Synthesis of Oligonucleotides Carrying Anchoring Groups and Their Use in the Preparation of Oligonucleotide–Gold Conjugates

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Dedicated to Prof. Dr. *Wolfgang Pfleiderer* on the occasion of his 75th birthday

Oligodeoxynucleotide conjugates **1**–**15** carrying anchoring groups such as amino, thiol, pyrrole, and carboxy groups were prepared. A post-synthetic modification protocol was developed. In this method 2′-deoxy-O^4-(*p*-nitrophenyl)uridine-3-phosphoramidite was prepared and incorporated in oligonucleotides. After assembly, the modified nucleoside was made to react with different amines carrying the anchoring groups. At the same time, protecting groups were removed to yield the desired oligonucleotide conjugates. In a second approach, amino, thiol, and carboxylic groups were introduced into the 3′-end of the oligonucleotides by preparing solid supports loaded with the appropriate amino acids. Oligonucleotide–gold conjugates were prepared and their binding properties were examined.

Introduction. – Oligonucleotides carrying gold nanoparticles were first described in 1996 [1][2]. These conjugates have been shown to form networked arrays [1][3][4] and to form predetermined dimeric and trimeric assemblies [2][5]. The special properties of gold nanoparticles linked to oligonucleotides has attracted large interest due to their potential use in gene analysis [6–11]. In addition, oligonucleotides have been linked to monodispersed CdSe/ZnS quantum dots [12][13] and core-shell Ag/Au nanoparticles [14].

The preparation of oligonucleotide–gold nanoparticle conjugates is usually performed by reaction of oligonucleotides carrying thiol or dithiane androsterone [8][9] with either citrate- [1][3][7–11][14] or phosphine- [5][15] stabilized gold nanoparticles or nanogold-carrying maleimido groups [2]. Dithiothreitol (DTT)-stabilized CdSe/ZnS quantum dots were also activated with carbonyldiimidazole to further react with oligonucleotides carrying amino groups [13]. Oligonucleotides carrying amino and thiol groups are extensively used for the preparation of conjugates that carry a large variety of functionalities including fluorochromes, biotin, intercalators, enzymes, and reactive groups [16], as well as for producing oligonucleotide microarrays [17]. Electrochemically-addressed DNA matrixes can also be produced by using oligonucleotides carrying pyrrole groups. These oligonucleotides can be anchored onto a conducting polymer film by electro-oxidization of a mixture of pyrrole and pyrrole-bearing oligonucleotides [18]. Finally, a description is given of the preparation

of oligonucleotides carrying a carboxy group. The specific reactivity of the carboxy group was further used to prepare oligonucleotide–daunomycin conjugates [19].

Anchoring groups such as amino and thiol groups can easily be introduced at the 5′-end of oligonucleotides by means of phosphoramidites derived from amino alcohols and mercapto alcohols [16]. The introduction of anchoring groups at the 3′-end is not so simple. Special linker molecules are needed to introduce the desired group between the succinyl linker and the oligonucleotide. Amino diols and dithio compounds are the most commonly used linkers for the preparation of oligonucleotides carrying amino and thiol groups at the 3′-end. Very little data is available on the preparation of oligonucleotides carrying pyrrole or carboxylic groups at the 3′-end [18][19].

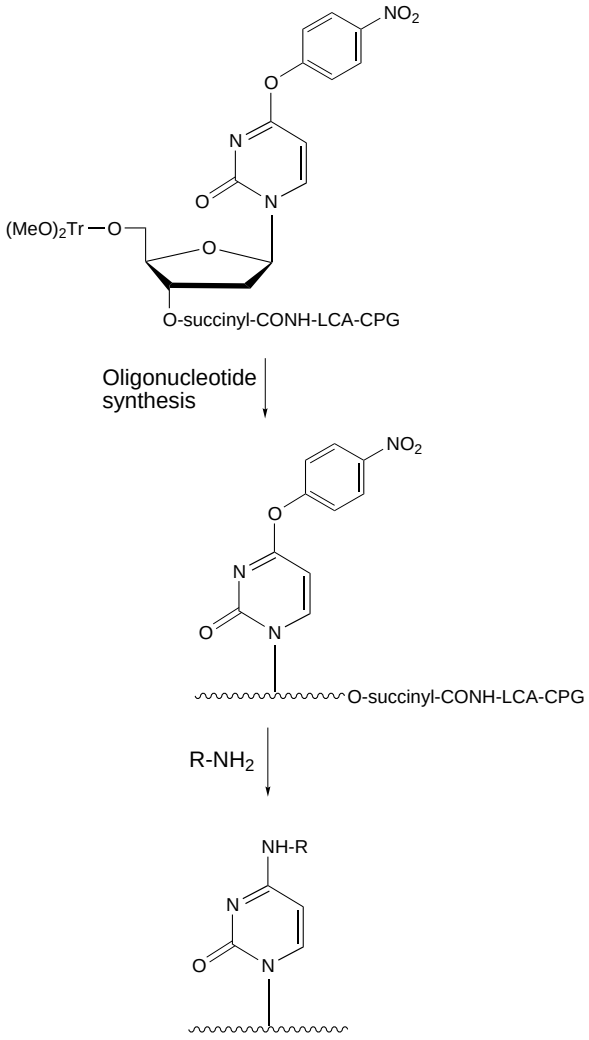
In this paper, we describe the use of post-synthetic modification protocols (or the convertible nucleoside approach [20]) to prepare oligonucleotides carrying amino, thiol, pyrrole, and carboxy groups at predetermined sites. This strategy involves a 2′-deoxyuridine derivative that carries a good leaving group (O^4-*p*-nitrophenyl, [21]) being incorporated at specific oligonucleotide sites and converted to a modified nucleobase, which carries the desired anchoring group by nucleophilic attack of the appropriate amine (*Scheme 1*), while the other protecting groups are also removed at the same time. In a second strategy, trifunctional amino acids were added at the 3′-end of the oligonucleotide through the use of special solid supports [22]. After the protecting groups are removed, the amino acid side chain generates the desired amino, thiol, or carboxy groups. Oligonucleotide–gold nanoparticle conjugates were also prepared, and a report is given of their binding properties.

