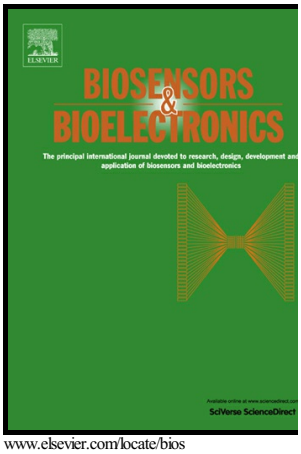

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Synthetic biology and biomimetic chemistry as converging technologies fostering a new generation of smart biosensors

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Abstract

Biosensors are powerful tunable systems able to switch between an ON/OFF status in response to an external stimulus. This extraordinary property could be engineered by adopting synthetic biology or biomimetic chemistry to obtain tailor-made biosensors having the desired requirements of robustness, sensitivity and detection range. Recent advances in both disciplines, in fact, allow to re-design the configuration of the sensing elements - either by modifying toggle switches and gene networks, or by producing synthetic entities mimicking key properties of natural molecules.

The present review considered the role of synthetic biology in sustaining biosensor technology, reporting examples from the literature and reflecting on the features that make it a useful tool for designing and constructing engineered biological systems for sensing application. Besides, a section dedicated to bioinspired synthetic molecules as powerful tools to enhance biosensor potential is reported, and treated as an extension of the concept of biomimetic chemistry, where organic synthesis is used to generate artificial molecules that mimic natural molecules. Thus, the design of synthetic molecules, such as aptamers, biomimetics, molecular imprinting polymers, peptide nucleic acids, and ribozymes were encompassed as "products" of biomimetic chemistry.

Keywords: biosensor, synthetic biology, biomimetic chemistry, rational design, bioinspired molecules

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1. Introduction

A large public awareness from biosensor community has driven research towards new technologies for the development of biosensors with smart features, competing with challenges associated to new sensing systems and their adaptation for commercial applications (Luong et al. 2008). Although innovations and improvements of this technology (Figure 1), biosensor market is still limited by a number of factors, such as long term stability, sensitivity, read-out time, miniaturization (Scognamiglio et al. 2010). In particular, the main challenges for the future generation of biosensors are the lowering of the detection limits and the increase of robustness. Synthetic biology could address these key issues allowing to improve biosensor potential in terms of sensitivity and stability, as well as selectivity and continuous analyses in complex mixtures, by extending the pristine response of biological systems and/or engineering them to provide new desired features (Andrianantoandro et al. 2006).

Synthetic biology emerged in the late 20th century to handle living matters by re-designing genes or proteins, metabolic pathways and complex biological systems, in order to understand the basis of biological mechanisms as well as to achieve new and advantageous functions (Sismour and Banner 2005). To realise that, the convergence of different technologies are required, such as genetic, chemistry, engineering, biology, mathematics and computer modelling. Due to its high multidisciplinary approach, synthetic biology has been interested in the last years by several concerns regarding the development of new bioinspired entities to be employed in producing new drugs, chemicals, fuels and materials from renewable biomass (Lee and Jonguk 2013). At the same strength of nanotechnology, synthetic biology accomplished a noteworthy attention in recent times showing its constantly increasing potential application in a wide range of fields, from pharmaceutical and biomedical, to biodefense, environmental, agrifood and so on (Figure 2). Nigel M. de S Cameron wrote: “When a hot technology prospect like synthetic biology gets the *New Yorker* treatment, it has plainly arrived - at least in the conversation of the cognoscenti” (de S Cameron and Caplan 2009). Indeed, the global market of synthetic biology was worth US$ 1.537.5 million in 2011 and it is expected to reach US$ 16.745 million by 2018 growing at a Compound Annual Growth Rate of 41.1% from 2010 to 2018 (Bhatia and Chugh 2013).

As previously debated, one of the innumerable approaches of synthetic biology regards the generation of novel biological systems, or the redesign of the existing ones, for the development of smart biological recognition elements for sensing application (Marner 2009). The ability to create new biological components has been rapidly improved by advances in technologies like nanotechnology, bioinformatics and molecular engineering to decode and rewrite genes, enzymes,

and networks, modifying them to alter their core function and structure, such as for example the substrate specificity of a molecule or an organism (Effendi et al. 2008). Emerging strategies have been adopted to exploit engineered organisms to produce a specific response to pollutants, or to design combinatorial library of bioinspired molecules, like peptidomimetics, molecular imprinting polymers, and aptamers, with tailored features concerning specificity and stability (Checa 2012).

New generations of biosensors with improved performance and wide application ranges emerged in the last years after the application of synthetic biology concepts to biosensor design. As an example, the design of genetic circuits, and consequently the development of synthetic cells, has led to the development of the first synthetic biosensors based on genetically modified organisms able to reveal a specific pollutant or an adverse environmental condition. Furthermore, several biosensors have been developed by altering the transcription of particular genes (transcriptional biosensors), varying gene expression (translational biosensors), or tuning signal-transduction pathways in response to a compound (posttranslational biosensors) (Khalil and Collins, 2010).

In this context, synthetic biological systems could pave the way to enlarge the potential of biosensors in several application fields. One of the areas where biosensor technology can attend significant progress is healthcare. Synthetic biology based biosensors can be employed for implantable sensing of a particular type of abnormality, like cancer or arterial disease. Similar biosensors could be part of a genetically engineered machine able to synthesise a drug to kill off the cancer or disperse an arterial plaque (Report: Synthetic Biology 2009; Neumann and Neumann-Staubitz 2010). Nevertheless, a number of interesting developments relating to environmental and agrifood applications concerns bioinspired sensors to detect pollutants in drinking water or food. Synthetic biosensors could be useful tools to reveal explosives and harmful chemicals, or may be integrated in purification process chains to produce clean water on large scales (Timothy et al. 2013).

