Poly(L-lysine)–graft–dextran copolymer: amazing effects on triplex stabilization under physiological pH and ionic conditions (in vitro)

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ABSTRACT

Triplex DNA formation involving unmodified triplex-forming oligonucleotides (TFOs) is very unstable under physiological conditions. Here, we report a novel strategy to stabilize both purine and pyrimidine motif triplex DNA within the rat α1 (I) collagen gene promoter under physiologically relevant conditions by a poly(L-lysine)–graft–dextran copolymer. Using an in vitro electrophoretic mobility shift assay, we show that the copolymer almost completely abrogates the inhibitory effects of physiological concentrations of monovalent cations, particularly potassium ion (K+), on purine motif triplex formation involving very low concentrations of an unmodified guanine-rich TFO. Of importance, pH dependency in pyrimidine motif triplex formation involving an unmodified cytosine-rich TFO is also significantly overcome by the copolymer. Finally, the triple-stabilizing efficiency of the copolymer is remarkably higher than that of other oligocations, like spermine and spermidine. We suggest that the ability of the graft copolymer to stabilize triplex DNA under physiologically relevant pH and salt concentrations will be a cue for further progress in the antigene strategy.

INTRODUCTION

Intermolecular triplex DNA formation by sequence-specific interaction of TFOs with the major groove of a short homopurine–homopyrimidine stretch in native duplex DNA can be a designed strategy (i.e. the antigene strategy) to create an artificial gene repressor for manipulation of gene expression, gene-targeted mutagenesis and inhibition of viral propagation (1–4). However, triplexes of either the purine motif (5,6) or the pyrimidine motif (4,7) are unstable under physiological conditions and thus impede their therapeutic application. For example, formation of pyrimidine motif triplex DNA needs conditions of low pH (pH < 6.0), because unmodified cytosine residues, if present in pyrimidine-rich TFOs, must be protonated to bind with the guanine (G) of the G:C duplex (4,8). In contrast, although the purine motif triplex DNA is pH-independent, triplexes involving guanine-rich (G-rich) TFOs are severely inhibited by physiological concentrations of certain monovalent cations (M+), especially K+ (9,10). To date, numerous strategies, such as modification or substitution of cytosines in pyrimidine-rich TFOs with non-natural bases and/or conjugation of triplex-specific or non-specific DNA intercalators with TFOs, have been reported to improve triplex stability through pH-independent triplex formation over that of regular TFOs (11–13). On the other hand, a chemical modification strategy with G-rich TFOs has partially overcome the inhibitory effect of K+ on purine motif triplex formation (14–19). Despite extensive efforts, significant stabilization of triplex DNA under physiological conditions is, however, yet to be achieved. We previously reported that a poly(L-lysine)–graft–dextran (PLL–g–Dex) copolymer (Fig. 1a), which hereafter will be designated the copolymer, significantly increased the thermal stability of the triplex structure with polynucleotides poly(dA)·2poly(dT) (20). Since triplexes with these synthetic polynucleotides do not contain any K+-sensitive G·G:C or pH-dependent C+·G:C base triplets (8–19), it is crucial to understand the triplex-stabilizing potency of the copolymer when such triplets are formed within a short target duplex of cellular DNA. To address this vital issue, here we report the effects of the copolymer on triplex formation in vitro using a 30 bp homopurine–homopyrimidine stretch (located between −141 and −170 bp) of the rat α1 (I) collagen gene promoter (21) as a target duplex and its specific purine- and pyrimidine-rich TFOs (Fig. 1b). Results from electrophoretic mobility shift assays (EMSAs) have revealed that the copolymer remarkably stabilizes purine motif triplex DNA, overcoming K+ inhibition almost up to 200 mM. The copolymer also stabilizes pyrimidine motif triplex DNA at pH 7.0, the physiologically relevant pH, involving very low concentrations of unmodified TFO. Finally, the triplex-stabilizing efficiency of the copolymer is extraordinarily higher than that of physiological concentrations of spermine and spermidine. The triplex-stabilizing ability of the copolymer under physiologically relevant conditions may provide a new research tool and will eventually enhance the possible in vivo applications of the antigene strategy for gene therapy.

