"Four-Potential" Ferrocene Labeling of PNA Oligomers via Click Chemistry

Nina Hüsken, Gilles Gasser, S. David Köster, and Nils Metzler-Nolte*

Lehrstuhl für Anorganische Chemie I - Bioanorganische Chemie, Fakultät für Chemie und Biochemie, Ruhr-Universität Bochum, Universitätsstrasse 150, D-44801 Bochum, Germany. Received March 20, 2009; Revised Manuscript Received June 4, 2009

The scope of the Cu(I)-catalyzed [2 + 3] azide/alkyne cycloaddition (CuAAC, click chemistry) as a key reaction for the conjugation of ferrocene derivatives to N-terminal functionalized PNA oligomers is explored herein (PNA: peptide nucleic acid). The facile solid-phase synthesis of N-terminal azide or alkyne-functionalized PNA oligomer precursors and their cycloaddition with azidoferrocene, ethynylferrocene, and *N*-(3-ethylpent-1-yn-3-yl)ferrocenecarboxamide (DEPA−ferrocene) on the solid phase are presented. While the click reaction with azidomethylferrocene worked equally well, the ferrocenylmethyl group is lost from the conjugate upon acid cleavage. However, the desired product was obtained via a post-SPPS conversion of the alkyne−PNA oligomer with azidomethylferrocene in solution. The synthesis of all ferrocene−PNA conjugates (trimer t_3-PNA, **3**, **4**, **5**, **6**; 12mer PNA, **10** - t c t a c a a g a c t c, **11** - t c t a c c g t a c t c) succeeded with excellent yields and purities, as determined by mass spectrometry and HPLC. Electrochemical studies of the trimer Fc−PNA conjugates **3**, **4**, **5**, and **6** with four different ferrocene moieties revealed quasi-reversible redox processes of the ferrocenyl redox couple $Fc^{0/+}$ and electrochemical half-wave potentials in a range of $E_{1/2} = -20$ mV to +270 mV vs $FcH^{0/+}$ (Fc: ferrocenyl, $C_{10}H_9Fe$). The observed potential differences $\Delta E_{1/2}^{min}$ are always greater than 60 mV for any given pair of Fc−PNA conjugates, thus allowing a reliable differentiation with sensitive electrochemical methods like e.g. square wave voltammetry (SWV). This is the electrochemical equivalent of "four-color" detection and is hence denoted "four-potential" labeling. Preparation and electrochemical investigation of the set of four structurally different and electrochemically distinguishable ferrocenyl groups conjugated to PNA oligomers, as exemplified by the conjugates **3**, **4**, **5**, and **6**, demonstrates the scope of the azide/alkyne cycloaddition for the labeling of PNA with electrochemically active ferrocenyl groups. Furthermore, it provides a PNA-based system for the electrochemical detection of single-nucleotide polymorphism (SNP) in DNA/RNA.

INTRODUCTION

Peptide nucleic acid (PNA) are nucleic acids analogues. In PNA, the negatively charged deoxyribose phosphodiester backbone of DNA and RNA is replaced by a neutral, pseudopeptide backbone of repeating *N*-(2-aminoethyl)glycine units (*1*−*9*). PNA have outstanding properties including faster hybridization with RNA, DNA, and PNA as well as higher binding affinity, mismatch sensitivity, and greater chemical and biological stability compared to natural nucleic acid oligomers (*10, 11*). These exceptional characteristics make PNA attractive for medicinal, biological, and analytical applications such as antigene/antisense therapy (*12, 13*), drug discovery (*14*), or molecular recognition of nucleic acids (*15*).

Promising and relatively new is the application of PNA as electrochemical biosensors for DNA or RNA (*16, 17*). A major advantage of using PNA for this purpose is their ability to reliably discriminate and therefore detect single-base mismatches in oligonucleotide sequences (*18*). Monolayers of PNA-modified transducer surfaces serve as the recognition device, while a reversible redox-active metal complex bound to the PNA sequence serves as the electrochemical probe. This type of biosensor offers the opportunity to monitor and detect the event of hybridization with various electroanalytical methods (*19*). A remaining challenge in the synthesis of an electrochemical PNA biosensor is the linkage of the electroactive metal complex to the PNA strand. In the past years, various examples of label-free electrochemical DNA detection with PNA were reported (*20*−*26*). In these studies, a charged metal complex or charged polymer interacts ionically with the negatively charged DNA backbone of the resulting PNA/DNA duplex. However, this method is limited by the lack of a defined bonding situation, which is crucial for the significance of the obtained electrochemical signal. In this perspective, a facile method to covalently conjugate a metal complex to a PNA strand is needed. Our group already presented the coupling of ferrocene carboxylic acid to the N-terminus of PNA oligomers on the solid phase (*27, 28*). Although this coupling method is efficient, it is limited to carboxylic acid functionalized metal complexes. An enhanced flexibility in the choice of organometallic starting materials would be very attractive, since not every metal complex can be

* Corresponding author. Fax: +49 (0)234 32 14378; Tel: +49 (0)234 32 28152; E-mail: Nils.Metzler-Nolte@ruhr-uni-bochum.de.

[1]Abbreviations: A, adenine; Ahx, amino hexanoic acid; DCM, dichloromethane; DMF, *N*,*N*-dimethyl formamide; DPV, differential pulse voltammetry; Bhoc, benzhydryloxycarbonyl; Boc, *tert*-butyloxycarbonyl; C, cytosine; CV, cyclic voltammetry; DEPA, diethylpropargylamine; DIPEA, diisopropylethylamine; ESI, electrospray ionization; Fc, ferrocenyl ($C_5H_5FeC_5H_4$); FcH, ferrocene (dicyclopentadienyl iron, Cp_2Fe, $(C_5H_5)_2Fe$); Fmoc, fluorenylmethoxycarbonyl; G, guanine; HATU, 2-(1*H*-7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxy-1*H*-benzotriazole; HPLC, high performance liquid chromatography; MALDI-TOF, matrix assisted laser desorption/ionization−time-of-flight; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MS, mass spectrometry; RP, reversed phase; R_t, retention time; PDA, photodiode array; SPPS, solid-phase peptide (or PNA) synthesis; PHB, photochemical hole burning; T, thymine; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TIS, triisopropyl silane; Trt, trityl. The common three-letter code for amino acids is used throughout. Nucleobases are denoted by their usual abbreviations in DNA; small letters however indicate PNA monomers.

10.1021/bc9001272 CCC: $40.75
Published on Web 07/08/2009

functionalized with a carboxylic acid, hence limiting the number of organometallic/metal complex PNA bioconjugates.

In order to provide new metal−PNA conjugates with different electrochemical potentials, we searched for an alternative method for the labeling of PNA with various ferrocene derivatives on the solid phase. We rapidly turned our attention to the so-called click chemistry methodology. "Click chemistry" describes a group of reactions that proceed with high yield and chemical specificity (*29*−*33*). The prototype "click reaction" is a Huisgen [2 + 3] dipolar azide/alkyne cycloaddition, and its Cu-catalyzed variant is valued as a key reaction for bioconjugation, since it is chemoselective, reliable, and takes places under mild conditions (*34*−*40*). For metal bioconjugates, this method enlarges the spectrum of metal complex starting materials to be possibly used, as the reagents may be azide- and alkyne-functionalized derivatives. We recently reported the use of click chemistry to generate peptide-functionalized self-assembled monolayers on Au surfaces, which are highly proteophobic (*41*). Furthermore, the first example of (multi)insertion by click chemistry of azidoferrocene into a PNA oligomer containing a new alkyne functionalized PNA monomer synthon was also presented recently (*42*).

In this paper, we report the facile N-terminal functionalization of PNA with azide or alkyne functionalities on the solid phase, and their subsequent conversion by click chemistry with a range of different alkynyl and azido ferrocene derivatives to give a small library of ferrocene−PNA bioconjugates. The metal complexes were chosen such that four readily discernible potentials result, and we therefore speak of "four-potential" labeling. In effect, this will provide the electrochemical equivalent of "four-color" detection, which is evidently important for sequencing applications and single nucleotide polymorphism (SNP) analysis. Hence, this study is of fundamental relevance for the development of new electrochemical DNA biosensors.

