SUPPORTING INFORMATION

Enzymatic formation of PEGylated oligonucleotides

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RUNNING TITLE

Enzymatic PEGylation of oligonucleotides

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Synthesis of PEG-Donors

To prepare PEG-Donors, $PEG2_{0KDa}$-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. As expected, both syntheses, performed according to well established protocols as reported in Materials and Methods, resulted fast, efficient and quantitative. As example, the HPLC elution profile of the synthesis of PEG_M-**d** is reported in Figure S1.

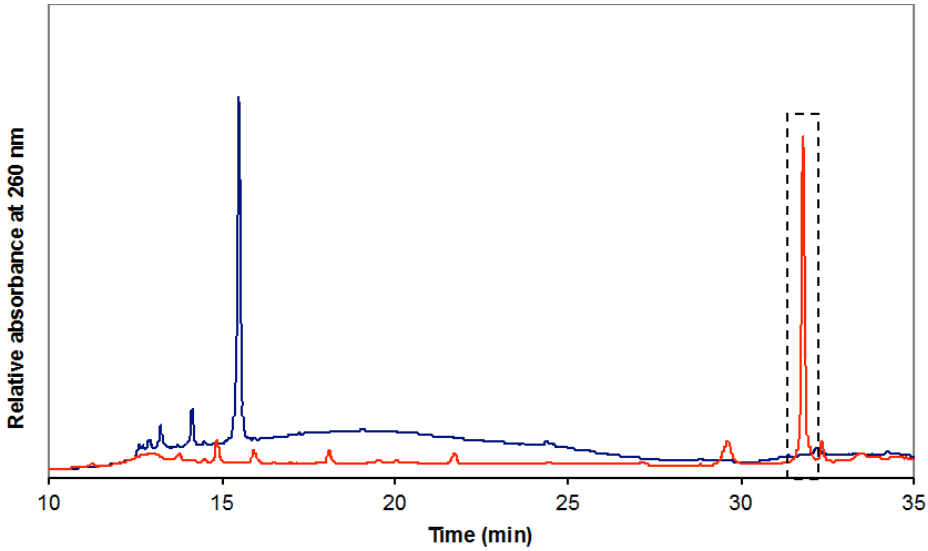

Figure S1. RP-HPLC characterization of PEG_M-d synthesis. Blue line: **d**, red line: reaction mixture of PEG_M-**d** synthesis. The peak included in the dotted rectangular represents the collected PEG_M-**d** during the HPLC purification step.

Optimization of ligation conditions

The ligation step of PEG_M-**D** was thoroughly optimized by investigating the effect of three different temperatures (10, 16 and 21°C) and the role of incremental incubation times. The ligation yields are summarized in Table S1 and an example of RP-HPLC elution profiles of the ligation at 21°C is reported in Figure S2.

The ligation at 21°C was faster than with the other temperatures. After 20 minutes at 21°C the reaction has almost come to completeness, since the maximum amount of PEG-Oligo detected by RP-HPLC (Table S1) was 50% of the starting Oligo-Acceptor **A**. During the RP-HPLC analysis the dsDNA PEG-Oligo may be denatured (that means not-annealed) and therefore the calculation of the ligation yields cannot reach the 100%. For this reason we investigated the ligation reaction at 21°C during time by gel electrophoresis.

Table S1: **PEG_M-D to A ligation yields by RP-HPLC at different annealing temperatures and ligation times.**

Temperature (°C)	Incubation time (min)				
	10	***20***	***30***	***60***	***120***
10	4.49[a]	23.71	23.09	43.31	50.94
16	3.28	29.14	33.35	38.02	40.99
21	1.59	45.85	42.23	47.50	50.46

[a] Yields (%) are calculated on the basis of peak area of ligated PEG-Oligo with respect to the starting area of peak A (Oligo-Acceptor).

