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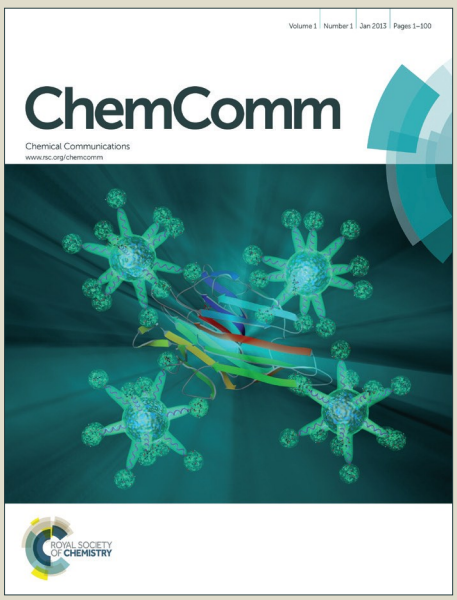

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FEATURE ARTICLE

Enzyme immunoassays as screening tools for catalysts and reaction discovery

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Enzyme immunoassays are incredibly powerful analytical tools for the quantifiable detection of target molecules in complex media. These techniques, which exploit the fantastic specific binding properties of antibodies, are fast, precise, selective and highly sensitive and thus perfectly adapted to high-throughput detection of important analytes. Although immunoassays have been used routinely by biologists for more than 50 years, especially for diagnostic purposes, it is only recently that chemists have used them to address pure chemical problems. In this feature article, we provide an overview of progress in the development of immunoassays and their use in two main fields of organic chemistry: the identification of efficient catalysts in libraries and the discovery of new chemical reactions.

1. Introduction

The development in the 1960s of methods for the labelling of antibodies and antigens with enzymes generated a whole new surge of research leading to numerous efficient immunoassay procedures. Because they can be used in a wide variety of different applications, the so-called enzyme-linked immunosorbent assays (ELISAs) play a dominant role today. In this method, antibodies or antigens are attached to a solid surface, for instance to the inside of a plastic microtitre plate. Automation and microtiter technologies then strongly impacted on the throughput of immunoassays. Nowadays, millions of immunoassays are run every day for diagnostic purposes and this success is fully justified. ELISAs are indeed very robust techniques that allow the fast and selective detection and quantification of a wide range of molecules, from large biomolecules to small haptens in complex media. Although routinely used by biologists and analysts, ELISAs were largely neglected by chemists for many years. It was only in the 1990s, when immunologists and organic chemists had to work together on catalytic antibody projects that the idea emerged of exploiting the fantastic analytical potential of ELISAs in basic research in chemistry.

As thousands of analyses can be done per day by ELISA, these techniques appeared particularly well adapted to combinatorial approaches, which needed powerful screening tools (Fig. 1). High-throughput screening of catalyst libraries appeared in the 1990s as a powerful approach to the rapid identification of new metal complexes, organocatalysts and enzymes with interesting catalytic properties. More recently, this type of approach based on a large number of experiments and high-throughput screening was also applied to reaction discovery. For both types of projects, immunoassays were found to be valuable screening tools with interesting advantages over other techniques.

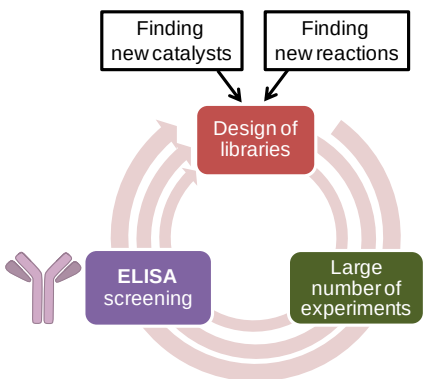

Fig. 1 Approaches based on ELISA screening for catalysts and reaction discovery.

This feature article summarises the achievements in this field. After a brief general presentation of ELISA techniques, their uses as a screening tool for catalysts and in reaction discovery are presented.

2. Immunoassays: background

Immunoassays make direct use of the ability of antibodies (Abs) to recognise a molecule they were raised against (antigen, Ag). The specific complex formed (antigen-antibody,

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AgAb) can be used for detection and/or quantification purposes, through measurement of a signal emitted by a label attached to either the antigen or the antibody, depending on the format of the assay.[1]

The production of antibodies involves the immune system of an animal after the administration of the antigen recognized as a foreign substance. Quite different animal (mainly rabbits and mice) immunization protocols may be used, depending on the administration route, the use of adjuvant, the frequency of injections and the injected dose. After a first injection aiming at initiating the stimulation of the immune system (primary response), several booster injections are performed leading to the production of high affinity antibodies in plasma. These antibodies result from the mobilization of different B lymphocytes, each producing a specific antibody for a unique epitope. Since this specificity panel comes from several clones of B lymphocytes, these antibodies are called polyclonal.

Monoclonal antibody production started at the end of the 70's and results from cellular culture operations allowing to select and separate lymphocytes B clones each producing a single type of antibody presenting unique and homogeneous specificity and affinity properties against a single epitope. Since B lymphocytes are unable to multiply, this is achieved by cellular hybridization process with myeloma cells (of the same animal species, generally mouse) possessing the capacity to indefinitely multiply. The resulting chimeric cells, named hybridomas, are thus selected on the double property to produce antibody and to multiply. The production of high amount of monoclonal antibody can be achieved either by in vitro culture (providing concentration range from 20 to sometimes 1000 µg/mL of antibody) or in vivo as ascitic fluids in the peritonea of immuno-deficient mice (antibody concentration until 10 mg/mL).

The binding of an antigen to an antibody leads to the formation of a specific complex of high affinity. This binding is reversible and reaches equilibrium in accordance with the law of mass action. All immunoassays are relative analytical methods using experimental standard curves plotted for several equilibria involving different known concentrations of antigen. Starting from this relationship, any unknown concentration in a sample can be measured using the same experimental conditions as for analysis of standard solutions. This relative measurement also means that it is not necessary to reach equilibrium to perform the assay. However, immunoassays require measurement of the concentration of the AgAb complex formed, which explains the use of labelling. Among numerous possible labels, enzymes (for enzyme immunoassay or ELISA) are now more widely used than radioisotopes (radioimmunoassays) or fluorescent dyes (fluoroimmunoassays). Among numerous variants of immunoassays, the most commonly used are competitive ELISA and sandwich ELISA (also called two-site immunometric ELISA).[2]

One of the main competitive ELISA formats, illustrated in Fig. 2a, involves a specific antibody (Ab), an enzyme labelled antigen (Ag*) and the same unlabelled antigen (Ag). Both Ab and Ag* are used at a constant and fixed concentration. Ag is used either at known concentrations to plot the standard curve, or at unknown concentrations in the samples. Ag* and Ag are both able to bind the same Ab and thus should compete for binding if the concentration of Ab is limited. To facilitate specific detection of the AbAg* complex, Ab is immobilised on a solid phase (generally the polystyrene surface of the wells of a microtitre plate), therefore allowing elimination of unbound Ag*. After incubation, the antibody-bound fraction of Ag* is thus separated from the free (unbound) Ag and Ag* by simple plate washing. Enzyme substrate is then added and the signal intensity of the enzyme product is measured. Since Ab and Ag* concentrations are constant, the concentration of the labelled complex AbAg* is inversely proportional to the concentration of Ag as shown by the theoretical dose-response curve in Fig. 2c. This competitive technique is also called "limited-reagent assay" since maximum sensitivity is reached when Ab and Ag* tend to zero.[3] A variant of this technique involves immobilisation of Ag (instead of Ab) and the use of an enzyme labelled antibody, Ab* (Fig. 2b). Competition of immobilised and free Ag towards Ab* binding sites decreases the enzymatic signal from the immobilised AgAb* complex.

Because they use only one antibody, competitive ELISAs can detect large entities such as proteins but also small molecules, called "haptens".

In the case of sandwich ELISA, at least two different antibodies that recognise and simultaneously bind two different complementary epitopes of the antigen are required. One antibody (Ab1) is immobilised on a solid phase, allowing the capture of the antigen in solution through a first epitope. The enzyme labelled antibody Ab2 (with a binding specificity different from that of the capture antibody) is added in solution to bind the antigen (captured or in solution) through another epitope (Fig. 3a).

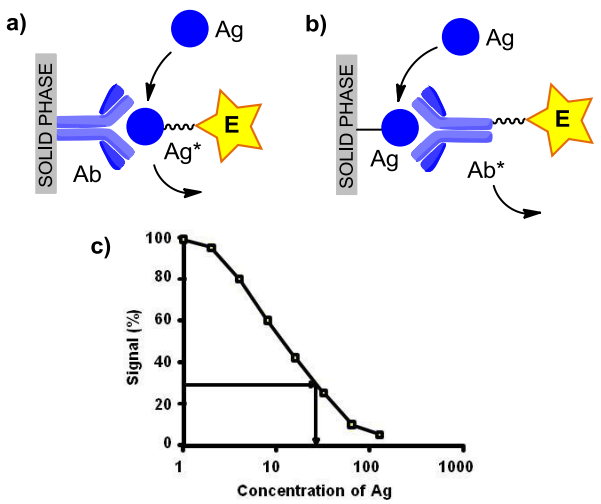


Fig. 2 Basic principle of competitive ELISAs. a) Ab is fixed on the solid phase, b) Ag is fixed on the solid phase, c) Example of a standard curve.

