Protocol

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Facile synthesis of peptide nucleic acids and peptide nucleic acid-peptide conjugates on an automated peptide synthesizer

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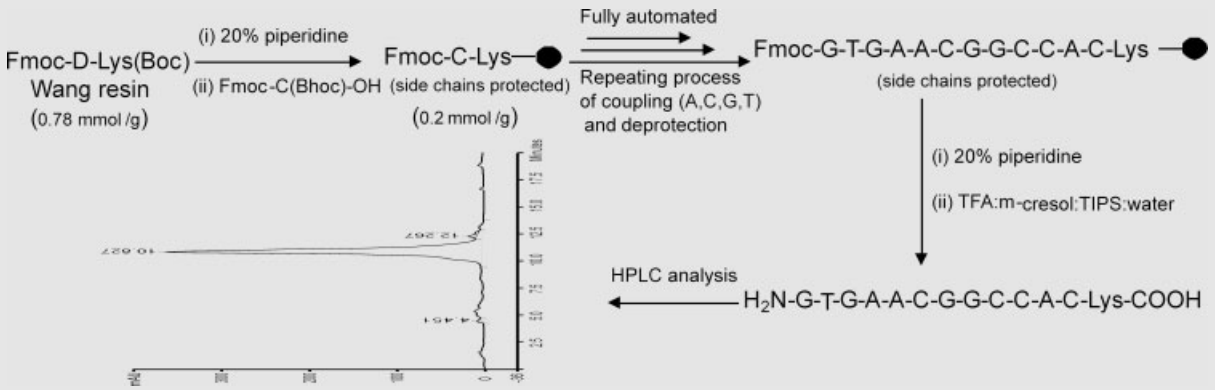
Peptide nucleic acids (PNAs) are DNA mimics with a neutral peptide backbone instead of the negatively charged sugar phosphates. PNAs exhibit several attractive features such as high chemical and thermal stability, resistance to enzymatic degradation, and stable binding to their RNA or DNA targets in a sequence-specific manner. Therefore, they are widely used in molecular diagnosis of antisense-targeted therapeutic drugs or probes and in pharmaceutical applications. However, the main hindrance to the effective use of PNAs is their poor uptake by cells as well as the difficult and laborious chemical synthesis. In order to achieve an efficient delivery of PNAs into cells, there are already many published reports of peptides being used for transport across the cell membrane. In this protocol, we describe the automated as well as cost-effective semi-automated synthesis of PNAs and PNA-peptide constructs on an automated peptide synthesizer. The facile synthesis of PNAs will be helpful in generating PNA libraries usable, e.g. for high-throughput screening in biomolecular studies. Efficient synthetic schemes, the automated procedure, the reduced consumption of costly reagents, and the high purity of the products are attractive features of the reported procedure. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide nucleic acid; peptide nucleic acid-peptide conjugates; automated peptide synthesis; solid phase methodology

REACTION SCHEME



GENERAL OPTIMIZED PROCEDURE

Fmoc-D-Lys(Boc)-Wang resin (50 mg, 0.78 mmol/g) was deprotected by 20% piperidine (2 ×, 5 min). Fmoc-C(Bhoc)-OH (14 mg, 0.5 equiv), HATU (7 mg, 0.45 equiv), and DIEA (7 µl, 1 equiv) were mixed in DMF (1 ml), activated for 3 min and then added to the resin. The reaction mixture was stirred for 1 h, washed twice with DMF, MeOH, and DCM each followed by capping with PNA-capping solution (2 ml, 5 min) containing 5% DIEA. Then the downloaded resin (37 mg, 0.2 mmol/g) was kept in the automatic synthesizer and coupling (0.5 ml of 58.8 mM different PNA monomers, 0.3 ml of 88.3 mM HATU, and 0.3 ml of 196.3 mM DIEA), washing (DMF, MeOH, and DCM), deprotection (20% piperidine, 2x5 min), and washing (DMF, MeOH, and DCM) steps were repeated automatically in a continuous way to obtain a 12-mer PNA construct. Freshly prepared cleavage cocktail [TFA, *m*-cresol, TIPS, water (90 : 5 : 2.5 : 2.5)] was added to the resin and the cleavage reaction was allowed to proceed for 2 h. The crude product was precipitated with diethyl ether and isolated by centrifugation. The pellet was washed twice with diethyl ether, dissolved, and purified by RP-HPLC.

