International Journal of Peptide Research and Therapeutics, Vol. 13, Nos. 1–2, June 2007 (© 2007) pp. 367–375
DOI: 10.1007/s10989-007-9096-x

Economical Parallel Oligonucleotide and Peptide Synthesizer – Pet Oligator

Michal Lebl,[1,3] Christine Pistek,[1] John Hachmann,[1] Petr Mudra,[2] Vaclav Pesek,[2] Vit Pokorny,[2] Pavel Poncar[2] and Karel Zenisek[2]

(Accepted April 3 2007; Online publication May 2, 2007)

Abstract We have developed a small benchtop oligonucleotide synthesizer which allows the scientist to prepare, rapidly and economically, up to 24 oligonucleotides in one batch. We have shown that this instrument can be used for peptide synthesis, as well. The instrument is based on the centrifugation method for solid–liquid separation.

KEY WORDS: automated synthesizer; centrifugation; parallel synthesis.

INTRODUCTION

Most automated synthesizers are based on Merrifield's solid-phase synthesis technology (Merrifield, 1963) and his pioneering concepts for automation of this process (Merrifield *et al*., 1966; Merrifield and Stewart, 1965). The majority of parallel synthesizers uses commercially available pipetting robots for the delivery of reagents and wash solutions to the synthetic compartments. The capacity of these synthesizers ranges from 12 to 384 compounds that can be synthesized in one run (for the review see e.g. (Lebl and Hachmann, 2005)).

Design of oligonucleotide synthesizers benefited from optimized chemistry developed in the 1980s. Phosphoramidite chemistry (Caruthers *et al*., 1987) allowed for very short coupling times performed on non-swelling support (controlled pore glass, CPG) packed in a column and percolated by reagents used for coupling, washing, oxidation, capping, and deblocking. Commercially available synthesizers were making one oligonucleotide at a time, with cycle time of about 6 min. Throughput of these machines was limited, and companies supplying custom oligonucleotides employed hundreds of these machines. Since the 1990s, several companies developed oligonucleotide and peptide parallel synthesizers utilizing 96 or 384 well filter plates (Cheng *et al*., 2002; Rayner *et al*., 1998), or synthesizers based on alternative technologies (Albert *et al*., 2003; Gao *et al*., 2001; Hughes *et al*., 2001; Komolpis *et al*., 2002; Lausted *et al*., 2004; Livesay *et al*., 2002; Pellois *et al*., 2002).

One of the fundamental processes in multiple solid-phase synthesis is the parallel removal of excess reagent and wash solutions from the solid support in all synthetic compartments. In most currently available synthesizers, this is achieved through the porous bottoms of the synthetic compartments, either by vacuum filtration or by application of pressure from the top of the compartments. These methods bear the inherent risk of clogging of one or more compartments, resulting in insufficient liquid removal from the clogged compartments, overflow, and, consequently, contamination of neighboring compartments. An alternative method employs aspiration of the liquid from the

Dedicated to the memory of Prof. Bruce Merrifield, who was the inspiration behind all of our efforts in building automatic synthesizers.

[1] Illumina, Inc., 9885 Towne Centre Drive, San Diego, CA, 92130, USA.
[2] Institute of Organic Chemistry and Biochemistry, Flemingovo nam. 2, Prague 6, Czech Republic.
[3] Correspondence should be addressed to: Tel.: +1-18-582024504; Fax: +1-18-582024680; e-mail: mlebl@illumina.com

surface (Krchnak *et al.*, 1997; Lebl *et al.*, 1999) but it is difficult to apply in ultra small scale synthesis.

We have devised a new technological concept for the automation of the solid-phase synthesis of large compound arrays (Lebl, 1999). The key feature of this technology is an alternative method for separation of the solid support from reagent solutions, termed "tilted plate centrifugation", which uses decantation by centrifugation as a means of liquid removal. Tilted plate centrifugation technology uses the wells of microtiter plates as synthetic compartments, thus enabling the parallel synthesis of large compound arrays (i.e., 3,072 compounds when eight 384-well plates are used). The plates are mounted on a centrifugal plate and slightly tilted down towards the center of centrifugation, thus generating a pocket in each well, in which the solid support is collected during centrifugation, while the supernatant solutions are expelled from the wells (Lebl *et al.*, 2001).

