Artificial enzyme-based biosensors

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During the last forty years, enzyme-base biosensors have had great success in the detection and quantification of various biologically-relevant molecules. However, native enzymes can sometimes be costly, delicate to manipulate or simply absent for a particular analyte. Hence, artificial or synthetic enzymes could be a useful alternative to natural proteins for the conception of new biosensors, since they can be, *a priori*, designed in their entirety, as well as more robust, available, chemically malleable and cheap, in comparison with their natural analogues. In this Perspective, we will provide a snapshot of this emerging research field.

1. Introduction

A biosensor is defined as "a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals"[1] (Fig. 1). Biosensing is a rapidly expanding field, with world market revenue estimated to reach £6 billion in 2013;[2] the major impetus coming from the health care industry, but with some pressure from other areas, such as food quality appraisal, environmental monitoring and the defence against terrorism. Research in this field is multi-disciplinary, spanning biological, physical and material chemistry.

Leland C. Clark, Jr. and Champ Lyons definitively pioneered the field of biosensing with a report in 1962 of the first glucose sensor using the enzyme glucose oxidase.[3] This protein converts its substrate, glucose, to gluconolactone in the presence of oxygen, and Clark, Jr. and Lyons demonstrated that the glucose in whole blood could be monitored by measuring the consumption of oxygen with an amperometric electrode. Within a decade, the Yellow Springs Instrument Company (now YSI Inc.) was able to commercialize a glucose analyzer based on Clark's concept of an enzyme-based biosensor. Significant research efforts have since been made in this field, and enzyme-based biosensors can now be successfully applied to the detection and quantification of numerous biologically-relevant molecules, such as neurotransmitters, pollutants or even noble gases. These works have been extensively reviewed in the literature.[4]

To be attractive, an enzyme-based biosensor must possess the following features: (i) specificity for target analytes, (ii) stability under normal storage conditions, (iii) reusability, (iv) accuracy and reproducibility of the response, and (v) reduced cost. In some cases, the limitations of enzyme-based biosensors are primarily those imposed by the nature of

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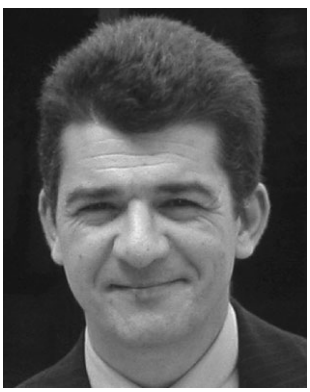

Pascal Dumy

Pascal Dumy obtained his PhD at the Université de Montpellier (France) in 1993. He then undertook postdoctoral research with Prof. Manfred Mutter at the Université de Lausanne (Switzerland), where he became an Assistant Professor in 1997. In 1998, he was appointed Full Professor at the Université Joseph Fourier de Grenoble (France), and in 2003, he became the Head of the Département de Chimie Moléculaire (UMR CNRS 5250). He was an appointed junior member of the Institut Universitaire de France (IUF) between 2000 and 2005. His main research interests include the chemistry of biomolecules, non-viral vectors for in vivo *targeting and molecular imaging, synthetic vaccines, and surface functionalisation.*

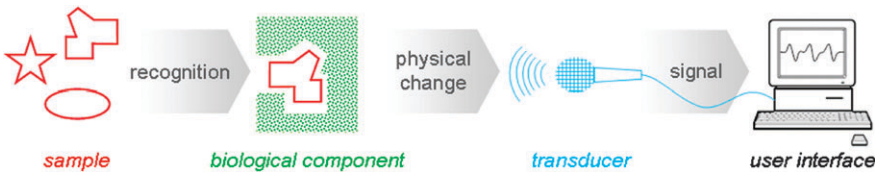

Fig. 1 A schematic representation of a biosensor.

