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TUTORIAL REVIEW

Sensing through signal amplification

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The naked eye detection of single molecules in a complex mixture is the ultimate detection limit. Since a single molecule is unable to generate a strong enough signal, sensing methodologies able to reach that limit by necessity need to rely on signal amplification. This *tutorial review* describes various molecular approaches towards signal amplification in which a single analyte molecule affects the properties of a multitude of reporter molecules. Sensing by advanced instrumentation or changes in the physical properties of materials are excluded. The review is divided into four parts (catalysts, macromolecules, metal surfaces and supramolecular aggregates) depending on the species responsible for generating reporter molecules. Although on first sight apparently very diverse in nature, the majority of approaches rely on two key concepts: catalysis and multivalency. The ability of a catalyst to convert a multitude of substrate molecules into product (defined by the turn over number) makes a catalyst an intrinsic signal amplifier in case the chemical conversion of the substrate is accompanied by a measurable change in physical properties. For sensing purposes, catalytic activity must depend on the interaction between the analyte and the catalyst. Sensing using multivalent structures such as polymers and functionalized nanoparticles relies on the ability of a single analyte molecule to affect the properties of a multitude of reporter molecules collected in the multivalent structure. Chemical sensing systems will be discussed with detection limits that indeed go down to a few molecules and can rival the best biological assays. It will be shown that the most sensitive methods rely on a cascade of amplification mechanisms.

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1. Introduction

The sensing of ultralow analyte concentrations is of crucial importance not just in the chemical laboratory, but also in many areas of society. The ability to detect proteins or other

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biomarkers with high sensitivity permits a faster disease diagnosis which increases the chances of successful treatment. DNA sensing and the ability to detect single nucleotide polymorphism (SNP) is emerging as key component of molecular diagnostics, but at the same time is also important in forensics science. The sensing of metal ions and (in)organic pollutants is a critical issue in assessing environmental pollution and also food contamination. The ability to detect explosives or residuals is a fundamental asset in assuring security against terrorism and also in the clearance of landmines or other explosives.

Analyte detection limits are continuously being pushed down by the gradual improvement of existing analytical technologies. In fact, the single molecule detection level can now be reached using various microscopies (TEM,[1] SEM,[2] AFM),[3] surface plasmon resonance (SPR)[4] and surface enhanced Raman spectroscopy (SERS),[5] the latter two also permitting an application for sensing purposes. Nonetheless, the ultimate challenge in sensing is the development of methodology that permits the naked eye detection of a single molecule in a complex mixture, that is, without the need for sophisticated instrumentation. Such a bottom-up approach to sensing is a perfect task for chemists as it requires the ability to master molecular properties both on the molecular and collective level.

The sensitivity of any analytical protocol is determined by the correlation between the analyte concentration and the strength of the output signal (Fig. 1). The importance of this correlation is very nicely illustrated by the development of biological assays. The first assays relied on the direct detection of the analyte, which implied that radioactive labels needed to be used to permit the generation of a detectable output signal.[6] Clearly, the use of radioactive isotopes is highly inconvenient and for that reason the introduction of enzyme-linked immunosorbent assays (ELISAs) was a very important breakthrough.[7–9] Shortly, in an ELISA a molecular recognition event by the analyte and a surface bound receptor co-immobilizes an enzyme, which is used for the generation of an easy detectable signal.[10] The conceptual novelty lies in the application of signal amplification: a single analyte molecule recruits an enzyme able to generate a multitude of reporter molecules, which is ultimately determined by the turn-over number of the enzyme. The increased concentration of reporter molecules renders detection using fluorescence and UV/Vis-spectroscopy feasible. Notwithstanding signal amplification, the lower detection limit for proteins using ELISAs is typically in the pM range, which is still far away from the single molecule level. The introduction of immuno-PCR made such concentration levels for the first time detectable.[11] In immuno-PCR assays the enzyme tag has been substituted for a DNA tag, which is subsequently amplified using the polymerase chain reaction (PCR).[12] The exponential growth curve of the PCR reaction allows for an up to 10^5 fold increase in sensitivity compared to ELISA. In the original paper, Cantor *et al.* reported on the reliable and reproducible detection of as few as 580 antigen molecules in 45 μl.[11]

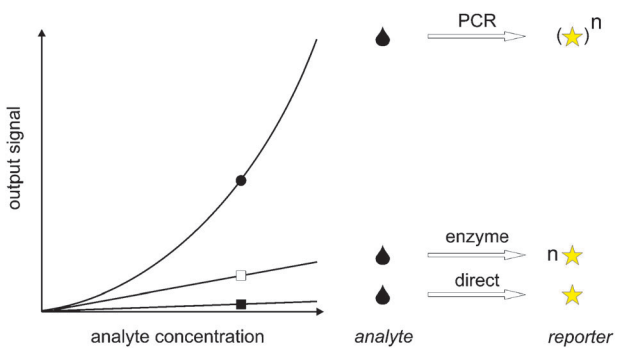

Fig. 1 Strength of the output signal as a function of the analyte concentration in case the signal results from a direct readout (■), is amplified by an enzyme (□) or amplified by means of PCR (●). It should be noted that the output signal is time-dependent when enzymes are used and cycle-dependent when PCR is used.

The development of biological assays provides a clear message: chemical approaches towards sensing need to rely on signal amplification in order to detect analytes at ultralow concentrations. In this tutorial review, it is our intent to highlight (and celebrate) the innovative methods that chemists have come up with to amplify weak input signals for sensing purposes. Some of these methods are entirely synthetic, others are hybrid methods relying also on the use of natural components such as enzymes. Rather than providing a detailed overview of a selected approach, it is our aim to illustrate the conceptual connectivity between very different methodologies. As boundary conditions we have chosen to focus on molecular signal amplification, which implies that systems will be discussed in which a single analyte molecule is able to affect the properties of a multitude of reporter molecules. This excludes a discussion on highly sensitive analytical techniques (SERS, SPR, *etc.*) or methods relying on changes in physical properties (due to nanoparticle aggregation for example). Nonetheless, the molecule-based approaches are so many and so versatile that only key examples from the literature are discussed, chosen by the criterion of permitting an illustration of the underlying concept and a discussion of the advantages and critical issues. Regrettably, this implies that many beautiful and elegant examples are left outside. References towards reviews giving extensive coverage of the respective areas are included. The review is divided in four parts (catalysts, macromolecules, metal surfaces, supramolecular aggregates) relying on the species responsible for signal amplification. These sections are preceded by a section in which signal amplification in the most frequently applied protocols is analysed quantitatively.

2. The efficiency of signal amplification

The vast majority of signal amplification strategies rely on some basic concepts, which are schematically depicted in Fig. 2. In all cases, the interaction of an analyte with a receptor initiates a process that results either in the formation of a large amount of reporter molecules through catalysis or affects the properties of multiple reporters through multivalency. In this section the various parameters that determine the maximum signal amplification that can be reached for each of these approaches will be discussed.

A discussion of the analyte-induced activation of a catalyst for signal generation was already provided by Anslyn and Zhu[13]

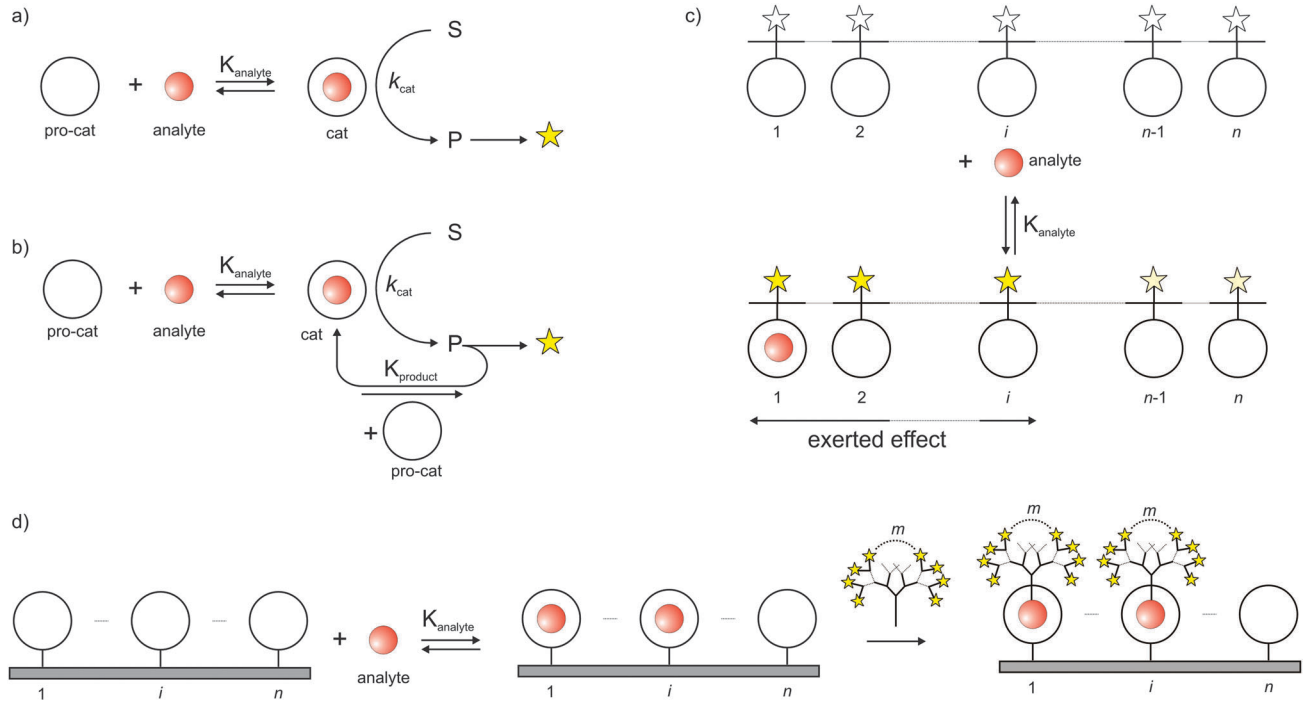

Fig. 2 Approaches towards signal amplification. (a) Catalytic amplification. An analyte activates a catalyst, which then produces multiple copies of a reporter molecule. (b) Catalytic self-amplification. An analyte activates a catalyst, which results in the production of reporter molecules. These not only generate a signal, but are also able to activate the catalyst. (c) Analyte-induced modification of a collective property. The binding of a single analyte molecule to a receptor affects the properties of neighbouring units through signal transduction. (d) Multivalent surfaces for binding of multiple analyte molecules. Recruitment of multiple reporters using multivalent scaffolds such as polymers, dendrimers or nanoparticles amplifies the signal.

of which we repeat here the key elements (Fig. 2a). Signal amplification is favoured by a high binding affinity between analyte and receptor (high K_{analyte}) and by working with an excess of pro-catalyst, thus generating one active catalyst per molecule of analyte ([cat] $\approx$ $[A]_0$). The linear relationship that is thus established facilitates also a quantitative correlation between analyte concentration and signal output. With regard to the catalytic part, three crucial issues (which are valid for any form of signal amplification relying on catalysis) are fundamental: a low background rate for the uncatalysed conversion of S into P ($k_{\text{uncat}} \ll k_{\text{cat}}$), the formation of a product P which can be easily detected, and the absence of product inhibition. Working at saturation conditions for the catalyst (at high [S]) gives that the rate of product P formation is given by:

$$\frac{d[P]}{dt} = k_{\text{cat}}[\text{cat}] = k_{\text{cat}}[A]_0 \qquad (1)$$

thus

$$[P]_t = k_{\text{cat}}[A]_0 t \qquad (2)$$

Analysis of eqn (2) leads to two observations. Under optimal conditions (quantitative complex formation between A and pro-cat and catalyst saturation concentrations of S) a linear relationship exists between the analyte concentration and the strength of the generated signal. Second, the strength of the signal is a function of time, *i.e.* measurements benefit from extended time intervals between analyte addition and signal measurements. Clearly, to avoid long delays, from a practical point of view this implies a preference for catalysts with a high turn-over frequency.