Centrifugation is the only truly parallel technique, which can be scaled up for processing volume or the number of simultaneously run reactions, without the limitation of overpressure or vacuum-driven filtration. We have applied the technique of tilted centrifugation in our design of oligonucleotide synthesizers. This concept allowed Illumina, Inc. to become a major player in the custom oligonucleotide market. At the same time, we felt that there is an unmet need for a synthesizer capable of parallel synthesis of only a fraction of microtiter plate well numbers, which would not consume reagents independently of the number of wells being processed (i.e. improve the economy of synthesis).

In order to ensure efficient liquid removal (i.e., no solution remaining in the wells after centrifugation), and at the same time to avoid any loss of solid support during centrifugation, the volume of the well-pockets should be equal to the volume of swollen resin in each well. Optimal well-pocket volume can be achieved by using plates with varying well volumes and/or modifying the tilt angle, as well as modifying the speed of rotation. The potential issue of well-to-well cross-contamination with reagent solution or resin is avoided by designing the synthesizer to have only one row of synthetic compartments on its perimeter. The development of the personal synthesizer comprised (i) the original rotor design, (ii) the building block delivery system, (iii) the bulk reagent delivery system, and (iv) the computer control system.

RESULTS AND DISCUSSION

Rotor design

The delivery into openings of the rotor did not require bringing the nozzles of the delivery banks extremely close to the plate. Therefore, we could design the machine with a stationary layout of nozzles and only one movable part – the rotor. The rectangular profile of the rotor well was satisfactory for retention of solid support of the synthesis. The size of the well-pocket was defined by the speed of rotation in the solvent removal step. The first generation of the rotor was constructed from Teflon with 25 openings on the perimeter. A total of 24 openings were used for the synthesis, and one opening went through the bottom of the rotor and was used as a priming position for the nozzles.

The second generation rotor was created by vacuum forming of a polypropylene sheet. Because of its very economical production ($0.50 per rotor), it was considered disposable, thus avoiding potential cross contamination of new product by product from the previous synthesis. Figure 1 shows these two versions of the rotor. The first rotor was attached to the motor shaft with a simple pin protruding to the side of the shaft and was secured in place by its own weight. The second generation of the synthesizer had the motor placed above the rotor and attachment of the vacuum-formed polypropylene rotor was achieved by a simple spring-loaded clip mechanism.

Reagent delivery system

The liquid delivery system is based on a stationary bank of nozzles placed in a circular fashion above the openings of the rotor with a pitch identical to double the distance between the wells. In this way, we have 12 positions for liquid delivery to the rotor wells. This number allows for use of six bases (A, C, G, T, and two modified bases), activator, wash solution (acetonitrile), oxidizer, two capping solutions (acetanhydride solution and base solution), and deblock solution.

We have tested this assembly both with a syringe pump operated system and with timed delivery of pressurized solutions through solenoid valves. The latter arrangement was shown to be superior both in performance and also in cost. The solutions are stored in pressurized (argon, 3 psi) glass vessels and solenoid valves in the bank are actuated according to a computer algorithm depending on the position of

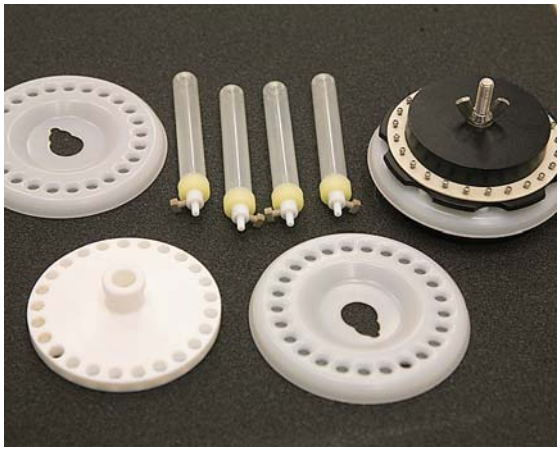

Fig. 1. Tools for up to 24 parallel syntheses. Bottom left – original Teflon rotor design, bottom right and upper left – polypropylene vacuum-formed disposable rotor. Upper center – cartridges for phosphoramidite dissolution. Upper right – tool for final deprotection of the CPG in rotor (arrangement of 25 teflon balls supported by polypropylene foam compressed by anodized aluminum plate against the rotor supported by another aluminum plate).

the well in the rotor under the nozzle. Every nozzle have to be calibrated for particular solution (different viscosities of solutions result in the need for different time to deliver the same volume) prior to synthesis. During one rotor revolution, all wells are consecutively placed under all nozzles, and all reagents are delivered. Due to the fact that all nozzles can be operated at the same time, the delivery of the reagents is very fast – one pass over the standard rotor delivering 25 μL of reagent can be achieved in less than 10 sec.