the enzyme itself, and include a lack of native enzymes corresponding to a target analyte acting as a substrate or inhibitor (see section 2.2), poor availability and/or expensiveness of the enzymes, and the highly specific conditions (*i.e.*, solvent, pH and temperature) in which they work. As a consequence, the use of biomimetic chemistry and artificial enzymes is an attractive alternative for the use of biotic enzymes in the design of new biosensors. The advantages of enzyme mimics are numerous: they can be, *a priori*, fully designable towards a target analyte, less fragile, more easily available (since they are synthetic objects), chemically more flexible to give them desirable properties (*e.g.*, for subsequent immobilisation) and cheaper, in comparison with their natural analogues. In this Perspective, we will provide a concise overview of the research field devoted to artificial enzyme-based biosensing, including recent results obtained in our laboratory.[5]

2. General considerations for the design of artificial enzyme-based biosensors

In this section, we intend to present a succinct guide for designing artificial enzyme-based biosensors in order to help researchers enter this research field.

2.1 What are artificial enzymes?

Like their natural homologues, artificial enzymes are catalysts that increase the rate of otherwise (very) slow reactions by lowering their activation energies.[6] Artificial enzymes must display an active site[7] that is responsible for the binding of the substrate, resulting in (i) the close proximity and correct orientation of the reactive functions of both the substrate and the artificial enzyme, and/or (ii) the stabilisation of the transition state by the active site's environment (Fig. 2).[8] The binding event can be the result of either (i) non-covalent interactions (*e.g.*, electrostatic interactions, see section 3.4) or (ii) a good three-dimensional fit (*e.g.*, the molecular sieve effect, see section 3.1) between the substrate and the artificial enzyme's active site.

Finally, a well-designed active site should also lead to substrate selectivity (*i.e.*, only a very narrow range of substrates can undergo catalysis), reaction selectivity (*i.e.*, catalysis of only one type of reaction) and, if necessary, stereoselectivity (*i.e.*, discrimination between the optical isomers of a substrate).

2.2 Operational mechanisms

Artificial enzyme-based biosensing is based on the measurement of the rate of a catalytic reaction and, hence, the analyte concentration. Biosensors primarily rely on two operational

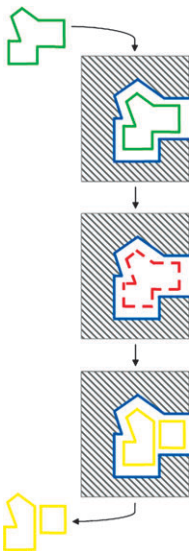

Fig. 2 The catalytic transformation of a substrate (green) into reaction products (yellow) through a transition state (red) mediated by an artificial enzyme (grey). The active site of the artificial enzyme is highlighted in blue.

mechanisms. The first involves the catalytic transformation of an analyte acting as a substrate for the artificial enzyme; the catalytic reaction being followed by monitoring either (i) the formation of a detectable reaction product (Fig. 3A) or (ii) the consumption of a detectable cosubstrate, for example oxygen (Fig. 3B). The second mechanism involves the detection of an analyte that acts as an inhibitor in the catalytic transformation of the artificial enzyme's substrate from a non-detectable form to a detectable one or *vice versa*

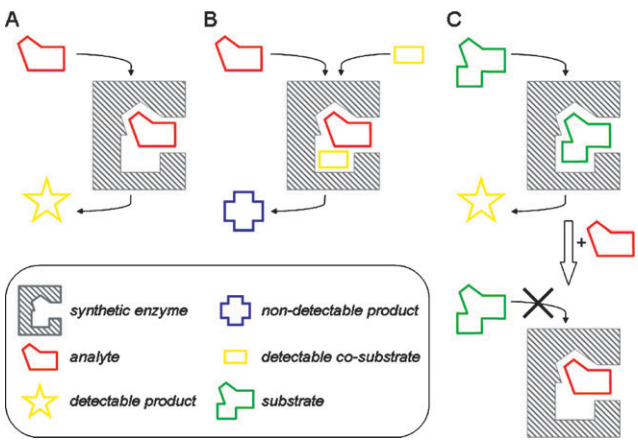

Fig. 3 Potential operational mechanisms for artificial enzyme-based biosensors.

