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A Chimeric Biocatalyst Combining Peptidic and Nucleic Acid Components Overcomes the Performance and Limitations of the Native Horseradish Peroxidase

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ABSTRACT

Chimeric peptide-DNAzyme (CPDzyme) is a novel design of an artificial peroxidase that relies on the covalent assembly of DNA (quadruplex-DNA, or G4), peptides and an enzyme cofactor (hemin) in a single scaffold. An accurate control of the assembly of these different partners allows for the design of the efficient CPDzyme prototype G4-Hemin-KHRRH, found to be >2,000-fold more active (in terms of conversion number k_{cat}) than the corresponding but non-covalent complex and, more importantly, >1.5-fold active than the corresponding native peroxidase (horseradish peroxidase, or HRP) when considering a single catalytic center. This unique performance originates in a series of improvements gradually made thanks to an accurate selection and arrangement of the different components of the CPDzyme, in order to benefit from synergistic interactions between them. The optimized prototype G4-Hemin-KHRRH is efficient and robust as it can be used under a wide range of non-physiologically relevant conditions (organic solvents, high temperature (95°C), in a wide range of pH (from 2 to 10)), thus compensating for the shortcomings of natural enzymes. Our approach thus opens broad prospects for the design of ever more efficient artificial enzymes.

INTRODUCTION

Scientists are devising different strategies to circumvent the deficiency of native enzymes, yet highly efficient but intrinsically fragile, including for instance directed evolution¹⁻³ or enzyme immobilization.^{4,5} Another strategy, currently in the limelight, is the design and use of artificial enzymes. The structure of native enzymes, including proteinaceous enzymes but also nucleic acid-based DNAzymes and ribozymes, relies on a handful of building blocks only, *i.e.*, amino acids, nucleotides, and redox molecules.⁶⁻¹⁰ The recent advances in the development of ever more efficient artificial enzymes stemmed from the deeper understanding of the catalytic mechanism of native enzymes^{7,11} and the application of this knowledge to recreate high-performance catalytic sites in synthetic biocatalysts.¹²⁻¹⁴

The usual routes to construct biomimetic enzymes rely on either the chemical optimization of naturally occurring catalysts¹⁵⁻¹⁷ or the design of new artificial catalysts on the basis of the structural insights gained from studying the enzyme active sites.¹⁸⁻²⁰ A typical example of this approach is the development of horseradish peroxidase (HRP) surrogates:¹³ HRP is a hemoprotein (*i.e.*, containing a heme prosthetic group) that is a well-established molecular tool for ELISA assays, immunohistochemical investigations and immunoblotting analyses. Besides protein engineering²¹⁻²⁶ and *de novo* design of enzyme mimics,²⁷⁻³³ a well-known HRP-mimicking system is G-quadruplex/hemin DNAzyme, originating in the association of a peculiar four-stranded DNA structure (G-quadruplex) with the HRP cofactor hemin. Alternative efforts have also been invested to provide hemin with a binding site in which it can be catalytically activated, using for example self-assembled peptides in the aim of encapsulating the catalytic center from the external deactivating aqueous environment^{34,35} or facilitating new catalytic reactions.³⁶ These works highlighted the potential of DNA and/or peptide building blocks to design artificial enzymatic systems, whose additional interest resides in their structural modularity *via* a well-established chemistry. This led us to envision that combining DNA and peptidic building blocks around the hemin cofactor within a single scaffold could provide an unprecedentedly active artificial peroxidase.

However, a bottleneck in the design of nature-inspired catalysts stands in the difficulty to recreate the highly complex three-dimensional organization of the enzyme active site. Some recently developed building blocks have expanded the possibility uniquely provided by mimetic enzymes:^{18,20,37} for example, efforts were invested to recreate and then optimize the hemin-iron catalytic center, boosting its activity by introducing additional ancillary iron-chelating moieties,³⁸⁻⁴⁰ which opens ways toward new applications,^{12,41} yet at the expense of an easy chemical access.^{17,42-45} In fact, this long-standing quest has provided interesting artificial

catalysts, although no system relying on a single catalytic site actually competes with natural enzymes.

We took up and met this challenge here, introducing a system named chimeric peptide-DNAzyme (CPDzyme), whose design hinges on a strategy in which finely selected building blocks are positioned in an optimized arrangement. This design relies on a combination of the building blocks described above (G-quadruplex, hemin, and either amino acids or oligopeptides) within a single scaffold, in a controlled arrangement thanks to the covalent links created between the different blocks. The best CPDzyme prototype, named G4-Hemin-KHRRH, surpasses HRP in k_{cat} (when considering a single catalytic site), thus establishing a new landmark in the artificial enzyme field. Its superiority over HRP was further substantiated by its implementation in reactions performed under harsh conditions such as high temperature (up to 95 °C), extreme pH (from 2 to 10) and non-aqueous media, which is precluded for HRP. This work thus provides a new design of artificial metalloenzymes and opens up a brand new field of industrially-relevant applications.

RESULTS

Preparation of Chimeric Peptide-DNAzyme. CPDzymes are devised as artificial catalysts that must be easily prepared and purified. To this end, our approach relies on the dissymmetric modification of the two carboxylate arms of the hemin and on an on-bead synthesis strategy using streptavidin-coated magnetic beads (MB-STV) to avoid time consuming and laborious synthesis and purification (Figure 1a). First, MB-STV was used to connect the chemically modified DNA sequence containing G-quadruplex-forming sequences (sequences details shown in Table S1). For example, the sequence biotin-T₉/iPCLink/TG₃TG₃TG₃TG₃-NH₂, termed bio-PC-G4-NH₂ (the G-quadruplex-forming sequence is underlined) is biotinylated on its 5' end to be immobilized on the MB *via* STV-biotin association, while its 3' end is modified by an amino group to allow for hemin linkage *via* a amide bond coupling. The other carboxylic arm of hemin is coupled to either amino acids or oligopeptides (see below) and the resulting CPDzyme is freed by UV irradiation, which cuts the internal photocleavage site (iPCLink). The main procedure of the CPDzymes preparation is schematically represented in Figure 1a, the full details are provided in the method section below. It should be noted that the STV molecule is a tetrameric protein with a size of 66 KDa, and the loading ratio preclude contacts between individual G4s once bound to the surface.⁴⁶ After CPDzyme isolation, its G-quadruplex is properly folded by addition of K⁺, confirmed by fluorescence titrations with thiazole orange (Figure S1);⁴⁷ a schematic representation of

CPDzyme is seen in Figure 1b, in which both carboxylate arms of hemin (green) are coupled with the folded G-quadruplex structure (grey) on one side, and an amino acid (here a histidine, in blue) on the other side.

