Enzyvitands: versatile evolvable colorimetric chembioreceptors

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Abstract Designing the perfect sensor is the dream of any chemist. Since decades, a wide diversity of synthetic receptors targeting analytes has been explored in chemistry. [1, 2, 3, 4, 5, 6, 7, 8, 9, 10] Their chemical optimization is hard and with no guarantee of success. [11] In this context, we propose a fast and self assembling colorimetric bio-chemical receptor coined Enzyvitand. It consists only of commercial chemicals. It relies on the reunification of combinatorial chemistry [12] , first and second coordination spheres interactions and indicators displacement assays. All harbored within a protein cavity [13]. The sensor is highly modular, cheap and evolvable. Thanks to its solved X-ray structure, we rationally designed it for the selectiv naked-eye recognition of dopamine over other neutrotransmitters through second coordination sphere. Hence, our sensor imitates a biological receptor for the recognition of neurotransmitters. Finally, it works in complex samples such as urine. Its immediate high versatility and evolvability is valuable for the selective detection of a wide assortment of analytes from small molecules up to micro-organisms. For the future, we anticipate new biotechnological or immunotherapeutic applications of our synthetic oligomer. [14, 15, 16, 17, 18, 19, 20, 21, 22]

1 Introduction

The literature is constalleted with descriptions of synthetic receptors for the detection of a plethora of analytes. They are divided in six categories (see Figure 1) such a for example: small organic molecule with; cyclophanes [23], crown ethers [24, 25], cryptands [26], cavitands [27, 28], calix[*n*]arenes [29], calix[*n*]pyrroles [30], cucurbit[*n*]uril [31], cyclodextrines [32, 33], pinwheels [34, 35], boronic acids [2, 3, 5, 6, 7, 10], inorganic molecules with for example: MOFS [36], molecular imprinted polymers, dendrimers, nanoparticules [37] and synthetic oligomers such as; aptamers or boronic acid synthetic code proteins. All these categories of sensors take advantage of the first coordination sphere to capture analytes, except synthetic oligomers. None of them are easily synthetically optimized thought.

Each requires synthesis steps that take time, are tedious and do not guarantee success. Based on this, by observing Nature more closely, we realize that bio-receptors such as membrane receptors [38, 39] are perfectly designed for the recognition of analytes. [39] Mention may in particular be made of membrane receptors for the recognition of neurotransmitters, for example dopaminergic receptors. [39, 40] These optimized receptors [41, 42] take advantage of the second coordination sphere given by a

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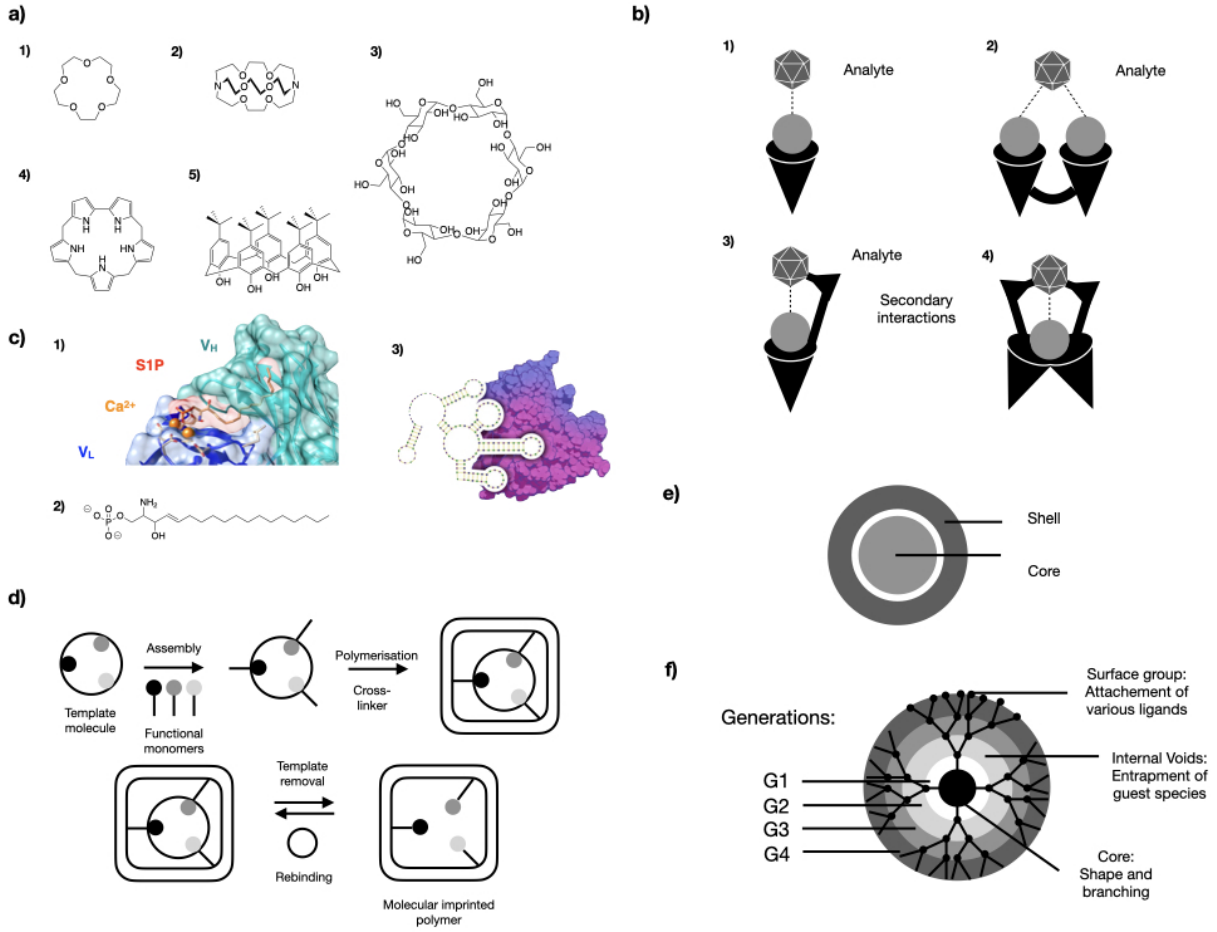