This review aimed to illustrate the last outcomes of synthetic biology in the development of new bioinspired systems for sensing application; therefore, examples of engineered complex living systems were provided to highlight the last trends in robustness/sensitivity improvement for biosensing claims.

On the other hand, taking into account the article by Bernadette Bensaude-Vincent (2009), which considers “synthetic biology” and “biomimetic chemistry” as “A Two-way Traffic Across the Borders” and discusses about what extent are these technologies symmetrical and do they converge, the proposed manuscript intended to account “synthetic biology” and “biomimetic chemistry” as converging technologies to envisage Biology as an inspiration to create new artificial entities for

biosensor application (i.e. novel engineered genetic organisms projected by synthetic biology, as well as new bioinspired molecules realised by biomimetic chemistry).

2. Design of complex living systems for biosensing application

2.1 Synthetic cell-based biosensors

Applications of cell-based biosensors are limited by several challenges, and most of them regards in particular a short shelf life in working conditions. On the other hand, single molecule-based biosensors may be more stable, but sometimes less functional compared to the whole cell counterpart (Zhao et al. 2006). Synthetic materials are likely to be a feature of these two extremes, as extensively demonstrated by the latest bibliography on artificial biosensing systems, which mimic cells in their detection capabilities, showing also improved features in terms of robustness and sensitivity. In addition, synthetic biosensors can dramatically ameliorate response time, compared to several time-consuming conventional methodologies. Moreover, based on tailored properties, a significant improvement on response selectivity could be obtained.

In the last years, many efforts have been reached in modelling, simulation, and experimental analyses to study the details of individual biological elements and apply them in biosensing field. In this context, genetic circuits, synthetic metabolic pathways and minimal cells were designed by manipulating natural components and realizing systems with desired features. Impressive improvements have been made in coupling gene networks with biological response systems for biosensor development (Lu et al. 2009, Siuti et al. 2013). The resulting synthetically projected microorganisms present a genetic circuit shaped on the basis of the natural cellular sensory to obtain a response that can be transduced into a reporter protein signal. In particular, genes encoding for specific proteins, called “reporter”, are expressed under the control of a synthetic sensory-regulatory circuit, in order to tune the amount of the reporter protein to the cellular response to a target analyte (figure 3).

In this context, bacteria have helped for long time as models in monitoring toxicity of environmental pollution or in drug analysis. The first example of engineered bacteria dates back from 1970, when cells of *Salmonella enterica* (subsp. *enterica serovar Typhimurium* str. L2) were used in mutagenicity tests of several chemicals (Ames et al. 1973). Since then, different design set-up for bioreporter network systems have been described in literature for sensing application. One of the most used network is the SOS response protein A (RecA)-LexA-regulated from *Escherichi coli*, which controls the DNA damage induced by UV radiation or chemicals. This response network

involves several genes and has aided to drive toxicity-inducible reporter gene expression (Khalil and Collins 2010). In particular, the SOS system allows the interplayed regulation of induced genes for the production of two proteins, the transcriptional repressor protein LexA, and RecA, the coprotease assisting the autocatalytic self-cleavage of LexA. UV radiation and many chemicals are able to interfere with DNA synthesis and cell division, leading to the accumulation of single stranded DNA. The rule of RecA protein consists in forming nucleoprotein filaments on single stranded and double stranded DNA, to protect it against damage. SOS-induced damage genes present a promoter/operator site specific for a 20-nucleotide “SOS- LexA-box", to which the LexA repressor protein is bound, preventing gene expression.

Based on a similar mechanism, Kobayashi et al. (2004) constructed a new programmable cell of *E. coli* for sensing application, where a genetic toggle switch was interfaced with the SOS signalling pathway responding to DNA damage, and a transgenic quorum sensing signalling pathway from *Vibrio fischeri*, able to detect acyl-homoserine lactone (AHL) molecules. This novel configuration allowed to obtain a synthetic cell that synthesizes a target protein when the cell population reaches a critical density. This study proved the suitability of synthetic biology for the production of engineered programmable cells hosting novel gene networks interfaced with regulatory circuits of the cell. Even though genetic engineering is a well-known technology, several novel programmed cells have been reported in the last years for specific purposes, including biosensing applications. In this context, the novelty of the article from Kobayashi et al. resides in the adoption of a modular approach able to standardise genetic circuits, becoming a starting point as general module in the construction of programmable cells for multipurpose biotechnological applications.

Quintero et al. (2007) describes the use of synthetic biology to develop a microbial biosensor machine, by means of assembling new protein promoters for iron uptake. They transformed bacterial cells with pSB1A3 vector containing promoters PI and PII from *Acidithiobacillus ferrooxidans rus* operon and a LacI regulated device coding a monomeric red fluorescent protein (mRFP1), for specific detection of iron ions. The response of the biosensor to different Fe II concentrations (0, 1, 50, 100 ppm) was measured by the expression of reporter protein, DNA fluorescence and/or concentration, bacterial growth, and redox potential (mV/pH) of the bacterial culture.

A major challenge in the development of such biosensors is standardization, crucial to guarantee consistent procedures for biosensor production and validation. Creating biosensors with solid characteristics of safety, selectivity, sensitivity and stability in a noisy cellular environment became mandatory for synthetic biologists using principles of engineering. Indeed, cellular environment is represented by a huge intercellular variability, being responsible to hamper

functionality of the system at colony level. The group of Prindle et al. (2012) overcome this challenge by projecting the synchronization of thousands of oscillating colony 'biopixels' through the use of synergistic intercellular quorum sensing within a colony and gas-phase redox signalling between colonies. Finally, they realised a liquid crystal display (LCD)-like macroscopic clock able to sense arsenic via modulation of the oscillatory period. This ability to coordinate cellular behaviour over large length scales leads to the construction of genetic biosensors capable of detecting heavy metals and pathogens in the field with fast and cost-effective procedures.