The actual concentration of CPDzyme was evaluated by UV-Vis absorption of hemin, and confirmed by an independent technique in which the peptides are replaced by DOTA-Gd to evaluate the bead loading by ICP-MS analysis (Figure S2). As seen in Figure S3, UV spectra indicated that 100 ng/ml of MB could be loaded with 308 nM of CPDzymes, and this result is in agreement with the data obtained by ICP-MS (1 ng/ml of MB contains *ca.* 3 nM of CPDzymes). Further, the successful synthesis of G4-Hemin and two representative CPDzymes discussed below were confirmed by mass spectrometric characterization (Figure S4). As each CPDzyme displays a single catalytic center, its catalytic activity can therefore be directly compared to the that of HRP that contains a single catalytic center as well.

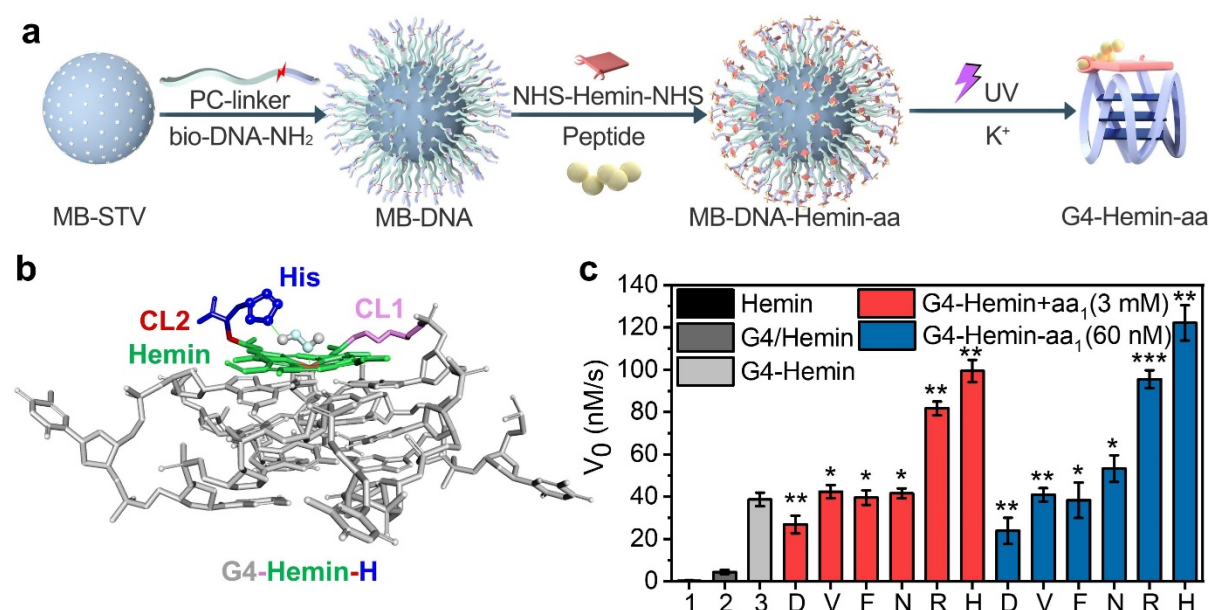


Figure 1. Synthesis, illustration and amino acids screening of CPDzyme. (a) Schematic representation of the preparation of CPDzyme (G4-Hemin-amino acid) and (b) of the structure of G4-Hemin-H, with an highlight of the covalent links between G-quadruplex and hemin (pink, CL1) and hemin and His (red, CL2). (c) Performance comparison of catalytic activity of non-covalent *versus* covalent systems: H_2O_2 -promoted oxidation of ABTS catalyzed by hemin alone (1), hemin in presence of G-quadruplex (G4/Hemin, 2), covalently linked G4-Hemin (60 nM, 3), G4-Hemin (60 nM) in presence of various amino acid (3 mM, red bars), the covalent assembly G4-Hemin-aa (60 nM, blue bars). Student' s t-tests are performed between G4-Hemin and CPDzymes: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

The desymmetrization of hemin and the screen of building blocks. It is difficult to recreate the environment of hemin within the HRP structure as it is tightly wrapped in an intricate amino acid network. This is why we decided to fully exploit the two carboxylate groups of hemin as they can constitute the bridges necessary to introduce suited blocks (Figure 1b),

that is, the G-quadruplex *via* the covalent link 1 (CL1, pink, the 3' end of DNA being modified with a C₆-NH₂ tail that is covalently linked to hemin upon activation as a NHS ester, [Figure S5](#)) on one side, and the oligopeptide (whose nature will be further discussed hereafter) *via* the covalent link 2 (CL2, red; *via* the naturally occurring NH₂ of amino acids, [Figure S5](#)) on the other side. The catalytic activity of CPDzymes was evaluated by the model oxidation reaction of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in the presence of the stoichiometric oxidant H₂O₂.⁴⁸⁻⁵⁰ The initial velocity (V_0) of the ABTS oxidation under various conditions is displayed in [Figure 1c](#): these results, in line with previous observations,^{51,52} confirm that covalently linking G-quadruplex and hemin (G4-Hemin, light grey bars) results in a better catalytic system (8.8-fold enhancement) than the non-covalent G4/Hemin assembly (dark grey bars).

