Eur Biophys J (1997) 26: 277–281

BIOPHYSICS LETTER

Matthieu Réfrégiers · Alain Laigle · Béatrice Jollès
Gavin V. Wheeler · Laurent Chinsky

Resonance Raman analysis of a fluorescently labeled oligonucleotide forming a very stable hairpin

Received: 13 December 1996 / Accepted: 7 April 1997

Abstract An oligodeoxynucleotide has been synthesized, which mimics an "antigene" oligonucleotide with a polypyrimidic stretch on its 5′ side and is protected on its 3′ side against nucelases by a naturally forming and very stable hairpin, 5′GCGAAGC3′. The in vitro degradation of the resulting oligonucleotide d(5′TTCTCGCGAAGC3′) has already been studied by fluorescence resonance energy transfer (FRET) (Réfrégiers et al. 1996, J Biomol Struct Dyn 14: 365–371). The technique required the grafting of fluorophores at both ends of the oligonucleotide. In the present work we have compared the hairpin formed in the presence and in the absence of such fluorophores. This was achieved by the study of the Raman spectra (excitation at 257 nm) of the oligodeoxynucleotides H, which forms the hairpin (5′TTCTCGCGAAGC3′), and a control C (5′TTCTCCGGAAGC3′) which is unable to form the hairpin. Resonance Raman spectroscopy with 257 nm excitation greatly favors the resonance of purines and therefore the study of the 3′ part of the oligonucleotides. The difference spectrum obtained from resonance Raman spectra of C and H showed marker peaks specific for hairpin formation. The search for these marker peaks in difference spectra involving the Raman spectrum of H labeled by fluorophores and either C or H proved that the fluorophores do not modify the structure of the hairpin but only the vibrations of the two terminal bases on which the fluorophores are grafted. The use of such labeling is then justified in order to allow oligonucleotides protected by a hairpin on their 3′ side to be studied by fluorescence spectroscopy.

Key words Antigene oligonucleotide · Hairpin · Resonance Raman spectroscopy

M. Réfrégiers · A. Laigle (✉) · B. Jollès · G. V. Wheeler
L. Chinsky
L.P.B.C. (CNRS URA 2056), Université Pierre et Marie Curie,
4 Place Jussieu, Case 138, F-75252 Paris Cedex 05, France
(Fax: 33 1 44 27 75 60; e-mail: laigle@lpbc.jussieu.fr)

Introduction

Oligonucleotides complementary to a specific DNA sequence (antigene strategy) or mRNA sequence (antisense strategy) are used to regulate gene expression (Agrawal 1992; Cohen 1991; Rapaport et al. 1992; Wickstrom 1992). A major limitation in their therapeutic use is their nuclease susceptibility (Crooke 1992; Wickstrom 1992). In serum and cells, the degradation is primarily due to 3′ exonucleases (Crooke 1992) and various modifications of oligonucleotides at their 3′-end reduce this problem (Agrawal and Goodchild 1987; Shaw et al. 1991; Zendegui et al. 1992). In recent years, it has been found that a set of short DNA fragments form extraordinarily stable hairpins with regard to thermal denaturation and nuclease degradation (Hirao et al. 1989, 1992, 1994; Khan and Coulson 1993; Poddevin et al. 1994; Tang et al. 1993; Varani 1995). The shortest oligonucleotide d(5′GCGAAGC3′) forms one of the most stable structures (Hirao et al. 1992, 1994; Yoshizawa et al. 1994). It is stabilized by two G-C Watson-Crick pairings and an atypical A-G base pair (Crooke 1996) (Figs. 1 a, b, [b according to Hirao et al. 1994]). We develop in our laboratory antigene and antisense strategies using oligonucleotides protected by such a hairpin. Moreover, for the purpose of following the degradation of the oligonucleotides in serum or inside cells by Fluorescence Resonance Energy Transfer (FRET), we added fluorophores at both ends of the oligonucleotides. Therefore, it is important to verify that the presence of these fluorophores does not perturb the structure and the stability of the hairpin. In this study, a polypyrimidic stretch mimicking an "antigene" oligonucleotide has been added at the 5′-end of the hairpin 5′GCGAAGC3′ or of the mixed structure 5′CGGAAGC3′. Tetramethylrhodamine was then grafted at the 5′-end and fluorescein at the 3′-end of the oligonucleotide.

