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Enzymatic Formation of PEGylated Oligonucleotides

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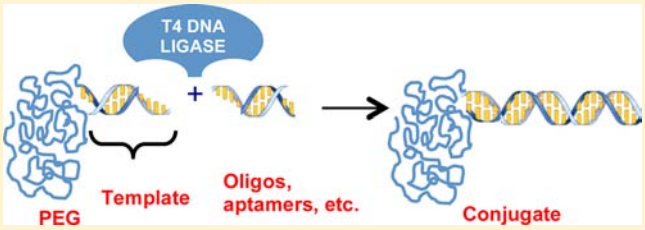
Supporting Information

ABSTRACT: Gene therapy, siRNA, and therapeutic aptamers attract great interest owing to their versatility to treat a wide range of diseases and their potential high selectivity. Unfortunately, oligonucleotide-based therapeutics suffer rapid degradation by nucleases, scarce cell internalization, and fast kidney clearance. To address these limitations, the covalent attachment by mild chemical reactions of an activated polyethylene glycol (PEG) is widely used to obtain PEGylated nucleic acids showing a more favorable pharmacokinetic profile. We describe here a method for the enzymatic formation of PEGylated nucleic acids employing T4 DNA ligase: the ligation protocol was set up and optimized allowing the complete achievement of PEGylated oligonucleotides amenable to further enzymatic reactions. The feasibility of this approach for bioconjugation was demonstrated employing a set of PEG-donors and oligonucleotide acceptors, differing in the chemical link between PEG and the oligonucleotide donor, and in the length, sequence, and structure of the oligonucleotides employed. The ligase reaction allowed us to obtain double-stranded as well as single-stranded oligonucleotides, thus demonstrating the applicability of the method to a variety of substrates suitable for diagnostic and therapeutic applications.



INTRODUCTION

PEGylation, the covalent attachment of polyethylene glycol (PEG)[1] has been proposed for prolonging the pharmacokinetic profile of nucleic acid materials in gene therapy, RNAi, and for the delivery of nucleic acid based therapeutics as aptamers.[2−4] PEGylated oligonucleotides and siRNAs have demonstrated an improved cell internalization and stability with respect to the free nucleic acids.[5,6] Presently, the PEGylation of nucleic acids is based on the covalent attachment of a properly activated PEGylating agent to the 3′ thiol- or amino-modified DNA or RNA oligonucleotide.[2,3] Consequently, the preparation of modified nucleic acids is an obligate step that might present some constraints, such as the formation of impurities[7,8] and additional steps with increasing costs especially when dealing with long sequences.

A simple method for the direct selective conjugation of PEG to nucleic acids would be desired. In the field of protein PEGylation, the attention has been recently directed toward the development of enzymatic methods of conjugation.[1,9] The use of enzymes to mediate the polymer conjugation has proven its efficiency also in comparison with known chemical approaches of coupling.[10,11] These new perspectives in protein PEGylation inspired us to develop a protocol allowing the enzymatic formation of PEGylated oligonucleotides. Following the scheme depicted in Figure 1, we employed T4 DNA ligase to obtain a PEGylated double-stranded DNA (PEG-Oligo) through the enzymatic conjugation of a short PEGylated oligonucleotide sequence, called the PEG-Donor, to a second double-stranded DNA, referred to as Oligo-Acceptor.

The PEG-Donor is a short oligo sequence conjugated to a 20 kDa PEG: this molecule can be obtained with standardized chemistries, a reduced synthetic cost, and would constitute the initial "building block" for the enzymatic ligation to the Oligo-Acceptor, a reaction taking place in a test tube in aqueous buffer without the use of organic solvents.

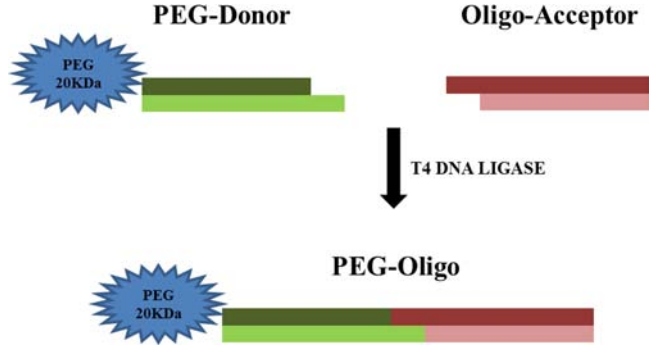


Figure 1. Scheme of the enzymatic formation of PEGylated oligonucleotides. A PEG-Donor, constituted of a 20 kDa PEG bearing a short oligonucleotide sequence, was enzymatically conjugated and transferred to a double-stranded DNA, the Oligo-Acceptor, to give a longer PEGylated double-stranded DNA (PEG-Oligo).

We optimized the reaction to obtain fast and efficiently the desired PEGylated DNA, as demonstrated by gel electrophoresis and chromatographic analysis, employing different PEG-Donors and different Oligo-Acceptors, as specified in detail in Table 1. As PEG-Donor we employed (i)

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Table 1. Oligonucleotides Used for Enzymatic Ligation[a]

Name	Sequence	5'-end Modification	Description	Graphical representation
d	5′-ACTATTCCCGGGTAATGA-3′	Thiol	18-mer single strand donor	5′-HS-ACTATTCCCGGGTAATGA-3′
d'	5′-AATTCATTACCCGGGAATAGT-3′	None	21-mer sequence complementary to **d**	5′-AATTCATTACCCGGGAATAGT-3′
d_F	5′-ACTATTCCCGGGTAATGA-3′	6-FAM	18-mer single strand donor	5′-FAM-ACTATTCCCGGGTAATGA-3′
D	5′-ACTATTCCCGGGTAATGA-3′ 3′-TGATAAGGGCCCATTACTTAA-5′	see **d** see **d'**	double strand (annealed **d**/**d'**) donor	5′-HS-ACTATTCCCGGGTAATGA-3′ 3′-TGATAAGGGCCCATTACTTAA-5′
D_F	5′-ACTATTCCCGGGTAATGA-3′ 3′-TGATAAGGGCCCATTACTTAA-5′	FAM at d_F None at **d'**	double strand (annealed d_F/**d'**) donor	5′-FAM-ACTATTCCCGGGTAATGA-3′ 3′-TGATAAGGGCCCATTACTTAA-5′
a	5′-ATTCCAAAGCTTCCCCCTA-3′	Phosphate	19-mer single strand acceptor	5′-ATTCCAAAGCTTCCCCCTA-3′
a'	5′-TAGGGGGAAGCTTTGG-3′	6-FAM	16-mer sequence complementary to **a**	5′-TAGGGGGAAGCTTTGG-FAM-3′
A	5′-ATTCCAAAGCTTCCCCCTA-3′ 3′-GGTTTCGAAGGGGGAT-5′	see **a** see **a'**	double strand (annealed **a**/**a'**) acceptor	5′-ATTCCAAAGCTTCCCCCTA-3′ 3′-GGTTTCGAAGGGGGAT-FAM-5′
s	5′-GGGTAATGA-3′	Thiol	9-mer single strand short donor	5′-HS-GGGTAATGA-3′
s'	5′-AATTCATTACCC-3′	Phosphate	12-mer sequence complementary to **s**	5′-AATTCATTACCC-3′
S	5′-GGGTAATGA-3′ 3′-CCCATTACTTAA-5′	see **s** see **s'**	double strand (annealed **s**/**s'**) short donor	5′-HS-GGGTAATGA-3′ 3′-CCCATTACTTAA-5′
t	5′-AAAAAATCATTA-3′	None	12-mer adapter (tray strategy)	5′-TGGAATTCATTA-3′
TBA	5′-TTTTTTGGTTGGTGTGGTTGG-3′	polyT (6T) tail	21nt(15+6nt) Thrombin Binding Aptamer	5′-TTTTTTGGTTGGTGTGGTTGG-3′