Next, the amino acid blocks were screened. As seen in [Figure S6](#) and [Figure 1c](#), the presence of an excess of amino acid (3 mM) affects the catalytic performances of G4-Hemin (60 nM) as a function of their nature: the negatively charged aspartate (Asp, D) slightly decreases the catalysis efficiency ([red bar](#), D), the neutral valine (Val, V), phenylalanine (Phe, F) and asparagine (Asn, N) have no effects ([red bars](#) V, F and N), while the positively charged arginine (Arg, R) and histidine (His, H) significantly enhanced the catalytic activity (2.5- and 3.2-fold attributed to single amino acid activation, [red bars](#) R and H), in line with the highly conserved amino acids in native enzymes.^{52,53} Interestingly, the strategy of covalently linking amino acids to G4-Hemin provides an efficient catalytic system as the performance of 60 nM G4-Hemin-aa₁ ([blue bars](#)) reaches that of 60 nM G4-Hemin + 3 mM amino acid ([red bars](#)). The most efficient systems comprised positively charged Arg and His.

Synergistic Relationship between the Different Building Blocks. It is known that cytosine, which was already used as a surrogate for HRP's distal His, must be positioned at an optimal distance from the catalytic center to be operative (which was again confirmed by the observations shown in [Figure S7](#)).^{43,45} We decided to increase the distance between the Arg/His amino acids and the hemin using lysine (Lys, K) as a bridge because its side chain is flexible and long enough to facilitate the proper orientation of the co-catalytic blocks. As seen in [Figure 2a](#) and [Figure S8](#), embedding a Lys in the CPDzyme scaffold improves its catalytic activity by *ca.* 2-fold. The best result was obtained with G4-Hemin-KH ([Figure 2b](#)), the role of Lys might be to allow for His to act as in the native enzyme, *i.e.*, to coordinate H₂O₂ right above the iron atom of hemin, thus facilitating the production of Compound I.⁵⁴⁻⁵⁶

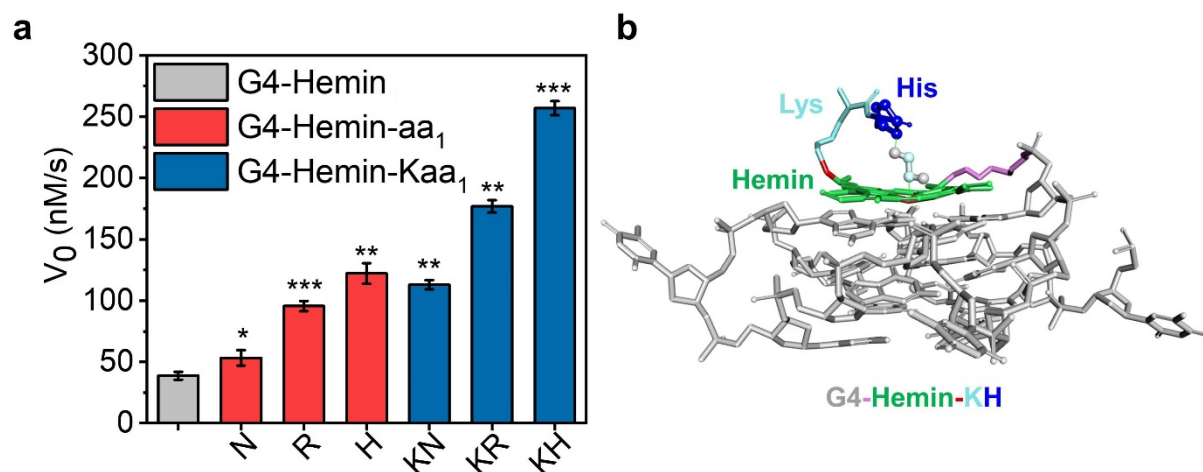


Figure 2. Embedding a lysine in the CPDzyme scaffold improves its catalytic activity. (a) Effect of Lys (K) spacer on the catalytic activity (H_2O_2 -promoted ABTS oxidation) in CPDzymes, estimated by G4-Hemin-aa₁ (red bars) *versus* G4-Hemin-Kaa₁ (blue bars). N, R and H stand for Asparagine, R for Arginine and H for Histidine. Note: G4-Hemin activity is shown here again for comparison. (b) Schematic representation of the structure of G4-Hemin-KH, where the Lys (light blue) serves as a spacer amino acid between His and hemin. Student' s t-tests are performed between G4-Hemin and CPDzymes: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Next, positively charged Arg and His were used to keep on improving the performance of CPDzymes (Figure 3a and Figure S9a). First, the simultaneous introduction of two histidines does not further improve the catalytic capacity of CPDzyme (G4-Hemin-KH *versus* G4-Hemin-KHH) (Figure 3a). Interesting effects could nevertheless be obtained by combining His and Arg: the best combination was G4-Hemin-KHR ($V_0 = 432 \pm 15$ nM/s; Figures 3a,b), which might originate in the ability of Arg to create a positive microenvironment that favors the formation of Compound I.^{7,9,11} Previous site-directed mutation studies have indeed demonstrated that the mutation of Arg in the native enzyme results in the loss of its catalytic activity, highlighting the important role of Arg from an evolution perspective.^{57,58} The proposed structure of Hemin-KHR displayed in Figure 3b reflects what is commonly found in native peroxidases,^{59,60} demonstrating that the concomitant presence of His and Arg, along with their precise organization in space, makes them synergistically interact to improve the catalytic efficiency of the resulting CPDzyme.

To go a step further, a close inspection of the structures of the peroxidases HRP and CcP (PDB IDs: 1W4Y and 4CVJ, Figure S10) allowed for selecting the next amino acid candidates. These structures indicate that His and Arg (*vide supra*) but also Asn could be used to optimize CPDzymes.^{61,62} The minor differences between HRP and CcP is a Phe41 for the former, and a Trp51 for the latter. As seen in Figures 3c-f and Figure S9b, the synergistic between His, Arg and Asn is strongly position-dependent: the best catalytic systems include continuous Arg and His, and presence of spacer between His and Asn. This phenomenon implies that Asn needs to

be accurately positioned to form hydrogen bonds with His (Figures 3c,d). Phe does not affect the CPDzyme performance, while Trp does, owing to its ability to be involved in H-bonds and participate to the catalytic process.⁶¹ Therefore, Trp leads to better catalytic performance (Figures 3c,e,f), the most optimized organization being G4-Hemin-KRHW, in which Arg and Trp are distributed on both sides of His (with $V_0 = 574 \pm 15$ nM/s), in a manner that is reminiscent of the catalytic microenvironment in CcP.

