Research Report

Automating Parallel Peptide Synthesis for the Production of PNA Library Arrays

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ABSTRACT

A system was established for the parallel synthesis of peptide library arrays in a fully automated manner. Synthesis takes place in blocks made of polyoxymethylene that hold during all synthesis steps a polypropylene membrane of 8 × 12 cm. Yields are in the nanomole range, obtained at a low consumption of reagents. The current setup is based on a commercially available pipetting robot and supports the generation of 1536 different oligomers/run. Much higher array densities are possible because the membranes are amicable to spot diameters of down to 200 μm, naturally at a cost of the absolute amount produced of each oligomer. The method was put to use for the creation of arrayed libraries of peptide nucleic acids (PNAs). These can be employed both as a source of PNA molecules applied individually in experimentation subsequent to their release or as intact oligomer arrays in hybridization analyses.

INTRODUCTION

A peptide nucleic acid (PNA) is a DNA analogue in which an N-(2-aminoethyl)glycine polyamide structure substitutes the sugar-phosphate backbone (2,12). It exhibits unique physical and chemical properties—being an achiral and uncharged biopolymer of high biological and chemical stability—and has a high but nevertheless specific binding affinity to complementary nucleic acids. For these reasons, PNA is applied as an agent in the fields of DNA-based diagnostics, therapy, and biotechnology (10,11). Chemically, synthesis is identical to the protocols used in normal peptide synthesis, mainly based on group protection by 9-fluorenylmethoxycarbonyl (Fmoc) or tert-butyloxycarbonyl (tBoc). Standard column-based peptide synthesizers produce 1–10 different molecules/run; some particular machines produce 48–96 different molecules/run. Synthesis of 20-mer molecules takes about three days to complete and yields product in the lower micromole range. Both for the purpose of producing larger numbers of PNA oligomers and for the application of PNA in hybridization-based assays, we initially took advantage of the SPOT methodology (4) for the generation of spatially addressable oligomer libraries (15). This combinatorial synthesis procedure consists of a mixture of sequential and parallel steps: distribution of activated monomers to defined areas (spots) on a contiguous porous support takes place in a sequential manner, while all subsequent chemical reactions, such as capping, washing, and deprotection, occur simultaneously at all molecules. The pores of the solid support act as micro-reaction chambers. High-boiling solvents with low vapor pressure allow sufficient reaction times and therefore high synthesis yields, which are comparable to those of standard peptide synthesizers. With this technology, some 1000 individual PNA sequences could be synthesized on a single pipetting device (9).

However, apart from rather long cycle times, the process has the major disadvantage that it requires manual interference during each cycle because the membranes have to be removed from the pipetting machine for the various washing steps. Subsequently, the membranes have to be refitted and aligned correctly to their initial positions before the next synthesis cycle can be started. The manual handling required is time consuming—synthesis of 20-mers takes about two weeks (9)—and error prone, especially with regard to the accuracy of the repeated refittings, which have immediate and potentially severe consequences to oligomer quality.

In this manuscript, a procedure is described for the array-based synthesis of PNA oligomers that requires no human interference after initial setup, combining parallelism of synthesis with automated control. The procedure is directly applicable to either Fmoc- or tBoc-protected monomers and thus supports current forms of peptide synthesis. The quality of the resulting biomolecules is improved in comparison to the SPOT protocol. Also, cycle times are shorter, with a concomitant reduction in the consumption of expensive reagents.

MATERIALS AND METHODS

Reagents

Fmoc-protected PNA monomers with the exocyclic amino groups of A, G, and C being blocked with the benzhydryloxycarbonyl (Bhoc) group, N-methyl pyrrolidinone (NMP), 1-hydroxy-7-azabenzotriazole (HOAt), and tetramethyl-fluoroformamidinium hexafluoro-phosphate (TFFH) were from PerSeptive Biosystems (Framingham, MA, USA). Dimethylformamide (DMF) and piperidine were from SDS (Peypin, France). Fmoc-β-Ala, N-methylimidazole (NMI), acetic acid anhydride, bromophenol blue, triisopropylsilane (TIPS), diisopropycarbodiimide (DIC), and other solvents were purchased from Fluka (Buchs, Switzerland) and used without further purification. Cy5-labeled DNA oligomers were from Interactiva (Ulm, Germany).