(Fig. 3C). In all cases, the rate of the catalytic reaction is related to the analyte concentration, provided that the reaction conditions and the artificial enzyme concentration are kept constant.

The detection limits of these sensors are obviously imposed by the artificial enzymes' catalytic parameters, which are the association constants of the different complexes and the rate at which the artificial enzymes catalyze the reactions. Together with the need for selectivity of the artificial enzymes' active site towards substrates/inhibitors, all of these requirements highlight the challenge of designing competitive artificial enzyme-based sensors with respect to those employing native enzymes.

2.3 Transduction

A key point in designing artificial enzyme-based biosensors is the choice of transducer, which makes use of a physical change accompanying the reaction for the detection and quantification of the analyte. This may be:

1. The electric current produced during a redox reaction, for instance when a potential applied between two electrodes is sufficient to oxidise or reduce one of the reactants or products of the catalysed reaction (amperometric biosensors).

2. The difference in the light absorption or luminescent properties between reactants and products that can be observed using a spectrometer (optical biosensors).

3. Changes in the distribution of charge, which are detected with ion-selective electrodes (potentiometric biosensors). For instance, a catalysed reaction that generates or absorbs hydrogen ions can be monitored using a pH meter.

4. The change in electrical conductivity of metal oxides when they are exposed to either a reactant or a product of the catalytic reaction (semiconducting biosensors).

5. The emission of light as the result of a catalysed chemical reaction (chemiluminescent biosensors).[9]

6. The heat produced or absorbed by a reaction, the temperature variation usually being determined by means of resistors that function by changing their electrical resistance with temperature (calorimetric biosensors).

7. The mass difference between the reactants and products, which can be detected using a quartz crystal microbalance (acoustic biosensors). This approach can typically be used if the product of a catalytic reaction is insoluble, resulting in its precipitation on the resonating crystal surface.

So far, only the first five transduction pathways have been exploited for the preparation of artificial enzyme-based biosensors (*vide infra*).

3. Classes of artificial enzyme-based biosensors

In this section, we present an assortment of abiotic enzymes that have recently been used for a sensing purpose, trying in each example to emphasize the interest in substituting an artificial for a native enzyme.

3.1 Inorganic material-based sensors

Zeolites are microporous solids composed of a framework of tetrahedral TO_4 building units (T = Si, Al, *etc*...).[10] The pore size within a zeolite's framework allows some molecules to pass through while others are excluded, resulting in a molecular sieve effect (Fig. 4). In addition, zeolites exhibit negatively-charged surfaces that are balanced by alkaline or alkaline-earth cations, which can be exchanged with other species, such as catalytically-active metal centres, leading to the formation of artificial enzymes.[11]

Trimboli and Dutta have reported a Pt-loaded zeolite Y that is able to catalyze the oxidation reaction of propane, yielding CO_2 and water, at temperatures ranging from 400 to 600 °C;[12] the reaction was monitored by measuring the change in resistance of a TiO_2 layer upon water exposure.[13] The sensor proved to be selective towards another gas, carbon monoxide, and, although not demonstrated in the paper, it should also be selective towards bulkier alkanes because of the molecular sieve properties of zeolites.

This work is remarkable since, to date, no native enzyme has been reported to catalyse the oxidative cleavage of unfunctionalized hydrocarbons.[14]

3.2 Polymer-based sensors

3.2.1 Molecular imprinted polymer (MIP)-based sensors. Molecular imprinting technology is based on the preparation of a polymer in the presence of a target template molecule, which leads to the formation of a cavity that is complementary to the target molecule after its removal from the polymer.[15] The incorporation of a catalytic centre within the polymer allows the construction of artificial enzymes.[16]

Sode *et al.* reported the development of a MIP-based dehydrogenase enzyme mimic that catalyzes the oxidative cleavage of fructosyl valine, a model compound for HbA1c that is an important indicator for diabetic control (Fig. 5).[17] The catalytic polymer was prepared from a fructosyl valine-type template, and from 1-vinylimidazole and 4-vinylphenylboronate as the catalytic centre and substrate recognition unit, respectively. The imidazole group acts as a general base catalyst in the presence of an electron acceptor, 1-methoxy-5-methylphenazinium methylsulfate (m-PMS), triggering the oxidative cleavage of the fructosyl valine. The electron acceptor, reduced during this step, is subsequently oxidized on the surface of an electrode, hence furnishing a measurable electrical signal.