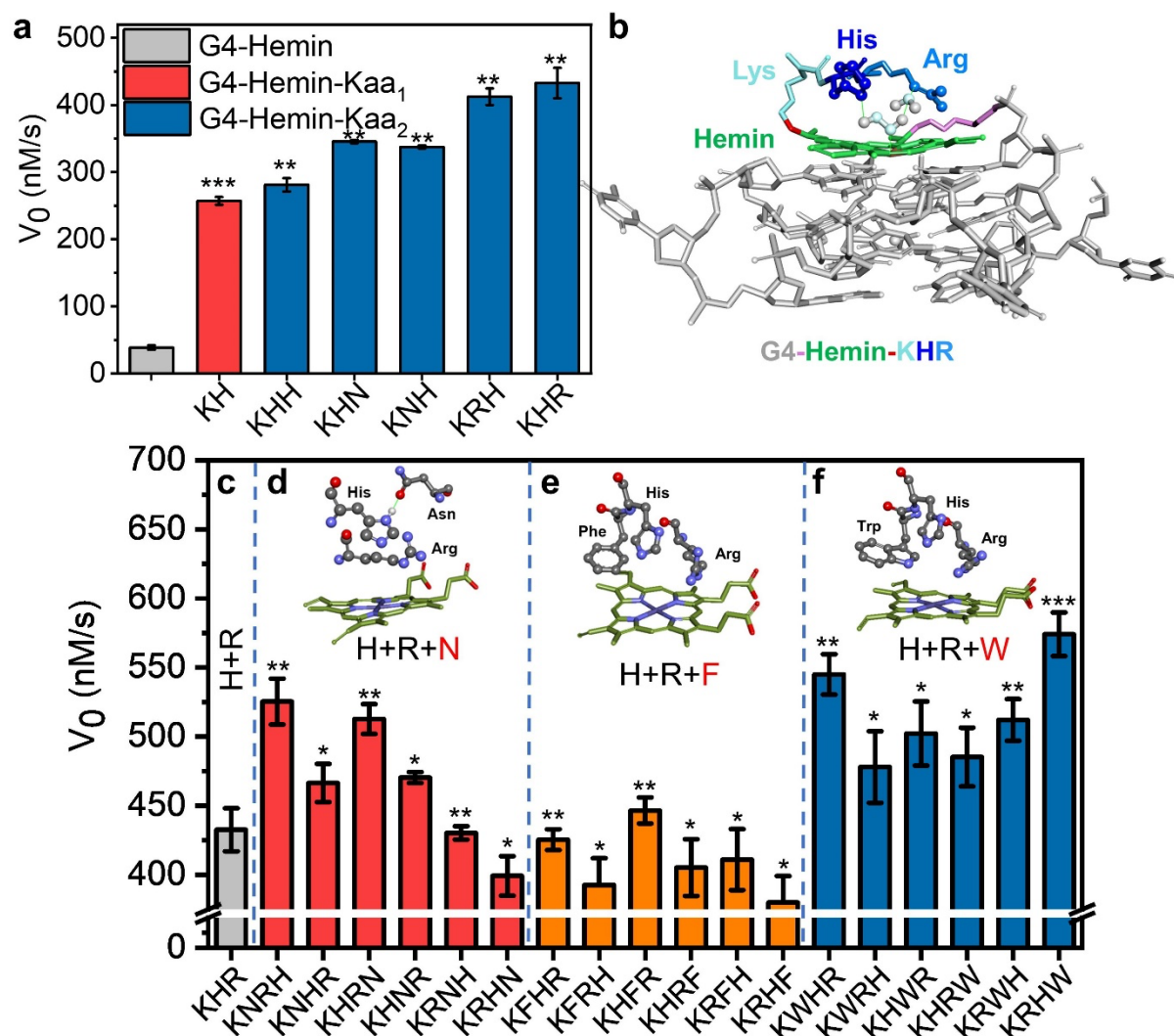


Figure 3. Biomimetic and synergistic catalytic design of CPDzymes. (a) Effect of 2-membered amino acid arms on the catalytic activity (H_2O_2 -promoted ABTS oxidation) of CPDzymes. Note: G4-Hemin activity is shown here again for comparison. Student' s t-tests are performed between G4-Hemin and CPDzymes: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. (b) Schematic representation of the structure of G4-Hemin-KHR, in which Arg (blue) assists His (dark blue) in coordinating H_2O_2 . (c-f) Effect of 3-membered amino acid arms on the catalytic activity of CPDzymes (with His and Arg in positions 1 and 2). For sake of comparison, the activity of G4-Hemin-KHR showed in Panel A is displayed in Panel C again. Student' s t-tests are performed between G4-Hemin-KHR and G4-Hemin-KHRN: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Boosting CPDzyme Catalytic Efficiency by Artificial Synergistic Catalytic Design. G4-Hemin-KRHW is an efficient CPDzyme but its activity could be still improved by further optimizing the G4-Hemin-KHR scaffold (Figures 3a,b). In this molecular organization, His acts both as a hydrogen bond partner of H₂O₂ and as an acid-base catalyst, making it the key amino acid for the initiation of the catalytic process. Further addition of His does not increase the catalytic activity (Figures 4a,b and Figure S11), while that of Arg increases it by *ca.* 1.5-fold (with V_0 from 432 ± 22 nM/s to 623 ± 29 nM/s). Indeed, further increasing the number of His does not enhance the catalytic activity, likely because of the lack of Arg to assist the catalysis of newly added His (*e.g.*, the close activities of KHR, KHRH, and KHRHH systems). In contrast, Arg may facilitate the reaction likely *via* the formation and stabilization of Compound I, allowing for reaching a V_0 value of *ca.* 600 nM/s with G4-Hemin-KHR + 2R (Figure 4c). The coordination of H₂O₂ seems to be central to the HRP activity: H₂O₂ must be present near the catalytic site and both His and Arg can help position it close to hemin. We further explored the effect of combinations formed by two His and two Arg on the activity of the CPDzymes: the activities of systems with two sets of His/Arg combinations are *ca.* 2-fold better than systems with a single set of His/Arg combination (Figure 4d). Also, the catalytic performance is better when the His are separated by Arg (*i.e.* HRHR, RHRH, HRRH > HHRR, RRHH, RHHR). The catalytic activity of G4-Hemin-KHRRH, with two HR pairs in mirror-inverted configuration, increased to 1009 ± 30 nM/s (Figure 4d), significantly higher than G4-Hemin-KRHW ($V_0 = 574 \pm 15$ nM/s). This result led us to propose the catalytic mechanism seen in Figure 4e: the two H₂O₂ are captured by two HR pairs and then delivered to hemin in a sequential manner, providing hemin with a double fuel system that allows for considerably increasing the velocity and efficiency of the whole system.

