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# Aptamer-Assisted Bioconjugation of Tyrosine Derivatives with hemin/G-quadruplex (hGQ) DNAzyme Nucleoapzyme Nanostructures

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Hemin/G-quadruplex (hGQ) DNAzymes are horseradish peroxidase-mimicking catalysts capable of the oxidation of a variety of substrates. We now implement aptamer-functionalized hGQ DNAzymes, also known as nucleoapzymes, to achieve increased bioconjugation of *N*-methyl luminol to tyrosine-containing residues and peptides. We found that the presence of a tyrosinamide-binding aptamer leads to a 12-fold increase in the catalytic rate constant ($k_{cat}$), and the saturation kinetics curves that were obtained provide evidence for the involvement of the substrate binding site in the reaction. The application of the best performing nucleoapzymes for the modification of Tyr-containing peptides reveals that (i) the aptamer also recognizes the ligand structure when this is embedded in a larger peptide structure, and (ii) distant residues in the peptide substrate can influence the conversion. As such, we show that nucleoapzymes display enzyme-like features and provide an additional tool in the toolbox of bioconjugation chemistry.

## Introduction

The enzymatic modification of bioactive molecules with chemical entities is a popular route for the preparation of molecular constructs with enhanced properties.[1] Although enzymes are unmatched when it comes to catalytic activity and selectivity, their use imposes constraints on the experimental conditions that can be used (*e.g.*, limited range in temperature and pH, limited tolerance for the presence of cosolvents) and on the chemical transformations that can be performed. Therefore, it is of interest to develop strategies that mimic the attractive features of enzyme-catalyzed bioconjugation reactions[2] while enabling unnatural chemical transformations.

Recently, we[3] and others[4] have shown that the horseradish peroxidase-mimicking hemin/G-quadruplex (hGQ) DNAzyme catalyst[5] has the ability to efficiently modify tyrosine (Tyr) residues in a variety of proteins with *N*-methyl luminol derivatives.[6] Applications of this hGQ DNAzyme as protein-modifying catalyst in a switchable nanostructure showed that the modification could also be performed in more advanced settings.[3] Although the hGQ nanostructure itself provided a basic level of substrate selectivity, its conjugation to structural elements that specifically recognize a substrate could, in principle, enable a higher level of selectivity.

A means to achieve this specific recognition could be involvement of an aptamer, and this basic concept of aptamer-assisted catalysis was recently uncovered.[7,8] For these so-called nucleoapzymes it was shown that the conjugation of an aptamer to a substrate-converting catalyst led to increased conversions. Even rational design of catalysts with improved properties[9] and incorporation in supramolecular micelle structures was shown.[10] However, in all cases the reaction entailed the oxidation or hydrolysis of one organic substrate, such as dopamine or ATP,[11] in the presence of $H_2O_2$ or $H_2O$, respectively. Whether aptamer-assisted conversions would also be useful for the conjugation of two organic substrates remained unknown, let alone the influence of mutations in the periphery of the reactive moiety of the substrates.

Therefore, in this paper we explored the potential of nucleoapzymes for bioconjugation. To this aim, we performed a detailed study of the catalyzed conjugation of the previously employed *N*-methyl-luminol (NML) derivative to tyrosine amino acid derivatives and several tyrosine-containing bioactive peptides[12,13,14,15] by tyrosinamide aptamer-functionalized hGQ DNAzyme nanostructures and $H_2O_2$. We investigate to which extent the reaction (i) is enhanced by the presence of an aptamer, (ii) is influenced by the relative positioning of the aptamer with respect to the hGQ DNAzyme, and (iii) is influenced by the presence of proximal amino acids in the

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peptide. Finally, we briefly discuss the potential of these novel enzyme-mimicking catalysts for bioconjugation reactions.

