Peptide Research

MARS – Multiple Automated Robotic Synthesizer for Continuous Flow of Peptides

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ABSTRACT

We have designed and constructed a multiple automated robotic synthesizer, the MARS. Its novel timing procedure for handling multiple synthetic tasks eliminates unnecessary respite time by keeping the robotic arm in continuous operation. Polypropylene syringes equipped at the bottom with polypropylene frits serve as physically independent reaction vessels. All operations are performed by the robotic arm, which is equipped with a specially designed gripper to hold a syringe and to aspirate and dispense liquid. Typically, the MARS synthesizes concurrently 5 to 15 peptides of different length, and once one peptide is finished it automatically starts the synthesis of the next peptide in the queue, assuring a continuous flow of peptides.

INTRODUCTION

Merrifield solid-phase peptide synthesis (9) is a process inherently amenable to automation. A number of synthesizers have been designed and constructed (14), and some are quite practical instruments for peptide production. A growing demand for synthetic peptides has led to the development of multiple-peptide synthesizers capable of synthesizing a number of peptides at the same time. Some of the manufacturers of these instruments are Advanced ChemTech/Zinsser (13), Abimed (3–5), Rainin, Spyder (8,12) and Shimadzu (10,11).

A cycle of peptide synthesis consists of several steps (operations). The timing of a particular operation during multiple synthesis is accomplished in the following way. A synthesizer performs only one distinct step in the synthetic cycle for all peptides at a given time; i.e., the synthesizer washes all peptides one by one, then deprotects them all one by one, etc. This concept is characterized by two aspects: batchwise operation and long respite times. As a result, once a run starts, the synthesis of a new peptide has to wait for completion of the ongoing synthesis. Since one step in the synthetic cycle is performed for all peptides at the same time, this timing protocol results in "respite" times during deprotection and coupling when the instrument waits for a chemical reaction to be completed and is not performing any operation. Moreover, washing is very time consuming, so that cycle times are prolonged.

We decided to build an instrument for multiple syntheses because we wanted to eliminate these two drawbacks. In doing so, we changed the timing concept of multiple syntheses from a batchwise operation to an individual timing protocol for each peptide synthesized. This was accomplished by using polypropylene syringes (7) as physically independent reaction vessels and a robotic arm as an operational device. The result is a multiple automated robotic synthesizer, the MARS.

EXPERIMENTAL

Materials and Methods

TentaGel RAM resin was obtained from Rapp Polymere (Tübingen, Germany). Fluorenylmethyloxycarbonyl (Fmoc) amino acids with standard side-chain-protecting groups were obtained from Advanced ChemTech (Louisville, KY, USA) or Propeptide (Vert-le-Petit, France). Diisopropyl carbodiimide (DIC), *N*-hydroxybenzotriazole (HOBt), phenol, piperidine and trifluoroacetic acid (TFA) were obtained from Aldrich Chemical (Milwaukee, WI, USA) or Sigma Chemical (St. Louis, MO, USA). High-purity solvents (Baxter, McGaw Park, IL, USA) were used without further purification.

Analytical HPLC was carried out on a Waters 625 LC system with a Waters 490E Programmable Multiwavelength Detector (Milford, MA, USA) using a Vydac Peptide and Protein C18 analytical column (4.6 × 250 mm, 5 µm, 1 mL/min) (The Separation Group, Hesperia, CA, USA). The analytical gradient was run from water containing 0.07% TFA to 60% of acetonitrile (MeCN)/water in 30 min. UV/VIS Absorption spectra were recorded on a Hewlett Packard HP 8452A Diode-Array spectrophotometer (Palo Alto, CA, USA) using a 1-cm quartz cuvette. Ion-spray mass spectra were obtained on a triple quadrupole PE-Sciex API III+ mass spectrometer (Perkin-Elmer/Sciex, Thornhill, Ontario, Canada) with an articulated ion-spray sample inlet system.