Examples of synthetic biosensors have been reported also as novel antimicrobial strategies against infectious pathogens. Saeidi et al. (2011) designed a synthetic genetic system allowing *E. coli* to sense and eradicate *Pseudomonas aeruginosa*. In details, the proposed system was able to: i) detect acyl homoserine lactones (AHLs) produced by *P. aeruginosa*; ii) produce the pyocin S5 proteins as detection response; and iii) lyse *E. coli* cells by E7 lysis protein to release pyocin S5 and kill *P. aeruginosa*.

Figure 4 illustrates a schematic representation of the genetic system designed by Saeidi et al. (2010). Based on the Type I quorum sensing mechanism of *P. aeruginosa*, the system was constituted by the tetR promoter, which constitutively produces a transcriptional factor, LasR, able to bind to AHL 3OC12HSL. The LasR-3OC12HSL complex, in turn, binds to the luxR promoter, used as inducible promoter in the sensing device, leading to the production of pyocin S5 and lysis E7 proteins within *E. coli*. At a threshold concentration, the lysis E7 protein perforates *E. coli* membrane and releases the accumulated pyocin S5, which diffuses toward the pathogen and damages its cellular integrity, thereby killing it.

Synthetic biosensors have been described as useful tools also to optimize assimilation of specific metabolites in microorganisms. A significant example was recently reported by Teo et al. (2015). The authors developed recombinant *Saccaromyces cerevisiae* strains, usually unable to ferment xylose, competent to sense and possibly assimilate xylose. In particular, they constructed a synthetic operon, in which native yeast constitutive promoters of PGK1 and PFY1 were cloned upstream of the XylR coding sequences, and the XylR operator sequence. Downstream to the XylR operator, the sequence encoding the yEGFP repoter protein, was cloned. In these systems, constitutively expressed XylR which binds to the operator sites in the synthetic hybrid promoter in the absence of xylose inducers to repress yEGFP transcription. In the presence of xylose, XylR affinity to the operator is reduced, resulting in an increase of yEGFP expression (Figure 5). This work demonstrated how genetically encoded biosensors can sense and translate intracellular concentration of biochemicals of interest into detectable and rated outputs, enabling on-demand

protein synthesis to help optimize cellular resource consumption and minimize cellular stress via flux balance in synthetic xylose utilization pathways.

In addition to whole cell sensors linked to reporter genes and actuator elements, RNA-based sensors have been similarly exploited to report the concentration of growth-limiting small molecules or sense toxicity in cellular behavior. As an example, riboswitches are RNA-based sensing-regulatory elements able to detect intracellular metabolite levels, which combine an RNA sensing function, encoded in an aptamer, to a gene-regulatory function. Upon aptamer binding to a target metabolite, a conformational change of the riboswitch occurs, modulating the activity of the gene-regulatory domain, conveying in changes in the levels of enzymes in related biosynthesis pathways (Michener 2012). Many research efforts have been conducted to design RNA devices based on the connection between sensing, regulatory components and mechanisms of action of integrated gene-regulatory components. One of the advantages in exploiting RNA-based sensing systems resides in the potential to adapt their sensing function *in vitro* to a wide range of small molecules by means of the *de novo* production of RNA aptamers.

A dominant example of riboswitch is represented by the fluorescent biosensors designed by Kellemberg et al. (2015) for the detection of Cyclic di-AMP (cdiA), a second messenger present in bacteria and Archaea. In the human pathogen *Listeria monocytogenes*, cdiA is an essential molecule able to regulate metabolic function and cell wall homeostasis. The biosensor was based on the fusion of the Spinach2 aptamer to ligand-binding domains of cdiA riboswitches, to visualize by flow cytometry assay intracellular cdiA levels in living *L. monocytogenes* strains and to determine the catalytic domain of the phosphodiesterase PdeA. Furthermore, the proposed biosensor was also exploited in different Gram-positive and Gram-negative bacteria as well as in Archaea, expanding the potential of RNA-based biosensors for in vivo metabolite imaging in different organisms, and enhancing the discovery of previously unidentified signaling domains.

Most of the exploited synthetic-based sensing approaches have been projected by means of optical transduction, being very sensitive, suitable for *in vivo* measurements, and feasible through simple instrumentation. Nevertheless, a huge amount of electrochemical-based synthetic biosensors has been designed, thanks to their uncountable advantages including robustness, high sensitivity and excellent detection limits, also with small volumes and turbid samples with optically interfering absorbing/fluorescing compounds. A crucial example is represented by the self-powering electrochemical biosensor called “ElectrEcoBlu” designed and realised by the team of Gu et al. (2010). The proposed engineered machine was based on the integration of a biosensor with a microbial fuel cell to transduce a pollution input into an easily measurable electrical output signal. The device consisted of a recognition element allowing to detect a range of input signals, and a

reporter element responsible for generating an electrical signal as an output. The recognition element of the sensing system involves a pollutant responsive transcriptional activator, XylR or DntR, able to bind BTEX chemicals (benzene, toluene, ethylbenzene and xylene) and dinitrotoluene, respectively. These biocomponents are associated to a reporter element, made of the enzymes S-adenosylmethionine dependent N-methyltransferase (PhzM) and flavin dependent hydroxylase (PhzS), capable to convert the precursor compound phenazine-1-carboxamide (PCA) to pyocyanin (PYO) in the biosynthetic pathway isolated from *Pseudomonas aeruginosa*. The construct was cloned into *E. coli* and into a non-pathogenic Pseudomonas strain. This kind of approach allowed to produce a biosensor with a novel reporter mechanism and provide an inexpensive and easy-to-use method to detect target analytes in real time, showing how the integration of converging techniques could lead to obtain new perceptions in the development of synthetic biochemical systems for biosensing application.

