

# Protein Recognition and Denaturation by Self-Assembling Fragments on a DNA Quadruplex Scaffold\*\*

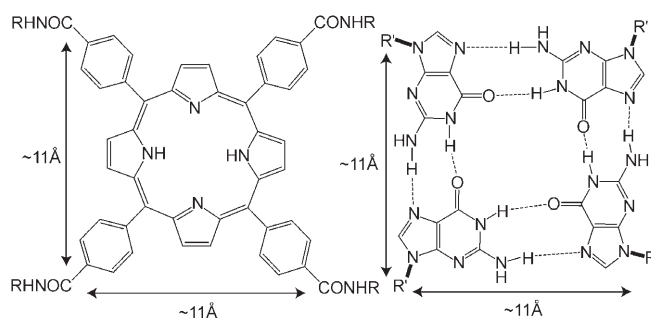
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Molecular self-assembly can be defined as a process by which small molecules associate through defined and designed interactions to form a larger aggregate with properties not possessed by the individual components.<sup>[1]</sup> Nature presents innumerable examples of large multiprotein aggregates that assemble through complementary exterior surface interactions and carry out critical functions in different organisms, from the complex photosynthetic reaction center<sup>[2]</sup> to simpler tetrameric hemoglobin<sup>[3]</sup> and from multisubunit enzymes such as polyketide synthases<sup>[4]</sup> to viral coat assemblies.<sup>[5]</sup> Similarly, complex oligonucleotide assemblies exemplified by the ribosome and lipid aggregates in bilayer membranes, represent functional assemblies formed by other biomolecules. However, outside the field of synthetic membrane chemistry, examples of artificial molecules that self-assemble into functional aggregates, particularly under aqueous conditions, are rare.<sup>[6]</sup>

There is great potential in the use of complementary oligonucleotide sequences to control the formation of aggregates that in turn can act as scaffolds for the formation of functional assemblies.<sup>[7]</sup> We,<sup>[8]</sup> and others,<sup>[9]</sup> have recently shown that functionalized and complementary DNA strands can form a duplex that presents two synthetic fragments on one end for binding to proximal sites on a target protein. These noncovalently assembled fragments mirror the strong protein binding properties of covalently linked bidentate ligands. In a further development of this strategy, we have now constructed scaffolds that present as many as four synthetic fragments for binding to a protein surface. We had previously reported that copper(II) complexes of polyanionic functionalized porphyrins are effective protein binding agents and trigger a conformational change in cytochrome *c* (cyt *c*).<sup>[10]</sup> The resultant cyt *c* adduct has markedly lowered thermal stability characterized by a decrease in the melting temperature of about 50 K.<sup>[11]</sup> This dramatic reduction in the

stability of the protein is attributed to preferential binding of the anionic porphyrins, through charge complementarity and hydrophobic interactions, to unfolded states of the protein.

The fourfold symmetrical structure of these functionalized tetracarboxyphenylporphyrins suggested that an alternative self-assembling scaffold might be provided by one end of an assembled guanosine tetraplex. These hydrogen-bonded tetramers project on one end a  $\pi$  surface comparable in size to tetraphenylporphyrin and which, when appropriately functionalized, should present four synthetic fragments in a similar manner (Scheme 1).



**Scheme 1.** Representation of the relative sizes of a G quartet and a tetraphenylporphyrin derivative.

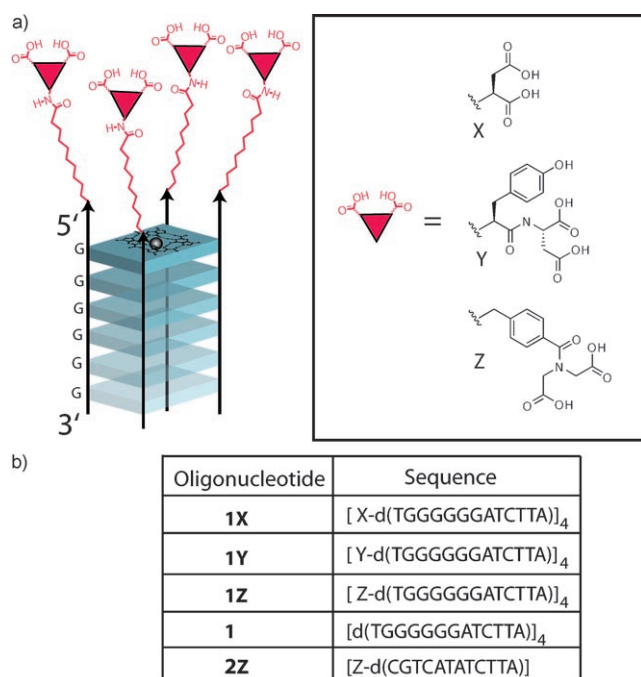
Quadruplexes arise from the  $\pi$  stacking of cyclic arrays of four hydrogen-bonded guanines (G quartet) in intermolecular and/or intramolecular Hoogsteen base-pair associations of one, two, or four strands in parallel and/or antiparallel orientations.<sup>[12]</sup> A single stretch of contiguous guanine bases has the propensity to form a four-stranded intermolecular parallel quadruplex in the presence of potassium ions.<sup>[13]</sup> Herein, we describe the use of quadruplexes to generate versatile supramolecular assemblies that orient, in a well-defined manner, four organic fragments on one end of a parallel quadruplex for protein recognition. An earlier, although nonfunctional example of a functionalized tetraplex was demonstrated by Aoyama and co-workers.<sup>[14]</sup>