Both the capture antibody and the enzyme labelled antibody should be in excess over the antigen to favour the formation of the ternary complex Ab1-Ag-Ab2, explaining why this

sandwich method is sometimes referred to as an excess-reagent assay. In these conditions, the amount of labelled antibody bound to the solid phase is directly proportional to the amount of antigen present. The excess unbound enzyme labelled antibody is eliminated by washing. The standard curves obtained for this assay are linear; the enzymatic signal is proportional to the concentration of Ag (Fig. 3b).

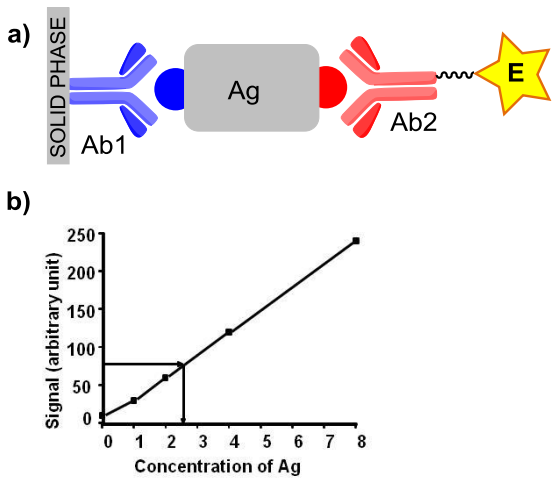


Fig. 3 Sandwich ELISA. a) Basic principle of the technique, b) Example of standard curve.

Due to the use of excess amounts of reagents, it has been theoretically calculated and experimentally shown that sandwich ELISA formats are always more sensitive (by a factor of 10 to 1000) than competitive assays performed with the same antibodies. However, the main limitation of sandwich ELISA is the requirement for two complementary antibodies with good affinity characteristics. It should be noted that polyclonal antibodies are rarely used in this format since the specific antibodies in the total antibody fraction of a serum only represent a few per cent, which is not in accordance with the excess reagents concept. Thus, unlike competitive ELISA, most sandwich ELISA formats use monoclonal antibodies, preparation of which is time-consuming and expensive. Furthermore sandwich ELISA require that analytes have at least two epitopes that are sufficiently far apart to be bound simultaneously, thus most haptens cannot be detected by this type of immunoassay.
Table 1 summarises the main advantages and drawbacks of the two ELISA techniques.

Table 1. Comparison of the two ELISA formats

Type of ELISA	**Competitive ELISA**	**Sandwich ELISA**
Development	Easy	Time-consuming
Resources	Low-cost (limited reagent assay)	High-cost (excess reagent method)
Labelled molecule	Antigen or Antibody	Antibody
Analyte	Small and large molecules	Large molecules
Antibodies required	1	2
Type of antibody	Polyclonal or Monoclonal	Monoclonal
Sensitivity	Medium to high	Very high
Specificity	Medium to high	Very high

Both types of ELISA techniques were used as analytical tools to monitor chemical transformations after several adaptations depending on the analytical needs.

3. Catalyst discovery by immunoassay

In the last two decades, high-throughput screening methods have become widely used for rapid identification of powerful catalysts in large libraries and have thus increased the efficiency of reaction development.[4] Among the numerous screening techniques developed and successfully used in the field,[5] immunoassays are attractive but still underexploited. This section, dedicated to this field, is categorized by type of catalysed reactions.

Immunoassay screening of catalysts for hydrolytic reactions

In the early 1990s, many research groups were involved in the development of catalytic antibodies as new enzyme mimics.[6] Typically, catalytic antibodies were obtained after immunisation with a stable synthetic analogue of the transition state of the target reaction coupled to a carrier protein. The resulting antibody repertoire was immortalised as hybridoma clones producing monoclonal antibodies (mAbs) at low concentrations (typically in the 30-300 nM range).[7] Specific and sensitive screening techniques were then needed to detect catalytic activities in culture supernatants of the thousands of different hybridomas in order to select the clones producing antibodies with enzyme-like properties. In this context, Tawfik et al. were the first to report the use of immunoassays to screen mAbs with esterase-like activities.[8] The technique, called catELISA, involves a substrate-protein conjugate immobilised on microtitre plates (Fig. 4). Upon catalysis the substrate is cleaved into two products, one remaining linked to the solid support and the second released in solution. A specific anti-product antibody is added to the microtitre plate and binds to the immobilised product. The product is then detected by addition of a secondary antibody labelled by an enzyme. The method is therefore a non-competitive ELISA, the enzymatic signal is thus proportional to the concentration of the product and, as a consequence, to the activity of the tested catalyst.

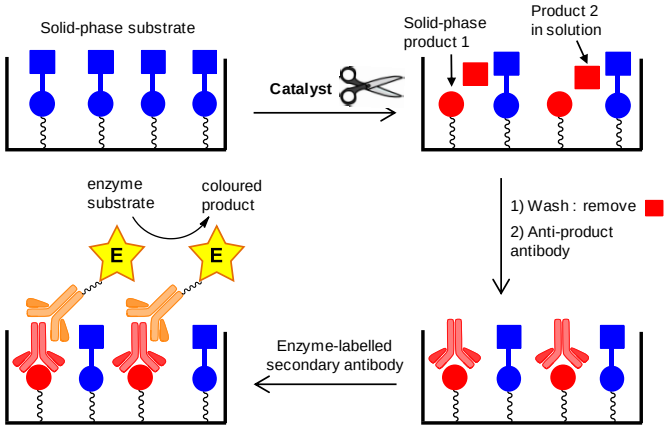


Fig. 4 Non-competitive catELISA. Catalysis and screening are run in the same microtiter plate.

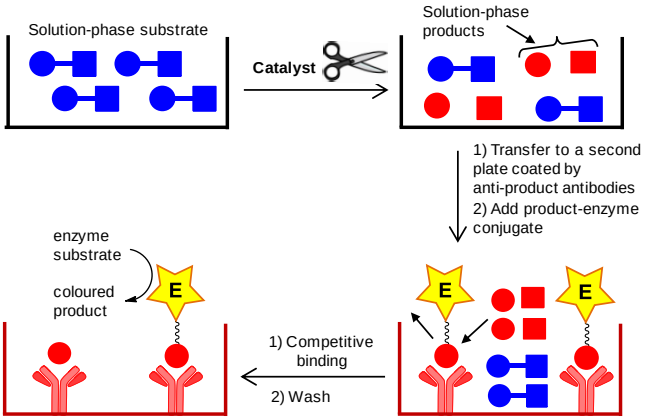
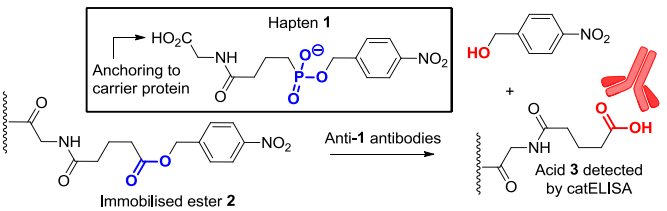


Fig. 5 Competitive catELISA. Catalysis and screening are run in two separate microtitre plates.

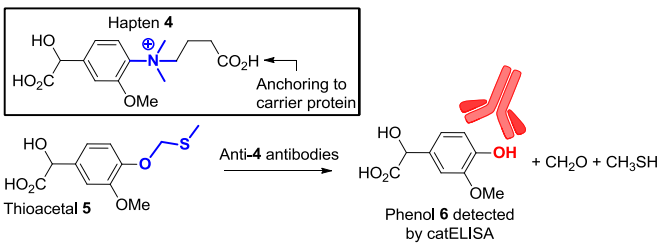
Employing this catELISA technique, the authors were able to screen 1570 hybridoma clones elicited against the phosphonate hapten **1** used as transition state analogue of the hydrolysis of ester **2** (Scheme 1). Two antibodies displaying significant esterase activity were identified.



Scheme 1 Esterase activity of antibodies raised against hapten **1** revealed by non-competitive catELISA.

The catELISA developed by Tawfik et al. is quite general and may be applied to any kind of reactions if specific anti-product antibodies are available. However, its major drawback is related to the fact that the tested catalysts must be active on immobilised substrates, which is not always the case. To avoid this drawback, Benedetti et al.[9] and our group[10] developed competitive catELISA (Fig. 5). In this case catalysis occurs in solution, and the crude is then transferred into a second plate previously coated by anti-product antibodies. A product-enzyme conjugate is added to the solution and competes with the product formed during the reaction for antibody binding sites. This binding competition leads to a low enzymatic signal when efficient catalysts, generating high product yields, are present.