2.2 Artificial liposomes

Liposomes have been largely employed as artificial cells in biosensing application, thanks to the possibility of combining the structural, chemical and lyotropic features of lipid membrane with the structuring capability of nanotechnology. A wide number of synthetic lipid nanostructures have been projected in the last years, which could find application in the study of biological processes as well as in biosensing. As an example, Kwakye et al. (2003) realised a semi-disposable microfluidic biosensor for the detection of pathogens via their nucleic acid sequences. They projected a polydimethylsiloxane (PDMS) mounted onto a reusable polymethylmethacrylate (PMMA) stand, for the immobilisation of two different DNA sequences employed as biorecognition elements, complementary to unique sequences on the target pathogen RNA. The first nucleic sequence was coupled to a dye and encapsulated in synthetic liposomes, while the second nucleic sequence was coupled to superparamagnetic beads. The implemented biosensing system proved the advantages of increased sensitivity and decreased analysis time. Indeed, the liposomes based biosensor showed a detection limit 100 times lower than the detection limit of a membrane based biosensor developed for Dengue fever virus. Moreover, a similar biosensor could become useful also as portable sensing system for in-field application. Indeed the reported biosensor can be integrated in an automatable fluidic system for sampling and treatment, as well as fluorescence detection be replaced by electrochemical detection, becoming suitable as microbiosensor for a portable analysis system.

A similar system was realised by Zhao and co-authors (2006), which developed an artificial cell-based biosensor able to mimic whole-cell in the detection of the toxic protein Listeriolysin O

(LLO). LLO is a pore-forming hemolysin secreted by the foodborne pathogen *Listeria monocytogenes* for bacterial virulence. The authors synthesised small unilamellar liposomes containing fluorescent dyes and immobilized them within porous silica, serving as cellular surrogates for membrane insertion and pore formation by LLO. In general, whole-cell biosensors harness the capability to sense their chemical and biological environment better than most engineered sensors, but could be less efficient in some applications requiring harsh growth conditions. Moreover, conventional methods for identification of *L. monocytogenes* in food is a culture-based procedure that takes 5-7 days, consequently increasing the probabilities to commercialise a contaminated food product before the problem has been identified. For these reasons, the materials implemented by Zhao et al. (2006) could be considered a good candidate to detect LLO, showing fast response (30 minutes) and high stability (at least 5 months in ambient conditions).

3. Bioinspired synthetic molecules to enhance biosensor potential

As highlighted by Eileen R. Choffnes et al. (2011) in her book "The science and applications of synthetic and systems biology", synthetic biosensors are entirely genetically encoded systems, based on transcription regulators able to bind the analyte of interest, resulting in either the induction or repression of a reporter gene encoding a specific protein as output signal. The following section deals with the use of "synthetic biology" as an extension of the concept of "biomimetic chemistry", in which artificial molecules are designed by organic synthesis to emulate the behaviour of parts of biology, typically proteins and enzymes (Benner and Sismour 2005; Sarikaya et al. 2003).

Many efforts have been made in the last years to meet the requirements of biosensors through the development of new materials inspired by nature. In the following chapters, design of synthetic molecules, such as aptamers, biomimetics, molecular imprinting polymers, peptide nucleic acids, and ribozymes will be debated as "products" of biomimetic chemistry able to provide a trustworthy sustenance to biosensing technology in solving some of the biosensing drawbacks related to sensitivity, stability, and reliability.

3.1 Biomimetics

The development of biosensors based on proteins and receptors as biological recognition elements is limited by several tasks. The design of protein-based arrays for high throughput analysis requires large-scale production and purification of the biological recognition elements, as well as

the set-up of labelling protocols with fluorescent dyes that represents a labour-intensive work. In addition, proteins or receptors are susceptible to denaturation, a relevant problem especially in microarray formats where a stable configuration of sensor molecules is mandatory.

In this context, biomimetics are achieving particular prominence as new entities inspired by biology, through an explosion of new discoveries in biology and genetic engineering (Lepora et al. 2013). Among bioinspired materials, biomimetic peptides could allow advances in any required biosensing systems, since they could be considered interesting alternatives to proteins or receptors, thanks to their tangible advantages. First of all, they do not require the laborious large-scale production and purification procedures as recombinant proteins, since peptide chains could be also available through automated chemical synthesis. Peptide sequences could lead to more stable tridimensional, and offer a larger chemical versatility to random and site-directed mutagenesis compared to proteins. For these reasons, synthetic peptides are ideal for biosensor design since they offer superior operational robustness compared to most proteins, and are well suited for long-term storage in dry, dissolved, and immobilized states.

Several biosensors have been extensively reported in literature demonstrating the potential of peptide rational design to overcome the challenges related to biosensor commercialisation (Scognamiglio et al. 2013). For example, Enander et al. (2008) demonstrated the ability of peptides combined with ratiometric fluorophores to ensure robustness in recognition and signalling in immobilized format quantification assays. The authors presented the design and functional evaluation of synthetic single labelled peptide constructs as a promising alternative to recombinant protein-based conjugates for biosensing of a target protein. In particular, a synthetic peptide pTMVP of 18 amino acid was produced and functionalised with 6-bromomethyl-2-(2-furanyl)-3-hydroxychromone, an environmentally sensitive fluorophore with a two-band emission, for the detection of Fab57P, a recombinant antibody fragment for the tobacco mosaic virus coat protein (TMVP).