Chemistry

Standard Fmoc/tBu chemistry was applied; polystyrene or polyethyleneglycol-grafted polystyrene resins were

Table 1. Robotic Steps for Washing, Deprotection and Coupling

Washing:

1. The robot picks up a syringe either from the incoming rack or the tumbler or from the holding position.
2. The robot moves the syringe to liquid waste and dispenses the liquid from the syringe (if the syringe is empty this step is skipped).
3. Piston pump #1 dispenses *x*1 mL of washing liquid (DMF).
4. The robot moves to delivery cup #1 and aspirates the solution and *y* mL of air.
5. The robot shakes the syringe for *z* min.

Add deprotection solution:

1. The robot moves the syringe to liquid waste and dispenses the liquid.
2. Piston pump #2 delivers the deprotection mixture into delivery cup #2.
3. The robot moves the syringe to delivery cup #2 and aspirates the solution and *y* mL of air.
4. The robot places the syringe into the tumbler.

Add coupling solution:

1. The robot places the resin-containing syringe into holding position.
2. The robot picks up the amino acid-containing syringe from its rack.
3. The robot moves the amino acid-containing syringe to delivery cup #1 and dispenses *x*2 mL.
4. The robot places the amino acid-containing syringe in its rack.
5. Piston pump #3 dispenses *x*3 mL of solution of activating reagents (DIC).
6. The robot picks up the resin-containing syringe from holding position.
7. The robot moves the resin-containing syringe to liquid waste and dispenses any liquid from the syringe.
8. The robot moves the resin-containing syringe to delivery cup #1 and aspirates the solution and *y* mL of air.
9. The robot places the resin-containing syringe into tumbler.
10. Piston pump #1 dispenses 10 mL of washing solvent (DMF) into delivery cup #1.
11. The piston pump #4 aspirates the liquid.
12. Steps 10 and 11 are repeated three times.

used as solid support. One synthetic cycle consisted of the following steps: *(i)* washing, dimethyl formamide (DMF), 3 times for 30 s; *(ii)* deprotection, 50% piperidine/DMF (vol/vol), 10 min; *(iii)* washing, DMF, 5 times for 30 s; *(iv)* coupling, 3-fold excess of DIC/HOBt activated Fmoc-protected amino acids, 2 h. After the last cycle, the synthesis was finished by washing, deprotection and washing (steps *(i)–(iii)*).

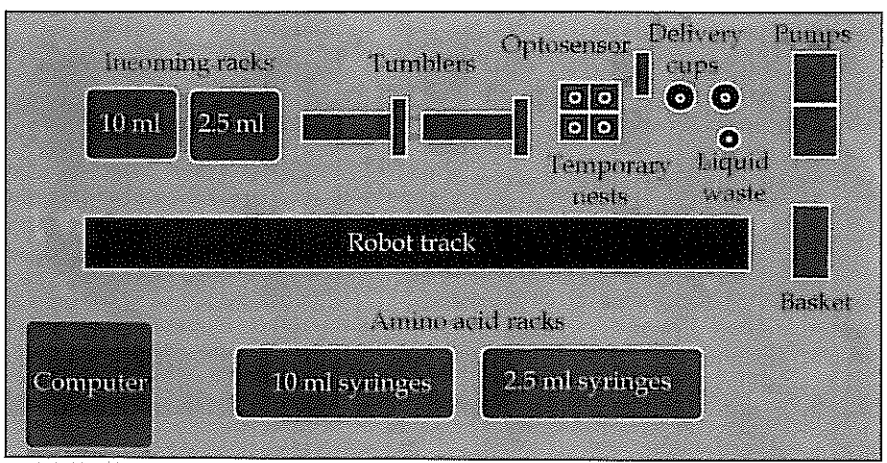

Figure 1. Robot table layout.