First used to identify esterase-like activity, competitive catELISA also allows the identification of other hydrolytic catalytic activities. A library of mAbs raised against hapten **4** (which is a stable analogue of the oxonium intermediate formed during the hydrolysis of thioacetal **5**) was screened by catELISA detection of phenol **6** leading to the identification of one mAb with thioacetal hydrolysis activity (scheme 2).[11] Protease activities have also been studied using competitive catELISA.[12]



Scheme 2 Catalytic activity of antibodies revealed by competitive catELISA.

Immunoassay screening of catalysts for enantioselective reactions

Because enantioselectivity of catalysts is difficult to predict, approaches based on high-throughput screening have largely been exploited in this field leading to the development of a series of techniques such as IR thermography,[13] capillary array electrophoresis[14] and electrospray ionisation,[15] in order to monitor enantioselective transformations.[16] Also, it has been known since the pioneering work of Karl Landsteiner[17] that antibodies allow stereoselective binding of chiral molecules. The capacity of antibodies to discriminate enantiomers was particularly exploited for the development of immunosensors to detect enantiomeric impurities.[18] In 2002 our team demonstrated that it can also be successfully used to screen enantioselective catalyst libraries.[19] The

principle of the screening method is based on two conjointly run catELISAs (Fig. 6). A first ELISA is run with a mAb that binds indiscriminately the two enantiomers of the product to determine the concentration of (*R*) + (*S*)-products, and a second is run with an enantiospecific anti-(*S*)-product mAb.

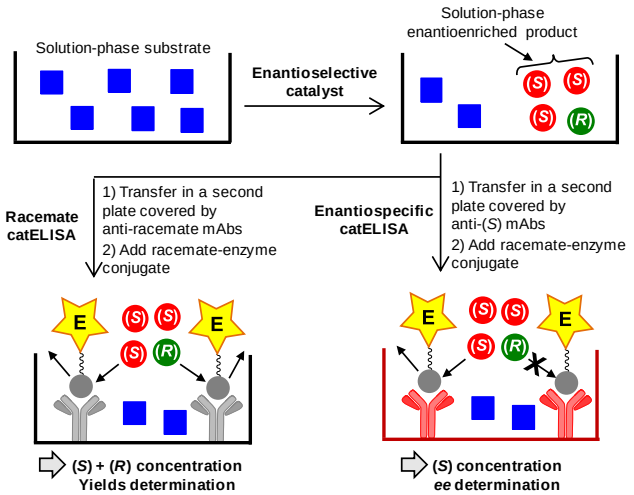


Fig. 6 Enantioselective catalysts screening by two competitive catELISAs.

This procedure was successfully used for the selection of catalysts for the enantioselective reduction of benzoylformic acid **7** into mandelic acid **8** used as a model reaction (Fig. 7). The development of the catELISAs was carried out with two mAbs raised against hapten **4** (see scheme 2), one binding equally the two enantiomers of **8**, and a second exhibiting high stereoselectivity toward (*S*)-**8**. The catalyst library was prepared by combining a set of 22 chiral diamine-based ligands and 4 different metal species; two hydrogen sources were used and solvent was DMF. This led to 176 different reactions which were screened in a few hours. Representative results are shown in Figure 7.

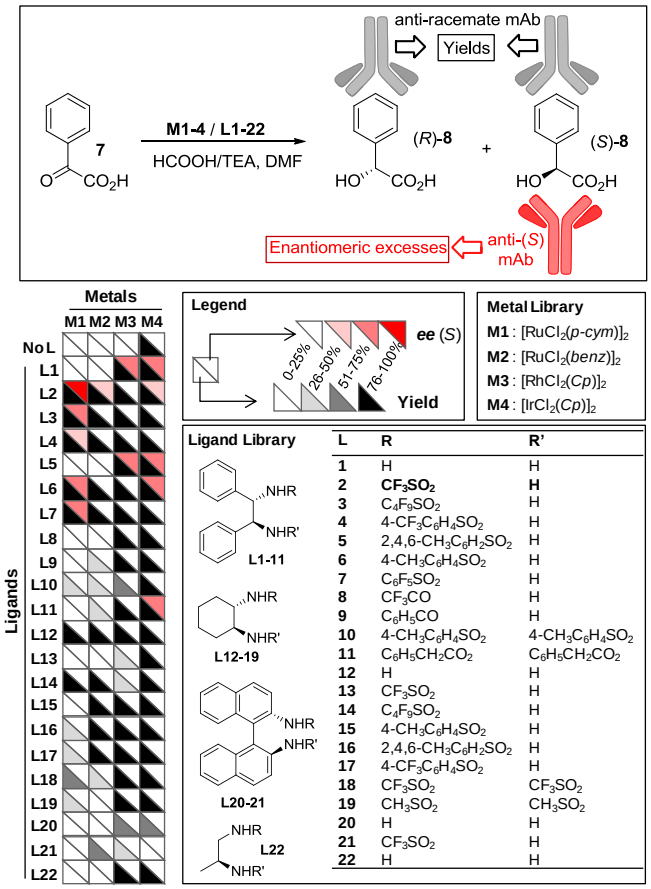
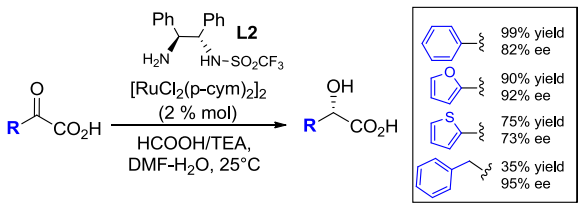


Fig. 7 Enantioselective reduction of benzoyl formic acid. catELISA screening results.

Only one combination (**M1**/**L2**) identified by the screening led to interesting results both in terms of yield and *ee*. This catalytic system was then used in flask on mmole amounts of several α-keto-acid substrates to look at the scope of the reaction. The results confirmed the interesting performances of the catalyst revealed by screening (scheme 3).



Scheme 3 Enantioselective reduction of α-keto-acids.

Comparison of HPLC and ELISA values of a set of randomly chosen samples confirmed the validity of the screening method. The precision of the *ee* determination was evaluated at ± 9%. This fairly broad variation in *ee* was mainly due to the use of the anti-racemate mAb which binds mandelic acid only with moderate affinity (K_d ~ 5 mM). To increase the precision of the catELISA technique, our group developed a new version of the screening technique by using two enantioselective anti-hapten **4** mAbs. In addition, the structure of the substrate was adapted to

form *p*-*t*Bu-mandelic acid **10**, a compound which binds the mAbs with good affinities (K_d ~ 1 µM). This new catELISA was then used to identify copper complexes able to promote the enantioselective Cu-carbenoid O-H insertion of water (Fig. 7).[20] Diazo substrate **9** was thus mixed with a series of copper complexes resulting from the combination of copper salts and 19 ligands (mainly BOX type ligands) in the presence of 3 equivalents of water. The formed α-hydro-esters were then saponified and the resulting mixtures assayed by two conjointly run catELISAs, one using an anti (*R*)-**10** mAb and a second using an anti (*S*)-**10** mAb. Part of the screening results is indicated in Fig. 7.

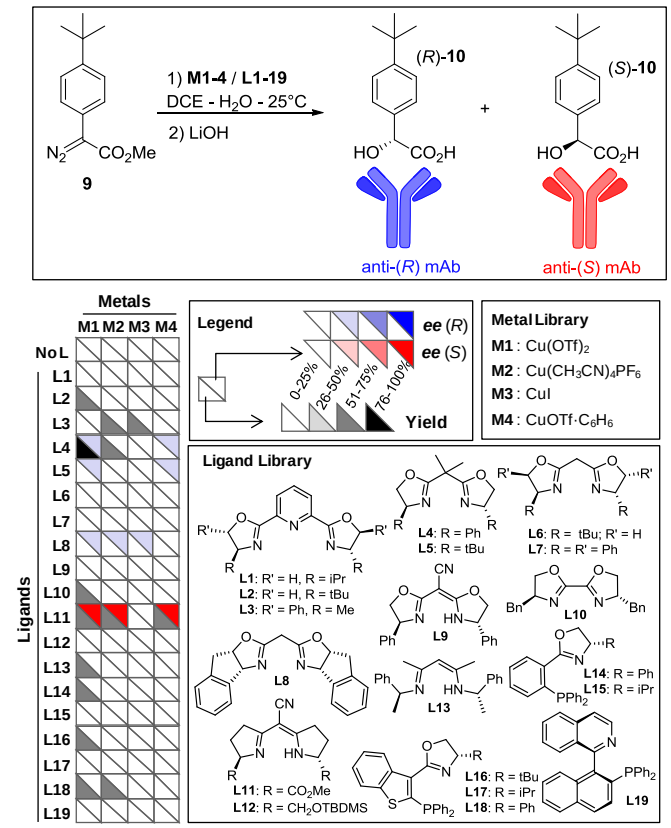
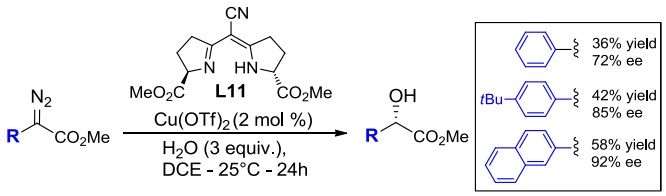


Fig. 7 Enantioselective formation of α-hydroacid **10** by Cu-carbenoid O-H insertion reaction. CatELISA screening results.

