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# Single nucleotide specific detection of DNA by native chemical ligation of fluorescence labeled PNA-probes

Christian Dose[b] and Oliver Seitz[a,*]

[a]*Institut für Chemie der Humboldt-Universität zu Berlin, Brook-Taylor-Strasse 2, 12489 Berlin, Germany*
[b]*California Institute of Technology, Division of Chemistry and Chemical Engineering, 91125 Pasadena, CA, USA*

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**Abstract**—DNA-directed chemical ligations provide the opportunity to diagnose DNA sequences with very high sequence specificity. Fluorescent labels have been attached to reactive probes to enable the homogeneous detection of DNA and RNA. However, it has frequently been found that the attachment of fluorescent labels results in decreases of ligation fidelity. Herein we describe the development of a fluorogenic ligation reaction that provides for $10^2$-fold to perfect sequence selectivity. The reaction is based on the isocysteine-mediated native chemical PNA ligation. It is shown that DNA-induced rate accelerations of ~43.000-fold can be obtained through subtle variations of the ligation conditions. PNA–thioesters and isocysteine–PNA conjugates were labeled with FAM and TMR fluorophores, respectively. For gaining rapid synthetic access, a convenient on-resin labeling approach was developed. A new PNA monomer featuring an Alloc-protected lysine side chain was synthesized and coupled in solid-phase PNA synthesis. In the event of a ligation reaction the two fluorophores are brought into proximity. It is shown that fluorescence resonance energy transfer provides a positive fluorescence signal which is specific for product formation rather than for loss of starting materials. Single base mutations can be detected within minutes and with very high sequence selectivity at optimized conditions.


## 1. Introduction

In the last 10 years tremendous progress has been achieved in developing non-enzymatic DNA-templated reactions.[1] Since the pioneering work of Orgel[2] and Shabarova[3] a variety of chemistries have been made available. Chemical reactions that are controlled by a nucleic acid template provide the opportunity to explore molecular evolution,[4,5] to tag or even amplify small-molecule libraries[6,7] and to construct DNA-based nanoarchitectures and nanodevices.[8,9] An important application of DNA-templated reactions is in diagnostics where the challenge arises to detect specific DNA or RNA sequences in a rapid and accurate manner.[10–34] In these chemical assays, product formation serves as an indicator for the presence of the oligonucleotide target. One of the advantages offered by non-enzymatic reaction techniques not provided by enzymatic reaction formats is the feasibility of performing reactions with DNA-analogues, on RNA templates, and/or within living cells.[35–40] Yet another important advantage concerns the sequence specificity of DNA-directed reactions. We have recently shown that a chemical ligation method, the native chemical PNA ligation, can discriminate a particular DNA from its single-base mutant by more than $10^3$-fold differences in ligation rates.[13]

An important issue in the design of DNA-diagnostic chemical reactions is the detection of the formed product. Gel-electrophoretic and HPLC-based separation techniques have most frequently been used as a precise yet time-consuming means of reaction monitoring. MALDI mass spectrometry has been demonstrated to enable rapid and accurate detection of reaction products if desired in a multiplexed format.[11,14,28,30] However, these methods are off-line methods. The majority of the read-out systems in bioassays are based upon fluorescence measurements, which enable real-time measurements and, thus, provide advantages as far as ease, sensitivity, and speed of detection are concerned. Surprisingly, DNA-directed reactions between fluorescently labeled oligonucleotides have been explored by only few groups.[10,18–24,33,34,41] Prior to an envisaged application in DNA diagnosis several issues

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* Corresponding author. Fax: +49 030 2093 7266; e-mail: oliver.seitz@chemie.hu-berlin.de

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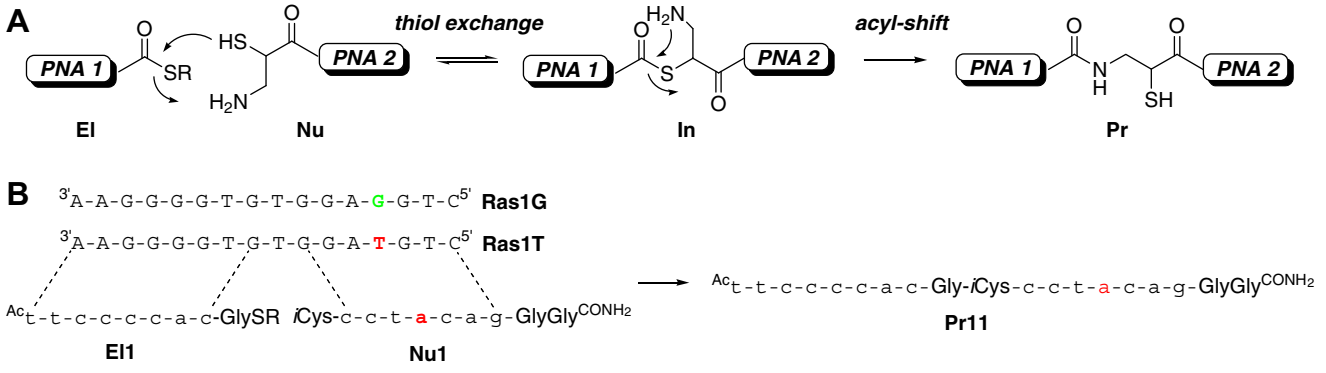
need to be addressed. First, the ligation chemistry must be compatible with the functional groups found in both oligonucleotides and fluorophores to avoid formation of by-products. Second, the fluorescent reporter groups should not interfere with probe-target hybridization, in order to maintain both a high rate ratio between the templated and the nontemplated reaction (required to reduce unspecific background signals) and high sequence fidelity. Third, read-out of the reaction should be specific for product formation rather than conversion of starting materials because consumption of starting materials may also originate from non-templated reactions such as hydrolysis. The previous fluorogenic detection chemistries have met many of these demands. For example, in Kool's powerful quenched autoligation probes (QUAL probes) an iodide leaving group has been replaced by a sulfonate based quencher group.[20] The sequence specificity of this reaction dropped from 180-fold for unlabeled ligation probes to 35-fold based on ligation on single mismatched templates.[16,18] The innovative DNA-triggered fluorophore and metal release systems reported by Taylor and Krämer, respectively, have 37-fold mismatch discrimination and less.[29,33,34] We sought for a fluorogenic method that provides for higher match/mismatch discrimination. We have recently introduced a PNA-based native chemical ligation system, which featured extraordinary $10^5$-fold DNA-triggered rate accelerations and very high $>10^3$-fold single nucleotide specificity.[12] We now report on the development of a fluorescence read-out system that is specific for product formation. The detection method is based on fluorescence resonance energy transfer (FRET). The required labeling involved the use of a novel protected lysine-containing PNA monomer which allowed convenient internal labeling of PNA-probes during solid phase synthesis. In this study different modes of fluorescence labeling are evaluated and it is shown that the length of the linker and the length of the ligation probe itself critically affect the responsiveness of FRET-based signaling. The study resulted in ligation probes that can signal the presence of target DNA by intensification of fluorescence within minutes and, perhaps most importantly, with very high sequence fidelity.

## 2. Results and discussion

### 2.1. Design and synthesis of the chemical ligation probes

DNA-directed chemical synthesis requires that reactive DNA oligomers or analogues are aligned by the template. Hence, the sequence fidelity of the reaction is primarily influenced by the specificity of the mutual recognition between reactive probes and the template.[26,42] Bearing in mind that the DNA-analogous peptide nucleic acids (PNA) bind complementary DNA and RNA with very high sequence specificity[43–45] we chose to use PNA and to equip PNA with reactive groups that confer efficient coupling reactions at physiological conditions.[14] Hallmarks of the ligation system shown in Scheme 1 are (i) ligation occurs opposite to an unpaired template base, which was demonstrated to confer higher sequence specificity than nick ligations[46] and (ii) use of isocysteine rather than cysteine in native chemical ligation to reduce the rate of the non-templated background reaction[47] and to facilitate catalytic turnover at low target concentrations.[12] The reaction of the electrophilic partner **El** and the nucleophilic probe **Nu** is assumed to proceed via the thiol exchange product **In** which is subject to a $S \rightarrow N$ acyl shift to form ligation product **Pr**. The reactive PNA-probes **El** and **Nu** were designed to target the carcinogenic single nucleotide G12V mutation of a 16mer *ras*-gene segment (Scheme 1B). The discrimination position was incorporated in the center position of 7mer probe **Nu** in order to maximize the influence on the sequence selectivity of hybridization and, hence, of the chemical ligation.[14]

The designed ligation required PNA bearing an *N*-terminal isocysteine residue. We decided to use Boc/Trt-protected isocysteine **1** as last building block in the solid-phase assembly of commercially available Boc/Cbz-protected PNA-monomers (Fig. 1). We optimized our previously reported synthesis,[47] which now provides Boc-*i*Cys(Trt) **1** in 4 steps starting from racemic thiomalic acid in 42% overall yield with only two chromatographic separations (see experimental part). The solid-phase synthesis of ligation probes **El1** and **Nu1** was described earlier.[12]




**Scheme 1.** (A) Reaction mechanism of the isocysteine-mediated native chemical PNA-ligation. (B) Typical PNA-probes and DNA sequences used in the template-controlled chemical ligation. R = $(CH_2)_2SO_3H$.