## Results and Discussion

At the start of this project, we determined the ability of hGQ DNAzymes to conjugate tyrosinamide **1** to azide-functionalized *N*-methyl luminol (NML) derivative **2**, resulting in the formation of tyrosine-NML conjugate **3** (Figure 1A); the azide moiety facilitates future two-step derivatization of NML-Tyr conjugates, *e.g.*, via alkyne-azide click chemistry. For this, we tested 20 different G-quadruplex forming sequences (see Supporting Table S1), originating from both biological and artificial sources, that together with hemin could form hemin/G-quadruplex DNAzymes. They consisted of at least four fundamentally different conformation types: intramolecular parallel, anti-parallel, hybrid/mixed and intermolecular parallel. For the quantification of the formed product, we used reversed phase HPLC-MS in the presence of benzoic acid as internal standard (see Supporting Table S2 and Figures S1–S3 for details). Although hemin alone was also able to catalyze the conjugation reaction, nearly all G-quadruplex-forming sequences could enhance the activity of hemin (Figure 1B, Supporting Table S3). Only the hGQ DNAzyme based on the human telomere (HT) repeat sequence did not display enhanced activity. In accordance with previous reports by us[3] and others,[16,17] the anti-parallel conformation of the hGQ DNAzyme is the least active, followed by the hybrid/mixed and intermolecular parallel structures, and with the parallel conformation being the most active. Differences in activity among DNAzymes with the same conformation type can be attributed to subtle differences in the interaction between the DNA G-tetrads and hemin, caused by the other nucleobases in the DNA sequence.[17] Whereas formation of Tyr-Tyr dimers and trimers was observed in the absence of NML – phenol-phenol coupling is known to occur with tyrosine residues[18,19] – this was not seen in the presence of NML. Moreover, previously reported modification of Tyr with two NML-derivatives –when a 10-fold excess of NML with respect to Tyr was used[4] – was not observed in our study in which we used stoichiometric ratios of Tyr, NML and $H_2O_2$.

Next, we explored whether fusion of a substrate-binding aptamer to the hGQ DNAzyme could generate a nucleoapzyme for enhanced tyrosine modification with NML. As the tyrosinamide-binding aptamer interacts with the planar aromatic ring of Tyr[20] and conjugation of Tyr to NML derivative **2** leads to a ring system with disrupted planarity (Supporting Figure S4), we assumed that the NML-Tyr conjugate would have reduced affinity for a tyrosine-binding aptamer. Therefore, we anticipated that minimal product inhibition would enable multiple turn-overs for each construct. To detect the increase in activity caused by the aptamer, we selected the hybrid/mixed type hGQ