Further increasing the length of peptide arms of G4-Hemin-KHRRH with H, R, HR, and RH combinations does not improve the efficiency of the catalysis (Figure S12), suggesting that the space within the catalytic center is limited. It is important to highlight that the G-quadruplex structure plays a central role in the catalysis (hemin coordination) as demonstrated by the significant decrease of the catalysis efficiency when the G-quadruplex-forming sequence is replaced by the control oligothymidylate T16 (Figure S13). Finally, we demonstrated that the catalytic proficiency of these CPDzymes is not substrate-dependent, as similar trends were observed with different substrates (Figure S14) including 3,3',5,5'-tetramethylbenzidine,

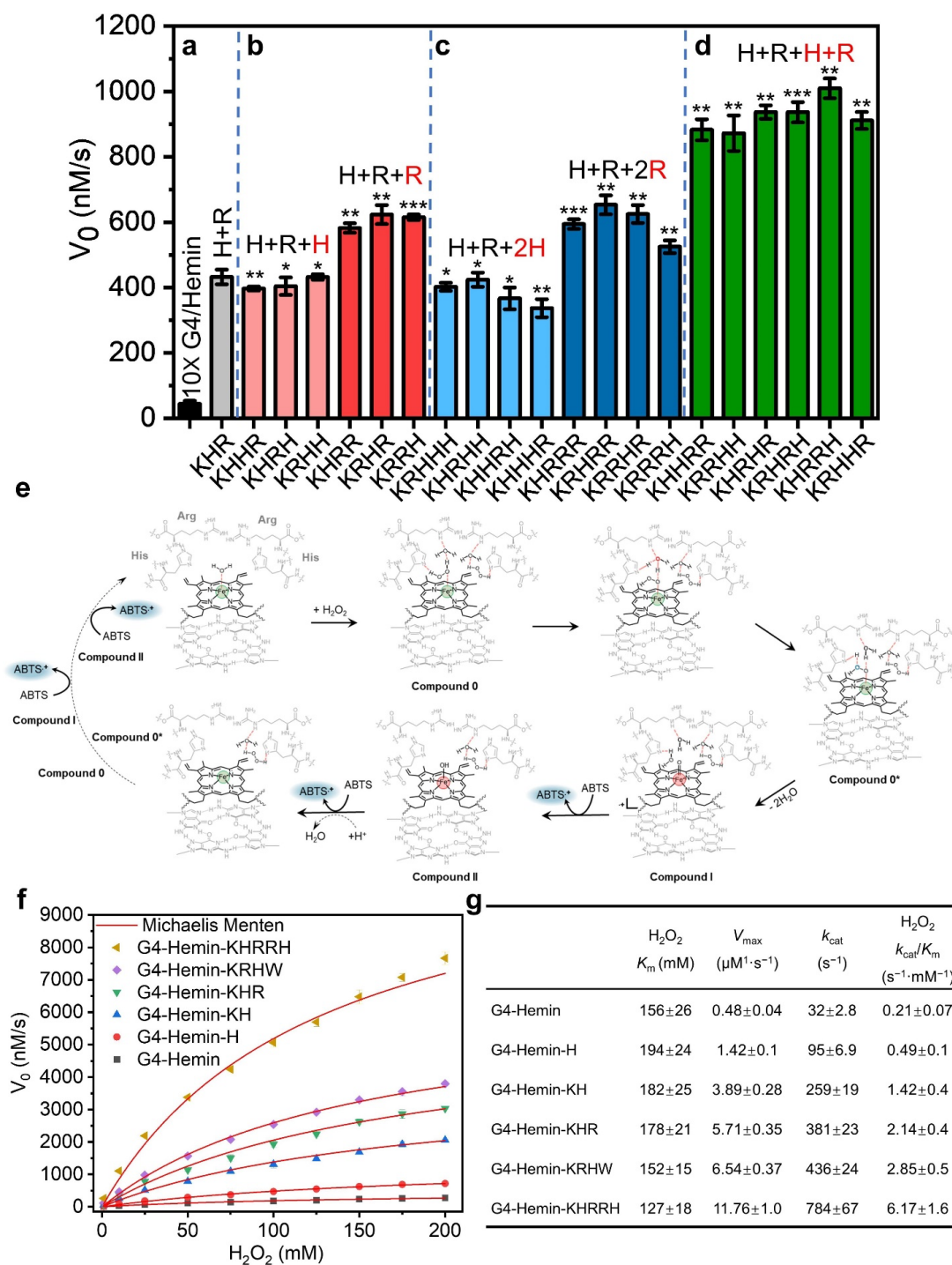


Figure 4. Artificial synergistic catalytic design, proposed rotational catalytic mechanism, and kinetics of CPDzymes. (a-d) Effect of 3-membered amino acid arms on the catalytic activity (H_2O_2 -promoted ABTS oxidation) of CPDzymes in which G4-Hemin-K is added of (b) 2H and R, or H and 2R, (c) 3H and R, or H and 3R and (d) 2H + 2R. Notes: the corresponding activity of G4/Hemin, too low to be seen, is 10X magnified (a) for sake of comparison. Student's t-tests are performed between G4-Hemin-KHR and G4-Hemin-K(HRN): * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. (e) Schematic representation of the catalytic cycle of G4-Hemin-KHRRH for the oxidation of ABTS by H_2O_2 . Green and red spheres represent Fe(III) and Fe(IV), respectively. (f) Saturation curves corresponding to the ABTS oxidation by G4-Hemin without or with amino acid ancillary arms (H, KH, KHR, KRHW and KHRRH) at different concentrations of H_2O_2 and (g) corresponding kinetic parameters. Experiments were performed in 10 mM Tris-HCl buffer (pH 7, 100 mM K^+) containing 15 nM CPDzymes and 5 mM ABTS.

