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Inhibition of Chymotrypsin by a Self-Assembled DNA Quadruplex Functionalized with Cyclic Peptide Binding Fragments

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Protein–protein interactions are crucial to many biological processes, including cell proliferation, signal transduction and apoptosis.[1–3] Modulation of protein–protein interactions through protein-surface recognition[4–7] by synthetic receptors can potentially lead to the discovery of novel therapeutic agents and cellular probes for investigating different biological pathways. However, unlike classical small molecule drug discovery where the design of leads that bind to enzyme active sites is straightforward, the disruption of protein–protein interactions is more challenging, because they usually involve the targeting of many charged and hydrophobic residues distributed over a large and often non-contiguous protein surface. Despite these demands, there has been some success in the identification of small molecules for the disruption of protein–protein interactions.[7–10] In certain cases, ligands based on different molecular scaffolds with large surface areas have been used.[4,11–14] For example, we have reported synthetic receptors based on calixarene and porphyrin derivatives that bind to protein surfaces and disrupt their function.[15–20] Rotello et al. successfully inhibited chymotrypsin activity through surface binding using nanoparticle-based receptors.[21–23] Kano et al. have enhanced the interactions of myoglobin with an anionic porphyrin through porphyrin self-assembly.[24] In the continuing search for smaller, more water soluble and versatile scaffolds for protein-surface recognition, we have begun to investigate the possibilities of self-assembly as a route to the controlled formation of binding agents.

Molecular self-assembly involves the defined and reversible organization of small molecules by non-covalent interactions into larger structures that possess properties not present in the individual components. Nature presents many examples of self-assembled functional structures, some of the most notable of which involve the aggregation of different DNA sequences into duplex, triplex or quadruplex assemblies.[25,26] There has been great interest in using duplex DNA as a scaffold to orientate synthetic fragments to direct organic reactions,[27] or to develop combinatorial libraries.[28,29] However, the use of a DNA quadruplex as a scaffold to template reactions or bring together multiple binding domains for protein-surface recognition is rare.[30]

We have previously reported that calixarene derivatives linked on the upper ring to four peptide loop fragments, **1X** and **1Y** (Figure 1a), can act as potent surface binding inhibitors of α-chymotrypsin (ChT).[31] The four-fold symmetrical structure of these calixarene-based receptors suggested that a G-quartet might be used as a self-assembling unit with a similar size and symmetry. In this article, we report the efficient binding and inhibition of ChT by a self-assembled DNA quadruplex with protein recognition fragments appended on the 5′-ends of the assembled G-quartet.

The peptide loops in this study were formed by the cyclization of different tetrapeptides across a 5-nitro-3-aminomethyl benzoic acid spacer. Following nitro group reduction, the cyclic peptides were tethered to the 5′-end of the quadruplex-forming oligonucleotides through a 4-aminomethyl benzoic acid spacer and a C10 linker (Figure 1b and c). Two peptide loop sequences were used, one containing two anionic residues in each loop (GDGD) thus forming quadruplex **2X**, and a second with one anionic and one hydrophobic residue (GDGY) to give quadruplex **2Y**. The peptide loops were constructed such that they are broadly complementary to the cationic and hydrophobic active site region of ChT, as previously reported[31] (Figure 1a). Compound **2** is a quadruplex without tethered loops to act as a control, and **3X**, contains a peptide loop tethered to a single strand of a sequence that is incapable of forming self-assembled structures.

The synthesis of the cyclic peptide precursors and their conjugation to the oligonucleotides is detailed in the Supporting Information. Functionalized parallel quadruplexes were prepared in buffer (10 mM Tris-HCl, 80 mM KCl,