The self-amplification scheme pushes catalytic signal amplification to a much higher level (Fig. 2b). Self-amplification occurs in case the product P is able to activate the pro-catalyst. This can occur in various ways, which will be evidenced by some examples discussed later, but in its most rudimentary form occurs because association of P to the pro-catalyst causes activation in a similar way as the analyte (defined by a high binding constant K_{product} and [pro-cat] $\gg$ $[P]_{\text{total}}$). The attractive point in terms of signal amplification is obviously the exponential increase in P (or signal) as a function of time. This can be expressed using a similar analysis as used for the linear catalyst activation. Again, optimal conditions for signal amplification are a high binding constant of both A (K_{analyte}) and P (K_{product}) for the pro-catalyst and the use of an excess concentration of pro-catalyst. In this case the rate of formation of P is expressed by:

$$\frac{d[P]}{dt} = k_{\text{cat}}[\text{cat}] = k_{\text{cat}}[A]_0 + k_{\text{cat}}[P(t)] \qquad (3)$$

in which the concentration of active catalyst is constituted by the initial amount of analyte A_0 present and the amount of product P formed, the concentration of which is dependent on time. Consequently, the concentration of P at a given time is given by:

$$[P]_t = k_{\text{cat}}[A]_0 t + [P]_0 e^{k_{\text{cat}} t} \qquad (4)$$

which immediately reveals the exponential growth of the reporter signal as a function of time. It is evident that in a self-amplification assay, the necessity of an extremely slow background reaction becomes highly important.

Contrary to catalytic signal amplification, signal amplification protocols relying on the use of multivalent systems bear no intrinsic kinetic element for signal generation. Nonetheless, a kinetic response may occur through, for instance, slow aggregation or slow conformational changes in case these are part of the signal production process. The first approach that will be treated is signal amplification originating from the ability of a single analyte molecule to affect the collective property of a macromolecule (see § 4.1).[14] Schematically this is represented in Fig. 2c, which represents a polymer composed of monomers able to complex an analyte, which generates a signal. Whereas a single receptor–analyte complex would affect only the signal generated by that isolated complex, signal amplification occurs in case analyte binding by a single receptor along the polymer chain is sensed by neighbouring monomers. The extent of signal amplification depends on the number of monomers, *i*, affected by the single interaction. A maximum signal amplification *n* is determined by the polymer length *n* in case a single analyte–receptor interaction is able to affect all monomers of the macromolecule. The amplification factor can even exceed *n* in case communication between polymer strands occurs, for instance in thin films or upon the formation of aggregates.

The final case of signal amplification is much related and involves the detection of analytes using immobilized receptors (Fig. 2d). Particular efficient signal amplification occurs in case signal generation originates from an interaction between the analyte and the surface (for example electrochemically). In such a case, the presence of multiple receptors on the surface by itself causes a linear increase of signal intensity as a function of bound analytes. Signal amplification (by a factor of *m*) occurs in case a single analyte–receptor complex brings a multitude (*m*) of signal generating units to the surface. This can be performed by using multivalent structures such as polymers, dendrimers or nanoparticles.

This section has illustrated the factors that determine signal amplification for the approaches that have been most frequently used. It will be evident from the examples that will be discussed in the following sections that spectacular results can be obtained when some of these concepts are combined in a single detection protocol.

3. Catalysts

The ability of a single catalyst to convert a multitude of substrate molecule into product (defined by the turn over number) makes a catalyst an intrinsic signal amplifier in case the chemical conversion is accompanied by a measurable change in physical properties (typically absorbance or fluorescence). For sensing purposes, an analyte needs to be able to up- or downregulate the activity of the catalyst. This can either occur through an alteration of the catalyst conformation (allosteric effect) or through a direct competition with the substrate for the catalytic centre (intrasteric effect).

3.1 Allosteric regulation of catalytic activity

Nature employs enzymes for catalysis and their extraordinary high activity in physiological conditions renders them ideal assay components for detecting biologically interesting targets. Nonetheless, enzymes need to be engineered to render them responsive to analytes that do not naturally interact with the enzyme. An elegant example was reported by Ghadiri *et al.* who reported on the use of a protease for the amplified sequence specific detection of ssDNA.[15] A construct was engineered with a 24-mer ssDNA probe separating a zinc-metalloprotease (Cereus neutral protease—CNP) and its phosphoramidite inhibitor (Fig. 3). The choice of the enzyme was motivated by its compatibility with the DNA tether and, importantly, its high intrinsic catalytic activity (k_{cat} = 165 s^{-1}) for a rapid signal generation. The working principle of this system lies in the fact that in the absence of a complementary DNA strand, the flexible ssDNA linker permits the inhibitor to block the active site of the enzyme. The presence of a complementary DNA strand results in the formation of a rigid duplex which causes the removal of the inhibitor from the enzyme. The enzyme is thus activated and generates a fluorescent product by hydrolysing an appropriate substrate. The approach was validated by the observation that the addition of a 100 pM solution of the complementary DNA strand gave a detectable signal after just 3 minutes. A 10 pM solution required an elongated period of 80 minutes to be detected. Sequence selectivity was demonstrated by the absence of fluorescent signal in case noncomplementary DNA was added even at 10 μM concentration.

A second class of catalysts that operate under ideal conditions for targeting bio-analytes are DNAzymes and ribozymes.

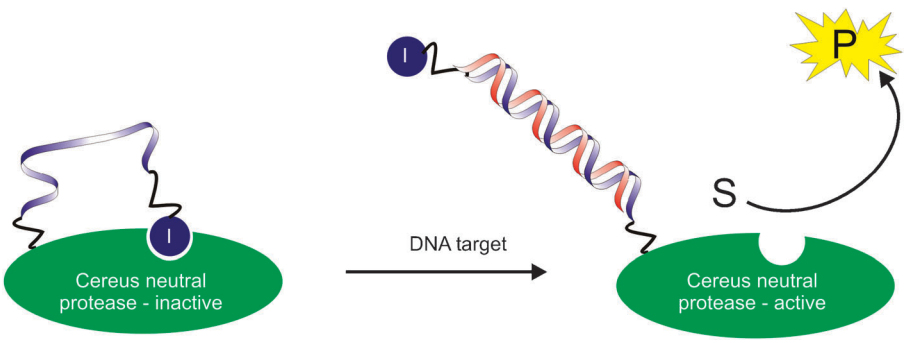

Fig. 3 Schematic representation of an inactivated inhibitor-DNA-enzyme, which is activated upon the addition of the complementary DNA target.[15]

These are nonnatural oligonucleotide-based catalysts, which, compared to enzymes, typically exhibit a higher thermal stability, can be easily conjugated to other elements, and may be prepared in large quantities using PCR. The underlying idea of using DNA (or RNA) as a source library relies on the enormous variation in the type of folded oligonucleotides available and the enormous combinatorial libraries that can be assessed (typically up to 10^{12} or 10^{14} members). DNAzymes and ribozymes are obtained through an exponential enrichment process (SELEX)[16,17] which relies on the isolation of the fraction of library members able to perform a given reaction (typically using affinity chromatography).[18] The enriched fraction is multiplied using PCR and screening is repeated. Repetitive cycles make that the library composition converges towards the catalytically active sequence, which is then isolated and characterized. Similar to enzymes, application for sensing requires that the activity of the DNAzyme or ribozyme depends on the presence of a co-factor, which is the analyte. The interesting aspect is that co-factor selectivity can be easily introduced by performing the selection procedure in the presence of the co-factor. This way, DNAzymes that require the presence of metal ions such as Pb^{2+}, Cu^{2+} or Zn^{2+} have been obtained.[19] In an elegant approach, Lu *et al.* added the final required element for detection using a DNAzyme, which is the generation of an easy detectable output signal.[20] The exploited DNAzyme is a single DNA strand able to cleave a complementary substrate DNA strand (Fig. 4). The substrate DNA strand was extended on both 3′ and 5′ ends with fragments that can hybridize with complementary oligonucleotide-fragments immobilized on Au nanoparticles. Consequently, these sticky ends cause aggregation of the Au nanoparticles resulting in a blue coloring of the solution. The addition of Pb^{2+} activates the DNAzyme which cleaves the substrate strand, thus breaking the linking unit between the nanoparticles. The resulting deaggregation is accompanied with a color shift to red characteristic of isolated Au nanoparticles, which can be detected with the naked eye. The lower detection limit of 500 nM Pb^{2+} illustrates the high sensitivity of this protocol. The addition of other divalent metal ions like Mg^{2+}, Cd^{2+}, Ni^{2+} *etc.* did not result in any color change. Importantly, the Pb^{2+} detection window could be tuned over several orders of magnitude by reducing the amount of DNAzyme and replacing it with a mutant. The same concept was used by Scrimin *et al.* for the development or a protease assay.[21] In their setup, peptides terminated at both ends with cysteinyl-groups induced aggregation of Au nanoparticles. The presence of enzymes (*e.g.* thrombin or lethal factor) able to cleave the internal enzyme-selective sequences resulted in the fragmentation of the peptides, thus preventing aggregation of the nanoparticles. The attractive feature of this approach is its high modularity: enzyme selectivity can be introduced in a straightforward manner by changing the internal peptide sequence of the cysteinyl-terminated peptide. Many DNAzymes have been developed including variants that produce colored reaction products or chemiluminescence, which allows for a direct measurement of activity.[22] Their ease of functionalization allows for an easy attachment to other assay components or surfaces, which will be shown in section 5 of this review. Nonetheless, a critical issue of DNAzymes is their tendency to suffer product inhibition, which effectively reduces their efficiency in generating multiple reporter molecules. In a recent contribution, Gianneschi *et al.* tackled this problem by employing a fluorescein labelled DNA-copolymer amphiphile which assembles into micellar aggregates with a diameter of around 20 nm.[23] The addition of DNAzyme causes the cleavage of the target bond which results in the release of a small DNA fragment containing the fluorescent tag. The advantage of this system is that the substrate molecules are present at a high local concentration within the micellar aggregates which favours displacement of the cleavage products from the DNAzyme. This permits turnover of the DNAzyme, which was indeed confirmed by the much higher

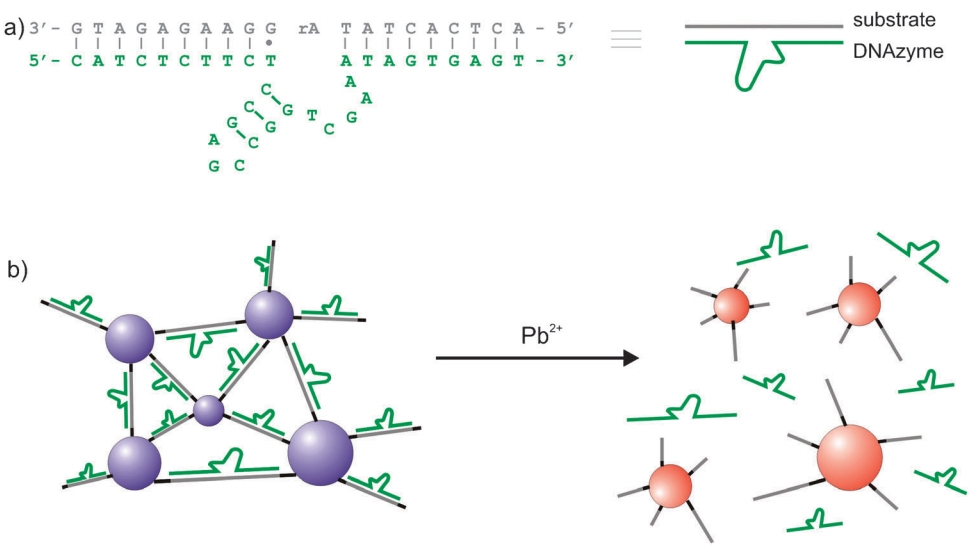

Fig. 4 (a) Secondary structure of the DNAzyme (green) and its substrate (grey) as employed by Lu *et al.* for the sensing of Pb^{2+}.[22] (b) Schematic representation of the sensing array. The substrate-strand (extended with 12 nucleobases on both the 3′ and 5′ termini) causes the aggregation of Au NPs (blue color). Complementary DNAzyme hybridizes with the substrate strand, but cleaves only in the presence of Pb^{2+}. Upon cleavages the Au NPs deaggregate resulting in a color change to red.

fluorescence intensity (after separating the micelles) compared to an analogous experiment using a substrate molecule unable to form micelles.