[a]Nomenclature, sequences, chemical modifications, and graphical representation of all oligonucleotides used.

oligonucleotide sequences of different length, respecting the requirement of substrate length for T4 DNA ligase activity,[12−14] and (ii) oligonucleotides with different chemistries of PEG-Donor conjugation, demonstrating efficient ligase reaction in all cases. The PEGylation by T4 DNA ligase was achieved with (iii) double-stranded and also with (iv) single-stranded Oligonucleotide-Acceptors, thus indicating the applicability of our method also for aptamer PEGylation.

We demonstrated that PEGylated oligos are amenable to enzymatic digestion by restriction endonucleases, showing that PEGylated DNA can be produced by enzymatic ligation and further manipulated with enzymatic activities common in molecular biology protocols. To the best of our knowledge, this is the first study on the use of enzymatic methods to PEGylate oligonucleotides: we believe that the method could be successfully applied to stimulate new applications in synthetic biology and gene delivery.

EXPERIMENTAL PROCEDURES

Materials. All oligonucleotides were synthesized by Metabion International AG (Martinsried, Germany) or Diatech Pharmacogenetics (Jesi, Italy), stored at −20 °C in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE), and used without further purification. Thiol modified oligos were deprotected as specified by the manufacturer. The set of appositely designed oligonucleotides, and their abbreviated names, sequences, and chemical modifications are reported in Table 1. Lower case names indicate single-stranded oligos, while capital letters designate the double-stranded form. Each oligonucleotide possesses specific features that will be explained through the text.

Single-stranded oligos were annealed with their complementary sequences (**d**+**d**′, $\mathbf{d_F}$+**d**′, **a**+**a**′, **s**+**s**′) to obtain the double-stranded **D**, $\mathbf{D_F}$, **A**, and **S**, respectively. The 5′-overhangs of **D**, $\mathbf{D_F}$, and **S** are cohesive to **A** and upon ligation represent an *Eco*RI restriction site. **TBA** is the 15-mer thrombin

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binding aptamer added of a polyT (6T) tail at the 5′-end that makes the aptamer partly complementary to **t**; the oligo **t** was used as an "adapter", thanks to its complementarity to **s** and **TBA** ends.

PEG maleimide 20 kDa ($PEG_{20\ kDa}$-Mal) and *ortho*-pyridine disulfide PEG 20 kDa ($PEG_{20\ kDa}$-OPSS) were purchased from IRIS Biotech GmBh (Marktredwitz, Germany). Acrylamide/Bis-acrylamide (19:1) 40% solution was purchased from VWR International PBI Srl (Milan, Italy); T4 Polynucleotide Kinase (T4 PNK), and Reaction Buffer A from Fermentas by Thermo Scientific (Waltham, MA, USA); *Eco*RI 10U/mL and REact 3 from Promega (Promega Italia srl); T4 DNA Ligase and SybrGreen II from Invitrogen (Life Technologies Ltd., Paisley, UK). All the other chemical reagents, including salts and solvents, were purchased from Sigma-Aldrich (Milan, Italy).

5′-OH Phosphorylation of Oligonucleotides. Chemically synthesized nucleic acids are not phosphorylated at the ends unless specified. Since phosphorylation of 5′-ends is strictly required for enzymatic ligation of nucleic acids by T4 DNA Ligase, the oligo **d**′ as well as **TBA** were enzymatically phosphorylated using T4 PNK. Twenty units of T4 PNK were added to 1 nmol of oligo in the presence of Reaction Buffer A (50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, pH 7.6) and ATP (10 nmol), in a total volume of 50 μL, at 37 °C for 1 h. The phosphorylated oligo was then recovered by precipitation: DNA solutions were added of 1/10 of the volume of 3 M sodium acetate pH 5.2 and of 3 volumes of cold EtOH. The solution was then placed at −80 °C for 15 min and centrifuged at 20 800 × *g* for 20 min at 4 °C. EtOH was removed and the DNA pellet rinsed once with 70% EtOH. The washed pellets were centrifuged again for 10 min at 20 800 × *g* at 4 °C. The EtOH was definitively removed and the pellets dried in a speed vacuum. After drying, pellets were resuspended in Milli-Q water in 9/10 of the original volume.

Synthesis of PEG-Donors. To synthesize the PEG-donors (PEG-**d** and the shorter PEG-**s**), 2 nmol of **d** and **s**, respectively, were mixed to a slight excess (molar ratio of oligo to PEG was 1:1.2) of $PEG_{20\ kDa}$-Maleimide ($PEG_{20\ kDa}$-Mal) in 0.1 M H_2NaPO_4, 2 mM EDTA, pH 7.0 for 30 min at room temperature. An alternative chemistry was used to obtain PEG_{SS}-**d**. The oligo **d** was mixed with $PEG_{20\ kDa}$-OPSS: the oligo to polymer molar ratio was 1:5 and the reaction was conducted in 0.1 M H_2NaPO_4, 2 mM EDTA, pH 7.0. Purification of PEG_{SS}-**d** was carried out after 24 h. The reactions were monitored by RP-HPLC. Analysis and purification of PEG-donors were performed with a C18 Jupiter (Phenomenex, USA; 5 μm, 300 Å, 250 × 4.60 mm), eluting at 1 mL/min (gradient: 0′ 10% B, 25′ 50% B, 27′ 90%, 29′ 10% B). Eluent A was 0.1 M triethylammonium acetate buffer (TEAA), pH 7.0, and ACN was the eluent B (50 °C). The effluent was monitored at 260 nm. As expected, the synthesis of PEG-donors was fast and efficient: the peak percentages corresponding to the PEG-donors (t_R = 32′) were around 90%, in terms of conversion yields. PEG-donors were collected, dried, and resuspended in Milli-Q water. The concentrations of conjugates were controlled on a NanoDrop ND-1000 UV−vis spectrophotometer (Thermo Fisher Scientific). The recovery yields, determined spectrophotometrically by measuring the oligo concentration, were above 80% for all PEG-donors.