The potential clinical value of a commercial biosensor for the diagnosis of hepatitis G virus (GBV-C) infection was demonstrated by Gómara et al. (2010). The authors synthesized chimeric multiple antigenic peptides (MAPs) containing epitopes from E2, NS4, and NS5 proteins of GBV-C, for the development of new systems to diagnose infections caused by the virus, allowing also to perform studies of the infection among the hemodialysed and hepatitis C virus (HCV)-infected population.

This clinical potential was also demonstrated by Villiers et al. (2015). This group projected a protein/peptide array to detect, by surface plasmon resonance imaging, anti-Delta genotype 1, 6, 8 specific antibodies among Hepatitis D Virus infected patients. In particular, they defined an array of recombinant proteins and mimetic peptides to provide a high throughput viral hepatitis diagnostic,

combining the potential of artificial molecules with the Surface Plasmon Resonance Imaging (SPRi), which is a high performing technique to obtain a wide sample screening.

The potential of combining mimetic peptides with nanotechnology was instead demonstrated by Prasuhn et al. (2010). They showed the simplicity of functionalise peptides with fluorescent probes and to covalently link them to terminal carboxyl groups on quantum dots (QDs) surface, highlighting the usefulness of joining peptides and QDs to create sensors for monitoring chemical compounds and biological processes. In particular, the authors functionalised QDs dye-labelled peptides to yield a Forster resonance energy transfer (FRET)-based sensor to monitor enzymatic activity of caspase 3, a key downstream effector of apoptosis, and Ca_2 ions that are ubiquitous in many biological processes.

The high performance of biomimetic-based sensors with respect to the conventional techniques were also described by Sanghavi et al. (2012), with the synthesis of a dimeric Cu(II) complex ($[Cu(\mu_2\text{-hep})(\text{hep-H})]_2*2ClO_4$) and its coupling with silver nanoparticles for the development of a sensor for determining dopamine (DA), levodopa (L-Dopa), epinephrine (EP) and norepinephrine (NE). The authors developed an adsorptive stripping square wave voltammetry (AdSSWV) based biosensor, establishing that similar sensors have several advantages in terms of high sensitivity, low cost and simple surface modification, in regard to conventional methods applied for the determination of catecholamines, such as high performance liquid chromatography, capillary electrophoresis, LC-MS/MS, chemiluminescence and fluorescence.

3.2 Molecular Imprinting Polymers (MIPs)

The *in vitro* diagnostics for biomedical, agrifood and environmental analysis rely mainly on the use of antibodies, employed in routine laboratory use for quantification of many analytes in biological fluids, as well as food products or environmental samples. Although highly specific for chemical and biological recognition, they could have short shelf-life, and high costs. Sometimes they also could show poor stability losing their tridimensional structure in working conditions, and consequently, immobilisation procedure could be challenging. Furthermore, there are several complications in producing antibodies against immune-suppressants or toxins because of their adverse effects on the immune response (Poma et al. 2010). Synthetic molecules like molecular imprinting polymers (MIPs), able to mimic the potential of antibodies with the advantages of high robustness and sensitivity, represent a great promise for the realisation of diagnostic tools (Piletsky et al. 2001a).

MIPs are synthetic materials that contain an artificial recognition site able to mimic a natural molecule in binding a highly specific target analyte. Several important features makes MIPs crucial entities as receptors in sensing application, showing high affinity, selectivity, stability, simplicity of preparation, and ease of adaptation to different sensor configurations (Piletsky et al. 2001ba). They can be prepared by the polymerisation of functional monomers around a template molecule, which represents the target, by means of covalent or non-covalent bonds. Upon polymerization, the target molecule is removed and a specific recognition sites results to be complementary to the target in size, shape and position of the functional groups (Figure 6).

The resulting synthetic molecule is able to mimic the biological activity of a natural receptor with enhanced specificity and stability, and can be involved in the development of ligand binding assays (Ye and Haupt 2004) or as selective sorbent in sensors (Patel et al. 2009). Due to their high thermal, chemical and mechanical tolerance, MIPs show resistance to enzymatic degradation, heat and low pH, good performance in aqueous and organic solvents. Although their preparation is relatively easy and cheap, the traditional bulk polymerization method is a time-consuming process, and the complete removal of the template molecule is often problematic resulting in low fidelity of the binding sites (Nicholls et al. 2001). Nevertheless, several biosensing systems were produced employing MIPs, with application in food and environmental field, clinical diagnostics and pharmaceutical sector. In particular, optical sensors combined with MIPs are often used, mainly with fluorescence transduction detection, due to its high sensitivity among the spectroscopic techniques. On the other hand, a significant number of articles on MIPs based sensors have been published also in association with conductometry, impedance, potentiometry, amperometry and voltammetry, since electrochemical device show high sensitivity and portability. A number of papers on MIPs based sensing devices were recently reviewed in the context of food and environmental science, for the detection of chemical species in ground and drinking water, such as monoamine naphthalenes (Valero-Navarro et al. 2009), phenols (Sergeyeva et al. 2010), microsystin-LR (Chianella et al. 2003), 2,4-dinitrophenol (Liu et al. 2012), parathion (Marx et al. 2004), for food contaminants monitoring, such as herbicides (Pardieu et al. 2009) and heavy metal (Liang et al. 2008). Recent analysis for biomedical and pharmaceutical applications include the monitoring of serotonin in human blood plasma (Peeters et al. 2012), creatinine (Subrahmanyam et al. 2001), and theophylline (Kindschy et al. 2007).

The high stability and reliability of the imprinting polymers as attractive alternatives to conventional antibodies or receptors were also demonstrated by Piletsky et al. (2001), in the design and production of MIPs specific for low-molecular weight analytes, such as epinephrine and atrazine, and proteins with different molecular weights, such as microperoxidase, horseradish

peroxidase, and lactoperoxidase. In particular, the authors set up a new method of microplates modification with imprinted polymers based on aniline and thiophene derivatives, in order to develop a platform for high throughput analysis showing practical significance for environmental analysis and drug screening.