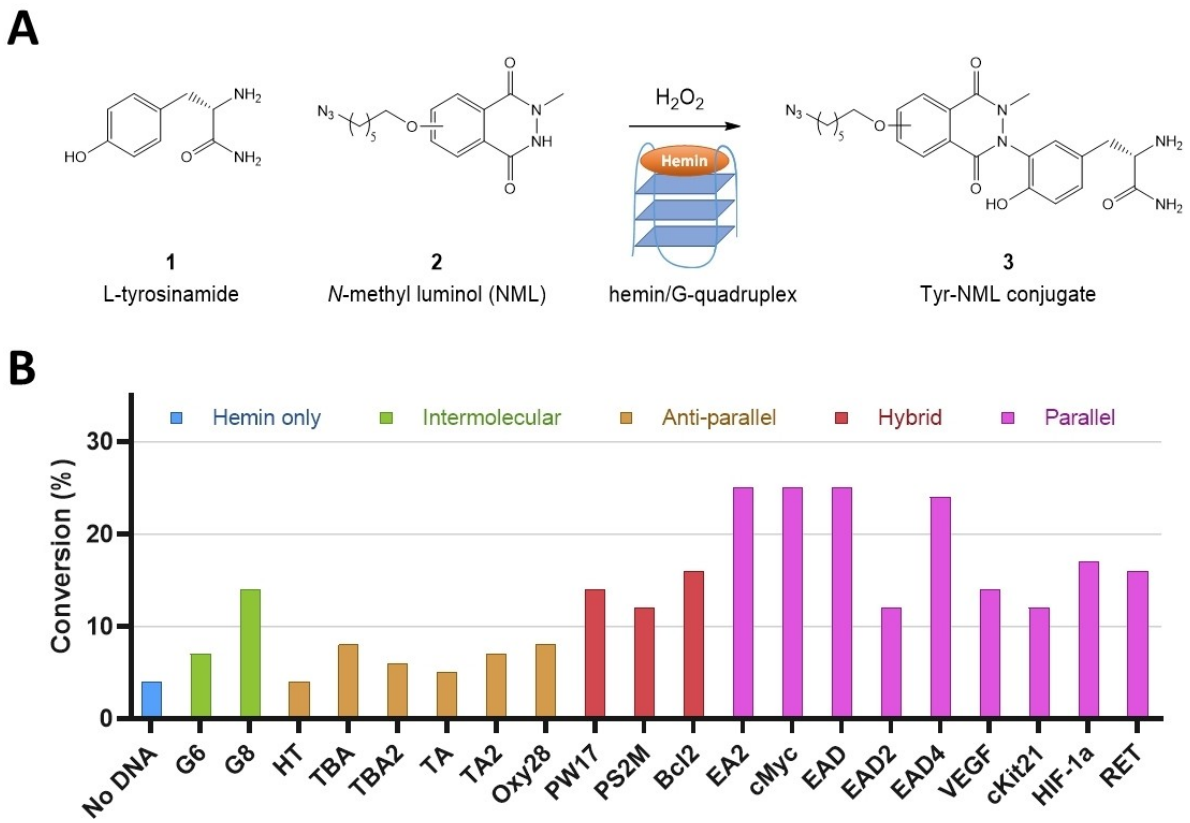


**Figure 1. A**: Conjugation reaction of tyrosinamide **1** to NML derivative **2** to form conjugate **3** under influence of a hemin/G-quadruplex (hGQ) DNAzyme and $H_2O_2$. **B**: Conversions for hemin and the various hGQ DNAzymes; no conversion was obtained in the absence of hemin. The different G-quadruplex topologies are indicated by the color code. *Conditions*: 5 μM hGQ DNAzyme, 100 μM L-tyrosinamide **1**, 100 μM NML **2**, and 100 μM $H_2O_2$, at 25 °C for 30 min.


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DNAzyme PW17, as this by itself showed intermediate peroxidase activity and thus enabled us to detect clear changes in reactivity as result of substrate binding. Since spatial positioning of the substrate binding site relative to the hGQ catalytic site was shown to be important for optimal catalysis,[7,9] a total of 18 nucleoapzyme constructs were tested. We designed the nucleoapzymes by extending the sequence of PW17 with that of the full or partial sequence of L-tyrosinamide-binding aptamer (TamBA): the full TamBA sequence (49 nucleotides; see Supporting Table S1) was placed either on the 3′ or the 5′-end of PW17 (Figure 2A); the partial sequences were used in a split-aptamer approach, where the two parts of the TamBA sequences were placed on either side of the PW17 sequence (Figure 2B).[21]

These nucleoapzyme constructs were tested for their ability to catalyze the conjugation of L-tyrosinamide (**1**) and NML (**2**). Importantly, TamBA itself did not increase the activity of hemin (Figure 2C, Supporting Table S4). The nucleoapzymes, however, showed 2–3 times higher conversion compared to the PW17 DNAzyme. The nucleoapzyme in which the full TamBA sequence was joined to the 3′-end of PW17 (PW17-TamBA) was slightly more active than the nucleoapzyme in which TamBA was joined to the 5′-end (TamBA-PW17). Higher conversions were also observed for the constructs that contained a split-aptamer, although all were in the same range as PW17-TamBA and TamBA-PW17. The highest conversions were observed for nucleoapzymes 10-PW17-39, 15-PW17-34, 27-PW17-22, 30-PW17-19 and 45-PW17-4, suggesting better substrate binding, or even alignment with respect to the catalytic hemin moiety, in these cases.

To prove that the tyrosinamide binding site of the aptamer indeed contributed to the reaction rate, we determined the modification rates at various substrate concentrations (Figure 2D, Supporting Table S5), since inclusion of a substrate binding site in the hGQ DNAzyme-forming sequence should lead to saturation kinetics. Analysis of the reaction curves indeed revealed saturation kinetics for nucleoapzymes PW17-TamBA and 27-PW17-22, whereas the PW17 DNAzyme and a nucleoapzyme consisting of the PW17 DNAzyme joined to a scrambled TamBA sequence (PW17-BaTAm) displayed almost linear kinetics (Table 1).[22] The small 1.8-fold higher activity of PW17-BaTAm compared to PW17 is attributed to flanking nucleobases that enhance the activity of the hGQ DNAzyme,[23] potentially assisted by electrostatic attraction between the cationic tyrosinamide substrate and the higher number of negative charges on the extended oligonucleotide. Importantly, the full-aptamer nucleoapzyme PW17-TamBA and split-aptamer nucleoapzyme 27-PW17-22 caused a substantially greater