PNA Synthesis on Polypropylene Membranes

Aminated membranes (0.45 µm pore size) with a hydrophilic spacer consisting of 10 polyethylene glycol units (AIMS, Braunschweig, Germany) were used as synthesis support. Molecules intended to stay on the membrane for hybridization experiments were attached via stable peptide bonds using Fmoc-β-Ala, while, otherwise, an acid-cleavable Fmoc-Rink-linker (Calbiochem-Novabiochem, Läufelfingen, Switzerland) was introduced (15). For pipetting, the ASP-222 robot of ABIMED (Langenfeld, Germany) was used.

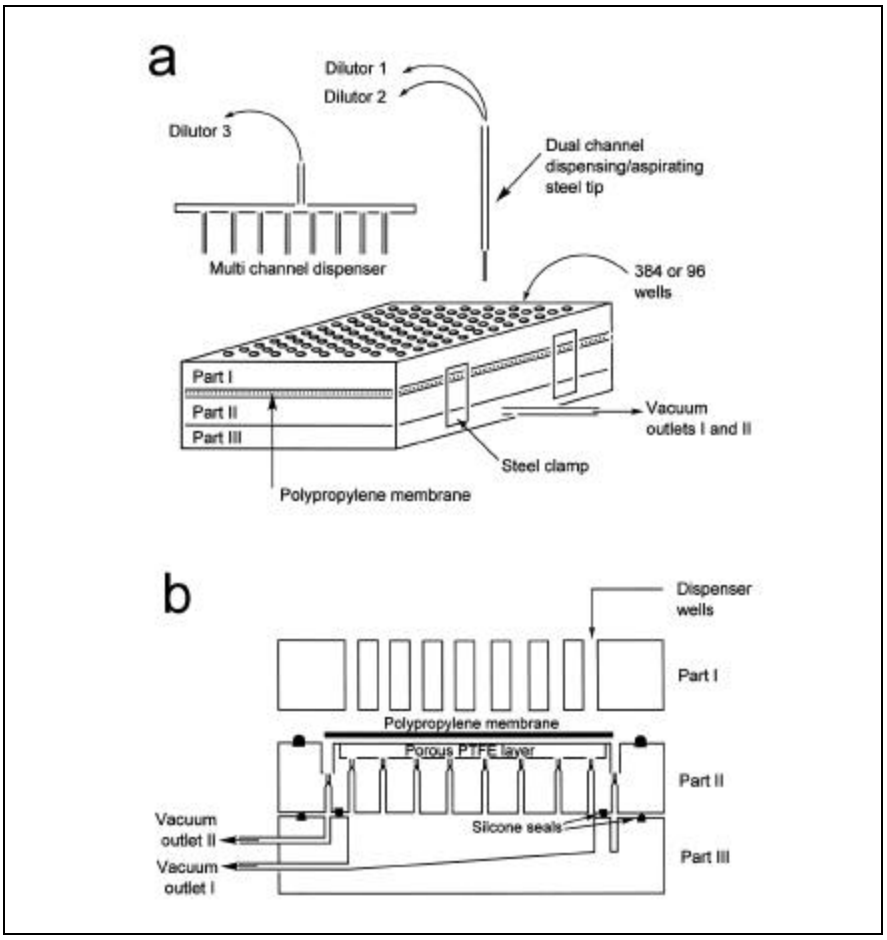

Figure 1. Schematic view (a) and cross-section (b) of the synthesis block. It consists of three parts made of polyoxymethylene held together by four steel clamps and sealed with silicone seals. The polypropylene membrane is sandwiched between parts I and II; vacuum is applied to part III. Distribution of reagents occurs via a double-channel dispenser needle and an 8- or 16-channel dispenser arm for 96- or 384-well blocks, respectively. Overall dimensions are 66 × 106 × 142 mm.