Noteworthy and in contrast with the native enzyme that oxidatively degrades fructosylamine compounds (*i.e.*, fructosyl amine oxidase),[18] the reported sensor displayed a clear selectivity for fructosyl valine towards similar analytes such as fructosyl alanine, fructosyl glycine, fructosyl phenylalanine and fructosyl ε-lysine.

Another MIP that displays both molecular recognition properties and the ability to catalyse a chemiluminescent reaction has been used by Lin and Yamada for the sensing of 1,10-phenanthroline (Fig. 6).[19] A ternary complex, 4-vinylpyridine/Cu(II)/1,10-phenanthroline, was employed as the functional monomer and divinylbenzene was used as the cross-linking agent. The MIP has a strong catalytic activity for the decomposition of H_2O_2, leading to the formation of a superoxide radical ion ($^{\bullet}O_2^-$), which is able to attack and oxidatively decompose the phenanthroline molecule with the production of light (maximum wavelength in the range

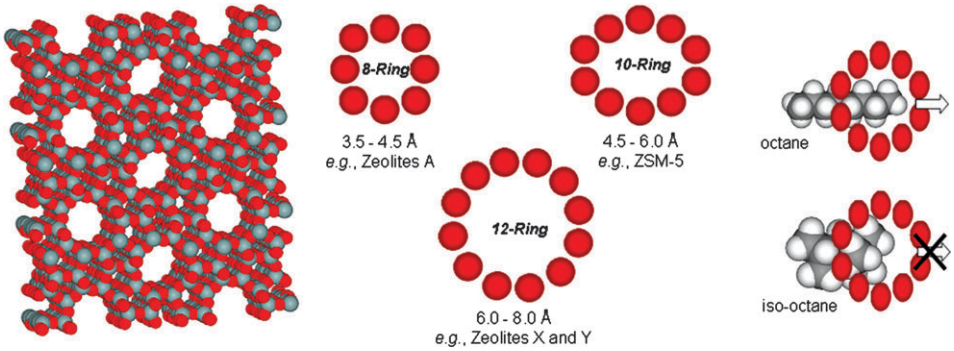

Fig. 4 Left: the microporous molecular structure of a famous zeolite, ZSM-5. Middle: the oxygen packing and pore sizes of several typical zeolites. Right: the molecular sieve effect.

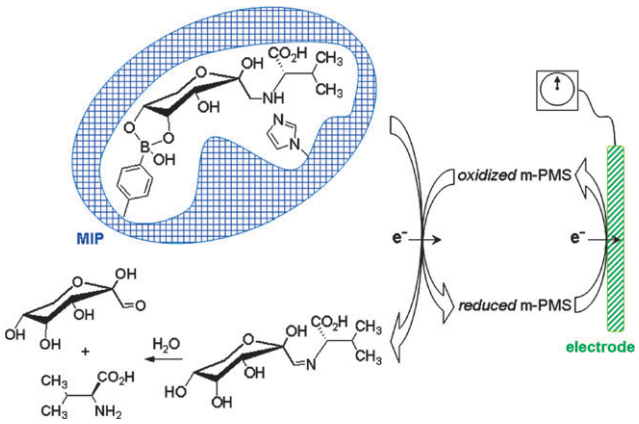

Fig. 5 Fructosyl valine sensing on a MIP-employing electrode.