Semicorin ligand **L11** and $Cu(OTf)_2$ appeared as the optimum combination for this reaction. This catalytic system was then tested in a series of experiments conducted in flask (scheme 4).

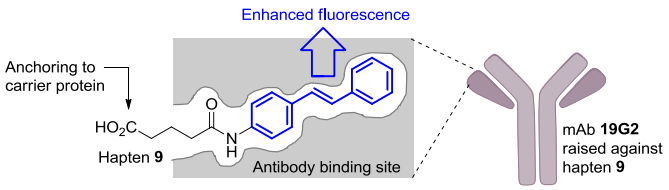


Scheme 4 Enantioselective Cu-carbenoid O-H insertion reaction with $Cu(OTf)_2$/**L11**.

The copper complex highlighted by the screening was found quite efficient in term of *ee* but yields remained moderate. Improvements in this reaction were later afforded by the group of Q-L. Zhou which developed a spirobisoxazoline ligand allowing the formation of α-hydroacids from diazo compounds with both high yields and *ee*.[21]

Comparison of HPLC and catELISA values proved the increased precision of the technique: *ee* were determined with ± 5% precision. Enantioselective catELISAs globally showed high throughput performances allowing the determination of hundreds of yields and *ee* per day. Furthermore, crudes can be analysed directly without any work-up. These techniques therefore present many advantages over others, but it requires at least one mAb displaying high enantioselective binding properties, which is the major drawback of the procedure given that enantioselective mAbs are not always easy to obtain.

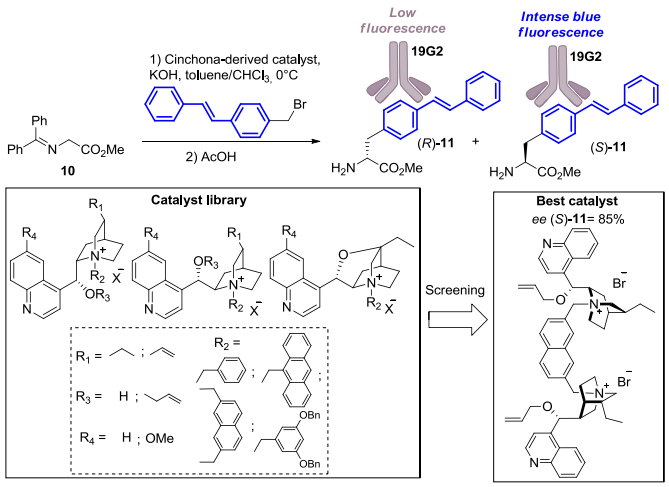
Using another strategy that does not require enantioselective mAbs, Matsushita and Janda *et al.* produced antibodies against the *trans*-stilbene hapten **9** that form hapten-antibody complexes with intense fluorescence properties (scheme 5).[22] Among the mAbs produced, **19G2** formed a complex with hapten **9** producing a blue fluorescence in high quantum yield (λ_{ex}= 327 nm, λ_{em} = 410 nm, Φ_f = 0.78).



Scheme 5 Structures of *trans*-stilbene hapten **9**.

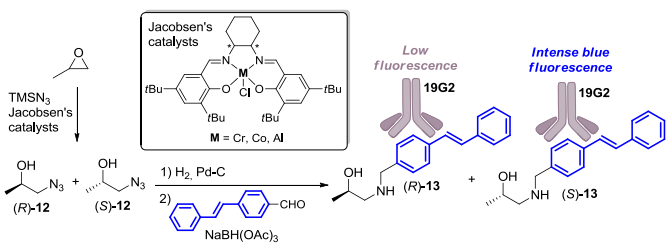
Although **19G2** was originally programmed to recognise the achiral hapten molecule **9**, the asymmetric environment of the antibody-combining site enabled specific detection of chiral molecules tagged by *trans*-stilbene. For example, both enantiomers of the amino-ester **11** could bind to **19G2**, but only the (*S*)-**11**-**19G2** complex resulted in blue fluorescence emission. Thanks to this enantioselective fluorescence detection, the authors screened a series of 35 putative organocatalysts derived from *Cinchona* alkaloids for the asymmetric α-alkylation of *N*-(diphenylmethylene)glycine methyl ester **10** (scheme 6).[23]

After alkylations, the benzophenone Schiff base group was hydrolysed and product mixtures of (*S*)-**11** and (*R*)-**11** were purified to determine yields. Then, *ee* values of each 35 reactions were determined in less than one hour using calibration curves with mAb **19G2**. Variation was ± 10% compared with chiral HPLC analysis.



Scheme 6 Fluorescent antibody-based screening of *Cinchona* alkaloid catalysts for the enantioselective alkylation of **10**.

The authors expanded the scope of their method to the detection of other chiral compounds and proved that the antibody **19G2** can also be used for the enantioselective detection of *trans*-stilbene (*S*)-amino alcohols.[24] The system was then used to screen a series of Jacobsen's catalysts for the asymmetric ring-opening reaction of an epoxide by $TMSN_3$ (scheme 7). The resulting mixtures of (*S*)-**12** and (*R*)-**12** azido alcohols were then reduced to the corresponding amino alcohols and further derivatised by the stilbene moiety through reductive amination to form mixtures of (*S*)-**13** and (*R*)-**13**, which could be analysed using **19G2.**



Scheme 7 Screening of Jacobsen's catalysts for the asymmetric ring-opening of 2-methyloxirane by $TMSN_3$ using **19G2**.

The main advantage of this strategy based on fluorescent product-antibody complexes is its simplicity and speed, but the need to attach a stilbene tag to the reactants is clearly a limitation.

Overall, the screening techniques described above suffer from the need to produce mAbs, which necessitates expensive and time-consuming techniques. Moreover, mAbs generally display very specific binding features such that the immunoassay system is applicable only to one or to a few analytes. As a consequence, the substrate scope of catalysed reactions cannot be evaluated using mAbs.

To address this deficiency, our group was interested in developing an immunoassay for asymmetric transformations using polyclonal antibodies (pAbs) instead of mAbs. Unlike mAbs, anti-hapten pAbs can be obtained very easily after immunisation of animals with a hapten-protein conjugate. Serum of these animals can be used directly, so neither hybridoma production nor culture cells are needed, thereby reducing very significantly the time and cost of ELISA development. But serum of immunised animals contains millions of antibodies, including hundreds of anti-hapten antibodies possibly displaying different binding properties. So, is it possible to obtain pAbs with sufficient enantioselectivity for a given chiral molecule? Pioneering work by B. S. Green et al.[25] demonstrated that such stereoselective pAbs can indeed be obtained by exposing an enantiomerically pure hapten to the immune system. To maximise the probability of obtaining stereoselective pAbs, the hapten should be attached to the carrier protein at the opposite site of the chiral centre.

Inspired by this work, our group immunised rabbits with BSA-coupled optically pure *N*-Bz-(*R*)- or (*S*)-lysine with the aim of producing stereoselective pAbs against *N*-Bz amino acids. Lysine haptens were linked to BSA through the amine function in order to maximise exposure of the chiral centre (Fig. 8). After a few weeks, rabbit sera were collected and assayed for their ability to bind *N*-Bz-(*R*)- and (*S*)-amino acids. As anticipated, the sera were found to contain pAbs displaying high chiral recognition but relaxed specificity for the side chain of *N*-Bz-amino acids. The stereoselective binding of these pAbs were in favour of the injected enantiomer by at least 2 orders of magnitude and affinities were in the 0.1-1 µM range for a large panel of *N*-Bz-amino acids.[26]

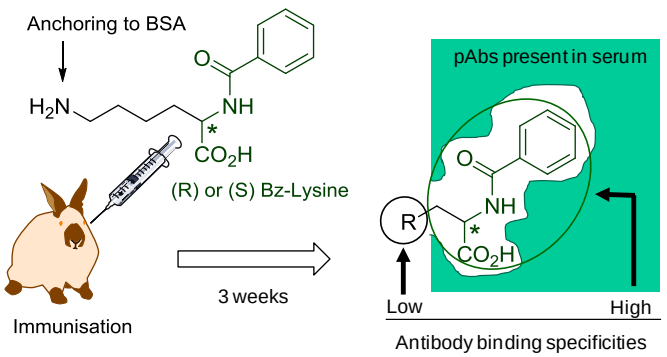


Fig. 8 Production of enantioselective pAbs.

The binding properties of the pAbs obtained allowed the development of a screening procedure for the selection of efficient and general enantioselective catalysts generating *N*-Bz-amino acids. This procedure was more particularly applied to the enantioselective ring opening of azlactones by water catalysed by lipases in organic solvents (Fig. 9).

