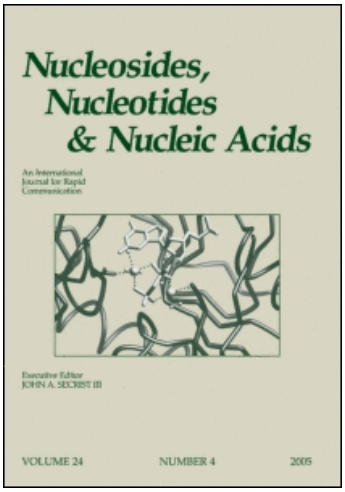

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Use of Oligonucleotide Conjugates in Creating Self-Assembling Supramolecular Systems

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USE OF OLIGONUCLEOTIDE CONJUGATES IN CREATING SELF-ASSEMBLING SUPRAMOLECULAR SYSTEMS

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Abstract: The chemistry of two types of oligonucleotide conjugates containing novel chromophores are described. One, containing a stilbenedicarboxamide bridge, generates unusually stable hairpin structures that are useful in assessing rates of electron transfer through the π system of a DNA double helix. The other, containing gold nanoparticle conjugates, provides a highly selective system for detecting nucleotide sequences in oligonucleotides.

Introduction. Oligonucleotides can be employed as frameworks for organizing and positioning covalently attached organic or inorganic substituents. Conversely, organic and inorganic units can serve as entities for modulating the properties of attached oligonucleotides. The interplay of oligonucleotides and conjugated groups in creating and controlling novel self-assembled structures is illustrated here with two quite different systems. In one, a relatively rigid, photoreactive substituent -- a stilbenedicarboxamide bridge -- is employed as cap for an oligonucleotide duplex. This bridge enhances the thermal stability of the duplex, and the oligonucleotides provide a framework that selectively positions the stilbene unit relative to the nucleotide bases. The construct affords a well defined system for studying and demonstrating electron transfer in the DNA duplex. In the other example, oligonucleotides are linked covalently through sulfur to gold nanoparticles. The oligonucleotides function as connectors in assembling the nanoparticles reversibly into large aggregates. Of particular interest is the finding that the nanoparticle oligonucleotide conjugates provide the basis for a simple and selective assay for nucleotide sequences in target oligonucleotides.

Stilbenedicarboxamide conjugates.[1-5] Stilbenedicarboxamide was selected for study as a bridging element since it is relatively rigid and is responsive to light in a region (λ_{max} 336 nm) that nucleotides do not absorb.[1,2] The former feature indicated that it might be especially

effective in stabilizing short duplex segments, and the latter opened opportunities for monitoring structural, conformational, and energetic changes spectrophotometrically. Oligonucleotides containing an extended stilbenedicarboxamide group, abbreviated "St", were prepared using phosphoramidite chemistry. Molecular models indicated that these units could span the distance across the end of a duplex without significant strain.

St = $-P(O)(O^-)O(CH_2)_3NHC(O)C_6H_4CH{=}CHC_6H_4C(O)NH(CH_2)_3OP(O)(O^-)-$

Thermal dissociation temperatures for several St conjugates are given in Table 1. Compounds **1-5** fold to give monomeric hairpin conformations at low temperature and open to random coiled structures on heating. The Tm values show that the St bridge is indeed very effective in stabilizing short oligonucleotide duplex segments. For comparison with other bridges, Tm's for T_6-X-A_6, where X = St, $-P(O)(O^-)O(CH_2)_6NHC(O)C_6H_4C(O)NH(CH_2)_6O-P(O)(O^-)-$ and $-P(O)(O^-)O(CH_2CH_2O)_3P(O)(O^-)-$, are 59 °C, 41 °C, and 35 °C, respectively. The open duplex, T_6:A_6 is unstable at 2 °C under the same conditions (0.1 M NaCl). The high thermal stability of the base-stacked conformation of cyclic compound **7**, which contains only four nucleotides, is especially striking.