Figure 1. Categories of sensors used for detection of analytes a) small organic molecules b) inorganic molecules c) synthetic oligomers d) Molecular imprinted polymers e) Nanoparticules f) Dendrimers

proteic environment for selective recognition. The active recognition site is decorated with amino acids that interact with the analyte to hold it captive. Dopamine, for example, is locked in the active site through it's catechol moiety via a serine microdomain. Whereas, the protonated amine of dopamine at physiological pH is locked with an ionic bond through the interaction with an aspartate conserved in all biogenic amine GPCRs. [43, 44] Therefore, inspired by chemical and biological sensors, we imagined to merge the best of both worlds to design a novel type synthetic oligomer. A sensor optimized by the first and second sphere of coordination for the exquisite recognition of analytes. We strove to add additional optical properties to it, in particular a color change during detection by using an indicator displacement as well as evolvable features for evolution processes.

In that purpose, we chose a well described protein with a deep cavity. [13] We then redesigned its active site by introducing ligand into it. This ligand carries a functional group and is anchored supramolecularly to the protein. On this functional group we added a coordination dependant indicator. When an analyte enters the active site, it attaches to the functional group and displaces the indicator. The change in color indicates interactions between the active site and the analyte. The second sphere of coordination acts as a screen. It selects the access to the analyte at the active site. More specifically, we have incorporated into the carbonic anhydrase, boronic acids [2, 3, 5, 7] coordinated with indicators. These colorimetric sensors were screened in the presence of neurotransmitters.

The results show that we have obtained a highly selective, cheap and easy to assemble dopamine sensor mimicking biological membrane receptors. Such a discovery allows us to imagine optimizations taking advantage of the broad possibilities such as the ligand and its fonctionnal group, the indicators, the

protein by mutation, the pH, the temperature and or the concentration of the reactions, etc. This can lead to dynamic combinatorial chemistry [12] , high throughput screening [45] and optimization processes via directed evolution protocols [46] using for example machine learning [47] . The immediate high adaptability and ability of our sensor to be evolved can be useful for the selective detection of a wide variety of analytes going from small molecules to microorganisms. This discovery therefore makes it possible to dream of new biotechnological or new immunotherapeutic applications [14, 15, 16, 17, 18, 19, 20, 21, 22] We show, proof of principle, that guided through the crystal structure of our receptor, we can create a cheap, easy to assemble, colorimetric biosensor for the selective naked-eye detection of dopamine which mimics a dopaminergic receptor.

2 Methods

2.1 First coordination sphere detection

Dissolving 4-sulfamoyl(phenyl)boronic acid (**1** *p*-BA, 250 μM) and pyrocatechol violet (**2** PCV, 250 μM) in 2-[4-(2- hydroxyethyl)-1 piperazinyl]ethanesulfonicacid (HEPES, 100 mM, pH=7.4) in a 1:1 mixture formed (*p*-BAPCV, 250 μM), which is water-soluble and orange-red at working micromolar concentrations (see Figure 2). By homology with other reported arylboronic acid described in literature, we hypothesized that *p*-BAPCV could be formed and used to assemble a receptor for the naked-eye detection of various vicinal diols such as carbohydrates as described for **17** (D)-fructose [2] (see Figure 4) or catechols [1] such as **9** dopamine (see Figure 2 and Figure 4).

As anticipated *p*-BAPCV can detect visually and spectrophotometrically both **17** (D)-Fructose and **9** dopamine with a concomitant color change using an indicator displacement assay (IDA) (see Figure 2). We determined the association constant of *p*-BAPCV with **9** dopamine using an indicator displacement assay and we obtained a K_{ass}=2.12 $\cdot$ 10^6 M^{-1} (see Table 1). [48] [2] Screening of various pHs ranging from pH=5 to pH=8 showed the best discrimination at pH=7.4 as reported in other publications. [2, 6]

2.1.1 Chemical diversity

To generate diversity in the composition of our sensors, we tested and used chemical optimization strategies. In such, combining two different sulfonamides boronic acids (**1** *p*-BA or **2** *m*-BA) with catechol based indicators (**3** PCV or **4** ARS) yielded four potential sensors for the detection of analytes. Of the four sensors tested only **1** *p*-BA was able to coordinate either **3** PCV or **4** ARS forming: *p*-BAPCV and *p*-BAARS. **2** *m*-BA did not reacted with both indicators except with a large excess of indicator (min 100 eq.) suggesting that the position of the boronic acid in the aryl-sulphonamid moiety plays a key role in the coordination process of the indicator. With both *p*-BAPCV and *p*-BAARS in hands, we tested the two chemical variants with **17** (D)-Fructose for recognition and both offered an indicator displacement assay allowing detection of the analyte and showing no special selectivity in the recognition process (see Figure 4). For the continuation of our study, we therefore selected *p*-BAPCV and *p*-BAARS.