More recently, a novel sensor for human cardiac troponin T (TnT) based on a molecularly-imprinted electro-synthesized polymer was fabricated and characterized by Karimian et al. (2013). The MIP-based sensor, able to detect TnT, a highly sensitive cardiac biomarker for myocardial infarction, showed high affinity in comparison with non-imprinted polymer (NIP) electrodes in both buffer and blood serum, being a reliable, sensitive and rapid system for the identification and diagnosis of acute myocardial infarction.

The potential of MIPs in building a foundation for the development of biomimetic sensors was likewise demonstrated by Jiang et al. (2015), highlighting how MIPs represent an interesting alternative to more traditional bioreceptors. They functionalised a thin gold-based chip with a MIPs film to detect histamine in real samples, obtaining a clear dose–response curve with a limit of detection of 25 μg L^{-1}, without any interferences in the presence of structural analogs.

3.3 Aptamers

Aptamers are short nucleic acid- or peptide-based artificial molecules, often termed as chemical antibodies, with specific binding affinity towards their target analytes (Colombo et al. 2015). Isolated from chemically synthesized combinatorial libraries, they are produced by chemical synthesis and purified to a very high degree. Finally, site-specific modifications can be introduced to incorporate particular reporters or linkers, as well as to increase stability, affinity and specificity (Mascini 2008). Many research efforts have been conducted on aptamers with respect to their remarkable properties, such as high specificity and affinity, longer shelf-life, easy manufacturing procedure, freedom to introduce chemical modifications for further improvement (Kanwar et al. 2015). For these reasons, aptamers appear ideal biological mimetic components for assembling reliable biosensors for specific recognition of a wide range of analytes, including small organics, peptides, proteins, viruses or cells, with exceptional binding constants from picomolar to nanomolar range (Tombelli, et al. 2007). Taking into account this efforts, aptamers expand the opportunity to develop a wealth of possible signal transduction systems, from optical to electrochemical, or mass based transducers.

In this context, several approaches were adopted to introduce labels into aptamers for optical sensing application. A simple set-up consists to label aptamers with a quencher and a fluorophore

aiding “light-on/off” strategies. The binding of ligand modifies the engineered molecule and brings the quencher and fluorophore in a different conformation, resulting in a fluorescence variation (figure 7 top).

A similar biosensor was developed by Pei (2010), which used this fluorescent sensor design strategy to develop a DNA aptasensor for IgE and vasopressin, and a RNA aptasensor for tobramycin and vasopressin, by employing fluorescein and dabcyl as fluorophore/quencher pair. These fluorescent aptasensors can be rapidly integrated into many sensor configurations and have been proven to be powerful molecular tools.

Often, a variety of ligands or solvents can interfere with quenching, leading to false signals. By exploiting the Forster Resonance Energy Transfer (FRET) of fluorescent molecules, it is possible to combine a couple of donor/acceptor fluorophores to the termini of the aptamer and obtain a ratiometric determination of the targets (Figure 7 bottom). As an example, Buranachai et al. (2012) developed a FRET based biosensor designing a DNA-based reconfigurable molecular aptamer beacons. The synthetic molecule consisted of an aptamer strand hybridized with an oligonucleotide doubly labelled with a fluorescent probes pair. The sensor showed a folded (high FRET) and an unfolded (low FRET) conformation depending on the absence or presence of the target analyte.

Analytical applications of aptamers have been rapidly moving also toward electrochemical biosensors due to the high sensitivity of the technique. Electroactive reporters such as methylene blue, ferrocene or ruthenium can be covalently conjugated or indirectly complexed to the aptamers in the case of electrochemical aptamer-based sensors. Target analyte monitoring can be performed by a “signal-on/off” due to the shielding of the redox reporter from the electrode. An example of electrochemical aptamer-based biosensors was described by Centi et al. (2007) for the detection of plasma proteins. In particular, the authors designed an aptamer-based sandwich assay for the electrochemical detection of thrombin in complex matrices, using a simple target-capturing step by aptamer-functionalized magnetic beads. The system showed high sensitivity and specificity in buffer, spiked serum, and plasma, opening the possibility of a real application to diagnostics for medical investigation.

An electrochemical aptamer-based biosensor for environmental application was also described by Pilehvar et al. (2012) for the detection of chloramphenicol. An unfolded ssDNA aptamer was synthesized and immobilized on a gold electrode surface. Target binding changes aptamer conformation from single strand to a hairpin structure, carrying the target close to the surface and generating electron transfer, providing LOD value in the nanomolar range when measured in milk samples.

Furthermore, a wide number of biosensors were developed employing: i) different transduction systems, such as Surface Plasmon Resonance (SPR) (Polonschii et al. 2010; Bini et al. 2011), FRET (Wang et al. 2011), Chemiluminescence Resonance Energy Transfer (CRET) (Liu et al. 2011), electrochemical impedance (Chen et al. 2010), ii) novel nanomaterials, including the SiO_2/gold nanoelectrodes projected by Ilyas et al. (2012), or the Au nanoparticles boron-doped diamond electrode designed by Song et al. (2012); iii) multi-plexing configurations, such as the rationally-designed modular allosteric aptamer sensor (MAAS) designed by Bang et al. (2013). Likewise, aptamer-based sensor were designed not only to detect chemical species of medical, agrifood and environmental interest, but also to monitor biological processes. For example, Xing (Xing et al. 2012) described a new graphene oxide based fluorescent aptamer-based sensor able to monitor the enzyme adenosine deaminase (ADA) activity. Zhang et al. (2012) also realized a fluorescence anisotropy aptamer based sensor for real time protein signalling in complex media of the oncoprotein human platelet-derived growth factor B-chain (PDGF-BB).