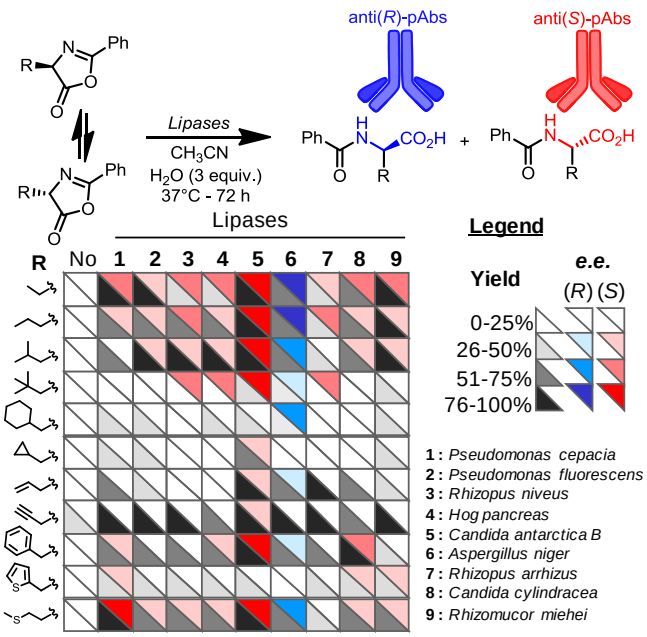


Fig. 9 Fingerprinting of lipases using catELISAs.

Eleven azlactones were reacted with 9 lipases and 3 equivalents of H_2O in 6 anhydrous solvents. A total of 660 experiments were thus conducted in parallel and screened in one day with two jointly run catELISAs (one using anti-(*R*) pAbs and the other using anti-(*S*) pAbs). The best results, obtained in CH_3CN, are summarised in Fig. 9. *Candida antarctica B* was the lipase that displayed broader substrate tolerance and generated the best yields and *ee* results. The superior activity of this enzyme was confirmed in experiments conducted in flask at the mmol scale with several azlactones (yields up to 93% and *ee*s up to 96%).

This immunoassay screening method presents at least three major advantages related to its sensitivity and to the use of stereoselective pAbs. First, small quantities of substrates and biocatalysts are needed. The sensitivity of the catELISA detection was such that 20 µL-scale reactions were run with only 10 µmols of azlactones and less than 0.1 mg of lipases. Second, it is cheap and fast to develop as compared with immunoassay screening using mAbs (we estimate that 1 month is the minimum time for development of an immunoassay using pAbs) and third, it allows exploration of the substrate scope of enantioselective catalysts. Since pAbs can be raised against virtually any kind of compound, this screening approach should be easy to extend to almost any kind of asymmetric reaction.

Immunoassay screening of catalysts for coupling reactions

The first immunoassay developed to monitor a coupling reaction was described by D. Hilvert and co-workers[27] who extended the catELISA technique developed by Tawfik to bimolecular reactions. The strategy of this bimolecular catELISA, outlined in Fig. 10, is based on the immobilisation of one of the two substrates on the surface of a 96-well microtitre plate, thus forming, upon catalysis, an immobilised coupling product detected by a specific anti-product mAb.

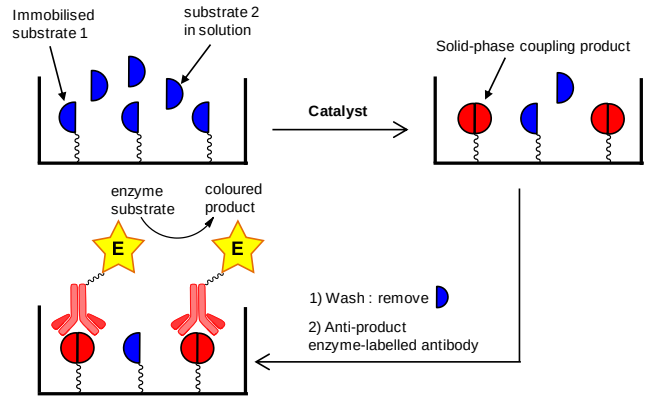
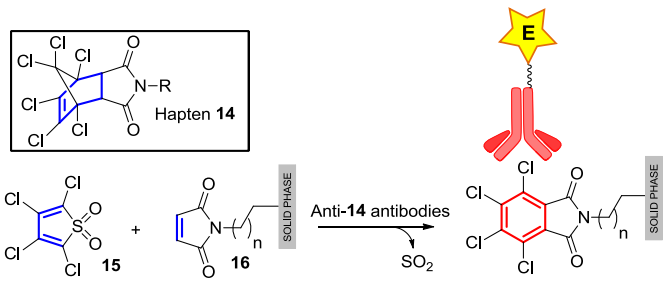


Fig. 10 Bimolecular catELISA.

This technique was applied to the screening of hybridoma supernatants of clones produced against hapten **14** used as a transition state analogue of the Diels-Alder reaction of tetrachlorothiophene **15** and *N*-derivatised maleimide **16** (Scheme 8). Four mAbs displaying Diels-Alderase activities were successfully identified among hundreds of clones. Interestingly, the authors showed that the use of catELISA was crucial for the discovery of catalytic antibodies. Indeed, due to their low concentrations in the supernatants and/or low efficiencies, several catalysts would not have been detected by other screening methods. This work showed that catELISA is particularly suitable for the detection of low-abundance catalysts. This may have important implications for the screening of libraries of biocatalyst variants.



Scheme 8 Diels-Alderase activity revealed by bimolecular catELISA.

The effectiveness of immunoassays in rapidly screening and identifying efficient catalysts for coupling reactions was thus demonstrated more than 20 years ago. However, bimolecular catELISA requires specific antibodies for each reaction under investigation, which is expensive and time-consuming.

To address this drawback, our group was interested in exploiting the advantages of sandwich immunoassays (see section 2) to develop a general screening tool for cross-coupling reactions. To be truly useful the method should allow the monitoring of any kind of coupling reaction always using the same pair of antibodies. The idea was to use antibodies

raised against small molecule tags, which can be easily linked to reactive functions **A** and **B** (Fig. 11).

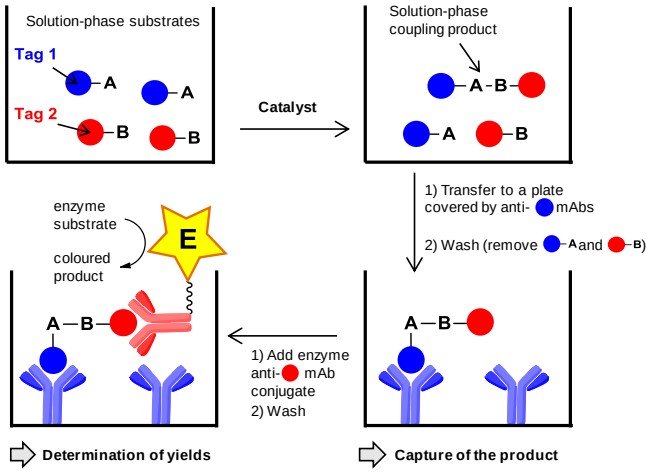


Fig. 11 General principle of sandwich-catELISA for the monitoring of coupling reactions. A, B = reactive functions.

In the presence of appropriate catalysts, covalent linkage between the two functions **A** and **B** should take place, thus generating a double tagged product. The reaction crude is then transferred into a microtitre plate coated by an anti-tag 1 mAb, inducing the capture of the product through the binding of tag 1. A second antibody, selective for tag 2 and attached to a signalling enzyme, is then added for the selective detection and quantification of the coupling product through the binding of tag 2 (Fig. 11).

In principle, sandwich-catELISA can determine yields of any kind of coupling reaction in a high-throughput manner always using the same pair of antibodies, therefore avoiding the drawbacks associated with mAb production. However, because the compound that has to be detected must be large enough to be bound simultaneously by two antibodies, sandwich immunoassays were considered unsuitable for haptens, thus prompting researchers to develop alternative strategies to detect small molecules.[28] Angiotensin II (octapeptide, MW = 1048 g.mol^{-1})[29] and ciguatoxin CTX3C (polycyclic ether, MW = 1023 g.mol^{-1})[30] are the smallest molecules detected by sandwich immunoassays described in the literature.