Fluorescein does not hinder the formation of a hairpin on the 3′-end of d(5′TTCTCGCGAAGC3′) (Réfrégiers et al. 1996). However, it is important to determine whether the structure of the hairpin is the same with and without

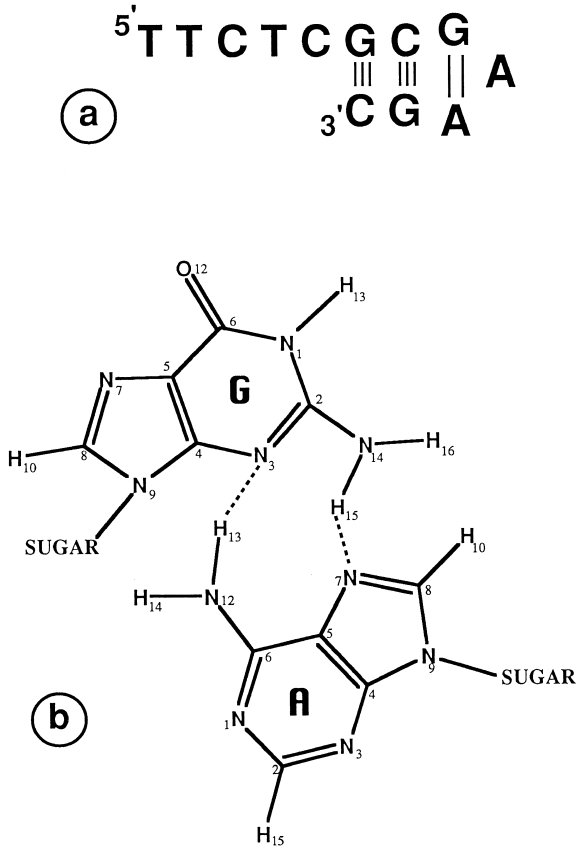

Fig. 1 **a** Schematic representation of the hairpin 5′TTCTCGCGAAGC. **b** The guanosine-adenosine atypical base pair (according to Hirao et al. 1994)

fluorophores. Resonance Raman spectroscopy (excitation wavelength = 257 nm) allows such a study. This technique is particularly well suited here since the 257 nm excitation will largely enhance the resonance of purines compared to pyrimidines and will therefore favor measurements of the vibrations of the bases on the 3′ side of the oligonucleotides which may form a hairpin.

Materials and methods

Oligonucleotides

Three oligodeoxynucleotides were synthesized by Genosys (GB): H (5′TTCTCGCGAAGC3′) containing the sequence 5′GCGAAGC3′ which forms the extraordinarily stable mini-hairpin (Hirao et al. 1992, 1994; Réfrégiers et al. 1996; Yoshizawa et al. 1994), C (5′TTCTCCGGAAGC3′) as a control which is unable to form the hairpin and $^{r}H^{f}$ which is the same as H plus tetramethylrhodamine at the 5′-end and fluorescein at the 3′-end. The oligonucleotide $^{r}H^{f}$ was purified by HPLC in order to remove unlabeled or singly labeled oligomers.

The oligonucleotides were solubilized at 10^{-2} M in a cacodylate buffer (10^{-2} M, pH 7) containing 5.10^{-2} M NaCl. Samples were heated for 20 minutes at 80 °C then slowly cooled down to 20 °C in order to favor hairpin formation (Sixou et al. 1994). The free fluorophores were solubilized in a cacodylate buffer (10^{-2} M, pH 7).