The oligonucleotide segments in duplex **6** serve to bring the stilbenedicarboxamide groups into proximity. Alignment of the aromatic groups on hybridization is demonstrated by the hypochromicity and shift of the major long wavelength absorption band (λ_{max} 336 nm to 330 nm), by the appearance of a strong excimer fluorescence band (λ_{max} 445 nm, Φ 0.32), and by a photoinduced addition reaction that generates a substituted cyclobutane cross-link (Φ 0.18).[1,3] On dissociation of the complex, either by alkali or heating, the spectra revert to those characteristic of the monomeric stilbenedicarboxamide groups, and photodimerization is no longer observed. When the two stilbenedicarboxamide units are aligned but separated by intervening nucleoside bases, as in cyclic compound **7**, the behavior is reversed; excimer fluorescence and photoinduced cross-linking are observed only after "denaturation" with alkali. Presumably in this case the cyclic compound undergoes a conformational change in the alkaline medium that brings the stilbenedicarboxamide groups into proximity.[5]

Fluorescence spectra for hairpin structures T_n-St-A_n, (n = 4, 6 and 12), **4**, and **5** are depicted in Figure 1. An interesting feature is that the spectra are identical for the T_n-St-A_n series but the fluorescence is strongly quenched when a CG pair lies near St. These data and an experiment showing that the C nucleotides in C_6-St-C_6 do not influence the fluorescence from St indicated that G was the quencher and led to a suggestion that electron transfer from G to a photoexcited stilbenedicarboxamide might be involved.[2]

Table 1. Stilbenedicarboxamide Oligonucleotide Conjugates

Oligomers		Tm °C[a]	Ref.
1	T_6-St-A_6	59	2
2	rU_6-St-rA_6	44	4
3	GCG-St-CGC	>80	2
4	TTCG-St-CGAA	>70	2
5	TTCT-St-AGAA	~57	2
6	GCTGA-St-AGTCT CGACT-St-TCAGA	39	1,3
7[b]	St $\begin{matrix}AG\\T_sC\end{matrix}$ St	>80	5

a. 0.1 M NaCl. b. The small s in formula 7 refers to an -OP(O)(O⁻)S- linkage.

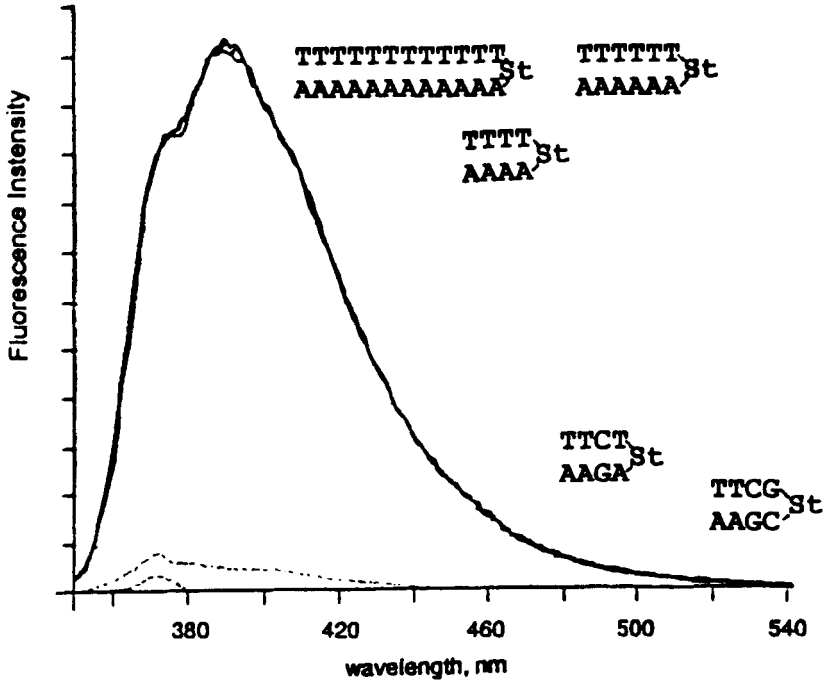


Figure 1. Fluorescence spectra for hairpin conjugates (The small peak at ~370 nm is an instrumental artifact)