Scope and Comments

Peptide nucleic acids (PNAs) are a DNA mimic consisting of the four common bases of DNA (Figure 1) on a pseudopeptide backbone [1] that makes them extremely stable in biological fluids. The four bases, i.e. adenine (A), cytosine (C), guanine (G), and thymine

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Abbreviations used: *Bhoc, benzhydryloxycarbonyl; DOTA, 1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid; equiv equivalent; FITC, fluorescein isothiocyanate; NMP, N-methylpyrrolidone; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl; TIPS, triisopropyl silane.*

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(T) are attached to the backbone by a methylene carbonyl linkage. The lack of charge repulsion and the flexibility of the backbone allow PNAs to bind to complementary DNA or RNA with higher thermodynamic stability than the corresponding DNA–DNA or DNA–RNA complexes [2,3]. Because of their unique biophysical properties [4] such as chemical stability, resistance to enzymatic degradation, and recognition of specific sequences of nucleic acids, PNAs have diverse applications in biomedical science, e.g. (i) therapeutic utilization as antisense agents [5,6], (ii) inhibition of gene expression [7], (iii) diagnostics [8] and drug delivery [9,10], (iv) molecular recognition [11], or (v) the use as biosensor [12]. However, the chemical synthesis of PNAs is a difficult and laborious process restricting their usability. In addition, the poor uptake of PNAs into cells is still considered as bottleneck in their effective application. Several reports have recently been published on the progress made in the development of peptidic delivery vectors to transport cargoes like PNAs inside cells [13,14]. Nevertheless, the coupling of cell-penetrating peptides increases the complexity of the synthesis even more. The aim of this protocol is to demonstrate the facile synthesis of PNAs and PNA-peptide conjugates on an automated peptide synthesizer.

Our group is involved in the development and synthesis of novel contrast agents for magnetic resonance imaging (MRI) in which PNAs act as a sensor for intracellular targeting and are supposed to bind with the corresponding mRNA in the cytosol. The syntheses of PNAs and their role in antisense targeting in our projects can be seen in recent publications of our group [15–17]. At the beginning of the project, we started the synthesis of PNAs on a manual synthesizer [18]. Later on, we developed and optimized the synthesis on an automatic peptide synthesizer. In this report, we will show the syntheses of three constructs (Table 1) (PNAs and a PNA-peptide conjugate) as model examples of a fully automated and semi-automated approach. The reason for presenting the semi-automated method also is to reduce the consumption of the expensive PNA monomers further on. In the semi-automated mode, no priming in the system takes place and thus there is no loss of PNA monomers during this process.

The concentration of the solutions of expensive PNA monomers, activator (HATU), and base (DIEA) was adjusted on the basis of the loading capacity and amount of used resin and the solutions were filled in 10 ml vials. HATU and DIEA had to be included in the programming of the synthetic sequence. For example, if a construct like ACGT has to be made, the sequence of the synthetic steps will be 651652653654 (starting from right corresponding to the *C*-terminus) where 1, 2, 3, 4, 5, and 6 represent A, C, G, T, HATU, and DIEA, respectively (Figure 2). The programmed method has been developed in a way that after the delivery of a building block, HATU is added and mixed for 3 min followed by DIEA delivery and mixing for 1 h. In the fully automated scheme, it was performed in a continuous way until the desired sequence was obtained. However, in the semi-automated mode, the single-shot delivery feature of the used peptide synthesizer was applied for the PNA monomers, so that the entire content of the PNA vials was delivered to the reaction vessel without priming. Thus, the reagent consumption was reduced further on but the synthetic scheme becomes discontinuous. If a construct like ACGTACGT has to be made, the system is continuous till the first four building blocks are coupled and then requires a refilling of the vials to be used for the next time. Positions of PNA monomers, HATU, and DIEA as well as the sequence programming and method developments were similar to those in the fully automatic mode. Importantly, HATU and DIEA were also placed in 10 ml vials and delivered in 0.3 ml aliquots without the single-shot function. In this way, laborious washing steps as well as the addition of activators and base were still fully automated and only the refilling of the vials for the monomers had to be done manually.