3.4 Peptide Nucleic Acids (PNAs)

DNA and RNA macromolecules play important regulatory roles in the cell processes by interacting with other nucleic acids, proteins and small molecules. In the last years, many efforts in synthetic biology were achieved to produce new synthetic components, inspired by the natural versatility of nucleic acids, to be applied as probes for detecting target genes responsible for diseases, or pollution in agrifood and environmental monitoring. DNA/RNA based biosensors are recognized as tools of high scientific and technological relevance in the development of microarray or lab-on-chip for gene expression pattern or detection of pathogens. Nevertheless, there are still critical challenges to improve sensitivity, selectivity and rapidity of the hybridisation detection. Among the possible strategies, new artificial molecules known as Peptide Nucleic Acids (PNAs) open promising ways to overcome these challenges.

For biosensing applications, synthetic DNA mimic PNAs could be advantageous as probe molecules, thanks to their unique physicochemical and biochemical properties. The use of PNA probes in electrochemical biosensors revealed higher sensitivity and specificity in comparison with nucleic acids. In addition, PNAs exhibit fast hybridization kinetics, independent by ionic strength, require shorter probe length than DNA, and are resistant to enzymatic degradation. Furthermore, the possibility to obtain a huge variety of different molecular structure enables to fine-tune mode of detection, in terms of sensitivity and detection range.

As an example, Jampasa et al. (2014) developed an electrochemical biosensor based on an immobilized anthraquinone-labeled pyrrolidinyl PNA (acpcPNA) probe for the selective detection of human papillomavirus (HPV) type 16 DNA. The probe, a 14-mer acpcPNA designed to recognize a specific 14-nucleotide region of HPV type 16 L1 gene, was labeled with the redox-active tag anthraquinone (AQ) and immobilized onto a chitosan-modified disposable screen-printed carbon electrode. This sensing platform was successfully applied to detect the HPV type 16 DNA exhibiting very high selectivity for the complementary 14 base oligonucleotide over the non-complementary oligonucleotides with sequences derived from HPV types 18, 31 and 33.

PNA specificity and stability were further enhanced when PNAs were combined to graphene oxide (GO) for the development of a FRET sensor. PNA was used as probe and graphene as quencher for the sequence-specific detection of double stranded DNA (dsDNA), as exhaustively emphasized in the work of Lee et al. (2014). In comparison with dsDNA sensors based on complementary DNA probes, PNAs in combination with GO allowed hybridization reactions with the target sequence avoiding the denaturation step, and provided fluorescence signals with very low background, high sensitivity and sequence selectivity even in the presence of serum proteins.

A similar biosensor based on PNAs immobilised on nanosized graphene oxide (NGO) was realised by Ryoo et al. (2013) to detect microRNA (miRNA) species acting in post-transcriptional regulation processes (Figure 8). miRNAs are considered crucial biomarkers since their abnormal expression levels is associated with many human diseases including cancer and diabetes. The group tightly bound graphene with PNA probes, resulting in fluorescence quenching. Fluorescence was recovered after the addition of target miRNA with high sensitivity, with a LOD as low as ~1 pM in living cells. In addition, the biosensor demonstrated to have many other advantages including high sequence specificity, high loading capacity on graphene surface, and resistance against nuclease-mediated degradation.

Bocková et al. (2015) demonstrated that the combination of DNA hairpin probes, mimicking the natural sequence of bacterial transposable elements (TE) involved in DNA transposition, with surface plasmon resonance (SPR) can provide a rapid and real-time monitoring of the activity of REP-associated tyrosine transposases (RAYTs), a class of Y1 nucleases related to the IS200/IS605 transposases associated with TE. The results of the RAYT-DNA interaction obtained by means of the SPR approach, compared with standard analysis, demonstrated a high suitability to detect and quantify crucial activities related to processes of DNA transposition.

3.5 Ribozymes

In vivo sensing systems depend on molecules which act as biomolecular switches, meaning that they undergo binding-mediated conformational changes to transduce chemical information into specific biochemical outputs. Due to the high performance of such systems for real time detection on highly complex matrices, several efforts have been delivered towards the construction of biologically inspired molecules as switches for artificial chemical sensors.

Recent advances in the field of nucleic acid engineering have seriously enhanced opportunity to design new switching sensors. In this context, rational design, directed evolution, and labelling chemistry procedures were integrated for the construction of synthetic ribozymes, RNA molecules that operate as molecular switches to regulate and catalyse a number of biochemical processes, including tRNA processing, mRNA splicing and regulation, structural assembly and chaperone-mediated folding. In addition, by computational approaches both natural and synthetic ribozymes and riboswitches have been converted to allosteric ribozymes (aptazymes) by inserting oligonucleotide sequences (aptamers) that regulate ribozyme activity upon ligand binding (Isaacs et al. 2006).

Several approaches included these molecules as alternative bioreceptors for biosensor development, in the detection of small analytes not amenable to antibody assays (Furchak et al. 2008), or as molecular sensors detecting the presence of effector molecules, or also for the quantitation of miRNA, gene expression, and viral RNAs (Hartig and Famulok 2008). To report some example, Chen (Chen and Ellington 2009) developed a quantitative and predictive model for projecting aptazymes as biosensors *in vitro* and as riboswitches *in vivo*. Ogawa (2011) adopted a strategy for producing various ligand-dependent riboswitches, available for detecting theophylline, tetracycline, and sulforhodamine B. A number of ribozyme based biosensors have been also reported for the detection of Hepatitis C Virus (HCV) (Levesque et al. 2010), L-histidine (Kong et al. 2011), adenosine (Sun et al. 2010), glutamate (Okumoto et al. 2005).