The 5'-end of the quadruplex-forming oligonucleotides was functionalized with various anionic and hydrophobic fragments to target both the hydrophobic patch and the invariant lysine residues near the heme edge domain of cyt *c* (Figure 1). A fully covalent version of this approach has employed synthetic porphyrins containing a variety of the same anionic substituents that show strong binding to, and denaturation of, cyt *c*.<sup>[11]</sup>

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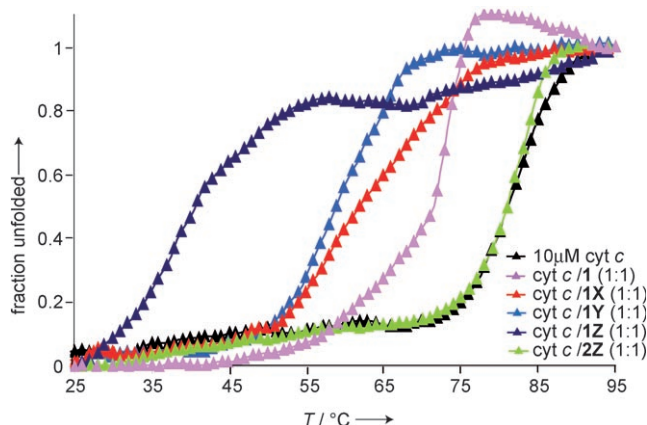
**Figure 1.** a) Functionalized parallel quadruplex with the different organic fragments used for the self-assembly shown as X, Y, and Z. b) Sequences of the designed oligonucleotides wherein quadruplex **1X**, **1Y**, and **1Z** are appended with X, Y, and Z fragments, respectively. Oligonucleotide **1** is an unfunctionalized quadruplex, and **2Z** is a functionalized single strand that is incapable of forming higher-ordered structures.

The organic fragments contained both an amino end for conjugation to the oligonucleotide and a carboxylic acid end for protein recognition. The synthesis of the carboxylic acid precursors X and Y and their conjugation to the oligonucleotide is outlined in the Supporting information. The synthesis of fragment Z has been previously reported.<sup>[11]</sup> The organic fragments were synthesized with the carboxylic acids protected as base-labile cyanoethyl esters for a single-step deprotection of the cyanoethyl groups along with the protecting groups on the DNA bases, and cleavage of the oligonucleotide from the solid support.

Functionalized parallel quadruplex formation was achieved in K<sup>+</sup> ion containing buffer (10 mM Tris-HCl, 80 mM KCl; pH 7.5; Tris = tris(hydroxymethyl)aminomethane) and confirmed by using nondenaturing gel electrophoresis and circular dichroism (CD) spectrophotometry. All the designed functionalized quadruplexes showed a positive CD peak at 266 nm and a negative peak at 242 nm, characteristic of a four-stranded parallel quadruplex.<sup>[15]</sup> Once formed, the quadruplexes show high thermal stability as evidenced from UV and CD denaturation studies (see the Supporting Information). If the functionalized quadruplexes mimic the behavior of the correspondingly functionalized porphyrins, binding to cyt *c* should result in an unraveling of the protein structure with a decrease in the  $\alpha$ -helical content, monitored by a loss of CD signal at 222 nm.

Single-stranded functionalized oligonucleotide **2Z** does not affect the melting temperature ( $T_m$ ) of cyt *c*, and the

parent unfunctionalized quadruplex **1** shows only a 9 K decrease in the  $T_m$  (Figure 2), presumably through non-specific oligoanionic effects of the phosphate backbone. The results are more pronounced with the functionalized quad-

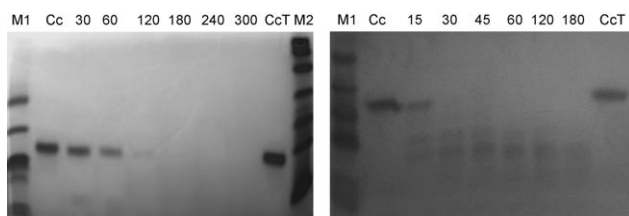


**Figure 2.** CD monitored thermal denaturation of cyt *c* at 222 nm in the presence of functionalized quadruplexes.

ruplexes **1X** and **1Y** in which the  $T_m$  is reduced by about 20 K and 25 K, respectively. However, the most dramatic effect is seen with **1Z**, which in its fully self-assembled state reduces the  $T_m$  for cyt *c* by about 45 K, a reduction of about 36 K and about 45 K when compared with the unfunctionalized tetraplex **1** and the unassembled functionalized single strand **2Z**, respectively. The enhanced denaturation effect of quadruplex **1Z** is presumably due to preferential binding to non-native states of the protein. There is a noteworthy decrease in the CD signal of cyt *c* at 95 °C, although the quadruplex peaks are unaffected indicating that the quadruplex structure is maintained at high temperatures (see the Supporting Information).