Ligation Reactions. A set of the appropriately designed double-stranded oligonucleotides **D** and **A** (Table 1) were ligated by enzymatic methods. Prior to ligation, an annealing step of the single-stranded oligo couples was performed to obtain the dsDNAs: equal amounts of the complementary strands of each pair were mixed, denatured at 95 °C for 5 min, and left to slowly cool to room temperature. The annealed dsDNAs were then incubated for 5 h at room temperature to allow the correct overlap of the compatible cohesive ends, and 4 U of T4 DNA Ligase was added to the combined dsDNAs (0.25 nmol **D** and 0.3 nmol **A**) in the Ligase Buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% PEG_{8KDa}) in a total volume of 40 μL. Different temperatures and times were analyzed as shown in the Supporting Information. The best conditions for the ligation reaction were 21 °C for a minimum of 90 min. The ligase reaction using as PEG-Donor PEG_{SS}-**D** was performed in the Ligase Buffer without DTT, to avoid the reduction of the disulfide S−S bridge of PEG-OPSS to **d**. When **S** was enzymatically ligated to **A**, the annealed dsDNAs were incubated together for 5 h at 4 °C and the ligase reaction was performed overnight at 16 °C to avoid the melting of the short dsDNA **S**. T4 DNA Ligase was employed also for the ligation of two single-stranded DNAs (**s** and **TBA**) using the "tray strategy" (oligo **t**, see Table 1): since oligo **t** is partly complementary to **s** and partly complementary to **TBA**, the three oligonucleotides were mixed, heated at 95 °C for 5 min, and left to slowly cool to room temperature. The annealed oligonucleotides were then added of 2 U of T4 DNA ligase in the ligase buffer and the reaction was performed overnight at 16 °C.

***Eco*RI Restriction.** Following the protocol of molecular biology as recommended by the supplier (Promega), the PEG-Oligo was incubated for 1 h at 37 °C with 20 U of *Eco*RI in the appropriate buffer REact3 (5 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 10 mM NaCl).

Gel Electrophoresis Analysis. The extent of ligation and restriction reactions was verified by polyacrylamide gel electrophoresis (PAGE). Samples were resolved by 20% native and/or denaturing (7 M urea) polyacrylamide gels containing TBE buffer (Tris-HCl 89 mM, borate 89 mM, EDTA 2 mM). Oligonucleotides in the gels were detected employing different detection systems: the fluorescence of FAM-modified oligonucleotides (λ_{exc} = 495 nm; λ_{em} = 520 nm) was visualized on the gels. Oligonucleotides were also stained with the fluorescent DNA binding dye SybrGreen II (Invitrogen) that preferentially binds to single-stranded DNA and emits fluorescence (λ_{exc} = 488 nm; λ_{em} = 522 nm) when in complex with DNA. Fluorescence in gel systems was detected on a Geliance 600 Imaging System (PerkinElmer).

RP-HPLC Analysis. The extent of ligation was also monitored by HPLC analyses using the elution conditions reported above for the synthesis of PEG-Donors, apart from the temperature of the column that was set at room temperature. The effluent was monitored by measuring the fluorescence of the reporter (λ_{exc} = 495 nm; λ_{em} = 520 nm).

RESULTS AND DISCUSSION

PEGylation of Double-Stranded Oligonucleotides by T4 DNA Ligase. Two different chemistries were used to prepare the PEG-donor substrates for the enzymatic ligation, the first yielding a physiologically stable conjugate, while the second resulted in an easily reducible building block. To do so, PEG_{20KDa}-Mal or PEG_{20KDa}-OPSS were conjugated to the 18-mer 5′-thiol modified oligo **d** to obtain PEG_M-**d** or PEG_{SS}-**d** donors, respectively (the low-case letter "**d**" designates the single-stranded form of the oligo). The synthesis of PEG_M-**d** was performed exploiting the Michael's addition, in which the

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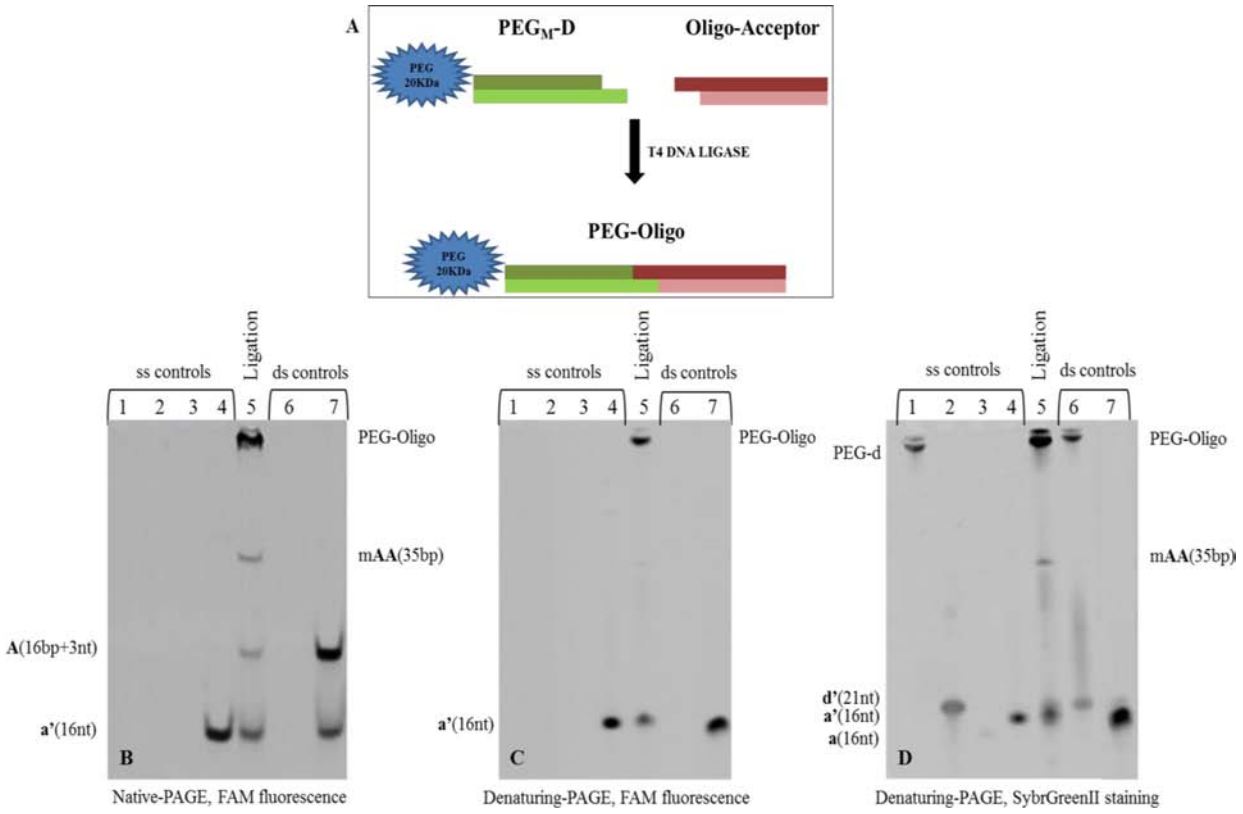

