Branched oligonucleotide-intercalator conjugate forming a parallel stranded structure inhibits HIV-1 integrase

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Abstract Integration of a DNA copy of the HIV-1 genome into chromosomal DNA of infected cells is a key step of viral replication. Integration is carried out by integrase, a viral protein which binds to both ends of viral DNA and catalyses reactions of the 3′-end processing and strand transfer. A 3′-3′ branched oligonucleotide functionalised by the intercalator oxazolopyridocarbazole at each 5′-end was found to inhibit integration in vitro. We show that both a specific (G,A) sequence and the OPC intercalating agent contribute to the capability of the branched oligonucleotide to form a parallel stranded structure responsible for the inhibition.
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Key words: Integration; HIV-1; Parallel stranded DNA; Oxazolopyridocarbazole; Branched oligonucleotide

1. Introduction

Integration of a double-stranded DNA copy of the human immunodeficiency virus (HIV)-1 genome into chromosomal DNA of newly infected cells is an essential step of the viral replicative cycle. Integration is carried out by a viral *pol*-encoded protein, the integrase (IN). This enzyme has been shown to be obligatory for HIV replication [1,2] and it has no known cellular counterpart. IN is therefore an attractive target for new selective anti-HIV therapies. Integrase directs two distinct reactions: (1) the 3′-processing of the linear reverse-transcribed DNA, removing a dinucleotide from each 3′-end and (2) the strand transfer, a one step transesterification which involves the nucleophilic attack of an internucleotidic phosphate of target DNA by the 3′-OH of the trimmed 3′-end, resulting in the covalent joining of donor into acceptor DNA [3]. These two reactions can be mimicked in vitro by using purified recombinant integrase [4] and oligonucleotide substrates. Development of these in vitro assays made the identification possible of several classes of integrase inhibitors [5,6]. They can be classified into three major different groups. The first group includes polyanionic compounds such as suramin which bind to the positively charged C-terminal domain of IN [7]. The second one gathers polyhydroxylated aromatic compounds, most of them possessing a catechol function,

which bind to the catalytic domain of IN (for a review see [2]). The third family comprises oligonucleotides which are the most potent inhibitors in vitro identified to date [8]. Two types of oligonucleotides inhibitors were reported: (1) tetrad forming oligonucleotides [9,10] and (2) triplex forming oligonucleotides (TFOs) [11,12]. Mechanisms of integration inhibition by these oligonucleotides are distinct: TFOs inhibit integration through the formation of a short triple helix on either U5 or U3 viral DNA extremities whereas tetrad-forming oligonucleotides exhibit a strong affinity for integrase itself due to their G-quadruplex structure. In both cases, IN binding to its cognate sequence is prevented and integration is therefore impaired.

In the present paper, we report the synthesis of a new bifunctionalised oligonucleotide. This conjugate is a 3′-3′ branched oligonucleotide with both 5′-extremities substituted by the oxazolopyridocarbazole (OPC) intercalating agent. Evidence that it inhibits integrase in vitro is presented. Moreover, as shown by UV and circular dichroism (CD) studies, this compound adopts a parallel duplex structure, which appears to be responsible for its specific inhibitory properties. Thus, parallel duplex forming oligonucleotides can be considered as a novel subgroup of the oligonucleotide family of integrase inhibitors.

2. Materials and methods

2.1. Synthesis of 3′-3′-branched oligodeoxyribonucleotides containing OPC

3′-3′-Branched oligonucleotides were synthesised on a modified polymer support (Fig. 1). To produce this support the 5′-monomethoxytrityl derivative of thymidine triazolide (660 mg, 1 mmol) synthesised as described in [13] was suspended with 4.5 g of CPG-500 polymer (100–200 mesh) in 30 ml of absolute pyridine and this mixture was then evaporated to oil. The oil was dissolved in 10 ml of absolute pyridine, then 2,4,6-triisopropylbenzenesulphonamide (1.5 g, 5 mmol) and *N*-methylimidazole (0.75 ml, 10 mmol) were added and the reaction mixture was degassed and kept for 2 h. The polymer was washed by acetonitrile (3 ml), treated with a 5% solution of 1-aminohexanol in acetonitrile for 10–15 min and then washed by 3 ml of acetonitrile. The derivative containing the unprotected hydroxyl function was blocked by the levulinyl protecting group. Loading of the modified nucleoside residue into the polymer was at a rate of 30 μmol/g.