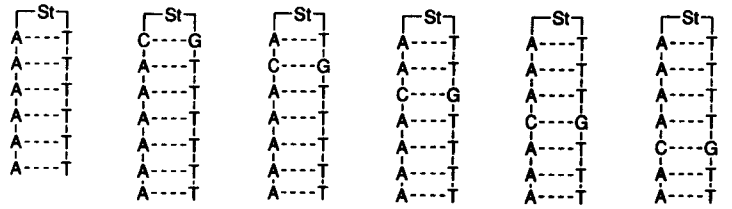
Electron transfer.[6] The question of the efficiency of electron transfer through the π system in double stranded DNA has been addressed by several research groups. Can DNA function as a "molecular wire"? Or is it an insulator? Current opinion is divided.[7] A convenient parameter for expressing the experimental data is β, the distance dependence of the electron transfer rate constant, which is defined by the equation $k_{ET} \propto \exp(-\beta R)$, where k_{ET} is the rate constant for electron transfer and R is the distance between the electron donor and acceptor. One research group has reported experiments indicating highly efficient long range electron transfer through double stranded oligonucleotides, with $\beta < 0.2$; several others have obtained data suggesting values in the range of 1, which indicates a poor conductor of electrons.[8] The interpretation of the data has been clouded by the fact that for some systems the geometry was not well defined, no systematic study of the effect of changing donor-acceptor distances has been made, and spectroscopic evidence for formation of the intermediates expected for an electron transfer process have been lacking.

Our work with the stilbenedicarboxamide oligonucleotide hairpins (Table 1, Figure 1) indicated that such structures might be useful in studying electron transfer in DNA. The duplex segments in these hairpins are unusually stable; the positions of the stilbene group and the nucleotide bases in the hairpins are reasonably well defined; both absorption and emission of light by the stilbene group can be readily monitored; spectral data indicate a unique role for guanine in quenching fluorescence from the stilbenedicarboxamide group; estimates for relevant oxidation-reduction potentials are consistent with a quenching mechanism involving electron transfer; and a range of distances between the putative donor (guanine) and acceptor (stilbenedicarboxamide) groups can be established by simply varying the nucleotide sequence in the hairpin stem. Accordingly, we synthesized the conjugates T_6-St-A_6, OGC, 1GC, 2GC, 3GC, and 4GC, and by steady state fluorescence spectroscopy, single photon counting, and transient absorption spectroscopy measured the fluorescence quantum yield, the fluorescence decay time, and the transient absorption spectra for each (Table 2).

In agreement with the earlier experiments, a GC pair placed next to the stilbene unit was found to quench essentially all fluorescence from the stilbene. The effect of the G base diminished with increasing distance between G and St; so that with four intervening T-A pairs (compound **4GC**) the quantum yield for fluorescence was 92% of that for dT_6-St-A_6. The data for the transient absorption were especially revealing. They showed formation of a short lived intermediate (λ_{max} 575 nm) that decayed to a longer lived intermediate exhibiting a narrower, differently shaped spectrum. On the basis of the spectra of model compounds, we concluded that the short lived intermediate was the singlet excited state and that the longer lived intermediate was the anion radical of the stilbenedicarboxamide group.

Table 2. Fluorescence and transient absorption spectral data [a]

Compound	Φ_f	fluorescence decay (ns)	transient decay time (ns) singlet	anion radical
DMS	0.16 ± 0.02		0.042 ± 0.005	
dT_6-St-dA_6	0.38 ± 0.04	2.2 ± 0.1	2.0 ± 0.4	
4GC	0.35 ± 0.04	1.8 ± 0.1	1.4 ± 0.2	>10
3GC	0.26 ± 0.03	1.4 ± 0.1	1.0 ± 0.2	>10
2GC	0.14 ± 0.02	0.5 ± 0.1	0.29 ± 0.03	4.0 ± 1
1GC	0.04 ± 0.01	< 0.2	0.005 ± 0.001	0.14 ± 0.01
0GC	<0.01	< 0.2	0.001 ± 0.0002	0.026 ± 0.003
DAS	<0.01	< 0.2	< 0.01	0.73 ± 0.05