Resonance Raman spectroscopy

The samples (400 µl of solution) in a quartz cuvette thermostated at 4 °C were stirred by a micro magnetic bar (Miskovsky et al. 1989); they were excited under grazing incidence at 257 nm, produced by doubling with a non linear temperature matched crystal the 514 nm frequency of a Lexel 95-4 argon ionized laser. The exciting intensity was controlled by the splitting of 5% of the incident light onto a monitoring photomultiplier. The Raman spectra were recorded in the 400 cm^{-1} – 1800 cm^{-1} range, using a Jobin-Yvon Ramanor HG-2S double monochromator. Each spectrum was the accumulation of five consecutive scans. These scans had the same intensity, proving the absence of photodegradation, which was also checked by comparing the absorption spectra before and after Raman analysis. The spectral resolution was 2 cm^{-1}. The spectra were normalized using the intense water band at 3450 cm^{-1}; the buffer contribution was then subtracted before aberrant point elimination and fast Fourier transform smoothing (Laigle et al. 1982 a). The difference spectra were calculated using normalized spectra of components at the same concentration, 10^{-2} M. Because of the inherent noise fluctuations of the method, we only discuss the main difference peaks which appear in the 1000 cm^{-1} – 1800 cm^{-1} range.

Results and discussion

The hairpin formed at the 3′-end of d(5′TTCTCGCGAAGC3′) strongly protects this oligonucleotide (called H) against enzymatic degradation as shown by PAGE experiments (Réfrégiers et al. 1996). This is not the case for the control C (5′TTCTCCGGAAGC3′), where the inversion between a cytosine and a guanine prevents the formation of the hairpin. H has on its 3′ side the sequence 5′GCGAAGC3′ which has been shown to form an extraordinarily stable hairpin (Hirao et al. 1992, 1994; Yoshizawa et al. 1994). The faster migration (Réfrégiers et al. 1996) of H compared to C on a native polyacrylamide gel is consistent with formation of a hairpin on its 3′side.

Figure 2 presents the resonance Raman spectra of H and C as well as the difference spectrum (C – H). With 257 nm wavelength excitation, the main Raman bands (1334 cm^{-1}, 1486 cm^{-1} and 1575 cm^{-1} in the case of C) are from adenosine and guanosine. Pyrimidines have low resonance intensities and are only observable around 1250 cm^{-1} and 1650 cm^{-1} and as a shoulder around 1370 cm^{-1} (Laigle et al. 1982 b). We are therefore in a very favorable situa-

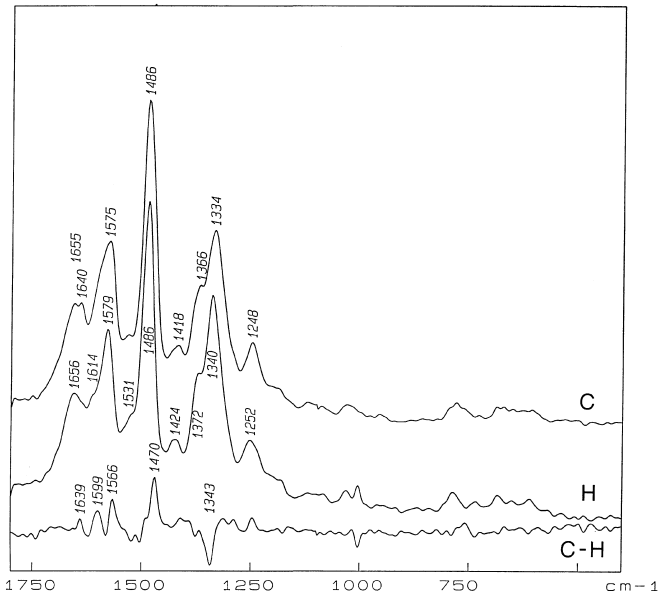

Fig. 2 Resonance Raman spectra of the oligonucleotides *C* (control 5′TTCTCCGGAAGC3′) and *H* (hairpin 5′TTCTCGCGAAGC3′) and difference spectrum (C−H), excitation wavelength = 257 nm

tion in which most of the spectrum is representative of the 3′ polypurine stretch, whether or not it forms a hairpin.