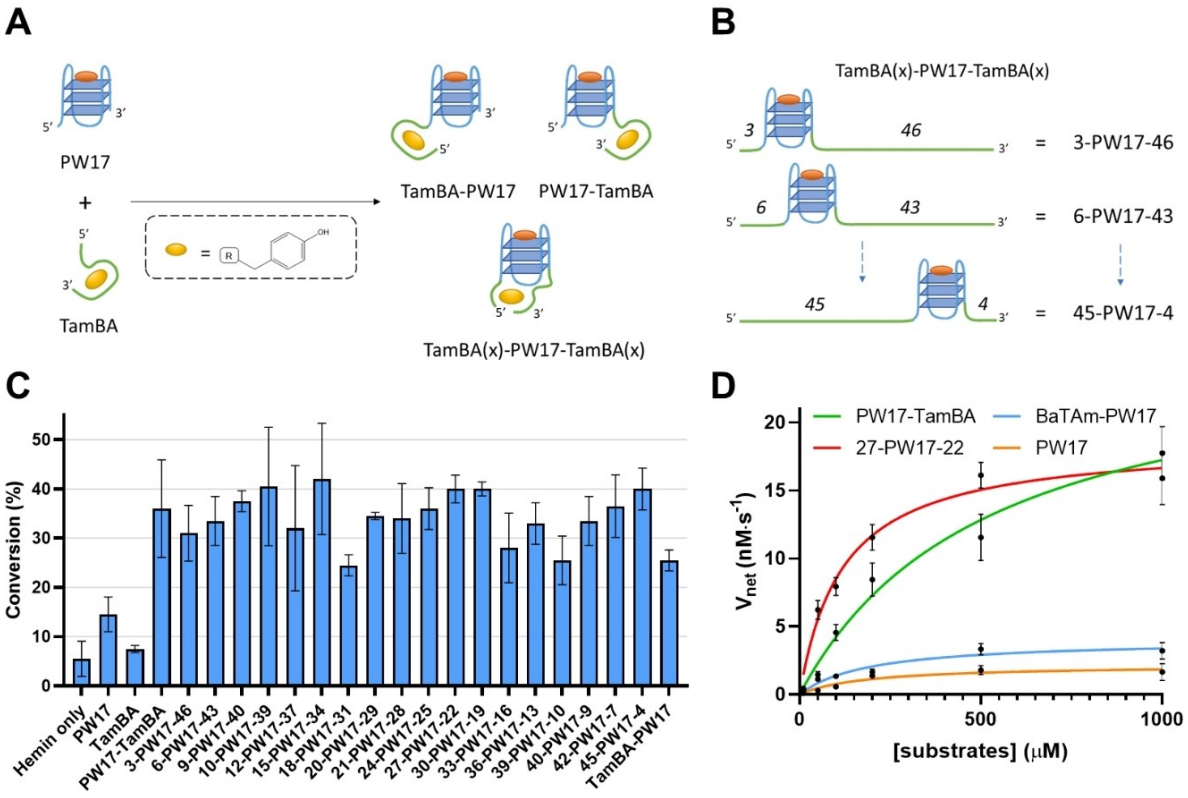


**Figure 2. A**: Schematic depiction of the PW17 DNAzyme, the TamBA aptamer and the tested nucleoapzyme constructs, including the 3′-end and 5′-end linked DNAzyme-aptamer construct and DNAzyme-split-aptamer constructs. **B**: Schematic depiction of the constructs based on the split-aptamer approach where the PW17 DNAzyme was incorporated at different positions in the aptamer sequence. The numbers indicate the number of nucleobases on either side. **C**: Conversions obtained by the various nucleoapzymes. *Conditions*: 5 μM catalyst, 100 μM tyrosinamide **1**, 100 μM NML **2**, and 100 μM $H_2O_2$ at 25 °C for 30 min. **D**: Michaelis-Menten kinetics of the isolated PW17 DNAzyme, the DNAzyme-aptamer conjugates PW17-TamBA and 27-PW17-22, and BaTAm-PW17 (5 μM catalyst). $V_{net} = V_{obs} - V_{hemin}$.


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**Table 1.** Kinetic parameters of catalyzed reaction between tyrosinamide **1** and NML **2** in the presence of various hGQ DNAzyme catalysts.[a]

| DNA construct | $k_{cat}$ [$10^{-3}$ $s^{-1}$] | $K_M$ [μM] | $k_{cat}/K_M$ [$10^{-3}$ $s^{-1}\cdot\mu M^{-1}$] | $V_{max}$ [$nM\cdot s^{-1}$] | Enhancement [PW17 = 1] |
|---|---|---|---|---|---|
| PW17 | 0.44 ± 0.07 | – | – | 2.2 ± 0.1 | – |
| PW17-TamBA | 5.25 ± 0.75 | 522 ± 155 | 0.010 | 26.2 ± 1.3 | 12 |
| 27-PW17-22 | 3.72 ± 0.23 | 119 ± 24 | 0.031 | 18.6 ± 0.9 | 8.5 |
| BaTAm-PW17 | 0.81 ± 0.11 | – | – | 4.0 ± 0.2 | 1.8 |