EXPERIMENTAL PROCEDURES

General Experimental Conditions. All reagents and HPLC-grade solvents were purchased from Acros (Geel, Belgium), Aldrich/Sigma/Fluka (Deisenhofen, Germany), E. Merck (Darmstadt, Germany), Novabiochem (Laufelfingen, Switzerland), and IRIS Biotech (Marktredwitz, Germany) and were used without further purification. The preloaded polystyrene resins were purchased from Rapp Polymers (Tübingen, Germany). Only L-amino acids were used throughout. PNA monomers were purchased from Link Technologies (Edinburgh, Scotland) and ASM (Hannover, Germany). All solutions were freshly prepared before use. HPLC[1] fractions of all products were frozen in liquid nitrogen and lyophilized using an Edwards Modulyo freeze−dryer. The preparation and handling of azide materials was performed at all stages on the smallest possible amounts and with routine precautions in order to minimize the risk of possible decomposition and explosions. Higher temperatures were avoided generally, and light was excluded during all stages of the handling of azidoferrocene.

General Analytical Procedures. MALDI-TOF mass spectra were measured on a Bruker Daltonics Autoflex in linear mode with positive polarity using sinapic acid as the matrix. ESI mass spectra were recorded on a Bruker Esquire 6000.

HPLC Analysis and Purification. HPLC analysis and purification was performed on a customized Varian ProStar 210 System, equipped with a PDA detector and auto sampler, using Varian DynaStar C-18 reverse-phase columns for analytical (250 × 4 mm, 8 μm) and preparative (250 × 21 mm, 8 μm) runs. Analytical (flow rate: 1.0 mL min^{-1}) and semipreparative (flow rate: 4.7 mL min^{-1}) runs were performed with a linear gradient of A (Millipore water containing 0.1% v/v TFA) and B (acetonitrile (Baker HPLC-grade), containing 0.1% v/v TFA. Analytical runs: t = 0 min 5% B, t = 20 min 80% B, t = 25 min 5% B. Preparative runs: t = 0 min 5% B, t = 12 min 15% B, t = 32 min 40% B, t = 50 min 80% B, t = 60 min 5% B. All samples were filtered before injection using a 0.22 μm syringe filter. Spectra were recorded at 254 nm and 25 °C; retention times (t_R [min]) were noted in each case.

Electrochemical Studies. Cyclic voltammetry and differential pulse voltammetry were performed on a Princeton Applied Research BES potentiostat operated by Princeton Applied Research MS DOS software M270. The measurements were perfomed with a home-made three-electrode microvolume electrochemical cell (*43*), placed in a Faraday cage consisting of copper hole sheet metal. For the measurements, a glassy carbon electrode with a geometric diameter of 2 mm was used as the stationary working electrode, a platinum wire was used as the counter electrode, and Ag/AgCl in 3 M KCl was used as the reference electrode. The Ag/AgCl reference electrode was permanently stored in aq KCl solution (3 M) if not in use. Measurements were carried out in a 1:1 (v/v) mixture of acetonitrile and MOPS buffer (0.15 M) at pH 7.4. Sodium perchlorate (0.2 M) was used as the supporting electrolyte. Solutions were degassed and filtered before use. The working electrode was polished with moistened carpet material before each measurement until DPV[1] of the pure electrolyte solution revealed a clean electrode surface. Ferrocene with the reversible redox couple $FcH^{0/+}$ was used as internal (**4**, **6**) or external (**3**, **5**) reference. To this end, ferrocene was measured three times before and after the respective measurement. Half-wave potentials $E_{1/2}$ and peak separation values ΔE_p were determined as described in Table 2. Return-to-forward peak current ratios I_{pc}/I_{pa} were determined according to the Nicholson procedure (*44*) on individual cyclic voltammograms by applying the following equation:

$$I_{pc}/I_{pa} = (I_{pc})_0/I_{pa} + 0.485(I_{pc})_0/I_{pa} + 0.086$$

Solid-Phase PNA Synthesis. SPPS was performed manually in 5 mL polypropylene one-way syringes as reaction vessels, which were equipped with a frit at the bottom. They were filled with 50 mg of polystyrene resin beads TentaGel R PHB Cys(Trt)Fmoc (0.18 mmol/g) or TentaGel S RAM Lys(Boc)-Fmoc (0.20 mmol/g). The resin was swollen in DMF before use for 1 h. All reactions were performed on a mechanical shaker with 400 rpm, soaking approximately 3−4 mL of freshly prepared solutions into the syringe. Fmoc/Bhoc protected PNA monomers (5 equiv) were preactivated in Eppendorf tubes before every coupling step for 2 min with HATU (4.5 equiv) in DMF, adding DIPEA and 2,6-lutidine (10 equiv each) (A(bhoc)-PNA-monomer, 5 min; C(bhoc)-PNA-monomer, 7 min). The amino acids Fmoc-Ahx and Fmoc-Lys(Boc)-OH were preactivated in an analogous manner with TBTU and HOBt·H_2O (4.5 equiv each). For each coupling step, the resin beads were treated with the activated acid under agitation (50 °C, 20 min) and subsequently washed with DMF. The coupling step was monitored with the Kaiser test. Double Fmoc deprotection was performed with piperidine (20%, v/v) in DMF (2 min + 10 min). The resin beads were then washed successively with DMF, DCM, and DMF. The whole procedure (deprotection, coupling, monitoring) was repeated for every PNA monomer until the PNA sequence was completed. The resin was washed and shrunk with methanol (30 min) and dried under vacuum. Final cleavage of the PNA oligomer from the resin and the deprotection of all Bhoc, Boc and Trt side chain protecting groups were simultaneously performed in TFA/TIS/phenol (85/5/10, v/v/v) (2 h + 1 h). Following the removal of TFA under reduced pressure, the crude product was precipitated with ice-cold diethyl ether, washed twice with ice-cold diethyl ether,

and each time centrifuged (10 min, 8000 rpm). The thus-produced crude oligomer was lyophilized in acetonitrile/water and analyzed by analytical HPLC, showing good purities already (90−98%) for all oligomers. For characterization with analytical HPLC and ESI or MALDI-TOF mass spectrometry, small oligomer quantities were purified by preparative HPLC. All yields given in the following are crude yields, with products likely containing at least traces of excess solvents, TFA, and salts.

Azide/Alkyne-Functionalized Carboxylic Acids and Ferrocene Derivatives. Propynoic acid as well as the ferrocene derivatives ethynylferrocene, ferrocene carboxylic acid, and ferrocenylmethanol were purchased from Acros (Geel, Belgium). All other ferrocene derivatives, namely azidoferrocene, DEPA−ferrocene, azidomethylferrocene, and 2-azidoacetic acid, were synthesized according to known and published synthetic routes. Analytical data of all compounds matched the previously reported data.

Preparation of Azide/Alkyne-Functionalized PNA Oligomers. N-Terminal azide and alkyne functionalizations of PNA oligomers were performed on polystyrene resins, which were preloaded with the respective PNA sequence, synthesized according to the general SPPS procedure. The solid-phase coupling of 2-azidoacetic acid and propynoic acid to the N-terminus of a PNA oligomer followed the Fmoc deprotection of the last PNA monomer of the respective PNA sequence.

Preparation of Propynoic Acid Functionalized PNA Oligomers 1 and 8. 50 mg (**1**, 10 μmol; **8**, 9 μmol) of a polystyrene resin, which was preloaded with the respective PNA sequence (**1**, H-t t t $LysNH_2$; **8**, H-t c t a c a a g a c t c Lys Ahx $CysNH_2$), was successively treated with a solution of DIPEA and 2,6-lutidine (each 10 equiv) in DMF (2 min) and then with a solution of propynoic acid (5 equiv) and HATU (4.5 equiv) in DMF at ambient temperature under agitation for 15 min. The resin was washed after each step with DMF, and the whole process was repeated eight times. When coupling was completed (monitoring with Kaiser test), the resin was washed successively with DMF, DCM, and DMF. The propynoic acid functionalized PNA oligomers were cleaved from the resin following the general procedure.