The advantage of using separate delivery nozzles for each reagent is that there is no need for extensive priming of the lines. However, we perform a small priming of the nozzles (10 μL) before each delivery cycle to guarantee that even the first delivery of reagent was not exposed to the fumes inside of the synthetic chamber.

The first generation synthesizer (fig. 2A) used larger-size Lee valves LFVA244032OH (The Lee Company, Westbrook, CN, USA), which were integrated into the top cover of the synthetic chamber (centrifuge lid) made of PEEK. Reagents were introduced and connected to the nozzles (PEEK tubing inserted in teflon sleeves press-fitted into the PEEK centrifuge cover) through threaded ports on the side of the cover (see fig. 3). This arrangement had the disadvantage of having to deal with the tubing connecting the flask with the solenoid valves. The second generation of the synthesizer actually integrated the storage compartment for the reagents with the solenoid valves. In this case, we used Lee valves INKX0502600AB and connected them directly to storage vessels, which were disposable polypropylene containers equipped with a stopcock (see fig. 1, 2B). The container was placed onto the machine, attached to gas pressure, the stopcock was opened, and reagent was ready to be delivered. Bulk reagents were attached to the valves by tubing as in the previous generation.

To test the potential of this type of synthesizer for peptide synthesis, we modified the reagent delivery system of the first generation synthesizer to allow the operator to manually interact with the instrument in every cycle. (The valves of the second generation machine did not allow the use of DMF in the system.) After deprotection and washing, the synthesizer presented individual wells under the inspection window and prompted the operator to deliver the appropriate amino acid solution. We have used two 30-min couplings with diisopropylcarbodiimide in the presence of *N*-hydroxybenzotriazole. We have synthesized four standard decapeptides (each being synthesized with six replicates) to evaluate the performance of the synthesizer. We have not observed any significant difference in quality of the products when compared with our synthesizer designed for the synthesis of 768 peptides in a batch.

Modification of this synthesizer for automated delivery using a single channel pipetting system should be trivial – the pipetting system delivers from multiple locations (stock solutions of amino acids) to one location in the centrifuge (inspection window, see fig. 3).

The synthetic compartment (drum)

The first generation machine had a stepper motor placed under the drum, and we had to be careful to seal off the motor from the harsh environment in the drum. The drum is constantly flushed with the flow of nitrogen, but every centrifugation step creates mists of reagents. Reagents are collected at the lowest part of the tilted drum and sent to the waste collection vessel. The disadvantages of this system are that it is harder to clean and the visual inspection of the synthetic progress can be done only by placing one well after another under the inspection window in the front of the instrument. The second generation instrument, with the motor placed above the drum, did not have problems with protecting the motor from the fumes, the drum was easily serviceable (it

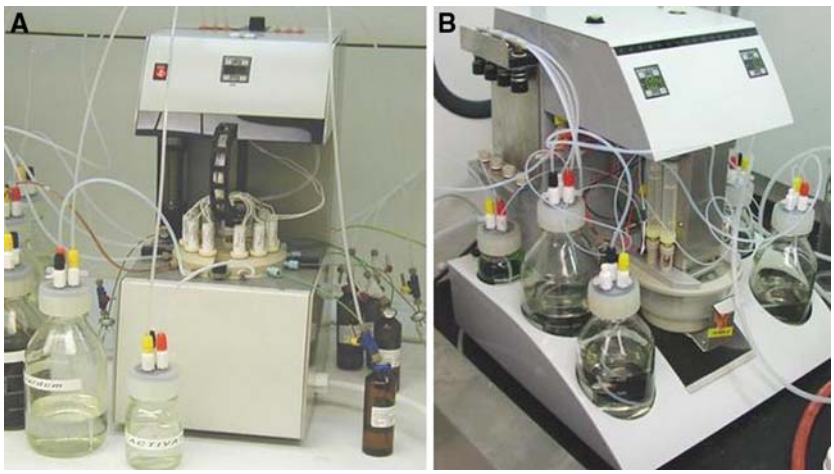

Fig. 2. Two versions of the synthesizer.