Hardware Description

The layout of the robot table is shown in Figure 1. The synthesizer was built using the Small Industrial Robot System (Model A251; CRS Plus, Harrington, Ontario, Canada) interfaced with an IBM™ PC and operated by the TCW software system (Hudson Total Control for Windows; Hudson Control Group, Springfield, NJ, USA). The robotic arm is equipped with a gripper that can take any syringe and transport it to any defined destination on the table. It can aspirate and dispense liquid by moving the syringe plunger. The gripper was designed to handle both 2.5- and 10-mL syringes. Syringes loaded with resin are placed in an incoming rack that can hold thirty 2.5-mL and thirty 10-mL syringes. Amino acid solutions are also stored in syringes, which are placed in two racks holding one hundred 2.5-mL and one hundred 10-mL syringes each. Solvent (DMF) and reagents (piperidine/DMF, DIC/DMF) are delivered from solvent reservoirs to delivery cups from which the liquid is aspirated by syringe. The first cup is used for all DMF washes and for the delivery of protected amino acids and coupling reagent (DIC); the second cup serves for the delivery of a deprotection mixture (piperidine/DMF). The system is equipped with four 10-mL piston pumps (Hamilton, Reno, NV, USA): Three pumps deliver liquid from reservoirs to cups (DMF, piperidine/DMF and DIC solutions), and the last one removes wash solvent from the cup, where amino acid and DIC are mixed. During the reactions (deprotection and coupling) the syringes are placed on a 2.5-mL or 10-mL tumbling rack, each one holding up to 18 syringes. Whenever the robotic arm picks up a new syringe, it passes the optical sensor to confirm that the syringe has been gripped. Syringes with finished peptides are placed into an output basket.

Robotic Procedures

A set of operations that leads to attachment of one amino acid to the resin is referred to as a synthetic cycle. In the synthesis of a peptide, the

synthetic cycle is repeated as many times as there are amino acids in a particular peptide (one cycle for each amino acid). One synthetic cycle consists of four basic operations: washing, deprotection, washing and coupling. However, from a programming point of view, it is advantageous to assemble one synthetic cycle so that it contains five different procedures: washing, add deprotection solution, deprotection, add coupling solution and coupling. The washing, add deprotection solution and add coupling solution procedures are composed of a set of robotic steps (see Table 1). Those steps are pre-programmed, but the operator can create a new procedure by "teaching" the robot what steps to perform. Since the deprotection and coupling procedures do not involve any robotic action, the robot can take care of another syringe when such procedures are performed.

Synthetic Protocol

The synthetic protocol is created by combining pre-programmed procedures (washing, add deprotection, deprotection, add coupling and coupling). Operational procedures (washing, add deprotection and add coupling) have the following variables that must be specified: *(i)* kind of liquid to be dispensed; *(ii)* volume of the liquid (excess of amino acid and reagent in the case of coupling); *(iii)* volume of air (it is necessary to aspirate some air into syringe to enable efficient mixing of resin with solvent); and *(iv)* time of washing or reaction.

The synthetic protocol is stored on disk. For a new synthesis, the operator can write a new protocol or retrieve an already existing protocol from the disk.

System Initialization

It is necessary to input the following information before the operation can start.

(i) Assignment of solvent and reagents to be delivered by the Hamilton piston pump (e.g., DMF, piperidine, DIC, etc.). The names of solvent and reagents must match the names used in the chemical protocol.

(ii) Concentration of reagent (DIC) in mmol/mL, which will be used to calculate the volume of reagent.

(iii) The positions and contents of the syringes holding the solution of amino acids using a three-letter code. The same code is used to enter the sequence of peptide, and it is case-sensitive (e.g., Ala means L-alanine, ala means D-alanine).

(iv) Concentration of amino acid solutions in mmol/mL and available volume.