*Oligomer **1***. TentaGel S RAM Lys(Boc)Fmoc (0.20 mmol/g) was used. **1** was obtained as a white solid. Yield: 86% (8.6 mg, 8.6 μmol). HPLC: t_R = 4.8 min. ESI-MS: m/z = 996.3 $[M+H]^+$ (100), 498.7 $[M+2H]^{2+}$ (71).

Oligomer **8.** TentaGel R RAM Cys(Trt)Fmoc (0.18 mmol/g) was used. **8** was obtained as a white solid. Yield: 79% (25.6 mg, 7.1 μmol). MALDI-TOF MS: m/z (%) = 3613.5 $[M+H]^+$ (100).

Preparation of 2-Azidoacetic Acid Functionalized PNA Oligomers 2 and 9. 2-Azidoacetic acid (5 equiv) was preactivated for 3 min in an Eppendorf tube with TBTU and HOBt·H_2O (4.5 equiv each) in DMF, with DIPEA and 2,6-lutidine (10 equiv each). 50 mg (**2**, 10 μmol; **9**, 9 μmol) of a polystyrene resin, which was preloaded with the respective PNA sequence (**2**, H-t t t $LysNH_2$; **9**, H-t c t a c c g t a c t c Lys Ahx $CysNH_2$), was treated with the activated acid under agitation at ambient temperature for 1 h. The resin was then successively washed with DMF, DCM, and DMF. The azide-functionalized PNA oligomer was cleaved from the resin following the general procedure.

*Oligomer **2***. TentaGel S RAM Lys(Boc)Fmoc (0.20 mmol/g) was used. **2** was obtained as a white solid. Yield: 83% (8.6 mg, 8.3 μmol). ESI-MS: m/z (%) = 1027.3 $[M+H]^+$ (100), 514.2 $[M+2H]^{2+}$ (91).

*Oligomer **9***. TentaGel R RAM Cys(Trt)Fmoc (0.18 mmol/g) was used. **9** was obtained as a white solid. Yield: 80% (26.0 mg, 7.2 μmol). MALDI-TOF MS: m/z (%) = 3609.6 $[M+H]^+$ (100).

Preparation of Fc-PNA Conjugate 7. The conjugation of ferrocene via peptide coupling was performed on 50 mg (10 μmol) of the resin TentaGel R PHB, which was preloaded with the 12-mer PNA sequence H-t c t a c g a g a c t c Lys Ahx $CysNH_2$, and follows the Fmoc deprotection of the last PNA monomer of this sequence. Ferrocenecarboxylic acid (5 equiv) was preactivated for 3 min in an Eppendorf tube adding HATU and HOBt·H_2O (4.5 equiv each) in DMF, with DIPEA and 2,6-lutidine (10 equiv each). The resin was treated with the activated ferrocenecarboxylic acid under agitation (20 min, 50 °C). The resin was then successively washed with DMF, DCM, and DMF. Cleavage from the resin following the general procedure yielded Fc-PNA conjugate **7** as a yellowish solid. Yield: 83% (31.3 mg, 8.3 μmol). HPLC: t_R = 12.5 min, 100%. MALDI-TOF MS: m/z (%) = 3785.5 $[M+H]^+$ (100).

Preparation of Fc-PNA-Conjugate 3. 2.00 mg (2.0 μmol) of PNA oligomer **1** was dissolved in an Eppendorf tube in 150 μL of a 1:1 (v/v) mixture of acetonitrile/water containing 10 equiv of DIPEA and 10 equiv of 2,6-lutidine. Successively, the following 1% solutions were added under nitrogen flow: 0.73 mg (3.0 μmol) of azidomethylferrocene in a 1:1 (v/v) mixture of acetonitrile/water, 1.2 mg (6.1 μmol) of sodium L-ascorbate in water, and 0.29 mg (2.0 μmol) of copper(I) bromide in acetonitrile. The reaction mixture was shaken for 2 days. Subsequently, acetonitrile was removed under reduced pressure. The remaining residue was treated with ice-cold diethyl ether, and the supernatant was removed from the resulting precipitate. After washing three times with diethyl ether and air-drying, **3** was obtained as a yellowish solid. Yield: 73% (1.8 mg, 1.5 μmol). MALDI-TOF MS: m/z (%) = 1279.0 $[M+H+CH_3CN]^+$ (100).

Preparation of Fc-PNA Conjugates 4, 5, 6, 10, 11. Conjugation of ferrocene via [2 + 3]-azide/alkyne cycloaddition was performed on polystyrene resins, which were preloaded with an alkyne or azide functionalized PNA oligomer under inert conditions. All solvents were taken out of crown cap bottles and Eppendorf tubes were flushed with nitrogen before use. Following the synthesis of the azide/alkyne-functionalized PNA oligomers, the resin beads were washed and shrunk with methanol (30 min) and completely dried under reduced pressure. The syringe was then flushed with nitrogen, and the resin was washed and swollen for 1 h with dry DMF. The following solutions were prepared: DIPEA and 2,6-lutidine (each 10 equiv) in DMF, 1% of an azide or alkyne functionalized ferrocene derivative (5 equiv) in DMF, and 1% copper(I) bromide (1 equiv) in acetonitrile. These solutions were successively introduced into the syringe containing the preloaded resin. This reaction mixture was shaken for the given reaction time at ambient temperature. Subsequently, the resin was successively washed with DMF, CH_2Cl_2, CH_3CN, CH_2Cl_2, and DMF. All ferrocene-labeled PNA oligomers were cleaved from the resin following the general procedure.

Preparation of Fc-PNA Conjugate 4. 50 mg (10 μmol) of the resin TentaGel S RAM (0.20 mmol/g), which was preloaded with the propynoic acid functionalized PNA oligomer **1**, was converted according to the general procedure with 11.4 mg (50 μmol, 5 equiv) of azidoferrocene in 2 days. Cleavage from the resin yielded **4** as a yellowish solid. Yield: 78% (8.6 mg, 7.0 μmol). HPLC: t_R = 13.7 min. ESI-MS: m/z (%) = 1223.2 $[M+H]^+$ (41), 612.2 $[M+2H]^{2+}$ (100).

Preparation of Fc-PNA Conjugate 5. 50 mg (10 μmol) of the resin TentaGel S RAM (0.20 mmol/g), which was preloaded with the azide-functionalized PNA oligomer **2**, was converted

according to the general procedure with 10.5 mg (50 μmol, 5 equiv) of ethynylferrocene in two days. Cleavage from the resin yielded **5** as a yellowish solid. Yield: 88% (10.9 mg, 8.8 μmol). HPLC: t_R = 13.2 min. ESI-MS: *m/z* (%) = 1237.2 $[M+H]^+$ (25), 619.2 $[M+2H]^{2+}$ (100).

Preparation of Fc-PNA Conjugate 6. 50 mg (10 μmol) of the resin TentaGel S RAM (0.20 mmol/g), which was preloaded with the azide-functionalized PNA oligomer **2**, was converted according to the general procedure with 16.2 mg (50 μmol, 5 equiv) of DEPA−ferrocene in two days. Cleavage from the resin following the general procedure yielded **6** as a yellowish solid. Yield: 91% (12.3 mg, 9.1 μmol). HPLC: t_R = 13.1 min, 100%. ESI-MS: *m/z* (%) = 1350.3 $[M+H]^+$ (22), 675.7 $[M+2H]^{2+}$ (100).

Preparation of Fc-PNA Conjugate 10. 50 mg (9 μmol) of the resin TentaGel R PHB Cys(Trt)Fmoc (0.18 mmol/g), which was preloaded with the propynoic acid functionalized PNA oligomer **8**, was converted according to the general procedure with 10.2 mg (45 μmol, 5 equiv) of azidoferrocene in 2 days. Cleavage from the resin following the general procedure yielded **10** as a yellowish/greenish solid. Yield: 74% (25.7 mg, 6.7 μmol). HPLC: t_R = 14.8 min, 100%. MALDI-TOF MS: *m/z* = 3841.1 $[M+H]^+$ (100).