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according to the manual instructions and freeze dried samples were finally dissolved in 10 mM Tris–acetate (pH 5.5 or 7.0). Calf thymus DNA (t-DNA) was obtained from Sigma-Aldrich Japan. Calf t-DNA (2 mg/ml) in TE (10 mM Tris–HCl, pH 8.0, and 1.0 mM EDTA) was sonicated, ethanol precipitated, dried and resuspended in H₂O (10 mg/ml) or in 500 mM Tris–acetate, pH 5.0 (6 mg/ml). t-DNA in H₂O was diluted (6 mg/ml) and added to the reactions without changing the buffer and salt concentrations of the reactions.

Preparation of the copolymer

Preparation of the PLL–g–Dex copolymer was described in detail previously (20,22). Briefly, the copolymer (Mn 2.5 × 10⁵, as free salt) was prepared by a reductive amination reaction between PLL·HBr (Mw = 4.5 × 10⁴; Peptide Institute Inc., Osaka, Japan) and Dextran T-10 (Mn = 5900; Pharmacia Biotech., Uppsala, Sweden) and added in all reactions at a copolymer/DNA charge ratio of 2:1 as described (20).

Electrophoretic mobility shift assays (EMSA)

All TFOs were heated at 65°C for 10 min to prevent self-aggregation and then quickly cooled on ice. Triplex formation was initiated by addition (in order) of 3 µl 3x buffer [135 mM Tris–acetate, pH 5.5 or 7.0, 30 mM MgCl₂ (buffer A) or containing 300 mM NaCl (buffer B)], 2 µl labeled duplex DNA (50 000 c.p.m., ~3 ng), 0–2 µl H₂O containing either KCl, graft copolymer or oligocations, 0–2 µl specific TFOs and 1–2 µl control TFO as carrier DNA to adjust to equimolar concentrations of TFO (specific TFOs + control TFO) in a final 9 µl reaction volume. Oligocations (sperrmine and spermidine) were obtained from Sigma-Aldrich Japan, diluted in H₂O and added to the reactions at the indicated final concentrations. At 1.0 mM spermine, the spermine/DNA ([amino groups]/[phosphate groups] [DNA]) charge ratio was 40 and it was >30 for spermidine. The buffer solutions were altered to examine triplex formation under various salt and specific TFO concentrations. To equilibrate triplex formation, reaction mixtures were incubated at 37°C for 6 h, then 2 µl 50% glycerol solution containing bromphenol blue were added and samples were directly loaded onto a 15% native polyacrylamide gel, prepared in buffer (50 mM Tris–acetate, pH 5.5 or 7.0, and 10 mM MgCl₂). If the copolymer or the oligocations were added to reactions during incubation, 1 µl respective reaction buffer with or without t-DNA (6 µg) was added before electrophoresis without changing (unless otherwise indicated) the buffer and salt concentrations. In some experiments, TFO alone or along with the copolymer and t-DNA were mixed with the target duplex at 4°C just before electrophoresis. Electrophoresis was performed at 8 V/cm for 16 h at 4°C in buffer (50 mM Tris–acetate, pH 5.5 or 7.0, and 10 mM MgCl₂) to confirm that the triplexes, either in the presence or absence of copolymer, were formed during incubation at 37°C but not during electrophoresis (data not shown, see Fig. 5). The dried gels were then exposed to Kodak Biomax film at ~80°C. Percent triplex formed and apparent dissociation constant (Kd) of triplex formation were basically calculated as described previously (17). Briefly, the amount of radioactivity present in the duplex and triplex forms was determined with the Ambis System. The percentage of the target duplex bound by specific TFOs was calculated using the following equation:

\[ \text{percentage of bound} = \frac{A_{\text{duplex}}}{A_{\text{duplex}} + A_{\text{triplex}}} \times 100 \]

where \( A_{\text{duplex}} \) and \( A_{\text{triplex}} \) are the radioactivities of the duplex and the triplex, respectively.