The allosteric control over truly synthetic catalysts is far less developed, which is a consequence of the fact that the simultaneous control over structure and activity in supramolecular constructs is still highly challenging. Nonetheless, significant progress has been made and several examples of allosterically controlled synthetic catalysts have been reported.[24] Evidently, the application of such systems for sensing requires just a small adjustment in perspective by considering the allosteric effector as the analyte.[13] For analytic purposes synthetic systems are very attractive, because applicable also under non-physiological conditions and because potentially easy to modulate and optimize. This potential was illustrated by a seminal contribution by Anslyn and Wu in which a synthetic system was developed for the detection of Cu(II) using catalytic signal amplification.[25] The output signal is generated by fluorescent indole product **2** obtained from the non-fluorescent aniline precursor **1** through a Heck cross coupling reaction catalyzed by Pd(0) (Fig. 5). The Pd(0)-catalyst is generated *in situ* by phosphine-mediated reduction of Pd(II). Importantly, the addition of cyclam, a tetraaza-macrocycle, results in complex formation with Pd(II) which effectively prevents formation of Pd(0) and, consequently, formation of the fluorescent product **2**. Here, cyclam takes the role of a regulatory element that controls the amount of free Pd(II) (and the catalytically active Pd(0) in the system) and, in doing so, controls the rate of fluorescent product formation. Analyte Cu(II) competes with Pd(II) for complex formation with cyclam and, therefore, its addition results in the displacement of Pd(II) from the Pd(II)–cyclam complex. In turn, the presence of liberated Pd(II) sets of the conversion of **1** into **2**. A fluorescence output signal could be detected for Cu(II) concentrations down to 30 nM, although this required relatively long response times (~1.5 h). The linear correlation between the initial rate of product formation and the amount of Cu^{II} added indicates that this sensing protocol in principle also permits a quantitative analysis of the analyte concentration.

A related approach was used by Kramer *et al.* for DNA sensing.[26] A single stranded 20mer DNA oligonucleotide was functionalized at both the 3′ and 5′ termini with terpyridine ligands. In the presence of Cu(II), both terpyridine moieties fold back and form an intrastrand complex with DNA. Addition of the complementary ssDNA target results in the formation of dsDNA and disrupts the intramolecular Cu(II)·$(tpy)_2$ complex. Under these conditions 1,10-phenantroline (phen) is able to scavenge the Cu(II) ion to constitute the active catalyst Cu(II)phen which catalyses the oxidation of 2′,7′-dichlorodihydrofluorescein into the fluorescent product 2′,7′-dichlorofluorescein. The DNA target could be detected down to 5 nM concentrations and a turnover number of 5 with respect to the DNA target was reported. The low turnover number was overcome in a second generation system by coupling Zn(II) displacement from the intrastrand complex to activation of apo-carbonic anhydrase.[27] It was calculated that a single DNA target strand was amplified into 10 000 CO_2 molecules in just 30 seconds.

Mirkin *et al.* reported on a synthetic catalytic amplification system, in which the allosteric control bears a much closer resemblance to enzymes, in the sense that the allosteric effector changes the conformation of the catalyst from an inactive into an active one.[28] Catalysts were prepared according to the so-called 'Weak-Link' approach. Catalysts of this type (for example **3**) are characterized by two structural domains containing Rh(I) metal centers and a catalytic domain in which two Zn(II) ions act cooperatively (Fig. 6). The 'Weak-Link' refers to the labile thioether–Rh(I) bond, which breaks in the presence of CO and Cl^-. Although the phosphine–Rh(I) bonds remains intact, their position changes from *syn* to *anti*, which induces an increased distance between the catalytic Zn(II) ions. It was observed that in the 'open' conformation, $\mathbf{3}_{open}$, the acyl transfer from acetic anhydride to pyridyl carbinol is accelerated up to 25 times through a catalytic pathway which involves both Zn(II) ions. This enabled the possibility to detect Cl^- ions through a catalytic readout, which was confirmed by measuring the rate of formation of 4-acetoxymethylpyridine as a function of the concentration of Cl^- ions (under CO saturation). To facilitate readout, the catalytic amplification step was coupled to a pH-sensitive fluorophore which permitted a visual and spectrophotometrical monitoring of the amplification process. Chloride concentrations down to 800 nM could be detected in a straightforward manner. Successively, it was shown that the system was also amenable for the detection of small molecules such as diimines and isocyanides.[29] In particular, the observation that also the acetate ion, *i.e.* the product

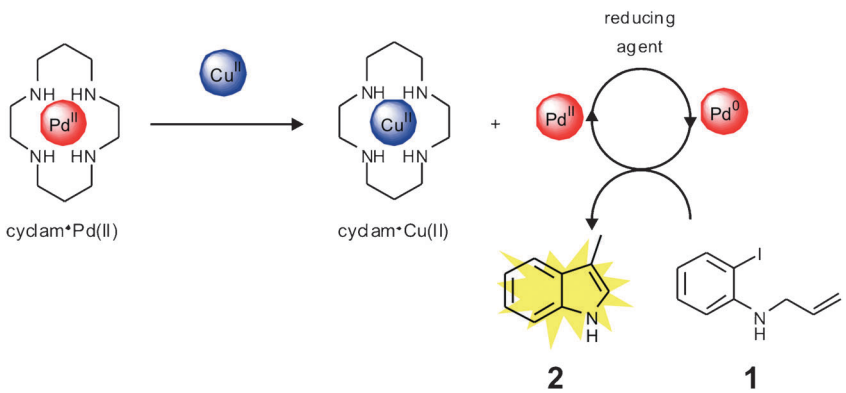

Fig. 5 Catalytic sensing of Cu(II). The addition of analyte Cu(II) displaces Pd(II) from the cyclam–Pd(II) complex. Subsequent reduction to Pd(0) initiates the catalytic conversion of **1** into the fluorescent reporter **2**.[25]

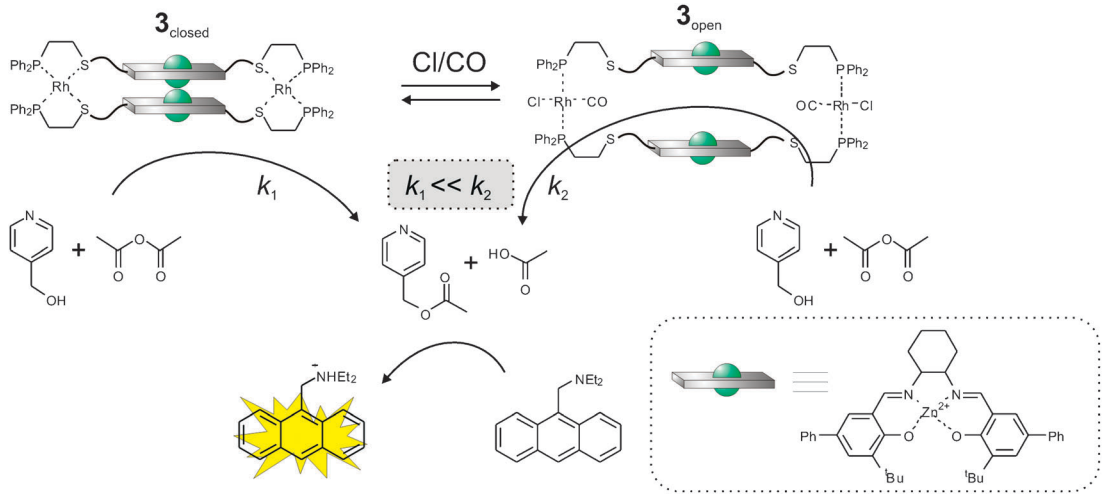

Fig. 6 The activity of catalyst **3** increases 25-fold in the presence of the analytes Cl^- and CO. The 'open' form of **3** enables a new catalytic pathway involving the cooperative action of two Zn^{2+}-metal centers.[28]

of the amplification reaction, had an allosteric effect created the possibility to chemically couple the allosteric and amplification processes.[30] In a process that can be regarded as truly artificial version of PCR, an initially small amount of acetate ion activates the catalyst, resulting in the production of more acetate, which activates more catalyst and so on. Plots of the substrate conversion against time as a function of the initial analyte concentration gave sigmoidal profiles, typical of PCR-like and self-replication processes (see below).

Self-replication or autocatalysis merits a special mentioning here, as it is one of the most powerful methods for chemical amplification.[31,32] It is used to describe systems in which a molecule catalyses its own formation resulting in an exponential growth of its concentration. Self-replication is considered to have played an essential role in the early development of life[33] and has been used to explain the homochirality of natural amino acids and sugars.[34] In particular, the observation by Soai *et al.* that the pyrimidine-containing secondary alcohol **4** is an asymmetric autocatalyst has sparked a strong interest for its spectacular efficiency.[35] The addition of **4** with a small enantiomeric excess (5%) to a mixture of diisopropylzinc and pyrimidine-5-carboxaldehyde results in the formation of more **4**, but enriched in the dominant (*S*)-enantiomer (39%) (Fig. 7) Repetitive cycles resulted in an impressive

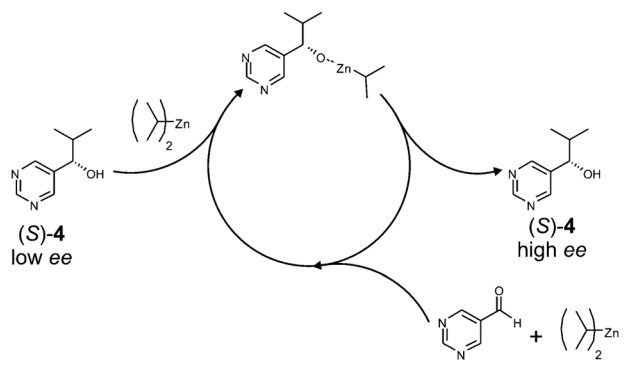

Fig. 7 Proposed reaction scheme for the asymmetric autocatalysis of (*S*)-**4**.[35]

enantiomeric excess of 85% after just three runs. The recent observation that the asymmetric amplification can be even triggered by chirality originating from $^{13}C/^{12}C$ carbon isotopes demonstrates the power of self-replication to observe small variances in the original sample.[36] Clearly, self-amplification is an intrinsic property of the reactive molecules themselves and therefore the amplification reports only on the intrinsic properties (typically enantiomeric excess) of those molecules.