[a] Reaction conditions: 5 μM hemin, 5 μM DNA, 10–1000 μM tyrosinamide **1** / NML **2** / $H_2O_2$ at 25 °C for 30 min. Buffer: 50 mM $Na_2HPO_4$, pH 7.0, 400 mM NaCl and 5 mM KCl.

enhancement, with 12-fold and 8.5-fold higher reaction rates, respectively. As it is likely that the ligand binding site in the aptamer is optimally formed in the PW17-TamBA construct and less optimal in the 27-PW17-22 construct, we conclude from the $K_M$ data that we obtained that the binding site is better aligned in the 27-PW17-22 construct than in the PW17-TamBA construct ($K_M$ values of 119 μM versus 522 μM, respectively). Even more, when we added L-phenylalaninamide, which also binds to TamBA ($K_D$ = 45 μM),[20] we found that formation of NML-Tyr (**3**) conjugate was inhibited (with 29% in the presence of 5 equiv. of L-phenylalaninamide), providing additional evidence for the involvement of the binding site in the conjugation reaction.

Interestingly, the $K_M$ value of the split-aptamer construct 27-PW17-22 was lower than that of PW17-TamBA, which indicates a closer interaction between the substrate binding site and the active center.[22] Additionally, removal of either the 5′- or 3′-end extension of the split-aptamer nucleoapzymes revealed that the 3′-end was more important for the rate enhancement (Supporting Figure S5, Supporting Table S6). Nevertheless, the conjugate that contained the intact aptamer sequence ultimately displayed a higher activity as the integrity of both the DNAzyme and the aptamer are likely better maintained than in the split-aptamer approach. Therefore, it can be concluded that incorporation of a substrate binding site in the PW17 DNAzyme leads to substantially higher reaction rates, especially at lower substrate concentrations. Also, when more active hGQ DNAzymes are conjugated to aptamers, *e.g.*, EAD, only marginal gains of only a few percentages were obtained, which indicates that other means to enhance the local concentration are required for those DNAzymes.

After this, we examined whether the modification of other tyrosine-based substrates with NML could also be increased by the presence of a tyrosinamide-binding aptamer (TamBA). For this, we chose hemin, the PW17 DNAzyme, and the two best performing nucleoapzymes: PW17-TamBA and 27-PW17-22, in combination of various tyrosine derivatives, *i.e.*, tyramine, L-tyrosine methyl ester, L-tyrosine ethyl ester and L-tyrosine *tert*-butyl ester (Figure 3A). As can be seen in Figure 3B (and Supporting Table S7), we found enhanced conversions for all derivatives when using either of the two nucleoapzymes. This supports the previous finding[20] that the aromatic ring of the Tyr residue is crucial for TamBA-substrate binding. However, the substrates with the ester functionality displayed slightly lower conversion when compared to tyrosinamide, which contains a carboxamide. This could be the result of the ester functionalities acting solely as H-bond acceptor and not as H-bond donor, whereas the carboxamide moiety is both H-bond donor and acceptor. Our results show that mutations in the backbone can thus influence substrate binding, but do not diminish the ability of the aptamer to bind the substrate. This can be explained by