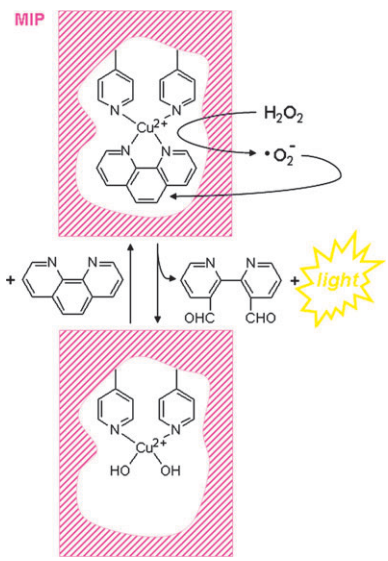

Fig. 6 Phenanthroline sensing based on the combination of molecular imprinting and chemiluminescence.

445–450 nm).[20] 1,10-Phenanthroline was destroyed during the chemiluminescent reaction, leaving a cavity and the copper binding site for a new analyte molecule. In addition, the sensor was shown to be selective for 1,10-phenanthroline against related heteroaromatic compounds such as 2,9-dimethyl-1,10-phenanthroline, 2-chloro-1,10-phenanthroline and 1,7-phenanthroline, indicating that these phenanthroline derivatives are accepted with difficulty into the catalytic cavity of the polymer.

Such sensing experiments were carried out in unbuffered solutions (*e.g.*, aqueous H_2O_2), which indicates that the field of activity of biomimetic catalysts can be extended to abiotic conditions, as opposed to most natural enzymes.

3.2.2 Self-assembled polymer-based sensors. The self-assembling of polymers, that is to say the combination of polymers that include complementary chemical functions (*e.g.*, opposite electrostatic charges), allows the formation of composite materials that exhibit three-dimensional structures and original properties to act, for instance, as membranes.[21]

Following this concept, Haruyama *et al.* designed and synthesized an artificial enzyme through the self-assembly of a cationic polyhistidine polymer, an anionic polystyrene sulfonate polymer and Cu(II) ions, leading to the formation within the composite material of multiple nanocavities that displayed phosphodiesterase activity towards biologically-relevant phosphates such as ATP, ADP and AMP (Fig. 7).[22] The release of phosphate anions could be monitored either amperometrically[23] or potentiometrically.[24] The authors showed that the hydrolytic activity significantly increased from AMP to ATP, and that the selectivity of the sensor was attributed to the easier penetration of the longer phosphate residues into the nanocavities (*i.e.*, the active sites of the artificial enzyme).

One potential application of such an artificial enzyme-based biosensor is in the field of biosurveillance, since ATP is a molecule found in every biological system and, as a consequence, is a good marker of microbial contamination by microorganisms during the preparation and processing of food. Furthermore, its cost of production is evidently negligible compared to that of already commercially-available ATP sensors, which typically rely on a chemiluminescence reaction involving the expensive luciferase enzyme.[25]

3.3 Macrocycle-based sensors

Cyclodextrins (CDs) are well known, naturally-occurring cyclic oligosaccharides composed of at least six α-1→4-linked α-D-glucopyranoside units (Fig. 8). CDs have found extensive use in clinical, industrial and environmental applications[26] since their cavities, of well-defined size, can selectively

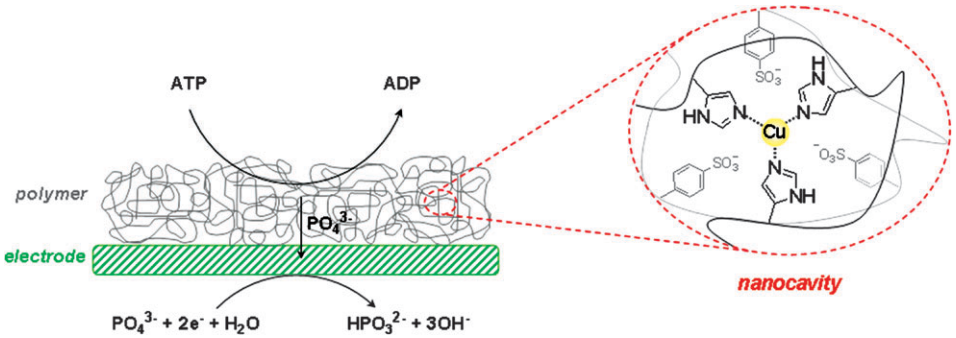

Fig. 7 A polymeric artificial phosphodiesterase for the amperometric detection and quantification of ATP.