MATERIALS AND METHODS

Oligonucleotides and thymus DNA

Oligodeoxynucleotides (ODNs) for target duplex DNA (T-1 and T-2) and the pyrimidine-rich TFO (Py) were purchased from Bio Source International and ODNs for purine-rich (Pu) and control (C) TFOs were from Grainers Japan Co. (Tokyo, Japan). All ODNs were further purified by gel electrophoresis on a 15% denaturing polyacrylamide gel, dissolved in 10 mM Tris–acetate (pH 5.5 or 7.0). After quantitation by UV spectroscopy, ODNs were stored at –30°C (pH 5.5 or 7.0). After quantitation by UV spectroscopy, ODNs were stored at –30°C. T-2 was end-labeled with [γ-32P]ATP (Amersham) and T4 polynucleotide kinase, purified by phenol/chloroform extraction and ethanol precipitation and then mixed with 1.2-fold molar excess of T-1 to prepare the labeled duplex. Labeled duplex free from unincorporated [γ-32P]ATP was purified on a Sephadex G25 column (Pharmacia Biotech).
% triplex = S_{triplex}/(S_{triplex} + S_{duplex}) × 100

where $S_{\text{duplex}}$ and $S_{\text{triplex}}$ represent the radioactive signal for the duplex and triplex bands, respectively. The $K_d$ of triplex formation was determined from the concentration of the TFO which caused half of the target duplex to shift to the triplex form. Half-maximal inhibitory concentration (IC$_{50}$) of KCl was calculated from the concentration of KCl that reduced triplex formation by 50% of that formed without KCl.

**RESULTS**

**Inhibition of purine motif triplex formation by M$^+$**

To explore the inhibitory effects of M$^+$ on purine motif triplex DNA, triplex formation was followed in the presence or absence of M$^+$. As shown in Figure 2a, in the absence of M$^+$, Na$^+$ and K$^+$, the percentage of stable triplex DNA increased with an increase in the amount of purine-rich TFO Pu (lanes 2, 5 and 8). About 86% triplex DNA is formed (lane 2) at 0.17 µM Pu (Fig. 1b). The apparent $K_d$ was $3 \times 10^{-8}$ M. A high concentration (17.0 µM) of control TFO, C (Fig. 1b), failed to form triplex DNA (lane 1), suggesting sequence specificity in triplex formation. Figure 2a shows that Na$^+$, at concentrations 3-fold higher than physiological levels (~33 mM), caused little inhibition of triplex formation (~15%) and only at low concentrations of Pu (cf. lanes 2 and 3 with 8 and 9, respectively), while the co-presence of physiologically relevant levels of KCl (150 mM) drastically diminished triplex formation (>90%) (lane 4). This inhibitory effect of K$^+$ on triplex formation was almost the same even in the absence of NaCl (data not shown). Note that the inhibitory effect of K$^+$ on triplex formation in the presence of M$^+$ has been summarized in Figure 2b, considering triplex formation in the absence of M$^+$ to be 100%. It would be quite difficult to introduce such a high concentration (17.0 µM) of TFO (lane 10) into the cells of a target tissue for therapeutic use. Therefore, it is of paramount importance to stabilize triplex DNA while using very low concentrations of TFOs.

**Copolymer stabilizes triplex DNA to overcome K$^+$ inhibition**

To determine whether the copolymer can stabilize triplex DNA at low concentrations of TFO, *in vitro* triplex formation involving unmodified Pu was tested in the presence or absence of 100 mM KCl (Fig. 3a). Since ionic interactions between the amino groups in the polycationic backbone, poly(l-lysine) (PLL), of the copolymer and the phosphate groups in DNA impede migration of the target duplex into the gel (lane 3), the copolymer–DNA interaction is, therefore, chased by adding an excess of calf t-DNA just before electrophoresis to get the target duplex into the gel (lane 4). Addition of t-DNA has no significant effect on duplex (lanes 1 and 2) or preformed triplex DNA (lanes 8 and 9).

As shown in Figure 3a, the presence of KCl during incubation severely decreased triplex formation at both 0.05 (lanes 5 and 7) and 0.5 µM (lanes 6 and 8) Pu, while almost no inhibitory effect of K$^+$ is observed if the copolymer is added under the same conditions (cf. lanes 5 with 7 and 10 and 6 with 8 and 11, respectively). This suggests that the copolymer efficiently stabilizes triplex DNA even at low concentrations of TFO.

A similar effect of the copolymer on triplex stabilization at 0.17 µM Pu and in the presence or absence of different concentrations of KCl is presented in Figure 3b. In the absence of the copolymer, an increase in KCl concentration causes a drastic decrease in triplex formation, with an IC$_{50}$ at 60 mM KCl, while in the presence of the copolymer, an IC$_{50}$ is not observed up to 200 mM KCl. Moreover, triplex formation (71%) at 100 mM NaCl alone is further increased (80%) in the presence of the copolymer. Taken together, the results strongly demonstrate that the copolymer can stabilize purine motif triplex DNA even though the final concentrations of M$^+$ are higher than the usual intracellular levels.