Taking into consideration only peaks more intense than the background noise, the difference spectrum between the resonance Raman spectra of C and H (C−H) shows three main positive peaks at 1470 cm^{-1}, 1566 cm^{-1} and 1599 cm^{-1} and one main negative peak at 1343 cm^{-1}. It is satisfying to notice that all these peaks can be explained in terms of vibrational modifications affecting the hairpin part of H (Fig. 1 a) as compared to C, i.e. (i) the C4−N3−C2−N1 part of guanosine and the N12−C6−C5−N7 part of adenosine which are involved in the non Watson-Crick G−A bonding (Fig. 1 b) which stabilizes the 5′GCGAAGC3′ mini-hairpin in the geometrical arrangement proposed by Hirao et al. (1994), (ii) the C6=O and N1H sites of guanosine which are involved in the Watson-Crick bonding. The numbering of atoms is the one used by Tsuboi et al. (1973).

The negative 1343 cm^{-1} peak results from (i) the shift of a Raman line from 1334 cm^{-1} to 1340 cm^{-1} between C and H, (ii) the enhancement of this line between C and H. By comparison with the spectra of the single mononucleotides (Jollès et al. 1985; Tomkova et al. 1995) we can assume that this peak is due to an adenosine modification and is assigned to C5N7 and C8N7 (Fodor et al. 1985) out of phase stretching vibrations.

The main resonance Raman band of DNA in the 1400 cm^{-1} − 1500 cm^{-1} range is located at 1486 cm^{-1} in both the C and H spectra. It corresponds to a wave of condensation and rarefaction along the long axis of the purines (Tsuboi et al. 1973). The involvement of guanine in this line could be the result of two Raman vibrations observed at 1480 cm^{-1} and 1468 cm^{-1} (Delabar and Majoube 1978). Tsuboi et al. (1973) calculated a guanosine N3C2 and N1C2 out of phase vibration at 1470 cm^{-1}. Therefore, we assume that the 1470 cm^{-1} peak appearing in the difference spectrum results from modification of the intensity of a 1470 cm^{-1} line, the main guanosine line remaining unchanged for H compared to C.

An adenosine C4C5 and C4N3 opposing stretching and a guanosine C4N3 + C5C4−N7C5 mainly contribute to the Raman line around 1580 cm^{-1} (Fodor et al. 1985). In the same range, Tsuboi described a shoulder at 1565 cm^{-1} observed both in the infrared and Raman spectra which has been assigned to the C6=O, C5−C6, and C4=C5 of guanosine (Tsuboi et al. 1973). We ourselves observed, beside a 1583 cm^{-1} line, a resonance Raman line around 1540 cm^{-1} when guanosine is methylated at N1 (Chinsky et al. 1987). Therefore, we assume that the contribution of guanosine to the line located at 1575 cm^{-1} in the case of C and at 1579 cm^{-1} in the case of H is composite. The intensity of a component in the 1540 cm^{-1} − 1570 cm^{-1} range is modified by the G−C Watson-Crick hydrogen bonds involving the guanosine's C6=O and N1H sites (Fig. 1 a). This results in the positive peak observed at 1566 cm^{-1} in the difference spectrum.

The 1599 cm^{-1} peak in the difference spectrum results from intensity modifications of a minor component in the 1580 cm^{-1} − 1620 cm^{-1} range. It could correspond to the H15N14H16 and C2N14 vibrations of a guanosine (Fodor et al. 1985). It is observed in resonance Raman in the case of concentrated guanosine at high resolution (Chinsky et al. 1987).