Results. – 1. *Synthesis of Oligonucleotides Carrying Anchoring Groups by the Post-Synthetic Modification Approach.* A derivative of 2′-deoxyuridine (dU) was selected as a convertible nucleoside [20]. For this purpose, the O^4-(*p*-nitrophenyl) derivative of dU was prepared, as described in [21]. The O^4-(*p*-nitrophenyl)dU derivative was incorporated on controlled-pore glass (CPG), in accordance with standard protocols [23][24]. The support carrying the O^4-(*p*-nitrophenyl)dU derivative was used to prepare two oligonucleotide sequences: *A*) 6mer: 5′-TAG CTU^{Np}-3′ and *B*) 17mer: TAG CTT GAC GAT AGG TU^{Np}-3′, U^{Np} = O^4-(p-nitrophenyl)dU.

Phosphoramidite derivatives protected with the (*tert*-butyl)phenoxyacetyl group were used to incorporate the natural bases. These groups are more labile than standard groups and are removed by amines at the same time that the O^4-(*p*-nitrophenyl)dU derivative is being modified. The use of these protecting groups prevents cytosine modification, which has been described under conditions when amines other than NH_3 are used to remove protecting groups in DNA synthesis [20]. A similar strategy has been successfully used to prepare N^2-substituted guanine derivatives [25].

Aliquots of the hexamer sequence *A* were treated with the following amines (1M aqueous solution): *I*) cystamine ($NH_2CH_2CH_2SSCH_2CH_2NH_2$), *II*) *N*-(2-aminoethyl)-pyrrole [26], *III*) 2,2′-[(ethane-1,2-diyl)bis(oxy)]bis(ethynamine) ($NH_2(CH_2CH_2O)_2$-$CH_2CH_2NH_2$), *IV*) hexane-1,6-diamine, *V*) triglycine ($NH_2(CH_2CONH)_2CH_2COOH$), and a thiol *VI*) 6-mercaptohexan-1-ol. The treatment with amine *I* yielded the hexamer **1** carrying a thiol group protected as a disulfide. Amine *II* generated the hexamer **2** carrying a pyrrole group. Amines *III* and *IV* generated hexamers **3** and **4** carrying an amino group linked through a polar (*III*) or apolar (*IV*) linker. Amine *V* produced the

Scheme 1. *Synthesis of Oligonucleotides Carrying Anchoring Groups by Postsynthetic Modification Protocol*



hexamer **5** carrying a carboxy group. The thiol *VI* was selected to check whether a thiol group could also be used to incorporate molecules into the hexamer sequence. After the treatment, the resulting solutions were concentrated and desalted on a *Sephadex G-25* column. The oligonucleotide-containing fractions were pooled and purified by reversed-phase (RP) HPLC. Mass spectrometry showed that the desired oligonucleotides **1**–**5** were obtained in all cases except one (*Table 1*). Treatment of the support with 6-mercaptohexan-1-ol did not yield any detectable oligonucleotide. Our belief is that the lack of product was due to 6-mercaptohexanol not being able to cleave the oligonucleotide-support bond. However, the strong yellow color of the solution did

Table 1. *Yields and Mass-Spectrometric Analysis of the Oligonucleotides Resulting from the Treatment of Hexanucleotide Support 5′-TAGCTU^{Np}-CPG-3′ with Amines or Thiols*

Compound	Amine/thiol used	Yield [%]	*M*	
			Found	Calc.
1	*I*) $(NH_2CH_2CH_2S)_2$	35	1903	1903
2	*II*) $NH_2CH_2CH_2$-pyrrole	10	1861	1862
3	*III*) $NH_2(CH_2CH_2O)_2CH_2CH_2NH_2$	30	1900	1899
4	*IV*) $NH_2(CH_2)_6NH_2$	25	1868	1867
5	*V*) $NH_2(CH_2CONH)_2CH_2COOH$	25	1941	1940
6	*VI*) $HS(CH_2)_6OH$[a])	15	1885	1885

[a]) NH_3 was needed to release the hexamer from the support, after it was treated with the thiol.

indicate that *p*-nitrophenol was released. After the thiol treatment, ammonia was used to break the linkage between the oligonucleotide and the support. The two-step treatment was successful, and the hexamer **6** was also obtained (*Table 1*). In addition to the desired oligonucleotide, there were variable amounts (10–20%) of the hexamer resulting from hydrolysis of the *p*-nitrophenyl group. To prevent the hydrolyzed product from forming, H_2O was replaced with dry MeCN to dissolve the amine *IV* (1M solution). In this case, only the desired hexamer was obtained, and no hydrolyzed product was detected by HPLC, enzyme digestion, or mass spectrometry.

Post-synthetic modification protocols were then applied to the 17mer sequence *B* to produce oligonucleotides carrying thiol, amino, and pyrrole groups. In this case, sequence *B* was treated with three amines: *I*) cystamine ($NH_2CH_2CH_2SSCH_2$-CH_2NH_2), *II*) *N*-(2-aminoethyl)pyrrole, and *III*) 2,2′-(ethylenedioxy)diethylamine ($NH_2(CH_2CH_2O)_2CH_2CH_2NH_2$). The resulting oligonucleotides were purified by HPLC under standard trityl-on, trityl-off conditions. Treatment of sequence *B* with diamine *I* yielded oligonucleotide **7** in a 42% yield (*Table 2*). This yield is similar to that obtained by standard methods. In the case of the diamine *III*, the yield was lower (22%), and much lower with amine *II*. Oligonucleotides **7**–**9** were characterized by mass spectrometry and by analysis of the nucleoside composition after enzyme degradation.