4. Summary and conclusions

During the past decade, a noticeable progress has been produced in the field of diagnostics along many fronts, in particular in the area of biosensing, both for single analyte detection methods or multi-array based technology. This pronounced interest has provided a wide plethora of information about biology of living entities or macromolecules. In the last years, the emerging of synthetic biology paved the way to create new biological systems and novel bioinspired material, allowing to

increase the potential of biosensors. Furthermore, the application of nanotechnology in biosensing technology in combination with synthetic biology, is creating a deep impact on the design of biological circuits, bio-production systems, biopolymers, biological machines, and consequently on the development of diagnostic tools for agrifood, environmental, pharmaceutical and biomedical application.

However, the spread of biosensors for commercial use is still limited. Thus, synthetic biology, together with nanotechnology, may help to obtain more reliable biological recognition elements with enhanced sensitivity and stability, which actually represents the main challenges to reach a commercial success for biosensors.

5. Future Perspectives

Synthetic biology has arisen as a stimulating and promising research field, collecting noteworthy consideration from the scientific community. This awareness rises from a number of remarkable characteristics of synthetic biology, having enormous potential to join converging technologies (biology, mathematics, physics and engineering) in solving a wide range of challenges in different application fields. Indeed, synthetic biology has proved high potentiality in the last years in programming pioneering functionality into engineered organisms for several purposes, including sensing, waste degradation, biofuel production, fine chemical production, and drug development.

The rationale resides in its huge potential to project new engineered organisms carrying out desired functions suitable for several uses. For example, novel genetic circuits have been projected to sense bioreactor environments to drive metabolic processes towards improved fine chemicals or biofuel production. Then again, new genetic toggle switches have been designed to program yeast cell flocculation and facilitate their separation to alcohol produced in industrial fermentation processes. Synthetic control systems have been also employed to extract synthesized products, such as novel biomaterials for various applications (e.g. bioplastics, biopolymers).

Nevertheless, there are still several engineering as well as ethical limitations regarding synthetic biology. For example, the predictability of evolution/adaptation of synthetic organisms is restricted. Actually, several parameters need to be considered when designing and evaluating a synthetic entity, such as population-level dynamical adaptation, noise in the course of gene expression, and continuing reproduction. Furthermore, risks related to the possibility that a designed organism can mutate into something different have to be taken into account, to avoid critical ethical and practical concerns.

From another point of view, many efforts are being conducted to improve the technological performances of synthetic bioinspired systems through joint and emerging technologies, such as microfluidics or graphene. Indeed, the development of synthetic biology can be accelerated through the use of microfluidic technology, which can provide unique income for dynamic profiling of gene expression/regulation with high resolution, highly sensitive on-chip and off-chip detection of metabolites, and whole-cell analysis (Vinuselvi et al. 2011). Furthermore, combining microfluidic platform with synthetic biology allows providing well separated compartments of cells with the ability to introduce rapid environmental perturbations, thus establishing the dynamics of gene networks in a single cell. In addition, microfluidics is able to increase number of samples to analyse and decrease cost of microscale multiplex trials.

At the same strength of microfluidics, the high versatile graphene technology is becoming an astonishing matter when blended to synthetic biology. The last decade has observed exponential advances in the generation of novel synthetic strategies based on graphene constructs combined with engineered organisms with multiple biological activities as well as with novel bioinspired molecules (e.g. such as anti-cancer drugs) (Servant et al. 2014).

The design and production of these sophisticated systems, based on the use of interdisciplinary technologies, has created important technological breakthroughs, allowing the progression in the development of a wide range of research and application fields, from pharmaceutical to biomedical diagnosis and therapeutics, as well as in the biotechnological and industrial sector.

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Figure captions

Figure 1. Biosensors: definition, market and application fields.

Figure 2. Scheme of the main application fields of synthetic biology.

Figure 3. Scheme of the bioreporter cell response to toxicity based on a synthetic sensory-regulatory circuit: in the presence of a desired toxic compounds or condition, the reporter gene is activated to encode a reporter protein assigned to the response.

Figure 4. Schematic representation of the genetic system projected by Saeidi et al. (2010). The designed genetic system is a synergistic combination of three modules that sense the presence of *P. aeruginosa*, produce the antibiotic pyocin S5 and deliver the antimicrobial by triggering lysis of the cell and killiing the pathogen when its presence is detected.

Figure 5. Schematic illustration of the xylose biosensor system by Teo el al. (2015). XylR repressor binds to synthetic hybrid promoter containing the respective XylR operator sequence to block transcription. Detection of xylose by XylR results in antagonization of XylR DNA binding, triggering transcription of the downstream reporter, yEGFP.

Figure 6. Schematic representation of the molecular imprinting process. A monomer mixture with chemical complementary to the template is allowed to form solution adducts through reversible covalent or non-covalent interactions. Polymerisation in the presence of a cross-linking agent is followed by removal of the template, leading to the defining of a recognition site with steric and functional complementary to the template.

Figure 7. Scheme of optical sensors based on quenching aptamers (top) and Forster resonance energy transfer (FRET) aptamers (bottom).

Figure 8. Scheme of the optical biosensors designed by Ryoo et al. (2013) based on a PNA immobilised on nanosized graphene oxide (NGO) to reveal microRNA (miRNA), in regulation processes of gene expression at the post-transcriptional level.

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Highlights

- The role of synthetic biology in sustaining biosensor technology was discussed
- The use of synthetic cells and bioinspired synthetic molecules for the development of novel biosensor systems was reviewed.
- Examples of synthetic-biosensors were reported, tailor-made to the requirements of robustness, sensitivity, and detection range.