These first results prove that (i) the addition of the 5′ pyrimidine stretch does not hinder the formation of a structure whose vibrational analysis is consistent with the hairpin proposed by Hirao (Fig. 1), (ii) the subtraction of two resonance Raman spectra allows small structural modifications to be followed.

To assess the structure of the hairpin in $^{r}H^{f}$ (same sequence as H plus the two fluorophores), it is necessary to subtract the contributions of rhodamine and fluorescein from the Raman spectrum of $^{r}H^{f}$. Figure 3 presents the UV resonance Raman spectra of $^{r}H^{f}$, rhodamine (r) and fluorescein (f) according to their contribution in $^{r}H^{f}$. This was carried out by determining, using a least squares method, the contributions of H, r and f which allow one to reconstruct a spectrum close to that of $^{r}H^{f}$. Figure 3 also presents H′($^{r}H^{f}$−r−f) obtained after subtraction of the contribution of the fluorophores. From now, the figures will concentrate on the 1000 cm^{-1} − 1800 cm^{-1} range since no significant lines appear in the region below 1000 cm^{-1} (see Fig. 2). The spectrum H′ looks like that of H (Fig. 2): (i) The purine band vibration located at 1334 cm^{-1} in the case of C (Fig. 2) is shifted to 1340 cm^{-1} in both the H and H′ spectra. (ii) In the 1550 cm^{-1} − 1660 cm^{-1} region, H′ shows a set of three lines as in the spectrum of H. The 1615 cm^{-1} band of the H′ spectrum is higher than the 1614 cm^{-1} band of the H spectrum but remains distinct from the 1586 cm^{-1} line (1579 cm^{-1} for the H spectrum) in contrast to what is observed in the C spectrum (a single line at 1575 cm^{-1}). Moreover, in the H′ spectrum, there is a single 1654 cm^{-1} band comparable to the 1656 cm^{-1} line

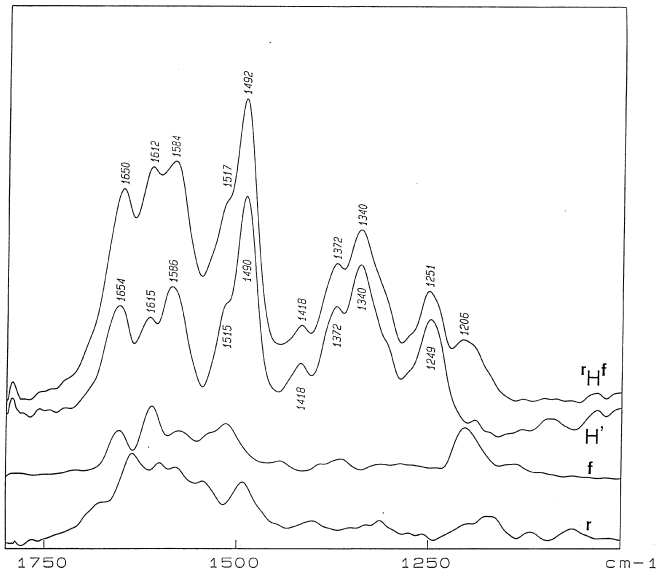

Fig. 3 Resonance Raman spectrum of ${}^{r}H^{f}$ (^{r}TTCTCGCGAAGC^{f}), calculated spectrum H' (${}^{r}H^{f}$ minus contributions of rhodamine *r* and fluorescein *f*), resonance Raman spectra of rhodamine *r* and fluorescein *f* according to their contributions in the spectrum of ${}^{r}H^{f}$, excitation wavelength = 257 nm