**Effects of the copolymer, its constituents and other oligocations on triplex stabilization**

The presence of high ambient concentrations of oligocations (also called polyanines) like spermine and spermidine in eukaryotic nuclei (23) and a report of their ability to stabilize purine motif triplex DNA (24) prompted us to compare the triplex-stabilizing efficiency of the copolymer with oligocations at physiological levels of K$^+$. Before that, triplex stabilization by the copolymer had been compared with its constituents, PLL and dextran chains,
Figure 3. Effects of the copolymer on purine motif triplex formation in the presence of M⁺. (a) Copolymer overrides the inhibitory effects of M⁺. Control TFO (C) was added with labeled duplex (−3 ng) alone (lanes 1–4) or containing 0.05 µM (lanes 5, 7 and 10) or 0.5 µM (lanes 6, 8, 9 and 11) Pu for a final 5 µM concentration of TFOs (C ± Pu) in each lane. Reaction mixtures (9 µl) in buffer B (pH 7.0) were incubated for 6 h at 37°C in the presence (+) or absence (−) of KCl and the copolymer (2.5 µg; copolymer/DNA charge ratio 2). After incubation, reaction buffer (1 µl) of respective salt concentrations with (+) or without (−)-t-DNA (6 µg) was added and the samples were electrophoresed to separate the duplex (D) and triplex (T) DNAs. (b) Analysis of purine motif triplex formation in the presence or absence of the copolymer and K⁺. Triplex formation was performed under the same conditions as described in (a) except that 0.17 µM Pu was used and percent triplex formed in the presence (△) or absence (○) of the copolymer was quantitated and plotted against the indicated millimolar concentrations of KCl.

Figure 4. Effects of the copolymer, its constituents and other oligocations on purine motif triplex stabilization in the presence of M⁺. (a) Comparison of the effect of the copolymer and its constituents on triplex stabilization. Triplex formation either in the presence (+) or absence (−) of KCl (150 mM), Pu (0.17 µM), 2.5 µg copolymer (lanes 1–6) or equivalent amount of PLL (lanes 7–10) and dextran (Dex) (lanes 11 and 12) was analyzed in buffer B (pH 7.0) as described in the legend to Figure 3. The duplex (D) and triplex (T) DNAs are indicated. (b) Comparison of the triplex-stabilizing efficiency between the copolymer, spermine (Spm) and spermidine (Spmd). Reactions were carried out identically as described in (a), except that reaction mixtures were incubated with (+) or without (−) copolymer (2.5 µg) (lanes 1–6), 0.2 mM Spm (lanes 7–10) and Spmd (lanes 11–14). The duplex (D) and triplex (T) DNAs are indicated.

Stabilization of pyrimidine motif triplex DNA at neutral pH

Since the pH dependence of the pyrimidine motif triplex DNA mainly constrains its in vivo applications (4,8), we subsequently explored the effects of the copolymer on pyrimidine motif triplex stabilization at pH 7.0. About 96% stable triplex DNA was formed when 1.7 µM unmodified pyrimidine-rich TFO, Py (Fig. 1b), was incubated with the target duplex at pH 5.5 (Fig. 5a, gel at pH 5.5, lane 4) and the presence of the copolymer further increased triplex formation (100%) (lane 6). Note that triplex DNA is formed neither at pH 5.5 if the mixture of Py and the target duplex is electrophoresed without incubation (lane 7) nor if the pH of the reactions incubated at pH 7.0 is changed to pH 5.5 in the presence or absence of t-DNA at the onset of electrophoresis (lanes 8 and 9; see figure legend). Of interest is that under the latter conditions, the presence of the copolymer during incubation at pH 7.0 produced 50% of the triplex DNA observed at pH 5.5 (lanes 6 and 11). However, all the pyrimidine motif triplexes completely disappeared if the same samples were electrophoresed at pH 7.0 (Fig. 5a, gel at pH 7.0). The same conditions did not affect the purine motif triplex DNA (lane 12) and further confirm the pH-dependence and pH-independence of the pyrimidine and the purine motif triplex DNAs, respectively. We can totally exclude the possibility that the copolymer-mediated stabilization of triplex DNA at pH 7.0 (lane 11) was due to the pH change of the reaction mixture, because triplex DNA was also undetectable at pH 5.5 when Py-t-DNA and the copolymer were added together with the target duplex just before electrophoresis (Fig 5b, lane 5).