2.2 Second coordination sphere detection

The catalytic Zn-active site present at the bottom of a deep hydrophobic funnel-shaped cavity of carbonic anhydrase endows high affinity for para-substituted aryl sulphonamids. [13] We thus speculated that upon tethering an aryl-sulfonamides boronic acid conjugated with an indicator may allow to anchor a recognition site within **5** bCA allowing an exchange between the indicator and selected molecules of choice (see Figure 2). In a first time, in order to confirm our hypothesis, we assembled the arylboronic acid **1** *p*-BA (250 μM) and **2** PCV (250 μM) in HEPES 100 mM, pH=7.4 and formed *p*-BAPCV (λ_{max}=465 nm) which is orange colour in a 1:1 mixture. We performed a titration of **1** with **2** in order to determine the binding constant of the host-guest receptor and obtained a K_{ass}=1.36 $\cdot$ 10^5 M^{-1} (see Table 1) with a 1:1 association.

In a second time, we added **5** bCA (1 eq.) to the 1:1 mix of *p*-BAPCV or *p*-BAARS (250 μM in HEPES pH=7.4). For *p*-BAPCV$\subset$ bCA, a bathochromic change in colour appeared immediately after the addition of the protein (see Figure 2). The orange solution (λ_{max} of=495 nm) of *p*-BAPCV turned intense pink with a λ_{max} of=525 nm (see Figures 2 and 4). Titration of **5** bCA with *p*-BAPCV shows a batochromic shift in