Finally, the amount of excess amine necessary for the deprotection-modification reactions was determined. The support with hexamer A (200 nmol) was treated with decreasing amounts of hexane-1,6-diamine in MeCN: 1 ml of a 1M solution and 0.1 ml of 1M, 0.1M, and 0.01M solutions (5000–5 molar amine/hexamer ratio). Good results were obtained with 5000–50 molar amine/hexamer ratio. At the 5 molar amine/hexamer

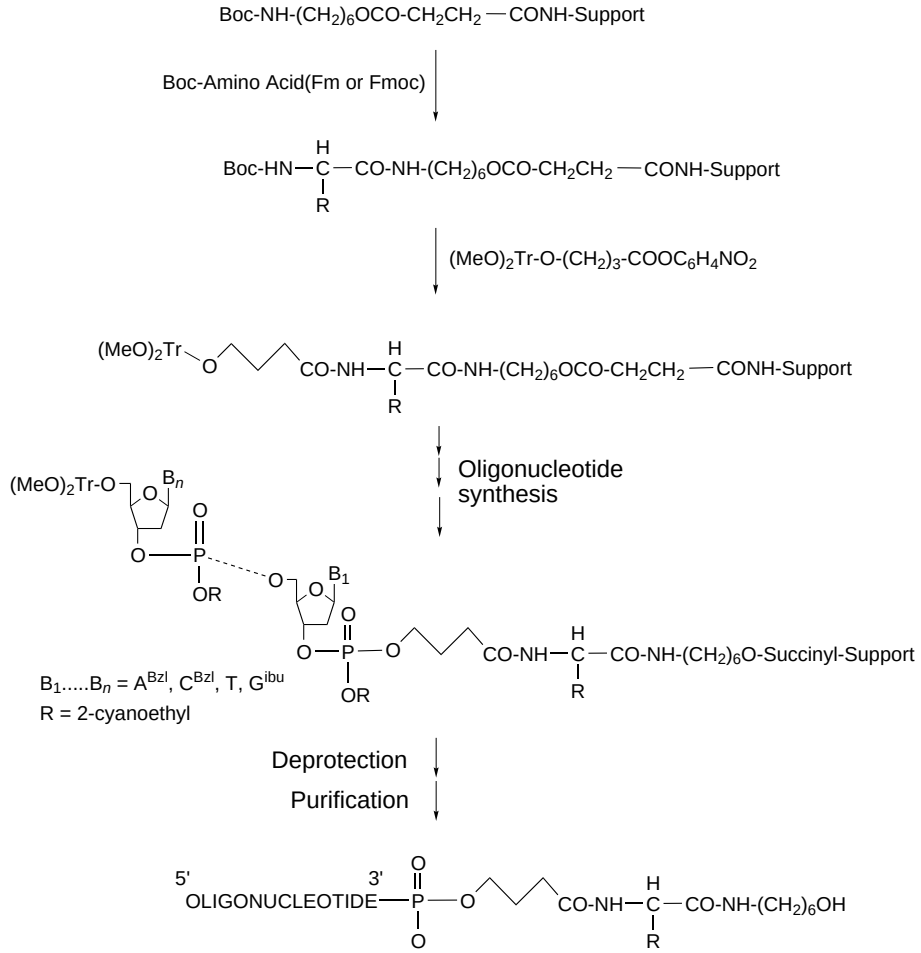
Table 2. *Yields and Mass-Spectrometric Analyses of Oligonucleotides Resulting from the Treatment of 17Mer-Support TAGCTTGACGATAGGTU^{Np}-CPG-3′ with Different Amines*

Compound	Amine/thiol used	Yield [%]	*M*	
			Found	Calc.
7	*I*) $(NH_2CH_2CH_2S)_2$	42	5361	5360
8	*II*) $NH_2CH_2CH_2$-pyrrole	7	5318	5318
9	*III*) $NH_2(CH_2CH_2O)_2CH_2CH_2NH_2$	22	5375	5376

ratio, the oligonucleotide was not released from the support. Subsequent treatment of the support with NH_3 gave a mixture of three oligonucleotides in an equimolar ratio: the desired oligonucleotide **4**, the hydrolyzed product (hexamer with dU), and the product of the reaction of the convertible nucleoside with NH_3 (hexamer with dC).

2. *Synthesis of Oligonucleotides Carrying Anchoring Groups with Trifunctional Amino Acids.* The use of trifunctional amino acids to introduce anchoring groups at the 3′-end of oligonucleotides was also studied. A recent protocol described for the stepwise synthesis of oligonucleotide–peptide chimeras [22] was selected. First, a special support carrying the succinate derivative of 6-aminohexan-1-ol linked to amino-functionalized supports by a succinyl linkage was prepared (*Scheme 2*). Two amino-functionalized supports were used: controled-pore glass (CPG) and polystyr-

Scheme 2. *Synthesis of Oligonucleotides Carrying Trifunctional Amino Acids at the 3′-End.* Lys(Fmoc): R = $(CH_2)_4NH$–Fmoc; Cys(Fm): R = CH_2S–Fm; Glu(Fm): R = $(CH_2)_2COO$–Fm.



ene-polyethyleneglycol (PEG-PS). The trifunctional amino acid was assembled on the 6-aminohexan-1-ol-succinyl-support by Boc chemistry. To protect side chains, the ε-amino group of lysine was protected with the (9*H*-fluoren-9-yl)methoxycarbonyl (Fmoc) group, and the carboxy group of glutamic acid and the thiol group were protected with the (9*H*-fluoren-1-yl)methyl (Fm) group. Coupling reactions were performed with Ph_3P and 2,2′-dithiobis(5-nitropyridine) [23], when CPG was used as solid support, and [(1*H*-benzotriazol-1-yl)oxy]tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)-mediated coupling was applied with PEG-PS. After the incorporation of the appropriate amino acid, assembly of the oligonucleotide chain requires the presence of a OH group. The 4-hydroxypropanoyl linker carrying the dimethoxytrityl [$(MeO)_2Tr$] group for protection of the OH group [27] was used. The progress of amino acid and linker incorporation on PEG-PS supports was monitored by the ninhydrin test [28]. This test was unreliable on CPG supports, and for this reason a capping step was introduced after each coupling reaction. Standard phosphoramidites were used for oligonucleotide assembly. For synthesis of the oligonucleotide part, the standard synthesis cycle was used in the case of a CPG support together with a special synthesis cycle at increased coupling time with PEG-PS. Although it has previously been reported that phosphoramidite coupling reactions on peptide-PEG-PS-supports are inefficient in MeCN due to poor swelling of the support, this problem was overcome with CH_2Cl_2 to dissolve the phosphoramidites [22]. However, attachment of just one single amino acid to the PEG-PS support produced no change in the solvents on phosphoramidite coupling.