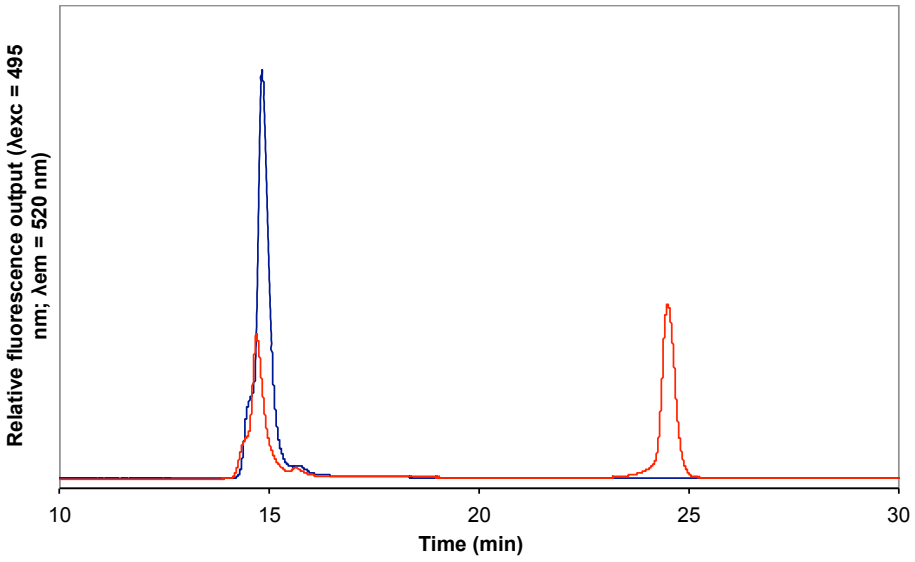

Figure S2. RP-HPLC characterization of the PEG_M-D to A ligation. Blue line: **A** before ligation. Red line: PEG_M-**D** to **A** ligation mixture of at 21°C after 2 h.

Mismatched dimer formation

Our experimental design included the formation of an EcoRI site in the ligated oligo for analytical purposes. We observed that the mismatched annealed ends of our oligos were stable enough at the temperature of the ligation to become substrate of T4 DNA Ligase. To test our hypothesis we verified whether **D** or **A** can self-anneal and self-ligate in the control experiment shown below. The dsDNA $\mathbf{D_F}$ (5'-FAM modified **d** annealed to the complementary **d'** oligonucleotide) and the dsDNA **A** (oligo **a** annealed to **a'**, see Table 1) were employed. The overhangs of $\mathbf{D_F}$ (AAT-) lead to a single nucleotide mismatch in the homo-dimer formation (See Figure S3.A), and, similarly, the overhangs of **a'** (TTA-) allows an imperfect pairing of **A** to **A** (Fig. S3.B). To investigate the activity of T4 DNA ligase in the presence of these mismatches the self ligations of **A** and of $\mathbf{D_F}$ were performed separately, as depicted in the cartoon in Figure S3.A and S3.B, in order to verify the formation of homo-dimers. Conditions were the same employed in the experiment with PEG_M-Donor (Figure 2). The ligation mixtures and the dsDNA controls were loaded on a 20% Native-PAGE (Figure S3.C) and the oligonucleotides were detected by the fluorescence of the reporter FAM.

In Native-PAGE (Figure S3.C), the $\mathbf{D_F}$ to $\mathbf{D_F}$ (lane 3) and the **A** to **A** (lane 4) ligation mixtures show the formation of the two homo-dimers of $\mathbf{D_F}$ and **A** despite a single nucleotide mismatch of the two overhangs. At the conditions employed for the ligation the formation of the mismatched **A** homo-dimer is more efficient than that of $\mathbf{D_F}$. Hence, we can conclude that T4 DNA ligase enzyme despite the presence of a single nucleotide mismatch can form the dimers of **A**, explaining the detection of the "unknown" band of Figure 3.

Our design, including the formation of an EcoRI for analytical purposes, led to annealing of the ends of **A** oligo at the temperature of the ligation to become substrate of T4 DNA Ligase. Further protocols will avoid the inclusion of this particular restriction site in our experimental design.