(TMB), dopamine and nicotinamide adenine dinucleotide (NADH) (Figure S15), luminol and Amplex Red (Figure S16) along with a series of organic dyes (further discussed below).

Kinetic Analyses and Comparison with HRP. To gain more insights into the way CPDzymes catalyze oxidation reactions, notably in comparison with the native HRP, the steady-state kinetics of Michaelis-Menten curves were investigated for after each round of optimization. First, the catalytic number (k_{cat}) of HRP used herein is 521 s^{-1} at pH 4.3 (Figure S17), which is in agreement with reported values.⁶³⁻⁶⁵ As displayed in Figures 4f and g, the k_{cat} value of G4-Hemin (32 s^{-1}) is better than that of the non-covalent G4/Hemin system (0.3 s^{-1}),⁴² highlighting the benefit of CL1. The k_{cat} of the smallest CPDzyme, *i.e.*, G4-Hemin-H, is also better (95 s^{-1}), which highlights the benefit of CL2. Next, the introduction of a Lys residue as a spacer boosts the k_{cat} of G4-Hemin-KH to 259 s^{-1} , demonstrating the critical role of a precise spatial organization. A combination of His and Arg further improves the k_{cat} of G4-Hemin-KHR to 381 s^{-1} , confirming the synergistic interaction of the HR pair. The introduction of additional amino acids makes the resulting CPDzyme even closer to HRP used herein (the k_{cat} value of G4-Hemin-KRHW is 436 s^{-1}). Finally, the k_{cat} of G4-Hemin-KHRRH, whose design relies on a combination of HR pairs, reaches 784 s^{-1} , which is about 1.5-fold higher than that of HRP, thus representing an unprecedented catalytic performance, found to be 2,600-fold higher than the non-covalent G4/Hemin system. These results reflect the catalytic synergy that can be reached by combining G-quadruplex, hemin and oligopeptides in a single scaffold. The other catalytic parameters (K_{m} and $k_{\text{cat}}/K_{\text{m}}$ values, Figure 4g) confirmed the exquisite performance of G4-Hemin-KHRRH: for example, K_{m} gradually decreases from 194 mM for G4-Hemin-H to 127 mM for G4-Hemin-KHRRH, and corresponding $k_{\text{cat}}/K_{\text{m}}$ increases from $0.49 \text{ s}^{-1} \text{ mM}^{-1}$ to $6.17 \text{ s}^{-1} \text{ mM}^{-1}$. Of note, these analyses are based on the variations of the H_2O_2 concentrations; we also varied the concentrations of ABTS and the collected results (Figure S18) confirm CPDzyme's efficiency is substrate-independent.

CPDzymes are More Practically Convenient than HRP. We demonstrated that G4-Hemin-KHRRH outperforms HRP in conditions in which the optimal enzymatic activity is ensured. We next assessed whether CPDzyme could perform catalytic transformations in conditions that are incompatible with the use of HRP, *i.e.*, in organic solvents, high temperatures and high/low pHs. First, G4-Hemin-KHRRH remains highly active in solutions with 50% (v/v) organic solvents (formamide, methanol, ethanol, acetone, acetonitrile, Figure S19 and Figure S20a) while HRP does not. The oxidative degradation of Basic Blue 9 (BB9,

Figure S14) in a 1:1 methanol:H₂O solution was used as a model reaction (Figures S19a,b and Figure S20b): the degradation of BB9 catalyzed by G4-Hemin-KHRRH reaches 77.7% within 20 min, while no reaction occurs without catalyst or with HRP. This degradation was also studied at high temperatures (Figures S19c,d and Figure S21), with 75.9% of degradation observed within 10 min at 95 °C with G4-Hemin-KHRRH, while no reaction occurs with HRP. Interestingly, G4-Hemin-KHRRH remains active (*ca.* 50%) after 24 h at 95 °C, displaying an impressive temperature-resistance (Figure S21). Finally, the degradation of two other dyes, acid blue 74 (AB74) and basic red 2 (BR2), was studied at pH 2 (the former) and 10 (the latter): we first verified that the catalytic capacity (ABTS oxidation) of G4-Hemin-KHRRH in pH = 2-10; results seen in Figures S22a and b show that CPDzyme is active over the whole pH range (with an optimized activity at pH 7), representing a significantly wider pH range than that tolerated by the HRP. Next, the degradation of AB74 was studied at pH 2 (Figures S19e,f): this degradation reaches 78.5% with CPDzyme within 20 min, which is far higher than that of HRP (24.4%). Then, the degradation of BR2 was studied at pH 10 (Figures S19g,h): 42.3% of BR2 degradation was obtained with G4-Hemin-KHRRH, while no reaction was monitored with HRP. This series of results (summarized in Table 1) demonstrates the superiority of CPDzymes over HRP as they can be used in a broad range of non-physiologically relevant conditions. Finally, to keep on increasing the practical convenience of CPDzymes, G4-Hemin-KHRRH was immobilized on magnetic beads (MB) and this conjugate was used in several rounds of ABTS oxidation. Quite satisfyingly, the resulting MB-CPDzyme has excellent stability and its catalytic activity is 93.8% maintained after 10 cycles (Figure S23).

Table 1. Dye degradation (%) by different catalysts under various conditions.

$\begin{matrix} b^* & a^* \\ c^* \end{matrix}$	50% methanol	95 °C ^d	pH 2	pH 10
background	1.0 ± 0.08	4.3 ± 0.4	1.0 ± 0.1	6.7 ± 0.6
HRP	1.4 ± 0.1	4.5 ± 0.5	24.4 ± 4.2	7.3 ± 0.7
G4-Hemin-KHRRH	77.7 ± 7.8	75.9 ± 9.8	78.5 ± 6.1	42.3 ± 7.4

*a: reaction conditions; b: degradation rate; c: different catalysts, d: % degradation after 10 min, all other conditions in 20 min.