The polymer (30 mg) was placed in a dismantled column to synthesise 3′-3′ branched oligonucleotides and the assembly began with the synthesis of sequences: 5′-GGAAAA-3′-(T*) for (GA and GA-OPC₂) and 5′-AAGGAA-3′-(T*) for (cGA and cGA-OPC₂), where T* indicates the modified thymidine residue attached to the CPG support (Fig. 1). After removal of the trityl group the column was off-supported from the synthesiser, washed by anhydrous *N,N*-dimethylformamide (DMF) (3×1 ml) and the 5′-hydroxyl was activated by the treatment with a solution of 120 mg of bis-(*N*-succinimidyl)-carbonate and 6 mg of 4-dimethylaminopyridine in 0.8 ml of anhy-

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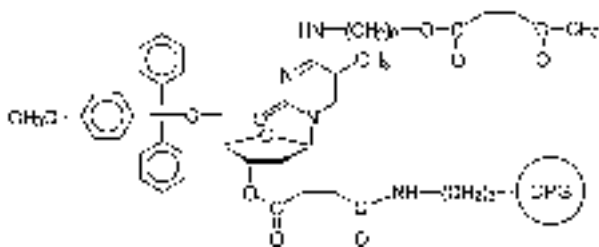E-mail: jfm@igr.fr

Fig. 1. Structure of CPG support containing a residue of modified thymidine used to synthesise 3′-3′-branched oligonucleotides.

drous DMF for 2 h. Then the polymer was washed by DMF (3×1 ml) and treated with a solution of 8 mg of amino containing OPC derivative (OPC-NH₂) prepared as previously described [14] in the mixture of 0.4 ml of DMF, 0.2 ml of pyridine, 0.1 ml of water and 0.035 ml of triethylamine at 50°C for 2 h. After this incubation the polymer was washed by DMF (5×1 ml), methanol (5×1 ml) and dried. The 5′-hydroxyl groups which did not react with OPC-NH₂ were then acetylated. The levulinyl protecting group was removed by treatment with 0.5 M hydrazine hydrate in pyridine/acetic acid (4:1) (5 ml) for 15 min. The column was again inserted into the device and synthesis of the second fragment of the oligonucleotides (GA-OPC₂ and cGA-OPC₂) was carried out as during routine procedures. After removal of the trityl group the column was off-supported from the synthesiser and coupling of the second OPC group was carried out as described above. After deprotection, the oligonucleotides were purified by RP HPLC in the acetonitrile gradient (0–40%) in 0.1 M ammonium acetate containing 5% of methanol.

Precise sequences of compounds are described in Fig. 2. Concentrations of conjugates were determined spectrophotometrically; the extinction coefficients for GA-OPC₂ and cGA-OPC₂ (ε_{260} = 237000 and 240300, respectively) and for GA and cGA (ε_{260} = 117000 and 120300, respectively) were used.

2.2. Assays for integrase activity

Different assays were carried out to investigate the effect of the conjugates on integration in vitro: (1) 3′-end processing of a 21-bp duplex, (2) strand transfer using a 21-bp duplex as both DNA substrate and target, and (3) strand transfer of a 492-bp mini-viral DNA into a plasmid target [15].

Sequences of the oligonucleotides (obtained from Eurogentec) used as DNA substrates are described in Fig. 2. All oligonucleotides were first purified on a 18% polyacrylamide/7 M urea gel. Oligonucleotides U5B, U3B, U5MB and U5B-2 were labelled at their 5′-end by T4 polynucleotide kinase (New England Biolabs) using [γ³²P]ATP (sp. act. 3000 Ci/mol NEN).

Two different integrase activities were assayed with the following substrates:

(1) 3′-End processing. 21-mer duplexes U5B/A and U3B/A corresponding to the sequences of U5 or U3 ends of the HIV-1 long terminal repeats (LTR), respectively, were used as DNA substrates. They were prepared by annealing ³²P-labelled U5B, U5MB or U3B with their complementary strand, respectively U5A, U5MA or U3A in 100 mM NaCl and resulting duplexes were finally desalted on a ChromaSpin-10 column (Clontech).