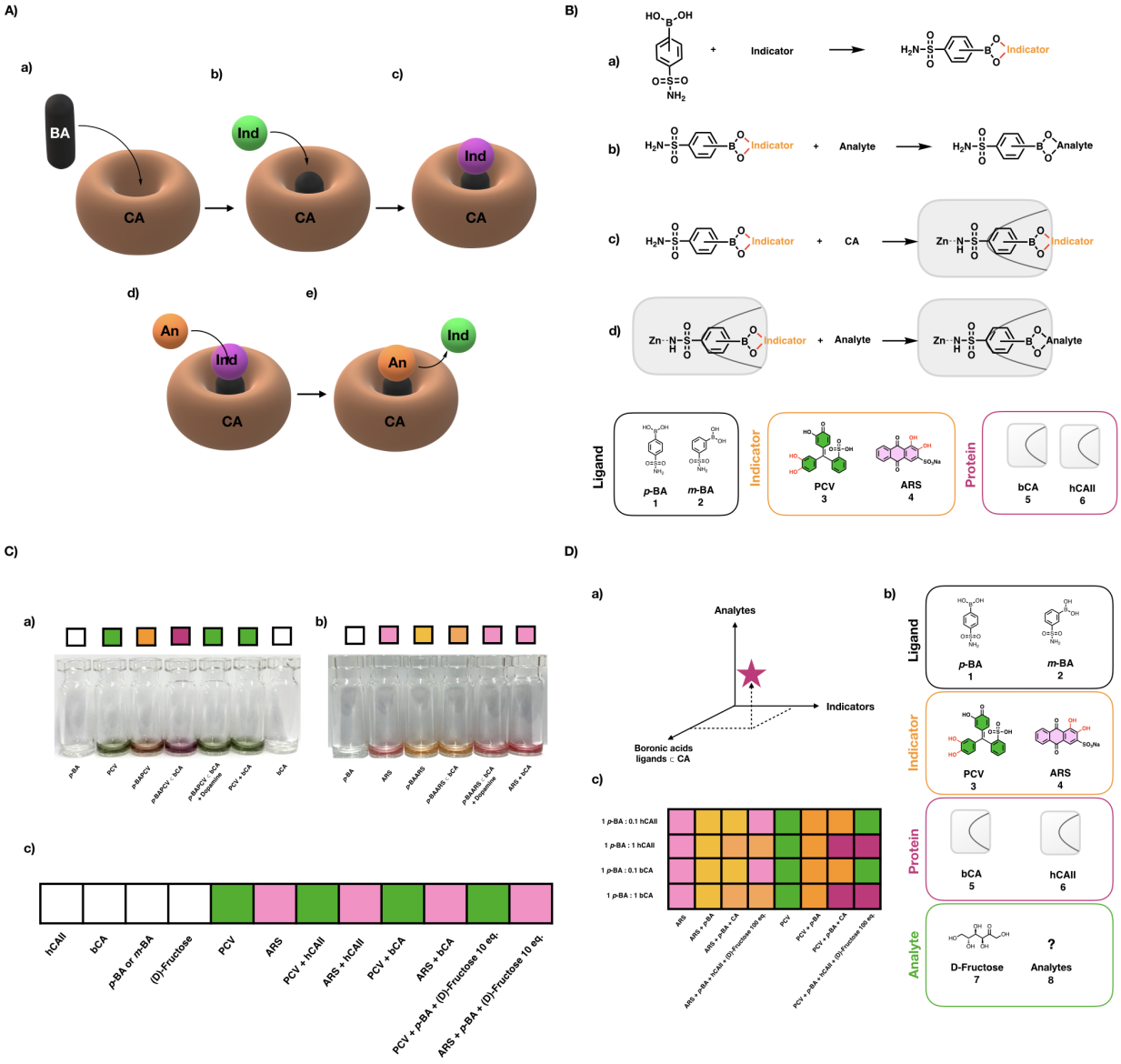

Figure 2. A) General principle of the supramolecular sensor: Addition of an arylsulfonamid boronic acid (BA) to carbonic anhydrase variants (CA) offers BA ⊂ CA. Addtion of an indicator (Ind) to the supramolecular construct allow the creation of BAInd ⊂ CA. Final addition of an analyte (An) allows an indicator displacement assay in the deep cavity of the protein liberating the indicator and forming BAInd ⊂ CA with a concomittant change in color **B)** Synthesis of the synthetic receptor; arylsulfonamidboronic acid **1** (250 μM) is added to catechol based indicator **3** or **4** (250 μM) in a 1:1 mixture, a naked eye change in color is observed. Upon addition of CA (either **5** bCA and **6** hCAII, 250 μM) to the mix, the synthetic receptor BAInd⊂ CA (250 μM) is formed and a change in color of the solution appears. The addition of analytes of interest **8** (10 eq., 2500 μM) allows a concomitant color change with an indicator displacement assay and forms BAAn ⊂ CA and releases the indicator **3** or **4**. **C)** a) When **1** or **2** is added to **3** or **4**, a coordination may occur of the indicator to the boronic acid generating a change in color. Addition of **5** bCA to the construct forms a colored solution indicating the formation of BAInd ⊂ bCA. Addition of a 10 eq. of **9** dopamine allows an indicator displacement assay releasing the indicator and with a concomitant change in color b) Control experiments. **D)** Optimisation strategy for the creation of a library of biosensors b) Building blocks for the creation of a biosensor library c) Results of the optimization screening: **2** PCV is green in solution at pH=7.4 (250 μM), upon addition of **1** *p*-BA 250 μM, the solution changes colour to orange forming *p*-BAPCV. The addition to the mix of **6** human carbonic anhydrase II (250 μM), generates a bathochromic shift in colour from orange to pink suggesting the entry of the complex inside the pocket of the protein and forming *p*-BAPCV ⊂ hCAII. The addition of an excess of **17** (D)-fructose (0.025 M, line 1 Cplx: 1 hCAII) to *p*-BAPCV ⊂ hCAII doesn't allow a displacement of the indicator (**3** PCV or **4** ARS) whereas outside of the protein (Line 1 Cplx : 0.1 hCAII) the indicator displacement assay occurs. It suggests that the protein prevents the entry of **17** (D)-fructose due to second coordination sphere interactions.

Table 1. Association constants of systems

System	K_{ass} [M^{-1}]
p-BA with PCV	$1.36 \cdot 10^5$
p-BAPCV with dopamine	$2.12 \cdot 10^6$
p-BAPCV with bCA	$2.08 \cdot 10^5$
p-BAPCV ⊂ bCA with dopamine	xxxxxxx

color from 495 nm to 525 nm and a Job Plot titration confirms a 1:1 association of the bio-receptor (see supporting information) and an association constant of $3.60 \cdot 10^5$ M^{-1} (see Table 1). Same conclusions where observed for *p*-BAARS ⊂ where the shift in color is from 480 nm for *p*-BAARS to 485 nm for *p*-BAARS ⊂ however less pronounced than for p-BAPCV⊂ bCA. The change in color confirms that the assembled orange colored p-BAPCV enters the protein. More interestingly, the order of the addition of **1** *p*-BA, **3** PCV and the protein **5** bCA didn't play any critical role. One can either form *p*-BAPCV before addition to **5** bCA or add *p*-BAPCV to the protein and add the indicator such as **3** PCV in a second time for coordination to *p*-BAPCV (see Figures 2 and 4) leading to an *indicator uptake-assay* which is known to improve the sensitivity of the assay compared to the displacement. [49] It suggests that CA can be used as a boronic proteic based platform for the recognition of analytes opening new routes for therapeutic proteins against catecholamines and that **3** PCV can be used as a sensor to probe the recognition of analytes coordinated to the boronic acid inside the active site of the protein.

2.2.1 Proteic diversity

In order to increase the diversity and induce selectivity in our sensors, we combined the two constructs *p*-BAPCV and *p*-BAARS with two variants of carbonic anhydrase such as **5** bCA (bovine carbonic anhydrase) and **6** hCAII (human carbonic anhydrase). The combination offered a four possibilities matrix ready to be screened with analytes; *p*-BAPCV ⊂ bCA, *p*-BAPCV ⊂ hCAII, *p*-BAARS ⊂ bCA, *p*-BAARS ⊂ hCAII.

2.3 Indicator displacement assay

We then tested our matrix of four sensors; *p*-BAPCV ⊂ bCA, *p*-BAPCV ⊂ hCAII, *p*-BAARS ⊂ bCA, *p*-BAARS ⊂ hCAII for the detection of **17** (D)-fructose troughs an IDA since recognition is described in literature outside a second coordination sphere. [2] The detection was not possible even in the presence of a large excess of **17** (D)-fructose (100 eq. of fructose compared to the proteic bio-congugates) (see Figure 4D) thus suggesting that the cavity of the protein prevents the entry of the sugar compared to the free assembled ligand-boronic complex *p*-BAPCV. In order to confirm the supra-molecular assembly, we performed endpoint assay using the complex *p*-BAARS and *p*-BAPCV with a 1 (250 μM) to 0.1 (25 μM) ratio of *p*-BAARS and *p*-BAPCV versus **5** bCA and an excess of **17** (D)-fructose (100 eq., 0.025 M) (see Figure 2). Color change outside the protein was observed both for **3** ARS and **4** PCV as indicators confirming that the excess of *p*-BAARS and *p*-BAPCV (250 μM) outside the protein reacts with **17** (D)-fructose (seeFigures 2 and 4). As a consequence and in order to explore further the detection ability of our construct, we decided to investigate various analytes for recognition.