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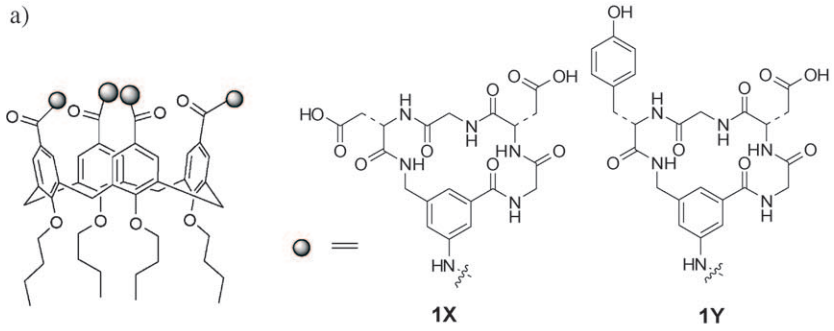
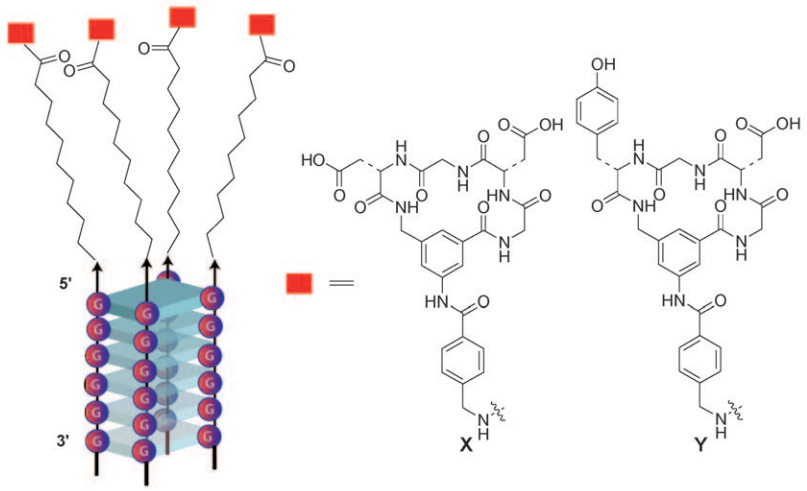
a)



b)



c)

Oligonucleotide	Sequence
2X	[X-d(TGGGGGGTTTT)]$_4$
2Y	[Y-d(TGGGGGGTTTT)]$_4$
2	[d(TGGGGGGTTTT)]$_4$
3X	[X-d(CGTCATATCTTA)]

Figure 1. a) Previously reported chymotrypsin inhibitors **1X** and **1Y**, which are calix[4]arene derivatives containing peptide loops GDGD and GDGY, respectively.[31] b) Functionalized parallel G-quadruplex with the synthetic fragments used for the self-assembly shown as **X** and **Y**. c) Sequences of the designed oligonucleotides wherein G-quadruplex **2X** and **2Y** are tethered with **X** and **Y** fragments, respectively. Compound **2** is an unfunctionalized quadruplex, and **3X** is a single strand that can not form a quadruplex appended with fragment **X**.

pH 7.5) with incubation times of 48 h.[32] The secondary structures of the complexes were confirmed by circular dichroism (CD) spectrophotometry (Figure S1). All the designed functionalized quadruplexes **2X**, **2Y** and **2** showed a positive ellipticity at 263 nm and a negative peak at 243 nm, which is the characteristic of tetramolecular parallel quadruplex.[33–35] In contrast, incubation of **3X** only showed a weak peak at 273 nm in the CD spectrum, indicating no quadruplex formation.

The inhibitory effects of the synthetic receptors on ChT activity were determined after various preincubation periods with the oligonucleotides in phosphate buffer followed by addition of the chromogenic substrate, *N*-benzoyl tyrosine *p*-nitroanilide (BTNA) (Figure 2).

Receptors **2X** and **2Y** strongly inhibited ChT activity, with **2X** showing more efficient inhibition. Receptors **2** and **3X** were found to be weak inhibitors. The decreased binding of **2Y** is expected because of the fewer anionic groups available to complement the positively charged ChT surface. However, electrostatic effects are not the only contributor to strong binding since **2**, an unfunctionalized G-quartet with a strongly negatively charged phosphate backbone, has only weak inhibition activity against ChT. The single-strand peptide loop adduct **3X** presents only a single monomeric peptide loop to the protein and displays the weakest inhibition. To test the selectivity of **2X** for the surface of ChT, we investigated its ability to inhibit a related serine protease, elastase. At concentration of **2X** up to 1200 nM, no measurable inhibition of elastase was seen (Figure S2).