T_6-St-A_6 OGC 1GC 2GC 3GC 4GC

a. DMS is N,N'-dimethylstilbenedicarboxamide, and DAS is N,N'-bis(N'''methyl-N'''-phenylamino-trimethylene)stilbenedicarboxamide. Models for the photoexcited singlet state and the anion radical of the St bridge were generated by irradiation of DMA and DAS, respectively.

Rate constants for the photoinduced electron transfers were calculated from the equation: $k_{et} = 1\backslash\tau_n - 1\backslash\tau_o$, where τ_n is the decay time for the nGC hairpins and τ_o, the decay time for dT_6-St-dA_6. Plots of the rate constants for decay of the singlet state and decay of the anion radical as a function of distance between the guanine residue and the stilbenedicarboxamide group are given in Figure 2. Although the forward and backward rates differ, the slope are the same and corresponds to a β value of ~0.6. This value shows that the DNA double helix is a poor medium for long range electron transfer between our donor and acceptor groups. As a conductor, DNA appears to be somewhat better than a protein; however is far from a "molecular wire".

Gold Nanoparticle Conjugates.[9,10] Although oligonucleotides conjugated to a variety of organic dyes, chromophores, hydrophobic substituents, and chelated metal ions have been

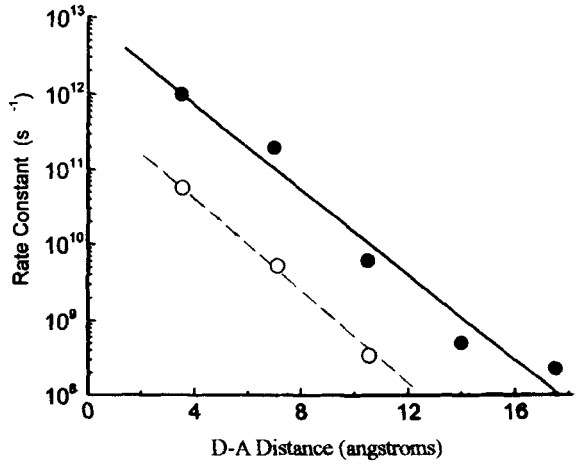


Figure 2. Distance dependence for rate constants for ET: (a) to the stilbenedicarboxamide excited singlet state, ——●——; and (b) from the stilbenedicarboxamide anion radical, -----○-----.

described, little if any work on oligonucleotides covalently bound to metal nanoparticles had been carried out when we began our studies on the chemistry of such systems. *Au priori*, it appeared that oligonucleotides might on one hand be useful in organizing nanoparticles into novel macroscopic aggregates, thereby generating new types of materials, and, on the other hand, that the physical properties of nanoparticles might be exploited in detecting specific DNA sequences. Several features of gold nanoparticles made them especially attractive for initial studies in this area. In particular, convenient methods were available for preparing relatively uniform gold particles in many different sizes; in absence of salts and a variety of reagents that react at the gold surface (e.g. pyridine and mercaptoethanol) the colloidal solutions were stable over long periods of time; the solutions were highly colored; and the color depended on the distance separating the nanoparticles and on the state of aggregation.[11] Furthermore, it had been shown than alkylthiols could be linked covalently through sulfur to gold nanoparticles in alcohol-water solution.[12] On the negative side, the gold colloids were known to be unstable, aggregating irreversibly, in salt solutions favorable for hybridization of oligonucleotides.