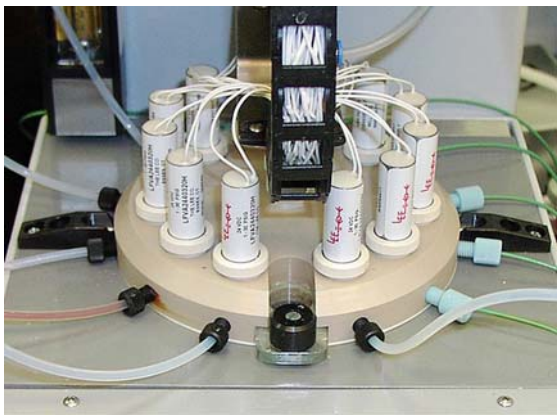

Fig. 3. PEEK cover of the synthesizer drum with integrated delivery nozzles, solenoid valves and glass inspection window. Phosphoramidites are introduced through peek tubing, activator and reagents use teflon tubing.

could be tilted down easily), and since it was made of polypropylene, synthetic progress could be checked at any moment. Figure 4 illustrates the monitoring of the deprotection step by observing dimethoxytrityl cation orange color.

The control system

The synthesizer control system utilizes a standard PC architecture, and the software is written in Visual Basic. It allows for the creation and editing of synthesis protocols (the step by step instructions driving the machine's hardware components). Instructions are written as English text statements representing the instruction and the parameters associated with that instruction. The interface also allows the loading, editing, saving, and running of protocols once they have been created. Figure 5 shows a screenshot of the window displayed during the synthesis. Programs resided on a Sony Vaio laptop computer (we have also successfully tested the version of the program residing on an Ipaq Pocket PC).

The PC communicates via RS-232 with two subcontroller systems to control the entire machine. The first system used a Microcon motion controller and a Pacific Scientific stepper motor. The second control subsystem used a valve controller board (VCB), which controlled the fast solenoid valves during the base dispensing process. This dedicated 8-bit controller downloads the sequence information and dispense volumes from the PC via an RS-232 link. The controller calculates the actual dispense time based upon an empirically determined calibration curve that correlates pressure and delivery volume to time (the amount of time the valve must be kept open to deliver the desired volume).

The dispense cycle is composed of moving the rotor under the dispensing head while the Microcon signals the VCB when the rotor is correctly positioned over sets of wells. At each trigger interval the VCB opens the valves for the desired solution in the desired well for the pre calculated time interval.

Synthetic results

The Pet Oligator system was used in hundreds of separate syntheses of up to 24 oligonucleotides in a batch, with lengths spanning from 25-mers up to 125-mers. We used mass spectroscopy and HPLC

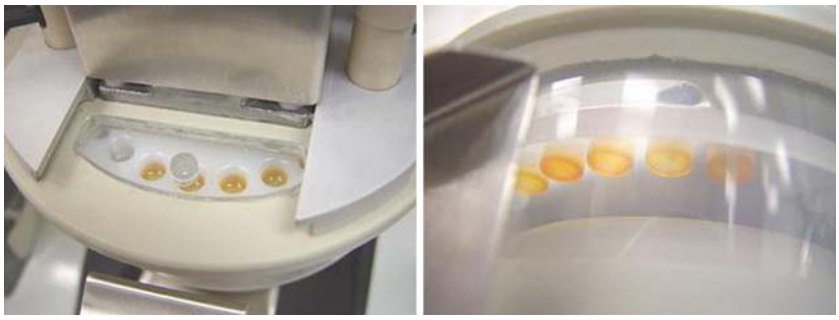

Fig. 4. Monitoring of the progress of deprotection step of oligonucleotide synthesis. Left – through the inspection window of the second generation synthesizer; right – from below through the wall of polypropylene drum.