It has been reported that spermine and spermidine can also stabilize pyrimidine motif triplex DNA at neutral pH (25,26). Increasing concentrations (0.2–1.0 mM) of these oligocations
were, therefore, tested for their ability to stabilize pyrimidine motif triplex DNA under the same conditions as used in the last experiment. Figure 5b shows that high concentrations (1.0 mM) of spermine (lane 11) but not spermidine (lane 13) slightly stabilized pyrimidine motif triplex formation at neutral pH. Different amounts of control TFO were added to labeled duplex (3 ng) alone (lanes 1–3) or containing 1.7 µM Py (lanes 4–6 and 8–11) or 0.17 µM Pu (lane 12) for a final 5 µM concentration of TFOs (C ± Py/Pu). The mixtures (9 µl) in buffer B were incubated in the presence (+) or absence (−) of the copolymer (2.5 µg) at pH 5.5 (lanes 1–6) or 7.0 (lanes 8–12). After incubation, samples were processed as described in the legend to Figure 3, except that 1 µl 500 mM Tris–acetate (pH 5.0) with (+) or without (−) 1-DNA (6 µg) was added in lanes 8–11 and 5 µl of each sample were then electrophoresed in pH 5.5 (gel at pH 5.5) or 7.0 (gel at pH 7.0) electrophoretic buffer. In lane 7, the reaction proceeded identically as lane 4 but without incubation. The duplex (D) and triplex (T) DNAs are indicated. (b) Comparison of the triplex-stabilizing efficiency between the copolymer, spermine (Spm) and spermidine (Spmd). Reactions for triplex formation were carried out as described in (a), except that the reaction mixtures were incubated at pH 5.5 (lanes 1–4) or 7.0 (lanes 6–13) with (+) or without (−) copolymer (2.5 µg) (lanes 1–9) and with 0.2 or 1.0 mM Spm (lanes 10 and 11) or Spmd (lanes 12 and 13) respectively. The samples were electrophoresed at pH 5.5 to separate the duplex (D) and triplex (T) DNAs. In lane 5, the reaction was processed identically as lane 6 of (a) but without incubation.

DISCUSSION

To our knowledge, we sought the first demonstration of designing polymer materials to stabilize triplex DNA of both motifs under physiologically relevant conditions using very low concentrations of unmodified TFOs (Figs 3–5). Moreover, the copolymer at a 20-fold less stabilizer/DNA charge ratio than that of other oligocations exhibits a significantly higher efficacy in stabilizing triplex DNA, consistent with our previous observations (20). In addition, we found that the inhibitory effect of K+ became more pronounced at a low molar excess of TFO to duplex (Figs 2 and 3). Therefore, despite chemical modifications and differences in nucleotide sequences, use of a high molar excess of TFO to target duplex in previous studies may have affected K+-insensitive triplex formation in vitro (14,17–19) and in vivo (27,28), as well as being the cause of the discrepancy with our observations for triplex stabilization by oligocations (24).

Since electrostatic repulsion between the TFO and the duplex plays a vital role in triplex destabilization under physiological conditions, a major target of using different oligocations and chemically modified and/or DNA intercalator-conjugated TFOs was, therefore, to minimize electrostatic repulsion between the TFO and duplex DNA. However, the triplex-stabilizing efficiency of oligocations is considerably reduced under physiological conditions due to their competitive replacement by co-existing M+ (26). If this were the case, a weak interaction of the oligocations with DNA might be involved in their low triplex-stabilizing ability under physiological conditions (Figs 4 and 5). In contrast, stabilization of internucleotide hydrogen bonding between the DNAs (TFO and target duplex) by shielding their repulsion effects through copolymer–DNA ionic interaction could be a plausible mechanism for copolymer-mediated stabilization of triplex DNA under physiological conditions. In addition to repulsion effects, intramolecular and intermolecular self-association through G quartet formation of G-rich TFOs have long been suggested as a mechanism for K+-mediated inhibition of purine motif triplex formation (29–31). Using very low concentrations of ODNs (0.17 µM Pu), we, like others (29,31), failed to detect G quartet formation under our reaction conditions (Ferdous et al., unpublished data). This suggests that G quartet formation in Pu is likely less sensitive to K+ and an additional mechanism(s) might be involved in triplex inhibition by K+. It has been reported that several factors, like concentration (29), length and sequence of G-rich ODNs (32), significantly affect their self-association and G quartet formation and M+ may inhibit triplex formation by a mechanism that does not involve G quartet formation (10), supporting our above conclusions.