DISCUSSION

Here, we proposed the concept of chimeric peptide-DNA enzyme (CPDzyme), a new biocatalytic system made of several building blocks (DNA, peptide, hemin), covalently linked in a precise manner which eventually lead to the first artificial enzyme that outperforms the

corresponding natural peroxidase HRP. As summarized in [Figure 5](#), four rounds of optimization were required to obtain a CPDzyme able to surpass the catalytic performance of HRP. This milestone was made possible by the double covalent functionalization of hemin, along with a series of cutting edge techniques that greatly facilitate CPDzyme synthetic access ([Figure 1a](#)).

In detail, the first round of optimizations was focused on the two covalent linkages CL1 and CL2 around the central hemin platform ([Figure 1b](#)). This approach allows for gathering the G-quadruplex pre-catalyst with its cofactor hemin on one side (CL1) and the amino acid/oligopeptide activators on the other side (CL2, [Figure S5](#)). CL1 not only ensures efficient and stable hemin stacking onto the terminal G-quartet of the G-quadruplex but also solves the problem of hemin dimerization usually observed with the conventional G-quadruplex/Hemin system. CL2 provides a suited catalytic microenvironment to hemin, close to that of HRP, playing the roles of iron auxiliary activators. Of note, although this strategy proves efficient, the lengths of CL1 and CL2 could be further optimized. The screening of different amino acids/oligopeptides reveals that imidazole (His)- and guanidine (Arg)-containing arms are critical for hemin activation, suggesting that the precise nature of these arms deserves to be further investigated.

The second round of optimization aimed at defining the optimal positions of the amino acid residues. We found that the introduction of a lysine on the hemin platform provides the best catalytic systems (evidenced by the significant difference between G4-Hemin-KH and G4-Hemin-H, [Figure 2](#)) likely due to its optimal length and flexibility.

Then, the effect of multi-amino acid arms on the CPDzyme performance was assessed during the third round of optimization ([Figure 5](#)). We found that His and Arg are both critical for the transition state stability⁶¹ while both Asn and Trp improved the catalytic performance of CPDzyme, but in a more modest manner ([Figure 3d-f](#)).

The fourth—and last—round of optimization was focused on the possible cooperativity between different amino acids pairs. This optimization led to the best CPDzyme prototype, G4-Hemin-KHRRH, which outperforms the native enzyme HRP (k_{cat} , [Figure 5](#)), thus marking a milestone in the field of mimetic enzymes. This ultimate improvement might be due to two His/Arg pairs working in a synchronized manner, rotating one after the other to bring H₂O₂ close to the catalytic center, as proposed in [Figure 4e](#). This rotational process greatly enhances the overall catalytic efficiency of the corresponding CPDzyme. It has to be noted, however, that the K_m (H₂O₂) of G4-Hemin-KHRRH lags way behind that of HRP (127 *versus* 0.11 mM, respectively, [Figure 4g](#) and [Figure S17d](#)), indicating that there is still room for improvements in catalytic design. For example, the design of an appropriate peptide to increase the binding of

CPDzyme to its substrates may further enhance the catalytic activity.¹⁴ As an example (Figure S24), the study of the binding of several CPDzymes to dopamine indicates that G4-Hemin-KHRRH displays the strongest binding, in line with its optimized activity. However, the relationship between binding ability and catalytic activity is not straightforward, which is also observed for the binding energy evaluated by docking experiments (Figure S25), suggesting that even if the binding strength is important for CPDzyme activity, it might not be the determinant factor.