Hybridization

Before hybridization of 5′-fluorescence-labeled DNA oligonucleotides, the membrane was incubated in 5 mL 15 mM NaCl, 1.5 mM Na-citrate, pH 7.5, 3% sodium N-lauroylsarcosine at 20°C. The buffer was changed, and Cy5-labeled oligomer was added to a concentration of 10 nM, followed by incubation for 20 min at the appropriate temperature, usually 20°C. The array was washed twice for 5 min with 10 mL buffer at the same temperature. The membrane was then dried between two cellulose sheets (Whatman, Maidstone, UK). A Fuji® Fluorescence Image Analyzer FLA 3000 (Tokyo, Japan) or a Storm™ 860 FluoroImager™ (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were used for detection of the fluorescence signals. Image analysis was carried out with the AIS software package (Imaging Research, Ontario, Canada).

RESULTS

Chamber for the Synthesis of PNA Arrays

The synthesis block consists of three parts (Figure 1) made of polyoxymethylene. The current design has 384 (16 × 24) vertical channels, each 3.0 mm in diameter. At the bottom of each channel, a polytetrafluoroethylene (PTFE) ring presses a polypropylene membrane to a porous polyethylene support of 6 mm thickness. Polypropylene was found to be the most appropriate support matrix, combining inertness to the solvents with a high loading capacity. In addition, its surface is amicable to the procedures involved in many subsequent experimental procedures, such as hybridization in our case, including the fact that its inherent fluorescence is low enough to use fluorescence labeling techniques. Because of these features, polypropylene had also been used successfully in the field of DNA arrays (1,8,13,14). Underneath the membrane layer, there are in part 2 of the synthesis block small holes of 0.8 mm diameter, through which reagents and solvents are drained by a vacuum of around 200 mbar applied to the outlet. The bottom

of the waste reservoir underneath slops toward the vacuum outlet for a fast and effective removal of liquid. Up to four such blocks could be mounted onto a single pipetting station.

Table 1. Protocol Applied for the Production of PNA Arrays on Polypropylene Membranes Using the 384-Well Synthesis Block

Synthesis Step	Reagents and Solvents	Time (min)	Volumes (mL)	Dilutor
2× coupling	100 mM Fmoc-PNA monomer in NMP	60	0.04	I/II
capping	20% acetic anhydride, 10% NMI in DMF	10	3.84	I/II
wash I	DMF	12	36.30	III
wash II	ethanol	6	14.70	III
wash III	petrolium ether	6	14.70	III
deprotection	20% piperidine in DMF	10	2.90	I
wash IV	DMF	24	72.60	III
wash V	ethanol	12	58.90	III
wash VI	petrolium ether	12	14.70	III
vacuum	-	20	-	-
Total		**202**	**218.68**	

The reagents and solvents are distributed by a dual-channel dispenser needle (dilutor I and II) or a multichannel dispenser arm (dilutor III).

In Situ Synthesis of PNA Oligomers

Activation of Fmoc-protected PNA monomers with DIC/HOAt in NMP was found to last for a few hours only (data not shown). Thus, the monomers could not be kept in an activated state during the entire synthesis process. To circumvent this problem, an aliquot of the relevant PNA monomer was activated just before the respective synthesis cycle. A double-channel dispensing needle connected to two individual dilutors usually took up equal volumes of 0.3 N PNA-monomer, 0.3 N HOAt and 0.4 N DIC in NMP and delivered them to a separate cartridge, one per monomer and synthesis cycle. The reagents were thoroughly mixed by aspiring and dispensing. After 5 min, 0.1 µL activated monomer was distributed to each well of the synthesis blocks. Once the first monomer was placed to all appropriate array positions, the other three monomers were dispensed in the same manner. In between, the needle was rinsed several times with DMF. After distribution of the last monomer, the whole procedure was repeated once to ensure high coupling yields, followed by a final incubation period of 5 min.

Subsequently, unreacted amino groups were inactivated with capping solution (20% acetic acid anhydride and 10% NMI in DMF, mixed 1:2 before use) delivered to the wells by a multichannel dispensing gadget that is attached to the same robot arm that holds the dispensing needle. This step was followed by incubation with DMF. Vacuum was applied, and the solvent was slowly drained through the porous membrane. Washing was repeated several times with ethanol and petrolium ether before a solution of 20% piperidine in DMF was used to cleave off the terminal Fmoc groups. The washing procedure was repeated with DMF, ethanol, and petrolium ether. Finally, a vacuum was applied for 20 min for drying the membrane before the next cycle was started. In total, cycle time was slightly less than 3.5 h. In Table 1, details on the particular reagent volumes and reaction times are given.

