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# Targeted TFO delivery to hepatic stellate cells

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ABSTRACT

Triplex-forming oligonucleotides (TFOs) represent an antigene approach for gene regulation through direct interaction with genomic DNA. While this strategy holds great promise owing to the fact that only two alleles need silencing to impact gene regulation, delivering TFOs to target cells in vivo is still a challenge. Our recent efforts have focused on conjugating TFOs to carrier molecules like cholesterol to enhance their cellular uptake and mannose-6-phosphate-bovine serum albumin (M6P–BSA) to target TFO delivery to hepatic stellate cells (HSCs) for treating liver fibrosis. These approaches however are rendered less effective owing to a lack of targeted delivery, as seen with lipid-conjugates, and the potential immune reactions due to repeated dosing with high molecular weight BSA conjugated TFO. In this review, we discuss our latest efforts to enhance the effectiveness of TFO for treating liver fibrosis. We have shown that conjugation of TFOs to M6P–HPMA can enhance TFO delivery to HSCs and has the potential to treat liver fibrosis by inhibiting collagen synthesis. This TFO conjugate shows negligible immunogenicity owing to the use of HPMA, one of the least immunogenic copolymers, thereby making it a suitable and more effective candidate for antifibrotic therapy.



## 1. Introduction

Hepatic fibrosis is the scarring response by the liver to chronic insults and damages, resulting in accumulation of extra extracellular matrix (ECM) [1]. Major causal factors for liver fibrosis include alcohol abuse, nonalcoholic steatohepatitis (NASH) and viral hepatitis [2]. During liver injury, the infiltrated leukocytes and resident macrophages, continually release reactive oxygen species (ROS), inflammatory cytokines and growth factors, leading to the transformation of quiescent, vitamin A storing hepatic stellate cells (HSCs) into proliferating α-smooth muscle actin (SMA) positive myofibroblast-like cells [3]. Activated HSCs, in addition to secreting excessive collagen in fibrotic liver, are also the source of inflammatory cytokines. This creates a positive feedback loop that ensures continual HSC activation, and leads to inflammation, and increased immune cell infiltration. Further influx of bone marrow-derived fibrocytes in the damaged liver tissue results in the transformation of other liver cell types like cholangiocytes into myofibroblasts via epithelial to mesenchymal transition (EMT), a process in which epithelial cells lose their phenotypic characteristics and acquire features of mesenchymal cells such fibroblasts [4]. All these pathogenic mechanisms result in the deposition of excess ECM, including type I collagen, which is the hallmark of fibrosis.

Excessive collagen synthesis interferes with the maintenance of normal liver function and prevents effective liver regeneration [2]. Strategies to treat liver fibrosis are based chiefly on reducing collagen synthesis [5], inhibiting the activation of HSCs to myofibroblasts [6], or protecting the injured hepatocytes by controlling inflammation [7]. In this review, we focus on triplex-forming oligonucleotide (TFO) which represents an antigene therapy and causes transcription inhibition [8]. Unlike antisense oligonucleotides (ODNs) and siRNA approaches, TFO can form triplex with the specific region of genomic DNA [9]. We have been able to enhance both the specific delivery to HSCs and the circulation time of this TFO by utilizing a bioconjugation strategy. Triplex formation with DNA prevents both the binding of transcription factors to the gene promoter and unwinding of the duplex during transcription, making this an attractive therapeutic strategy for managing and treating various diseases, including fibrosis of the liver and other organs.

## 2. Triplex formation for transcription inhibition to treat liver fibrosis

Although DNA normally exists in a duplex form, under some circumstances it can assume triple helical (triplex) structures, which are either intramolecular or intermolecular. The intermolecular triplexes, formed by the addition of a sequence-specific third strand to the major groove of the duplex DNA, have the potential to serve as selective gene regulators [10]. Several genes contain potential triplex-forming homopurine/homopyrimidine sequences, which can be the targets for gene regulation by TFOs.

Sequence composition and organization are essential for both triplex formation and its therapeutic effectiveness. Previous research,

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both *in vitro* and *in vivo*, has shown that antiparallel TFO was more efficient than parallel TFOs in reducing collagen gene expression [11,12]. TFOs occupy the major groove of duplex DNA forming purine or pyrimidine motif triplex structures with the native purine strand. The most stable triplexes are formed when the third strand binds in antiparallel orientation to the homologous native strand. It has been shown that formation of triplexes with various promoters could result in transcription inhibition [13]. TFOs, usually 13–20 nucleotides long, are composed of either polypurine or polypyrimidine and have binding specificity only towards the purine-rich strand of their target DNA duplex in the major groove. Further, TFOs containing C and T nucleotides bind in a parallel while those containing G and A or T nucleotides bind in an antiparallel orientation to the target strand, respectively (Fig. 1) [2]. Because only purines are able to further establish two hydrogen bonds in the major groove of DNA, successful TFO design must follow precise principle that requires consecutive purines on the same strand for stable binding [14,15].