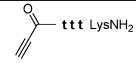
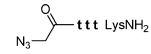
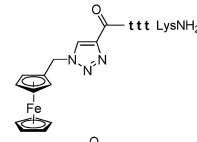
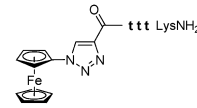
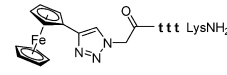
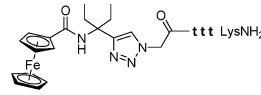
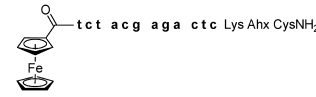
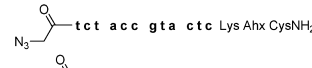
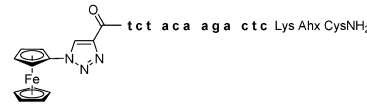
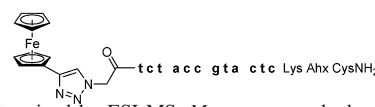
Preparation of Fc-PNA Conjugate 11. 50 mg (9 μmol) of the resin TentaGel R PHB Cys(Trt)Fmoc (0.18 mmol/g), which was preloaded with the azide-functionalized PNA oligomer **9**, was converted according to the general procedure with 9.45 mg (45 μmol, 5 equiv) of ethynylferrocene in 2 days. Cleavage from the resin following the general procedure yielded **11** as a yellowish/greenish solid. Yield: 76% (26.1 mg, 6.8 μmol). HPLC: t_R = 13.7 min, 100%. MALDI-TOF MS: *m/z* (%) = 3819.8 $[M+H]^+$ (100).

RESULTS AND DISCUSSION

As a proof of principle, all synthetic reactions and electrochemical studies were first undertaken on a trimer PNA model sequence H-t-t-t-Lys-NH_2. Selected reactions were subsequently carried out on 12mer bacterial PNA sequences to verify the compatibility of the key reactions with mixed-sequence PNA oligomers and various protecting groups.

Synthesis of Azide- or Alkyne-Functionalized PNA Oligomers 1, 2, 8, 9. All PNA oligomers were synthesized by manual standard solid-phase synthesis using Fmoc-protected PNA monomers (see Table 1 for a summary of the sequences synthesized in this work and Schemes 1 and 2 for synthesis details). Oligomers with the trimer model sequence H-t-t-t-Lys-NH_2 were synthesized on Fmoc-Lys(Boc) preloaded TentaGel S RAM resin, while 12mer oligomers were synthesized on Fmoc-Cys(Trt) preloaded TentalGel R PHB resin. Additionally, the Fmoc-protected amino acids lysine (with Boc-side chain protection) and aminohexanoic acid were introduced to increase the solubility of PNAs and as a spacer molecule, respectively. For the synthesis of **2** and **9**, an azide function was introduced by coupling 2-azidoacetic acid (*45*) to the Fmoc-deprotected N-terminus of the PNA oligomer. Correspondingly, for the synthesis of **1** and **8**, an alkyne function was introduced by peptide coupling of propynoic acid to the *N*-terminus. The coupling of 2-azidoacetic acid proceeded under conditions analogous to those for the coupling of α-amino acids and therefore offers the possibility of automation. On the contrary, the coupling of propynoic acid under standard conditions had to be repeated several times until complete conversion was achieved as shown by a negative Kaiser test (*46*). Cleavage from the resin with TFA/TIS/phenol 85:5:10 (v/v/v), precipitation with diethyl ether followed by centrifugation gave products **1**, **2**, **8**, and **9** in good yield and purity. The ESI mass spectra of compounds **1** and **2** show both the $[M+H]^+$ and $[M+2H]^{2+}$ peaks, while the MALDI spectra of **8** and **9** show only the monocationic $[M+H]^+$ peak. Mass spectrometry of the crude products only revealed product mass peaks and no peaks corresponding to truncated sequence fragments. This, in combination with clean HPL chromatograms, proves that all reactions including the coupling of the alkynyl and azido carboxylic acids occurred with nearly complete conversion. However, comparison of mass spectra and analytical HPL chromatograms show that the azide-functionalized PNA oligomers **2** and **9** are not completely stable toward our routine HPL chromatography conditions.

Table 1. Sequences and MS Data of PNA Oligomers 1−11

PNA	Sequence	M_{calc}	M_{found}
1		995.4	996.3[a]
2		1026.4	1027.3[a]
3		1237.1	1279.0[b]
4		1222.4	1223.2[a]
5		1236.5	1237.2[a]
6		1349.5	1350.3[a]
7		3785.6	3785.5[b]
8		3609.6	3613.5[b]
9		3607.6	3609.6[b]
10		3836.6	3841.2[b]
11		3817.6	3819.8[b]

[a] Determined by ESI MS. M_{calc} corresponds the calculated monoisotopic mass. [b] Determined by MALDI-TOF MS. M_{calc} corresponds to the calculated average mass.

Synthesis of Fc-PNA Conjugates 3−7, 10, 11. The conversion of the azide or alkyne functionalized PNA oligomers **1**, **2**, **8**, and **9** in a [2 + 3] azide/alkyne cycloaddition was performed with different alkyne or azide functionalized ferrocene derivatives, namely, the commercially available ferrocene derivative ethynylferrocene and the synthesized derivatives azidoferrocene (*47*), azidomethylferrocene (*48*), and the diethylpropargylamine ferrocene derivative *N*-(3-ethylpent-1-yn-3-yl)ferrocene-carboxamide (DEPA−ferrocene) (*49*). The cycloaddition reactions with ethynylferrocene, azidoferrocene, and DEPA−ferrocene were performed on the solid support, starting from the synthesized azide or alkyne functionalized PNA oligomers **1**, **2**, **8**, or **9** (Schemes 1 and 2). The reactions were carried out in syringes with a frit at the bottom under argon in thoroughly dried solvents using similar reaction conditions to those recently reported by our group for the synthesis of mono- and multiferrocene-

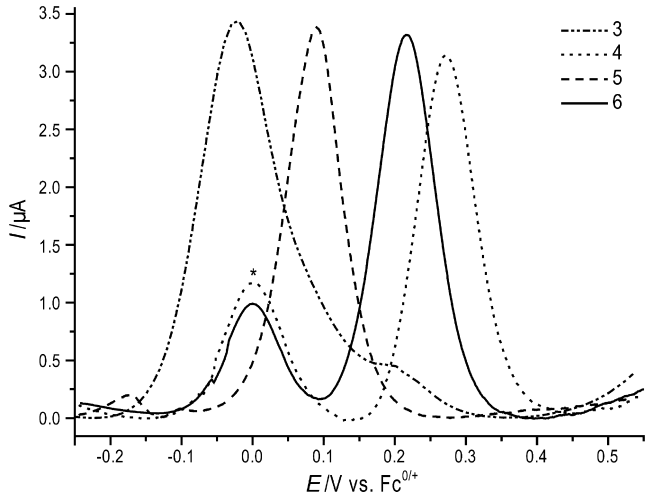


Figure 1. Overlay of differential pulse voltammograms (anodic scan; background subtracted) of Fc conjugates of PNA trimers **3**, **4**, **5**, and **6** at a glassy carbon electrode in 1:1 (v/v) mixture of CH_3CN and MOPS-buffer (0.15 M, pH 7.4) containing $NaClO_4$ as the supporting electrolyte (0.2 M). The experiments were undertaken at a scan rate of 4 mV/s with ferrocene as internal reference for **4** and **6** (smaller signals around 0 V, marked by an asterisk) and as external reference for **3** and **5**.