Final Deprotection and Release of Individual Oligomers

After synthesis, the membranes were removed from the blocks. Spots with oligomers attached via an acid-cleavable Rink handle were punched out and concomitantly transferred to microplate wells. For final deprotection, a mixture made of TIPS, dichloromethane, and trifluoroacetic acid (TFA) at a ratio of 5:70:25 was freshly prepared, and 20 µL were added to each well. After an incubation of 15 min at room temperature, the supernatants were transferred to another microplate, and fresh solution was added to the membranes. The two fractions were combined and lyophilized. For neutralization, 100 mM triethylammoniumacetate buffer (pH 7.0) was added, and drying was repeated. The dry material was stored at 4°C. Before subsequent analyses, the PNA molecules were dissolved in 0.1% aqueous TFA or pure water.

PNA oligomers attached to the membrane via a stable peptide bond were deprotected in the same manner as above, only with the difference that after the treatment with TIPS/dichloromethane/TFA the membranes were washed in dichloromethane, DMF, water, and ethanol (4). They were stored at 4°C or used directly in hybridization experiments.

Quality Assessment

During synthesis, coupling yields could be determined by visualizing spots with a solution of 0.01% bromophenol blue in DMF (6). This procedure had been found to correlate well with the actual yield (15). The color intensity resulting from this assay weakened with the increasing number of synthesis cycles. From this, an average coupling yield of around 96% could be estimated. On average, the loading of the membranes was found to be about 50 nmol/cm^2, as determined by a trityl-assay (9) and probe hybridization. The quality of the oligomers was additionally controlled by hybridization analysis (Figure 2) in direct comparison to oligomer libraries generated by the standard SPOT protocol; in a few cases, such results were confirmed by mass spectrometry. The observed signals were also compared to the predicted melting temperatures calculated as described (5); it should be noted, however,

Table 2. Sequences Used in Hybridization Experiments

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Array Position:                28         17           3
PNA octamers:     (C-)   ACTCTCGG   AGCCTGTA     ACAGACTC   (-N)
                         ||||||||   ||||||||     ||||||||
DNA octamers:     (5′-)  TGAGAGCC   TCGGACAT     TGTCTGAG   (-3′)
DNA 37-mer         5′- TCTGAGAGCCCACTCGGACATGTTTACTGTCTGAGGT -3′


Array Position:          33           47        58
PNA octamers:     (N-)   ACTCTCGG     CTGTACAA   ACAGACTC   (-C)
                         ||||||||     ||||||||   ||||||||
DNA octamers:     (3′-)  TGAGAGCC     GACATGTT   TGTCTGAG   (-5′)
DNA rev37-mer:     3′- TCTGAGAGCCCACTCGGACATGTTTACTGTCTGAGGT -5′
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Two 37-mer sequences were picked that were inverse in their sequence. All 60 possible octamer sub-sequences were synthesized on PNA arrays and hybridized with the respective DNA octamer. The positions of six exemplary oligonucleotides are indicated, whose hybridization results are presented in Figure 3.

that the predicted values can only provide a rough estimate of the actual intensities because various aspects of hybridization are unaccounted for in the equation used for their calculation. Oligomer quality was found to be significantly more variable in the case of the SPOT synthesis procedure, most likely because of the repeated refittings of the support matrices to the pipetting device. Every inaccuracy in positioning immediately translated into imperfect extension of a part of the oligomers placed around the edges of the spots. Also, the individual spots can vary in size, again leading to variation in condensation yields during oligomer synthesis. The over-proportional signal intensities in spots 23 and 24 of the SPOT array in Figure 2, for instance, were caused by large spot areas that most probably resulted from a transfer of volumes larger than normal at the beginning of synthesis. While increasing the total amount of oligomer, this simultaneously reduced the oligomer quality significantly. These factors were eliminated in the automated procedure, resulting in improved reproducibility across the entire reaction area.