Figure 2. Enzymatic formation of PEGylated DNA. Scheme of ligation of **PEG$_M$-D** to Oligo Acceptor (A). Native-PAGE (20%): the oligonucleotides on the gel system were visualized by the fluorescence of FAM (B). Denaturing-PAGE (20%, 7 M Urea): the oligonucleotides on the gel system were visualized by the fluorescence of FAM (C). Denaturing-PAGE (20%, 7 M urea): the oligonucleotides on the gel system were visualized by staining with SybrGreen II (D). Single-stranded and double-stranded controls were loaded as well as the ligation mixture: 1. **PEG$_M$-d**; 2. Oligo **d**′; 3. Oligo **a**; 4. Oligo **a**′ (5′-FAM modified); 5. **PEG$_M$-D** + **A** Ligation mixture; 6. dsDNA PEG-Donor (**PEG$_M$-D**); 7. Oligo-Acceptor, dsDNA **A**.

thiol group reacts with a double bond forming a physiologically stable thioether linkage. PEG$_{SS}$-**d** was formed by the conjugation of PEG-OPSS to **d,** leading to the disulfide (S−S) bond formation easily cleavable by a reducing agent such as dithiothreitol (DTT).[6] As expected, both syntheses, performed according to established protocols, were fast and efficient (see Supporting Infomation and Figure S1).

Having prepared the starting building blocks, we initially set up the enzymatic reaction with T4 DNA ligase using the double-stranded PEG$_M$-**D**, characterized by the physiologically stable thioether linkage to PEG, and the double-stranded Oligo-Acceptor **A**. PEG$_M$-**d** was therefore annealed to its phosphorylated complementary sequence **d**′ (see Table 1) to obtain the double-stranded PEG$_M$-Donor (PEG$_M$-**D**), designed to possess an overhang of three nucleotides (AAT- at the 5′-end of **d**′) that makes it cohesive to the Oligo-Acceptor **A** (see Table 1). **A** is a dsDNA formed by the annealing of the 19-mer sequence **a** to its complementary 16-mer sequence **a**′, and exhibits three important features: (i) the overhang (TTA-) at the 5′-end of **a**, cohesive to the PEG$_M$-**D**, (ii) an *Eco*RI restriction site created upon ligation with the PEG-Donor, and (iii) a fluorescent reporter (6-carboxyfluorescein, FAM) at the 5′-end of **a**′, that allows the fluorescent detection of **A** in the gels and in the HPLC system, and functions as a marker for the PEGylation of the Oligo-Acceptor.

The ligation step shown in Figure 2A was thoroughly optimized by investigating the effect of different temperatures and the role of incremental incubation times, as detailed in the Supporting Information (Table S1). The results of the ligation reaction performed at 21 °C for 90 min and analyzed by gel electrophoresis are shown in Figure 2B−D: the samples were loaded on a 20% native-PAGE (Figure 2B) and on a 20% denaturing-PAGE (Figure 2C and D); oligonucleotides in the gel system were detected by the fluorescence of the reporter FAM (Figure 2B and C) and by staining with SybrGreen II to visualize also the nonfluorescent bands (Figure 2D).

In native-PAGE (Figure 2B) the reaction mixture (lane 5), loaded between the single-stranded (ss controls, lanes 1−4) and the double-stranded controls (ds controls, lanes 6−7), shows that the enzymatic ligation of PEG$_M$-**D** to **A** resulted in the incorporation of the fluorescent acceptor (**a**′) into the limited mobility band at the top of the gel (PEG-Oligo). Since PEGylated oligonucleotides have a large hydrodynamic volume, the fluorescent band with the lower electrophoretic mobility in the PAGE system corresponds to the PEG-Oligo, which became fluorescent after ligation, thus indicating the success of the ligase reaction. Of the three fluorescent bands of lane 5, Figure 2B, the two faster correspond to the unreacted Oligo-Acceptor **a**′ (compare with lane 4 control) and to the double-stranded unreacted **A** (compare with lane 7 control), present in excess in the ligation reaction. The fourth discrete fluorescent band with intermediate electrophoretic mobility (m**AA**) corresponds to a new fluorescent DNA species, unknown, resulting from the action of ligase. To further verify the outcome of the reaction, all controls and samples were loaded on the 20% denaturing-PAGE gels shown in Figure 2C,D. In