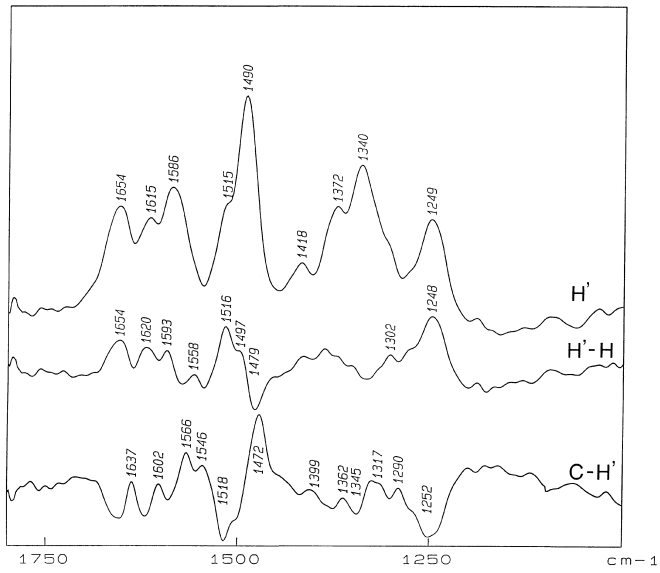

Fig. 4 H' (${}^{r}H^{f}-r-f$) and the difference spectra ($H'-H$) and ($C-H'$)

of the H spectrum but different from the doublet in the C spectrum (1655 cm^{-1} and 1640 cm^{-1}).

Further information may be obtained from the difference spectra (C−H′) (i) and (H′−H) (ii) (Fig. 4). In (C−H′), H′ plays the role of H as compared to (C−H) and in (H′−H), H′ plays the role of C.

(i) Apart from the 1518 cm^{-1} and 1252 cm^{-1} negative peaks which will be explained below, (C−H′) is comparable to (C−H): negative peak at 1345 cm^{-1} (although smaller than the 1343 cm^{-1} peak in (C−H)), positive peaks at 1472 cm^{-1}, 1566 cm^{-1}, 1602 cm^{-1} and 1637 cm^{-1} (very similar to the 1470 cm^{-1}, 1566 cm^{-1}, 1599 cm^{-1} and 1639 cm^{-1} peaks in (C−H)). Therefore, within the limits of the method we can assume that the structure of H′ is very close to that of H: the hairpin has the same structure with and without fluorophores.

(ii) In contrast, (H′−H) (where H′ plays the role of C as compared to (C−H) in Fig. 2) is quite different from (C−H): there is no negative peak around 1340 cm^{-1}, the peak around 1470 cm^{-1} is now negative, new positive peaks appear at 1497 cm^{-1} and 1516 cm^{-1} and all other positive peaks are shifted. (H′−H) shows that the structure of ${}^{r}H^{f}$ is different from that of the control C, which reinforces our previous assumption. (H′−H) has an additional significance: it shows that there are some vibrational differences between the hairpin with (H′) and without (H) fluorophores. Since the contribution of the fluorophores has already been removed in H′ (H′ = ${}^{r}H^{f}-r-f$), the difference spectrum is interpreted in terms of modifications of the base vibrations. The peak at 1248 cm^{-1} corresponds to an enhancement of the line around 1250 cm^{-1} between H and H′. This is a pyrimidine line essentially due to thymidine (Laigle et al. 1982 b). The shift of the 1486 cm^{-1} line up to 1490 cm^{-1} in H′ causes a negative peak at 1479 cm^{-1} and a positive peak at 1497 cm^{-1}. Neither purines or thymidine have vibrations around 1500 cm^{-1} but cytidine present two resonance Raman bands in this range (Fodor et al. 1985). The increase in H′ of the intensity of the first one located at 1500 cm^{-1} could cause the shift of the 1486 cm^{-1} line and the increase of the second one located near 1530 cm^{-1} could cause the positive peak at 1516 cm^{-1}. In both cases, stretching vibrations involving the N1 site of cytidine are implicated (Fodor et al. 1985; Tsuboi et al. 1973). The difference at 1593 cm^{-1} is more difficult to interpret: it seems to be the C4−C5 and C5−C6 vibrations of a cytidine coupled to a NH2 scissoring vibration (Tsuboi et al. 1973). The difference at 1620 cm^{-1} is due to the bending of the NH2 of a cytidine (Tsuboi et al. 1973). Finally, the difference at 1654 cm^{-1} results from the strong enhancement of a thymidine line corresponding to a C4=O−C4C5 vibration (Jollès et al. 1985; Tomkova et al. 1995).