The syntheses of the constructs **1** and **2** were performed fully automatically. Two different resins with two different antisense sequences were chosen to verify the versatility of the presented protocol. More importantly, both the syntheses were performed in parallel which saves time and can be useful in generating PNA libraries easily. The synthesis was started on Wang or Rink amide resins which were downloaded in manual mode in order to avoid aggregation. Construct **3** was synthesized to demonstrate the semi-automated synthesis of a PNA-peptide conjugate. This synthesis was started on Fmoc-D-Arg(Pbf)-Wang resin, downloaded by adding the first amino acid of the sequence (Lys) in the manual mode. The remaining amino acid building blocks and the linker were assembled in the fully automatic mode. Afterwards, the synthesis was continued with the coupling of the PNA sequence in the semi-automated mode. After the assembly of all PNA monomers, the remaining specific building blocks required for our study [e.g. DOTA tris(*tert*-butyl), FITC] were added manually.

After the complete synthesis, the products were cleaved from the resin, precipitated in diethyl ether, purified by HPLC, and molecular masses were confirmed by ESI-MS.

Recently, a manual procedure was published [19], in which the authors have applied a 20-min reaction time (with a double coupling for G monomer) and only a 2.5-fold excess of PNA monomers to obtain the optimum results. We, however, employed a 1-h reaction time, did not perform a double coupling, and added a fourfold excess of PNA monomers to ensure the reaction went to completion. The final isolated yields of the two PNA molecules by our scheme (89 and 86%) are superior to the best result of reported synthesis by Avitabile *et al*. (75%). Nevertheless, the scheme reported in this article might also be tested for the automatic synthesis of PNA on a peptide synthesizer using our protocol.

In conclusion, we have developed and optimized a facile synthesis scheme to achieve PNAs and PNA-peptide constructs in high purity in an automated peptide synthesizer. The applicability to various PNA sequences on different resins (**1** and **2**) as well as to the PNA-peptide construct (**3)** demonstrated the versatility of the presented protocol in fully automated as well as semi-automated synthesis. Costs can be minimized by applying the single-shot delivery feature of the used peptide synthesizer. The laborious washing steps as well as additions of activators and base were simplified in both semi- and fully automated schemes.

Experimental Procedure

Materials

All reagents were used in HPLC or peptide synthesis grade. DMF, DCM, TFA, and methanol were obtained from Acros Organics (Heidelberg, Germany). All standard protected Fmoc amino acid derivatives were obtained from Novabiochem (Nottingham, UK). HATU, PNA-capping solution and aminoethoxyethoxyacetic acid (AEEA) linker were obtained from Applied Biosystems (Darmstadt, Germany). PNA monomers were obtained from Link Technologies (Bellshill, UK).

Methods

All syntheses were performed by Fmoc solid phase chemistry using a Prelude™ peptide synthesizer (peptides & elephants GmbH,