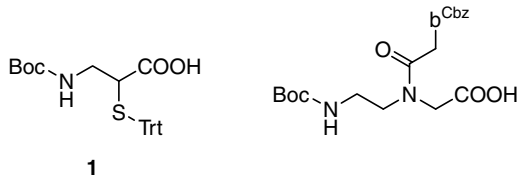



**Figure 1.** Boc/Trt-protected isocysteine **1** and Boc-protected PNA-monomers used in PNA solid-phase synthesis. b = adenine (a), guanine (g), cytosine (c) or thymine (t, non-protected); Trt, trityl.

### 2.2. Template-directed PNA-ligation reactions

In native chemical ligation-like reactions, thiols are added to maintain a reducing environment and to generate thioesters with higher reactivity.[48,49] We previously used benzylmercaptan (BnSH) as additive.[13] However, the low aqueous solubility, toxicity, and strong odour of BnSH render the handling unpleasant. We explored sodium 2-mercaptoethanesulfonate (MESNa) as alternative. Chemical ligation reactions of PNA-probes **El1** and **Nu1** were carried out at 25 °C in a pH 7.4 buffer containing 10 mM NaCl, 10 mM $NaH_2PO_4$, and 10 mM MESNa. HPLC-analysis revealed that the template-independent bimolecular ligation proceeded slowly ($k_{init} = 1.8 \times 10^{-13}\ M^{-1}\ s^{-1}$) at 1 μM concentration furnishing only 1.6% product **Pr11** after 24 h. Remarkably, on match DNA **Ras1T** product **Pr11** was formed extremely fast reaching the 50% stage within 90 s (Fig. 2A). Comparison of the initial rates revealed that the ligation is 43.195-fold accelerated on target DNA **Ras1T**. The initial rate $k_{init} = 8 \times 10^{-9}\ M^{-1}\ s^{-1}$ of the templated reaction including MESNa as additive is almost 4-fold higher than the initial rate $k_{init} = 2 \times 10^{-9}\ M^{-1}\ s^{-1}$ determined previously for the reaction in presence of BnSH. This enhanced ligation rate was surprising due to identical reactivities of the benzyl and ethanesulfonic-thioester in non-templated ligations at higher concentration (Fig. 2B and Table 1).

Next, we examined the sequence fidelity of the ligation reactions. The reaction of **El1** with **Nu1** on mismatched DNA **Ras1G** gave 6.4% yield after 60 min. Comparison of the initial rates indicated that the reaction proceeded 450-fold faster on matched DNA than on singly mismatched DNA. This match/mismatch discrimination is significantly lower than the 3450-fold selectivity determined for the ligation in presence of BnSH. Reductions of the salt concentration from 150 mM NaCl to 10 mM used in reactions in presence of BnSH resulted in decreases of the sequence specificity (190-fold). This was expected due to the reported tendency for unselective binding of PNA at low ion concentration.[50] To increase the sequence fidelity, the *i*Cys containing probe was shortened by one nucleobase, which resulted in 1350-fold single nucleotide discrimination measured in the ligation of *i*Cys-cctaca-GlyGly$^{CONH_2}$ **Nu2** and PNA–thioester **El1**.

### 2.3. Design and synthesis of fluorescence labeled PNA-ligation probes

The use of two chromophores that communicate in a distance-dependent manner allows real-time measurements of binding events or chemical reactions. In the context of DNA-directed ligation reactions, a product specific fluorescence signaling is desired. We chose a fluorescence signaling method based on fluorescence resonance energy transfer (FRET) between a 6-carboxyfluorescein (FAM) labeled probe and a 5-tetramethylrhodamine (TMR) labeled probe (Scheme 2). In this detection system, the fluorescence donor FAM and the fluorescence acceptor TMR will be brought into proximity upon ligation. This would result in decreases of FAM fluorescence with concomitant enhancement of TMR emission.

To harvest the full potential of FRET it is necessary to attach the fluorophores in close proximity. However, labeling at the ligation site should be avoided in order to prevent collisional quenching of both donor and acceptor emission. A spacing of 10–12 nucleotides between the two labels will align the fluorophores on one

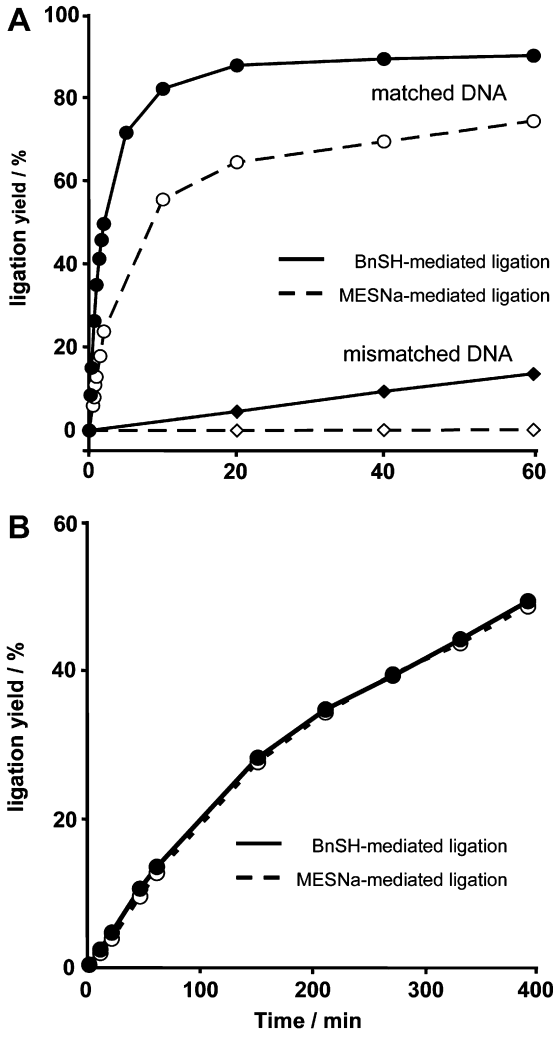
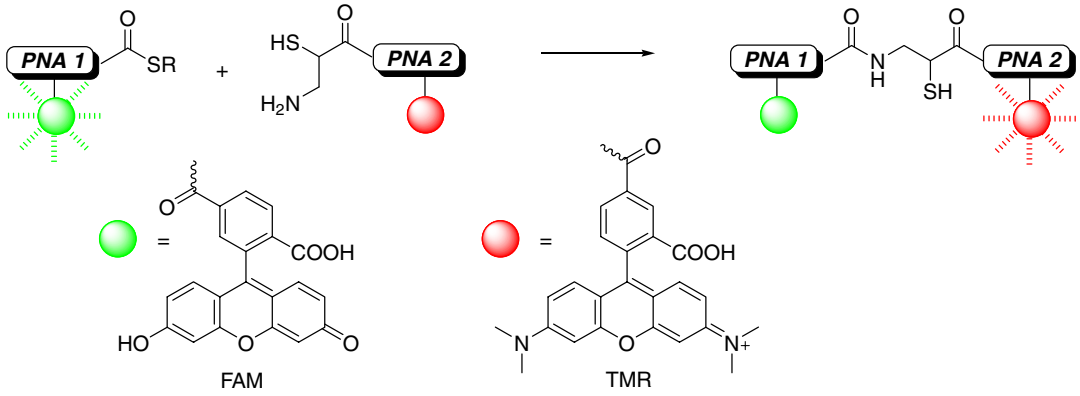



**Figure 2.** Time courses of the *i*Cys-mediated PNA-ligation. (A) 1 μM **El1** and **Nu1** on matched DNA **Ras1T** (cycles) or single mismatched DNA **Ras1G** (diamonds). Conditions: 10 mM NaCl, 10 mM $Na_2HPO_4$, 10 mM MESNa (filled symbols) or satd BnSH (open symbols), pH 7.4, 25 °C. (B) 100 μM **El1** and **Nu1**. Conditions: 100 mM $Na_2HPO_4$, 10 mM MESNa (open cycles) or satd BnSH (filled cycles), pH 7.4, 25 °C.

**Table 1.** Properties of the *i*Cys-mediated native chemical PNA-ligation on matched DNA **Ras1T** and single mismatched DNA **Ras1G**

| | $k_{init}$ ($\times 10^{-11}$ M $s^{-1}$) | Yield[a] (%) |
|---|---|---|
| Reaction | **El1** + **Nu1**, sat BnSH, 10 mM $Na_2HPO_4$, 10 mM NaCl | |
| Matched DNA | 206.2 | 74 |
| Mismatched DNA | 0.06 | 0.2 |
| Selectivity[b] | 3450:1 | 370:1 |
| Reaction | **El1** + **Nu1**, 10 mM MESNa, 10 mM $Na_2HPO_4$, 10 mM NaCl | |
| Matched DNA | 724.7 | 92 |
| Mismatched DNA | 3.81 | 13.7 |
| Selectivity[b] | 190:1 | 7:1 |
| Reaction | **El1** + **Nu1**, 10 mM MESNa, 10 mM $Na_2HPO_4$, 150 mM NaCl | |
| Matched DNA | 794.8 | 93 |
| Mismatched DNA | 1.77 | 6.4 |
| Selectivity[b] | 450:1 | 15:1 |
| Reaction | **El1** + **Nu2**, 10 mM MESNa, 10 mM $Na_2HPO_4$, 150 mM NaCl | |
| Matched DNA | 229.1 | 70 |
| Mismatched DNA | 0.17 | 0.6 |
| Selectivity[b] | 1350:1 | 117:1 |