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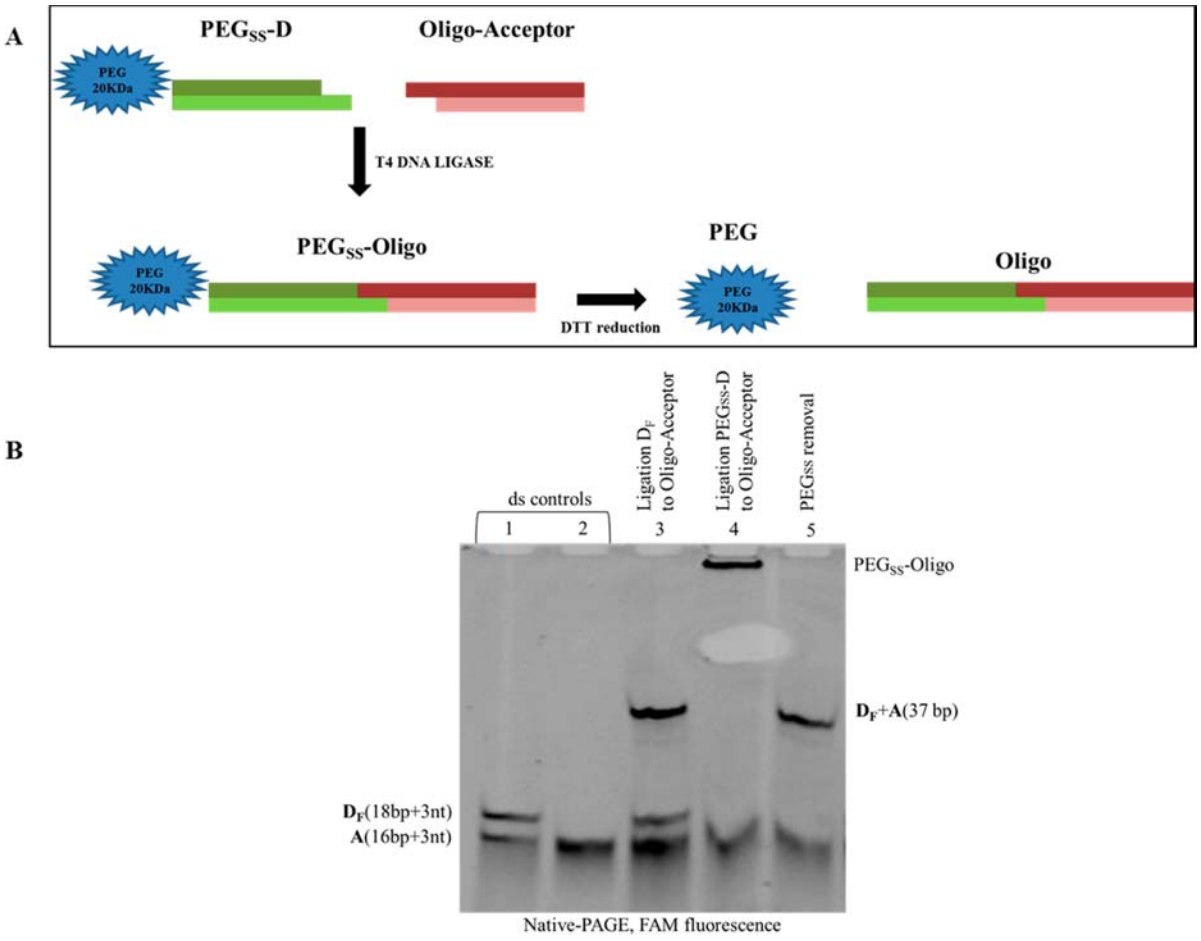

Figure 3. Different PEG-Donors can be enzymatically ligated to DNA. Scheme of the ligation reaction of PEG_{SS}-Donor to the Oligo-Acceptor followed by PEG removal (A). Analysis by Native-PAGE (20%): the oligonucleotides on the gel system were visualized by the fluorescence of FAM (B). Double-stranded (ds) controls were loaded with the ligation mixtures: 1. dsDNA $\mathbf{D_F}$ + Oligo-Acceptor **A**; 2. dsDNA PEG_{SS}-Donor $\mathbf{PEG_{SS}}$-**D** + Oligo-Acceptor **A**; 3. $\mathbf{D_F}$ + **A** Ligation mixture; 4. $\mathbf{PEG_{SS}}$-**D** + **A** Ligation mixture; 5. $\mathbf{PEG_{SS}}$-**Oligo** after DTT treatment and PEG removal.

Figure 2C, lane 5, the double-stranded **A** disappears, as expected, being denatured to **a** (nonfluorescent) and into **a**′; the band corresponding to the PEG-Oligo remains at the lower mobility, consistently with the presence of PEG becoming fluorescent after the ligase reaction. The faint band with an electrophoretic mobility intermediate between the unreacted oligos and the PEG-Oligo product is also visible in these conditions. The staining with SybrGreen II of the denaturing gel, shown in Figure 2D, allows the detection of the nonfluorescent bands (see controls: lane 1, 2, 3, 6). In these conditions, the m**AA** band of lane 5, which is clearly a secondary product of T4 DNA ligase activity, is again evident. We hypothesize that this non-PEGylated band corresponds to the ligation of **A** to itself through a mismatched overhang, a secondary reaction consistent with literature findings, resulting in a mismatched homodimer m**AA**.[15,16] The experimental evidence supporting this hypothesis are presented in Figure S3 of Supporting Information. Given that the *Eco*RI site making possible the mismatch formation was introduced only for analytical purposes, the mismatched homodimer formation could be easily avoided by other experimental setups (see below).

Different PEG Donors Can Be Enzymatically Ligated to Oligo-Acceptors. In the previous experiments PEG_{20KDa}-Mal was used for the design of the PEG donor moiety: the success of ligation was proven by the transfer of fluorescence from the Oligo-Acceptor to the ligated PEG-Oligo product in the electrophoretic system and in HPLC analysis (not shown). However, the mobilities of PEGylated donors (PEG_M-**d** and PEG_M-**D**) are identical to those of the ligated PEG-Oligo due to the presence of bulky PEG: the fluorescence of the PEG-Oligo was the only marker for the ligation of **A** into PEG.

To rule out the possibility that incorporation of the fluorescence of the PEGylated-Oligo is due to the adsorption into PEG of unligated **A** rather than to its covalent ligation to PEG-Donor, we set up a new experiment designed to fulfill two goals: (i) to show conclusively that the ligated PEG is formed by the covalent addition of the Oligo-Acceptor to the PEG-Donor, and (ii) to show that PEGylated donors obtained with different chemistries can be substrate for the enzymatic ligation reaction. To answer to these questions, $PEG_{20\ kDa}$-OPSS was conjugated to **d** to give a PEG-donor in which the oligo is linked to the polymer through a disulfide bond (PEG_{SS}-**d**). The double-stranded PEG_{SS}-**D**, obtained by annealing PEG_{SS}-**d** to **d**′, was ligated to the Oligo-Acceptor following the same procedure used for the PEG_M-Donor ligation, but excluding DTT from the buffer to avoid the untimely reduction of the disulfide bond during the ligation reaction. Once the ligation was complete the disulfide bond was reduced by DTT (0.1 M DTT pH 7.0 for 3 h) allowing the removal of PEG from the ligated PEG-Oligo, as represented in the cartoon of Figure 3A. The ligated oligo (37 base pairs) released from PEG should