In the context of the sandwich-catELISA strategy outlined in Fig. 11, the minimum size of the spacer (which includes the coupling functions **A-B**) separating the two tags is important and must be determined. A systematic study was therefore carried out to identify a pair of mAbs raised against small tags and able to form ternary complexes with small double tagged molecules. The model study was conducted with two series of mAbs raised against histamine and guaiacol derivatives, respectively, and whose binding properties were previously characterised (Fig. 12).[31]

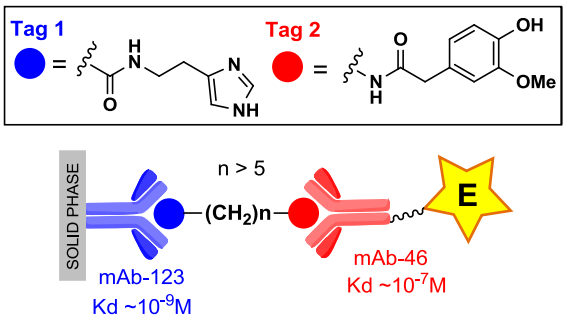


Fig. 12 Schematic diagram of sandwich ELISA for double tagged compounds. Specific anti-tag1 mAbs (blue) are immobilised on solid support, anti-tag2 mAbs (red) are conjugated with an enzyme reporter.

Combinatorial association of the two series of mAbs with compounds bearing both histamine (tag 1) and guaiacol (tag 2) moieties separated by spacers of different sizes clearly indicated that a spacer of 10 carbon atoms is enough to allow sandwich immunoassays for more than 85% of the 192 pairs of antibodies tested.[32] Among the tested antibodies, the mAb-123/mAb-46 combination allowed the detection of a double tagged product whose tags were separated by a spacer as small as 5 methylene groups. However, to allow precise determination of yields, the spacer separating tags 1 and 2 should be constituted by at least 8 atoms. This particular pair of mAbs was therefore selected to develop the sandwich-catELISA.

The Sonogashira reaction was chosen as a model coupling reaction. The terminal alkyne and iodo-aryl functions were therefore attached to tag 1 and tag 2, respectively, in a manner that the two tags of the Sonogashira product were separated by 9 carbon atom (Fig. 13a). Control experiments proved that the Sonogashira coupling product is detected in a dose-dependent manner with high precision, sensitivity and specificity (Fig. 13c). Wells of a microtitre plate were directly coated with mAb-203, while mAb-46 was conjugated to the enzyme acetylcholinesterase (AChE) according to known protocols.[33] Following a conventional sandwich immunoassay protocol using the Ellman reagent as colorimetric enzymatic substrate,[34] the Sonogashira product was detected down to 10 nM levels (detection limit: ~0.5 nM), while none of the starting reagents provided any detectable signal.

A catalyst library prepared by combining a series of 12 palladium species (4 homogeneous and 8 heterogeneous) with 8 ligands and 8 Cu, Ag or Au cocatalysts was tested in 12 different reaction conditions (6 bases and 2 solvents). Boc protection of the imidazole part of tag 1 was used in order to avoid possible interference with catalysts. The high sensitivity and selectivity of the sandwich-catELISA avoided any interference due to substrates, solvent or catalysts, thus permitting direct measurement of reaction yields without work-up of the crude mixtures (Fig. 13b). The 9216 parallel reactions were thus carried out in microtitre plates, quenched by TFA (Boc deprotection) and directly screened following transfer and

dilution on sandwich catELISA plates.[35] The whole process took 3 weeks to complete.

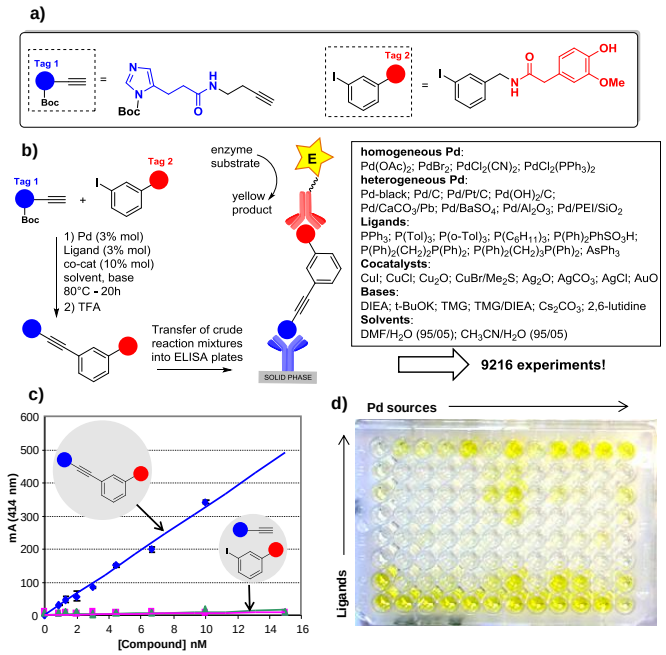


Fig. 13 Screening of Pd-catalysed Sonogashira reactions by sandwich-catELISA: a) structures of tagged reactants, b) target reaction and catalyst library, c) standard calibration curves of coupling product and starting reactants, d) example of a screening plate.

Interestingly, the best catalytic system highlighted by the screening was heterogeneous. The highest yield was indeed obtained by the combination Pd/C, CuBr.Me_2S, PPh_3, TMG in CH_3CN/H_2O. The technique was validated by comparing ELISA and HPLC values from 68 representative samples (Fig. 14a), precision of yield determination was evaluated as ± 3%.
The performances of the catalytic system identified by screening were then confirmed on non-tagged substrates (Fig. 14b).

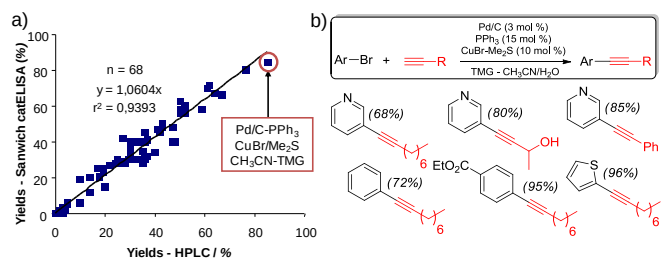


Fig. 14 Heterogeneous Pd-catalyst highlighted by screening: a) correlation between yields determined by sandwich-catELISA and by HPLC on 68 samples, b) scope of the catalyst identified by screening.

This study demonstrated the capacity of sandwich-catELISA to screen a high number of coupling reactions in a reasonable period of time: the throughput was ~500 reactions run and screened per day for one person.

4. Reaction discovery by immunoassays

The search for new chemical reactions is one of the cornerstones of organic chemistry. Reaction development has historically been guided by total synthesis or interest in developing general chemical processes of broad utility for chemists. Traditional approaches to reaction discovery therefore focus on one transformation at a time with a series of experiments designed on a rational basis. However, many chemical transformations were discovered serendipitously, leading to important, unanticipated breakthroughs in chemistry.[36] As an alternative to rational approaches based on mechanistic hypotheses or preconceived notions, "forced serendipity" strategies using the systematic evaluation of a large number of reactions by robust high-throughput screening methods have recently emerged. Pioneering work in this field was carried out by L. Weber and co-workers who reported the discovery of a new Ugi-type reaction after screening 10.000 reaction mixtures by LC-MS.[37] More recently, D. W. C. MacMillan's group discovered a photoredox-catalysed C-H arylation reaction using robotic GC-MS equipment.[38] A copper-catalysed alkyne hydroamination and two nickel-catalysed hydroarylation reactions were also recently discovered by J. F. Hartwig and co-workers using simple MS equipment.[39] Several new interesting reactions were also successfully identified by J. A. Porco Jr., A. B. Beeler and co-workers who developed a so-called "multidimensional" strategy[40] using a platform for microfluidic reaction screening.[41] A Pd-catalysed coupling reaction of alkylamides and alkenes and a photo-catalysed reduction of arylazides were discovered by D. R. Liu et al. using DNA-based technology.[42] Using a MALDI-TOF-MS screening method, S. A. Kozmin et al. discovered two new benzannulation reactions with 2-pyrones and quinoline *N*-oxides and a phosphine-catalysed three-component condensation.[43] The basic principle behind all of these investigations is very simple: the more reactions that are run, the higher the probability of discovery. However, two distinct fundamental approaches were followed depending mostly on the presence or absence of design in the choice of reactants. If substrates are chosen randomly, the discovery process is unbiased in design and free of preconceived ideas in attempts to identify new reactions. Conversely, when substrates are chosen by design the screening process will explore a specific chemical reactivity space. These screening approaches to reaction discovery have recently been summarised in an excellent review.[44]
In this context, our group was interested in evaluating the sandwich-catELISA technique in the field of reaction discovery.[45] Our strategy (Fig. 15) was based on a four-step procedure: 1) parallel reactions resulting from combinations of tagged functions **A** and **B** and metal catalysts, 2) quenching and transfer of reaction crudes to determine cross-coupling yields by sandwich-catELISA, 3) validation of hits by LC/MS analysis and 4) evaluation of hits by reproducing the active combination with non-tagged functions.