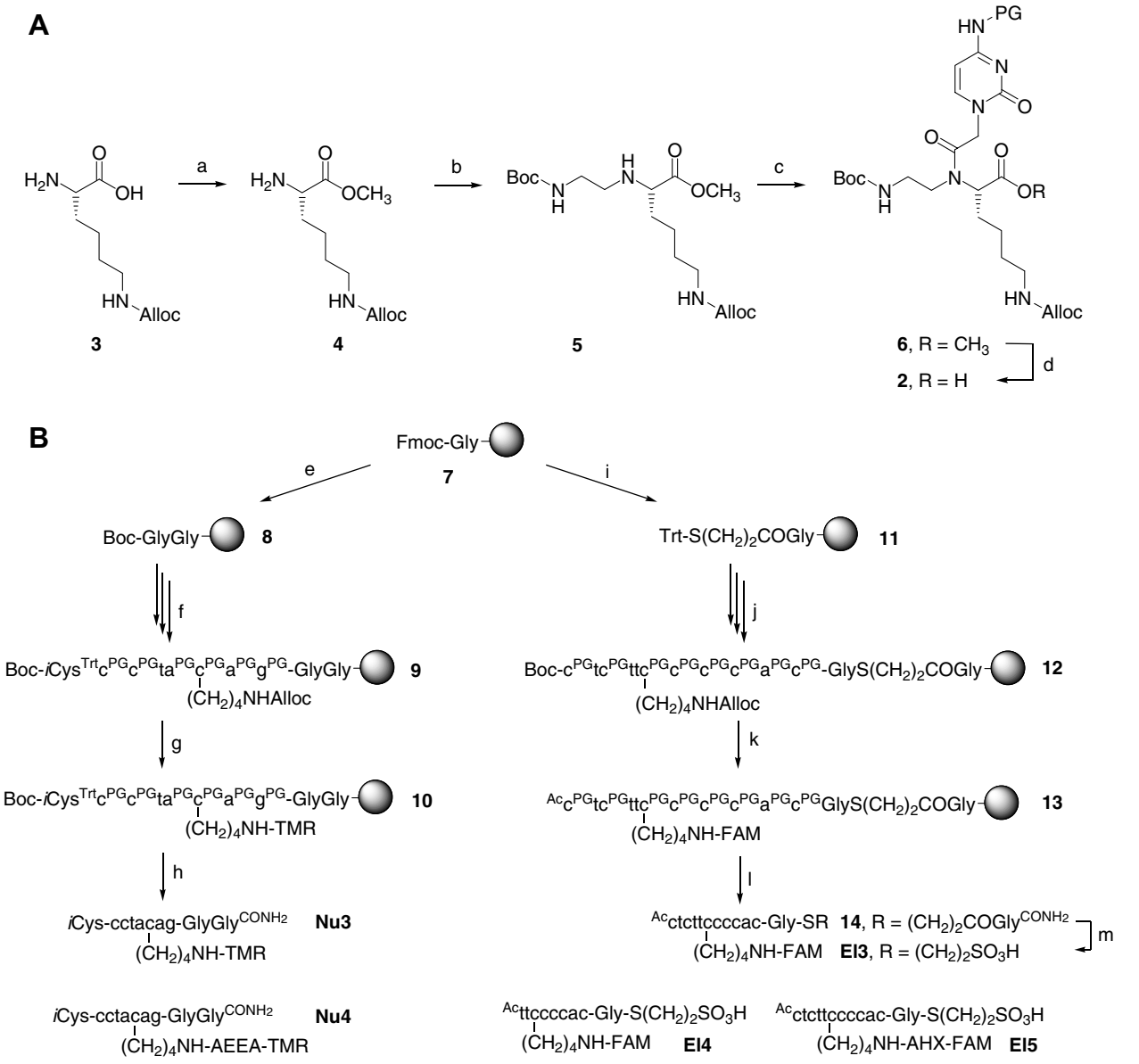
[a] Ligation yields after 60 min.
[b] Ratio between the initial rates of product formation or the ligation yields on matched DNA **Ras1T** and single mismatched DNA **Ras1G**.




**Scheme 2.** Ligation of probes labeled with FAM and TMR to reveal fluorescence resonance energy transfer (FRET) upon ligation.

side of the PNA–DNA helix in a 30–40 Å distance. Sixteen base pairs are required to provide a unique sequence in the human genome. To accommodate both constraints internal positions must be labeled. PNA offers the opportunity to conveniently install internal conjugation sites at the backbone of a PNA-monomer. For example, cysteine and lysine side chains have been incorporated by using the respective amino acid building blocks in PNA monomer synthesis.[47,51] We favored the introduction of a flexible lysine side chain to the PNA-backbone which may avoid the necessity of coupling additional linkers. Appella and Englund have recently reported the synthesis of a PNA-monomer containing a lysine side chain at the γ-position.[52] The fluorophore was attached to the PNA-backbone on solid phase after removal of a Fmoc-group of the lysine side chain. The reported protecting group strategy is not suited for the synthesis of labeled PNA–thioesters which would be rapidly cleaved from the solid support during treatment with base required for Fmoc-cleavage. To enable on-resin labeling in the solid-phase synthesis of PNA–thioesters the allyloxycarbonyl (Alloc)-group was used as an orthogonal protecting group for the ε-amino function of PNA monomer **2** (Scheme 3A). The synthesis of the triply protected PNA building block **2** was commenced from known Alloc-protected L-lysine **3**.[53] Treatment of **3** with thionylchloride in dry methanol provided the hydrochloride of methyl ester **4**. Reductive amination with Boc-protected glycinal, itself being obtained from *N*-Boc-glycine in high yield through a two-step 'weinreb amide' formation/lithium aluminum hydride reduction reaction sequence, furnished backbone building block **5** in 78% yield. The nucleobase portion, 1-*N*-carboxymethyl-4-*N*-Cbz-cytosin,[54] was coupled as a mixed anhydride by using pivaloyl chloride activation. Finally, the methyl ester in **6** was saponified upon treatment with aqueous LiOH in THF, which delivered the desired PNA-monomer **2** in 24% overall yield.

The lysine-residue containing PNA monomer **2** was used in the solid phase PNA-synthesis of three different PNA–thioester probes **El2**, **El3**, and **El4** labeled with the FRET donor FAM and two different *i*Cys-PNA-probes **Nu3** and **Nu4** containing the acceptor fluorophore TMR (Scheme 3B). Appended FAM has been reported to decrease the thermal stability of PNA–DNA duplexes.[55] The additional nucleotides in PNA **El3** served to compensate for such losses of target affin-




**Scheme 3.** (A) Synthesis of the lysine-containing PNA-monomer **2**. (a) thionylchloride, $CH_3OH$, 95%; (b) Boc-HN-$CH_2$-CHO, $NaBH_3CN$, $CH_3OH$ 74%; (c) 1-carboxymethyl-4-Cbz-cytosin, $ClCO_2(CH_3)_3$, NMM, DMF/$CH_3CN$ (1:1), 39%; (d) LiOH, $CH_3OH$, 87%; (B) Solid-phase synthesis of fluorescent labeled PNA-oligomers. (e) 1—DMF/piperidine, 2—Boc-Gly-OH/PyBOP/NMM/DMF, 3—$Ac_2O$/pyridine; (f) 1—TFA/*m*-cresole, 2—Boc-$b^{Cbz}$-OH or **2** or **1**/PyBOP/NMM/DMF, 3—$Ac_2O$/pyridine; (g) 1—$Pd(PPh_3)_4$/$(CH_3)_2NH{\cdot}BH_3$/DCM, 2—5-TMR/PyBOP/NMM/DMF; (h) 1—TFA/*m*-cresole, 2—TFA/TFMSA/*m*-cresole; (i) 1—DMF/piperidine, 2—Trt$S(CH_2)_2COOH$/PyBOP/NMM/DMF, 3—$Ac_2O$/pyridine; (j) 1—TFA/*m*-cresole, 2—Boc-$b^{Cbz}$-OH or **2**/PyBOP/NMM/DMF, 3—$Ac_2O$/pyridine; (k) 1—$Pd(PPh_3)_4$/$(CH_3)_2NH{\cdot}BH_3$/DCM, 2—6-FAM/PyBOP/NMM/DMF; (l) TFA/TFMSA/*m*-cresole; (m) 100 mM $Na_2HPO_4$, 10 mM MESNa, pH 7.4. PG = Cbz.

ity. The long tethers in probes **El4** and **Nu4** were incorporated with the intention to enhance the orientation factor (and, hence, FRET) by increasing the flexibility of the fluorescent groups. The synthesis of *i*Cys-containing PNA **Nu3** was commenced from Fmoc-Gly-loaded MBHA-resin **7**. The Boc/Cbz-protected PNA-monomers and the lysine containing building block **2** were coupled by an iterative procedure of (i) acidolytic Boc-cleavage, (ii) coupling of 4 equiv building block for 30 min in DMF after preactivation by PyBOP in presence of *N*-methylmorpholine (NMM), and (iii) capping with acetic anhydride in pyridine.[56] After coupling of Boc/Trt-protected isocysteine **1** to the resin the Alloc-group in **9** was removed by $Pd^0$-catalyzed reaction in presence of $(CH_3)_2NH{\cdot}BH_3$ as allyl scavenger.[57] Solid phase labeling of the lysine side chain was carried out by adding pre-activated 5-carboxytetramethylrhodamine (TMR) to the resin. For the introduction of the tether in probe **Nu4**, Alloc removal of **9** was followed by an acylation with Fmoc-aminoethyloxyethyloxyacetic acid (Fmoc-AEEA) in PyBOP-induced coupling reaction. The Fmoc-group was removed and the liberated amine subjected to on-resin labeling as described. Finally, treatment with TFMSA/TFA/*m*-cresol released the desired PNA-oligomers **Nu3** and **Nu4** in 3.5% and 4.4 % overall yield, respectively, after HPLC purification.