Hexanucleotide sequences **10** and **11** were assembled according to *Scheme 2* on CPG and PEG-PS supports carrying Fm-protected Glu (*Table 3*). Following assembly of the sequences, standard NH_3 deprotection was performed. In both cases, HPLC analysis of the resulting products gave one main product with the expected mass (*Table 3*). Supports carrying hexanucleotides **10** and **11** were alternatively treated first with a 0.5M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) solution (to remove the Fmoc group), followed by NH_3 to prevent glutamine formation. HPLC Profiles of the products obtained with or without DBU treatment were identical, indicating that glutamine formation did not happen at a detectable level ($< 10\%$) under standard NH_3 deprotection. Likewise, oligonucleotides **12** and **13** were also assembled on CPG

Table 3. *Oligonucleotide Sequences Carrying Trifunctional Amino Acids at the 3′-End Prepared in the Present Study*

Compound	Sequence 5′ → 3′	Solid support[a])	Yield [%]	*M*	
				Found	Calc.
10	CCCCCC	Glu(Fm)CPG	22	2066	2067
11	TTTTTT	Glu(Fm)PEG-PS	20	2154	2157
12	GAGCTACTGAGTTACTGAGC	Glu(Fm)CPG	43	n.d.	
13	GCTCAGTAACTCAGTAGCTC	Glu(Fm)CPG	40	n.d.	
14	CCCC	Lys(Fmoc)PEG-PS	35	1486	1488
15	TCTCAACTCGTA	Cys(Fm)CPG	40	3947	3948

[a]) Abbreviations: CPG: controlled-pore glass, PEG-PS: polyethyleneglycol-polystyrene, Fm: (9*H*-fluoren-9-yl)methyl, Fmoc: (9*H*-fluoren-1-yl)methoxycarbonyl, n.d.: not determined.

supports with glutamic acid as the amino acid component. These oligonucleotides were obtained in similar yields and purity (tested by gel electrophoresis, data not shown) as unmodified oligonucleotides. The carboxy group-functionalized oligonucleotides **12** and **13** are used for anchoring oligonucleotides to amino-modified gold electrodes (work in progress).

Attempts to synthesize the hexanucleotide sequence C_6 on CPG support carrying Fmoc-protected Lys were not successful. This was possibly due to the poor coupling efficiency of the linkers and the amino acid. On the contrary, the tetranucleotide sequence **14** was assembled without any problems on PEG-PS carrying Lys(Fmoc) and, after NH_3 deprotection, gave a main product with the correct mass (*Table 3*).

Finally, the dodecanucleotide sequence **15** containing Fm-protected Cys resulted from analogous synthetic steps. In this case, deprotection was performed with a mixture of concentrated NH_3 and 50 mM DTT to prevent disulfide formation. A single peak by HPLC showing the expected mass was obtained (*Table 3*), and no dimer formation was observed even after several days with the HPLC-purified product kept in solution without DTT.

3. *Synthesis and Properties of Oligonucleotide-Gold Nanoparticle Conjugates.* First, phosphine-stabilized gold nanoparticles (1.4 nm) carrying either a carboxy group active ester (*N*-hydroxy-2,5-dioxopyrrolidine-3-sulfonic acid sodium salt, sulfo-NHS) or a maleimido group (NANOGOLD, *Nanoprobes Inc.*, USA) were selected to form defined conjugates carrying one oligonucleotide per particle (NANOGOLD particles statistically carry one reactive molecule incorporated into the phosphine shelf). Conjugation of maleimido-NANOGOLD to cysteine-oligonucleotide **15** was performed in 10% aqueous i-PrOH. 0.1M Carbonate buffer (pH 9.0) was used for the conjugation of sulfo-NHS-NANOGOLD to amino-oligonucleotide **9**. Conjugates were analyzed and purified by 2% agarose-gel electrophoresis (*Fig. 1*) as described in [5][15]. NANOGOLD is uncharged and, for this reason, remained near the wells, whereas oligonucleotide-NANOGOLD conjugates are negatively charged and moved towards the anode. Conjugation efficiency was judged to be similar for both the cysteine-oligonucleotide **15** and the mercapto-oligonucleotide **16** prepared with commercially available chemicals. These conjugates will be used for the self-assembly of well-defined gold nanoparticle architectures in solution [2][5][11] (work in progress).

In addition, oligonucleotides carrying thiol groups (**15**–**19**, *Tables 3* and *4*) were reacted with citrate-stabilized Au nanoparticles (13 nm) to obtain Au nanoparticles with several oligonucleotide molecules per nanoparticle [7]. Melting experiments with duplexes formed by complementary oligonucleotides **16** and **17** were performed with detection by UV/VIS absorption spectroscopy. Marked changes in the optical absorption spectra, observed as a function of increasing temperature, included a substantial increase in the absorption at 260 nm and a shift (40 nm) in the maximum position of the surface plasmon absorption mode of the Au nanoparticles (*Fig. 2, a*). Both these features are in good agreement with previous studies, which assign these spectral changes to the binding of the derivatized Au nanoparticles with their complements [7]. *Fig. 2, b* shows the temperature-dependent melting of duplexes formed by sequences **16** and **17** at 260 nm with and without Au nanoparticles attached. When nanoparticles are present, melting transitions occur over a small temperature range and with a large absorbance change, producing sharp, well-defined melting transitions. In