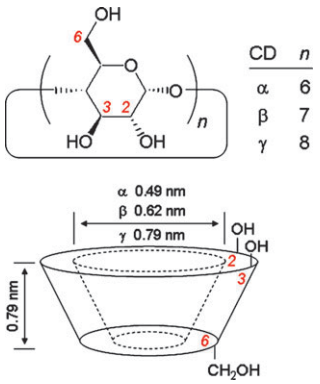

Fig. 8 The general chemical structure of a cyclodextrin (top) and its schematic three-dimensional representation (bottom).

incorporate a wide range of guest molecules, mainly through hydrophobic interactions and hydrogen bonding.[27] Therefore, a suitably modified cyclodextrin may bind a guest molecule and consequently catalyse its chemical transformation, mimicking an enzyme-catalysed reaction.[28]

Kataki and Morgan have shown that a simple β-CD derivative with a nicotinamide group attached to its secondary face could behave like a dehydrogenase enzyme, which achieves oxidation reactions of alcoholic functions by the transfer of hydrogen from a reduced substrate to an electron acceptor, the coenzyme nicotinamide adenine dinucleotide (NAD^+) (Fig. 9).[29] The catalytic reaction was monitored by measuring the current while the applied potential was varied (cyclic voltammetry), and a substrate concentration dependence of the magnitude of the current was observed. In contrast to branched alcohols like propranalol, it appeared that only linear substrates such as dopamine[30] were small enough to fit within the β-CD cavity, along with the nicotinamide group, therefore allowing the oxidation reaction to occur. In addition, immobilisation of the artificial enzyme in a sol–gel matrix did not modify its catalytic properties, indicating that a re-usable alcohol sensor is feasible.

A significant aspect of this study lies in the fact that, on the contrary to the simple nicotinamide derivative used here, coenzyme NAD^+ is unstable and impractical,[31] highlighting the benefits one can gain by using abiotic catalysts, since they can be more robust than their natural analogues.

Other chemical modifications can obviously confer new catalytic properties on CDs. For instance, the addition of an imidazolyl group at the C3 position of a β-CD led to the dramatic acceleration of the hydrolysis reaction of nitrophenyl acetate at a neutral pH relative to the unmodified β-CD (Fig. 10).[32] The catalytic properties of this β-chymotrypsin mimic were exploited for the quantification of nitrophenyl acetate in the micromolar concentration range either spectrometrically[32a] or amperometrically.[32b] In addition, it was confirmed that the catalytic activity of this miniature enzyme was significantly different for the *ortho*-, *meta*- and *para*-isomers of nitrophenyl acetate,[33] therefore allowing their discrimination. The scope of application of such sensors is wide, since many chemicals commonly used in industrialized societies—including detergents, antioxidants and agricultural chemicals—contain aromatic esters, which can undergo enzymatic or bacterial degradation to produce toxic phenol derivatives that accumulate in food, soil and water.[34]

Native protease β-chymotrypsin achieves only a modest rate acceleration of the hydrolysis reaction of nitrophenyl acetate in buffered aqueous solutions at an optimum pH of 8.2. In

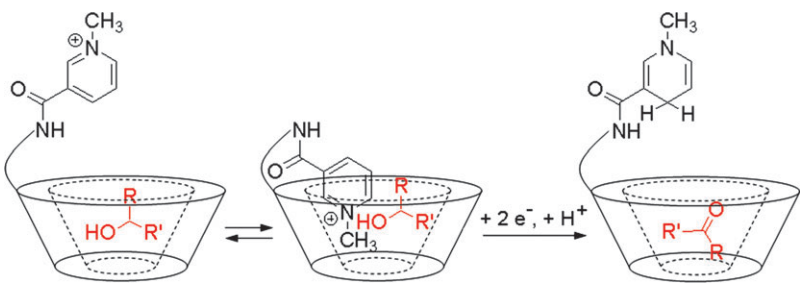

Fig. 9 The dehydrogenase mimic-catalyzed oxidation of alcohols.