The solid phase synthesis of thioesters **El2**, **El3**, and **El4** was performed according to the Boc-strategy by using mercaptopropionyl resin **11**.[58] The subsequent steps were performed as described in the synthesis of **Nu3** and **Nu4**. Several attempts delivered desired PNA **El3** in unacceptably low yields. We speculated that $(CH_3)_2NH{\cdot}BH_3$, which was used as nucleophilic allyl scavenger, would trigger thioester cleavage from the solid support during the Alloc-removal step. Indeed, application of dimethylbarbituric acid (DMB) as less nucleophilic alternative[59] followed by fluorescent labeling, subsequent cleavage from the solid support, and HPLC purification provided the desired PNA–thioester **14** in 6.9% overall yield. The synthesis of **El4** involved the coupling of Boc-protected aminohexanoic acid (Boc-AHX) to the lysine side chain in **12**. Subsequent Boc-removal, on-resin coupling of FAM, and final cleavage delivered **El4** in 8.9% overall yield after preparative HPLC. Finally, the propionic amide thioesters obtained by solid phase PNA-synthesis were quantitatively converted into the more reactive ethanesulfonic acid thioesters **El2**, **El3**, and **El4** by the treatment with 100 mM $NaH_2PO_4$ phosphate buffer (pH 7.4) containing 10 mM MESNa.

### 2.4. FRET-based read-out of the iNCL

The chemical ligation of fluorescence labeled PNA-probes **El2** and **Nu3** was performed on matched DNA template **Ras2T** as described for the unlabeled probes **El1** and **Nu1**. The reaction was analyzed by fluorescence spectroscopy (Fig. 3A). The fluorescence spectrum that was measured after 60 min reaction time showed the expected changes. However, the fluorescence response (decrease of FAM emission and increase of TMR emission) was considered as small when compared to alternative FRET-based assays.[55] The melting analysis of FAM-labeled PNA–DNA duplex **El2·Ras2T** failed to show a sigmoidal melting curve; in contrast to the unlabeled PNA·DNA-duplex **El1·Ras2T**, which displayed a $T_M$ = 33 °C. Apparantly, attachment of carboxyfluorescein to PNA interfered in PNA·DNA binding. The three additional nucleotides in FAM-probe **El3** improved the affinity for template **Ras2T** as suggested by the $T_M$ = 38 °C for the **El3·Ras2T** duplex. The comparison with $T_M$ = 49 °C measured for a duplex comprised of DNA **Ras2T** and nonmodified PNA $^{Ac}$ctcttccccac-Gly$^{CONH_2}$ confirmed the FAM-induced destabilization of PNA·DNA duplexes. Such duplex destabilization was not observed for TMR labeling. The $T_M$ = 36 °C for the TMR-labeled *i*Cys-PNA·DNA duplex **Nu3·Ras2T** was almost as high as the $T_M$ = 38 °C for the non-labeled *i*Cys-PNA·DNA duplex **Nu1·Ras2T**. These results indicate that at 25 °C both extended FAM-PNA thioester **El3** ($T_M$ = 38 °C) and TMR-*i*Cys-PNA **Nu3** ($T_M$ = 36 °C) can be aligned and, thus, ligated by the template. In fact, addition of match DNA **Ras2T** to **El3** and **Nu3** triggered a notable change in fluorescence characterized by a significant decrease of the FAM emission at 523 nm and a simultaneous increase of the TMR emission at 585 nm (Fig. 3B). This clearly demonstrated the expected FRET between both fluorophores.

The incorporation of spacers is a commonly applied means to improve the efficiency of FRET. Figure 3C shows the fluorescence spectra of flexibly tethered fluorophores in FAM-PNA thioester **El4** and TMR-*i*Cys-PNA **Nu4** before and 60 min after ligation. It became apparent that the fluorescence response was lower than in ligation of **El3** and **Nu3**. The significantly smaller TMR emission measured for ligation of **El4** and **nu4** may be explained by a higher propensity for collisional quenching between fluorophores that are connected via long (Lys side chain + spacer) and flexible tethers.

Among the three studied FRET ligation systems, ligation of **El3** with **Nu3** clearly provided the highest responsiveness of fluorescence. We next investigated the influence of the FRET labels on the sequence fidelity of the native chemical ligation. Toward this end,

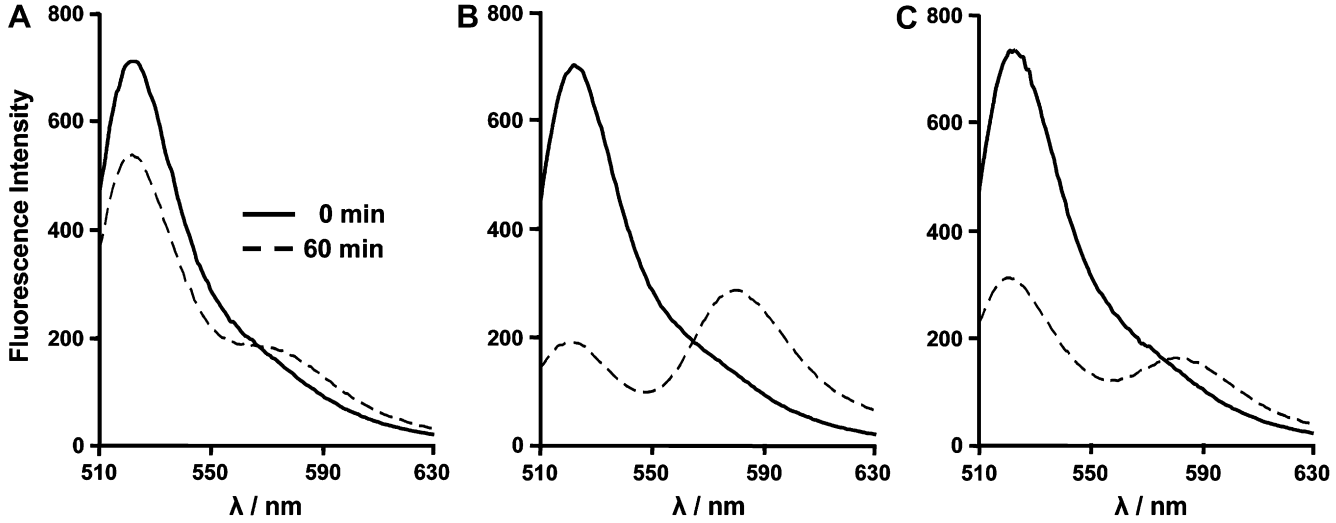



**Figure 3.** Fluorescence monitoring of the *i*Cys-mediated PNA ligation at 0 min (—) and after 60 min (– –). (A) Reactions of **El2** and **Nu3** on matched DNA **XX**, (B) **El3** and **Nu3** and (C) **El4** and **Nu4** on matched DNA $^{3'}$GAGAAGGGGTGTGGA***T***GTC$^{5'}$,**Ras2T.** Conditions: 1 μM probes and template, 150 mM NaCl, 10 mM $NaH_2PO_4$, 10 mM MESNa, 25 °C, pH 7.4.

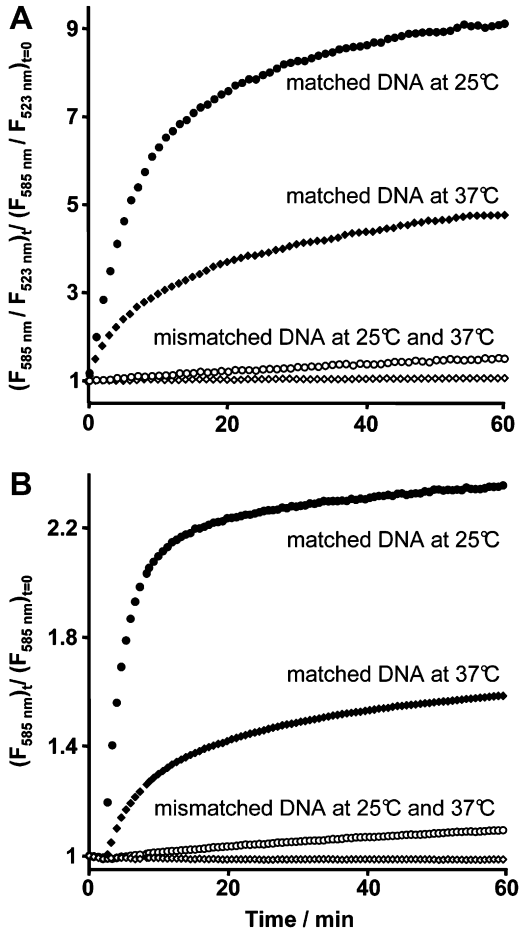



**Figure 4.** Time course of (A) the ratio between fluorescence intensities at 585 nm and 523 nm and (B) fluorescence intensity at 585 nm measured for the ligation of **El3** and **Nu3** on matched DNA **Ras2T** (filled symbols) and single mismatched DNA **Ras2G** (open symbols) at 25 °C (cycles) and 37 °C (diamonds).

matched DNA **Ras2T** or single mismatched DNA $^{3'}$GAGAAGGGGTGTGGA***G***GTC$^{5'}$, **Ras2G**, was added to reactive probes **El3** and **Nu3**, and the ligation followed by means of the ratio of the donor/acceptor fluorescence intensities. The time course of the relative $F_{585}/F_{523}$ ratio shows a rapid signal increase in the presence of matched DNA reaching 9-fold enhancement after 60 min (Fig. 4A). The ligation on single mismatched template was a rather inefficient reaction. A comparison of the initial fluorescence signaling rates revealed that at 25 °C the reaction on single mismatched DNA was 100-fold slower than on matched DNA (Table 2). Expectedly, increasing the reaction temperature from 25 °C to 37 °C improved the match/mismatch discrimination to 380-fold; however, at the cost of the signaling rate.