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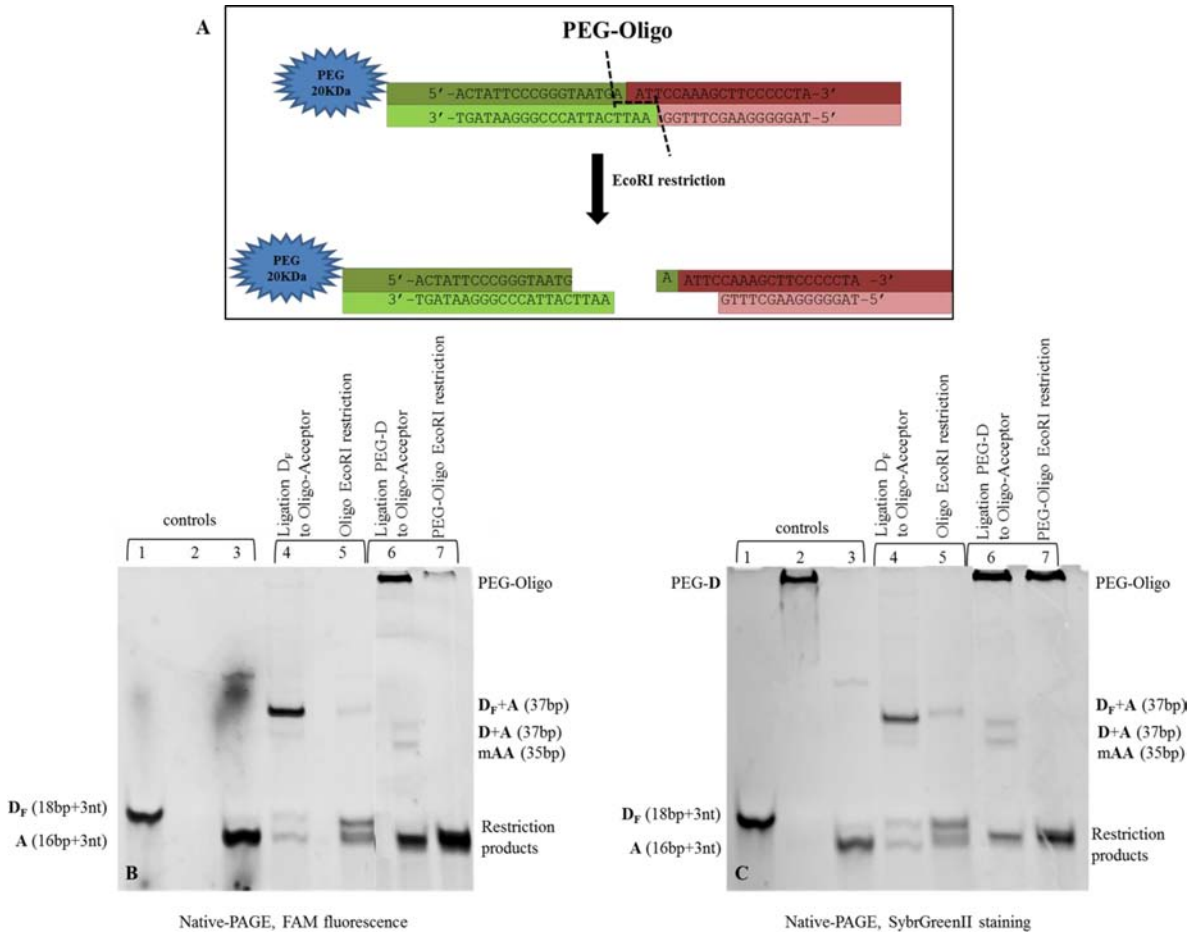

Figure 4. PEG-Oligo can be restricted by *Eco*RI. Scheme of the experiment (A). Analysis by native-PAGE (20%): the oligonucleotides on the gel system were visualized by the fluorescence of FAM (B) and by staining with SybrGreen II (C). Controls (lanes 1−3), ligation mixtures (lanes 4 and 6), and the restriction mixtures (lanes 5 and 7) were loaded: 1. dsDNA Donor $\mathbf{D_F}$; 2. dsDNA Donor **PEG-D**; 3. Oligo-Acceptor dsDNA **A**; 4. $\mathbf{D_F}$ + **A** Ligation mixture; 5. $\mathbf{D_F}$-**A** Restriction mixture (*Eco*RI); 6. **PEG-D** + **A** Ligation mixture; 7. **PEG**-Oligo Restriction mixture (*Eco*RI).

migrate in the polyacrylamide gel as a discrete band, easily distinguishable from the unligated shorter species. Before the enzymatic reaction we prepared the appropriate controls, namely, $\mathbf{D_F}$ (Table 1), having the same oligonucleotide sequences of **D** but conjugated with FAM to allow fluorescent detection (Figure 3B, lane 1), and the ligated $\mathbf{D_F}$-**A** control useful to locate the fluorescent 37 bp band (Figure 3B, lane 3). The outcome of the enzymatic PEGylation, before and after the reduction treatment, was analyzed by gel electrophoresis (Figure 3B) on a 20% native-PAGE.

In lane 4, corresponding to the PEGylation of the Oligo-Acceptor using the PEG-Donor PEG_{SS}-**D**, a fluorescent band (PEG_{SS}-Oligo) with limited electrophoretic mobility can be detected, as expected, along with the excess **A** present in the reaction mix. The PEG_{SS}-Oligo thus obtained was treated with DTT to remove the PEG by the reduction of the disulfide bond and the sample was loaded in lane 5: PEG release from the dsDNA is evidenced by the appearance of fluorescent dsDNA with the same electrophoretic mobility of the 37 bp control (lane 3), corresponding to the expected **D**−**A** product of covalent ligation and validating the experimental results. We can therefore affirm that (i) the PEG-Donor and the Oligo-Acceptor are effectively ligated, (ii) the PEGylation of the Oligo-Acceptor is successful also using the PEG_{SS}-**D** Donor, and (iii) PEG can be removed from the dsDNA obtained with the T4 DNA Ligase mediated reaction provided that the adequate chemistry is employed in the design of the PEG-Donor.

PEG-Oligo Restriction by *Eco*RI. The set of oligonucleotides used in the present work was properly designed with the aim to obtain, after the ligation reaction, a dsDNA that could represent the substrate for the restriction enzyme *Eco*RI. *Eco*RI specifically recognizes the palindromic sequence GAATTC formed upon ligation and cuts the phosphodiester bond between the G and the A on both DNA strands. As depicted in the scheme in Figure 4A, the PEG-Oligo obtained by the enzymatic method was restricted by treatment with *Eco*RI; the outcome of the restriction reaction was analyzed by a 20% native-PAGE and detected by the fluorescence reporter FAM (Figure 4B) and by staining with SybrGreen II (Figure 4C).