(v) For each peptide the following data have to be entered:

- Peptide code (used as a reference only).
- Amino acid sequence of the peptide using three-letter codes. From this entry the number of cycles the syringe has to go through and the kinds of amino acids added will be determined. The sequences can be either typed or imported from a file in ASCII format.
- The size of the syringe for the peptide synthesis (2.5 mL or 10 mL).
- The amount of resin in grams in this syringe.
- The loading capacity of the resin in mmol/g.
- Final deprotection (yes or no). "No" means that the synthesis will end with the first washing; "yes" means that the synthesis will include washing, deprotection and washing.

After the information has been entered, the status "not ready" is assigned to each peptide. Then the computer calculates how much of each amino acid and reagent is needed to prepare all peptides. The amount of each amino acid is determined according to the formula:

$$\text{Volume of amino acid solution (mL)} = \frac{\text{Amount of resin} \times \text{loading} \times \text{excess}}{\text{Concentration of amino acid solution}}$$

The amount of the activating reagent is determined in a similar way. If there are enough amino acids and reagents for a particular peptide synthesis, its status is changed to "ready". After starting the run, the system takes the first syringe that has the status "ready" for synthesis.

During the run, the computer shows the status of all syntheses (peptide codes, sequences, current cycle and step number) and the operation currently being performed. A new peptide sequence can be entered at any time from the keyboard or imported from a file. When a peptide is finished, the computer deletes it from the table and starts synthesis of the next peptide (the first peptide with "ready" status in the table). The computer stores the peptide code, peptide sequence, name of synthetic protocol and date of synthesis in a file.

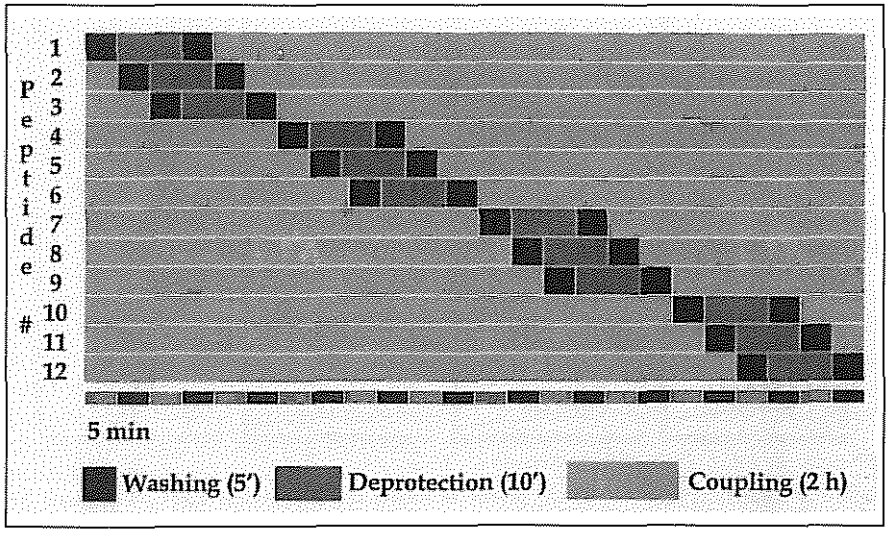

Figure 2. Time schedule of individual operations during multiple synthesis on the MARS.

RESULTS AND DISCUSSION

The simplest and cheapest reactor for solid-phase peptide synthesis is a plastic syringe equipped with a frit to keep the resin inside the syringe (7). Polypropylene is sufficiently chemically inert, and it is compatible with all solvents and reagents used in solid-phase peptide synthesis, including trifluoroacetic acid. We have been using polypropylene syringes for peptide synthesis successfully for many years and the only frustration is the boring

repetition of aspirating and dispensing the liquid.

Synthesis in plastic syringes has one important aspect. The reaction vessel, the syringe, can be handled independently. It can enter or leave the automated synthetic process at any time without interrupting the robot run. This feature has allowed us to apply an individual timing protocol for each peptide being synthesized. Only one peptide is handled at a same time. This timing procedure eliminates any unnecessary respite time, since the robotic arm is continuously in operation. When one peptide is finished, the robot automatically takes the next peptide for synthesis.