An alternative method to read-out product formation is to directly measure the fluorescence enhancement of TMR emission at 585 nm. Figure 4B shows that the addition of match DNA triggered a rapid increase of the fluorescence intensity at 585 nm. It was found that the match/mismatch discrimination calculated from initial TMR signal rates is higher than the discrimination determined from the initial $F_{585}/F_{523}$ rates (126:1 vs 100:1, Table 2). Most noticeable were the results obtained for ligation at 37 °C. The TMR signal remained unchanged when attempting ligation in presence of mismatched DNA. One possible explanation for the higher specificity of TMR signaling than of $F_{585}/F_{523}$ signaling is that, at 470 nm excitation, increases of the acceptor emission can only occur due to FRET from the excited donor fluorophore. In contrast, decreases of donor emission can also originate from non-ligation processes such as quenching by target nucleobases. The kinetics of $F_{585}/F_{523}$ and TMR signaling indicate that FRET is indeed the result of a ligation since adjacent hybridization, expected to occur with milliseconds,[60] without ligation would produce an almost instantaneous change of fluorescence spectra.

It is instructive to compare the sequence specificity of the FRET-ligation system with the sequence specificity observed in the ligation of unlabeled probes. The *i*Cys-PNA conjugates probe the SNP-site and are the probes that are expected to have a major influence on sequence specificity of ligation. The TMR-labeled *i*Cys-PNA **Nu3** ($T_M$ = 36 °C) and the non-labeled *i*Cys-PNA **Nu1** ($T_M$ = 38 °C) showed comparable target affinities. Nevertheless, at 25 °C the ligation of unlabeled probes occurred with higher match/mismatch discrimination (450:1 vs 127:1) than ligation of labeled probes. Such negative effects of fluorescent reporter groups on ligation selectivity are known.[21] However, the sequence specificity of fluorescence signaling at 25 °C was still of the order of $10^2$-fold which is in the range of the best unlabeled DNA ligation methods.[16,28]

In the literature there is a vast body of data on DNA detection probes that produce fluorescence signals upon hybridization. Typical examples are probes that draw upon the distance-dependent interaction of two chromophores such as in Molecular Beacons[61] and Adjacent Probes[62] or probes that rely on environmentally sensitive fluorophores such as those found in HyBeacons,[63] Light-Up Probes,[64] and FIT-Probes.[65,66] However, hybridiza-

**Table 2.** Initial rates of fluorescence signaling by ligation of PNA-probes **El3** and **Nu3** on matched DNA **Ras2T** and single mismatched DNA **Ras2G**

| Reaction | $k_{init}$ rel. $F_{585}/F_{523}$ ($\times 10^{-4}$ min$^{-1}$) | | $k_{init}$ rel. $F_{585}$ ($\times 10^{-4}$ min$^{-1}$) | |
|---|---|---|---|---|
| | **El3 + Nu3** | | | |
| | 25 °C | 37 °C | 25 °C | 37 °C |
| Matched DNA **Ras2T** | 8424 | 5091 | 3150 | 559 |
| Mismatched DNA **Ras2G** | 84.3 | 13.4 | 25.1 | −0.8 |
| Selectivity[a] | 100:1 | 380:1 | 126:1 | Beyond detection limit |

[a] Ratio between the initial rates of fluorescence monitoring on matched DNA **Ras2T** and single mismatched DNA **Ras2G**.

tion alone usually is not sufficient to distinguish matched from singly mismatched targets with greater than 10fold selectivity. In contrast, ligation reactions typically employ two short probes rather than hybridization of one long probe. Hybridization of short probes, and thus ligation, proceeds with higher sequence fidelity than hybridization of long probes. Few laboratories have investigated chemical reactions to generate fluorescence signals due to the presence of oligonucleotide sequences. Taylor showed techniques in which the fluorescence signal is triggered by hydrolysis of acyl-quenched fluorophors.[33] The template-directed deacylation proceeded within hours with 30-fold match/mismatch selectivity. Kool and coworkers introduced a powerful ligation format which was used in multiple approaches like FRET-probes,[18] QUAL-probes[19,20,22,23] or the QUAL-FRET probe design[24] even within living cells. Each detection technique needed hours rather than minutes to provide intensive fluorescence signals. The introduction of the fluorescent reporters reduced the sequence selectivity from 180-fold to 12-fold. We have also noted decreases of match/mismatch discrimination upon attachment of fluorophores to the ligation probes. However, the selectivity of FRET-detected native chemical PNA ligation presented by us is of the order of 100-fold at 25 °C and beyond the limit of detection at optimized conditions.

## 3. Conclusions

We have investigated the *i*Cys-mediated native chemical PNA-ligation which offers an efficient alternative to the commonly used enzymatic and chemical methods for the detection of single base mutations in DNA. The ligation method is characterized by dramatic >40.000-fold increases of reaction rate in presence of perfectly matched DNA templates. The sequence selectivity is in the range of $10^3$-fold. We have also shown that speed and sequence selectivity of the ligation chemistry can be influenced by changing the thiol leaving group. Moreover, we have introduced the synthesis of a lysine-residue containing PNA-monomer which allowed rapid internal labeling of PNA-probes on the solid phase. It has been shown that fluorescence resonance energy transfer (FRET) upon ligation is a convenient and precise means of detecting single base mutations in DNA, with signaling of mismatched target being below the detection limit at optimized conditions. In future work we will take advantage of the high sequence selectivity of native chemical PNA ligation in the establishment of a ligation format usable for real-time PCR.

## 4. Experimental

### 4.1. General procedures and materials

Commercial reagents were used without further purification. Solvents were dried by standard methods and freshly distilled prior to use. DMF was purchased in peptide synthesis grade. PNA monomers and PyBOP were purchased from PerSeptive Biosystems. Resins and protected amino acids were purchased from Novabiochem. 6-Carboxyfluoresceine (FAM), 5-Carboxytetramethylrhodamine (TMR), and Boc-6-Ahx-OH were purchased from Fluka. Water was taken from a MembraPure Astacus Water Purification System from MembraPure GmbH (Bodenheim). Flash chromatography was performed using Merck Silicagel 60. TLC was performed with Merck aluminum-backed Silicagel 60 $F_{254}$ plates. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, concentrations are given in units of g 100 $mL^{-1}$. Melting points were measured using a Boetius apparatus of Nagena-Rapido in open glass plates and are not corrected. The concentrations of the stock solutions of the oligonucleotides were determined by measuring the optical density at $\lambda = 260$ nm on a Varian Cary 100 UV/vis spectrometer. The specific absorption coefficients $\varepsilon$ of the oligonucleotides were calculated using the nearest-neighbor-method. DNA was purchased from MWG or Biotez (Berlin) in high purity salt free (HPSF) quality and was purified by HPLC if necessary. $^1$H- and $^{13}$C NMR spectra were recorded on a Bruker DPX-300 spectrometer at room temperature. The signals of the residual protonated solvents ($CDCl_3$ or DMSO-$d_6$) were used as reference signals. Coupling constants $J$ are reported in Hz. Solid-phase synthesis was carried out in PE syringes, equipped with Teflon filters and Teflon piston, purchased from MultiSynTech.

### 4.2. $T_M$-Measurements

UV melting curves were measured at 260 nm by using a Varian Cary 100 spectrometer equipped with a peltier block. A degassed aqueous solution of 10 mM NaCl and 10 mM $NaH_2PO_4$ adjusted at pH 7.0 using 2 M NaOH was used as buffer. The oligonucleotides were mixed to 1:1 stoichiometry and the solutions adjusted to a final duplex concentration of 1 μM. Prior to analysis, the samples were heated to 90 °C and cooled within 3 h to a starting temperature of 15 °C. The samples were heated to 90 °C with a rate of 1 °C/min. $T_M$-values were defined as the maximum of the first derivative of the melting curve.

### 4.3. Mass spectrometry

High resolution mass spectra were measured on a Finnigan LTQ-FT mass spectrometer by electrospray ionization. FAB-MS was recorded on a JOEL JMS-SX102A machine, applied matrices were given for each compound. MALDI-TOF mass spectra were measured on a Voyager-DE™ Pro Biospectrometry Workstation of PerSeptive Biosystems. A 10% solution of sinapinic acid in MeCN/1%TFA (1:1) was used for generating the probe–matrix mixture.

### 4.4. HPLC chromatography

Analytical HPLC was performed on a Merck-Hitachi Elite LaChrom machine using a RP-C18-A5μ 'Polaris' column (PN 2000-250x0.46, Varian). Detection of the signals was achieved with a photodiode array detector at 280 nm. Eluents A (0.1% TFA in water + 1% MeCN) and B (0.1% TFA in MeCN + 1% water) were used in a

linear gradient at 55 °C with a flow rate of 1 mL/min. Gradient: 3% → 30% B in 30 min (Gradient A) or 3% → 40% B in 20 min (Gradient B). Preparative HPLC was performed on a Gilson Nebula 321 Series using a semipreparative Column (VP 250/10 Nucleosil (100-7) HD or SP 125/10 Nucleodur Gravity (5μ)/ Macherey& Nagel, Düren). Flow rate 3.5 mL/min or 6 mL/min at 55 °C.