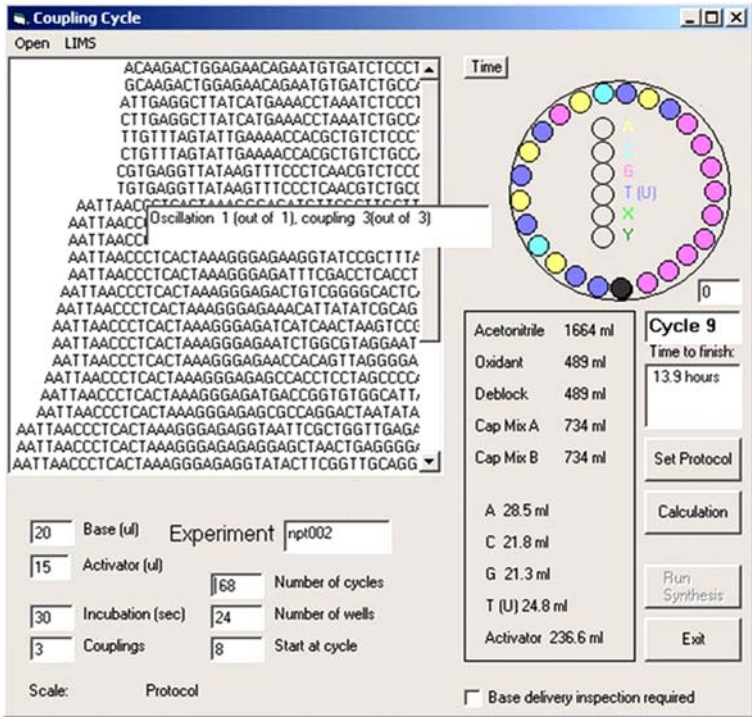

Fig. 5. Screenshot of the computer synthesis window. (Screenshot for test synthesis of 68-mers using three 30 sec couplings).

for evaluation of quality of produced compounds, and we found this small synthesizer equal to or better performing than both individual commercial synthesizers or multiple synthesizers built at Illumina. Several traces of synthetic 25-mers are given in fig. 6.

In semiautomatic peptide synthesis, we achieved comparable purity of model peptide sequences with that obtained from a fully automatic centrifugal synthesizer. The HPLC traces shown in fig. 7 can be compared with results achieved in our comprehensive study evaluating coupling reagents in high throughput peptide synthesizer (Hachmann and Lebl, 2006b).

MATERIALS AND METHODS

Oligonucleotide synthesis

Cap A solution (tetrahydrofuran (THF)/acetic anhydride (9:1), Cap B solution (10% *N*-methylimidazole in THF/pyridine (9:1), Oxidizer solution (0.02 M iodine in THF/pyridine/H_2O (89.6:0.04:10)

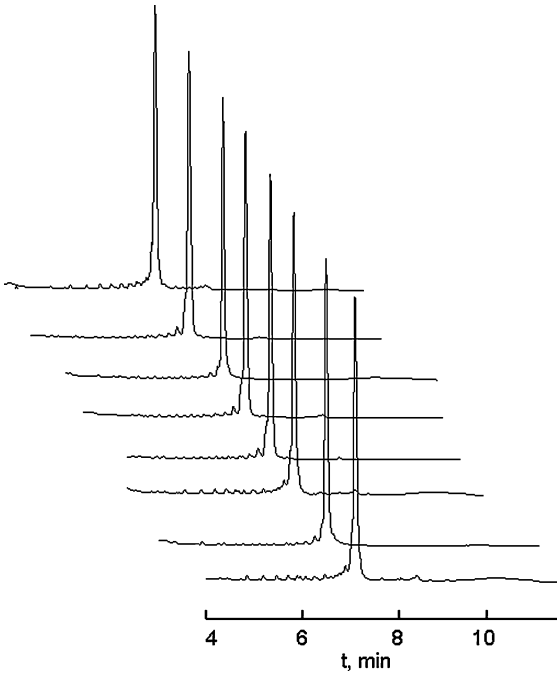

Fig. 6. HPLC traces of 25-mer oligonucleotides. For HPLC conditions see experimental part.