Hybridization Assays

To test the specificity of interaction with DNA, a PNA library of 60 octamers was synthesized as part of a larger array, each oligomer sequence overlapping by seven bases with its two immediate neighbors and thus representing all possible octamer sub-sequences contained in two 37-mer sequences, each 37-mer actually being the inverse of the other (Table 2). Octamer DNA oligonucleotides were used in individual hybridization experiments (Figure 3). Only interaction between complementary sequences of antiparallel orientation was observed (Figure 3, a–c). The poor signal of the oligonucleotide d(TTGTACAG) (Figure 3c) was not surprising and could also not be increased by reduction of the hybridization stringency. Its central stretch of self-complementary sequence leads to hybridization between the oligonucleotides in solution, decreasing substantially the free concentration of single-stranded molecules able to hybridize to the PNA complement on the array. Because of the relatively slow kinetics of PNA-DNA nucleation and the short incubation period, this effect could not be offset by the higher thermodynamical stability of the PNA-DNA duplex. The oligonucleotides d(TGAGAGCC) and d(CCGAGAGT) clearly cross-hybridized with each other's complementary PNA octamers (Figure 3, d and e) because they share the core GAGAG sequence. The octamer d(TGTCTGAG) hybridized to three positions of the array (Figure 3f). In addition to its full-match annealing at around position 7 of the PNA array, the oligonucleotide bound also via a GTCTG partial match to the PNA molecules at positions 32 and 33 as well as positions that overlap with the binding site of oligonucleotide d(TGAGAGCC) (Figure 3d). In all these cases, 4–5 consecutive base pairs contributed sufficient energy for a duplex formation at 20°C. This indicates that PNA oligomers are particularly advantageous for hybridization to arrays of short sequences because of the relatively high rate of discrimination between short sequences at, nevertheless, good thermal stability. Parallel hybridization, potentially possible because of the PNA's achiral nature, could not be detected for several full-match sequences in this and several other experiments and was therefore ruled out as causing molecule interaction on PNA arrays.

DISCUSSION

Combinatorial approaches are prerequisite to many high-throughput screening techniques. For their efficient application, however, reasonably large numbers of molecules and, by virtue of this, automation in their production are essential. Here, a robust procedure is presented for the automated chemical synthesis and presentation of PNA oligomers and peptides. The reliability of the system was demonstrated here by synthesis and analysis of PNA oligomers. However, results from peptide syntheses confirmed the findings (not presented). The major advantage over the SPOT protocol is the automation of the process, which not only directly translates into much increased speed of synthesis—now comparable to column-based systems—but also improved the quality of the oligomers produced across each entire reaction area of the membrane. In comparison to column-based systems, many more oligomers can be synthesized in parallel at a low consumption of reagents per PNA molecule. Also, they are readily presented for subsequent analyses. With the current pipetting setup, 1536 individual peptide/PNA oligomers could be synthesized in parallel. Larger numbers are a matter of reduced bore sizes in the blocks because spots with diameters of down to 200 µm were found to be workable with the polypropylene membranes used (data not shown). Even with the distance between spots being as big as the spots themselves, a single membrane of 8×12 cm could hold as many as 60 000 oligomers. Prerequisite to such densities is a pipetting system of appropriate spatial resolution. However, there exist commercial devices able to perform at this level.

With a reactive anchor group coupled to the end of each PNA oligomer as a last step of synthesis, full-length molecules could be isolated and bound to appropriately activated surfaces, very similar to a process recently described for DNA-oligonucleotides (7). This could be advantageous, especially for the synthesis of long oligomers, because of the intrinsic purification effect for full-length molecules. Also, by this means, the more macroscopic library arrays synthesized with the pipetting technology described here could act as a material source for the generation of multiple copies of microarrays of very high probe density. Alternatively, control procedures for high-resolution synthesis such as photolithography (3)

