AUTOMATED TECHNOLOGIES FOR DRUG DISCOVERY:
ROBOTIC EQUIMOLAR PEPTIDE MIXTURE SYNTHESIS

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ABSTRACT

A fully automated peptide synthesizer has been constructed that is capable of the simultaneous synthesis of up to 36 individual peptides, and the synthesis of equimolar peptide mixtures using standard solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The instrument consists of an array of synthesis vessels, a cleavage/deprotection station, a series of solenoid valves to control liquid flow, and a Zymark robot to deliver solvents and reagents. Following peptide synthesis, resin samples are cleaved/deprotected and extracted to provide the peptides in aqueous solution. These samples can then be screened in a variety of receptor-binding assays. Details of the automated synthesis and workup procedure are presented.
INTRODUCTION

The chemical diversity and step-wise synthesis of short peptides has made them attractive candidates for the rapid generation of biologically-active lead compounds in drug discovery programs. In recent years chemists and biologists have devised multiple and mixed peptide approaches to achieve sequence diversity (1). Multiple peptide synthesis technology allows for a substantial increase in the number of individual peptides that can be generated and screened, as compared to conventional synthesizer technology. The Geysen pin method (2), the Houghten tea-bag method (3) and the use of cellulose discs (4) allow for the parallel synthesis of several hundred peptides in a period of weeks. Recently, automated instruments have been designed that are capable of synthesizing up to 96 individual peptides simultaneously (5-6). Light-directed spatially addressable parallel chemical synthesis (7), a method requiring the use of photolithographic equipment, allows for the simultaneous synthesis of $\sim 10^4$ peptides on a solid phase. Resin-bound synthetic peptide libraries have been generated that contain $\sim 10^6$ ligands (8). Finally, epitope libraries, where peptide sequences are displayed on the surface of filamentous bacteriophage particles (9-11), are a source of great peptide diversity ($10^7$-$10^8$ distinct sequences). Although the latter three methods generate a large number of compounds, the C-termini of these peptides are anchored to a surface, thus preventing assay of the free peptide in solution. The qualitative screening of free-peptide libraries that contain $\sim 10^6$ ligands has recently been reported (12).

We describe here a fully automated procedure, using standard Fmoc chemistry and polystyrene resin, that allows the synthesis of solution-phase peptide libraries (13-14). The components of these libraries are synthesized in equimolar proportions by physically separating the solid support (resin) into $n$ equal aliquots, coupling a unique amino acid to each aliquot, and then mixing all the resin aliquots (15) (Figure 1). After removal from the resin, the peptide mixtures are characterized by amino acid analysis and mass spectrometry to verify their composition. The mixtures can then be screened in a competition binding assay to ascertain whether they contain high affinity components. Affinity selection methods can then be used to directly identify the high affinity mixture components (16).
Figure 1. Resin-splitting scheme for the synthesis of equimolar peptide mixtures.

ROBOTIC EQUIMOLAR PEPTIDE MIXTURE SYNTHESIS

Peptide mixtures can be synthesized by two different methods. In one method, mixtures are synthesized by coupling a mixture of amino acids to a single batch of resin (17-19). In order to synthesize mixtures of known composition, however, this method requires knowledge of individual coupling rate constants since the amino acids are competing for a single batch of resin (19). In another method, called the "resin-splitting" method (Figure 1), mixtures of known composition can be readily prepared by physically separating the solid support into equal aliquots, coupling a unique amino acid to each aliquot, and then mixing all the resin aliquots (8, 12, 13, 15).

The robotic synthesizer was designed to perform the fully automated synthesis of equimolar peptide mixtures by the resin-splitting method. The EPM synthesizer was designed around a Zymate XP Laboratory Automation System.
(Zymark Corp., Hopkinton, MA) which consists of a central robotic arm that can exchange hands, thereby providing a variety of functions. The synthesizer also consists of an array of fritted reaction vessels, a resin mixing vessel, a series of solenoid valves, a rack to store amino acid solutions, pressurized solvent bottles, a syringe pump, a vacuum trap, a computer controller and an integrated cleavage/deprotection station (Figure 2).