A very exciting application relying on the instalment of an auto-catalytic cycle was very recently disclosed by Phillips and Baker[37] Their assay for Pd(II) relied on the use of two components: a detection agent **5** that is activated in the presence of Pd(II) (*in situ* reduced to Pd(0)), which results in the release of two fluoride anions (Fig. 8). The fluoride anions then catalyse the dissociation of the signal amplification reagent **6**, generating a yellow reporter and two new fluoride anions. In the auto-catalytic cycle that is installed, the amount of catalytic fluoride anion in the system increases exponentially and, consequently, also the concentration of reporter. The assay was able to generate a visibly detectable colour down to 0.36 ppm Pd(II). The attractiveness of this system lies in its potential adaptability to a wide variety of reagents.

3.2 Intrasteric regulation of catalysts

The term intrasteric regulation is used to describe the auto-regulation of enzymes through the use of pseudo-substrates (competitive inhibitors) that act directly at the active site.[38] Sensing based on intrasteric regulation requires a disactivation of the pseudo-substrate by the analyte either through chemical alteration or scavenging. Very recently, Rotello *et al.* presented a highly innovative sensing system referred to as enzyme-amplified array sensing.[39] Key components of the assay are the enzyme β-galactosidase (β-Gal) responsible for signal generation by hydrolyzing the fluorogenic substrate 4-methylumbelliferyl-β-D-galactopyranoside (MUG) and monolayer-protected Au colloids (Au MPCs) which inhibit the activity of β-Gal through complex formation with the enzyme (Fig. 9). The Au MPCs are displaced from β-Gal upon the addition of analyte proteins, which restores catalytic

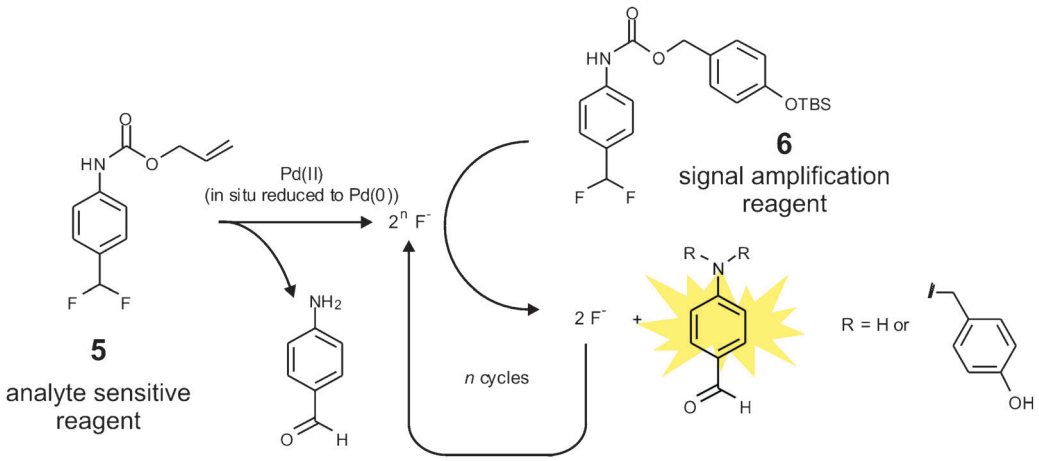

Fig. 8 Sensing array for Pd(II) relying on autocatalytic amplification.[37]

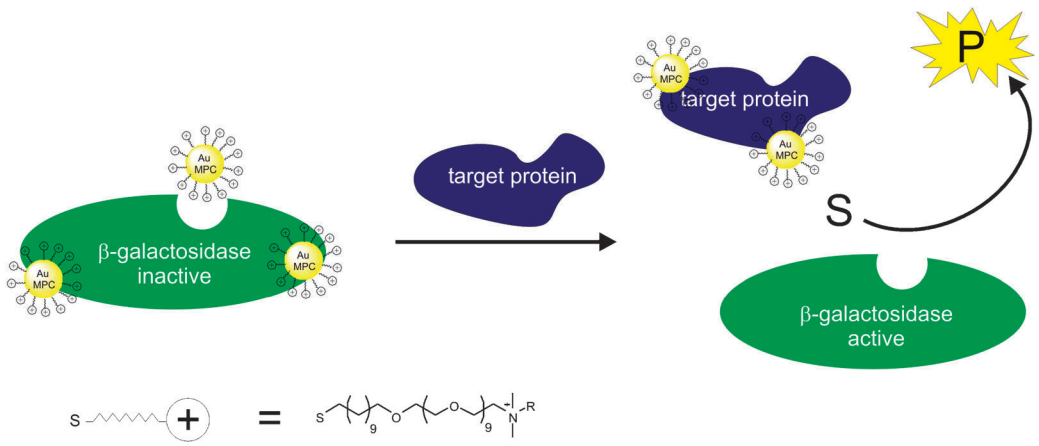

Fig. 9 Schematic representation of the Au MPC assay developed by Rotello *et al.*[39] Au nanoparticles covered with charged headgroups inhibit the enzyme β-galactosidase. Au MPCs are displaced from the β-galactosidase surface upon the addition of a target protein, which restores the catalytic activity of β-galactosidase.

activity generating a fluorescent signal. Complexation between the positively charged Au MPCs and β-Gal is driven by electrostatics and the effectiveness of enzyme inhibition was evidenced by the progressive decrease in enzymatic activity in the presence of increasing concentrations of Au MPCs. Given the aspecific nature of the interaction between Au MPCs and β-Gal, a clear challenge in this set up is the issue of analyte selectivity. This was resolved by using a displacement assay for sensing the analyte proteins. It was observed that the inhibitory power of a series of six Au MPCs containing differently charged headgroups was dependent on the nature of the headgroup (linear–branched, aliphatic–aromatic). The addition of a set of nine analyte proteins with varying isoelectric points to the individual β-Gal/Au MPC complexes gave different responses resulting in specific response patterns for each analyte protein. On this basis a fluorescent displacement assay was set up in which the overall response was correlated to the analyte input through linear discriminant analysis (LDA). A canonical score plot of the three major factors revealed nine distinct clusters for each of the analytes. Importantly, enzymatic amplification of the input signal permitted differentiation of the analytes in the 1 nM range, which is significantly lower compared to other methods (1–350 μM). An identification accuracy of 92% was obtained from a screening of 60 samples of unknown analyte composition at 1 nM concentration. The ability to perform these analyses also in desalted human urine marks a big step towards a diagnostic application.

Prins, Scrimin *et al.* have recently reported on the use of catalytic Au MPCs for the detection of enzyme activity.[40] Au MPC **7** highly efficiently catalyzes the transphoshorylation of 2-hydroxypropyl-4-nitrophenyl phosphate (HPNPP) exhibiting a rate acceleration over 30.000 (k_{cat}/k_{uncat}) under saturation conditions in buffer (pH 7.5) (Fig. 10).[41] Catalysis originates from the cooperative action of two TACN·Zn(II) complexes on the periphery of Au MPC **7**. It was observed that oligoanionic species, such as the tripeptide AcNH-DDD-OH and ATP, act as inhibitors for catalysis because of complex formation with the positively charged surface. Binding studies revealed that the free energy of binding between inhibitor and Au MPC **7** increases linearly as a function of the number of negative charges. This implies that the system could act as a sensor for enzymes that are able to switch-off the inhibitory ability of the inhibitors by converting these into smaller fragments. This was illustrated by incubating a 5 μM solution of AcNH-DDD-OH

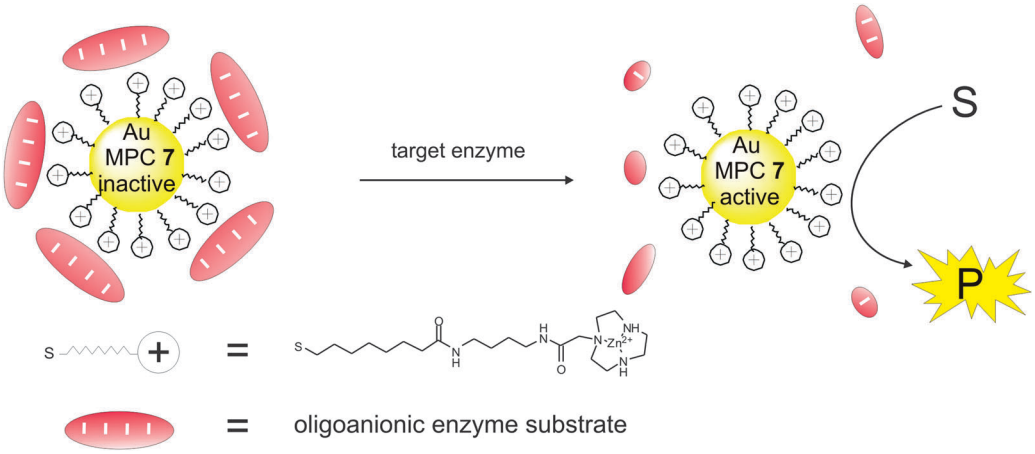

Fig. 10 The catalytic activity of Au MPC **7** is inhibited in the presence of oligoanions (such as tri-Asp or ATP).[40] The addition of an enzyme able to hydrolyze the substrate in small fragments restores the catalytic activity of Au MPC **7**.

with the non-specific protease Subtilisin A in concentrations down to 66 nM for 1 hour. Measurement of the absorbance at 405 nm after the addition of Au MPC **7** and HPNPP gave indeed a correlation between the amount of enzyme present and the rate of hydrolysis of HPNPP. Because relying on a cascade of two catalytic events, the final strength of the output signal depends on two time frames: first, the exposure time of the substrate to the enzyme (or, alternatively and more important from an analytical point of view, the concentration of enzyme present) and, second, the time permitted for production of the *p*-nitrophenol reporter molecule. The system has general applicability in the sense that enzyme selectivity can be introduced by varying the peptide sequence of the inhibitor. A signal amplification factor of 18 (compared to a conventional assay) was obtained targeting caspase 1.

4. Macromolecules

Macromolecules are attractive components in a signal amplification assay for several reasons.[42] Through the covalent connectivity of monomers within the macromolecule a single interaction between a monomeric subunit and the analyte can be 'sensed' by many neighbouring units, which by itself increases the sensitivity. This is particularly important in case the macromolecule possesses a collective property, which is absent in the monomers. A maximum signal amplification is obtained in case the interaction of an analyte with a single subunit is able to affect that collective property. Alternative applications of macromolecules for amplified sensing rely either on the analyte-triggered dissociation or growth of the macromolecule.