Following preliminary studies showing that nucleotides could be attached to gold surfaces *via* alkylsulfur bridges,[13] we succeeded in loading 13 nm gold nanoparticles with mercaptoalkyloligonucleotides and demonstrating that in the presence of target oligonucleotides in 1 M NaCl the nanoparticles would assemble reversibly, accompanied by a change in color.[9] However, work with these nanoparticle conjugates was complicated by the fact that the nanoparticles would aggregate irreversibly in absence of the target oligonucleotides at the high salt concentrations needed for hybridization in this system. This problem was solved by an improved preparative procedure that afforded more extensively loaded nanoparticles, which were stable in solutions containing up to 1 M NaCl.[10] For a representative system (probes **8** and **9** and target **10**, Table 3), hybridization of the nanoparticle conjugates with the target oligonucleotide in 0.1 M NaCl was rapidly achieved either by warming the mixture to ~50 °C or by quick freezing and thawing (~5 min). Hybridization of the probes with the target could be followed by a change in color of the colloidal solution from red to reddish purple, by the decrease in absorbance at 260 nm or the increase in absorbance at 620 nm, or by spotting a sample on a C-18 silica gel tlc plate; a blue spot signified hybridization and a pink spot, free nanoparticles. The color change may be attributed to the reversible assembly of the separated nanoparticles into aggregates in which duplex oligonucleotide segments function as inter-particle linkers. A striking feature is the sharpness of the transition. The range for the break in the thermal dissociation curve is ~5 °C, and the transition from pink to blue observed in the spot test occurs within 1 °C. In these systems the oligonucleotides serve to organize the nanoparticles and the nanoparticles function as reporters for hybridization of the oligonucleotides.

The sharpness of the melting transitions enables one to discriminate easily between closely related oligonucleotide targets. A representative example is illustrated in Table 3. In each case the target was hybridized with a mixture of gold-oligonucleotide probes **8** and **9** by quick freezing and thawing. The samples were then held for a few minutes in a water bath at the indicated temperatures (25-60 °C) and spotted on a C-18 silica plate. Blue spots, indicative of hybridization, were obtained for targets **10**, **11**, and **12** held at 25 °C. With an increases in the temperature the complexes formed by the mismatched and short probe sequences dissociated, so that at 53 °C only the target with the fully complementary sequence afforded a blue spot. Controls in which the target was omitted or contained extensive mismatched bases in one strand gave pink spots at all temperatures.

It is noteworthy that the nanoparticle probes can distinguished between 30-nt targets differing by a single base. This high selectivity probably reflects the fact that the signal for

Table 3. Probes and Targets for Hybridization Tests

Probes[a]	Spot Tests; T °C[b]				
	25	38	42	53	60
Au Au 3'-TCGTACCAGCTATCC TTTGCTGAGATCGCG **8** **9**					
Targets					
5'-AGCATGGTCGATAGGAAACGACTCTAGCGC **10** (Fully Complementary)	**bl**	**bl**	**bl**	**bl**	pk
5'-AGCATGGT***T***GATAGGAAACGACTCTAGCGC **11** (1 Base Mismatch)	**bl**	**bl**	**bl**	pk	pk
5'- GTCGATAGGAAACGACTCTAGCGC **12** (6 Base Deletion)	**bl**	**bl**	pk	pk	pk
5'-AGCATG***TT T***GATAGGAAACGACTCTAGCGC **13** (2 Base Mismatch)	**bl**	pk	pk	pk	pk
5'-AGCATGGTCGATAGGA***TGGCAACTATA***CGC **14** (Extensive Mismatch)	pk	pk	pk	pk	pk
no target	pk	pk	pk	pk	pk

a. Each gold nanoparticle has many thiololigonucleotide molecules attached; for simplicity only one strand is shown here. Also the probes contained 13-nt spacers at the 5'ends, represented here by dashed lines. b. For spot tests, 5 μL of the hybridized nanoparticle solution was spotted on a C-18 silica tlc plate; **bl** indicates a blue spot and pk, a pink spot, after drying.

hybridization depends on alignment of two relatively short oligonucleotide probes and on formation of a network of particles held together by the hybridization product. Further studies on potential applications of the chemistry of nanoparticle-oligonucleotide conjugates are in progress.

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