(2) Strand transfer. (a) Homologous integration into a short duplex. To assay only the strand transfer, a pre-processed substrate U5B-2/A corresponding to the viral U5 LTR end after 3′-end processing was used. The ³²P-labelled oligonucleotide U5B-2 was annealed to U5A and desalted as described above. The same duplex represents both donor and acceptor DNA. (b) Heterologous integration into a long DNA fragment. A 492-bp U3U5 DNA which is generated by NdeI restriction of the pU3U5 vector as previously described [15] was used as donor substrate. Plasmid pSP70 was used as target DNA.

Recombinant integrase protein was a generous gift of Rhône-Poulenc-Rorer and was purified as described [16]. The same reaction conditions were used for both 3′-processing and strand transfer. Different amounts of oligonucleotides (GA, cGA, GA-OPC₂, cGA-OPC₂) were incubated in 20 μl with 0.3 pmol of the corresponding ³²P-labelled DNA substrate in the presence of 1 pmol of integrase in buffer containing 20 mM Tris, pH 7.5, 30 mM NaCl, 0.5 mM spermine (Sigma), 10% glycerol, 10 mM DTT, 0.01% NP40, supplemented

with 8 mM MnCl₂ and 2 mM MgCl₂ at 30°C. For heterologous integration assay, 40 ng pSP70 vector was also added in the reaction mixture. Reaction was stopped by adding 80 μl of a stop solution (0.3 M sodium acetate, pH 7.5) and the DNA fragments were phenol-purified and precipitated with ethanol. For 3′-end processing and homologous strand transfer, DNA fragments were suspended in 7 M urea and separated on a 18% polyacrylamide/7 M urea gel. For heterologous strand transfer, DNA fragments were dissolved in 5% glycerol and separated on a 1% agarose gel. Finally, gels were analysed on a STORM 840™ phosphorimager (Molecular Dynamics). IC_{50} values were calculated with Image QuaNT™ 4.1 software.

2.3. Assay for integrase binding to its substrate

The same reaction mixtures prepared for integrase activity assay were also used for the integrase binding assay. They were incubated at 30°C for 15 min, at room temperature for 15 more minutes and finally incubated at 4°C before being loaded on a 8% non-denaturating acrylamide gel containing 0.2% glycerol. Electrophoresis was performed with a running buffer containing 50 mM Tris-Borate pH 7.5, 1 mM EDTA for 2 h at 4°C (5.6 V/cm). Analysis and quantification were performed on Molecular Dynamics STORM 840 phosphorimager.

2.4. UV absorption spectroscopy

Thermal denaturation profiles were performed with 1.5 μM oligonucleotide-OPC conjugates in 10 mM Tris-HCl buffer at pH 7.5, 10 mM MgCl₂ and 2.5 mM spermine. Absorbance versus temperature curves were recorded on a Uvikon 933 spectrophotometer. The temperature of the bath was increased or decreased at a rate of 0.3°C/min from 8 to 68°C by a Neslab RTE Bath/Circulator.

2.5. Circular dichroism (CD)

CD spectra were recorded on a modified dichrograph Mark V. Samples were placed in a quartz cell (200 μl, 1 mm path length) thermostated at various temperatures. In typical experiments, 40 μM of conjugates was dissolved in 20 mM Tris, pH 7,0 10 mM MgCl₂, 2 mM MnCl₂ and 2.5 mM spermine.

3. Results and discussion

3.1. Inhibition of HIV-1 Integration

Anti-integrase effect of conjugates GA-OPC₂, cGA-OPC₂, GA and cGA (see Fig. 2 for structures) were initially investigated in two different assays which measure the extent of 3′-processing and strand transfer using either a blunt-ended 21-mer duplex U5B/A or a 3′-processed duplex U5B-2/A, respec-
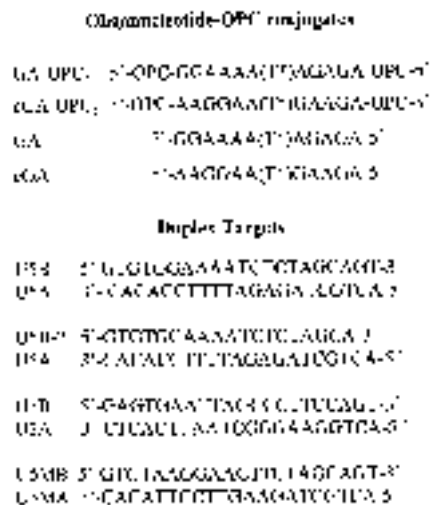