4.1 Conjugated or chiral polymers

The collective properties of macromolecules that have been mainly exploited for amplified sensing are electron conductivity in conjugated polymers and chirality expressed by helical polymers. In the past decades, conjugated polymers have been widely exploited as chemosensor components, because of their unique electronic and photophysical properties.[43,44] In particular, the electrical conductivity is highly sensitive to very small perturbations induced by the interaction of an analyte with recognition units in the polymer. In addition, the fluorescence emission of conjugated polymers is often dominated by energy migration to local minima in their band structures. Swager *et al.* have applied these properties in a so-called 'molecular wire approach' towards the development of fluorescent chemosensors.[14,45] The molecular wire is a strongly fluorescent cyclophane-containing conjugated polymer **8** with a phenyleneethylene-backbone (Fig. 11). Fluorescence quenching occurs upon the addition of the electron transfer quenching agent paraquat $\mathbf{PQ^{2+}}$. A dramatic quenching enhancement was observed compared to the same concentration of monomeric cyclophane units. For example, the addition of 3.45×10^{-4} M of $\mathbf{PQ^{2+}}$ to a 3.69×10^{-6} M concentration of cyclophane **9** resulted in a 30% quenching of fluorescence for the monomer, but a near quantitative quenching in case the cyclophane was incorporated in the polymer. The improved quenching enhancement in the molecular wire originates from the migration of the excited state through the polymer backbone thus permitting a sampling of a multitude of cyclophane receptors, rather than just one. In case only

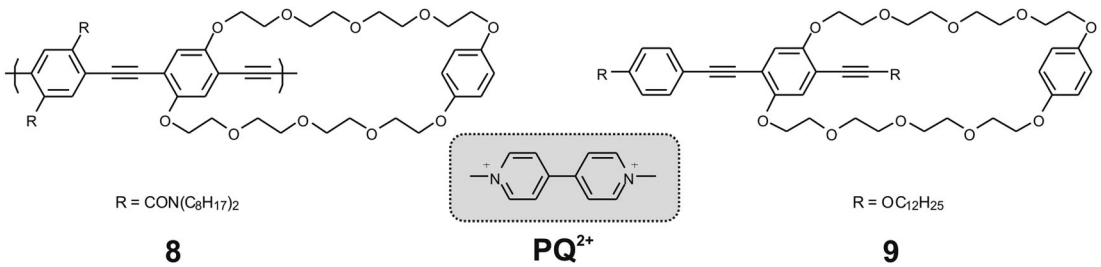

Fig. 11 Conjugated cyclophane-containing polymer **8** used for sensing of $\mathbf{PQ^{2+}}$ and the monomeric reference unit **9**.[14]

one of the receptors has formed a complex with $\mathbf{PQ}^{2+}$ quenching of the excited state occurs. Quantitative fluorescence studies confirmed that quenching occurs through a static process in accord with host–guest complexation. In this setup, quenching enhancement is favoured both by a long lifetime of the excited state and a high rate of energy migration in the polymer backbone as this permits a sampling of more receptor units. In an ideal case, where the association constants for complex formation are additive and the diffusion length of the excitation state exceeds the length of the polymer, the maximum signal enhancement is determined by the degree of polymerization. A further increase in sensitivity was obtained in case the polymers were deposited as films, because in the solid state also communication between polymer backbones is possible.[46]

Boudreau, Leclerc *et al.* provided an example in which polymer fluorescence was turned on, rather than quenched, upon the addition of analyte.[47,48] The principal constituent of their system is the water soluble, cationic polythiophene **10**, of which the chromogenic and fluorogenic properties strongly change upon the addition of single stranded or double stranded oligonucleotides (Fig. 12). For example, a yellow-to-red color change and a complete fluorescence quenching is observed upon the stoichiometric addition of the capture probe 5′-CATGATTGAACCATCCACCA-3′. This is ascribed towards the formation of a planar, highly conjugated form of the polythiophene backbone. The addition of the complementary DNA target strand results in a blue shift and increase in fluorescence. Spectroscopic data suggest the formation of a superstructure in which the oligothiophene is wrapped in a helical fashion around the DNA duplex (less conjugated, less planar). The sensing system gives excellent results in terms of sensitivity and selectivity. A two bp-mismatch 20mer DNA sequence was unable to induce triple helix formation, whereas a single bp-mismatch could be discriminated under kinetic monitoring. For 20mer oligonucleotide targets a lower detection limit of around 310 molecules per 150 μl sample (3.6×10^{-18} M) could be achieved. Importantly, the multivalency of the system permitted the application of a new readout mechanism based on FRET (Förster resonance energy transfer).[49] Labelling of the DNA capture probe with a fluorophore (Alexa Fluor 456)

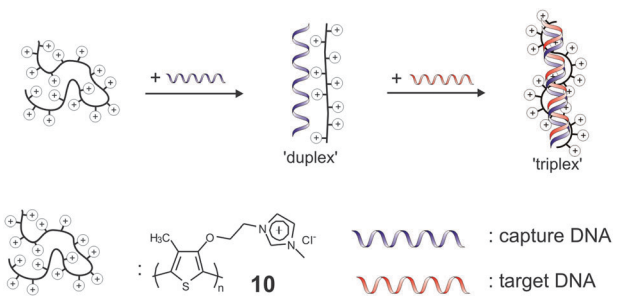

Fig. 12 Addition of a capture DNA strand induces a planar, highly conjugated conformation of polythiophene **10**. The change in conformation is accompanied with a yellow to red color change and a complete fluorescence quenching. Upon addition of the complementary target DNA strand a triplex structure is formed in which **10** is wrapped in a helical fashion around the DNA duplex, resulting in a blue shift and increase in fluorescence.[47]

that absorbs at the emission wave length of the polymer (530 nm) results in the instalment of FRET upon formation of the triple helix. Interestingly, starting from a large number of probes ($\sim 10^{10}$ copies), the addition of just 30 copies of the 20mer target in 3 ml could be easily detected. A lower detection limit of 5 molecules in 3 ml (corresponding to 3 zM) was estimated for this system. As pointed out by the authors, signal amplification not only arises from the large optical density of the polymer, but presumably also from a fast and efficient energy transfer from the helical and well-structured polythiophenes to many neighbouring chromophores. This process is referred to as 'superlighting' or 'fluorescence chain reaction (FCR)'. Evidence for the formation of nano-aggregates of the duplexes (also after hybridization) was obtained from dynamic light scattering measurement, which indicate that energy transfer between different oligothiophenes could indeed play a role in the signal amplification process.

In the early 1990s, seminal contributions by Green *et al.* revealed the fascinating correlation between the macromolecular helicity of polymers and the chirality of its constituent monomers.[50] On one hand it was observed that helical polyisocyanates had a propensity to favour a single handedness in case a small amount of enantiopure monomer (less than 1%) was copolymerized with an excess of achiral monomer. This phenomenon is now commonly referred to as the 'sergeants-and-soldiers principle'.[51] Secondly, it was shown that a polymer comprised of a mixture of *R*- and *S*-monomers with a small enantiomeric excess (down to 12%) expressed macromolecular helicity as if the polymer was composed of the major enantiomer only ('majority rule').[52] Both observations have been frequently observed in a variety of systems and illustrate the potential of chiral macromolecules or supramolecular assemblies to report on small energetic differences caused by the monomers. Nonetheless, for sensing purposes these effects need to be induced by external analytes through noncovalent interactions with the chiral polymer.[53] In that respect, key contributions were made by Yashima *et al.* studying the chiroptical properties of polyacetylenes containing pendant functional groups able to form noncovalent complexes with analytes. Typically, the addition of analytes induces a preferred handedness in the polymer resulting in an intense circular dichroism (CD), which is the characteristic output signal for these systems. Here, the working principle is exemplified discussing a system that was developed to detect very small enantiomeric excesses in α-amino acids.[54] A stereoregular *cis-transoidal* poly(phenylacetylene) ($M_n = 19.7 \times 10^4$) containing peripheral crown ethers was used (**11**), in which the helical polyacetylene backbone serves as the chromogenic reporter unit and the bulky crown ethers serve both as amino acid receptors and for the rigidification of the polymer in order to improve cooperative behaviour (Fig. 13). The addition of either one of the 19 natural amino acids (with the exception of L-Pro) in DMSO resulted in intense ICDs, reaching a constant value upon the addition of 1 equivalent of amino acid (with respect to the crown ether) at −10 °C. For L-Ala it was observed that the addition of 0.1 equivalent was sufficient for the nearly completely induction of single handedness in the polymer and even for 0.01 equivalents an appreciable ICD was obtained. This is an illustration of the 'sergeants-and-soldiers'

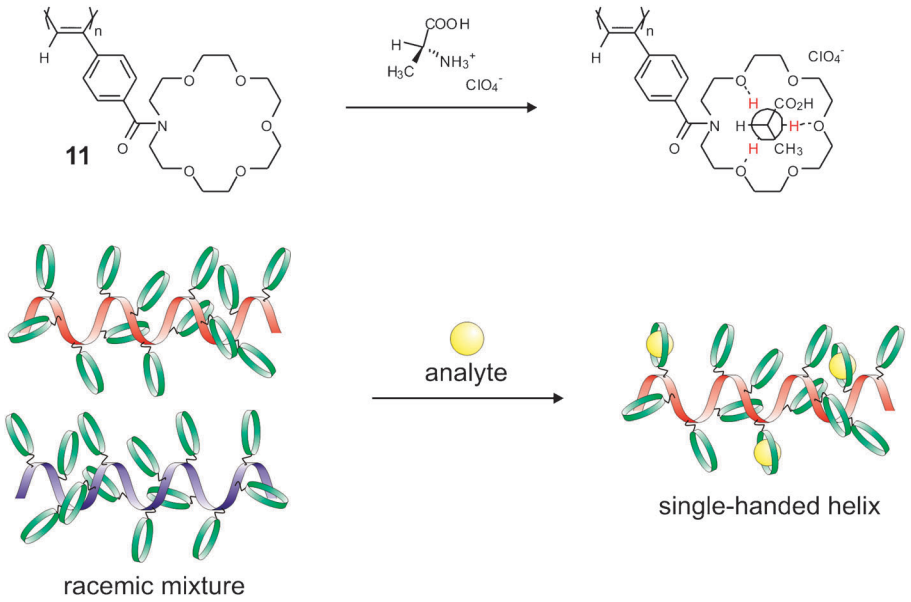

Fig. 13 (a) Molecular structure of chiral polymer **11** and complex formation with L-Ala. (b) The addition of small amounts of either one of the 19 natural amino acids (except L-Pro) is sufficient to induce a single handedness in the polymer.[54] For clarity reasons the polymers with opposite handedness have been given different colors.

principle in which the empty crown ethers follow the handedness induced by the occasional crown ether/L-Ala complex. A lower detection limit of 70 ng of L-Ala per ml was determined at 25 °C, corresponding to 0.005 equivalents per crown-ethers, which makes it one of the most sensible synthetic amino acid receptors. Subsequently, the ability of the system to report on the enantiomeric excess (*ee*) of amino acids according to the 'majority rule' was studied. An *ee* of L-Ala as low as 5% was sufficient to induce a CD of the same intensity as the enantiopure sample. The impressive ability of this system to reliably amplify chiral information was evidenced by the observed linear relationship between the *ee* of Ala mixtures (in the range from 0.1 to 0.005% !) and the measured CD intensity.