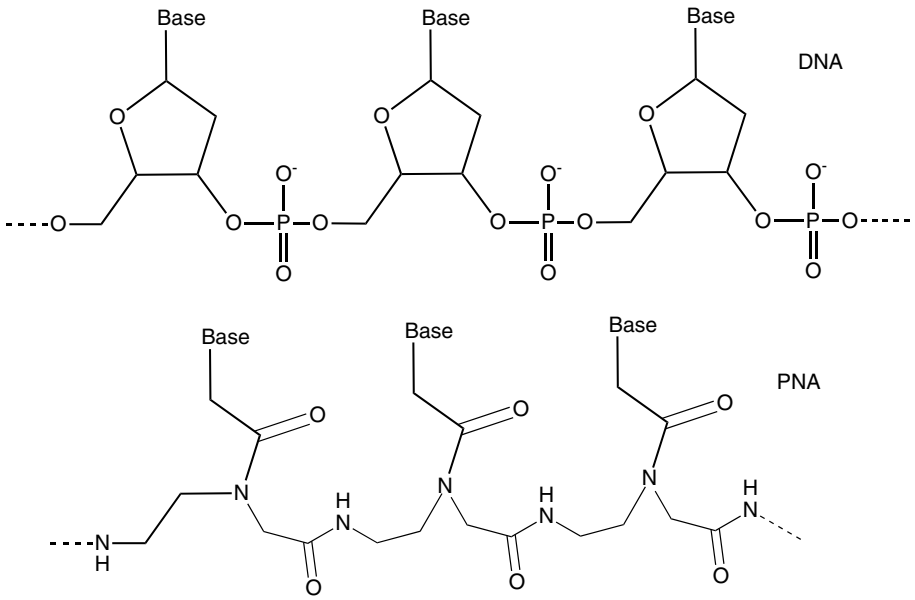

Figure 1. Comparison of chemical structures of DNA and PNA. Base indicates adenine, cytosine, guanine, or thymine.

Table 1. Building block sequence of PNAs and PNA-peptide conjugates

Constructs	Sequence
1	H_2N-GTGAACGGCCAC-Lys-COOH
2	H_2N-AGCGCCTGTACC-CO-NH_2
3	DOTA-Lys(FITC)-GTGAACGGCCAC-linker*-r-r-r-q-r-r-k-k-r-COOH

* AEEA.

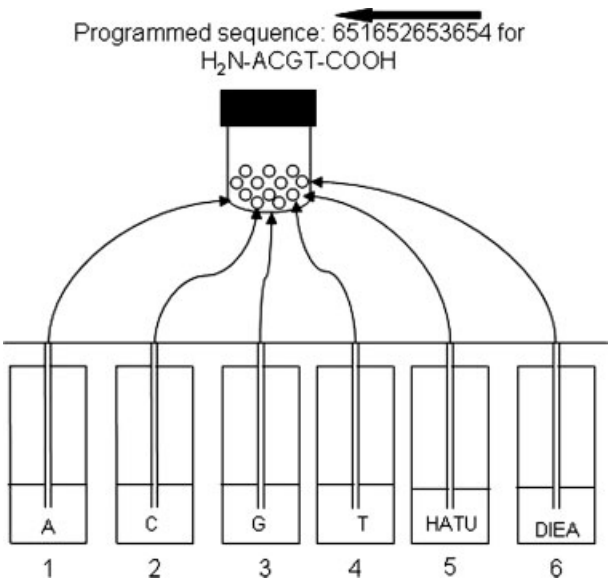

Figure 2. Schematic representation of the synthesis of an exemplary PNA sequence. 1, 2, 3, 4, 5, and 6 are Fmoc-A(Bhoc)-OH, Fmoc-C(Bhoc)-OH, Fmoc-G(Bhoc)-OH, Fmoc-T-OH, HATU, and DIEA, respectively. Washing and deprotection steps are included in the programmed method.

Potsdam, Germany) capable of performing up to six syntheses in parallel. The scale of the synthesis was 30–150 mg resin with a loading capacity of 0.1–0.2 mmol/g. The resins were downloaded by partial coupling of the first building block followed by capping of the unreacted free α-amino group. The capping was carried out by PNA-capping solution. Fmoc/Bhoc chemistry was used with the commercially available PNA monomers, with the backbone amine protected by Fmoc and the exocyclic amines of the nucleobases (A, C, G) protected with Bhoc group (Fmoc-A(Bhoc)-OH, Fmoc-C(Bhoc)-OH, Fmoc-G(Bhoc)-OH, and Fmoc-T-OH). Each cycle of elongation consisted of (i) Fmoc deprotection with 20% piperidine in DMF, 5 min, 2×, (ii) washing with DMF/NMP (2 : 1), MeOH, DCM, (iii) 3 min preactivation followed by 1 h coupling, and (iv) washing with DMF/NMP (2 : 1), MeOH, DCM twice. The molar ratio of resin/building block/HATU/DIEA was 1 : 4 : 3.6 : 8. The PNA monomers were dissolved in DMF/NMP (2 : 1) solution. All other building blocks were dissolved in DMF. Solutions of 0.5 ml of building block, 0.3 ml of HATU, and 0.3 ml of DIEA were mixed with the resin in the reaction vessel for each coupling. The mixing was carried out by nitrogen bubbling.