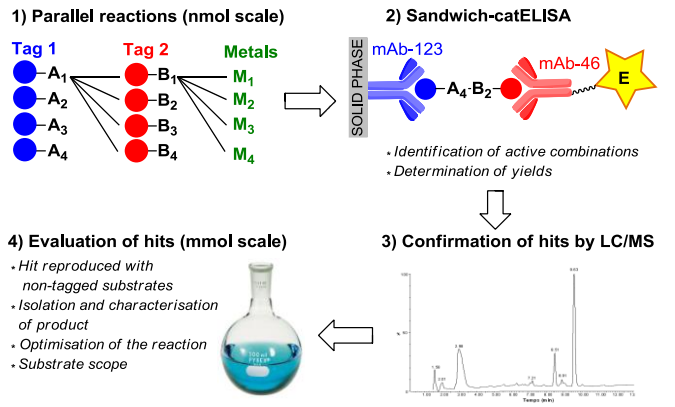


Fig. 15 Principle of the immunoassay-based reaction discovery process.

The core experiment was conducted with 21 tagged functions **A** and 16 tagged functions **B** (Fig. 16), most containing classic functional groups (alcohol, amine...) and some less common (skipped diyne, *N*-OH thiourea...); alkanes were also used as negative controls. No particular design was applied to select these reactive functions. The substrates were combined in a parallel manner and exposed to one set of reaction conditions with or without transition metals and with or without TEA in DMA at 50°C. These combinations led to a total of 3360 parallel reactions performed in 96-well plates. Following treatment with a TBAF/HCl solution (quenching and tag deprotection), the crudes were transferred to microtitre plates coated with anti-tag1 mAb-123. After washing, the enzyme-labelled anti-tag 2 mAb-46 was added and the enzymatic activity detected. The whole process took 5 days (3 for parallel reactions and 2 for screening) to highlight 44 new coupling reactions (Fig. 16).

Among these hits, only 9 gave acceptable yields and were therefore reproduced in flasks with non-tagged substrates. Seven reactions gave complex mixtures containing several coupling products and by-products, but 2 reactions led to clean formation of coupling products. These two reactions were therefore optimised in flasks to achieve exploitable chemical transformations. The first one was a Cu(II)-catalysed coupling of phenols with thioureas leading to isoureas at room temperature (scheme 9a). This reaction was further extended to the one-pot preparation of aminobenzoxazoles from aminophenols (scheme 9b). The second reaction was a Cu(II)-promoted coupling reaction of *N*-hydroxythioureas with terminal alkynes to give 2-iminothiazolidines. Despite numerous optimisation efforts, yields remained moderate, but the process was highly regioselective and always yielded only one regioisomer (scheme 9c).

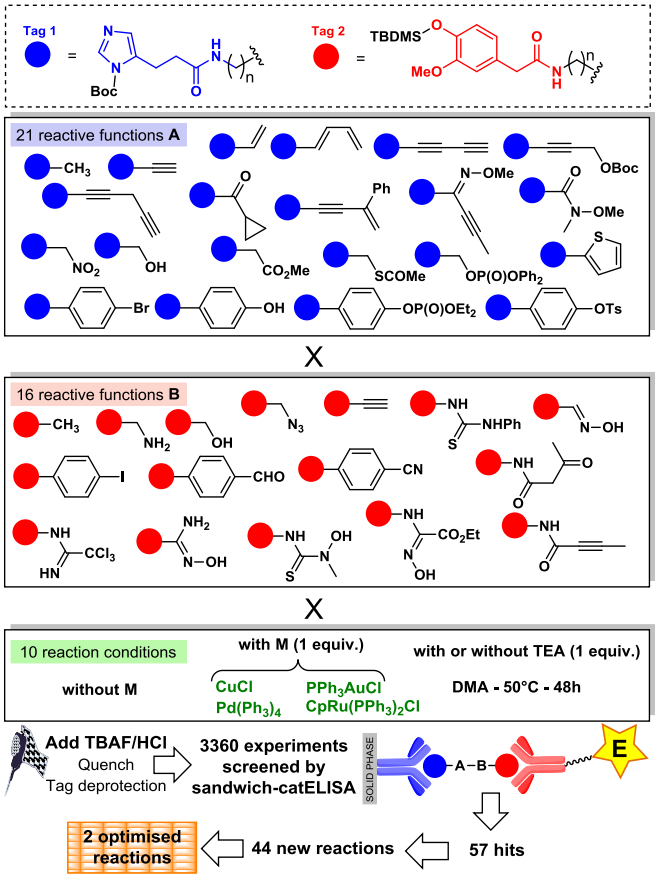
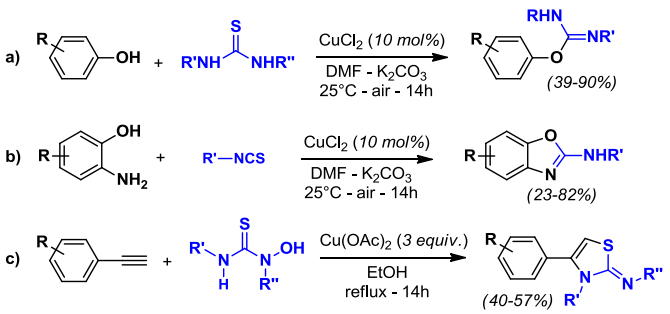


Fig. 16 List of combinations explored by sandwich-catELISA and global screening results.



Scheme 9 New reactions identified by sandwich-catELISA and further optimised in flasks. a) reaction 1: copper-catalysed formation of isoureas from thioureas and phenols, b) extension of reaction 1 to aminobenzoxazole synthesis; c) reaction 2: copper-promoted formation of 2-iminothiazolidines.

Feedback on this study underlined the success of the screening strategy, as two reactions of valuable synthetic interest were discovered, but also some drawbacks. Indeed, although the parallel reactions sandwich-catELISA high-throughput screening and hit confirmation by LC/MS (steps 1, 2 and 3 in Fig. 15) took only a few days, hit optimisations (step 4 in Fig. 15) were very time-consuming and, in fact, corresponded to the rate-limiting step of the overall discovery process. Furthermore, since the screening strategy is only based on reaction yields obtained with specific substrates, it does not guarantee the synthetic utility of the discovered reaction.

A more powerful approach to reaction discovery would be to increase the level of selection such that only powerful robust reactions can be discovered. Such highly demanding selection should therefore be based not only on reaction yields but also on other parameters that evaluate the scope and limitations of the discovered reaction. For this purpose, F. Glorius and co-workers developed a screening protocol using common GC analytical techniques that allows the rapid and robust evaluation of two major points that make a reaction useful: the functional group tolerance and the stability of chemical motifs under the reaction conditions.[46] In addition to these considerations, biocompatibility and bioorthogonality are two other criteria that strongly influence the extent and impact of a given chemical reaction. A reaction sharing all of these qualities belongs to the limited number of so-called "click" reactions[47] whose utility is not limited to synthetic chemistry but extends to the fields of material science and biotechnology.[48]

Because sandwich-catELISA is based on the highly specific binding properties of antibodies, it allows the quantification of cross-coupling products either in organic mixtures of compounds bearing diverse functional groups or in biological fluids. The technique should thus be able to evaluate the efficiency of coupling reactions in these complex media and to give information on both the tolerance to functional groups and the biocompatibility of the discovered reactions.

With the aim of discovering a novel reaction that fulfils most of the criteria of "click" chemistry, our group decided to exploit the full potential of sandwich-catELISA by designing a series of successive screenings that should select only efficient, chemoselective and biocompatible reactions.[49] In this new approach, the tagged reactants were not chosen randomly but were designed to undergo [3 + 2] cycloaddition reactions (Fig. 17).

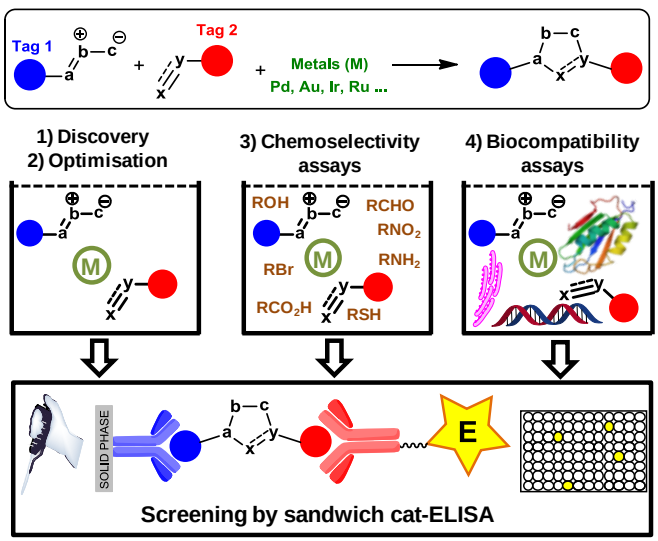


Fig. 17 Principle of the immunoassay-based process leading to the discovery of [3+2] cycloaddition reactions.

The sandwich-catELISA technique is not only used to identify active combinations of tagged dipoles, dipolarophiles and transition metals, but also to optimise the discovered reactions, and to assess their kinetics, chemoselectivity and biocompatibility. Only the reactions that successfully pass through all of these selection steps are studied in flasks with non-tagged functions.

