are user friendly; the well-known glucose electrode is a typical example. Nowadays it is also possible to print many different working electrodes by

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using screen-printing technology, allowing multi-analyte detection in one measurement shot. However, the dilemma of disposable or multi-use sensing is still open: savings provided by reusable sensors should not be exceeded by the expenses of necessary maintenance. The choice of the device to be used depends on the final user: test strips are mainly used in home monitoring, in the doctor's consulting room and in small clinical laboratories, whereas reusable devices are better used in clinical laboratories (as bench instruments) or in bedside instrumentation in hospitals.

The choice between disposable or multi-use systems could also be driven by the specific assay format: in general, for affinity biosensors in which a regeneration step of the affinity interaction is often necessary, a disposable device could be more useful in order to decrease the number of steps for the measurements and the time required for analysing different samples. Anyway, in this specific field, a good compromise should be found between the reduction of the analysis time and the cost of the measurement because the chips/transducers for affinity biosensors are often very expensive and multiple use of the same chip could be more convenient.

Real samples

Applications dealing with 'real' clinical samples are still rare. Many papers describing the use of biosensors in this field have only exemplary character. Detailed data as well as validation with established methods for particular parameters are missing in most cases. The analytical potential of biosensors in the medical diagnostics field still has to be strengthened by the demonstration of their applicability to real matrices testing.

The main problem connected with the lack of experimental data on the real samples is the difficult 'communication' between technologists and hospitals. A better connection between the researcher in the biosensor field and physicians in hospitals could assure a real understanding of physicians' needs, the choice of correct applications and the availability of real samples for biosensor optimisation and validation.

New receptors: aptamers

The commercial success of biosensors different from the glucose one has been partially hampered by the lack of suitable biological recognition molecules that are inexpensive to produce and stable enough to withstand storage. This problem becomes particularly acute when designing high-density analytical arrays to support future needs in medical diagnostics, functional genomics and proteomics. DNA technology has furnished one powerful way to increase natural diversity and libraries of new receptors for integration into sensors. These new biomimetic receptors, such as aptamers, may provide a viable alternative to solving these problems.

The aptamer science in its complexity is still evolving. The therapeutic use of aptamers is now well established, as the first aptamer was approved for clinical use in 2004. On the contrary, the different fields of analytical chemistry, when dealing with systems based on biomolecular interactions, are still under the supremacy of immunoassays but deep analytical studies are now demonstrating that some of the limitations of these conventional assays can be circumvented by alternative recognition reagents such as aptamers.

Aptamer receptors, owing to their production by chemical synthesis, have several advantages that make them very promising in analytical applications such as the

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development of assays for diagnostics. Several important aspects have to be examined in detail when using aptamers as immobilised ligand, such as the immobilisation process and the assay protocol. Moreover, several findings demonstrated that aptamers could have differing susceptibility to assay protocols and that optimal operating conditions could be different from one aptamer to another.

If all these important aspects are taken into consideration, the application of aptamers as biocomponents in diagnostic assays offers a multitude of advantages, such as the possibility of easily regenerating the function of immobilised aptamers and the possibility of using different detection methods owing to easy labelling.

Despite the application of nucleic acids in the 'genomics' being well established, the possibility of selecting aptamers for a wide range of targets has opened the possibility of also using these nucleic acid molecules for proteomics with very low detection limits reaching the attomolar limit. This is important because amplification is becoming an easy task and large dilution of the sample is then allowed.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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