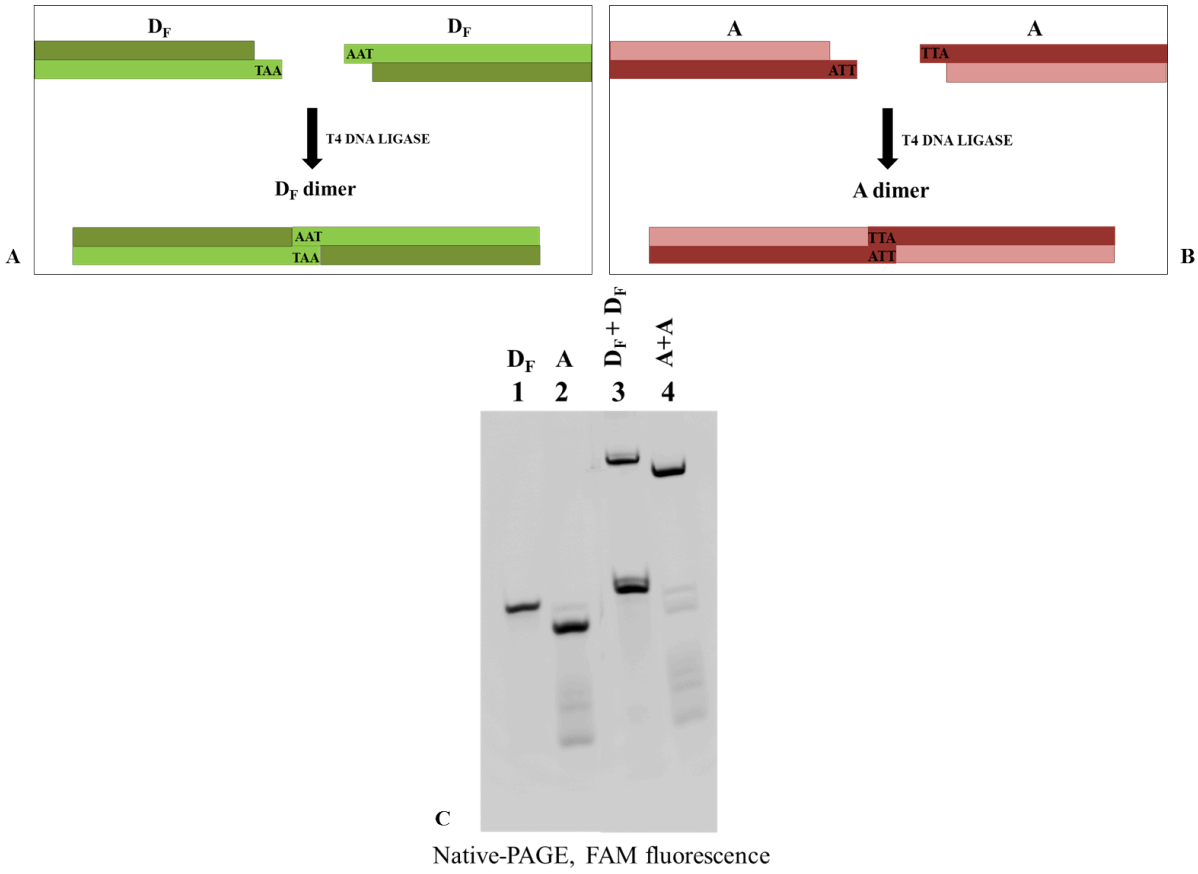

Figure S3. Scheme of the formation of the mismatched homo-dimers. Scheme for the mismatched homo-dimer D_F (Panel A). Scheme for the mismatched homo-dimer A (Panel B). The oligonucleotides on the Native-PAGE (20%) were visualized by the fluorescence of FAM (Panel C). Double stranded controls were loaded as well as the ligation mixtures in this order: **1.** dsDNA **D_F**; **2.** dsDNA **A**; **3. D_F + D_F** Ligation mixture; **4. A + A** Ligation mixture.