This work was conducted with 11 tagged dipoles, 8 tagged dipolarophiles and 31 transition metals (Fig. 18). Control experiments in the absence of metal were also carried out in parallel. Reactants were combined in a parallel manner at 50°C in DMF leading to a total of 2816 parallel reactions performed in 96-well plates. After 2 days of screening, 41 new coupling reactions were identified. Optimisations were then carried out by assaying each reaction with 4 different metal salts, 8 ligands, 4 bases and 8 solvents. A new run of ELISA screening indicated that only 10 reactions were successfully optimised. Chemoselectivity and biocompatibility assays were then performed first in the presence of 1 equiv. of a mixture of nucleophiles (amine, thiol, acid...) or electrophiles (Michael acceptors, bromoalkane, aldehyde...) and, second, in the presence of concentrated protein, DNA or sugar solutions, in cell media as well as in blood plasma (Fig. 18).

The screening results highlighted 4 particular transformations, 3 displaying only limited chemoselectivity and bioorthogonality and one which successfully passed all the selection steps. Efforts were thus focused on these 4 reactions, which were reproduced in flasks to identify the structure of the coupling products. Pyridinium salts were found to undergo fast 1,3-dipolar cycloaddition at room temperature with aromatic nitriles in the presence of 20 mol % of cationic rhodium to generate imidazolopyridines. Yields and substrate scope were, however, disappointing (Scheme 10a). Under Pd-catalysis, sydnones react with alkenes through a dehydrogenative Heck-type coupling to afford 4-alkenyl-sydnones, but in moderate yields and poor regioselectivity (Scheme 10b). This reaction was recently improved by C. Kuang and co-workers.[50]

An interesting [3+2] cycloaddition reaction of azides with bromoalkynes catalysed by iridium dimer complexes was discovered, but unfortunately gave poor yields of a mixture of 1,4- and 1,5-bromotriazoles under the optimised conditions identified by screening. Further optimisation studies conducted in flasks, however, identified $[Ir(cod)OMe]_2$ as the optimum catalyst for this transformation, which regioselectively affords pure 1,4-bromotriazoles in good yields at room temperature (Scheme 10c).[51]

The best reaction identified by sandwich-catELISA screening was the Cu-catalysed sydnone-alkyne cycloaddition reaction, which we called CuSAC. The thermal version of this reaction has been known since Huisgen's work[52] and was later successfully exploited notably by the group of J. P. A. Harrity,[53] but high temperatures are required and specific alkynes are needed to avoid formation of mixtures of 1,3- and 1,4-pyrazoles.

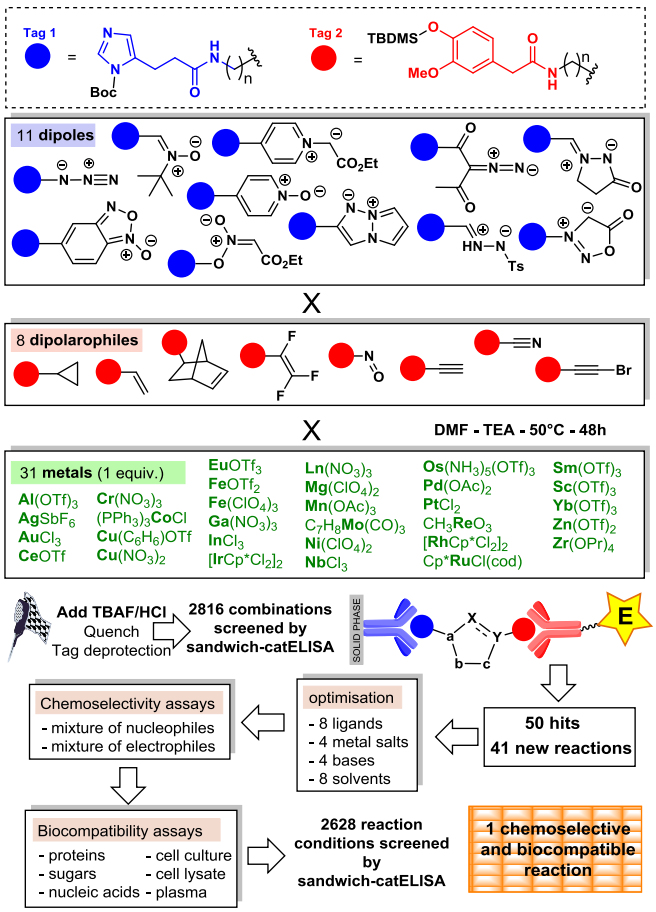
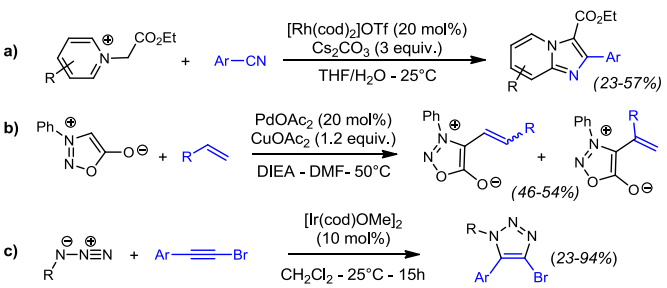
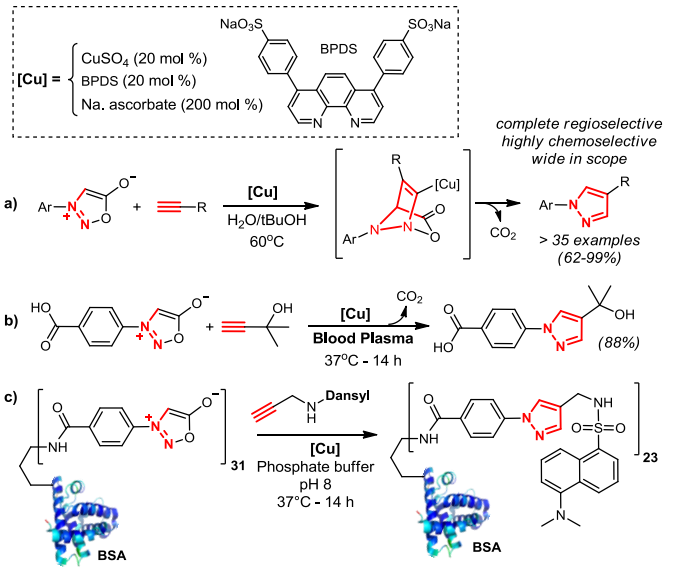


Fig. 18 Principle of the immunoassay-based reaction discovery process.



Scheme 10 Reactions discovered by sandwich-catELISA that are of synthetic interest but which display only limited chemoselectivity and biocompatibility.

The addition of copper-phenanthroline complexes is highly beneficial to this reaction: yields are usually very high, regioselectivity is total and, unlike the thermal mode, tolerance to chemical and biological functional groups is almost perfect, and reaction conditions are simple and mild (organic or aqueous solvents, temperatures from 30 to 60 °C, Scheme 11a). Furthermore, the reaction performed well in complex biological media, such as plasma (Scheme 11b), and was successfully applied to the fluorescent labelling of a model protein (Scheme 11c). The CuSAC reaction was recently used by our group to develop a practical synthesis of pyrazoles from arylglycines[54] and extended to the preparation of 1,4,5-trisubstitued pyrazoles.[55] The Cu-free version of this reaction was also recently developed and used for bioconjugation applications by J. W. Chin et al.[56] and by our group.[57]



Scheme 11 Cu-catalysed sydnone-alkyne cycloaddition reaction (CuSAC).

Overall, we considered that the reaction discovery strategy based on the exploration of a designed series of [3+2] cycloaddition reactions was more efficient than our previous strategy using randomly chosen substrates, because the design of reactants oriented the discovery in a good place and because sandwich-catELISA was used at its full potential. Indeed, the multiple screening, not only used for evaluation of reaction efficiency, but also for functional group tolerance and biocompatibility, increased the probability of discovering a chemical reaction of broad utility both for organic synthesis and biological applications. Such a strategy might be widely applied to identify new important reactions in the future.

5. Conclusions and perspectives

Many chemists are unfamiliar with immunoassays and probably think they are complicated or expensive to set up or both. In this review we have tried to show that ELISAs, on the contrary, should be considered as easy-to-use, powerful techniques for problem-solving in ambitious chemical research projects. We illustrate this with examples of catalysts and reaction discoveries and optimisation using immunoassay screening tools. However, immunoassays have yet to be fully exploited and their future possible applications in chemical fields are numerous. For example, the high specificity and sensitivity (up to the attomolar range) of immunoassays are evident advantages which can be fully exploited to detect low-abundance biocatalysts produced by phage display or expressed in microorganisms. A key advantage of immunoassays is that they can easily be miniaturised and automated, thus enabling unprecedented levels of throughput. In addition, the recent rise of microfluidic technologies presents great opportunities to enable small-scale and rapid chemical reactions.[58] The

combination of these two techniques already affords promising platforms for multiple, sensitive, and automatic diagnostics,[59] but may also be highly profitable in the future in the discovery of both catalysts and chemical reactions.

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