Both **2X** and **2Y** exhibited slow binding inhibition of ChT. This effect can derive from a number of mechanisms,[36] although a two-step Equation (1) is frequently encountered, where an initial rapid binding step is followed by a slower

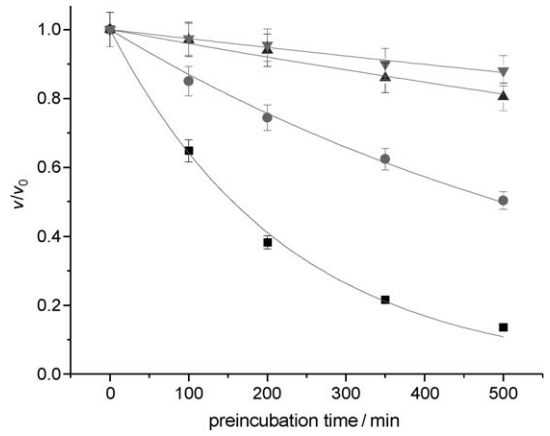


Figure 2. Time course of ChT activity after preincubation with 1200 nM oligonucleotides in phosphate buffer ([ChT] = 350 nM, [BTNA] = 0.14 mM); ■: **2X**, ●: **2Y**, ▲: **2**, ▼: **3X**. Lines are provided to lead the eye.

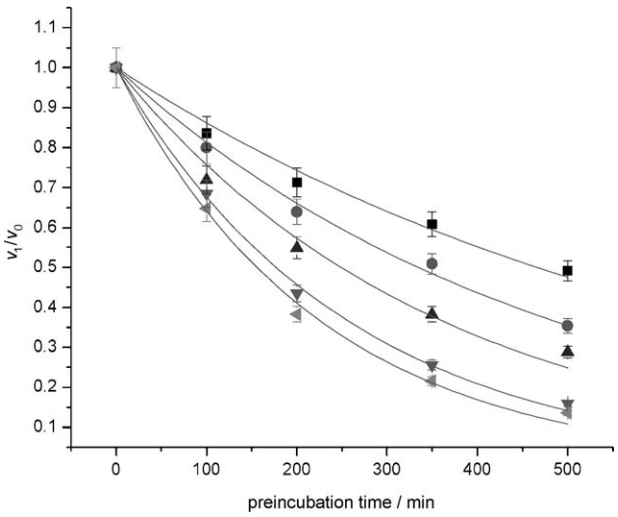


Figure 3. Plot of fractional velocity vs preincubation time for different concentrations of **2X**; ■: 100, ●: 200, ▲: 300, ▼: 600, ◀: 1200 nM. Concentration of chymotrypsin was fixed as 350 nM in pH 7.4, 5 mM potassium phosphate buffer. Substrate was BTNA at the fixed concentration of 0.14 mM.

conformational rearrangement to form the enzyme/inhibitor complex E·I.

$$E + I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E \cdot I \quad (1)$$

This binding mechanism was investigated for **2X** by preincubation of the enzyme with the inhibitor followed by the measurement of initial velocities for substrate hydrolysis as a function of preincubation time. The inhibition under these circumstances is described by the following equation:

$$\frac{v_i}{v_0} = \exp(-k_{obs}t) \quad (2)$$

v_i and v_0 are the initial velocities of inhibited and uninhibited reaction, respectively. k_{obs} is the apparent first-order rate constant for the interconversion between the initial and the steady state. The fractional velocities for the hydrolysis of *N*-benzoyl tyrosine *p*-nitroanilide by ChT as a function of preincubation time for different concentrations of receptor **2X** were plotted (Figure 3).

The correlation of k_{obs}, obtained from each curve in Figure 3, against [**2X**] was plotted (Figure 4). The nonlinear plot is indicative of the two-step mechanism of inhibition shown in Equation (1). The apparent dissociation constant K_i^{app} was then determined by nonlinear curve fitting of the plot using the following equation:

$$k_{obs} = k_6 + \frac{k_5[I]}{K_i^{app} + I} \quad (3)$$

The weaker inhibitor **2Y** was also analyzed using the same approach (see Supporting Information). For **2** and **3X**, time dependent effects could not be determined because the inhibition is weak (less than 20% in 5 h of incubation). Lineweaver–Burk analysis was applied to the measurement of the inhibition constants and all the apparent dissociation constants (K_i^{app}) of the receptors are collected in Table 1. To