All differences between the spectra of H and H′ can be assigned to vibrational modifications of thymidine and cytidine. It seems logical to consider that these vibrational changes are only related to chemical bonding of fluorescein and rhodamine to cytosine and thymine and concern the first thymine on which rhodamine is grafted and the last cytosine on which fluorescein is attached.

This looks as if the labeling of cytosine and thymine increases their resonance with 257 nm excitation. For instance, the 1249 cm^{-1} line in H′ has a resonance Raman intensity similar to that of purine lines. Moreover, the 1516 cm^{-1} line in the difference spectrum H′−H (which has been assigned to the intensity increase of a cytosine line usually not observable in resonance Raman with 257 nm excitation wavelength) is now observable in the H′ spectrum before subtraction.

The enhancement of the lines at 1248 cm^{-1} and 1516 cm^{-1} from H to H′, which is due to the attachment of fluorophores at the ends of the oligonucleotide, is consequently the cause of the positive peaks at 1516 cm^{-1} and 1248 cm^{-1} in (H′–H) as well as of the negative peaks at 1518 cm^{-1} and 1252 cm^{-1} in (C–H′). This reinforces the similarity between (C–H′) and (C–H).

In conclusion, we determined four resonance Raman marker bands for the non Watson-Crick base pair stabilizing the 5′TTCTCGCGAAGC3′ hairpin (1343 cm^{-1}, 1470 cm^{-1}, 1566 cm^{-1} and 1599 cm^{-1}). The observation of these marker bands proved that the attachment of fluorophores at both ends of the oligonucleotide does not modify the structure of the hairpin which forms itself; it only modifies the vibrations of the two end bases. The use of such attached fluorophores is then justified in order to allow the study of oligonucleotides by fluorescence spectroscopy.

References

Agrawal S (1992) Antisense oligonucleotides as antiviral agents. Trends Biotech 10:152–158

Agrawal S, Goodchild J (1987) Oligodeoxynucleosides methylphosphonates: Synthesis and enzymic degradation. Tetrahedron Lett 6:3539–3542

Chinsky L, Jolles B, Laigle A, Turpin P (1987) Resonance Raman studies of guanine derivatives. J Raman Spectrosc 18:195–198

Cohen JS (1991) Oligonucleotides as therapeutic agents. Pharmacol Ther 52:211–225

Crooke ST (1992) Therapeutic applications of oligonucleotides. Annu Rev Pharmacol Toxicol 32:329–376

Crooke ST (1996) Progress in antisense therapeutics. Med Res Rev 16:319–344

Delabar JM, Majoube M (1978) Infrared and Raman spectroscopic study of 15N and D-substituted guanines. Spectrochim Acta 34 A:129–140

Fodor SP, Rava RP, Hays TR, Spiro TG (1985) Ultraviolet resonance Raman spectroscopy of the nucleotides with 266-, 240-, 218-, and 200-nm pulsed laser excitation. J Am Chem Soc 107:1520–1529

Hirao I, Nishimura Y, Naraoka T, Watanabe K, Arata T, Miura K (1989) Extraordinary stable structure of short single-stranded DNA containing a specific base sequence: d(GCGAAAGC). Nucleic Acids Res 17:2223–2231

Hirao I, Nishimura Y, Tagawa Y, Watanabe K, Miura K (1992) Extraordinarily stable mini-hairpins – Electrophoretical and thermal properties of the various sequence variants of d(GCGAAAGC) and their effect on DNA sequencing. Nucleic Acids Res 20:3891–3896