Automated PNA synthesis

The synthesis of **1** was started on Fmoc-D-Lys(Boc)-Wang resin (50 mg, 0.78 mmol/g). The Fmoc protecting group was removed by 20% piperidine in DMF twice for 5 min each. Afterwards, downloading of the resin was achieved by partial coupling to free amino groups as follows: Fmoc-C(Bhoc)-OH (14 mg, 0.5 equiv), HATU (7 mg, 0.45 equiv), and DIEA (7 µl, 1 equiv) were mixed in DMF (1 ml) and activated for 3 min and then added to the resin. The reaction mixture was stirred for 1 h and washed with DMF, MeOH, and DCM. The unreacted free amino groups were capped by adding PNA-capping solution (2 ml, 5 min) containing 5% DIEA. The resin was washed and dried. Downloading was measured in an UV spectrometer at the wavelength of 290 nm. The downloaded resin (37 mg, 0.2 mmol/g) was kept in the automated synthesizer and coupling (0.5 ml of 58.8 mM of each PNA monomers, 0.3 ml of 88.3 mM HATU and 0.3 ml of 196.3 mM DIEA), washing (DMF, MeOH,

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and DCM), deprotection (20% piperidine, 5 min, 2×), and washing steps (DMF, MeOH, and DCM) were repeated automatically in a continuous way to obtain the 12-mer PNA construct.

The synthesis of **2** was started on Rink amide resin (275 mg, 0.70 mmol/g) which was downloaded to 0.18 mmol/g by Fmoc-C(Bhoc)-OH in the same way as mentioned above. The downloaded resin (40 mg) was kept in the automatic synthesizer and synthesis was started in parallel to construct **1**. The concentration of PNA monomers, activators, and base as well as the cycle of coupling, washing, and deprotection steps were exactly the same as for **1.**

Semi-automated synthesis

The synthesis of **3** was started on Fmoc-D-Arg(Pbf) Wang resin (200 mg, 0.51 mmol/g), which was downloaded to 0.16 mmol/g by Fmoc-Lys (Boc)-OH. The downloaded resin (110 mg) was kept in the automated synthesizer and the peptide synthesis was performed in automatic mode. However, PNA synthesis was continued by applying the single-shot delivery feature of the machine in the semi-automatic mode. In this case, once the monomers were used up in the synthesis, they have to be refilled manually for the next coupling steps as explained above. The method became discontinuous and the cycle of refilling continues till the desired sequence was achieved. For individual coupling steps, 51 mg of Fmoc-A(Bhoc)-OH, 50 mg of Fmoc-C(Bhoc)-OH, 52 mg of Fmoc-G(Bhoc)-OH, or 36 mg of Fmoc-T-OH were dissolved in 500 µl DMF/NMP (2 : 1). The addition of activator (0.3 ml of 211.2 mM HATU) and base (0.3 ml of 469 mM DIEA), washing steps (DMF, MeOH, and DCM), and deprotection (20% piperidine, 5 min, 2×), were the same as for **1**. The remaining specific building blocks (like DOTA and FITC) for this synthesis were added manually.

Cleavage

When the synthesis was completed, a freshly prepared cleavage cocktail (TFA, *m*-cresol, TIPS, water 90 : 5 : 2.5 : 2.5) (2.5 ml for **1** and **2**, 3.5 ml for **3**) was added to all three types of resins and the cleavage reaction was allowed to proceed for 2 h for **1** and **2** and 4 h for **3**. The product was precipitated with diethyl ether (20 ml) and isolated by centrifugation to form a pellet. The pellet was washed twice with diethyl ether, dissolved, and purified by RP-HPLC to yield **1** (22.5 mg, 89%), **2** (20 mg, 86%), and **3** (55 mg, 55%).