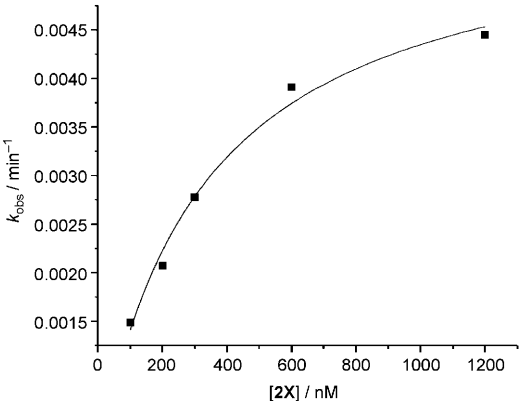


Figure 4. Plot of k_{obs} vs [**2X**].

compare the inhibition activities, the K_i^{app} of the calixarene-scaffold based tetra-loop receptors **1X** and **1Y** (Figure 1a)[31] for the inhibition α-chymotrypsin are also included in the Table.

Among the receptors, **2X** displayed the highest inhibition potency for ChT. Surprisingly, **2Y** was also found to be a very good inhibitor, compared to **1Y** (a calixarene derivative) reported earlier, which is at least 20 times less potent. There might be two reasons for this improvement. First, the longer alkyl linkers on the quadruplexes may deliver the peptide loops to a larger surface area on the protein than calixarene-based receptors. Second, the superiority of the

Table 1. Summary of apparent dissociation constants (K_i^{app}) for the inhibition of ChT by receptors.

	2X	**2Y**	**2**	**3X**	**1X**[31]	**1Y**[31]
K_i^{app} [μM]	0.33	1.8	>12	>24	1.4[37]	43[37]

quadruplex scaffold may be due, in part to the negatively charged oligophosphate scaffold contributing additional electrostatic contact to a protein surface that has a predominant positive charge. The possible role of the oligonucleotides in binding to the protein is supported by the observation that the G-quartet without projecting functional groups (**2**) and single DNA strand (**3X**) still show some inhibition activity, albeit weak compared to that of **2X**.

In studies on the binding of functionalized nanoparticles, Rotello et al.[23] detected significant denaturation of the protein ChT on contact with its cationic surface. To further determine the effects of oligonucleotide appended receptors on the conformation of ChT, CD experiments were carried out (Figure 5). The CD spectrum observed for ChT in 5 mM potassium phosphate buffer is the same as that reported by Rotello.[23] Compared to ChT alone, incubation of the protein with **2X** for 12 h at room temperature leads to a increase in the intensity of the minimum at 201 nm, and a decrease in the intensity of the characteristic minimum at 229 nm, indicating a degree of conformational change in ChT.[23] Under our current experimental conditions, complete denaturation is not observed.

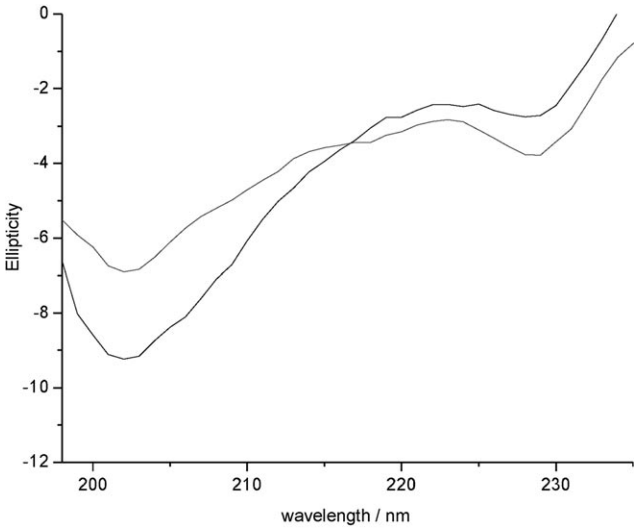


Figure 5. CD of ChT (——) ([ChT] = [**2X**] = 2.5 μM) after a 12 h incubation. The spectrum of ChT + **2X** (——) was normalized by subtracting the CD spectrum of 2.5 μM **2X**.

In summary, we have shown that G-quadruplex-scaffold-based receptors are effective inhibitors of ChT by protein-surface recognition. This may provide a general strategy to generate synthetic combinatorial libraries of receptors to target different classes of proteins. Probing the asymmetric nature of various protein surfaces by DNA quadruplexes with different binding groups is underway.

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Keywords: chymotrypsin · DNA structures · incubation · inhibition · receptors

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