### 4.5. Preloading of Fmoc-Gly onto the MBHA-linker

Resin loadings were aimed at approximately 0.3 mmol/g by adding the resin in excess. First, the resin was washed (5 × DCM, 3 min 5% DIPEA/DCM, 5 × DCM, 5 × DMF). For preactivation of the Fmoc–glycine, 1 equiv of PyBOP and 4 equiv of *N*-methylmorpholine were added to a solution in DMF (0.1 M). After 2 min of preactivation, the mixture was added to the resin. After 12 h the resin was washed (5 × DMF, 5 × DCM, 5 × DMF). For capping, the resin was treated with acetic anhydride/pyridine (1:9, 2 × 1 mL) for 5 min, washed (5 × DMF, 5 × DCM, 5 × DMF, 5 × DCM), and finally dried in vacuo. Loading was determined by photometrical analysis.

### 4.6. Solid-phase PNA synthesis according to the Boc/Z strategy

*General washing procedure*: 5 × DMF (1 mL), 5 × DCM (1 mL), 5 × DMF (1 mL). *Boc-cleavage*: After treatment with TFA/*m*-cresol (19:1, 2 × 5 min, 1 mL) the resin was washed following the general washing procedure. *Coupling*: After preactivation of 4 equiv of the corresponding building block (final concentration 0.1 M in DMF) for 2 min using 4 equiv PyBOP and 6 equiv *N*-methylmorpholine, the solution was added to the resin. After 30 min the resin was washed following the general washing procedure. *Capping*: Acetic anhydride/pyridine (1:9, 1 mL) was added to the resin. After 5 min the resin was washed following the general washing procedure. *Cleavage from the solid support*: TFA/TFMSA/*m*-cresol (8:2:1, 500 μL) was added for 10 min and the resin was washed with DMF (5 × 1 mL) and DCM (5 × 1 mL). After 3 h, the resin was washed with TFA (2 × 500 μL). The combined solutions were concentrated under reduced pressure before addition of cold diethyl ether. The precipitated crude product was dissolved in water, analyzed by HPLC and MALDI-TOF/MS (sinapinic acid), and purified by preparative HPLC.

### 4.7. Nontemplated PNA ligations at high concentrations

The ligation buffer was comprised of an aqueous solution of $NaH_2PO_4$ (100 mM) and was freshly prepared prior to use. The pH was adjusted to 7.4 by using 2 M NaOH solution. Subsequent manipulations were carried out by avoiding unnecessary exposure to oxygen. The ligation buffer containing BnSH (4% v/v) or MESNa (10 mM) was placed in Eppendorf tubes and the appropriate amount of the PNA thioester (100 μM) was added for conversion into the desired thioester. After separation of residual BnSH by centrifugation and disposal of the organic layer the thiol containing PNA-conjugate (100 μM) was added and the reaction mixture was allowed to react for the denoted time by vortexing at 25 °C. Reaction aliquots of the reactions were quenched by adding TFA (5 μL) and analyzed by analytical HPLC.

### 4.8. Template controlled PNA ligation reactions

Aqueous stock solutions were prepared to contain 1–5 mM concentrations of DNA template or PNA. The ligation buffer was comprised of an aqueous solution of NaCl (10 mM or 150 mM), $NaH_2PO_4$ (10 mM), BnSH (4% v/v) or MESNa (10 mM) and was freshly prepared prior to use. The pH was adjusted to 7.4 by using 2 M NaOH solution. Subsequent manipulations were carried out by avoiding unnecessary exposure to oxygen. The ligation buffer was placed in Eppendorf tubes and the appropriate amount of the PNA thioester conjugate and the DNA-template were added. After conversion into the desired thioester, the thiol containing PNA-conjugates were added and the reaction mixture (1:1:1 molar ratio) was allowed to react for the denoted time by vortexing at 25 °C. Aliquots (100 μL) of the reactions were quenched by adding TFA (5 μL) and analyzed by analytical HPLC.

### 4.9. Fluorescence resonance energy transfer measurements

Fluorescence spectra were recorded on a Varian Cary Eclipse fluorescence spectrometer (excitation: 470 nm; excitation slit width: 5 nm; emission slit width: 5 nm). The ligation buffer was a degassed aqueous solution containing NaCl (150 mM), $NaH_2PO_4$ (10 mM), MESNa (10 mM) and was freshly prepared prior to use. The pH was adjusted to 7.4 by using 2 M NaOH solution. Subsequent manipulations were carried out by avoiding unnecessary exposure to oxygen. The ligation buffer was placed in a fluorescence quartz cuvette (1 mL) purchased from Helma and the appropriate amount of PNA thioester, thiol containing PNA-conjugate, and DNA-template were added. The fluorescence signals were measured for the denoted time.

**4.9.1. Isocysteine-hydrochloride.** A suspension of (2-Oxo-1-oxa-4-thia-spiro[4.4]non-3-yl)-acetic acid (3 g, 13.9 mmol) and triethylamine (2 mL, 14.3 mmol) in dry toluene (50 mL) was stirred at room temperature until the solid was completely dissolved. The solution was cooled to 0 °C and diphenylphosphorylazide (3.08 mL, 14.3 mmol) was added dropwise. After stirring over 2 h at room temperature, the solution was heated up to 85 °C until the evolution of $N_2$ ceased. The reaction mixture was cooled to room temperature and EtOAc (40 mL) was added. The organic phase was washed with satd $NaHCO_3$ (30 mL), dest. $H_2O$ (30 mL), dried ($MgSO_4$), and concentrated under reduced pressure. The residue was dissolved in 6 N HCL (50 mL) and refluxed for 1.5 h. The aqueous solution was washed with diethyl ether (3 × 30 mL) and evaporated under reduced pressure. The crude product was used for the next synthesis step.

**4.9.2. (*S*-Trt)-isocysteine.** Crude isocysteine-hydrochloride was dissolved in TFA (50 mL) and tritylalcohol (3.9 g, 15.0 mmol) was added portionwise. After 30 min stirring at room temperature, the solvent was evaporated under reduced pressure. Diethyl ether (50 mL) was added and the solution was adjusted to pH 5 by the addition of 0.2 N sodium acetate. The originated solid was filtered and subsequently stirred in acetone (40 mL) at 40 °C for 30 min. The suspension was cooled to 0 °C and the solid was filtered and dried under reduced pressure to afford 2.89 g (61% over two steps) of the desired product as a white solid. $C_{22}H_{21}NO_2S$; mp = 170–172 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, 25 °C): $\delta$ 1.79–1.82 (m, 2H), 2.94–2.98 (m, 1H), 7.19–7.51 (m, 15H) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$, 25 °C): $\delta$ 40.7 ($CH_2$), 46.6 (CH), 67.1 (Cq), 127.2 (3 × CH), 128.5 (6 × CH), 129.8 (6 × CH), 144.9 (Cq), 171.4 (Cq, COOH) ppm; HRMS-FAB, *m*-NBA, pos.: Anal. calcd: 363.1293, found: 363.1298.

**4.9.3. *N*-Boc-(*S*-Trt)-isocysteine.** To a solution of Trt-*S*-isocysteine (2.89 g, 7.95 mmol) and triethylamine (1.23 mL, 9.54 mmol) in THF/$H_2O$ (45:35 mL) was added $Boc_2O$ (2.08 g, 9.54 mmol) in THF (10 mL) dropwise. The reaction mixture was stirred for 3 h at room temperature and $H_2O$ (40 mL) was added. Additionally the pH was adjusted to ~4 by adding concentrated acetic acid and the aqueous solution was extracted with ethyl acetate (3 × 40 mL) and dried ($MgSO_4$). The solvent was evaporated under reduced pressure and the residue was purified by silica gel flash chromatography (DCM/methanol, 95:5 + 1% formic acid) to afford 3.7 g (100%) of the desired product as white solid. $C_{27}H_{29}NO_4S$; $R_f$ = 0.32 (dichloromethane/methanol, 95:5 + 1% formic acid); mp = 58–60 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, 25 °C): $\delta$ 1.30 (s, 9H), 2.35–2.42 (m, 1H), 3.01 (s, 2H), 6.78 (s, 1H), 7.22–7.37 (m, 15H), 12.54 (s, 1H) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$, 25 °C): $\delta$ 28.07 (3 × $CH_3$), 42.26 ($CH_2$), 46.94 (CH), 67.28 (Cq), 77.60 (Cq), 126.81 (3 × CH), 127.97 (6 × CH), 129.10 (6 × CH), 144.04 (3 × Cq), 154.93 (Cq), 172.15 (Cq) ppm; HRMS-FAB, *m*-NBA, pos.: Anal. calcd: 463.1817, found: 463.1835.

**4.9.4. *i*Cys-cctacag-GlyGly$^{CONH_2}$ (Nu1).** Starting from Fmoc–glycine loaded MBHA-Resin **XX** (loading 0.3 mmol/g, 17 mg) a white solid was obtained by standard solid phase PNA synthesis described previously. $C_{81}H_{106}N_{44}O_{23}S$; yield: $OD_{260}$ = 60.4; 912 nmol, 9.2%; MALDI-TOF/MS (*m*/*z*): Anal. calcd for $[M+H]^+$: 2097, found: 2096; HPLC: $t_R$ = 12.3 min (Gradient A).