Hirao I, Kawai G, Yoshizawa S, Nishimura Y, Ishido Y, Watanabe K, Miura K (1994) Most compact hairpin-turn structure exerted by a short DNA fragment, D(GCGAAGC) in solution – An extraordinarily stable structure resistant to nucleases and heat. Nucleic Acids Res 22:576–582

Jollès B, Laigle A, Chinsky L, Turpin P-Y (1985) The poly dA strand of poly dA.poly dT adopts an A-form in solution: a UV resonance Raman study. Nucleic Acids Res 13:2075–2085

Khan IM, Coulson JM (1993) A novel method to stabilise antisense oligonucleotides against exonuclease degradation. Nucleic Acids Res 21:2957–2958

Laigle A, Chinsky L, Turpin PY (1982a) Resonance Raman spectroscopy of biomolecules in low concentrated solution: signal/noise improvement and background rejection by a fast Fourier transform treatment. Stud Biophys 89:99–101

Laigle A, Chinsky L, Turpin PY (1982b) Recognition of base pairs by polar peptides in double stranded DNA. Nucleic Acids Res 10:1707–1720

Miskovsky P, Chinsky L, Laigle A, Turpin P (1989) The Z-conformation of Poly(dA-dT). Poly(dA-dT) in solution as studied by ultraviolet resonance Raman spectroscopy. J Biomol Struct Dyn 7:623–637

Poddevin B, Meguenni S, Elias I, Vasseur M, Blumenfeld M (1994) Improved anti-herpes simplex virus type I activity of a phosphodiester antisense oligonucleotide containing a 3′-terminal hairpin-like structure. Antisense Res Dev 4:147–154

Rapaport E, Misiura K, Agrawal S, Zamecnik PC (1992) Antimalarial activities of oligodeoxynucleotide phosphorothioates in chloroquine-resistant Plasmodium falciparum. Proc Nat Acad Sci USA 89:8577–8580

Réfrégiers M, Laigle A, Jollès B, Chinsky L (1996) Fluorescence resonance energy transfer analysis of the degradation of an oligonucleotide protected by a very stable hairpin. J Biomol Struct Dyn 14:365–371

Shaw JP, Kent K, Bird J, Fishback J, Froehler B (1991) Modified deoxyoligonucleotides stable to exonuclease degradation in serum. Nucleic Acids Res 19:747–750

Sixou S, Szoka FC, Green GA, Giusti B, Zon G, Chin DJ (1994) Intracellular oligonucleotide hybridization detected by fluorescence resonance energy transfer (Fret). Nucleic Acids Res 22: 662–668

Tang JY, Temsami J, Agrawal S (1993) Self-stabilized antisense oligodeoxynucleotide phosphorothionate: properties and anti-HIV activity. Nucleic Acids Res 21:2729–2735

Tomkova A, Miskovsky P, Chinsky L, Turpin P-Y (1995) UV Resonance Raman contribution to structure determination of the X form of double-stranded (poly(dA-dT). J Mol Struct 344:11–20

Tsuboi M, Takahashi S, Harada I (1973) Infrared and Raman spectra of nucleic acids – vibrations in bases-residues, vol 2. Academic Press, New-York

Varani G (1995) Exceptionally stable nucleic acid hairpins. Annu Rev Biophys Biomol Struc 24:379–404

Wickstrom E (1992) Strategies for administering targeted therapeutic oligodeoxynucleotides. Trends Biotech 10:281–287

Yoshizawa S, Ueda T, Ishido Y, Miura K, Watanabe K, Hirao I (1994) Nuclease resistance of an extraordinarily thermostable mini-hairpin DNA fragment, d(GCGAAGC) and its application to in vitro protein synthesis. Nucleic Acids Res 22:2217–2221

Zendegui JG, Vasquez KM, Tinsley JH, Kessler DJ, Hogen ME (1992) In vivo stability and kinetics of absorption and disposition of 3′ phosphopropyl amine oligonucleotides. Nucleic Acids Res 20:307–314