2.4 Crystal structure of the bio-sensor

In order to do so and predict further the interactions of potential analytes with our biosensor, we set out to crystallize it. hCAII Crystals were obtained using sitting-drop vapor diffusion at 20 °C in 24-well plates as described [50]. Plate-shaped crystals matured within one week in the reservoir containing 2.6 M $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 8.5.**1** *p*-BA was soaked inside the enzyme subsquentially. The soaked crystals were incubated at 20 °C for 2 h, followed by flash freezing in liquid nitrogen. Diffraction data was collected at the beamline X06SA at the Swiss Light Source (SLS, Villigen). Datasets were processed and merged with XDS [51]. The phases were solved by molecular replacement using Phaser [52] with hCAII structure (PDB code: 3ZP9) as the search model. The model building was done in COOT [53] and

refined with Phenix [54] (X-ray data and refinement statistics are given in the supporting information). The obtained crystal structure (see Figure 3) was compared with the reported structure of hCAII coordinated to **18** 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylsulfamide [55] (PDB code: 3MNU, see Figure 3). Attempts to obtain crystal structure of *p*-BA ⊂ hCAII in conjugation with **3** PCV or **9** dopamine were not possible probably due to disruption of crystals in the soaking process.

2.5 Screening of analytes

With the obtained crystal structure (PDB code: 6XVH) in hands (see Figure 3) we reasoned that molecules with a geometric similarity with the cavity should enter more easily the pocket of CA compared to **17** (D)-fructose. [2] In so, we were particularly interested in the close lying aminoacids to the boronic acids moiety such as: T-200, L-198, F-131, V-135, C-206. Remarkably, we observed that F-131 could interact with potential tested analytes entering the activ site of our construct.

In addition, for screening of analytes of interest, we concentrated our efforts towards medically relevant and compatibles geometries molecules such as : **9** dopamine, **10** (*S*)-DOPA, **11** (*R*)-norepinephrine, **12** (*R*)-epinephrine, **13** GABA, **14** (*S*)-glutamic acid, **15** homovanilic acid, **16** DOPAC, **17** (D)-Fructose. (see Figure 4). Finally, we selected *p*-BAPCV ⊂ bCA and *p*-BAPCV ⊂ hCA II for screening since best discrimination with the naked eye is obtained with these systems (going from pink to green).

3 Results

The results of the screening showed that *p*-BAPCV (250 μM) is able to recognize the catechol based neurotransmitters tested (small excess of 10 eq., 2500 μM) showing no special selectivity (see Figure 2 and Figure 4). **13** GABA or **14** glutamic acid for example where not recognized even with a large excess of analyte (10 or 100 eq.). *p*-BAPCV ⊂ bCA and *p*-BAPCV ⊂ hCAII are not able to recognize **17** (D)-fructose even with a large excess (100 eq. compared to the bioreceptor *p*-BAPCV) suggesting that the second coordination sphere plays a critical role in preventing the recognition of the analyte (seeFigures 2 and 4). *p*-BAPCV ⊂ bCA and *p*-BAPCV ⊂ hCAII recognize preferentially **9** dopamine over **11** (*R*)-norephinephrine or**12** (*R*)-ephinephrine and other catechol based neurotransmitter even with significant structural similarities suggesting that second coordination sphere plays an Determining role in the selectivity process in the cavity of the protein (see Figure 4). Hence, **15** Homovanilic acid is not recognized by either *p*-BAPCV ⊂ bCA or *p*-BAPCV ⊂ hCAII suggesting that the catechol moiety plays a critical role for the recognition of the analyte. *p*-BAPCV ⊂ bCA II and *p*-BAPCV ⊂ hCAII follows the same trend in recognition of analytes suggesting that the active sites of both proteins are similar and pursue similar recognition patterns (see Figure 4). **9** dopamine is preferentially recognized over **16** DOPAC suggesting that the functional group at the tale of the neurotransmitter interacts with the second coordination of CA. A positive charge plays a key role in the recognition process (**9** dopamine) over a negative charge (**16** DOPAC).

3.1 Detection of analytes discussion

In order to understand the mechanism of selection discriminating the recognition between **9** dopamine and **16** DOPAC we sought to manually dock **9** dopamine inside *p*-BA ⊂ hCAII. Upon manual bond rotation we found that F-131 was close lying to the tail of **9** dopamine (see Figure 3). We then realized that the close proximity of an aromatic ring to an amine may lead to an NH/π interaction that could stabilize the interaction between **9** dopamine and *p*-BA ⊂ hCAII. We therefore suggest a mechanism of recognition with an NH/π interaction F-131 (see Figure 4). It is remarkable to mention the similarity of our artificial system with natural ones. The serine microdomain present in the dopaminergic receptors that interacts with the catechol moiety of dopamine ressemble the boronic acid of our receptor. In addition, dopamine is stabilized in the natural transmembranar receptor via an ionic bond taking advantage of aspartate to the amine moiety. In our system, the stabilisation of dopamine occurs with an NH/π via F-131.