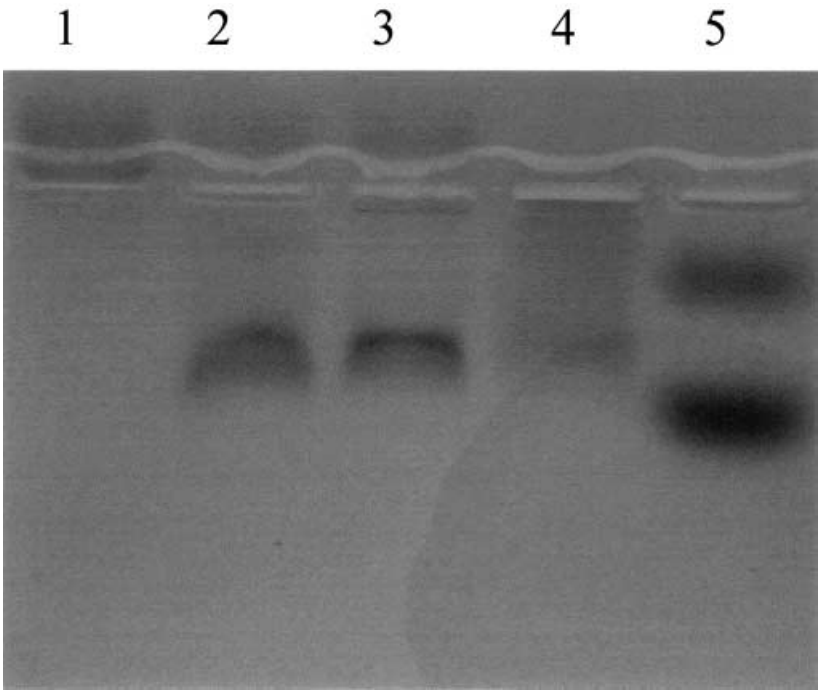


Fig. 1. *2% Agarose gel* (0.5 × *Tris*-Borate-EDTA (TBE)) *of oligonucleotide–Au nanoparticle conjugates.* The gel was run at 80 V for 20 min. *Lane 1:* maleimido-NANOGOLD, *Lane 2:* reaction of cysteine-oligonucleotide **15** with maleimido-NANOGOLD, *Lane 3:* reaction of thiol-oligonucleotide **16** with maleimido-NANOGOLD, *Lane 4:* reaction of amino-oligonucleotide **9** with sulfo-NHS-NANOGOLD, *Lane 5:* bromophenol blue and xylenecyanol dyes.

Table 4. *Oligonucleotide Sequences Prepared by Standard Methods*

Compound	Sequence 5′ → 3′
16	Thiol-hexyl-CGAGTCATTGAGTCATCGAG
17	Thiol-hexyl-CTCGATGACTCAATGACTCG
18	TCTCAACTCGTA-propyl-thiol
19	Thiol-hexyl-CGCATTCAGGAT
20	TACGAGTTGAGAATCCTGAATGCG
21	TACGAGTTGAGACATCCTGAATGCG
22	TACGAGTTGAGACCATCCTGAATGCG
23	TACGAGTTGAGACCCATCCTGAATGCG

contrast, unmodified oligonucleotide duplexes melt over broad temperature ranges and produce comparatively small absorption changes.

Further studies were carried out to measure the effects of introducing a gap in the stability of a three-component oligonucleotide system, **20**–**23**. Two short oligonucleotides, **18** and **19**, attached to Au nanoparticles were hybridized to complementary oligonucleotides **20**–**23** with an increasing number of bases inserted into the target oligonucleotide sequence. As can be seen from *Fig. 3*, the introduction of unpaired

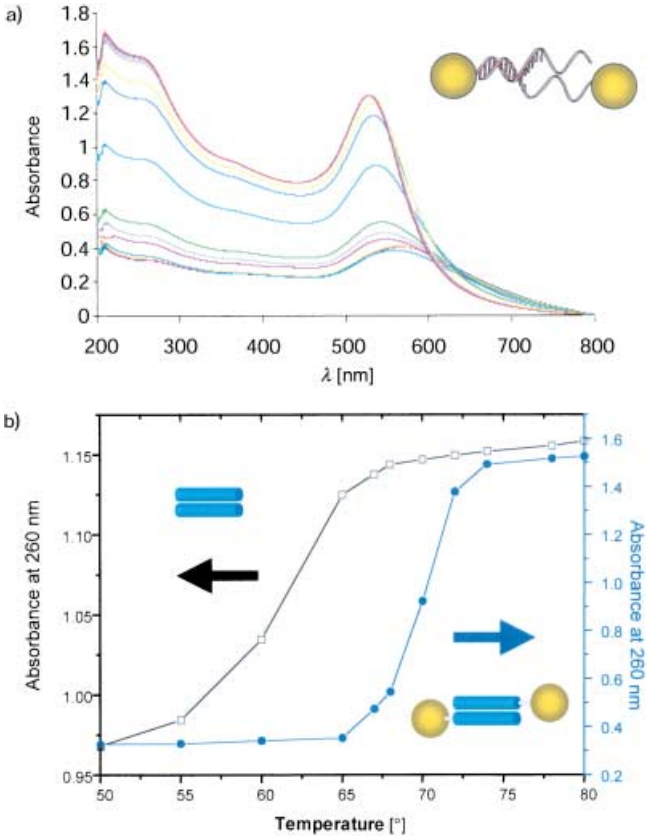


Fig. 2. *Hybridization and melting of oligonucleotide–Au nanoparticle conjugates. a*) UV Spectra of duplex formed with oligonucleotide–Au nanoparticle conjugates **16** and **17**. *b*) Melting profiles at 260 nm of duplex formed by sequences **16** and **17** with and without Au nanoparticles.

bases into the target oligonucleotide results in a systematic lowering of the melting temperature. Again, the absorbance changes that occur during the melting of the three component oliognucleotide–nanoparticle conjugates are of greater magnitude and occur over narrower temperature ranges than with unmodified oligonucleotides. Similar results were obtained when oligonucleotide **18** was replaced by cysteine-oligonucleotide **15** (*Fig. 4*). Melting temperatures obtained with cysteine-oligonucleotide **15** were the same (within an error of $\pm 0.3°$) as melting temperatures obtained with oligonucleotide **18** prepared with commercially available compounds (*Table 5*).

Discussion. – Oligonucleotides carrying several anchoring groups were prepared. The results obtained showed that O^4-(*p*-nitrophenyl)dU is a versatile compound for the preparation of modified oligonucleotides carrying a variety of additional functional groups, including thiol, amino, pyrrole, and carboxy groups, on the nucleobase. The O^4-(*p*-nitrophenyl)dU-modified oligonucleotides allow the different linker molecules to