Table 2. Half-Wave Potentials ($E_{1/2}$), Peak Separation Values (ΔE_p), and Peak Current Ratios (I_{pc}/I_{pa}) of Fc-PNA Trimer Conjugates 3, 4, 5, and 6 as Determined by Cyclic Voltammetry[a]

no.	scan rate/ mVs^{-1}	$E_{1/2}$ vs $FcH^{0/+}$/ mV	ΔE_p/ mV	I_{pa}/ μA	I_{pc}/ μA	I_{sp}/ μA	I_{pc}/I_{pa}
3	250	−14	+114	−9.45	+5.83	−9.14	1.17
4	250	+269	+87	−3.73	+1.22	−3.05	0.81
5	250	+97	+79	−5.41	+2.88	−3.39	0.92
6	250	+208	+80	−2.66	+1.40	−1.88	0.96
FcH	250	0	+72	−10.67	+7.17	−4.98	0.99

[a] Data for ferrocene (FcH) measured under identical conditions are included for comparison. E_{pa} = anodic peak potential; E_{pc} = cathodic peak potential; half-wave potential $E_{1/2} = (E_{pa} + E_{pc})/2$; I_{pa} = anodic peak current; I_{pc} = cathodic peak current; peak separation value $\Delta E_p = E_{pa} - E_{pc}$; I_{sp} = current at switching potential; peak current ratios I_{pc}/I_{pa} determined as described in experimental section.

Table 3. Peak Potentials (E_p), Peak Currents (I_p), and Peak Widths ($w_{1/2}$) of Fc-PNA Trimer Conjugates 3, 4, 5, and 6 as Determined by Differential Pulse Voltammetry[a]

no	ΔE_p vs $FcH^{0/+}$ /mV	I_p /μA	$w_{1/2}$ /mV
3	−20	−3.92	131
4	+272	−3.14	103
5	+89	−8.46	90
6	+212	−3.32	100

[a] Scan rate 4 mVs^{-1}; voltage range 0−800 mV; modulation amplitude 10 mV; pulse width = 50 ms.

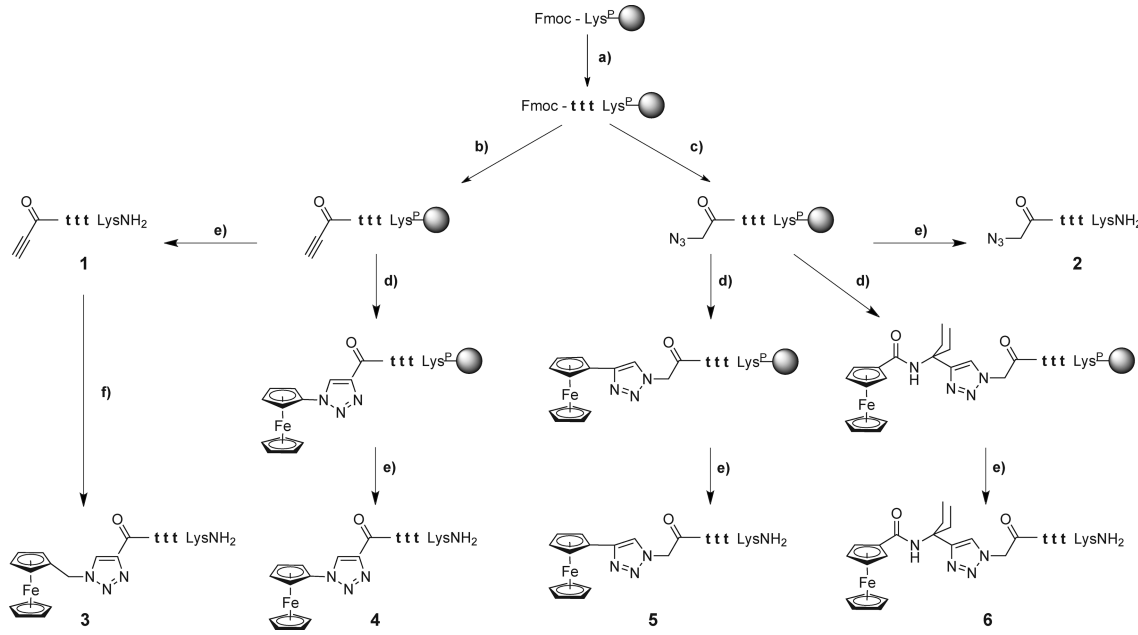
containing peptides (*50*) and PNA oligomers (*42*). Hence, the preloaded resins were treated with a reagent mixture containing copper(I) bromide as the catalyst and the respective ferrocene derivative. The reactions were carried out in DMF with small amounts of acetonitrile as cosolvent to achieve complete solubility of copper(I) bromide. After cleavage of the oligomer from the resin with TFA/TIS/phenol 85:5:10 (v/v/v), precipitation with ice-cold diethyl ether and lyophilization of the acetonitrile/water solutions, the crude products of **4**, **5**, **6**, **10**, and **11** were obtained as slightly orange powders.

The ESI mass spectra of products **4**, **5**, and **6** show the molecular peak $[M+H]^+$ with low intensity and the calculated peak $[M+2H]^{2+}$ as the base peak. ESI-MS of the crude products only revealed product peaks and not even trace signals of the azide or alkyne functionalized PNA precursors. This, again in combination with HPL chromatography of the crude products, proves that all cycloadditions occurred with practically complete conversion. Furthermore, it indicates that all synthesized Fc-PNA bioconjugates **4**, **5**, and **6** are completely stable to the fairly harsh (for organometallic compounds) cleavage conditions. MALDI-TOF spectra of 12mer Fc-PNA conjugates **10** and **11** reveal as well only peaks $[M+H]^+$ of the clicked products, as found during our previous investigations (*42*). No peaks originating from the azide and alkyne precursors could be detected. HPLC purification was performed to reduce the salt content of the crude products. An overall comparison of the trimer models **4**, **5**, and **6** with analytical data for the 12mer PNA conjugates **10** and **11** did not show any general difference in conversion, purity, or yield. This underscores that the solid-phase conversion of azide- or alkyne-functionalized PNA oligomers in a [2 + 3] cycloaddition with the chosen alkyne or azide ferrocene derivatives, respectively, is compatible with all four PNA monomers and qualified for the ferrocene labeling of even longer mixed-sequence PNA oligomers.

The synthesis of Fc-PNA conjugate **7** succeeded according to the literature procedure (*51*). Comparison of MALDI-TOF spectra and HPL chromatograms of the crude product **7** with those of the crude products **10** and **11** shows that the conjugation of a ferrocene moiety by click chemistry (**10**, **11**) yields comparable conversion and purity like the known conjugation of ferrocene carboxylic acid by peptide coupling (**7**). Click chemistry is even superior to the more traditional peptide coupling procedure in that it allows conjugation of a larger range of chemically diverse, commercially available, or easy-to-synthesize ferrocene derivatives. Furthermore, identical procedures and reagent concentrations for the click chemistry labeling with ethynylferrocene, azidoferrocene, and DEPA−ferrocene offer the possibility for automation. This flexibility is crucial for the preparation of PNA biosensors, since it allows the synthesis of metal−PNA conjugates with different electrochemical potentials.

Besides the successful introduction of azidoferrocene, ethynylferrocene, and DEPA−ferrocene, also an N-terminal labeling by click chemistry with azidomethyl ferrocene was attempted on a resin which was preloaded with the alkynyl functionalized PNA oligomer **1**. After cleavage and precipitation with cold ether, the product was characterized by ESI-MS. A major peak at m/z = 1039.3 corresponds to the PNA t_3-trimer, carrying a triazole ring at the N-terminus but *without* an attached ferrocene moiety. We thus conclude that the click reaction had taken place but yielded a product that is not stable to the relatively harsh PNA cleavage conditions as recently reported by Spiccia et al. in their endeavor to synthesize a ferrocenyl PNA monomer (see above and Experimental Procedures) (*52, 53*). We assume that the acidic cleavage conditions first cause a protonation of triazole N-3. In the presence of nucleophiles, the N−C bond is then cleaved with formation of the relatively stable ferrocenyl methyl cation, which finally forms ferrocenylmethanol. Following this line of argument, it is only the strongly acidic cleavage conditions that cause decomposition of the desired oligomer **3**. To exclude the possibility of inherent instability of **3**, the click reaction of azidomethyl ferrocene with the PNA trimer **1** was performed in solution as a postsynthetic labeling strategy. This finally succeeded and gave Fc-PNA conjugate **3** in good yield and purity. The MALDI-TOF mass spectrum of **3** after precipitation and washing revealed just one major peak corresponding to the $[M+H+CH_3CN]^+$ molecular ion. This synthetic work thus reveals the scope of click chemistry for the labeling of PNA oligomers with organometallic complexes. Since an instability toward harsh reaction conditions, e.g., the strong acidic PNA cleavage conditions, is a characteristic of many classes of metal complexes, click chemistry as a key reaction