**4.9.5. *i*Cys-cctaca-GlyGly$^{CONH_2}$ (Nu2).** Starting from Fmoc–glycine loaded MBHA-Resin **XX** (loading 0.3 mmol/g, 17 mg) a white solid was obtained by standard solid phase PNA synthesis described previously. $C_{70}H_{93}N_{37}O_{20}S$; yield: $OD_{260}$ = 33.8, 597 nmol, 11.9%; MALDI-TOF/MS (*m*/*z*): Anal. calcd for $[M+H]^+$: 1806, found: 1806; HPLC: $t_R$ = 13.2 min (Gradient A).

**4.9.6. $^{AcHN}$ttccccac-GlyS$(CH_2)_2^{CONH_2}$ (El1).** Starting from Fmoc–glycine loaded MBHA-Resin **XX** (loading 0.3 mmol/g, 17 mg) a white solid was obtained by standard solid phase PNA synthesis described previously. $C_{90}H_{118}N_{42}O_{28}S$; yield: $OD_{260}$ = 151.4; 2.24 μmol, 23%; MALDI-TOF/MS (*m*/*z*): Anal. calcd for $[M+H]^+$: 2269, found: 2269; HPLC: $t_R$ = 13.2 min (Gradient A).

**4.9.7. *N*-Boc-glycinal.** A solution of Boc-glycine (5 g, 28.5 mmol) and *N*-methylmorpholine (6.9 mL, 62.8 mmol) in 200 mL dichloromethane was cooled to −15 °C (ice/methanol). Isobutylchloroformiat (4.1 mL, 31.4 mmol) was added dropwise. After stirring for 15 min *N*,*O*-dimethylhydroxylamine hydrochloride (2.8 g, 28.5 mmol) was added and stirring was continued for 16 h at room temperature. The solution was washed with 0.2 M $KHSO_4$ (40 mL) and the aqueous layer extracted with dichloromethane (3 × 40 mL). The combined organic phases were dried ($MgSO_4$) and the solvent was evaporated under reduced pressure. The residue was purified by silica gel flash chromatography (cyclohexane/ethyl acetate, 1:1) to afford 6.1 g (98%) of the Weinrebamide as colorless powder. A stirred solution of the Weinrebamide (2.1 g, 9.67 mmol) in dry THF (70 mL) was cooled to 0 °C. Lithium aluminum hydride in THF (12.1 mL, 12.1 mmol) was added in portions and after 30 min 0.2 M $KHSO_4$ (20 mL) was added. The organic compounds were extracted with diethyl ether (3 × 30 mL). The combined organic phases were washed with 1 M HCl (3 × 10 mL), brine (3 × 10 mL) and dried ($MgSO_4$). The solvent was evaporated under reduced pressure and the residue was purified by silica gel flash chromatography (cyclohexane/ethyl acetate, 1:2) to afford 1.32 g (86%) of the title compound as colorless oil. $C_7H_{13}NO_3$; $R_f$ = 0.43 (cyclohexane/ethyl acetate, 1:2); $^1$H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta$ 1.45 (s, 9H), 4.06–4.08 (d, *J* = 4.94, 2H), 5.21 (s, 1H), 9.64 (s, 1H) ppm; $^{13}$C NMR (75 MHz, $CDCl_3$, 25 °C): $\delta$ 28.26 (3 × $CH_3$), 51.40 ($CH_2$), 80.18 (Cq), 173.61 (Cq), 197.07 (CHO) ppm; HR-FTMS: M+$Na^+$/pos.: Anal. calcd: 160.0968, found: 160.0966.

**4.9.8. Lysine-(ε-*N*-Alloc)-methylester-hydrochloride (4).** A stirred suspension of Lysine-(ε-*N*-Alloc)-OH (3.56 g, 13.4 mmol) in dry MeOH (150 mL) was cooled to 0 °C and thionylchloride (4.9 ml g, 67 mmol) was added dropwise. The reaction mixture was stirred for 20 h at room temperature and the solvent was evaporated to 10 mL under reduced pressure. The product was precipitated by adding cold diethyl ether (100 mL). The solid was filtered, washed with diethyl ether (2 × 10 mL), and dried under reduced pressure to afford 3.62 g (95%) of **4** as colorless solid. $C_{11}H_{20}N_2O_4$; mp = 125–127 °C; $[\alpha]_D^{25}$ +18.1°; $^1$H NMR (300 MHz, MeOH-$d_4$, 25 °C): $\delta$ 1.33–1.56 (m, 4H), 1.80–1.96 (m, 2H), 3.09 (t, *J* = 6.5, 2H), 3.80 (s, 3H), 4.01 (t, *J* = 6.4, 1H), 4.48 (d, *J* = 5.3, 2H), 5.12–5.29 (ddd, *J* = 1.1, *J* = 10.5, *J* = 1.6, *J* = 17.2, 2H), 5.83–5.96 (m, 1H) ppm; $^{13}$C NMR (75 MHz, MeOH-$d_4$, 25 °C): $\delta$ 23.14 ($CH_2$), 30.40 ($CH_2$), 31.17 ($CH_2$), 41.15 ($CH_2$), 53.70 ($CH_3$), 53.92 (CH), 66.33 ($CH_2$), 117.45 ($CH_2$), 134.56 (CH), 158.92 (Cq), 171.03 (Cq) ppm; HR-FTMS: M+$Na^+$/pos.: Anal. calcd: 245.1496, found: 245.1497.

**4.9.9. α-*N*-(Boc-aminoethyl)-(ε-*N*-Alloc)-lysine methylester (5).** To a stirred solution of Boc-glycinal (0.29 g, 1.82 mmol) in dry methanol (50 mL) were added **4** (1.53 g, 5.46 mmol) and $NaBH_3CN$ (0.14 g, 2.18 mmol) at 0 °C. The reaction mixture was stirred for 14 h at room temperature and the solvent was evaporated under reduced pressure. The residue was purified by silica gel flash chromatography (ethyl acetate/cyclohexane = 5:1) to afford 0.52 g (74%) of **5** as colorless oil. $C_{18}H_{33}N_3O_6$; $R_f$ = 0.2 (ethyl acetate/cyclohexane, 5:1); $[\alpha]_D^{25}$ −1.9°; $^1$H NMR (300 MHz, $CDCl_3$, 25 °C): δ 1.34–1.71 (m, 15H), 2.52–2.60 (m, 1H) and 2.71–2.79 (m, 1H), 3.13–3.25 (m, 5H), 3.71 (s, 3H), 4.53 (d, *J* = 5.4, 2H), 5.17–5.31 (ddd, *J* = 0.9, *J* = 10.4, *J* = 1.4, *J* = 17.2, 2H), 5.84–5.97 (m, 1H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$, 25 °C): δ 22.80 ($CH_2$), 28.37 (3 × $CH_3$), 29.57 ($CH_2$), 32.74 ($CH_2$), 40.15 ($CH_2$), 40.64 ($CH_2$), 47.47 ($CH_2$), 51.85 ($CH_3$), 60.79 (CH), 65.39 ($CH_2$), 79.23 (Cq), 117.54 ($CH_2$), 132.92 (CH), 156.06 (Cq), 156.22 (Cq), 175.36 (Cq) ppm; HR-FTMS: M+H$^+$/pos.: Anal. calcd: 388.2442, found: 388.2445.

**4.9.10. α-*N*-(Boc-aminoethyl)-α-*N*-(2-(4-Cbz-cytosine-1-yl)-acetyl)-(ε-*N*-Alloc)-lysine methylester (6).** To a solution of 4-Cbz-1-carboxymethylcytosine (0.37 g, 1.23 mmol) in dry DMF/$CH_3CN$ (10 mL) were added *N*-methylmorpholine (0.54 mL, 4.92 mmol) and pivaloylchloride (0.17 mL, 1.35 mmol) at −15 °C (ice/methanol). After 20 min **5** (0.32 g, 0.82 mmol) in dry DMF (5 mL) was added dropwise and the reaction mixture was stirred for 12 h at room temperature. Ethyl acetate (50 mL) was added and the organic phase was washed with 0.1 N HCl (3 × 20 mL). The combined organic phases were dried ($MgSO_4$) and the solvent evaporated under reduced pressure. The residue was purified by silica gel flash chromatography (ethyl acetate) to afford 0.22 g (39%) of **6** as colorless powder. $C_{32}H_{44}N_6O_{10}$; $R_f$ = 0.2 (ethyl acetate); mp = 64–66 °C; $[\alpha]_D^{25}$ −21.5°; $^1$H NMR (300 MHz, $CDCl_3$, 25 °C) both rotamers: δ 1.28–1.62 (m, 15H), 1.96 (q, *J* = 7.36, *J* = 14.4, 2H), 3.13–3.39 (m, 5H), 3.70 (s, 3H), 4.16 (t, 1H), 4.52 (d, *J* = 5.4, 2H), 4.62–4.86 (dd, *J* = 15.7,*J* = 15.5), 5.15–5.30 (m, 4H), 5.40 (s, 1H), 5.58 (s, 1H), 5.82–5.96 (m, 1H), 7.22 (d, *J* = 6.2, 1H), 7.34–7.38 (m, 5H), 7.63 (d, *J* = 6.8, 1H) ppm; $^{13}$C NMR: (75 MHz, $CDCl_3$, 25 °C) both rotamers: δ 23.31 ($CH_2$), 28.08 ($CH_2$), 28.42 ($CH_3$), 29.32 ($CH_2$), 39.27 ($CH_2$), 40.34 ($CH_2$), 47.70 ($CH_2$), 50.44 ($CH_2$), 52.57 ($CH_3$), 60.45 (CH), 65.31 ($CH_2$), 68.05 ($CH_2$), 77.24 (Cq), 79.87 (Cq), 95.18 (Cq), 95.25 (Cq), 117.37 ($CH_2$), 128.23 (2× CH), 128.28 (CH), 128.67 (2× CH), 133.17 (CH), 134.91 (Cq), 150.30 (CH), 156.05 (Cq), 156.54 (Cq), 162.50 (Cq), 162.57 (Cq), 167.17 (Cq), 171.61 (Cq), 171.66 (Cq), 181.40 (Cq), 187.84 (CH) ppm; HR-FTMS: M+H$^+$/pos.: Anal. calcd: 673.3200, found: 673.3198.