Fig. 2. Oligonucleotide-OPC conjugates and duplex target sequences. U5B/A and U5B-2/A mimic the U5 LTR end, U3B/A the U3 LTR and U5MB/A a mutated U5 LTR.
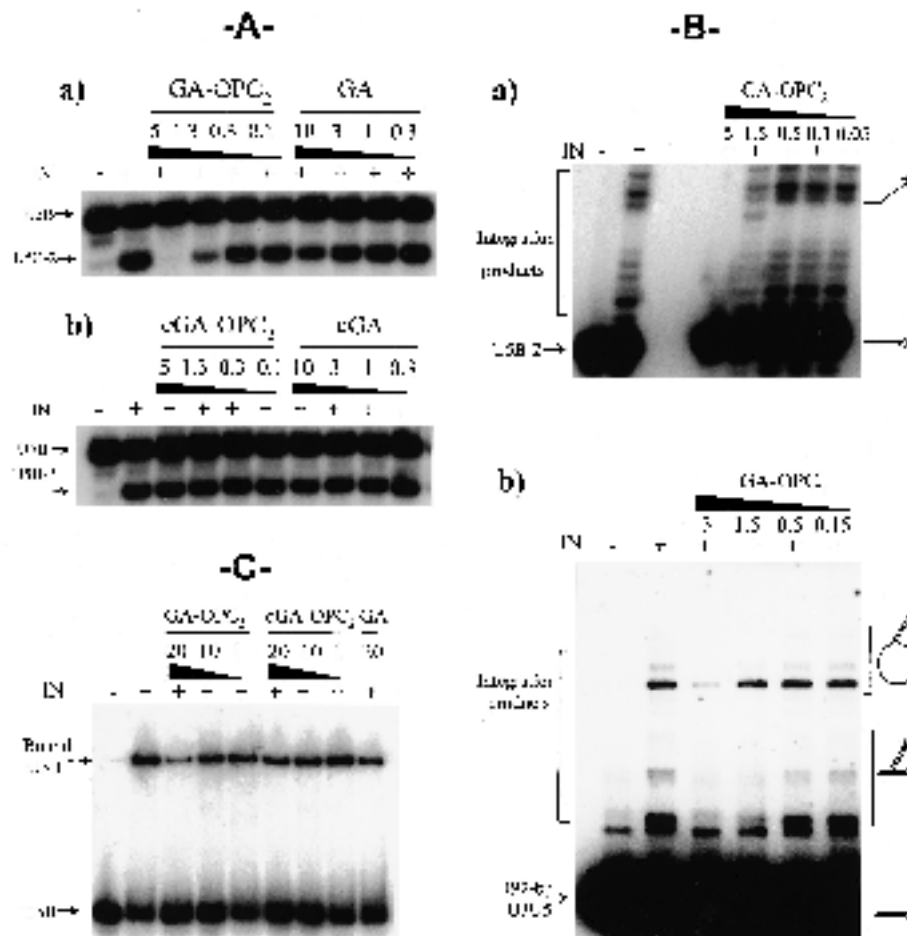
Fig. 3. Activity and binding assay of IN in presence of oligonucleotide-OPC conjugates. Corresponding concentrations are shown in µM. A: 3′-Processing assay on U5B/A target in the presence of GA-OPC$_2$ and GA (a) or cGA-OPC$_2$ and cGA (b). B: Strand transfer assay in the presence of GA-OPC$_2$ on U5B-2/A target (a) and on the long U3U5 fragment (b). C: Integrase binding on its target U5 in the presence of GA-OPC$_2$, cGA-OPC$_2$ and GA.