Scheme 1. Synthesis of Fc-PNA Trimer Conjugates 3, 4, 5, and 6 and Corresponding Azide- and Alkyne-Functionalized PNA Precursors 1 and 2[a]



[a] (a) Standard Fmoc solid-phase PNA synthesis. (b) Coupling of propynoic acid, HATU, DIPEA, 2,6-lutidine in DMF. (c) Coupling of 2-azidoacetic acid, TBTU, HOBt·H_2O, DIPEA, 2,6-lutidine in DMF. (d) Click reaction on solid support: Ferrocene derivative (**4**, azidoferrocene; **5**, ethynylferrocene; **6**, DEPA−ferrocene), DIPEA, 2,6-lutidine in DMF; CuBr in CH_3CN. (e) Cleavage: TFA/TIS/Phenol. (f) Click reaction in solution: Azidomethylferrocene, DIPEA, 2,6-lutidine, CuBr in H_2O/CH_3CN. See experimental section for details, "Lys[P]" denotes N-Boc protected lysine.

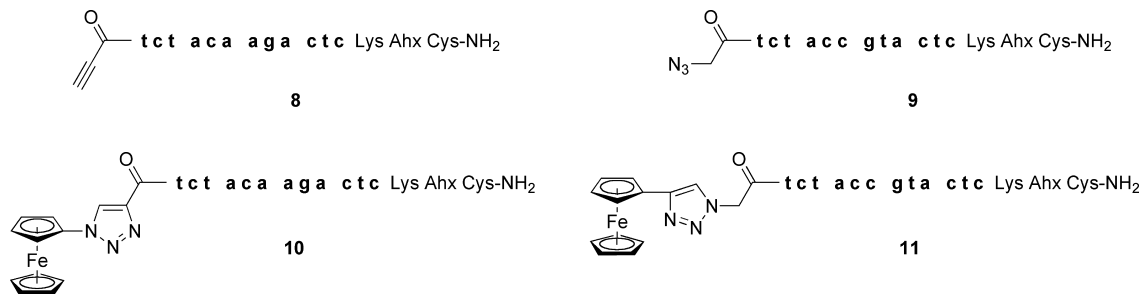
in the synthesis of metal-PNA conjugates is not just applicable as part of the solid-phase synthesis scheme but furthermore offers a reliable alternative as a postsynthetic labeling strategy, effectively yielding the same products.

Electrochemical Studies. The electrochemical behavior of the Fc-PNA conjugates **3**, **4**, **5**, and **6** with the trimer model sequence H-t-t-t-Lys-NH_2 was examined by cyclic voltammetry (CV) and differential pulse voltammetry (DPV). Each of these four compounds contains a ferrocene moiety that was attached to the N-terminus of the t_3-PNA sequence via one of the four specific click reactions described above. Fc-PNA trimer conjugates **3**, **4**, **5**, and **6** present structurally comparable compounds that only show differences in the chemical environment of the ferrocene moiety. An electrochemical microvolume cell with a stationary glassy carbon working electrode, Ag/AgCl in aq KCl (3 M) as reference electrode, and a platinum wire as counter electrode was used. All electrochemical measurements were undertaken with 80 μL of 0.8 mM solutions of the respective Fc-PNA bioconjugate in a 1:1 (v/v) mixture of acetonitrile and MOPS buffer (0.15 M, pH = 7.4) with $NaClO_4$ (0.2 M) as the supporting electrolyte. CV and DPV of ferrocene were performed in the same electrolyte solution, and the electrochemical half-wave potential $E_{1/2}$ of the redox couple ferrocene/ferrocenium $FcH^{0/+}$ vs Ag/AgCl ($E_{1/2}$ = 270 mV) was set to 0 mV as the reference potential for all measurements. Ferrocene was used as an internal reference for the DPV measurements of **4** and **6**. For the DPV measurements of **3** and **5** and for all CV measurements, ferrocene was used as an external reference to avoid an overlap of the corresponding redox processes (Figure 1).

Cyclic voltammograms of all Fc-PNA conjugates revealed one-electron waves with quasi-reversible Nernst behavior. Peak separations ΔE_p between +80 mV and +114 mV were observed, which are 8 to 42 mV higher than the ΔE_p value obtained with the same electrochemical setup for the fully reversible redox couple $FcH^{0/+}$(ΔE_p = +72 mV). Fc-PNA conjugates **5** and **6** yield current ratios I_p^{ox}/I_p^{red} close to unity, while conjugate **3** shows a higher value of 1.17 and conjugate **4** shows a lower value of 0.81, both still being sufficiently close to unity for practical biosensor applications.

The electrochemical half-wave potentials $E_{1/2}$ (vs $FcH^{0/+}$) are determined by CV and spread over 280 mV, ranging from $E_{1/2}$ = −14 mV for compound **3** to $E_{1/2}$ = +269 mV for compound **4**. These electrochemical potentials could be confirmed with DPV. Oligomer **6** revealed a half-wave potential of $E_{1/2}$ = +208 mV vs. $FcH^{0/+}$. This value corresponds well to the literature

Scheme 2. Azide- and Alkyne-Functionalized PNA Oligomers 8 and 9 and Fc-PNA Conjugates 10 and 11 with Bacterial 12mer PNA Sequences[a]



[a] See experimental section for details of the syntheses.

values for PNA with a peptidic bound ferrocene moiety (*51*), which was expected, since oligomer **6** is derived from a propargylamido ferrocene derivative and therefore contains a Cp-bound carboxamido function. Compound **4** reveals a potential of $E_{1/2} = +269$ mV vs $FcH^{0/+}$, showing a shift to positive potentials of 61 mV compared to **6**. In contrast, a potential of $E_{1/2} = +97$ mV vs $FcH^{0/+}$ was determined for oligomer **5**, a shift of 111 mV to negative potentials compared to **6**. Both conjugates **4** and **5** contain ferrocene moieties carrying the triazole structure in α-position to the Cp ring. The ferrocene moiety of **4** is bound to a nitrogen atom (N-1), whereas the ferrocene moiety of **5** is bound to a C_{sp2} atom (C-4) of the respective triazole ring. Independent of any effects of a possible Cp/triazole-conjugation, it can be assumed that differences in the electronegativity of the respective Cp-bound triazole ring atoms have a strong influence on the determined differences in the half-wave potentials of **4** and **5** (*54*). In contrast, the ferrocene moiety of oligomer **3** is disconnected from the triazole ring via a methylene bridge. The determined half-wave potential of **3** just exhibits a small shift to negative potentials compared to ferrocene/ferrocenium ($E_{1/2} = -14$ mV vs $FcH^{0/+}$), which is comparable to literature values for methyl or methylene substituted ferrocene derivatives (*55*).

The minimum differences in the electrochemical half-wave potentials $\Delta E_{1/2}^{min}$ of all Fc-PNA trimer conjugates **3**, **4**, **5**, and **6** were determined from Table 2. Conjugates **4** and **6** show a minimum value of $\Delta E_{1/2}^{min}$ (4, 6) = 61 mV, whereas conjugates **6** and **5** as well as **5** and **3** reveal values of $\Delta E_{1/2}^{min}$ (5, 6) = 111 mV (= $\Delta E_{1/2}^{min}$ (3, 5)). An overlay of differential pulse voltammograms of the four Fc-PNA trimer conjugates **3**, **4**, **5**, and **6** nicely demonstrates the differences in the electrochemical potentials (Figure 1). Generally, potential differences in this range are significant for a reliable resolution with sensitive electrochemical methods like differential pulse voltammetry (DPV) or square wave voltammetry (SWV). Figure 1 reveals that, even with consideration of the respective peak width $w_{1/2}$, ranging from 90 mV for **5** to 131 mV for **3** (see Table 3), a good resolution of the electrochemical potentials is possible, and therefore, a reliable distinction of the Fc-PNA conjugates **3**, **4**, **5**, and **6** with DPV is ensured.