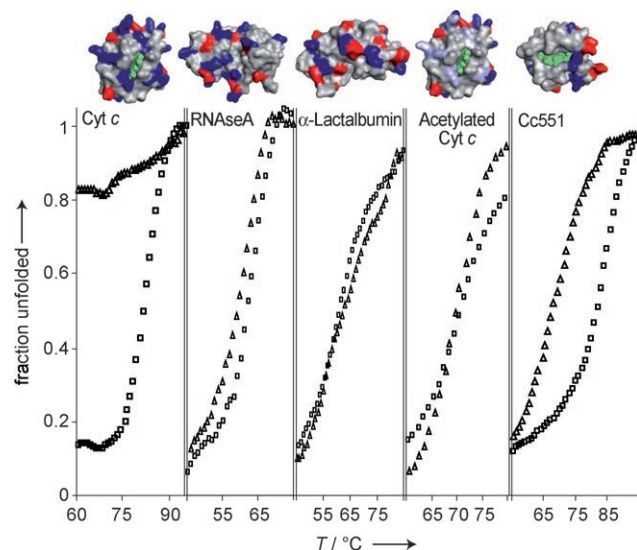
Cyt *c* has a well-folded, compact structure and hence is resistant to proteolytic digestion. However, even a partial unfolding of the protein on denaturation would lead to an increased susceptibility to proteolysis. Such selective degradation of target proteins by destabilization of the native state is an attractive goal. The effect of quadruplexes **1Y** and **1Z** in unfolding cyt *c* was assessed by proteolytic digestion of the quadruplex/cyt *c* complexes with trypsin at 37 °C. In the presence of quadruplexes **1Y** and **1Z**, tryptic digestion of cyt *c* is accelerated and is complete in 120 min and 30 min, respectively (Figure 3). In contrast, there is no appreciable digestion of cyt *c* after 5 h in the absence of the quadruplex. Furthermore, the structure dependence of this effect is evident from the significantly slower rate of proteolysis observed in the presence of the functionalized single-stranded oligonucleotide **2Z** and the unfunctionalized quadruplex **1** (see the Supporting Information).

The denaturation and acceleration of proteolysis of cyt *c* observed with oligoanionic-functionalized quadruplexes strongly suggests a discrete interaction of the quadruplexes based on hydrophobic and electrostatic complementarity. This is further substantiated by the selectivity observed against a range of other protein targets. As cyt *c* has a



**Figure 3.** SDS-PAGE of trypsin-digested quadruplex–cyt *c* complexes. Left: Cyt *c* treated with quadruplex **1Y** causes complete proteolysis within 120 min. Right: Proteolytic digestion is complete in 30 min for cyt *c* treated with quadruplex **1Z**. Cc = cyt *c*, M1 and M2 = protein markers, CcT = trypsin-treated cyt *c* for 300 min. The times for digestion are shown in minutes. Proteolytic digestion was performed with 10  $\mu$ M cyt *c* and 40  $\mu$ M quadruplex.

physiological isoelectric point (pI) of 10.5, the selectivity of quadruplex **1Z** was compared with RNaseA (pI 9.0),  $\alpha$ -lactalbumin (pI 5.0), and most importantly, a modified version of cyt *c* (acetylated cyt *c*) in which certain critical lysine  $\epsilon$ -amine residues are acetylated resulting in a different charge distribution on the surface of the protein (Figure 4). There is



**Figure 4.** Thermal denaturation profiles of various proteins in the presence of 1 equivalent of quadruplex **1Z** (10  $\mu$ M protein, 10  $\mu$ M quadruplex **1Z**, all in 10 mM Tris-HCl, 80 mM KCl; pH 7.5). □: denaturation of the proteins; △: denaturation of the complex.

no appreciable change in the  $T_m$  of these proteins, with only cyt c551 (modified cyt *c*) showing a 15 K decrease in the  $T_m$ . The lack of any effect on closely related proteins indicates that the surface charge complementarity plays an important role in the selectivity of the quadruplexes for cyt *c*.

The results presented herein underline the appeal of hydrogen-bond-based self-assembly for the formation of functional aggregates. In particular, designed oligonucleotide sequences allow the opportunity to “dial in” to each

component a defined stoichiometry and structure for the aggregate. This in turn imparts the functional property of protein binding and denaturation not seen in the individual functionalized strands or unfunctionalized aggregates. We are currently extending this approach to oligonucleotide aggregates with different stoichiometries of strand association and more complex symmetries of fragment positioning across the face of the aggregate to probe the asymmetric nature of various protein surfaces.

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