3.2 Test of the best sensor in a complex sample

In order to test our best sensor *p*-BAPCV ⊂ bCA in real conditions, we decided test the detection of **9** dopamine in human urine. Urine is known to contain traces of **9** dopamine due to metabolic activities in

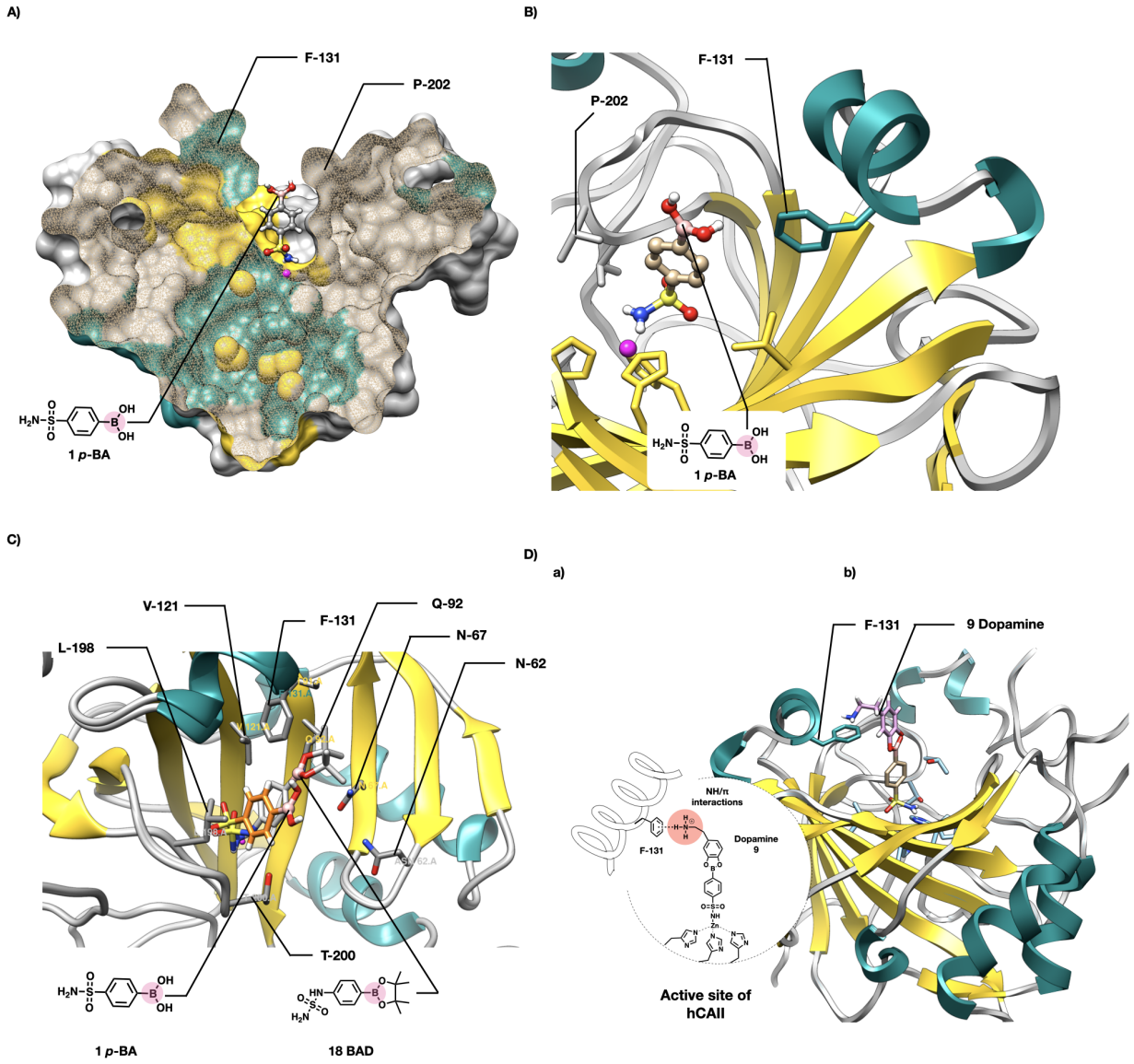

Figure 3. **A)** Surface representation sectioning of the X-ray structure **1** *p*-BA (shown in sticks) inside hCAII (PDB code: 6XVH).Of particular interest is the close lying aminoacid (F-131) to the boronic acid center of the supramolecular construct. **B)** a) Close-up view of *p*-BA inside hCAII b) statistic table of the crystal structure *p*-BA ⊂ hCAII **C)** Superposition of hCAII in conjugation with **18** BAD (PDB code: 3MNU) and hCAII with **1** *p*-BA (PDB code: 6XVH). Of particular interest are the aminoacids close to the binding site such as N-62, T-200, V-121, F-131. **D)** a) Proposed mechanisms for the recognition of dopamine inside *p*-BA ⊂ hCAII, a cataionic NH/π should occurs with close proximity of F-131 b) Manual docking of dopamine inside the active site of *p*-BA ⊂ hCAII.

the human body but not detectable in the range of our sensor. In a first time, we tested *p*-BAPCV in the presence of pure urine from two different persons. Naked-eye detection of **9** dopamine traces was not possible but slight disturbance appeared on the sensor. Urea is known to denature proteins at very high concentration. However, it is remarkable to mention the robustness of our sensor given the complexity of the sample tested.

In a second time, we spiked urine with 5 eq. and 10 eq. of **9** dopamine versus *p*-BAPCV ⊂ bCA for recognition. A concomitant change in color appeared immediately proving the possible determination of **9** dopamine inside complex samples such as human urine (see Figure 5). It allows to conclude that our sensor can be used in complex samples for the detection of **9** dopamine.