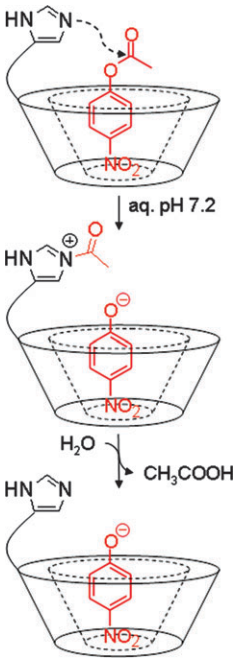

Fig. 10 The β-chymotrypsin mimic-catalyzed hydrolysis of phenolic esters.

contrast, the reported modified β-CD can work at wide range of pH values and displays good solubility in aqueous, as well as in organic media.

3.4 Peptide-based sensors

The activity and selectivity of native enzymes rely on complex folding, resulting in a precise spatial arrangement of a small number of catalytically important amino acids. Thus, the presentation of these functional groups on small peptides with well defined secondary structures is an attractive choice for the preparation of effective enzyme mimics.[35]

We recently showed that a cyclic decapeptide, which displays—in a spatially controlled manner[36]—three positive charges (*via* the presence of lysines) and a nucleophilic/basic centre (*via* the presence of a histidine residue), was able to catalyse the hydrolysis of a negatively-charged colourless fluorogenic ester, yielding a yellow-coloured fluorescent product in water and at physiological pH (Fig. 11).[37,38]

The catalytic activity was credited to either nucleophilic or general base catalysis, assisted by electrostatic molecular recognition of the substrate (Fig. 12).

The catalytic decapeptide displayed different inhibition behaviours in presence of anionic metabolites and, on the contrary to the other negatively-charged potential inhibitors tested, ADP strongly slowed down the formation of the fluorescent product by virtue of cooperative inhibition;[40] hence allowing its spectroscopic or visual detection (Fig. 13). This kind of sensor may find future use in biological applications, such as in the detection of protein kinase activity.

We then decided to apply this strategy of detection to the sensing of heparin, an anionic and polymeric glycosaminoglycan that is clinically used as an anticoagulant, and is administered at dosing levels as low as 80 nM.[41] Since short cyclic or linear peptides that incorporate several lysine/arginine

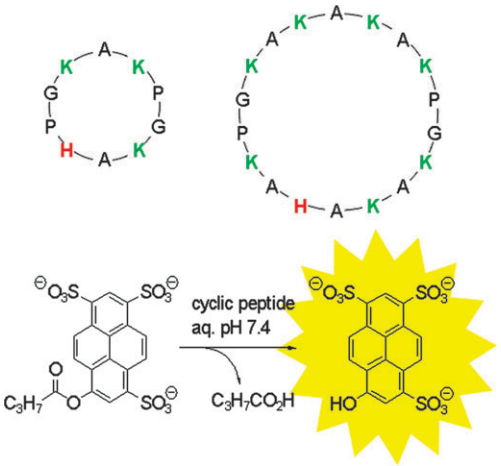

Fig. 11 Top: catalytic deca- and octadecapeptides; nucleophilic/basic and cationic amino acids are highlighted in red and green, respectively. Bottom: the miniature esterase-catalyzed hydrolysis of a colourless fluorogenic ester to give a fluorescent yellow-coloured product.

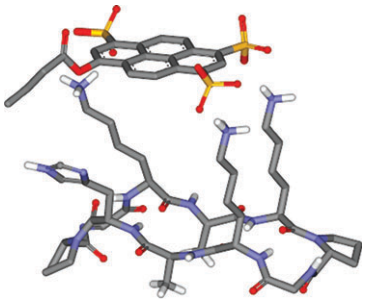

Fig. 12 A hypothetical complex between the catalytic decapeptide and the fluorogenic ester.[39] Such a recognition event results in the bringing of the nucleophilic/basic peptide residue and the substrate's ester function into close proximity.