CONCLUSIONS

In summary, the facile, reliable, and high-yielding preparation of new Fc-containing PNA oligomers was accomplished in this work using the click chemistry methodology. In contrast to all earlier work which had used ferrocene carboxylic acid as an electroactive label for PNA oligomers (*56*−*59*), the synthetic method used here allows the formation of a small library of new ferrocenyl PNA bioconjugates through the use of different ferrocenyl starting materials, but employing the same "click" chemistry. Accordingly, different electrochemical potentials could be encoded into PNA oligomer sequences by using one and the same type of reaction. Using the same conditions for the introduction of different labels is a bonus for automation, e.g., for an on-chip or parallel library synthesis. A similar approach was utilized by Seela et al. for the labeling of all four nucleobases with coumarin dyes by click chemistry (*60*). Our electrochemical analysis shows that the four ferrocenyl−triazole PNA oligomers described exhibit electrochemical potentials with potential differences bigger than 60 mV, which can be reliably distinguished by electrochemical methods like DPV. This is unprecedented and of significant interest for a potential application of PNA as electrochemical nucleic acid biosensors. In principle at least, the use of four different potentials in electrochemical labels (called "four-potential" labeling) corresponds to the "four-color" detection with four different dyes in classical DNA analysis. Kuhr and co-workers were the first to show a related electrochemical "four-potential" detection scheme with ferrocene-labeled DNA (*61, 62*). Apart from the fact that different chemistry had to be used in Kuhr's work for the introduction of all four ferrocene labels into the DNA oligomers, PNA clearly has advantages for DNA biosensors because of its higher affinity and superior mismatch discrimination. Investigations of the electrochemical responses of these oligomers with (non)cDNA/RNA sequences is currently underway in our laboratories and will be published in due course.

ACKNOWLEDGMENT

N.H. is the recipient of a Ruhr-University Research School fellowship for doctoral students. G.G. thanks the Alexander von Humboldt Foundation for a research fellowship. The authors thank Prof. Mallayan Palaniandavar for helpful discussions on the electrochemical measurements and Prof. Wolfgang Schuhmann and his co-workers for experimental help and assistance in the preparation of the glassy carbon electrodes and the microvolume electrochemical cell.

Supporting Information Available: ESI-MS and MALDI-MS data for all oligomers, HLPC, cyclic voltammograms. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Nielsen, P. E., Egholm, M., Berg, R. H., and Buchardt, O. (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science 254*, 1497–1500.

(2) Egholm, M., Nielsen, P. E., Buchardt, O., and Berg, R. H. (1992) Recognition of guanine and adenine in DNA by cytosine and thymine containing peptide nucleic acids (PNA). *J. Am. Chem. Soc. 114*, 9677–9678.

(3) Egholm, M., Buchardt, O., Nielsen, P. E., and Berg, R. H. (1992) Peptide nucleic acids (PNA). Oligonucleotide analogs with an achiral peptide backbone. *J. Am. Chem. Soc. 114*, 1895–1897.

(4) Uhlmann, E., Will, D. W., Breipohl, G., Peyman, A., Langner, D., Knolle, J., and O'Malley, G. (1997) Synthesis of polyamide nucleic acids (PNAs), PNA/DNA-chimeras and phosphonic ester nucleic acids (PHONAs). *Nucleosides Nucleotides 16*, 603–608.

(5) Uhlmann, E. (1998) Peptide nucleic acids (PNA) and PNA-DNA chimeras. From high binding affinity towards biological function. *Biol. Chem. 379*, 1045–1052.

(6) Larsen, H. J., Bentin, T., and Nielsen, P. E. (1999) Antisense properties of peptide nucleic acid. *Biochim. Biophys. Acta 1489*, 159–166.

(7) Soomets, U., Hällbrink, M., and Langel, Ü. (1999) Antisense properties of peptide nucleic acids. *Front. Bioscience 4*, d782–786.

(8) Koppelhus, U., and Nielsen, P. E. (2003) Cellular delivery of peptide nucleic acid (PNA). *Adv. Drug. Delivery Rev. 55*, 267–280.

(9) Metzler-Nolte, N. (2006) Conjugates of peptides and PNA with organometallic complexes: syntheses and applications. *Bioorganometallics* (Jaouen, G., Ed.) pp 125−179, Wiley-VCH, Weinheim.

(10) Ratilainen, T., Holmén, A., Tuite, E., Haaima, G., Christensen, L., Nielsen, P. E., and Nordén, B. (1998) Hybridization of peptide nucleic acid. *Biochemistry 37*, 12331–12342.

(11) Ratilainen, T., Holmén, A., Tuite, E., Nielsen, P. E., and Nordén, B. (2000) Thermodynamics of sequence-specific binding of PNA to DNA. *Biochemistry 39*, 7781–7791.

(12) Wang, G., and Xu, X. S. (2004) Peptide nucleic acid (PNA) binding-mediated gene regulation. *Cell Res. 14*, 111–116.

(13) Knudsen, H., and Nielsen, P. E. (1996) Antisense properties of duplex- and triplex-forming PNAs. *Nucleic Acids Res. 24*, 494–500.