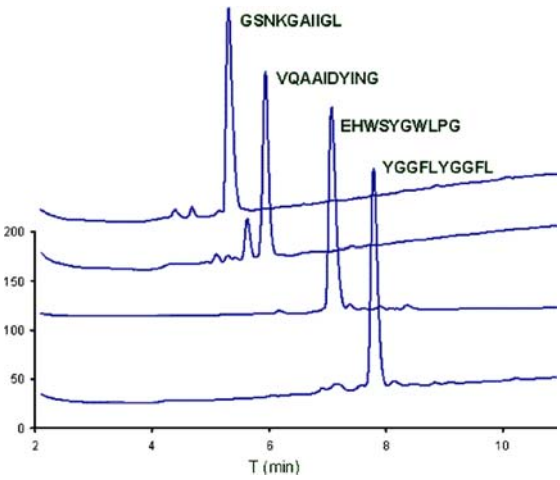

Fig. 7. HPLC traces of model peptides (beta-amyloid 25–34 GSNKGAIIGL, acyl carrier protein 65–74 VQAAIDYING, LHRH analog EHWSYGWLPG, and enkephalin dimer YGGFLYGGFL).

and Activator solution (4,5-dicyanoimidazole in acetonitrile) were purchased and the vendor's bottles were attached directly to the instrument. Low water content (< 30 ppm) acetonitrile was purchased in 4 L bottles. To each bottle a large Trap Pak™ (ABI) was added and the bottle was attached to the machine. Deblock solution was prepared by addition of 100 mL dichloroacetic acid (DCA) to 4 L dichloromethane, and this bottle was also attached to the instrument. All reagents were from Glen Research, Sterling, VA.

The phosphoramidites, d-(Bz)A-CEP, d-(Ac) C-CEP, d-(iBu)G-CEP, and d-T-CEP (Bz = N^6—benzoyl, Ac = N^4—acetyl, iBu = N^2–isobutyryl, CEP = 3′-*O*-cyanoethyl- *N*,*N*-diisopropylaminophosphine) were purchased in serum bottles (Glen Research, Sterling, VA) and were dissolved in anhydrous acetonitrile transferred by syringe from an Aldrich Sure-Seal™ bottle. When dissolved, they were transferred to an oven-dried 60 mL serum bottle, to which a small Trap-Pak™ (ABI) had been added before sealing. Again using a syringe, additional anhydrous acetonitrile was added to make the final volume. The amidite bottles were then attached to the instrument.

The instrument was connected to a pressure line containing dry nitrogen. The bottles containing the reagents were pressurized to 3.0 psi. During the run, dry nitrogen was allowed to flow through the reaction chamber at 5 L per minute.

The solid support used was the Universal Support from Glen Research (Sterling, VA). For oligomers of 40 bases in length or less, 500 Å pore size CPG (controlled pore glass) was used. For longer sequences we used 1000 Å pore size support. The CPG support was plated onto a metal ring, which had holes drilled in it calibrated to contain approximately 4 mg of the support. The 500 Å CPG with typical loadings (≈50 μmol/g) resulted in a 200 nmol synthesis per well, and 1000 Å support (≈33 μmol/g) gave ≈133 nmol per well.

The rotor was inverted and placed over the ring. The assembly was inverted again so that the support was deposited into the rotor wells. The rotor was then placed in the synthesizer.

The phosphoramidite synthesis protocol was used (see Table I). The instrument allowed the cycle to be paused during the deblock step so that the dimethoxytrityl (DMT) color could be inspected visually. The synthesis cycle was repeated until the complete oligonucleotide was synthesized and then stopped. The final detritylation step was conducted by using a separate program. This allowed the operator to observe the final deblock color. The rotor was removed from the instrument and used in the cleavage and deprotection step.

Table I. Synthetic protocol used in the synthesis of oligonucleotides

Step	Volume	Incubation	Repeat
Deblock	60 μL	30 sec	5 times
Wash (ACN)	60 μL	1 sec	7 times
Prime amidites and activator	–	–	4 times
Couple	20 μL amidite 15 μL activator	30 sec (agitation)	3 times
1st Cap	30 μL Cap A, 30 μL Cap B	1 sec	5 times
Oxidizer	60 μL	1 sec	5 times
Wash (ACN)	60 μL	1 sec	3 times
2nd Cap	30 μL Cap A, 30 μL Cap B	1 sec	5 times
Wash (ACN)	60 μL	1 sec	5 times

After synthesis, the oligonucleotides were cleaved from CPG and the protecting groups removed by treatment in either concentrated ammonium hydroxide or 40% aqueous methylamine. Solution was pipetted into the wells by the user, and the wells were capped by an assembly of teflon balls supported by foam polypropylene – see fig. 1. Assembly was placed in the oven for 8 h at 80 °C (ammonium hydroxide) or at 55 °C (methylamine).