Analytics and Purification

HPLC was performed on a Varian PrepStar instrument (Australia) equipped with PrepStar SD-1 pump heads. UV absorbance was measured using a ProStar 335 photodiode array detector. Analytical HPLC was performed on a Varian Polaris C18-ether column (4.6 × 250 mm, 5 µm), over 20 min (**1** and **2**) or 30 min (**3**) with a flow of 1 ml/min using a linear gradient of 20–90% B when buffer A was 0.1% TFA in water and buffer B was 0.1% TFA in acetonitrile.

The purification was performed on a Varian Polaris C18-ether column (10.0 × 250 mm, 5 µm) over 30 min with a flow of 3 ml/min and using a linear gradient of 20–90% B when buffer A was 0.1% TFA in water and buffer B was 0.1% TFA in acetonitrile.

The wavelength of the UV detector was set to 260 nm for both analytical and semi-preparative purification. Figures 3 and 4 display the HPLC chromatograms of **1** and **3**, respectively. High purity of crude product **1** can be seen in the inserted

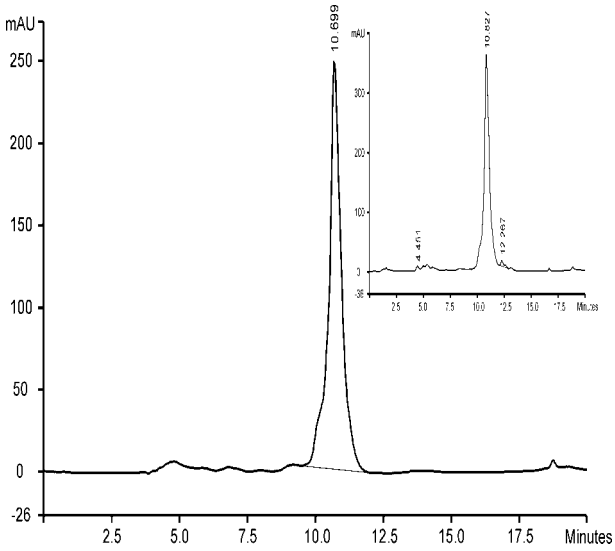

Figure 3. HPLC analysis of H_2N-GTGAACGGCCAC-Lys-COOH. Inserted is the chromatogram of crude product.

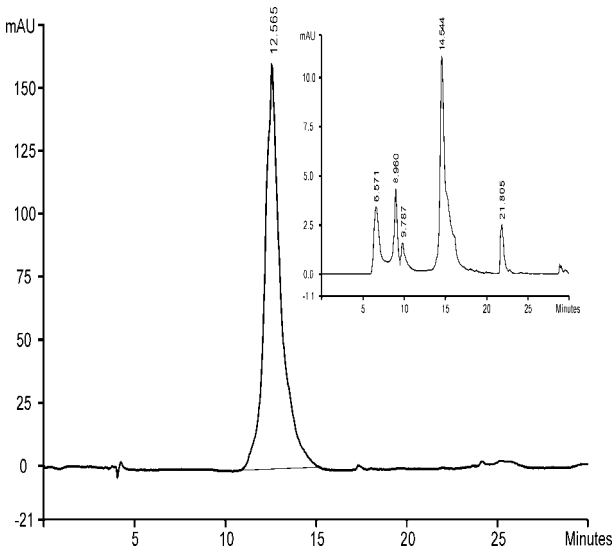

Figure 4. HPLC analysis of DOTA-Lys(FITC)-GTGAACGGCCAC-linker*-r-r-r-q-r-r-k-k-r-COOH. Inserted is the chromatogram of crude product. * AEEA.

chromatograms. In Figure 4, the inserted HPLC chromatogram of crude product **3** exhibits some byproducts which were easily separated by HPLC. As in manual synthesis, the longer the sequence the more byproducts were formed in automated synthesis.

After purification by HPLC, the constructs were characterized by ESI-MS on an Agilent 1100 Series LC/MSD Trap system (Boeblingen, Germany): nebulizer, 20.0 psi; dry gas, 5.0 l/min; dry temperature, 250 °C; compound stability, 100–1000%; trap drive level, 100%.