It is important to notice that the above approaches towards sensing are not limited to covalent polymers. It is well known that also noncovalently bound macromolecular systems (such as supramolecular polymers or self-assembled nanotubes) can express collective properties including conductivity[55] and chirality.[56] These systems are appealing because of their ease of formation and modulation. Nonetheless, it has to be considered that the communication in supramolecular macromolecules occurs through noncovalent interactions which for that purpose are much less efficient than rigid covalent bonds. In that respect, it is worth noticing that very strong 'sergeants-and-soldiers' effects have indeed been observed in supramolecular polymers, indicating that these systems might indeed prove useful platforms for signal amplification.[57] The difference in kinetics between guest exchange and helix inversion may serve as an additional tool to obtain strong signal amplification in supramolecular aggregates.[58]

4.2 Self-immolative dendrimers

Self-immolative dendrimers are designed to fully disassemble through a domino-like cascade of cleavage reactions induced by a single reaction that occurs at the dendritic core.[59] Such materials are interesting as prodrugs, because multiple peripheral drug molecules can be locally released upon a single enzymatic cleavage. In an elegant approach, Sabat and Sella have exploited self-immolative dendrimers for the exponential signal amplification of an input signal.[60] Their approach, referred to as dendritic chain reaction, relies on the use of AB_3 dendron **12** designed to detect the analyte hydrogen peroxide (Fig. 14). Dendron **12** contains a phenyl boronic acid as analyte sensitive trigger, two choline units and one *p*-nitroaniline reporter unit. In the presence of hydrogen peroxide the boronic acid is oxidized, which initiates a series of intramolecular cascade reactions which ultimately leads towards the release of the two cholines and yellow *p*-nitroaniline. The two choline units are oxidised by choline oxidase (COX), which gives four new hydroperoxide molecules as side product. In turn, these trigger the destruction of four more dendrons **12**, which is the source of the exponential amplification. In fact, sigmoidal curves characteristic of exponential growth were observed when the release of *p*-nitroaniline was measured in the presence of low initial concentration of hydrogen peroxide. For an initial concentration of 5 μM of hydrogen peroxide an output signal could be generated that was 53 times stronger than a conventional probe. As illustrated by the authors, the system can be adapted for other analytes in a relatively straightforward manner, especially by separating the trigger and reporter release on two different dendrons.[61] Nonetheless, considering that spontaneous hydrolysis triggers the same cascade events, the clear challenge in this approach is the development of dendrimers that self-destruct only in the presence of analyte.

4.3 Polymerization-based amplification

A final approach towards the use of polymers for detecting molecular recognition events was reported recently by Bowman *et al*. and is fascinating for its conceptual simplicity,

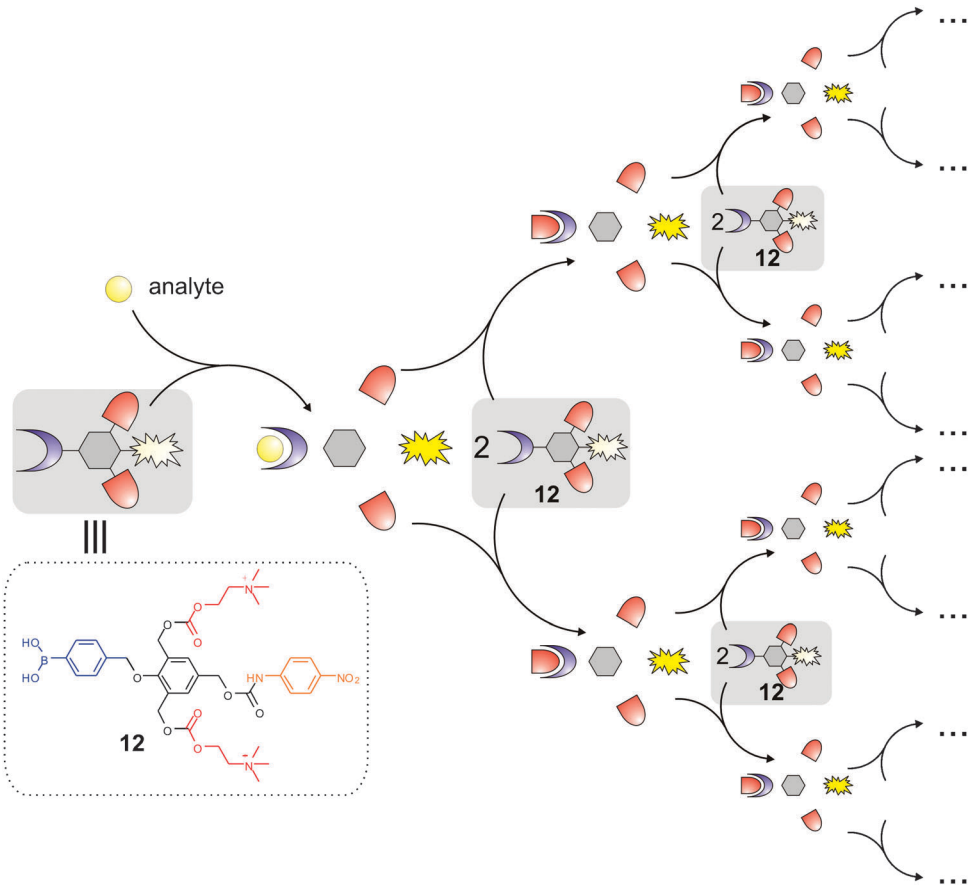

Fig. 14 Schematic representation of the dendritic chain reaction. The presence of an initial analyte triggers the cleavage of **12**.[60] This set of a cascade of reactions leading towards an exponential increase of *p*-nitroaniline reporter molecules (indicated as yellow stars).

elegancy and performance in terms of signal amplification.[62] The central thought behind this approach is that polymers are large enough to be detected with the naked eye. So, confining polymer formation to a local spot where a molecular recognition

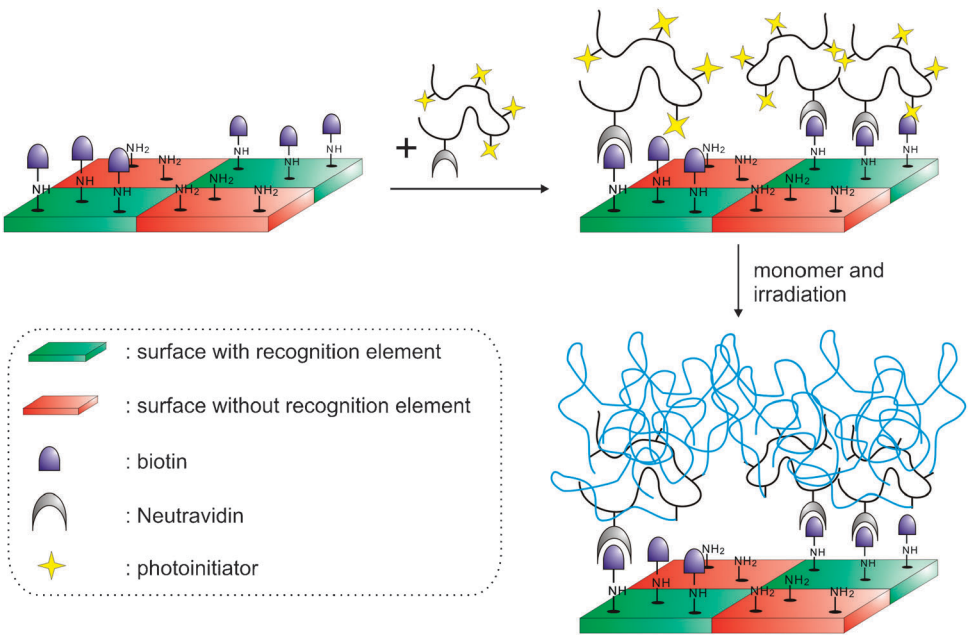

Fig. 15 Schematic representation of polymerization-based signal amplification.[62] Surfaces labelled with biotin (green) recruit macromolecules containing Neutravidin (for biotin binding) and ~100 photoinitiators. Polymerization is then started by adding monomer solution and applying irradiation. In the absence of the target (red surfaces) polymer growth is not observed.

takes place provides for its straightforward visualization. A key component of such an assay is a molecule that is capable both of molecular recognition *and* of initiating a (radical) polymerization reaction. For that purpose, water-soluble photoinitiators and Neutravidin (a biotin receptor) were coupled to a high-molecular-weight copolymer of acrylic acid and acrylamide (Fig. 15). Measurements revealed a substitution grade of around 140 initiators and 1–2 Neutravidins per macromolecule. These macrophotoinitiators were tested for their ability to bind to biotin labeled oligonucleotide sequences immobilized on a surface. Exposure of the surface to the proper irradiation under polymerization conditions resulted only in polymer growth in case biotinylated oligonucleotides were present (5 and 0.5 fmol). Polymer films reached thicknesses exceeding 100 nm and could be visually detected. Dilution studies revealed that a visible amount of polymer was formed even in case only a mere 1000 biotinylated oligonucleotides were present. This is 3 orders of magnitude less than that could be detected using a comparative enzymatic assay. Evidently, this methodology is not suitable for quantification purposes as the 'strength' of the output signal cannot be related to the number of initiating binding events. Nonetheless, as stated by the authors it is a highly practical approach to rapidly detect whether a certain analyte is present above a tunable threshold value.

5. Metal surfaces

5.1. Nanoparticles

The past decades have witnessed a burst in the application of metal and semiconductor nanoparticles for sensing and diagnostics.[63–65] These are particularly adapted for applications in these fields because of their small size (1–100 nm) and concomitant large surface-to-volume ratio, their extraordinary physical properties which can be chemically tuned and related in a rational fashion to their size, shape and composition, and the possibility for a controlled surface functionalization with a large variety of functional groups.[66] Numerous detection methods for various analytes such as metal ions, DNA, proteins, and other biomarkers have been developed relying on analyte induced changes in the physical properties of the nanoparticles which can be detected optically or electrochemically for instance.[63–65] In particular, the change in surface plasmon resonance (SPR) of Au nanoparticles upon aggregation is a highly applied detection probe as it is accompanied by an easily detectable color change from red (separated Au NPs) to blue (aggregated Au NPs).[66,67] Similarly, the analyte-induced growth of the inorganic nanoparticles, and concomitant changes in their physical properties, has proven to be a very sensitive probe.[68] The utility of nanoparticles as assay components is evidenced by the fact that it allows for DNA detection at pM concentrations without using PCR.[69] Nonetheless, most of these sensing approaches fall outside the scope of this review which focuses on systems in which a single analyte molecule affects the behaviour of multiple reporter molecules. It will emerge that the combination of nanoparticles and signal amplification enables detection limits down to the single molecule level.

In particular, in this section it is our intention to illustrate nanoparticle based signal amplification which relies on their multivalent nature. It was already shown in the catalysis section (3.2) that the multivalency of nanoparticles by itself allows strong binding interactions with (bio)targets, but the examples presented here exploit multivalency in a different manner. The basic concept is that confinement of many molecules on the nanoparticle surface connects those molecules in terms of spatial position. Thus, signal amplification can occur in a very straightforward manner in case the interaction of an analyte with a limited number of surface molecules determines the collective behaviour of the full system. It will be shown that this in practice means that an analyte triggers the immobilization of the nanoparticles on a surface employing only a few surface molecules. The remaining large excess of molecules serves for the generation of an output signal.