All controls were loaded in lanes 1−5: the substrates in lanes 1−3, the 37 bp ligated $\mathbf{D_F}$-**A** (fluorescent) in lane 4, and $\mathbf{D_F}$ + **A** after the restriction reaction in lane 5. The results of the experiment are in lanes 6 (after ligation) and 7 (after restriction): we see that *Eco*RI is able to recognize and cut the Oligo-PEG substrate, since, observing lane 7 in Figure 4B, the limited mobility band corresponding to the PEG-Oligo in lane 6 disappears and at the same time the fluorescence of the band of the Oligo-Acceptor increases. The products of the *Eco*RI cleavage are a PEGylated nonfluorescent dsDNA and a fluorescent dsDNA that differs from the Oligo-Acceptor only by one nucleotide (4 nt AATT overhang resulting from the

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*Eco*RI restriction). In Figure 4C, the gel was stained with SybrGreen II to visualize the nonfluorescent bands: in lane 7, in particular, the band corresponding to the PEGylated nonfluorescent product of restriction is evident (compare with lane 7, Figure 4B). *Eco*RI digestion of PEG-Oligo is very efficient despite the presence of PEG, as the enzyme in these conditions also cleaves the mismatched dimers.[17]

Enzymatic Formation of PEGylated Oligonucleotides with Shorter PEG-Donors. The enzymatic formation of PEGylated Oligo-Acceptor was also performed using as PEG-Donor a 20 kDa PEG bearing a shorter oligonucleotide sequence of half-length compared to the PEG-Donors previously used (Figures 2–4). $PEG_{20\ kDa}$-Mal was conjugated to the 9-mer 5′-thiol modified oligo **s** (Table 1) to obtain the short PEG-**s**. PEG-**s** was annealed to the 12-mer oligonucleotide **s**′ to obtain the short double-stranded PEG-**S** (see Table 1) possessing the three nucleotide overhang cohesive to the Oligo-Acceptor **A**. PEG-**S** and **A** were enzymatically ligated by T4 DNA Ligase as schematically depicted in Figure 5A. The results of the ligation reaction were analyzed by gel electrophoresis: the samples were applied on a 20% denaturing-PAGE and bands were visualized by staining with SybrGreen II (Figure 5B).

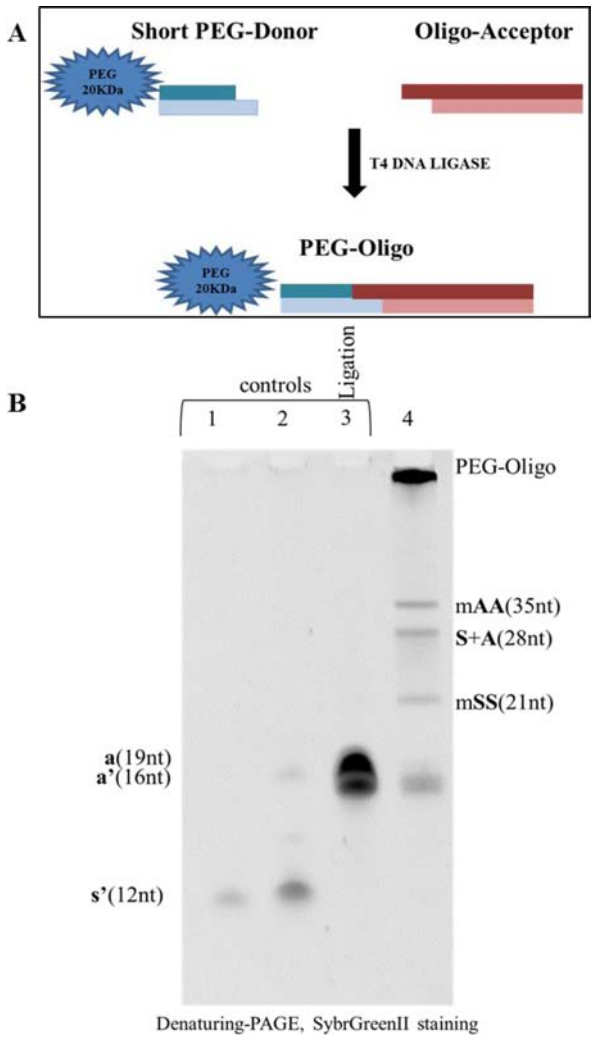

Figure 5. Short PEG-Donor can be enzymatically ligated to DNA. Scheme of the ligation reaction of the short PEG-Donor to the Oligo-Acceptor (A). Denaturing-PAGE (20%, 7 M Urea): the oligonucleotides on the gel system were visualized by staining with SybrGreen II (B). Single-stranded and double-stranded controls were loaded as well as the ligation mixture: 1. dsDNA Donor **PEG-S**; 2. dsDNA Donor **S**; 3. Oligo-Acceptor, dsDNA **A**; 4. short **PEG-S** + **A** Ligation mixture.

The ligation mixture loaded in lane 4 allows the detection of a band with low electrophoretic mobility relative to controls, confirming that the PEG-Oligo was formed and that the enzymatic ligation of the short PEG-Donor to the Oligo-Acceptor was achieved. The ligation reaction was performed at 16 °C instead than 21 °C due to the lower T_m of the **S** oligo: in these conditions we notice along with the PEGylated primary product the appearance of secondary products due to formation of mismatched dimers, as previously evidenced, demonstrating that secondary reactions do not depend on PEG but on the design of the ends of PEG-Donors and Acceptors and the temperature of annealing-ligation. An additional secondary band corresponds to a non-PEGylated oligonucleotide resulting from the ligation of **S** to **A**. Since the PEG conjugation to **S** is stable, this secondary product originates from unreacted **s** that became unspecifically absorbed into the PEG polymer, and copurified with the covalent PEG-**s** product in the HPLC purification step. This gel witnesses the power of the ligation method also in revealing otherwise undetectable impurities originating from the chromatographic purification of the high molecular weight polymer.

Enzymatic Formation of PEGylated Thrombin Binding Aptamer. The short PEG-Donor PEG-**s** was finally employed for the PEGylation of the DNA 15-mer thrombin binding aptamer (**TBA**). **TBA** is a well-known aptamer that recognizes specifically the fibrinogen binding domain of human thrombin.[18] PEG-**s**, directly used as single-stranded DNA (ssDNA) short PEG-Donor, and the ssDNA **TBA** sequence, synthesized by adding a polyT (6T) tail at the 5′-end (21 nt, see Table 1), were enzymatically ligated by T4 DNA Ligase through the use of a 12-mer adapter in what we call the "tray strategy" shown in Figure 6A. The sequence of the adapter (oligo **t**, shown in pink in Figure 6A) was designed to be complementary to 6 nucleotides at the 3′-end of **s** and to 6 nucleotides at the 5′-end of **TBA**: **t** anneals at the same time with **s** and with **TBA**, joining the two otherwise blunt ends of the ss-oligos and forming a double helix, which is the substrate of T4 DNA ligase. The ligation of the two ssDNA oligonucleotides by the T4 DNA Ligase was performed as schematically depicted in Figure 6A. The results of the aptamer PEGylation, analyzed by gel electrophoresis, are shown in Figure 6B: the samples were loaded onto a 20% native PAGE; oligonucleotides in the gel system were detected by staining with SybrGreen II.