tively. The 3′-processing reaction liberates the 3′-terminal dinucleotide, producing a 19-mer oligonucleotide (U5B-2) from a 21-mer substrate (U5B). The strand transfer reaction is a transesterification resulting in the insertion of one 3′-processed oligonucleotide into another oligonucleotide, yielding higher molecular weight products with slower migration than the 19-mer U5B-2 on acrylamide gel. As shown on Fig. 3Aa (left) and Ba, the conjugate GA-OPC$_2$ exhibited a significant inhibition of both 3′-processing and strand transfer with an IC_{50} of roughly 1.5µM. The same inhibition efficiency was also observed for the heterologous transfer of the ^{32}P-labelled 492-bp U3U5 DNA fragment into the plasmid pSP70 (Fig. 3Bb). In sharp contrast, the oligonucleotide GA alone without OPC inhibited neither 3′-processing (Fig. 3Aa, right) nor strand transfer (data not shown). Moreover, the control conjugate cGA-OPC$_2$ having a different nucleotide sequence did not show any inhibition either (Fig. 3Ab). Thus, the inhibition effect observed happens to be both sequence specific and OPC dependent. It is worthy to note that a similar inhibition effect was observed when GA-OPC$_2$ was mixed with IN before or after addition of its substrate DNA.

3.2. Inhibition of integrase binding to its cognate substrate

To investigate the mechanism of inhibition further, we used a gel shift approach to determine the influence of the conju-

gates on the binding of IN to its substrate. The complex of integrase with its labelled substrate ^{32}P-U5B/A remained on the top of a 8% acrylamide gel running under non-denaturing conditions whereas the non-bound substrate migrated much faster (Fig. 3C). Increasing concentrations of conjugate GA-OPC$_2$ were incubated with integrase and ^{32}P-U5B/A. The amount of the ^{32}P-labelled complex characterised by a slow migration decreased as the concentration of the conjugate increased (Fig. 3c). This result strongly suggests that the specific conjugate GA-OPC$_2$ prevented the integrase fixation to its substrate. Conversely, the conjugate cGA-OPC$_2$ that showed no inhibitory activity, did not influence the integrase binding (Fig. 3C). Finally, addition of the inactive, non-conjugated oligonucleotide GA at concentrations up to 20 µM had no effect on integrase-DNA complex formation either. Altogether, these results indicate that the anti-integrase activity that was observed specifically with GA-OPC$_2$ can be ascribed to its capability of blocking IN binding to its DNA substrate.

3.3. GA-OPC adopts a parallel stranded structure

Two different mechanisms have been previously summoned to explain integrase inhibition by (G,A)- and (G,T)-containing oligonucleotides: The first one involves formation of a short triple-helix on the viral DNA extremity spanning over the

integrase binding site. This hypothesis was first considered since a molecular modelling study suggested that the branched oligonucleotide OPC-5′-GGAAAA3′-(CHOH)$_3$-3′-AGAGA-5′-OPC may form a thermodynamically stable triple helix on the U5 sequence [17]. To address that possibility, GA-OPC$_2$ was assayed on the 3′-processing of either a mutated LTR U5 extremity or the U3 DNA extremity (see Fig. 2). In both cases, the substrate oligonucleotide did not contain a sequence suitable for the triplex formation of this conjugate. Inhibition efficiency of GA-OPC$_2$ with these substrates was similar to the one found in the wild-type (wt) U5 sequence (data not shown). Thus, the GA-OPC$_2$ inhibitory activity could not be a result of a triplex formation with the U5 DNA substrate. The second mechanism is based on the capability of G-rich oligonucleotides to form G-quadruplex containing intra- or intermolecular structures. Because of their strong affinity for IN, these structured oligonucleotides prevent the enzyme from binding to its substrate. In order to detect a possible autostructure of GA-OPC, we carried out thermal denaturation experiments at 295 nm. In fact, the variation of temperature-dependent absorbance at this particular wavelength is sufficient to monitor DNA structures involving non-Watson-Crick bonds between guanine residues [18]. More interestingly, this variation is inverted as compared with the melting profile at 260 nm of a classical duplex, thus allowing the unambiguous identification of such structures. As shown in Fig. 4, a temperature increase yielded a melting profile for GA-OPC$_2$ with an absorbance decrease as high as 15%. The T_m estimated from this profile was 37.5°C. In contrast, we did not observe any melting transition at 295 nm with the three other oligonucleotides which did not inhibit integration. Therefore, the intrinsic autostructure of GA-OPC$_2$ depends both on sequence and intercalator OPC and appears to be responsible for its inhibitory effect.