It is important to note that despite stable ionic interaction of the copolymer with DNA, it forms a soluble complex and stabilizes both triplex and duplex DNA without affecting their native structures (20,22). This virtue of the copolymer makes it unlike other polyvalent cations, like poly(t-arginine) and PLL, which irreversibly interact with DNA, form insoluble complexes and also change the native structure of DNA through compaction into a globular conformation (20,33,34). These effects of polyvalent cations could be a simple interpretation of the PLL–DNA interaction as possibly observed in Figure 4a. It suggests that the comb-type structure of the copolymer is important for reversible interaction with and stabilization of duplex (22) and triplex DNA (Fig. 3a; 20). Although the PLL backbone is generally considered important for ionic interaction with DNA (Fig. 3), two roles can be assumed for the grafted dextran (Dex) chains. First, they might interfere with close contact of DNA with the PLL backbone and thereby decrease hydration and compaction, which are presumably responsible for irreversible complex formation between DNA and polyvalent cations. Secondly, as the copolymer consists of 90 wt% Dex and 10 wt% PLL, for ionic interaction with PLL both the TFO and the target duplex must merge through the Dex-enriched phases, which have a low dielectric constant. Such low dielectric constant environments have been suggested to

Figure 5. Effects of the copolymer, spermine and spermidine on pyrimidine motif triplex formation at neutral pH. (a) The copolymer stabilizes pyrimidine motif triplex DNA at neutral pH. Different amounts of control TFO were added to labeled duplex (3 ng) alone (lanes 1–3) or containing 1.7 µM Py (lanes 4–6 and 8–11) or 0.17 µM Pu (lane 12) for a final 5 µM concentration of TFOs (C ± Py/Pu). The mixtures (9 µl) in buffer B were incubated in the presence (+) or absence (−) of the copolymer (2.5 µg) at pH 5.5 (lanes 1–6) or 7.0 (lanes 8–12). After incubation, samples were processed as described in the legend to Figure 3, except that 1 µl 500 mM Tris–acetate (pH 5.0) with (+) or without (−) 1-DNA (6 µg) was added in lanes 8–11 and 5 µl of each sample were then electrophoresed in pH 5.5 (gel at pH 5.5) or 7.0 (gel at pH 7.0) electrophoretic buffer. In lane 7, the reaction proceeded identically as lane 4 but without incubation. The duplex (D) and triplex (T) DNAs are indicated. (b) Comparison of the triplex-stabilizing efficiency between the copolymer, spermine (Spm) and spermidine (Spmd). Reactions for triplex formation were carried out as described in (a), except that the reaction mixtures were incubated at pH 5.5 (lanes 1–4) or 7.0 (lanes 6–13) with (+) or without (−) copolymer (2.5 µg) (lanes 1–9) and with 0.2 or 1.0 mM Spm (lanes 10 and 11) or Spmd (lanes 12 and 13) respectively. The samples were electrophoresed at pH 5.5 to separate the duplex (D) and triplex (T) DNAs. In lane 5, the reaction was processed identically as lane 6 of (a) but without incubation.
enhance hydrogen bonding between base pairs and increase triplex stability (4). Accordingly, Dex chains may play an important role in stabilizing intranucleotide hydrogen bonding, leading to promotion of intranucleotide recognition (i.e. duplex/triplex formation). This conclusion is in good agreement with our previous observations (22) and is further supported by recent kinetic analysis which reveals that K+ significantly decreases the on-rate of triplex formation while the copolymer increases it massively (Ferdous et al., unpublished data). Despite this, additional experiments, such as thermodynamic analysis, especially to estimate the activation energy for TFO binding with the duplex with or without the copolymer, are required to demonstrate the precise mechanistic contributions of the copolymer on triplex formation. Finally, it has been reported that overexpression of collagen type I genes is clinically significant in hepatic cirrhosis and is further supported by recent observations (22) and is further supported by recent observations (22) and is further supported by recent observations (22).

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