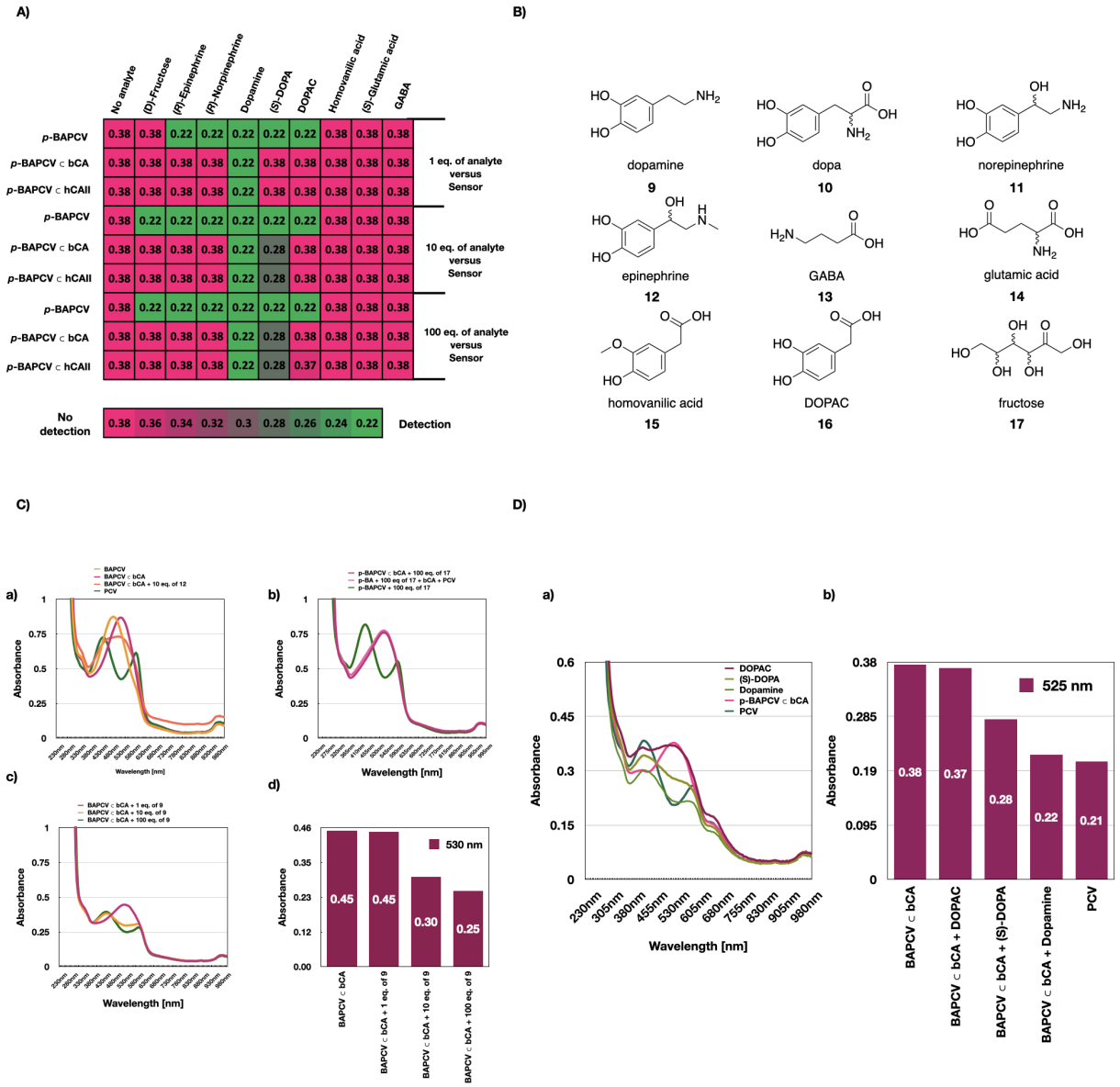

Figure 4. A) Screening of the synthetic bio-inspired receptor *p*-BAPCV ⊂ bCA for recognition of neurotransmitters. *p*-BAPCV (250 μM), outside the protein is orange whereas inside the protein (CA, 250 μM), the construct (*p*-BAPCV ⊂ bCA) is pink. Addition of a small excess (2500 μM, 10 eq. versus sensor) of the analyte to the boronic acid outside of the protein may allow an indicator displacement assay. When *p*-BAPCV (250 μM) is incorporated inside either **5** bCA or **6** hCA II (250 μM) forming *p*-BAPCV ⊂ bCA only **9** dopamine can be fully recognized even with a large excess (0.025 M, 100 eq.) of the other analytes. **B)** Analytes used in the screening of this paper **C)** Absorbance spectras of the screening a) the incorporation of *p*-BAPCV (250 μM) into bCA (250 μM) allows a bathochromic shift in colour from λ_{max}=495 nm to λ_{max} =525 nm. Recognition of (*R*)-epinephrine **11** (100 eq., 0.025 M) at a concentration 100 eq. higher than **5** *p*-BA allows a poor recognition b) addition of **17** (D)-fructose to *p*-BAPCV ⊂ bCA doesn't allows an IDA either c) addition of a small excess (10 eq., 2500 μM) of dopamine **9** to *p*-BAPCV ⊂ bCA allows an indicator displacement assay d) Decreasing absorbance upon addition of various catechol based analytes such of **9** dopamine (from 1 eq. to 100 eq.) to *p*-BAPCV ⊂ bCA D) a) Absorbance spectras of *p*-BAPCV ⊂ bCA in addition with various analytes b) Absorbance of *p*-BAPCV ⊂ bCA with various analytes at 525 nm.