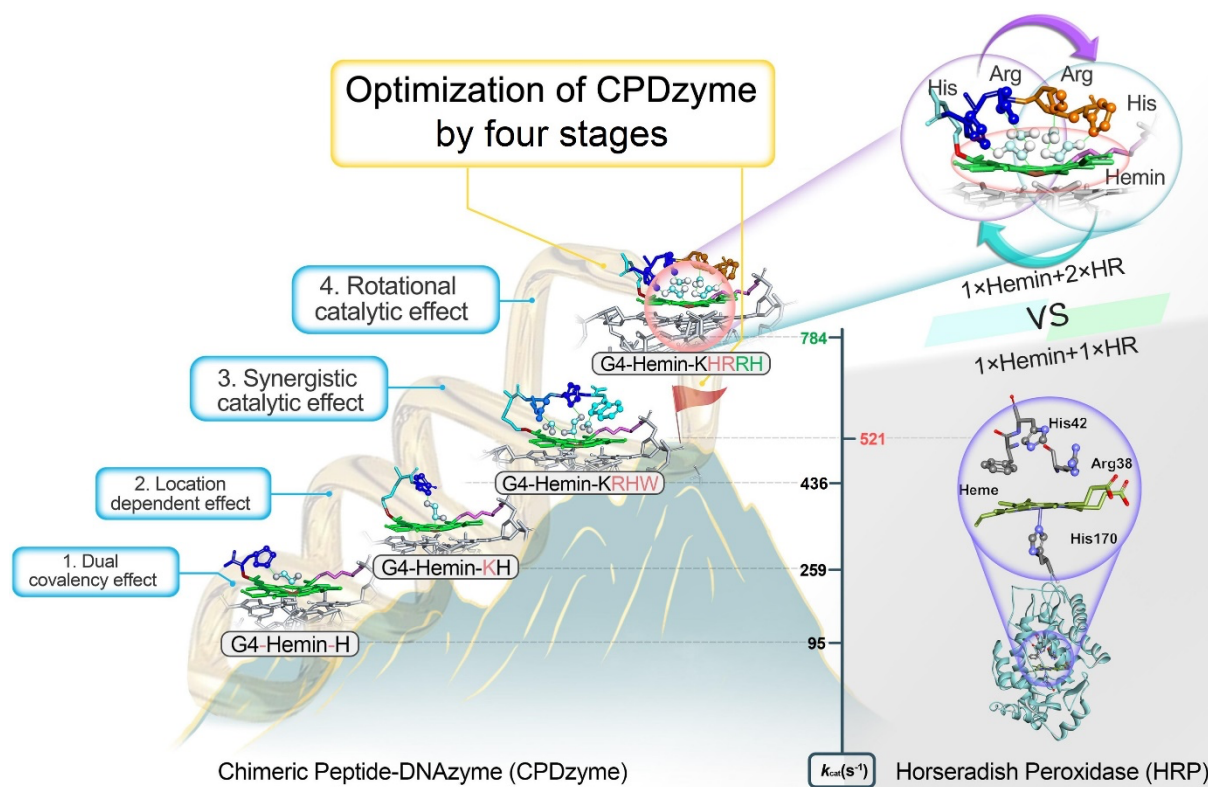


Figure 5. The catalytic efficiency of CPDzyme surpasses HRP. Schematic representation of the structure of horseradish peroxidase (HRP, lower right corner) *versus* the optimized CPDzyme G4-Hemin-KHRRH (upper right corner) and of the various rounds of optimization that led from G4-Hemin-H to the best CPDzyme prototype G4-Hemin-KHRRH, illustrated by an gradual increase of the k_{cat} values from 95 (G4-Hemin-H) to 259 (G4-Hemin-KH), 436 (G4-Hemin-KHRW) and 784 s^{-1} (G4-Hemin-KHRRH) *versus* 521 s^{-1} for HRP.

In conclusion, the assembly of the three blocks, including hemin (the natural cofactor of HRP), a peculiar DNA structure referred to as a G-quadruplex (known to display high affinity for hemin and to activate its catalytic properties) and peptides (to recreate the microenvironment found in the active site of HRP) allows for the design of CPDzyme that, after four rounds of optimization, competes with HRP. This optimized system displays great practical advantages over HRP, being operable in harsh experimental conditions (mixtures of organic solvents, high temperatures, high/low pHs, etc.) and easily recycled without loss of efficiency (10 cycles). We showed here the strategic relevance of CPDzyme through the oxidation of a series of dyes

(BB9, AB74, BR2), which are common pollutants found in the wastewater of the textile industry. Efforts must now be invested to gain mechanistic insights into the way CPDzymes perform their catalytic transformation, in the ultimate goal of designing ever more efficient system (notably optimizing the G-quadruplex unit, which was only partly investigated here). The concept of CPDzyme described here thus paves the way for future improvements of applications involving either hemoproteins or G-quadruplex-based DNAzyme, which are both abundant and diverse in the literature, thus representing the surest pledge for a dazzling future for nanobiocatalysis.

MATERIALS AND METHODS

Preparation of CPDzyme. As illustrated in [Figure 1a](#), the streptavidin-coated magnetic beads (MB) and the 10-fold excess of biotinylated DNA strands were mixed in 10 mM B&W buffer (pH 7.0, 500 μ M EDTA, 1mM K^+) and shaken at 25 °C for 3 hours. MB were then isolated by magnetic separation, rinsed (thrice), resuspended in 10mM HEPES (pH 7.0) buffer and added of a 100-fold excess of pre-mixed NHS-Hemin-NHS, amino acid / peptide, and 0.2 mg/ml DMAP (the volume of the aqueous and organic phases were adjusted to reach 1:1 ratio) and the mixture was stirred at 0°C overnight. A magnetic separation (as above) allowed to isolated the MB-G4-Hemin-aa conjugates, which were irradiated at Ultraviolet light for 30 min to free the CPDzyme (photocut molecular breakage), which was then properly folded by adding potassium to the buffer.

Measurement of CPDzyme Catalytic Activity (V_0). CPDzymes, ABTS (1 mM) and H_2O_2 (1 mM) were mixed in 10 mM Tris-HCl buffer (pH 7.0, 100 mM K^+). The catalytic activity measurement was followed by monitoring the absorbance of the oxidized ABTS ($ABTS^{\cdot+}$) which has a typical UV-Vis signature at 420 nm using a Cary100 (Agilent) spectrophotometer for 60 s at 25 °C. Three other substrates, TMB, dopamine and NADH, were also tested (see [Figure S14](#)): the absorbance of the oxidized TMB ($TMB^{\cdot+}$) is monitored at 652 nm, that of dopamine at 480 nm and NADH at 340 nm, all reactions being recorded for 60 s. The extinction coefficient for $ABTS^{\cdot+}$ at 420 nm was 36,000 $M^{-1}cm^{-1}$; $TMB^{\cdot+}$ at 652 nm was 39,000 $M^{-1}cm^{-1}$; dopamine oxidation product at 480 nm was 3,058 $M^{-1}cm^{-1}$; and NADH at 340 nm was 6220 $M^{-1}cm^{-1}$. The initial rate (V_0 , nM/s) of the oxidation reaction was obtained from the slope of the initial linear portion (the first 5 s) of the plot of absorbance *versus* reaction time. All kinetic results were obtained from triplicate experiments.

Kinetic Analysis. The oxidation reaction kinetics were established using a steady-state assay, with initial reaction rates varying with the concentrations of substrates. The kinetic parameters were calculated according to the Michaelis–Menten equation: $V_0 = (V_{\max} * [S]) / (K_m + [S])$, where V_0 is the initial reaction rate, V_{\max} is the maximum reaction rate, $[S]$ is the concentration of substrate, K_m is the Michaelis constant. $K_{\text{cat}} = V_{\max} / [E_0]$ is the catalytic number, in which $[E_0]$ is the concentration of the catalyst.

ASSOCIATED CONTENT

Supporting Information includes **Materials and reagents, Supplementary methods, Figures S1-S23, and Tables S1.**

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NOTES

The authors declare no competing financial interest.

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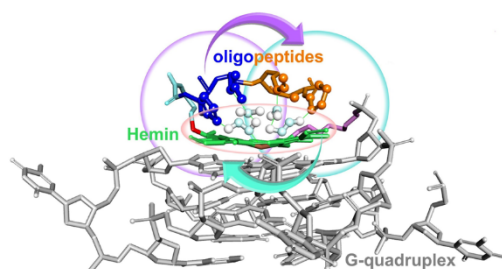
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Table of Contents (TOC)



High active and robust CPDzyme

A chimeric peptide-DNAzyme (CPDzyme), which relies on the covalent assembly of G-quadruplex, hemin and oligopeptides, surpasses HRP in catalytic number and far more chemically stable than HRP.