Type I collagen consists of two α1(I) and one α2(I) polypeptide chains encoded by the α1(I) and α2(I) genes and synthesized in a 2:1 ratio. The polypyrimidine/polypurine sequence (C1) of α1(I) collagen gene can form triplexes if a third strand is added. It is a major structural component of ECM and thus an ideal target for the treatment of liver fibrosis.

It has been demonstrated that up-regulated expression of type I collagen by activated HSCs can be at both the transcriptional and post-transcriptional levels [16]. It was further shown that both the synthesis and stability of α1(I) collagen mRNA was significantly increased during hepatic fibrosis. Therefore, it may be possible to prevent and potentially reverse fibrosis by inhibiting the transcription of type α1(I) collagen gene. Mammalian α1(I) collagen gene promoter contains two contiguous 30-bp polypurine tract, C1 and C2, located at −141 to −170 and −171 to −200 upstream from the transcription initial site [17]. Studies have also demonstrated that 18-, 25-, and 30-mer antiparallel phosphorothioate (APS) TFOs specific for C1 tract, inhibit transcription in cultured fibroblasts by forming triplex with the genomic DNA [12]. Using this approach, we have demonstrated good correlation between the extent of triplex formation with the degree of transcription inhibition in naked genomic DNA, isolated nuclei of HSC-T6 cells and whole cells [18]. Further, in a rat model of dinitrosamine (DMN) induced liver fibrosis, the 25-mer and 18-mer TFOs, designed to be specific for the upstream nucleotide sequence from −141 to −165, were effective in preventing collagen accumulation and in improving liver function tests [10].

Systemic delivery of TFOs has been applied for treating both genetic and acquired diseases. The major advantage of TFOs over antisense ODNs and siRNAs is their ability to interact with genomic DNA and shutting down transcription rather than silencing mRNA translation, for which hundreds or thousands of copies per cell may be present. Furthermore, specific mRNAs are continuously transcribed from genomic DNA, even though those in the cytoplasm may have been silenced. Therefore, inhibition of gene transcription using TFOs might offer significant advantage over other gene therapy techniques, at least in some cases.

The TFO investigated in our laboratory has two advantages compared to other TFO. Firstly, this TFO is a polypurine lacking any CpG motifs. In a study by Woolridge et al. [19], DNA with CpG motifs were shown to trigger innate immune defense mechanisms. This stimulation of immunostimulation may actually worsen liver fibrosis instead of curing it. Secondly, the TFO utilized in our studies can form DNA triplex under physiological conditions, thus facilitating triplex formation at target sites. The resulting triplex is thus more stable and makes gene silencing more efficient. Taken together, the proposed TFO against α1(I) collagen can be used as a potent antifibrotic drug. Our experimental data confirmed our hypothesis when it was demonstrated that administration of this TFO to DMN-induced hepatic fibrosis in rat can abrogate collagen accumulation and alleviate fibrosis. Compared to

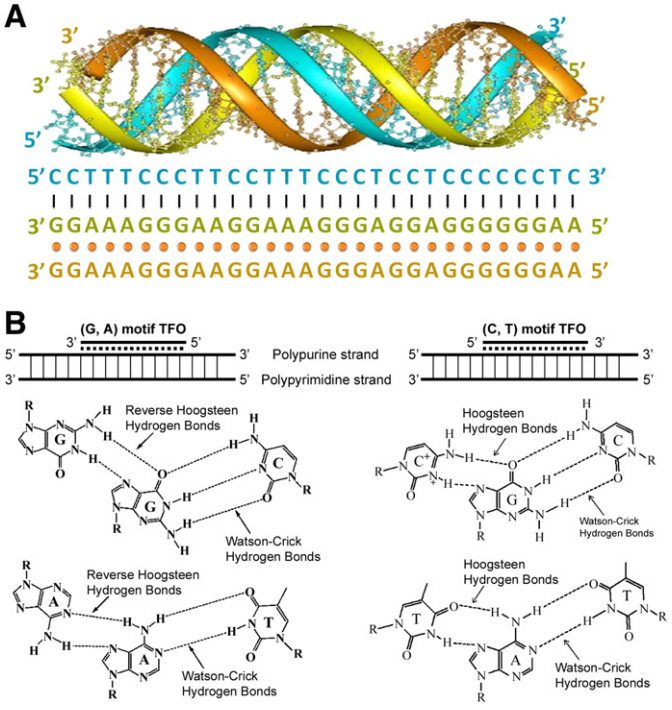


**Fig. 1.** Triplex-forming oligonucleotides. A. The blue and green bands refer to the double strands of DNA molecule. The red one is triplex-forming oligonucleotides (TFOs). B. Principles of triplex formation. A third polynucleotide sequence can bind to double-stranded DNA at the major grove to form triplex structure via formation of Hoogsteen/reverse Hoogsteen hydrogen bonds. TFOs can only bind to polypurine strand of target DNA by either (G, A)-motif or (C, T)-motif.