(14) Good, L., and Nielsen, P. E. (1997) Progress in developing PNA as a gene-targeted drug. *Antisense Nucl. Acid Drug Dev. 7*, 431–437.
(15) Wang, J., Palecek, E., Nielsen, P. E., Rivas, G., Cai, X., Shiraishi, H., Dontha, N., Luo, D., and Farias, P. A. M. (1996) Peptide nucleic acid probes for sequence-specific DNA biosensors. *J. Am. Chem. Soc. 118*, 7667–7670.
(16) Wang, J. (1998) DNA biosensors based on peptide nucleic acid (PNA) recognition layers. A review. *Biosens. Bioelectron. 13*, 757–762.
(17) Wang, J. (1999) PNA biosensors for nucleic acid detection. *Peptide Nucleic Acids* (Nielsen, P. E., Egholm, M., Ed.) pp 155−161, Horizon Scientific Press, Wymondham.
(18) Mateo-Martí, E., Briones, C., Pradier, C. M., and Martín-Gago, J. A. (2007) A DNA biosensor based on peptide nucleic acids on gold surfaces. *Biosens. Bioelectron. 22*, 1926–1932.
(19) Wang, J. (1999) PNA biosensors for nucleic acid detection. *Curr. Issues Mol. Biol. 1*, 117–122.
(20) Steichen, M., Decrem, Y., Godfroid, E., and Buess-Herman, C. (2007) Electrochemical DNA hybridization detection using peptide nucleic acids and $[Ru(NH_3)_6]^{3+}$ on gold electrodes. *Biosens. Bioelectron. 22*, 2237–2243.
(21) Aoki, H., and Umezawa, Y. (2002) High sensitive ion-channel sensors for detection of oligonucleotides using PNA modified gold electrodes. *Electroanalysis 14*, 1405–1410.
(22) Degefa, T. H., and Kwak, J. (2008) Electrochemical impedance sensing of DNA at PNA self assembled monolayer. *J. Electroanal. Chem. 612*, 37–41.
(23) Liu, J., Tian, S., Nielsen, P. E., and Knoll, W. (2005) In situ hybridization of PNA/DNA studied label-free by electrochemical impedance spectroscopy. *Chem. Commun.* 2969–2971.
(24) Le Floch, F., Ho, H.-A., Harding-Lepage, P., Bédard, M., Neagu-Plesu, R., and Leclerc, M. (2005) Ferrocene-functionalized cationic polythiophene for the label-free electrochemical detection of DNA. *Adv. Mater. 17*, 1251–1254.
(25) Wang, Y., Li, C., Li, X., Li, Y., and Kraatz, H.-B. (2008) Unlabeled hairpin-DNA probe for the detection of single-nucleotide mismatches by electrochemical impedance spectroscopy. *Anal. Chem. 80*, 2255–2260.
(26) Li, X., Lee, J. S., and Kraatz, H.-B. (2006) Electrochemical detection of single-nucleotide mismatches using an electrode microarray. *Anal. Chem. 78*, 6096–6101.
(27) Hess, A., and Metzler-Nolte, N. (1999) Transition metal labels on peptide nucleic acid (PNA) monomers. *Chem. Commun.* 885–886.
(28) Verheijen, J. C., van der Marel, G. A., van Boom, J. H., and Metzler-Nolte, N. (2000) Transition metal derivatives of peptide nucleic acid (PNA) oligomers-synthesis, characterization, and DNA binding. *Bioconjugate Chem. 11*, 741–743.
(29) Kolb, H. C., Finn, M. G., and Sharpless, K. B. (2001) Click chemistry: diverse chemical function from a few good reactions. *Angew. Chem., Int. Ed. 40*, 2004–2021.
(30) Huisgen, R. (1963) 1,3-Dipolar cycloadditions. *Angew. Chem. 75*, 604–637.
(31) Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem., Int. Ed. 41*, 2596–2599.
(32) Tornøe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem. 67*, 3057–3064.
(33) Moses, J. E., and Moorhouse, A. D. (2007) The growing applications of click chemistry. *Chem. Soc. Rev. 36*, 1249–1262.
(34) van Berkel, S. S., Dirks, A. J., Debets, M. F., van Delft, F. L., Cornelissen, J. J. L. M., Nolte, R. J. M., and Rutjes, F. P. J. T. (2007) Metal-free triazole formation as a tool for bioconjugation. *ChemBioChem 8*, 1504–1508.
(35) Laughlin, S. T., Baskin, J. M., Amacher, S. L., and Bertozzi, C. R. (2008) In vivo imaging of membrane-associated glycans in developing zebrafish. *Science 320*, 664–667.
(36) Gramlich, P. M. E., Warncke, S., Gierlich, J., and Carell, T. (2008) Click-click-click: single to triple modification of DNA. *Angew. Chem., Int. Ed. 47*, 3442–3444.
(37) Gramlich, P. M. E., Wirges, C. T., Manetto, A., and Carell, T. (2008) Postsynthetic DNA modification through the copper-catalyzed azide-alkyne cycloadditon reaction. *Angew. Chem., Int. Ed. 47*, 8350–8358.
(38) Wang, Q., Chan, T. R., Hilgraf, R., Fokin, V. V., Sharpless, K. B., and Finn, M. G. (2003) Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J. Am. Chem. Soc. 125*, 3192–3193.
(39) Aucagne, V., and Leigh, D. A. (2006) Chemoselective formation of successive triazole linkages in one pot: "click-click" chemistry. *Org. Lett. 8*, 4505–4507.
(40) Speers, A. E., and Cravatt, B. F. (2004) Profiling enzyme activities in vivo using click chemistry methods. *Chem. Biol. 11*, 535–546.
(41) Chelmowski, R., Köster, S. D., Kerstan, A., Prekelt, A., Grunwald, C., Winkler, T., Metzler-Nolte, N., Terfort, A., and Wöll, C. (2008) Peptide-based SAMs that resist the adsorption of proteins. *J. Am. Chem. Soc. 130*, 14952–14953.
(42) Gasser, G., Hüsken, N., Köster, S. D., and Metzler-Nolte, N. (2008) Synthesis of organometallic PNA oligomers by click chemistry. *Chem. Commun.* 3675–3677.
(43) Habermüller, K., and Schuhmann, W. (1998) A low-volume electrochemical cell for the deposition of conducting polymers and entrapment of enzymes. *Electroanalysis 10*, 1281–1284.
(44) Nicholson, R. S. (1965) Theory and application of cyclic voltammetry for measurement of electrode reaction kinetics. *Anal. Chem. 37*, 1351–1355.
(45) Alvarez, S. G., and Alvarez, M. T. (1997) A practical procedure for the synthesis of alkyl azides at ambient temperature in dimethyl sulfoxide in high purity and yield. *Synthesis* 413–414.
(46) Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem. 34*, 595–598.
(47) Sheinker, Y. N., Senyavina, L. B., and Zheltova, V. N. (1965) Position and intensity of the absorption band due to antisymmetric valence vibration of the N_3 group in the infrared spectra of organic azides. *Dokl. Akad. Nauk SSSR 160*, 1339–1342.
(48) Casas-Solvas, J. M., Vargas-Berenguel, A., Capitán-Vallvey, L. F., and Santoyo-González, F. (2004) Convenient methods for the synthesis of ferrocene-carbohydrate conjugates. *Org. Lett. 6*, 3687–3690.
(49) Brosch, O., Weyhermüller, T., and Metzler-Nolte, N. (2000) A two-step palladium-catalyzed coupling scheme for the synthesis of ferrocene-labeled amino acids. *Eur. J. Inorg. Chem.* 323–330.
(50) Köster, S. D., Dittrich, J., Gasser, G., Hüsken, N., Henao Castañeda, I. C., Jios, J. L., Della Védova, C. O., and Metzler-Nolte, N. (2008) Spectroscopic and electrochemical studies of ferrocenyl triazole amino acid and peptide bioconjugates synthesized by click chemistry. *Organometallics 27*, 6326–6332.
(51) Maurer, A., Kraatz, H.-B., and Metzler-Nolte, N. (2005) Synthesis and electrochemical characterization of metallocene-PNA oligomers. *Eur. J. Inorg. Chem.* 3207–3210.
(52) Gasser, G., Belousoff, M. J., Bond, A. M., and Spiccia, L. (2006) Facile synthesis and detailed characterization of a new ferrocenyl uracil peptide nucleic acid monomer. *J. Org. Chem. 71*, 7565–7573.
(53) Gasser, G., and Spiccia, L. (2008) Synthesis of a ferrocenyl uracil PNA monomer for insertion into PNA sequences. *J. Organomet. Chem. 693*, 2478–2482.
(54) Batterjee, S. M., Marzouk, M. I., Aazab, M. E., and El-Hashash, M. A. (2003) The electrochemistry of some ferrocene

derivatives: redox potential and substituent effects. *Appl. Organomet. Chem. 17*, 291–297.

(55) Gubin, S. P. (1970) Application of electrochemical methods in the organometallic chemistry of transition metal p-complexes. *Pure Appl. Chem. 23*, 463–487.

(56) Salmain, M., Metzler-Nolte, N. (2008) *The bioorganometallic chemistry of ferrocene. Ferrocenes* (Stepnicka, P., Ed.) pp 499−639, John Wiley & Sons, Chichester.

(57) van Staveren, D. R., and Metzler-Nolte, N. (2004) Bioorganometallic chemistry of ferrocene. *Chem. Rev. 104*, 5931–5985.

(58) Metzler-Nolte, N. (2006) Bioorganometallic Chemistry. *Comprehensive Organometallic Chemistry III* (Parkin, G., Ed.) pp 883−920, Elsevier, Amsterdam.

(59) Metzler-Nolte, N. (2004) Modifizierte DNA, PNA und ihre Anwendungen in der Medizin und Biotechnologie. *Molekulare Biotechnologie* (Wink, M., Ed.) pp 579−593, Wiley-VCH, Weinheim.

(60) Seela, F., Sirivolu, V. R., and Chittepu, P. (2008) The modification of DNA with octadiynyl side chains: synthesis, base pairing and formation of fluorescent coumarin dye conjugates of four nucleobases by the alkyne-azide "click" reaction. *Bioconjugate Chem. 19*, 211.

(61) Brazill, S. A., Kim, P. H., and Kuhr, W. G. (2001) Capillary gel electrophoresis with sinusoidal voltammetric detection: a strategy to allow four-"color" DNA sequencing. *Anal. Chem. 73*, 4882–4890.

(62) Brazill, S. A., and Kuhr, W. G. (2002) A single base extension technique for the analysis of known mutations utilizing capillary gel electrophoresis with electrochemical detection. *Anal. Chem. 74*, 3421–3428.

BC9001272