In a seminal contribution Mirkin *et al.* magnificently applied this concept for the development of a highly sensitive protein detection method.[70] As is the case for immuno-PCR, proteins are not detected directly, but tagged to a DNA sequence (a so called bio-barcode) which is used for read out (Fig. 16). The original assay developed for the detection of the prostate-specific antigen (PSA) relies on the use of two probes: (i) 13–30 nm-sized gold nanoparticles (NP) containing numerous copies of hybridized oligonucleotides (the bio-barcodes) and polyclonal antibodies for PSA recognition, (ii) 1 μm sized magnetic polyamine microparticles (MMP) containing a magnetic iron oxide core functionalized with PSA monoclonal antibodies. The protocol relies on the initial binding of PSA to the MMPs followed by the subsequent capture of multiple NPs on the surface of each MMP. The resulting superstructures embed the key element of this approach concerning signal amplification, since they combine a limited number of PSA targets with a multitude of bio-barcodes, *i.e.* reporter molecules. These superstructures are then isolated through the application of a magnetic field and the bio-barcodes are released through dehybridization and quantified. Among various DNA detection methods (gel electrophoresis and fluorescence labelling) a scanometric DNA detection method combined an easy implementation with high sensitivity. Shortly, this detection relies on the capture of the bio-barcodes on DNA-chips to which subsequently oligonucleotide containing Au NP probes are attached, which are then enlarged through the reduction of Ag^+ on the Au colloids.[71] Final detection occurs through measurement of light scattering from the developed spots using specialized instrumentation. Target PSA concentrations down to 30 attomolar (30×10^{-18} M) could be detected. The lower detection limit could be further pushed down to 3 attomolar by performing PCR on the released bio-barcodes prior to their quantification. These results are highly impressive considering that conventional assays for the detection of PSA have sensitivity limits in the order of 3 picomolar, which is 6 orders of magnitude higher. The ultrahigh sensitivity of this approach was further evidenced by showing that it allows DNA detection at concentration levels normally accessible only with PCR.[72] Here, rather than PSA it is the target DNA that is sandwiched between the bio-barcode containing nanoparticles and the

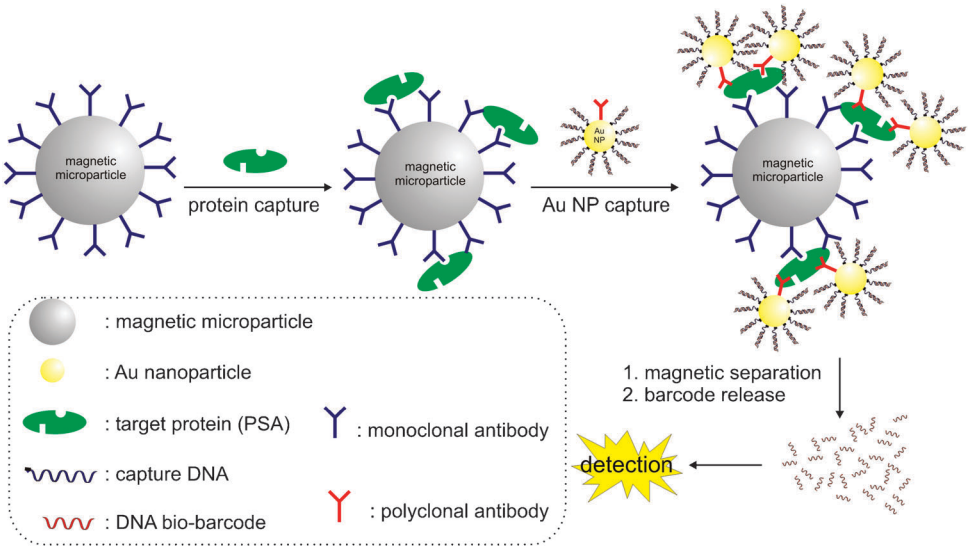

Fig. 16 Schematic representation of bio-barcode based signal amplification.[70] The target protein is captured on magnetic microparticles functionalized with monoclonal antibodies. Next, Au MPCs functionalized with small amounts of polyclonal antibodies and numerous DNA barcodes bind to the protein target. The aggregated systems are isolated and the DNA barcodes released and quantified.

magnetic microparticles. To maximize signal amplification rather large (30 nm) Au NPs were used accommodating up to 360 oligonucleotides with a $\sim 70:1$ ratio of bio-barcodes to target binding DNA. A lower DNA detection limit of 500 zeptomolar (300×10^{-21} M) was reported, corresponding to around 10 copies of target DNA per 30 μl sample !

5.2 Flat surfaces

Highly sensitive analytical techniques such as SERS, SPR, and electrochemical detection all rely on interactions occurring between the analyte and a metal surface. In particular SERS can give a signal amplification as high as 10^{10}, which permits detection even at the single molecule level.[5] However, as will be illustrated in this section, also these sensitive surface-based detection methodologies benefit from the use of signal amplification strategies to improve sensitivity. In particular, we will illustrate different approaches aimed at improving the detection of oligonucleotides using sandwich assays (Fig. 17a).[73] The sandwich assay relies on the capture of the target oligonucleotide by a *capture* oligonucleotide probe immobilized on a surface. Subsequently, a second probe labelled with a signalling unit is added to hybridize with the target probe. Depending on the method of analytical detection, the signalling

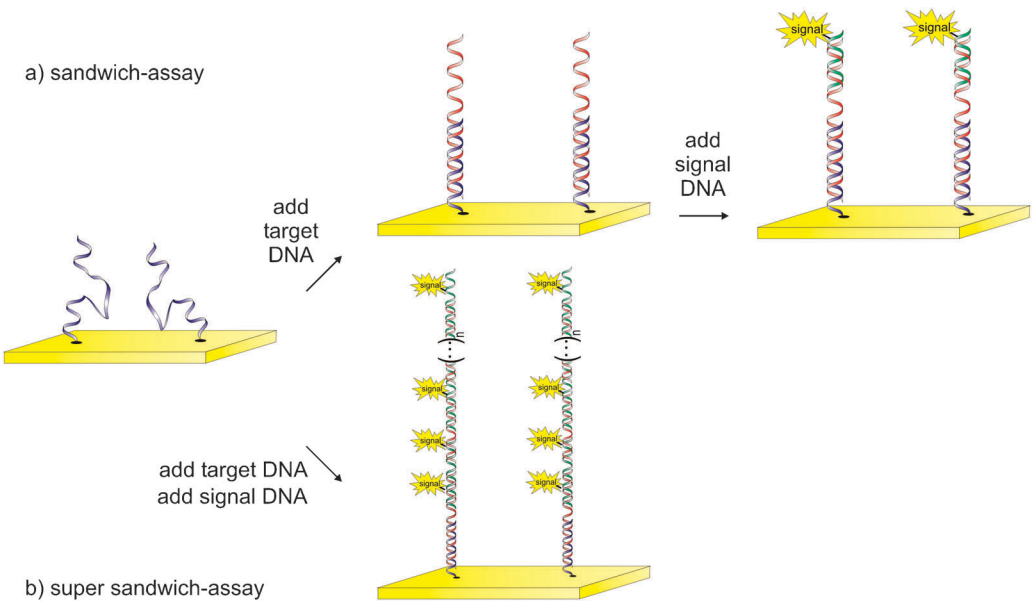

Fig. 17 (a) In the classical sandwich assay the target DNA oligonucleotide strand is captured by the complementary capture strand immobilized on a surface. Subsequent hybridization with a DNA strand containing a signalling unit completes the assay. (b) The super sandwich assay reported by Zuo, Plaxco, Heeger *et al.* relies on the use of signalling probes able to hybridize to complementary regions on two distinct target sequences resulting in the formation of DNA polymers composed of alternating target and signalling DNA sequences.[75]

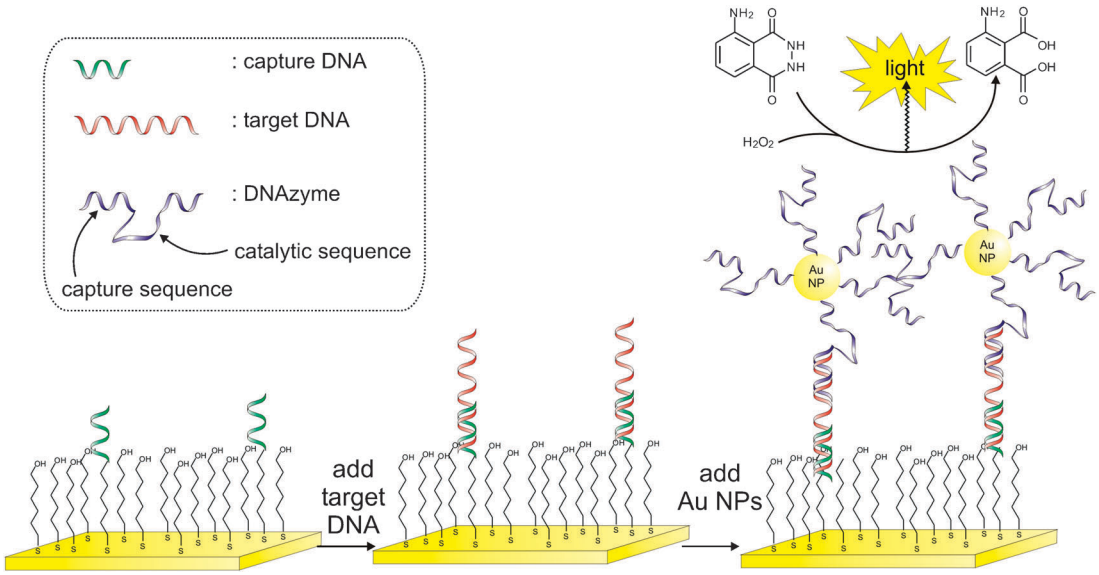

Fig. 18 Amplified chemiluminescence detection of DNA using DNAzyme functionalized Au NPs.[77]

unit can either be a catalyst, fluorophore or electrochemically active unit. Main advantages of the sandwich assay are, first, that it does not require labelling of the target probe and, second, no signal is generated in the absence of the target. Clearly, the sandwich assays bears much resemblance to ELISA, which relies on the surface-immobilization of an enzyme following a recognition event between a surface bound receptor and an analyte, often in the form of sandwich type complexes. Similar to ELISA, a limitation of sandwich assays in terms of sensitivity is the fact that each recognition event results in the capture of only one signalling unit. Methodology that allows multiple signalling units to be captured through a single binding event allows sensitivity limits to be pushed further down. Here, this is illustrated by two examples relying on different read out mechanisms: electrochemically or catalytically.

The electrochemical detection of analytes is highly appealing because signal generation is straightforward and does not require sophisticated instrumentation.[74] An electrochemical readout requires the presence of a redox-active moiety in the signal probe that induces a flow of electrons from the electrode upon capture by the target. In a very recent study, Zuo, Plaxco, Heeger *et al.* illustrated a simple, but ingenious approach to improve the sensitivity of the sandwich assay (Fig. 17b).[75] The key novelty of this so-called super sandwich assay is the use of signal probes (DNA sequences functionalized with the redox-active compound methylene blue) able to hybridize to two regions on the target DNA. However, the probes are engineered such that hybridization occurs more readily between complementary regions on two distinct target sequences rather than two regions on the same target molecule (inspired by the work by Matile *et al.*,[76] see § 6). Consequently, an extended superstructure composed of alternating target molecules and signalling units is formed on the surface of the electrode. The capture of a large number of methylene blue moieties results in strongly enhanced Faraday current compared to a classical sandwich assay. The use of the super sandwich assay permitted target DNA detection down to 100 fM, which is 3 orders of magnitude lower than with a classical assay.