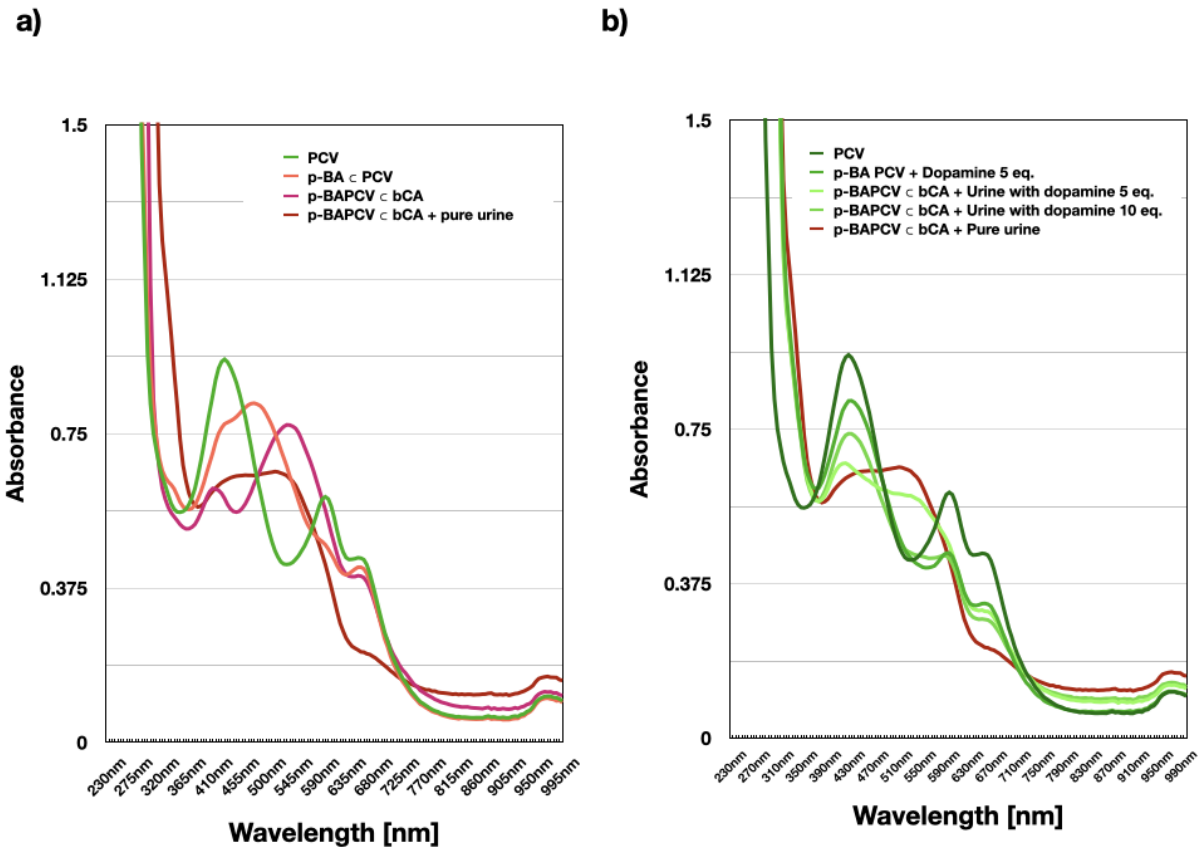

Figure 5. a) *p*-BAPCV ⊂ bCA in the presence of urine b) *p*-BAPCV ⊂ bCA in the presence of urine and spiked with **9** dopamine.

4 Conclusions

Bio-molecular systems such as enzymes normally are dedicated to substrate transformations and allows very specific catalytic processes that depends on the composition of the active site. Here we have shown that such an active site can be completely redesigned by the introduction of a specific ligand bearing a functional group. In specific, we have shown that boronic acids can be introduced supramolecularly into a proteic environment and coordinated to indicators allowing specific detection of analytes using a displacement assay, for example dopamine. Despite their limited detection limits, these bio-receptors are distinguished by their easy to assemble, excellent selectivity, commercial disponibility, high modularity and evolvable features. Key to our construction strategy was the reimagination of the active site of carbonic anhydrase within a new structural context; a new boronic acid ligand for coordination of various analytes surrounded by a selective second coordination sphere. This example expands the generanal use of enzymes transforming them into exquisite biosensors. It could potentially be used for detection of various analytes going from small molecules to microorganisms mimicking natural antibodies.

5 Competing interests

The authors declare no competing interests

6 Contributions

Thibaud Rossel conducted the research, performed the experiments and wrote the manuscript. Bing Zhang produced crystals solved the X-ray structure. Raphael Gobat helped with the crystals production and with solving the X-ray structure.

7 Experimental section

All chemicals were purchased by Sigma Aldrich and used without further purification. hCAII was synthesized by Thomas R. Ward group University of Basel Switzerland.

A typical experiment (such as Figure 4**C** and **D** is realized as follows (all solutions are freshly prepared prior to experiment):

25 μL of a 250 μM solution of (*m* or *p*)-BA in HEPES pH=7.4 100 mM is added to a 25 μL of a 250 μM solution of indicator (PCV or ARS) in a 96 well plate (same buffer). Upon change in color, 25 μL of CA is added to the mix and change in color is observed (same buffer). Addition of 25 μL of analyte allows an indicator displacement assay and a concomitant change in color. The absorbance spectra of the wells are measured in a 96 well plate reader TECAN safire 2 for analysis and comparison.

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