**Figure 2.** The Equimolar Peptide Mixture Synthesizer consists of a robotic arm, a synthesis station and a cleavage/deprotection station.
The user inputs the peptide sequence information, adds resin to the appropriate reaction vessels, prepares the amino acid and activator solutions, and fills the solvent reservoirs. The robot then adds the appropriate amino acid solutions (stored under a blanket of argon) to the desired reaction vessels with a pipetting hand. The hand changes pipet tips when necessary to avoid cross-contamination. Small volumes of activating reagents are then added via a gripping hand which holds a reagent line connected to a syringe pump. Thorough mixing of the reaction mixture is achieved by the bubbling of argon gas through the fritted bottom (Figure 3). The reagents are removed by the application of a vacuum, which draws the liquid through the frit. Fresh wash solvent is added by the gripping hand which dispenses the solvent from a pressurized reagent line. Liquid flow is regulated by Teflon solenoid valves (General Valve, Fairfield, NJ) which are controlled from a digital I/O board (GW Instruments, Somerville, MA) inside the Macintosh (Apple Computer, Cupertino, CA). The entire plumbing and solvent path is constructed from Teflon, siliconized glass, polyethylene, or polypropylene. The Macintosh software for control of the solenoid valves and for interfacing with the Zymark controller was written in Microsoft QuickBasic and C languages.

The EPM synthesizer uses a custom hand fitted with a 30 mL syringe to perform the task of resin separation and mixing (Figure 4). The standard 1% crosslinked polystyrene resin is transferred as a free-flowing slurry in 60% dichloroethane/dimethylformamide. The density of this solvent system allows for a uniform suspension of the resin and an equimolar distribution of resin by volume, with minimal interruption of the synthesizer chemistry.

RESIN DISTRIBUTION

Prior to distribution of resin into the reaction vessels, the resin is mixed as a slurry in the resin-mixing chamber (Figure 5). Here, transfer solvent (60% dichloroethane/dimethylformamide) is added to obtain the resin slurry. A level sensor is used to obtain a pre-determined volume of resin-slurry despite changes in the resin volume throughout the synthesis. The resin-mixing chamber is initially filled with a slight excess of solvent, the solvent is then drained to the sensor level by opening the bottom valve to vacuum. The slurry is mixed by opening the valve
Figure 3. Thorough mixing is achieved by the bubbling of argon gas through the fritted bottom of each reaction vessel.

Figure 4. Resin is transferred into equal portions as a slurry in 60% methylene chloride/dimethylformamide.
to argon pressure. Solvent is dispensed from a pressurized line containing eight radial holes which spray the walls.

Distribution of the resin is done with the 30mL syringe hand. Transfer solvent is added to the resin in the mixer to make the resin slurry. The resin slurry must be evenly distributed to each of the reaction vessels in order to obtain an equimolar mixer. An accuracy of ±4% can be achieved in resin transfer (Figure 6). In a similar manner, resin samples are recombined by dispensing transfer solvent into the individual reaction vessels and transferring the slurry with the 30mL syringe hand to the mixer. All transfer steps are repeated 3 times to ensure quantitative resin transfer.

![Figure 5. Prior to distribution of the resin into the reaction vessels, the resin is mixed as a slurry in the resin-mixing chamber.](image)

![Figure 6. Accuracy of automated resin distribution as performed by the isopycnic slurry method.](image)
PLUMBING CONFIGURATION

The plumbing configuration in Figure 7 allows for the individual control of any number of reaction vessels between 1-36 with a minimum number of solenoid valves. This is essential since a different number of vessels may be required in each cycle of the synthesis. The configuration allows each reaction vessel to be mixed by argon bubbling, or to be drained by the application of vacuum. Each row is controlled by one 2-solenoid, 3-way valve. In one of the rows, the reaction vessels are individually controlled by 2-way (normally closed) solenoid valves. The solenoid valves, plumbing, reaction vessels and vacuum/pressure manifolds are constructed from solvent inert materials such as Teflon, polypropylene, polyethylene and glass. Each of the solenoid valves are energized by a series of relays which are controlled by a Macintosh.

![Diagram of plumbing system]

Figure 7. Peptide synthesis rack plumbing.

PRESSURE AND VACUUM SYSTEMS

A schematic for the pressure and vacuum system of the robotic peptide synthesizer is shown in Figure 8. The pressure system is maintained at 7-8 psi of argon gas, and is used for the delivery of solvents through spigot lines, and the mixing of resins in the reaction vessels. The vacuum system is maintained at 28-29" Hg and collects solvent waste in the 4L vacuum trap. The 4L trap can be back flushed with argon pressure into a 25L carboy to prevent overfilling.
Figure 8. Pressure and vacuum systems for the robotic peptide synthesizer (C = common; NC = normally closed).