Although it is widely known that G-rich oligonucleotides can form G-quadruplex structures, alternating (G,A) oligonucleotides were now shown to form preferentially parallel stranded structures [19,20]. In order to solve the nature of the autostructure unambiguously, CD studies were performed. Under similar salt conditions as integration experiments, GA-OPC$_2$ displayed a CD spectrum with a strong positive Cotton effect at 265 nm and a negative one at 240 nm (Fig. 5, curve 1). The positive band is much stronger for GA-OPC$_2$ than for the three other compounds cGA-OPC$_2$, GA and cGA. The CD spectrum of GA-OPC$_2$ corresponds to CD spectra observed for parallel duplexes formed by alternating d(G-A)
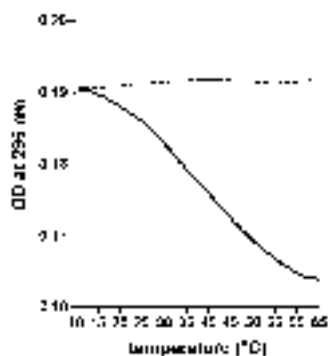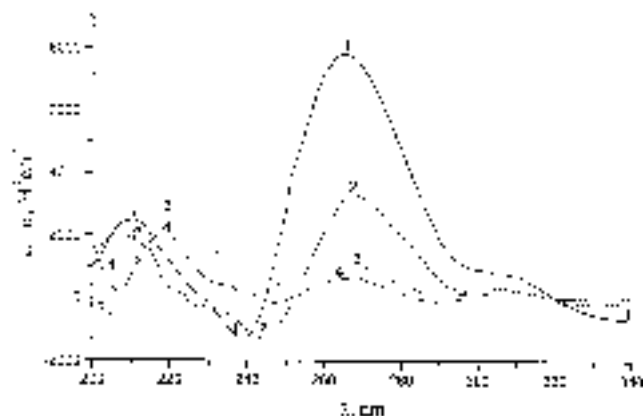

Fig. 5. CD spectrum of GA-OPC$_2$ at 22°C (1) and 65°C (2); cGA at 22°C (3) and 65°C (4).

sequences [21], [22]. Moreover, the notable absence of a positive CD peak at 295 nm shows that the structure does not contain antiparallel G-quartets. Furthermore, while quartet structures were observed in the presence of monovalent cations such as K$^+$ or Na$^+$ [23–25], these cations were not necessary to detect the GA-OPC$_2$ intermolecular structure. Finally, when the GA-OPC$_2$ conjugate was heated to 65°C, the maximum amplitude of its spectrum decreased and the positive band shifted from 265 to 270 nm (Fig. 5, curve 2) whereas cGA exhibits a maximum amplitude at 265 nm, which remained unchanged after heating (Fig. 5, curve 3 and 4). In agreement with the absence of autostructure of cGA-OPC$_2$ and GA, their respective spectra were not temperature dependent either (data not shown).

Thus, taken together, CD and UV-absorption experiments results provide a compelling evidence for the presence of an autostructure, relevant to the anti-integrase activity observed with GA-OPC$_2$, and strongly suggest the involvement of a parallel stranded duplex, stable at neutral pH and 10 mM MgCl$_2$. Both the intercalating agent OPC and a specific sequence are required for autostructure formation. OPC requirement is in good agreement with the previous report of a stabilisation of autostructure formed by 5′-end substituted d(TGGGAG) oligonucleotides [26].

Finally, to reinforce the correlation between the GA-OPC$_2$ structure and its inhibitory activity, integration experiments were carried out at a different temperature. Since a T_m of 37.5°C was obtained from melting experiments, a decrease of the inhibition efficiency should occur when the reaction temperature increased. When the integration assay was performed at 37°C instead of 30°C, we readily observed a decrease of the conjugate efficiency since IC$_{50}$ shifted from 1.5 to 5 μM. This result brings new evidence that the efficiency of integrase inhibition was dependent on the oligonucleotide autostructure.

In conclusion, specific inhibition of HIV-1 integrase by GA-OPC$_2$ versus controls led us to the conclusion that integrase is able to interact with parallel structured oligonucleotides. To our knowledge, this is the first report of an oligonucleotide capable to inhibit integrase by such a mechanism.

Fig. 4. Thermal denaturation profile at 295 nm of GA-OPC$_2$ (—) and cGA-OPC$_2$ (···).

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