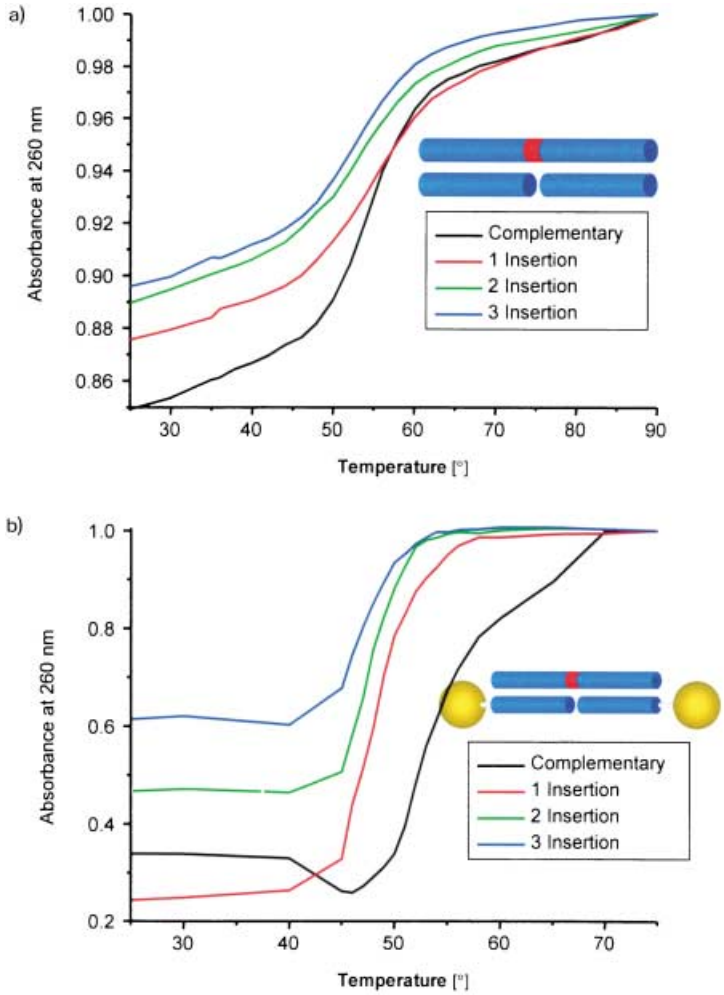


Fig. 3. *Melting profiles at 260 nm of thiol-modified oligonucleotide–Au nanoparticle conjugates. a*) Target oligonucleotide sequences **20**–**23** with an increasing number of inserted bases were mixed with unmodified oligonucleotide sequences **18** and **19**. *b*) Target oligonucleotide sequences **20**–**23** were mixed with thiolated oligonucleotide sequences **18** and **19** attached to Au nanoparticles (0.3M NaCl and 10 mM sodium phosphate pH 7).

be fixed at specific sites. This may be an advantage for finding the optimal characteristics of linker molecules without having to prepare a large number of derivatives. Moreover, side reactions may occur when aqueous conditions are used. Changes to anhydrous solvents may have a positive effect. Although the convertible nucleoside was located in this study at the 3′-end, this methodology allows the convertible nucleoside to be assembled at any position on the oligonucleotide. 2′-Deoxy-O^4-(2,4,6-trimethylphenyl)uridine was previously used for similar purposes

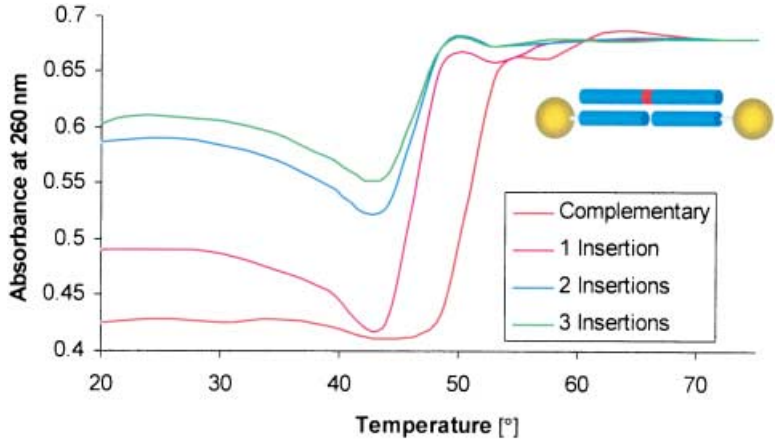


Fig. 4. *Melting profiles at 260 nm of cysteine-modified oligonucleotide-Au nanoparticle conjugate.* Target oligonucleotide sequences **20**–**23** with an increasing number of inserted bases were mixed with oligonucleotides **15** and **19** attached to Au nanoparticles (0.3M NaCl and 10 mM sodium phosphate pH 7).

Table 5. *Comparison of Melting Temperatures* (T_m) Obtained with a DNA Assembly Made of Three Components, a Target Oligonucleotide Sequence, **20**–**23**, *with an Increasing Number of Inserted Bases and Two Short Oligonucleotide–Au Nanoparticle Conjugates.* In the first column, thiolated oligonucleotides **18** and **19** were used while cysteine-oligonucleotide **15** and thiolated-oligonucleotide **19** were used in the next column. Conditions: 0.3M NaCl and 10 mM sodium phosphate pH 7.

Template	Number of insertions	Oligonucleotide **18** T_m [°]	Oligonucleotide **15** T_m [°]
20	0	51.8	51.2
21	1	47.6	47.5
22	2	47.4	47.0
23	3	46.4	46.7

[20], and the use of a *p*-nitrophenyl as a more-active leaving group in combination with the more-labile (*tert*-butyl)phenoxyacetyl (Tac) group for amino protection is preferred because the deprotection-modification reaction can be performed in a single step. Moreover, the intense yellow color of the resulting *p*-nitrophenolate ion allows the progress of nucleophilic substitution to be monitored.

The use of trifunctional amino acids to incorporate amino, thiol, and carboxy groups at the 3′-end of oligonucleotides was also studied. The use of base-labile protecting groups for the side-chain functions of amino acids was compatible with oligonucleotide synthesis. The α-amino groups of the amino acids were protected with the Boc group, but these groups were removed prior to the oligonucleotide being assembled. Two linkers were needed to connect the amino acid to the desired oligonucleotide, and 4-hydroxybutanoic acid and 6-aminohexan-1-ol, inexpensive compounds, were applied as starting materials. CPG and PEG-PS supports, in general, gave good results and were compatible with oligonucleotide synthesis without any change to the solvent used on the phosphoramidite coupling reactions.

Finally, the hybridization properties of oligonucleotide–Au nanoparticle conjugates were investigated, and it was shown that the conjugates, in general, gave sharper and more-defined melting transitions than unmodified oligonucleotides.