The sensitivity of a catalytic assay can be increased in case a single recognition event results in the immobilization of multiple catalysts rather than one. For that purpose, nanoparticles are a perfect platform which was demonstrated by Willner *et al.*[77] They engineered Au NPs functionalized with multiple (96) copies of a ssDNA strand containing an internal DNAzyme sequence able to generate chemiluminescence in the presence of hemin, O_2 and luminol, and a terminal DNA sequence complementary to the ssDNA strand to be detected (Fig. 18). In the first instance, the analyte is hybridized to a single strand immobilized on a Au surface resulting in the exposure of a sticky end able to capture the DNAzyme-functionalized Au NPs. The subsequent addition of the DNAzyme substrates then results in chemiluminescence which can be readily detected. Compared to ELISA, a second amplification step is present in the sense that a single recognition event now can recruit in principle up to 96 catalysts, determined by the surface coverage of the Au nanoparticles. The assay was applied for the detection of telomerase activity, which is a versatile marker for cancer cells. For this purpose, a Au surface was functionalized with a DNA sequence which is telomerized in the presence of HeLa cells extracts (containing telomerase). Since the extent of telomers formed on the surface is controlled by the concentration of telomerase in the cell extracts, the amount of DNAzyme labeled Au nanoparticles that are captured on the surface, and thus the intensity of the generated light, is directly related to the concentration of telomerase. In fact, a linear relation was observed between the intensity of the emitted light and the number of HeLa cells within a range between 1000 and 10 000. The sensitivity of the reported method was 10^2–10^4 fold higher compared to chemiluminescence assays for DNA analysis or telomerase assays. A conceptually related approach has recently been used by Wang *et al.* for the detection of α-fetoprotein (AFP), which is a protein cancer biomarker. Their approach relied on the AFP induced aggregation of DNAzyme-functionalized Au NPs to magnetic micronanoparticles functionalized with AFP monoclonal antibodies.[78]

An alternative approach to improve the performance of the sandwich assay was developed by Taton, Mirkin *et al.*[79] Here, multiple target DNA sequences are captured by capture probes immobilized on SiO_2 in between two microelectrodes (60 nm Au at 5 nm Ti) at 20 μM distance. The target sequences contain additional sequences which are complementary to oligonucleotides immobilized on Au nanoparticles. Consequently, the presence of target causes the gap between the electrodes to be filled by Au nanoparticles. The reduction of Ag^+ on the seeding Au nanoparticles was required to connect the two electrodes and thus to generate a signal. Target DNA at concentrations down to 500 fM could be detected with a point mutation selectivity factor of around 100 000 : 1.

6. Supramolecular aggregates

The final section is dedicated to signal amplification techniques that involve the use of supramolecular aggregates. As will emerge, supramolecular aggregates are easily accessible through self-assembly processes and can be easily modulated to adapt the system for different applications. Two examples will be discussed by Matile *et al.* who has been particularly involved in the development of conceptually new approaches towards sensing and signal amplification relying on the use of vesicles containing fluorescent dyes at such high concentrations to induce self-quenching.[80] The presence of synthetic transmembrane pores permits a release of the fluorescent dyes from the interior of the vesicles, resulting in an increase in fluorescence intensity (Fig. 19). This system can be used to sense the presence of analytes able to block the pores and thus inhibit the generation of fluorescent signal. In terms of sensing, a more interesting situation arises in case an external effector (for instance an enzyme) is able to transform a good pore blocker into a poor one, which results in a fluorescent signal. Matile *et al.* first illustrated this concept by engineering synthetic pores based on rigid-rod β-barrels in the lipid bilayers of large unilamellar vesicles (LUVs) composed of egg yolk phosphatidylcholine loaded with 5(6)-carboxyfluorescein.[81] The β-barrels spontaneously form through the self-assembly of *p*-octiphenyl units containing small pending peptides.[82] The pores were designed such to contain arginine–histidine dyads lining up in the interior of the pore. Fluorescence titration experiments revealed that ATP bound inside the probes with a dissociation constant of around 2 μM compared to 66 μM for less charged AMP. Consequently, the system was able to report on the presence of potato apyrase, a nonspecific adenosine triphosphatase that converts ATP in the poor pore blockers AMP and pyrophosphate. Advantages of the assay include the use of straightforward detection methodology and the use of unlabelled enzyme substrates. The system can be easily adapted to various enzymes simply by changing the substrates. An obvious limitation of this first setup is posed by the fact that the enzyme substrate and product need to have different affinities for the pores. This limitation was overcome by using so-called reactive signal amplifiers, which are compounds that upon selective reaction with the products of enzymatic reactions turn into pore blockers thus reducing the fluorescence intensity.

The same group reported on another vesicle-based system for the sensing of ATP.[76] The working principle is the ability of DNA to act, in the presence of suitable counteractions, as a transporter of cations across lipophilic membranes. The DNA induced removal of the cationic quencher *p*-xylene-bis-pyridinium bromide from the interior of a vesicle restores the fluorescence of the entrapped 8-hydroxy-1,3,6-pyrene-trisulfonate fluorophore. Multivalency is important here, as the activity of the polyion transporters increases with the number of charges. Thus, dsDNA is a better cation transporter

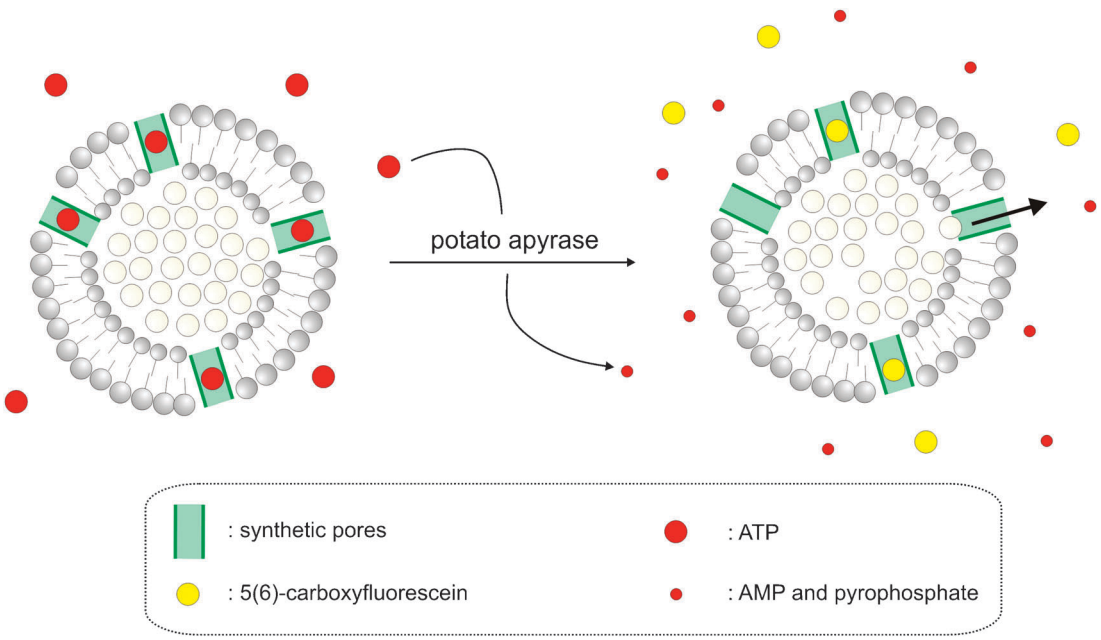

Fig. 19 ATP blocks the synthetic pores in the vesicle bilayer preventing the export of the internal 5(6)-carboxyfluorescein. Potato apyrase converts ATP into the poor blockers AMP and pyrophosphate and, consequently, the fluorophore exits the vesicle resulting in an increase in fluorescence intensity.[81]

compared to ssDNA restoring higher levels of fluorescence. In order to make the system responsive to an analyte, a DNA aptamer for ATP was used. The way the aptamer is selected ensures its activity as ssDNA. Importantly, the high affinity of the analyte for the aptamer induces the dissociation of the double stranded helix formed between the aptamer and the complementary anti-aptamer strand. Together, these two characteristics can be used for signal generation: the presence of ATP disrupts the double stranded DNA helix which lowers the efficiency of DNA as a transporter unit. The result is a lower fluorescence intensity, which was indeed validated by initial experiments. Nonetheless, optimization of the system to improve the output signal was not straightforward. The use of shorter anti-aptamer strands appeared a logical way to favour dissociation of dsDNA by the analyte. However, the lower valency of the dsDNA reduced its capacity as a cation transporter. The solution was found by extending both the aptamer and anti-aptamer strand with sticky ends resulting in the formation of a supramolecular DNA-polymer with excellent transporting capabilities. Access by the analyte to the binding sites in the polymer was less hindered compared to the simple duplex, adding also to a higher sensitivity and selectivity. The characteristics of aptamers (poor sensitivity, excellent selectivity) are reflected by the obtained results for this system (ATP detection in the low mM range, but with excellent selectivity over GTP).

7. Conclusions

Chemists have developed a myriad of approaches for the detection of small amounts of analytes through signal amplification. Although on first sight very diverse in nature, the majority of them are based on two key concepts: catalysis and multivalency. Catalysis is frequently used for the production of reporter molecules. Although frequently enzymes are used, it has been shown that also manmade catalysts can be inserted in an analytical assay. In that respect, the possibility to operate also under non-physiological conditions is an important achievement. Importantly, the first system displaying an exponential signal growth curve due to a positive feedback loop with the catalyst product has been reported. Considering the strong current interest in self-replicators, it is reasonable to expect that such artificial PCR-like amplification mechanisms are going to play an important role in synthetic analytic assays. Clearly, the critical point in such hypersensitive systems is to avoid spontaneous activation in the absence of analyte.

The second pillar is multivalency in its widest definition and relies on the ability of an analyte to modify the chemical properties of a cluster of reporter molecules, be them covalently or noncovalently connected in a macromolecule or aggregate, respectively, or embedded on a metal surface. For sensing applications, a particularly favourable situation occurs in case the multivalent system has a collective property (conductivity or chirality or even size) which is altered by the interaction of the analyte with a single subunit.

The examples collected in this review demonstrate that it is now in some cases possible to detect analytes almost at the single molecule level. It is important to notice that the best results are obtained in systems that rely on cascades of amplification events. In this regard, assays containing nanoparticle-based components play a relevant role. These objects are readily accessible and functionalizable and, importantly, can play multiple roles in signal amplification processes. Their multivalent nature itself has an amplification factor embedded, which can be coupled in a straightforward manner with other amplification mechanisms relying on catalysis or catalytic growth of the inorganic core itself.

It should be noted though that the best results in terms of signal amplification have been obtained for oligonucleotides. These are attractive targets, because molecular recognition between complementary strands is extremely well-defined and also robust in terms of affinity. Secondly, it is noted that most amplification protocols rely on hybrid systems, *i.e.* relying on both biological and synthetic components. From these observations emerges the challenge for chemists to develop amplification protocols for non-natural analytes that have much less-defined interactions with complementary receptors. Screening under non-physiological conditions discards the use of biological components, such as enzymes, in the assay. The potential of (conductive) polymers in this area results clearly from the examples discussed in this review.

In conclusion, chemists have acquired the skills to set up assays that can compete with biological assays (ELISA, but also immuno-PCR) in terms of sensitivity and move beyond the boundaries of physiological conditions. The future will see an implementation of these signal amplification techniques for the multiplexed detection of analytes in the areas mentioned at the beginning of this review through naked-eye detection.

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