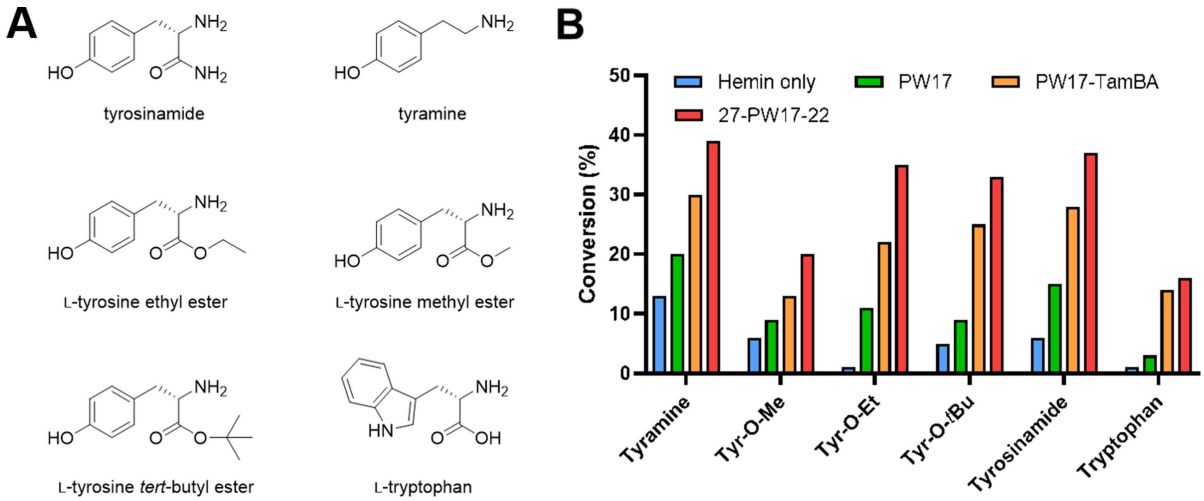


**Figure 3. A**: The substrates tested in the aptamer-enhanced conjugation reaction. **B**: Conversions for the various nucleoapzyme constructs. *Conditions*: 5 μM catalyst, 100 μM of the respective tyrosine derivative, 100 μM NML **2**, and 100 μM $H_2O_2$ at 25 °C for 30 min. Buffer: 50 mM $Na_2HPO_4$, pH 7.0, 400 mM NaCl and 5 mM KCl.


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the low binding affinity of TamBA for tyrosinamide and phenylalaninamide (both have a $K_d = 45$ μM),[20] which makes it likely that TamBA binds substrates that contain a planar aromatic ring. This is highlighted by the fact that we discovered that L-tryptophan – which contains an aromatic indole ring instead of a phenol moiety – was not only also modified with NML (see Supplementary Figure S5), but that this process was enhanced by the presence of aptamers. Specifically, whereas hemin or the PW17 DNAzyme hardly modified this amino acid residue, the nucleoapzymes gave significantly higher conversions (14–16%). Although this result supports our earlier finding that Trp can also be modified by hGQ DNAzyme-catalyzed NML conjugation,[3] we have not yet been able to identify the site of modification with atomic precision. Still, conversions on tyrosine almost reach 40%, which indicates that the aptamer is more efficient in assisting transformations on this residue.

Following these encouraging results, we tested our systems on larger substrates, *i.e.* tyrosine-containing peptides. For these studies, we first modified the opioid neuroactive peptides Leu-enkephalin and Met-enkephalin with NML **2** using the 22 different hGQ DNAzymes (Figure 4A). We observed that for these peptides, not all hGQ DNAzymes were able to reach higher conversions than hemin (Figure 4B, Supporting Tables S8 and S9). Especially the intermolecular parallel and intramolecular anti-parallel structures did not show substantially enhanced activity when hemin was bound to G-quadruplex structures. A notable exception was the G8 DNAzyme, which doubled the conversion of Met-enkephalin compared to hemin. For the hybrid and intramolecular parallel sequences, conversions all reached over 30% for both substrates.

In previous studies, it was demonstrated that DNAzyme-catalyzed modification of Tyr-containing peptides can show peptide sequence dependence, for example by recognizing a specific sequence motif (YPR) that is required for conversion.[27,28] However, as the peptides in this study only differ at the 5$^{th}$ position with respect to the Tyr residue (Figure 4A), we expected limited influence of the Leu-to-Met mutation on the conversions. Surprisingly, comparison of the two peptides showed that this is not the case, as conversions between Leu-enkephalin and Met-enkephalin differ when choosing different DNAzymes. Specifically, the G8 and PW17 DNAzymes convert twice as much Met-enkephalin as Leu-enkephalin. This suggests that for some hGQ DNAzymes the interaction between the DNAzyme nanostructure and the microenvironment caused by distant residues in the peptide sequence influence the reactivity of Tyr residues, which thus increases substrate specificity. As such, the family of hGQ DNAzymes display an essential feature also found in enzymes, *i.e.*, the ability to discriminate between substrates using very subtle interactions.