Lanes 1 and 2 show the mobility of the unreacted controls (lane 1) and of the ligase reaction performed with **s** and **TBA** through the annealing with **t** (lane 2). The 9-mer oligo **s** is too short to be stained by SybrGreen II, but in lane 2 a new band corresponding to the **s**+**TBA** ligation product (9 + 21 nt) can be detected. The oligonucleotide mixture containing PEG-**s**, **TBA** and **t** was loaded in lane 3, while lane 4 corresponds to the ligation of PEG-**s** to **TBA** according to the Scheme in Figure 6A: since PEG-**s** cannot be detected by SybrGreen in this gel system (see lane 3), the new low mobility band in lane 4 corresponds to the new longer PEGylated oligo, corresponding to the ligation of PEG to the aptamer **TBA** through the short PEG-Donor. We do not observe mismatched bands in this ligation, thanks to a different experimental design of oligos lacking the −AAT cohesive end. As already mentioned above, a

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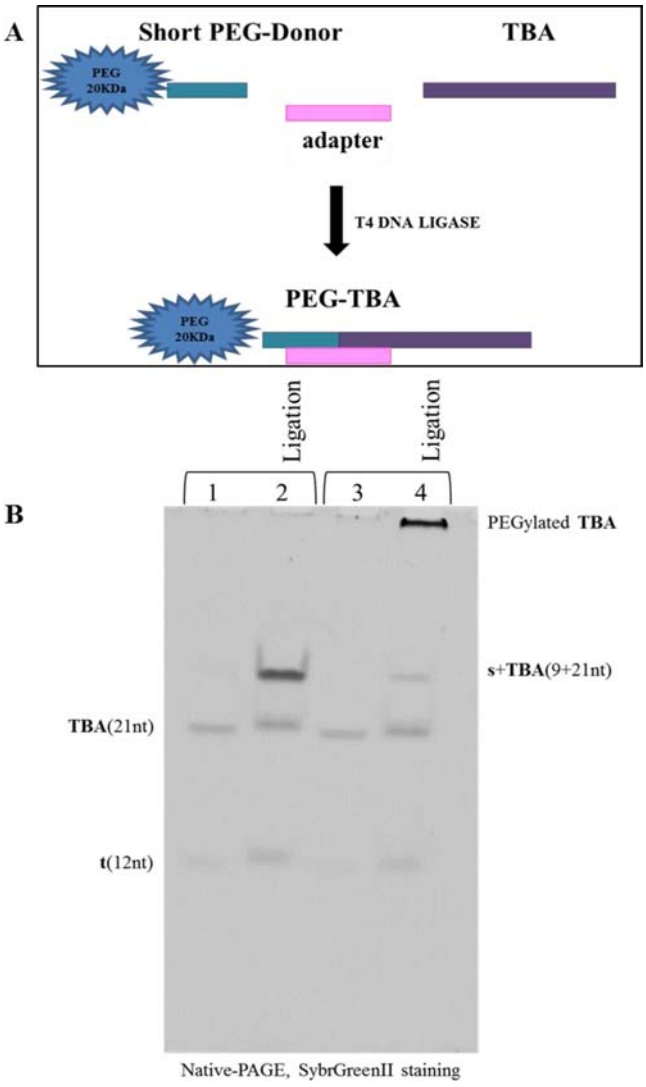

Figure 6. Enzymatic formation of PEGylated thrombin binding aptamer. Scheme of ligation of the short PEG-Donor to the thrombin binding aptamer TBA (A). Analysis by Native-PAGE (20%): the oligonucleotides on the gel system were visualized by staining with SybrGreen II (B). 1. Oligo **s** + oligo **t** + **TBA** mixed but not ligated; 2. Oligo **s** + oligo **t** + **TBA** Ligation Mixture; 3. **PEG-s** + oligo **t** + **TBA** mixed but not ligated; 4. **PEG-s** + oligo **t** + **TBA** Ligation Mixture.

minor **s**+**TBA** product derives from the ligation of PEG-**s** to the aptamer, evidencing the presence of a contaminating undetectable free **s** adsorbed into PEG after the chemical preparation of substrates.

CONCLUSIONS

PEGylation has recently become an established and highly refined technology for the delivery of therapeutic oligonucleotides.[1] The positive results obtained with Macugen, a PEGylated aptamer inhibiting the vascular endothelium growth factor (VEGF), are encouraging the use of PEG for this purpose.[1,19] Taking advantage of the results recently achieved in enzymatic PEGylation of proteins,[1] we focused on the use of enzymes to mediate the site-selective conjugation of PEG to oligonucleotides. We developed a new and versatile method for the PEGylation of nucleic acids that employs T4 DNA ligase enzyme. The ligase reaction was optimized employing different chemistries in the design of our PEG donor moiety, and we have proven the success of ligation by following the transfer of fluorescence from the Oligo-Acceptor to PEG as well as observing the removal of PEG after the ligation by chemical reduction of the disulfide linkage. The enzymatic restriction of the PEG-Oligo was also achieved, suggesting easy accessibility of the PEGylated product to chemical as well as to biological manipulation. Hence, enzymatic formation of PEGylated oligonucleotides does not lower the activity of enzymes commonly used in molecular biology. On the other hand, this result does not contrast the several reports showing that PEGylation protects oligos in vivo against the action of nucleases. In fact, in this experiment the concentration of the enzyme was higher that those of nucleases in vivo.

The enzymatic formation of PEGylated Oligo-Acceptor was also performed using a shorter 9-mer PEG-Donor which places the substrates to be joined in an asymmetric fashion. The enzymatic ligation of PEG-**s** to the Oligo-Acceptor was successful, but the presence of unwanted secondary products due to the lower temperature required for ligation limits its applicability when the designed ends are cohesive. Although this secondary reaction further demonstrates that PEG-Donors in the reaction mix do not influence the catalytic activity of T4 DNA ligase, an accurate design of the ends of PEG-Donors and Acceptors is needed to avoid unwanted dimerization reactions. Finally, we performed the enzymatic formation of PEGylated **TBA** aptamer that represents one of the possible applications of our system.[20,21] In this case the single-stranded PEG-**s** was ligated to an aptamer by T4 DNA ligase through what we call "the tray strategy", designing an adapter which is not consumed during the reaction but allows the formation of a dsDNA intermediate for the enzyme catalysis. The success of the enzymatic formation of PEGylated **TBA** aptamer reveals the feasibility of the method not only for the PEGylation of double-stranded oligonucleotides, but also for single-stranded nucleic acid sequences. Potentially, this enzymatic method for PEGylation of oligonucleotides can be applied to every DNA or RNA sequence, opening new applications in synthetic biology and affordable methods for the delivery of therapeutic oligonucleotides.

ASSOCIATED CONTENT

Supporting Information

Synthesis of PEG-Donors, Optimization of ligation, Mismatched dimer formation Table S1 and Figures S1, S2, and S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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