This work was supported by the *Commission of the European Union* as part of the *Information Societies Technology Programme* (IST-1999-11974), by the *Dirección General de Investigación Científica* (BQU2000-0649) and *The Generalitat de Catalunya* (2000-SGR-0018, 2001-SGR-0049). We are grateful to *Blanca SanSegundo* and *Sonia Campo* for their assistance in the preparation of agarose gels.

Experimental Part

General. Phosphoramidites and ancillary reagents used for oligonucleotide synthesis were from *Applied Biosystems* (*PE Biosystems Hispania S.A.,* Spain), *Cruachem* (*Cruachem Ltd.*, Scotland) and *Glen Research* (*Glen Research Inc.*, USA). Amino acid derivatives were from *Bachem* (*Bachem AG*, Switzerland) and *Novabiochem* (*Calbiochem-Novabiochem AG*, Switzerland). The rest of the chemicals were purchased from *Aldrich*, *Sigma*, or *Fluka* (*Sigma-Aldrich Química S.A.*, Spain). Long-chain alkylamine controlled-pore glass (LCA-*CPG*) was purchased from *CPG* (*CPG, Inc.*, New Jersey, USA). Amino-polyethyleneglycol-polystyrene (PEG-PS) was purchased from *PerSeptive* (*PerSeptive Biosystems*, now *Applied Biosystems*, USA). Solvents were from *S.D.S.* (*S.D.S.*, France). $(MeO)_2$Tr-Protected 2′-deoxy-O^4-(*p*-nitrophenyl)uridine was synthesized according to the procedure in [21]. Au Nanoparticles (13 nm, citrate stabilized) were prepared as described in [7], and NANOGOLD (1.4 nm, phosphine stabilized) carrying maleimido and sulfo-NHS ester functionalities were purchased from *Nanoprobes* (*Nanoprobes, Inc.*, USA).

Instrumentation. UV Spectra were recorded on a *UV-2301PC* and a *2401 Shimadzu* spectrophotometer. Mass spectra (matrix-assisted laser desorption ionization time-of-flight, MALDI-TOF) were provided by *Eurogentec* and the mass-spectrometry service at the University of Barcelona.

Preparation of Solid Supports. Solid-support carrying 2′-deoxy-O^4-(*p*-nitrophenyl)uridine was prepared by reacting the 3′-hemisuccinate derivative of 5′-[$(MeO)_2$Tr-]-2′-deoxy-O^4-(*p*-nitrophenyl)uridine [24] with LCA-*CPG* with Ph_3P and 2,2′-dithiobis(5-nitropyridine) [23] for condensation.

Solid supports (CPG and PEG-PS) carrying amino acids were prepared by sequential addition of the following compounds: *1*) N^6-Boc-6-aminohexan-1-ol hemisuccinate prepared by reaction of commercially available N^6-Boc-6-aminohexan-1-ol with succinic anhydride and DMAP as reported in [24]; *2*) either Boc-Glu(Fm)-OH, or Boc-Lys(Fmoc)-OH, or Boc-Cys(Fm)-OH and *3*) O^4-[$(MeO)_2$Tr]-4-hydroxybutanoic acid *p*-nitrophenyl ester [27]. The addition of Boc-amino acids on PEG-PS was performed in DMF with a 5-fold excess of the appropriate amino acid, a 5-fold excess of PyBOP, and a 10-fold excess of EtN(i-Pr)$_2$ for 1 h at r.t.. The removal of the Boc protecting group was performed with 40% CF_3COOH in CH_2Cl_2. A neutralization step with 5% EtN(i-Pr)$_2$ in CH_2Cl_2 and several washing steps were introduced between removal of the Boc group and coupling. The coupling of the *p*-nitrophenyl ester of [$(MeO)_2$Tr]-protected 4-hydroxybutanoic acid was performed in the presence of 1 equiv. of *N*-hydroxy-1*H*-benzotriazole (HOBt) with a 5-fold excess. A negative ninhydrine test was obtained after several hours at r.t. As a precaution, acetylation of unreacted amino groups was performed by a 10-min treatment of the support with a mixture of Ac_2O/EtN(i-Pr)$_2$/DMF 1:1.7:15.3 ml. The addition of Boc-amino acids to CPG supports was performed with Ph_3P and 2,2′-dithiobis(5-nitropyridine) as coupling activating agents [23]. The coupling of the *p*-nitrophenyl ester of [$(MeO)_2$Tr]-protected 4-hydroxybutanoic acid was performed as described for PEG-PS supports.

Oligonucleotide Synthesis. Oligonucleotide sequences were synthesized on an *Applied Biosystems* DNA synthesizer model *392* (*Applied Biosystems,* USA). 5′-[$(MeO)_2$Tr]-, [(*tert*-butyl)phenoxyacetyl]-protected 3′-(2-cyanoethyl *N,N*-diisopropylphosphoramidites) were used for the synthesis of oligonucleotides carrying the 2′-deoxy-O^4-(*p*-nitrophenyl)uridine moiety. The rest of the sequences were prepared with standard (Bz- or ibu-protected) 3′-phosphoramidites. Standard 1-μmol-scale synthesis cycles were used. Supports were prepared as described below. Coupling efficiencies were higher than 98%.

In addition to compounds **1**–**15**, oligonucleotides carrying thiol groups at the 5′-end, **16**, **17**, and **19** and at the 3′-end, **18**, were prepared with the phosphoramidite of [$(MeO)_2$Tr]-protected 6-hydroxyhexyl disulfide and the CPG support functionalized with [$(MeO)_2$Tr]-protected 3-hydroxypropyl disulfide (*Glen Research*, USA).