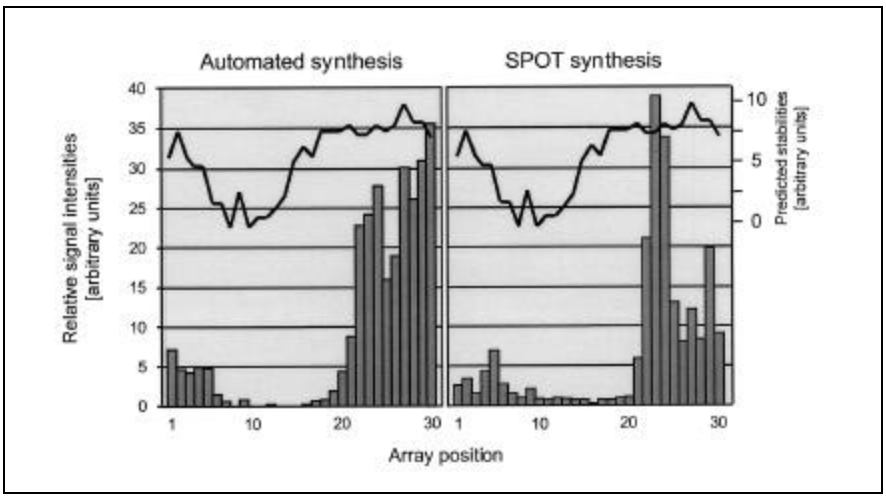

Figure 2. Synthesis quality. Direct comparison of signal intensities obtained on PNA arrays generated by the automated synthesis procedure described in this manuscript (left panel) and the SPOT protocol (right panel). The membrane-bound PNA octamers—representing the sequence 5′-TGGAGTGTGTCATTTGTACAGGCTCACCCGAGAGTCT-3′—were hybridized with a mixture of complementary DNA targets. Signal intensities vary because of different dissociation temperatures of the individual sequences. However, the signals on the array produced by the SPOT-procedure deviate significantly more from the predicted values (black line), indicating additional sources of variation.

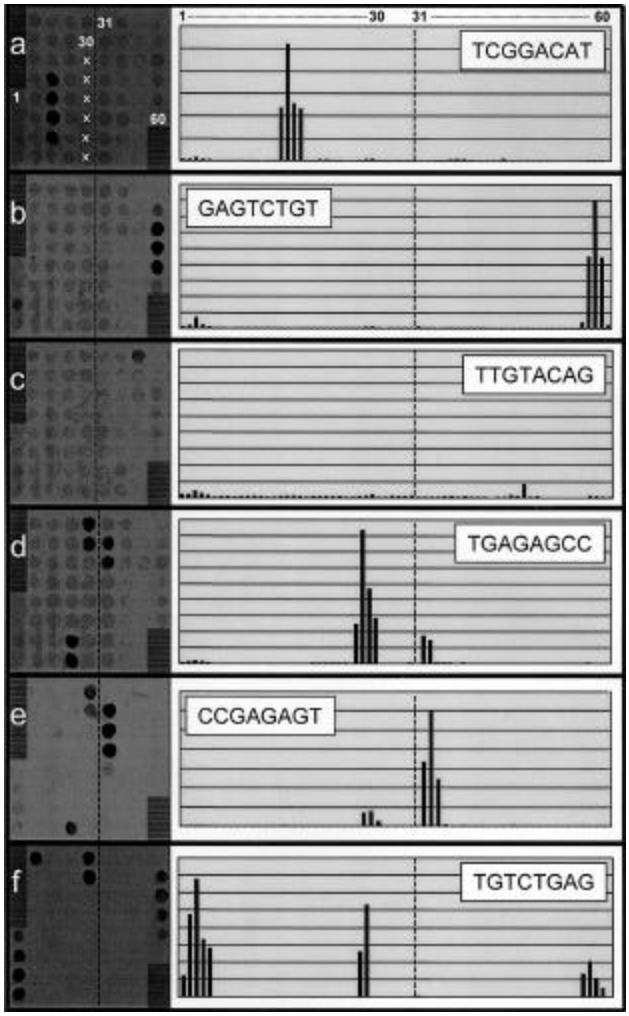

Figure 3. Hybridization of individual octamer oligonucleotides to a PNA array. The panels to the left present the actual fluorescence images obtained from scanning the array are shown; irrelevant regions spots are darkened. The panels to the right present a quantification of signal intensities. Also, the respective oligonucleotide sequence is shown. The signal in panel c is weak, but nevertheless specific to the full PNA complement of the oligonucleotide. In panel a, a few spots on the image and the respective peaks in the graph are named for better orientation. The six positions labeled with "x" are control spots of entirely unrelated sequence.

could be used for array production upon availability of monomers with light-sensitive protection groups. We are following both routes toward the establishments of microarrays of both PNA and peptide libraries.

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