Table 2. Purity of Crude Peptides

Peptide	HPLC Panel	Tea Bag	Inclusion Volume	Standard Automation	MARS
YAFGYPS	A	87.3	75.0	91.0	90.5
DPAFNSWG	B	60.3	67.8	35.6	54.6
YGGFMRRV	C	72.1	81.8	83.1	97.9
WAGGDASGE	D	68.7	92.3	71.3	68.0
GNLWATGHFM	E	41.2	74.8	62.4	48.8
ARPGYLAFPRM	F	40.8	79.4	68.1	94.5
LEEEEEAYGWMDF	G	31.0	58.3	51.3	59.8
YMFHLMD	I	65.8	67.5	56.7	91.9

Note: Tea bag synthesis (6) was performed on a Compas synthesizer (12); inclusion volume synthesis used only as little liquid as necessary to soak the insoluble support; standard automation synthesis was done on the ACT 396 synthesizer (ChemTech, Louisville, KY, USA).

Description of Operation

The MARS was designed to perform synthesis in plastic syringes essentially the same way as it would be done by a chemist at the bench. As an example, the operation of the synthesizer is described here using Fmoc/tBu chemistry, 10 min deprotection time and 2 h coupling time.

The robotic arm takes the syringe loaded with resin from the incoming rack and brings it to the delivery cup; the piston pump delivers liquid into the cup, the gripper moves the syringe plunger to aspirate the liquid plus a small amount of air from the cup, the robotic arm shakes the syringe to mix the resin with the liquid (30 s), the robotic arm takes the syringe to the waste position and dispenses the liquid to waste. This procedure repeats as many times as necessary (typically, washing before deprotection is done 3 times; before coupling, it is done 5 times). Then the piston pump dispenses the deprotection mixture into the second delivery cup, the syringe is taken to the second cup, the liquid is aspirated and the syringe is put on the tumbler. Complete washing takes about 5 min, and the arm is busy for this period of time. While the resin is being deprotected (typically 10 min), the arm is free and typically picks up the next syringe, performs washing and starts deprotection (see the timing chart presented in Figure 2). Since in this example there is still 5 min left before the resin in the first syringe is finished being deprotected, the arm takes the third syringe, washes the resin and starts deprotection. Then the arm takes the first syringe (the deprotection time is over), washes the resin (typically 5 times) and puts the syringe into a temporary nest position. The robotic arm takes the syringe, containing the solution of amino acid that is to be coupled, from the amino acid rack, delivers a calculated volume into the first cup and returns the syringe to its original position. In the meantime, the piston pump delivers a solution of DIC. The robotic arm takes the syringe from the temporary nest position, aspirates the activated amino acid from the delivery cup and puts the syringe on the tumbling rack. Then the arm takes the second syringe that is in the deprotection step. In the meantime, the first cup is washed using DMF. The arm performs washing and prepares for coupling in the second syringe. Once all three syringes are in the coupling step, the arm takes the fourth syringe and starts washing and deprotection. The arm will take as many syringes as it can handle