PEPTIDE CLEAVAGE AND DEPROTECTION

Peptides synthesized by standard solid-phase chemistry require side-chain deprotection and cleavage from the resin to obtain the free peptide. For peptides synthesized by Fmoc chemistry, this can be accomplished by treating the peptide-resin with trifluoroacetic acid (TFA) and a cocktail of scavengers to prevent unwanted side reactions. Recovery of the peptide from this mixture is accomplished by aqueous-ether extraction, which is somewhat labor intensive.

An apparatus of our own design has been constructed which fully automates the side-chain deprotection, cleavage and ether extraction of the peptide-resin samples (14). The robotic apparatus consists of a cleavage station and an evaporation/extraction station (Figure 9). The robotic arm delivers solvents/reagents and transfers peptide samples to these stations.
CLEAVAGE STATION AND COLLECTION RACK

In the cleavage station the peptide-resin samples are treated with a TFA/scavenger cocktail and filtered. The cleavage station consists of a polypropylene receptacle that forms a leak-tight seal with a disposable cleavage vessel delivered by the robot arm. Reagent mixing and draining are controlled by a solenoid valve that can open the vessel to a nitrogen source or to a drain path (C = common, NC = normally closed).

Figure 9. Deprotection/ Cleavage station. The robotic peptide-resin deprotection/ cleavage apparatus consists of two substations; (1) cleavage/ deprotection substation and (2) evaporation/ extraction sub-station. The trifluoroacetic acid reactions occur in disposable polypropylene vessels in the cleavage substation (left), and the evaporation and extraction procedures occur in a glass tube in the evaporation/ extraction substation (right).

A multiple-reagent spigot is used to deliver a variety of reagents and can form a gas-tight seal when pressed against the top of the cleavage vessel. Contents of the cleavage vessel can be directed to either an extraction tube or to a waste receptacle in the collection rack (Figure 10). The collection rack is controlled by a pneumatic air cylinder.

EVAPORATION/ EXTRACTION STATION

In this station the filtered peptide solution is concentrated (by evaporation), diluted into aqueous solution with 10% HOAc and extracted with ether to remove impurities. The evaporation/extraction station (Figure 11) has two positions that
can be accessed by the reagent-delivery tubes. Evaporation occurs in the heating block position (left) with the delivery of nitrogen gas. Aqueous acetic acid can also be delivered to this station. Aqueous-ether continuous extraction occurs in the adjacent position. Ether is delivered from the diaphragm pump at a flow rate of 2 mL/min, flows through the aqueous sample and out the sidearm, carrying away the contaminants.

Figure 10. Collection rack and cleavage station (M = Multi-port solenoid valve).

Figure 11. Evaporation/extraction station (P = Diaphragm pump).
CHARACTERIZATION OF ROBOTICALLY PREPARED PEPTIDES

After the peptides have been cleaved/deprotected, they are routinely analyzed by reversed-phase C18 HPLC and amino acid analysis. As a demonstration of the instruments ability to accurately synthesize an equimolar peptide mixture, a simple eight-component mixture was prepared. The mixture consists of eight decapeptides where one position was varied among eight hydrophobic amino acids. Amino acid analysis data (Figure 12) of this eight component mixture shows that it is indeed equimolar. An HPLC analysis trace (Figure 13) of the same mixture clearly shows the expected eight components.

![Graph showing amino acid percentages](image)

**Figure 12.** Amino acid analysis of an 8-component peptide mixture RAXHTTGRII, X=F,Y,W,L,I,nL,P,V.

![HPLC trace](image)

**Figure 13.** Reverse-phase C18 HPLC analysis of an 8-component peptide mixture: Ac-RAXHTTGRII-NH2, X=F,W,Y,L,I,V,nL,P.
CONCLUSION

A fully automated peptide synthesizer has been constructed that is capable of generating up to 36 individual peptides or equimolar peptide mixtures. In addition to performing repetitive amino acid couplings in high yield, we have demonstrated the automated quantitative transfer of peptide-resin particles. The open stationary vessel design, active resin mixing with argon, and resin transfer by the isopycnic slurry method, allow resin to be divided accurately into equal portions by volume. The equimolar peptide mixture synthesizer automates and otherwise laborious method for the generation of synthetic polymer libraries. These libraries hold great promise for the rapid discovery of new diagnostic and therapeutic agents.

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