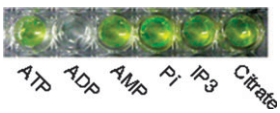

Fig. 13 Photography of the kinetic assay wells, in which were performed decapeptide-catalysed hydrolysis reactions of the fluorogenic ester in presence of different anionic metabolites, acting as inhibitors.

residues are known to efficiently bind heparin through electrostatic interactions,[42] we initially imagined that the highly anionic nature of heparin would make it an efficient inhibitor for our decapeptidic enzyme mimic, thus allowing its sensing. However, kinetic studies revealed that it was not able to bind the ester substrate and therefore to catalyze its hydrolysis under the low concentration conditions necessary for the detection of heparin in clinical settings. We therefore designed and prepared a catalytically-active cyclic octadecapeptide (Fig. 11), which was build upon the success of the previous miniature esterase, except that it incorporates a larger number of cationic lysine residues (*i.e.*, seven), leading to enhanced molecular recognition with the substrate.[43] Using this optimized peptide, heparin could be detected with a remarkable 13 nM sensitivity, which is among the lowest detection

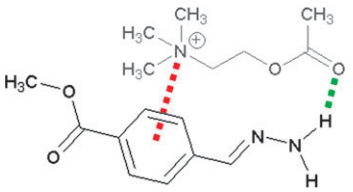

Fig. 14 Molecular recognition between an acetylcholine esterase mimic (black) and acetylcholine (grey). Probable hydrogen bond and cation-π interactions are highlighted in green and red, respectively.

limits reported so far for fluorescent heparin sensors, and is markedly below the lowest administered doses of heparin.

This study highlights the convenience of using enzyme mimics in biosensors, since they are easily adaptable (*i.e.*, chemically malleable) to give them desirable properties.

3.5 Organic molecule-based sensors

Recently, Sarkar *et al.* designed and synthesized a simple, small organic molecule, 4-[(1*E*)-ethanehydrazonoyl]benzoic acid, which behaves like an acetylcholine esterase mimic.[44,45] The miniature enzyme was actually able to bind acetylcholine, most likely *via* hydrogen bond and cation-π interactions, and then to catalyse its hydrolysis under physiological conditions, probably through a mechanism that involves the terminal nitrogen atom of the miniature enzyme in general base (or nucleophilic) catalysis (Fig. 14).

After immobilization of the artificial enzyme on an electrode surface, the resulting amperometric biosensor for acetylcholine gave a response that was comparable to that of an acetylcholine esterase-based electrode. Moreover, the authors showed that the signal's magnitude dropped at high acetylcholine concentrations when using the native enzyme-based biosensor, most likely because of the inhibitory effect of the substrate; whereas this effect was not observed with the enzyme mimic-based biosensor.

4. Conclusion and perspectives

Although it is obvious that artificial enzymes can hardly reach the levels of efficiency and selectivity of natural catalysts, we have shown in this Perspective that these synthetic objects can—in some cases—overcome their limitations. We have also shown that a variety of enzyme mimics, spanning from inorganic clusters to small organic molecules, can be used for a biosensing purpose, and the wide range of artificial enzymes reported in the literature (*e.g.*, catalytic dendrimers,[46] synthetic pores[47] or capsules[48]) definitively emphasise the extensive possibilities for discovering new artificial enzyme-based biosensors.

While designing artificial enzymes from scratch remains a formidable and stimulating challenge for chemists, a recent successful trend is the use of selection approaches,[14,49] in recognition of the fact that the *de novo* design of enzyme mimics is often both imperfect and time consuming. Among these selection approaches, a particularly promising one inspired by Nature is dynamic combinatorial chemistry.[50] Introducing a transition state analogue of a chemical transformation within a thermodynamically-controlled library of receptors can shift the equilibrium towards potential catalysts,[51] in a selection and amplification process that is, after all, the way in which native enzymes have evolved their sophisticated functions. Investigations in this direction are currently under way in our laboratory.

Whatever the approach chosen, one can be optimistic for the future of artificial enzyme-based biosensing, given the promising degrees of success met so far in this emerging research field.

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