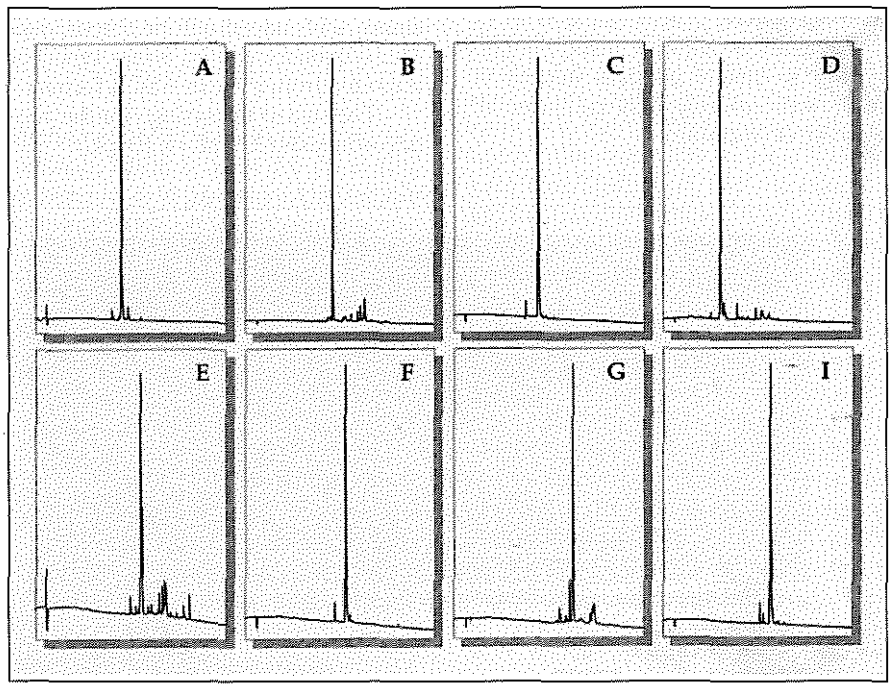

Figure 3. Analytical gradient HPLC profile of model crude peptides: YAFGYPS (Panel A), DPAFNSWG (B), YGGFMRRV (C), WAGGDASGE (D), GNLWATGHFM (E), ARPGYLAFPRM (F), LEEEEEAYGWMDF (G) and YMFHLMD (I).

before the coupling time in the first syringe expires. Now the system is in a full load and all procedures repeat until the sequence of the shortest peptide is assembled. Figure 2 shows the timing schedule. Twelve peptides can be synthesized at the same time when the coupling time is set for 2 h.

When the first peptide is finished, the robot takes the next syringe with status "ready" from the queue for synthesis. This arrangement allows for creation of a list of peptides arranged according to their priorities. The list can be changed any time and the operator can insert a peptide into any position in the list. There is also an option that allows for removal of any peptide that is currently being synthesized from the synthesis. This option is useful when, for example, one of the peptides in the synthesis has lost its urgency status.

It is interesting that shortening the coupling time will not increase the throughput of the synthesizer to any appreciable extent. However, the peptides will be synthesized faster, as there will be fewer syringes in operation at the same time.

The robotic process is inherently very flexible and any chemistry that involves transfer of liquid and/or syringe can be programmed. To thoroughly test the reliability and throughput of the synthesizer, we programmed the robot for Fmoc/tBu chemistry. The chemistry is well-documented (2). Since we have synthesized hundreds of peptides by this methodology, we are able to compare the yield and purity of crude peptides made by the MARS. We describe here the results of a model synthesis of eight peptides. These peptide syntheses have been used to compare the efficiency of different synthetic methods (1) (the results are shown in Table 2). The purity of all crude peptides synthesized by the MARS was comparable to or better than prior syntheses, as judged from the analytical gradient HPLC tracings (Figure 3). All crude peptides were analyzed on a mass spectrometer and the correct molecular weights were confirmed.

The maximum amount of peptide that can be synthesized with the current version is limited only by the size of the syringe. A 10-mL syringe can be charged with up to 500 mg of resin; considering a typical loading of 0.5 mmol/g resin, the yield can be 0.25 mmol of peptide (ca. 300 mg of crude decapeptide). Typical throughput is 10 decapeptides per day.

CONCLUSIONS

The multiple automated robotic synthesizer, the MARS, is characterized by a novel timing procedure for handling multiple synthetic tasks and independent reaction vessels. As a result of this design, the MARS possesses the following advantages: *(i)* Continuous synthesis update that enables one to change the waiting queue of peptides at any time without interrupting the run; *(ii)* short turn-around time because of the elimination of instrument respite time; *(iii)* flexibility in the use of different chemical protocols; and *(iv)* ability to use independent reaction vessels, which allows the operator to handle each synthesis independently, and the reaction vessels and syringes can be of different sizes.

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