The rotor wells were extracted with 4×150 μL water using a Packard Multiprobe™ (Perkin Elmer Life and Analytical Sciences, Boston, MA) robotic dilutor. The scheme of the operation on the robotic bed is shown in fig. 8. The cleaved oligonucleotide solutions were transferred to a 1 mL $\times$ 96 well microtiter plate. The oligonucleotide solutions were analyzed without additional purification. For HPLC analysis, 50 μL of the extract was transferred to an Evergreen (Evergreen Scientific, Los Angeles, CA) 96 well v-bottom microtiter plate and was diluted by addition of 150 μL water.

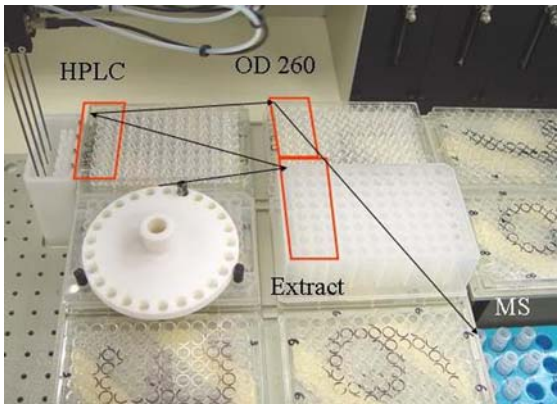

Fig. 8. Scheme of robotic extraction and sampling. Solution from the rotor was extracted into the deep well plate (Extract), from which a sample was taken and diluted for HPLC (HPLC), the fraction of which was taken to the plate for reading the optical density (OD260) and to vials for mass spectroscopy (MS).

A 20 μL aliquot of each well was taken and diluted to 200 μL in a BD Falcon Microtest™ 96 well plate (VWR International, West Chester, PA) with a UV transparent film bottom. The plates were read at 260 nm in a Tecan SpectraFluor Plus plate reader.

HPLC analysis was carried out with an Agilent 1100 HPLC system using a Dionex (Sunnyvale, CA) DNAPac™ PA-100 4×250 mm anion exchange column. The runs were carried out at room temperature. Buffer A consisted of 0.02 M NaOH, pH 12.0 ± 0.1, and buffer B was 0.02 M NaOH, pH 12.0 ± 0.1, 2.0 M NaCl. The flow rate was 1.5 mL/min, gradient 5–55% of B in 15 min, and the absorbance was monitored at 260 nm.

Peptide synthesis

Fmoc amino acids, DIC, and Rink resin (0.4 mmol/g) were purchased from Novabiochem (EMD Biosciences, Inc., San Diego, CA, USA) or Advanced ChemTech (Louisville, KY, USA). Solvents were from VWR International, Inc. (West Chester, PA, USA). 4–Methylpiperidine was from Sigma-Aldrich (Milwaukee, WI, USA).

Rink resin (300 mg) was added into mixture of DMF and DCM (10 mL total) to form nonsedimenting suspension which was distributed into the wells of the rotor. The rotor was placed into the synthesizer. An additional 100 μL of DMF was added into the wells (beads sedimented) and the rotor was centrifuged. The standard synthetic protocol was followed (Table II) (wash three times with DMF, 2 and 15 min with 25% 4–methylpiperidine in DMF (Hachmann and Lebl, 2006a), 6 times with DMF, and once with 0.01% bromophenol blue in 1% HOBt in DMF (Krchnak *et al.*, 1988)). Software was modified so that the synthesizer stoped after washing and deprotection and waited for the operator to manually pipet the appropriate amino acid solution (0.3 M solution in 0.3 M HOBt in DMF) and