After mapping the abilities of the hGQ DNAzymes, we subjected Leu-enkephalin and Met-enkephalin to NML-conjugation using our nucleoapzymes and studied the effect of the aptamer sequence on the conversions (Figure 4C, Supporting Tables S10 and S11). We note that the peptides that we selected can be viewed as C-terminally extended versions of tyrosinamide. As expected, most of the nucleoapzyme constructs achieved substantially higher conversions compared to the PW17 DNAzyme alone. Specifically, whereas PW17 reached a conversion of 22 ± 6% for Leu-enkephalin and 25 ± 10% for Met-enkephalin, PW17-TamBA increased the conversions to 30 ± 3% for Leu-enkephalin and 40 ± 15% for Met-enkephalin. The most active nucleoapzymes include 15-PW17-34, 27-PW17-22, 30-PW17-19 and 45-PW17-4, a finding that matches our data from the modification of tyrosinamide. Importantly, analysis of Met-enkephalin by mass spectrometry did not reveal any oxidized Met, proving that the oxidative nature of the hGQ DNAzyme does not lead to compromising side-reactions on this residue. Although a limited 2.5-fold enhancement of the conversion of tyrosine residues in peptides was observed, it demonstrates that a tyrosinamide-specific aptamer assist in the conjugation of NML to Tyr-containing peptides. Proof that this process is indeed assisted by substrate-binding can be derived from the saturation curves obtained for Leu-enkephalin in the presence of PW17-TamBA and 27-PW17-22 (Figure 4D and Table 2, Supporting Table S12). Whereas PW17 and BaTAm-PW17 displayed linear curves, PW17-TamBA and 27-PW17-22 displayed typical saturation kinetics curves, supporting the notion that a substrate binding site is involved in the reaction. Furthermore, a distinct difference in the rate of conjugation of Met-enkephalin to NML was observed between 27-PW17-22 and PW17-TamBA (Figure 4E, Supporting Table S13), indicating that the split-aptamer approach can be used to enhance the interaction between peptide substrate and aptamer. As this effect was not seen for Leu-enkephalin, we conclude that this is highly sequence specific and shows that, indeed, our bioconjugation nucleoapzymes can interact with substrates via subtle interactions that, likely, can be optimized further.[26]

**Table 2.** Kinetic parameters of catalyzed reaction between Leu-enkephalin and NML **2** in the presence of various hGQ DNAzyme catalysts.[a]

| DNA construct | $k_{cat}$ [$10^{-3}$ $s^{-1}$] | $K_M$ [μM] | $k_{cat}/K_M$ [$10^{-3}$ $s^{-1} \cdot \mu M^{-1}$] | $V_{max}$ [nM·$s^{-1}$] | enhancement [PW17 = 1] |
|---|---|---|---|---|---|
| PW17 | 0.47 ± 0.1 | – | – | 2.3 ± 0.1 | – |
| PW17-TamBA | 3.6 ± 0.5 | 5027 ± 1247 | 0.0007 | 18.1 ± 0.9 | 7.8 |
| 27-PW17-22 | 2.1 ± 0.4 | 1533 ± 659 | 0.0014 | 10.3 ± 0.5 | 4.4 |
| BaTAm-PW17 | 1.2 ± 0.4 | – | – | 6.0 ± 0.3 | 2.6 |

[a] Reaction conditions: 5 μM hemin, 5 μM DNA, 100–5000 μM Leu-enkephalin / NML **2** / $H_2O_2$ at 25 °C for 30 min. Buffer: 50 mM $Na_2HPO_4$, pH 7.0, 400 mM NaCl and 5 mM KCl.