Deprotection of Oligonucleotides. Oligonucleotide supports carrying 2′-deoxy-O^4-(*p*-nitrophenyl)uridine were treated with 1 ml (or less) of 1M aq. solns. of the appropriate amine (*Tables 1* and *2)* at 55° overnight, and

the solns. were filtered and concentrated to dryness. The soln. of *N*-(2-aminoethyl)pyrrole contained 10% of dioxane. Cystamine was obtained from commercial sources as the HCl form; 1 equiv. of Et_3N was added to the cystamine soln. to neutralize the HCl. Two different deprotection protocols were used for oligonucleotide supports carrying lysine and glutamic acid at the 3′-end. In the first protocol, supports were treated directly with 1 ml of NH_3 (overnight, 55°). In the second protocol, supports were treated with 1 ml of 0.5M DBU soln. in MeCN for 30 min at r.t. The support was washed in MeCN, 1% Et_3N in MeCN, and MeCN and dried. The resulting support was then treated with 1 ml of conc. NH_3 (overnight, 55°). Oligonucleotide supports carrying cysteine at the 3′-end or oligonucleotides carrying the 6-mercaptohexyl group at the 5′-end were treated overnight with 1 ml of 50 mM dithio-DL-threitol (DTT) in conc. NH_3 55°.

Purification of Oligonucleotides. The resulting products were dissolved in H_2O, and the soln. was desalted on a *Sephadex G-25* column (*NAP-10*, *Pharmacia*, Sweden). The oligonucleotide-containing fractions were analyzed and purified by HPLC, as follows: column: *PRP-1*, 10 μm (*Hamilton*, USA 305 × 7 mm), flow rate 3 ml/min. A 20-min linear gradient from 15 to 55% MeCN over 100 mM aq. triethylammonium acetate was used for the oligonucleotide carrying the $(MeO)_2$Tr group. After removal of the $(MeO)_2$Tr group with 80% AcOH (30 min), the resulting oligonucleotides were purified on the same column with a 20-min linear gradient from 5 to 25% MeCN over 100 mM aq. triethylammonium acetate. Purified oligonucleotides were analyzed by MALDI-TOF-MS. The results are shown in *Tables 1–3*.

Enzymatic Analysis of Oligonucleotides. Oligonucleotides prepared by postsynthetic modification (0.5–1 OD units) were incubated in 50 mM T*ris*-HCl pH 8.0 and 10 mM $MgCl_2$ with snake venom phosphodiesterase (*Pharmacia Biotech*, Sweden) and bacterial alkaline phosphatase (*Pharmacia Biotech*, Sweden) in a total volume of 0.03 ml at 37° overnight. The resulting mixture was diluted and analyzed by HPLC: column: *C-18 Nucleosil*, 5 μm (250 × 4 mm), flow rate 1 ml/min. A 20-min linear gradient from 5 to 22% MeCN over 20 mM aqueous triethylammonium acetate was used, followed by 5 min of isocratic conditions at 22% MeCN. Retention times observed under these conditions were dC 3.6 min, dU (hydrolysis product) 4.3 min, dG 6.1 min, T 7.0 min, and dA 9.7 min. Modified dC derivatives: N^4-(2-pyrrolylethyl)-dC 15.8 min, N^4-{2-[2-(2-aminoethyl)oxyethyl]oxyethyl}-dC 11.4 min, N^4-[2-(2′-aminoethyl)dithioethyl]-dC 8.1 min, N^4-(6-aminohexyl)-dC 12.6 min, S^4-(6-hydroxyhexyl)-4-thio-dU 24.0 min (UV max at 330 nm).

Preparation of Au Nanoparticles and Oligonucleotides Prior to Conjugation. Au Nanoparticles were prepared by the citrate reduction of $HAuCl_4$ as described in [7]. Nanoparticles were analyzed by UV/VIS absorption spectroscopy (*Shimadzu 2401* spectrophotometer) and TEM (*JEOL JEM 1200-EX*). Oligonucleotides were desalted prior to use on a *NAP-10* (*Pharmacia*) column with a 10 mM sodium phosphate buffer (pH 7) as solvent. The resulting fractions were analyzed by UV/VIS.

Preparation of Oligonucleotide-Au Conjugates. Conjugation of maleimido-NANOGOLD to thiol-oligonucleotides was performed by following the recommendations of the suppliers. Aliquotes of lyophilized maleimido-NANOGOLD (6 nmols) were mixed with thiol-oligonucleotides (6 nmols) dissolved in 10% aq. i-PrOH. The resulting mixtures were kept overnight at r.t., and the resulting solns. were stored in the refrigerator until further use. A similar protocol was used for the conjugation of amino-oligonucleotides to sulfo-NHS-NANOGOLD. In this case, 0.1M carbonate buffer (pH 9.0) was used as a reaction buffer. Conjugates were purified by 2% agarose-gel electrophoresis (0.5 × TBE) as described in [5][15].

Furthermore, oligonucleotides carrying thiol groups were also conjugated to citrate-stabilized Au nanoparticles. The desalted thiolated oligonucleotide was removed from the freezer and allowed to thaw. The optical absorbance of both the oligonucleotide and nanoparticle samples were measured. The nanoparticles and oligonucleotide were mixed in appropriate amounts, and the entire soln. was brought to 10 mM sodium phosphate buffer (pH 7). After 1 d, the sol. was brought to 0.1M NaCl concentration and allowed to stand at r.t. for another 40 h. After this time, the soln. was centrifuged at 13,200 rpm for 30 min. Supernatant was removed, and the reddish solid at the bottom was dispersed in 0.1M NaCl and 10 mM sodium phosphate buffer (pH 7) soln. (the volume added was similar to that removed). This procedure was repeated by applying 0.3M NaCl and 10 mM phosphate buffer (pH 7). The soln. was analyzed by UV/VIS spectroscopy, and the absorbance at 260 nm was recorded for future use. The sol. was stored at r.t.

Melting Experiments. Melting experiments were carried out with equimolar amounts of each oligonucleotide-Au nanoparticle conjugate in 0.3M NaCl, 10 mM sodium phosphate buffer, pH 7. The soln. was heated to 90° for 5 min and then allowed to cool to r.t. for 48 h. The soln. was transferred to a stoppered 1-cm path-length cuvette, and UV/VIS spectra were recorded at 5° intervals, while the sample was heated from 20–90° at 5°/5 min. Heating of the UV cuvette was performed with a *Grant W6-KD* precision thermostatic circulator attached to a *Shimadzu* constant-temp. cell holder. Calibration spectra were recorded to determine the heat transfer efficiency to the cuvette. The data were collected as wavelength *vs.* absorbance spectra, and a plot of

temp. *vs.* absorbance at 260 nm was drawn. The melting temperature of the systems was determined from these graphs.

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Received May 5, 2002