Construct **1**: The detected molecular ions at m/z = 1705.1 $[(M+2H)^{2+}]$, 1136.8 $[(M+3H)^{3+}]$, 853.0 $[(M+4H)^{4+}]$, and 682.7 $[(M+5H)^{5+}]$ (Figure 5) were consistent with the calculated mass of the desired product 3408.27.

Construct **2**: The detected molecular ions at m/z = 1615.7 $[(M+2H)^{2+}]$, 1077.4 $[(M+3H)^{3+}]$, and 808.4 $[(M+4H)^{4+}]$ were

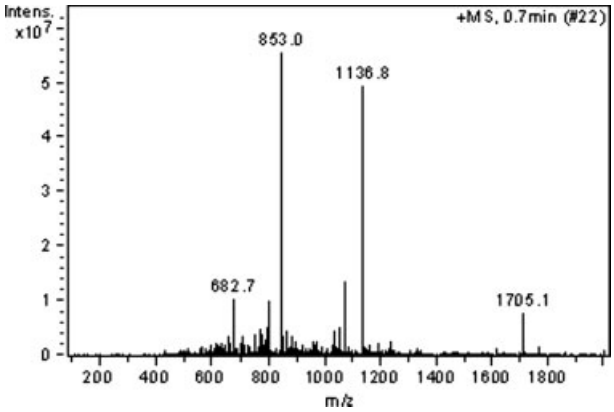

Figure 5. ESI-MS analysis of H_2N-GTGAACGGCCAC-Lys-COOH.

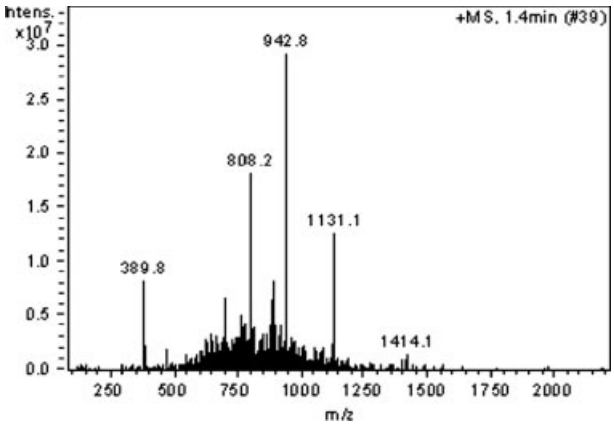

Figure 6. ESI-MS analysis of DOTA-Lys(FITC)-GTGAACGGCCAC-linker*-r-r-r-q-r-r-k-k-r-COOH. * AEEA.

consistent with the calculated mass of the desired product 3230.08 (spectra not shown).

Construct **3**: The detected molecular ions at $m/z = 1414.1$ $[(M+4H)^{4+}]$, 1131.1 $[(M+5H)^{5+}]$, 942.8 $[(M+6H)^{6+}]$, and 808.2 $[(M+7H)^{7+}]$ (Figure 6) were consistent with the calculated mass of the desired product 5650.8.

Limitations

As seen in the HPLC chromatogram of crude **3**, in the course of the synthesis of larger molecules (e.g. longer PNA sequences or PNA-peptide conjugates) more byproducts are formed reducing the yield. Therefore, a relatively long sequence might not be obtained in high yield. However, the separation of the product from these impurities could be easily performed to obtain the PNA conjugate in high purity.

In addition, one has to be aware that in the automated mode, the success of coupling and deprotection can generally not be checked when the cycle is running. Thus, the failure of an intermediate reaction step might emerge only at the end of the synthesis.

A method to reduce the required amount of PNA monomers like the single-shot delivery feature reported in this protocol might not be available in all automated peptide synthesizer models. Therefore, this cost-saving option will be restricted to peptide synthesizers which permit the delivery of small amounts of reagents without priming. In addition, the use of the single-shot feature is leading to a discontinuous, semi-automatic protocol. There are only seven single-shot positions available in the used peptide synthesizer, of which two are used for the activator and base. Thus, only five can be used for PNA monomers. Therefore, not more than a 5-mer sequence can be automatically synthesized at once. As a result, refilling has to be done in the semi-automated mode.

Acknowledgements

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