**4.9.11. α-*N*-(Boc-aminoethyl)-α-*N*-(2-(4-Cbz-cytosine-1-yl)-acetyl)-(ε-*N*-Alloc)-lysine (2).** To a solution of **6** (0.49 g, 0.73 mmol) in THF (10 mL) was added dropwise 1 N LiOH (4 mL) at 0 °C. After stirring for 4 h at room temperature the solution was acidified by adding 1 N HCl to pH 2–3. Water (20 mL) was added and the aqueous phase was extracted with ethyl acetate (3 × 50 mL). The combined organic phases were dried ($MgSO_4$) and the solvent was evaporated under reduced pressure. The residue was purified by silica gel flash chromatography (ethyl acetate/methanol/formic acid, 94:5:1) to afford 0.42 g (87%) of **2** as colorless powder. $C_{31}H_{42}N_6O_{10}$; $R_f$ = 0.15 (ethyl acetate/methanol/formic acid, 94:5:1); mp = 74–77 °C; $[\alpha]_D^{25}$ −13.1°; $^1$H NMR (300 MHz, DMSO-$d_6$, 25 °C) both rotamers: δ 1.22–1.29 (m, 2H), 1.38–1.45 (m, 11H), 1.67–2.01 (m, 2H), 2.92–3.05 (m, 2H), 3.17–3.47 (m, 4H), 4.32–4.45 (m, 3H), 4.72, 4.81 (s, 2H), 5.13–5.29 (m, 4H), 5.83–5.96 (m, 1H), 7.01–7.03 (d, *J* = 7.3, 1H), 7.34–7.43 (m, 5H), 7.95–7.97 (d, *J* = 7.3, 1H) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$, 25 °C) both rotamers: δ 23.05 ($CH_2$), 28.10 ($CH_3$), 28.19 ($CH_2$), 29.10 ($CH_2$), 38.99 ($CH_2$), 39.94 ($CH_2$), 45.72 ($CH_2$), 49.83 ($CH_2$), 50.15 ($CH_2$), 58.77 (CH), 64.03 ($CH_2$), 66.40 ($CH_2$), 77.97 (Cq), 93.72 (CH), 109.46 (Cq), 116.77 ($CH_2$), 127.85 (CH), 128.07 (CH), 128.40 (CH), 133.81 (CH), 150.96 (CH), 153.12 (Cq), 154.89 (Cq), 155.63 (Cq), 155.82 (Cq), 158.34 (Cq), 163.10 (Cq), 167.17 (Cq), 172.12 (Cq) ppm; HR-FTMS: M+H$^+$/pos.: Anal. calcd: 659.3035, found: 659.3037.

**4.9.12. *i*Cys-cctac($(CH_2)_4$NH-TMR)ag-GlyGly$^{CONH_2}$ (Nu3).** Starting from Fmoc–glycine loaded MBHA-Resin **7** (loading 0.3 mmol/g, 8.5 mg) the linear solid phase PNA synthesis was accomplished according to the Boc/Z strategy as described previously. The resin was washed with argon saturated DCM (5 × 1 mL) and the Alloc group was deprotected by adding 1 equiv $Pd(PPh_3)_4$ and 6 equiv $Me_2NH{\cdot}BH_3$ in argon saturated DCM (2 × 1 mL) to the resin for 30 min. Subsequently the resin was washed with DCM (5×), DMF (5×), dioxane/$H_2O$ (2×, 9:1), methanol (2×), DMF (5×), DCM (5×), and DMF (5×). After preactivation of 4 equiv 5-carboxytetramethylrhodamine (final concentration 0.1 M in DMF) for 3 min using 4 equiv PyBOP and 6 equiv *N*-methylmorpholine, the solution was added to the resin for 60 min. The resin was washed following the general washing procedure. Cleavage from the solid support was accomplished following the general cleavage protocol according to the Boc/Z strategy described previously. Synthesis yield was determined by measuring the UV absorption at 565 nm ($\varepsilon_{565nm}$ = 91.000 L mol$^{-1}$ cm$^{-1}$) in phosphate buffer (100 mM $NaH_2PO_4$, pH 7.4) at 25 °C. $C_{110}H_{135}N_{47}O_{27}S$; yield: $OD_{260}$ = 8.6, 88 nmol, 3.5%; MALDI-TOF/MS (*m*/*z*): Anal. calcd for [M+H]$^+$: 2580, found: 2579; HPLC: $t_R$ = 16.2 min (Gradient B).

**4.9.13. *i*Cys-cctac($(CH_2)_4$NH-AEEA-TMR)ag-GlyGly$^{CONH_2}$ (Nu4).** The synthesis was accomplished according to the previously described procedure using pre-activated Fmoc-aminoethyloxyethyloxyacetic acid as building block for the coupling reaction after Alloc-deprotection. $C_{116}H_{146}N_{48}O_{30}S$; yield: $OD_{260}$ = 10.8, 110 nmol, 4.4%; MALDI-TOF/MS (*m*/*z*): Anal. calcd for [M+H]$^+$: 2726, found: 2726; HPLC: $t_R$ = 16.6 min (Gradient B).

**4.9.14. $^{AcHN}$ctcttc($(CH_2)_4$NH-FAM)cccac-Gly-S$(CH_2)_2^{SO_3H}$ (El2).** Starting from Fmoc–glycine loaded MBHA-Resin **7** (loading 0.3 mmol/g, 8.5 mg) the linear solid phase PNA synthesis was accomplished according to the Boc/Z strategy as described previously. The resin

was washed with argon saturated DCM (5 × 1 mL) and the Alloc group was deprotected by adding 1 equiv $Pd(PPh_3)_4$ and 6 equiv DMB in argon saturated DCM (2 × 1 mL) to the resin for 30 min. Subsequently the resin was washed with DCM (5×), DMF (5×), dioxane/$H_2O$ (2×, 9:1), methanol (2×), DMF (5×), DCM (5×), and DMF (5×). After preactivation of 4 equiv 6-carboxyfluoresceine (final concentration 0.1 M in DMF) for 3 min using 4 equiv PyBOP and 6 equiv *N*-methylmorpholine, the solution was added to the resin for 60 min. The resin was washed following the general washing procedure. Cleavage from the solid support was accomplished following the general cleavage protocol according to the Boc/Z strategy described previously. The product fractions of the semi-preparative HPLC containing the propylamide thioester were dissolved in phosphate buffer (100 mM $NaH_2PO_4$, 10 mM MESNa, pH 7.4) and the solution was vortexed for 90 min at 25 °C. The reaction was stopped by adding TFA (30% v/v) and the solvent was evaporated under reduced pressure. The residue was dissolved in $H_2O$ containing 0.1% TFA (100 μL) and was purified by HPLC. The product fractions were concentrated under reduced pressure and the residue dissolved in $H_2O$. Synthesis yield was determined by measuring the UV absorption at 490 nm ($\varepsilon_{490\ nm}$ = 83.000 L mol$^{-1}$ cm$^{-1}$) in phosphate buffer (100 mM $NaH_2PO_4$, pH 7.4) at 25 °C. $C_{145}H_{175}N_{56}O_{46}S_2$; yield: $OD_{260}$ = 8.8, 81 nmol, 3.2%; MALDI-TOF/MS (*m*/*z*): Anal. calcd for $[M+H]^+$: 3504, found: 3504; HPLC: $t_R$ = 14.7 min (Gradient B).

**4.9.15. $^{AcHN}$ttc((CH$_2$)$_4$NH-FAM)cccac-Gly-S(CH$_2$)$_2^{SO_3H}$ (El3).** The synthesis was accomplished according to the previously described procedure. $C_{117}H_{140}N_{44}O_{35}S$; yield: $OD_{260}$ = 29.9, 343 nmol, 6.9%; MALDI-TOF/MS (*m*/*z*): Anal. calcd for $[M+H]^+$: 2699, found: 2699; HPLC: $t_R$ = 16.8 min (Gradient A).

**4.9.16. $^{AcHN}$ctcttc((CH$_2$)$_4$NH-AHX-FAM)cccac-Gly-S(CH$_2$)$_2^{SO_3H}$ (El4).** The synthesis was accomplished according to the previously described procedure using pre-activated Boc-AHX-OH as building block for the coupling reaction after Alloc-deprotection. $C_{151}H_{186}N_{57}O_{47}S_2$; yield: $OD_{260}$ = 24.2, 222 nmol, 8.9%; MALDI-TOF/MS (*m*/*z*): Anal. calcd for $[M+H]^+$: 3617, found: 3617; HPLC: $t_R$ = 15.7 min (Gradient B).

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