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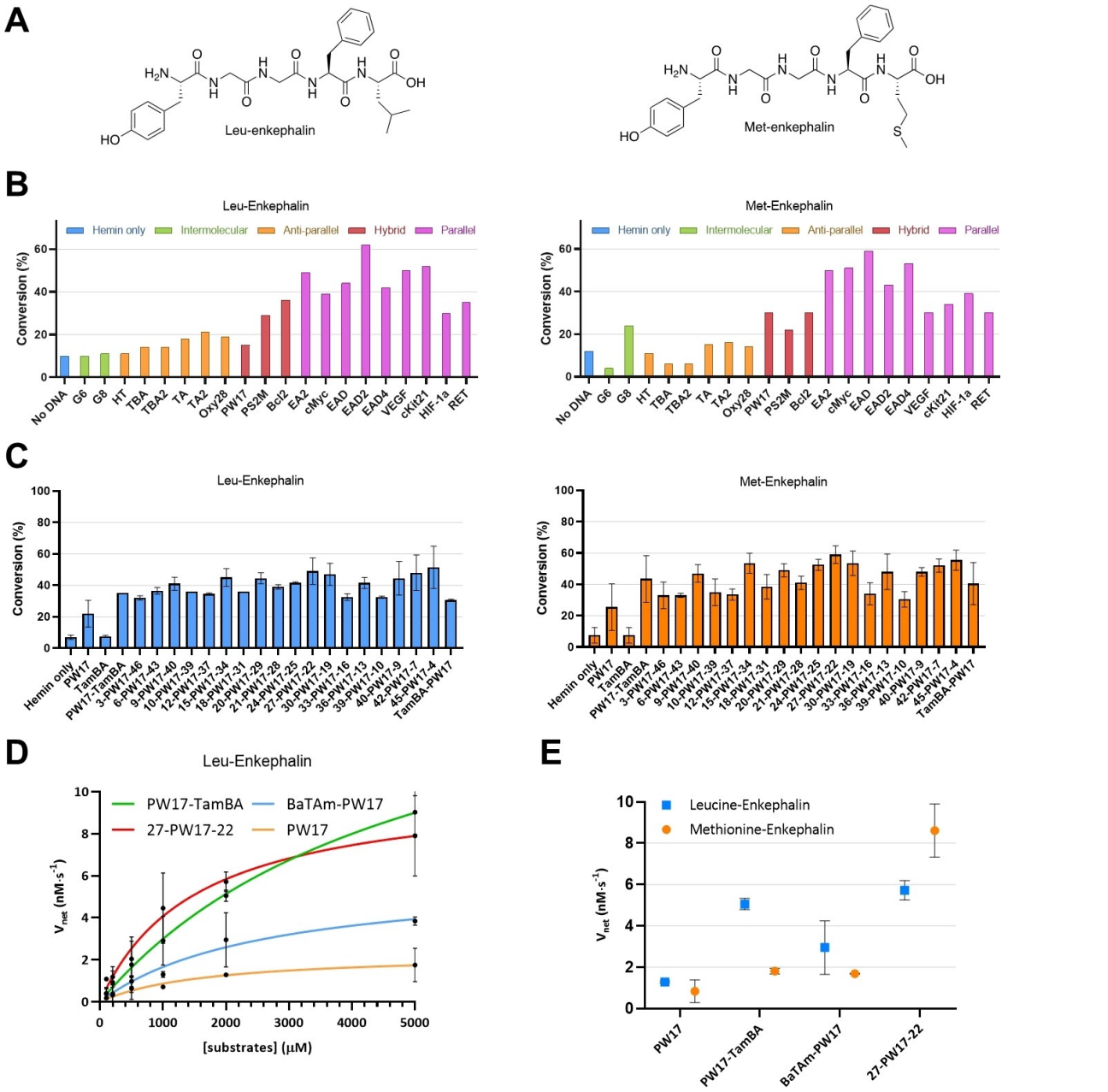


**Figure 4. A**: Structures of the enkephalin peptide substrates. **B**: Measured conversions for hemin and the various hGQ DNAzymes. The different G-quadruplex topologies are indicated by the color code. **C**: Measured conversions for hemin and the various DNAzyme-aptamer constructs. *Conditions*: 5 μM catalyst, 100 μM tyrosinamide **1**, 100 μM NML-$N_3$ **2**, and 100 μM $H_2O_2$ at 25 °C for 30 min. **D**: Michaelis-Menten kinetics of the isolated PW17 DNAzyme, the DNAzyme-aptamer conjugates PW17-TamBA and 27-PW17-22, and PW17-BaTAm, *i.e.*, BaTAm-PW17 (5 μM catalyst). $V_{net} = V_{obs}$-$V_{hemin}$. **E**: Rates of the conjugation of either Met-enkephalin or Leu-enkephalin in the presence of PW17, PW17-TamBA, 27-PW17-22 and BaTAm-PW17. *Conditions*: 5 μM catalyst, 2000 μM substrate, 2000 μM NML **2**, and 2000 μM $H_2O_2$.

## Conclusions

The bioconjugation of tyrosine derivatives and *N*-methyl luminol by means of peroxidase-mimicking hemin/G-quadruplex (hGQ) DNAzymes can be enhanced up to 12-fold by extending the DNAzyme with an aptamer sequence that binds the substrate. The observed saturation kinetics for the DNAzyme-aptamer constructs reveal that the binding site of the aptamer indeed assists in the bioconjugation reaction. Interestingly, different conversion efficiencies were obtained for the opioid peptides Leu- and Met-enkephalin, indicating that residues distally positioned from the site of conjugation can influence the reaction, thus pointing to the potential of high substrate specificity. We therefore expect that the current results encourage applications of nucleoapzymes in selective bioconjugation reactions of tyrosine residues,[6,27,28] and such studies are currently ongoing in our laboratories.


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## Conflict of Interest

The authors declare no conflict of interest.

**Keywords:** bioorganic chemistry · conjugation · DNA · G-Quadruplexes · peptides

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