Table II. Synthetic protocol used in the synthesis of peptides

Step	Volume	Incubation	Repeat
25% MePip/DMF	60 μL	60 sec	2 times
25% MePip/DMF	60 μL	15 min(agitation)	1 time
DMF	60 μL	15 sec	6 times
0.01%BB, 1% HOBt/DMF	60 μL	15 sec	1 time
Couple (manual reagent addition)	25 μL 0.3 M AA in 0.3 M HOBt/DMF25 μL 1 M DIC/DMF	30 min (agitation)	2 times
DMF	60 μL	15 sec	3 times

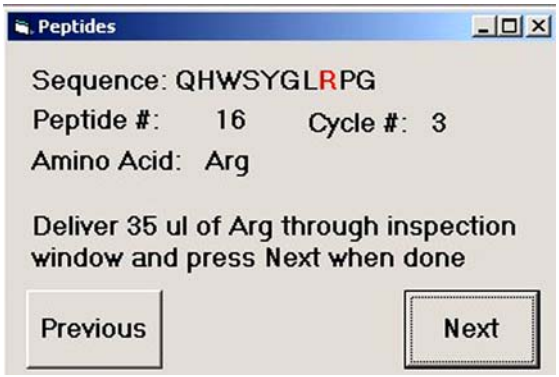

Fig. 9. Peptide synthesis prompt in semi manual synthesis.

coupling reagent (1 M DIC in DMF) into the wells of the rotor. The program prompts the operator with the information about the well, which is under the inspection window and does not go into the next step of the synthesis until all wells were served (see fig. 9). The rotor was oscillated 5 times and rested for 50 sec. (During oscillation the plate is rotated at a speed at which the liquid does not overflow the wall of the well and solid support moves towards the outer side of the well. When the rotation is stopped, liquid returns to horizontal position and beads distribute at the well bottom, mixing thus the well content.) This procedure was repeated 30 times. Plate was centrifuged and addition of amino acids and reagents were repeated. After another 30 cycles of oscillation and resting the reagents were removed by centrifugation and washing and deprotection was repeated to prepare plate for the next cycle of synthesis.

At the end of the synthesis, rotor was dried in vacuo and 150 μL of mixture K (King *et al.*, 1990) (TFA/thioanisol/water/phenol/EDT: 82.5:5:5:5:2.5 v/v) was added. The rotor was capped by the teflon ball assembly (see above) and shaken on the plate shaker for 3 h. The suspension was transferred to filter plate (Orochem Technologies, Lombard, IL). The eluate in the deep well plate (VWR) was precipitated by ether (600 μL), and after standing in refrigerator for 3 h, the pellet was formed by centrifugation, supernatant removed by a surface suction device (Krchnak *et al.*, 1997; Lebl *et al.*, 1999) and pellet was resuspended in ether (600 μL) and centrifuged again. The process of supernatant removal and resuspension was repeated 3 times. The product was dried in Speedvac (ThermoSavant, Waltham, MA), dissolved in 200 μL of H_2O, or 50% dimethylsulfoxide (DMSO)-50% H_2O

Fig. 10. Prof. Merrifield in our laboratory in San Diego in 2001.

and samples of 20 μL were added to 180 μL of water. Twenty microliters were injected onto an HPLC column (Waters, Milford MA, μBondapak, C18, 10 μm particle, 125 Å pore, 3.9×150 mm, gradient 0.05% TFA in H_2O to 70% Acetonitrile, 0.05% TFA in 15 min, flow rate 1.5 mL/min, detection by UV at 217 nm). LC-MS was performed at HT-Labs (San Diego, CA, USA) using the same gradient.

CONCLUSION

Centrifugation can be used as a basis for construction of extremely economical high throughput solid phase synthesizers. The greatest advantage of centrifugation, in contrast to filtration, is the independence of individual wells in the solvent removal operation, allowing for virtually unlimited scaling of the operation. The described small footprint benchtop synthesizer has shown its performance in both oligonucleotide and peptide synthesis. It can become an inexpensive alternative to presently available large and expensive synthesizers.

ACKNOWLEDGEMENTS

We were proud to have had an opportunity to show our synthesizers to Dr. Bruce Merrifield during his visit to San Diego in 2001. Bruce was thoroughly impressed with the throughput of our machines and gave us his full support (fig. 10). The project was financially supported by NIH SBIR Phase I and II Grants #R43 GM61511-01 and #R43 GM58981-01.

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