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High-Throughput Peptide Synthesis

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Summary

The methodologies of high-throughput peptide synthesis are overviewed and discussed. Particular focus is given to the techniques applicable to laboratories with a limited budget. Automated solutions for synthetic problems are also discussed.

Key Words: Automation; parallel synthesis; solid phase synthesis; manual synthesizer; centrifugation; review.

1. Introduction

According to the Protein Biochips 2003 Report, the protein biochip market is underserved, despite growing from $70 million in 2001 to $100 million in 2002. The report projects that the market will grow to more than $400 million in 2007, a compound annual growth rate of more than 35%. This need for peptide synthesis technology is being driven by the increasing availability of sequence information. The initial draft of the human genome sequence has been finished and results were published recently *(1,2)*. With this explosion in sequence information has come the development of new technologies that take advantage of this knowledge. Some of these technologies have a high dependence on the availability of peptides—for example, array-based technologies that allow the parallel analysis and quantitation of thousands of proteins at a time.

The current worldwide supply for custom-made peptides is estimated to be around $10 billion (this amount includes pharmaceutically important peptides). Principal components of the market are peptides for studies of protein–protein interactions, finding antibody epitopes, and analogs of biologically active peptides and potential drugs. Added to this market is a significant business in peptide synthesizers, reagents, and supports. Large-scale proteomics research is

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Table 1
Leading Suppliers of Custom Peptides

Company	Country	Price ($/amino acid residue)
Peptron	Daejeon, South Korea	10
New England Peptide	Gardner, MA, USA	15
Genemed Synthesis	South San Francisco, CA, USA	15
American Peptide Company	Sunnyvale, CA, USA	16
Invitrogen	Carlsbad, CA, USA	18
Sigma/Genosys	The Woodslands, TX, USA	18
AnyGen	Kwang-ju, Korea	20
AnaSpec	San Jose, CA, USA	25

not yet a primary driver of this growth. One particular area that will stimulate the growth of the peptide market is the isotope-coded affinity tagging (ICAT) approach to protein profiling *(3,4)*. While there are many different methods for performing protein profiling, none of them is as efficient and multiplexable as ICAT. To unequivocally identify and quantify a particular protein, two or three specific peptides are required. With an estimated 500,000 common proteins present in the human proteome, just one application will require 1.5 million peptides. The present cost of custom peptide synthesis (*see* **Table 1**) precludes the wide application of this technology. At $15 per amino acid residue, the cost of necessary peptides would be approx $225 million. As technologies capable of bringing the cost of custom peptides to a level of $0.5 per amino acid residue, or lower (i.e., 30 times lower than current running costs) are becoming available, ICAT studies of the whole proteome become feasible.

As evident from previous paragraphs, peptide synthesis technology is of major strategic importance in the field of proteomics. Currently, there are various instruments for automatic or even parallel synthesis of peptides. While these technologies meet the modest requirements of most experiments today, they are inadequate for the manufacturing needs looming in the very near future. Current synthesis technologies do not meet the need for the cost-effective manufacture of large numbers of peptides (tens of thousands to millions of sequences).

1.1. Parallel Synthesis

Merrifield's idea of the synthesis of peptides on solid support *(5–9)* fundamentally changed the thinking of peptide chemists (even though, depending on the personality of the scientists, the adoption of the solid phase methodology sometimes required a very long "induction period"). Later, as it became

apparent that the transformation of sometimes unpredictable behavior of synthetic intermediates into predictable behavior of solid support with attached organic moieties makes synthetic process and purification of intermediates very simple, organic chemists worked hard to show that almost all types of organic transformations can be performed on solid support and solid phase synthesis was broadly embraced by chemists in all fields.

Solid supports allow an easy realization of parallel synthesis. It requires just the compartmentalization of solid phase and the individualized delivery of reactants. This concept of compartmentalization was later pushed to its limit by the realization that each particle of solid support can serve as an individual compartment in which individual peptides can be produced to create libraries of millions of peptides or any other molecular species *(10)*. This one-bead–one-compound concept is covered in detail elsewhere *(10,11)* and will not be discussed here in detail.

2. Methods

2.1. Manual Approaches to High-Throughput Synthesis

The requirements of epitope mapping were addressed by the synthesis of peptide arrays on polyethylene "pins." This technique, pioneered by Geysen and colleagues *(12–14)*, utilizes solid support in the form of rods functionalized with a layer of "synthesis friendly"–swellable polymer, on which the repetitive coupling reaction is performed. These pins are arranged in a grid mapping the microtiter plate format and coupling is realized by dipping this grid into the plate preloaded with appropriate activated amino acids. Washing and the deprotection reaction do not require segregation of individual pins, and can be realized by simple dipping of the array into the common container. The problem of limited amounts of peptide prepared on individual pins was overcome by the introduction of "crowns," or "lanterns" having higher surface areas available for synthesis *(15)*. The original paper described the utilization of the pin-bound peptide for the evaluation of the antibody binding, but the release of peptides into solution became more popular, enabling peptides to be used for other analytical evaluations.

The manual aspect of pin synthesis is simplified by the utilization of a computer-driven light box *(16)* indicating that amino acid should be pipetted into which position by lighting up LEDs under appropriate wells. A very simple and helpful technique is overlaying the microtiter plate with a grid preprinted with the amino acid labels. Delivery of each building block is verified by puncturing the paper overlay, thus preventing skipping the well or delivering multiple doses into each well. Further simplification of the delivery of appropriate building blocks can be achieved by application of pipetted robots. A simple

Excel macro generating pipetting tables from a list of sequences is available online (www.5z.com/mlebl/macros.html). The area of grafted solid supports in peptide synthesis was recently reviewed *(17)*.

Another extremely flexible parallel synthesis approach was developed by Houghten et al. *(18,19)*. This so-called "tea-bag" synthesis utilizes the compartmentalization of polystyrene-based solid support into individual polypropylene mesh bags, which are labeled by either alphanumeric or bar codes *(20)*, or into which a radiofrequency tag is added *(21,22)*. The bags are re-sorted prior to each coupling reaction and all bags requiring the same amino acid are placed into the same reaction vessel. After completion of amino acid coupling, all tea-bags are combined into a larger vessel and washing and common reactions (e.g., removal of amino terminal protecting group) are performed simultaneously. The size of individual bags ranges from 25 mg to several grams. Manual sorting allows batches of up to several hundred peptides to be synthesized in parallel. Automated sorting of "NanoKans" (permeable microreactors in the form of tiny cans, filled with the resin and labeled by two-dimensional bar codes; (*see* http://www.irori.com/Products/irori_tech_nanokan.html) enabled the synthesis of thousands of compounds in parallel. This technology is marketed by Discovery Partners International (http://www.discoverypartners.com).

Parallel synthesis in tea-bags would not be as valuable if there was no method for the parallel cleavage of products from the solid support. For this reason, a 24-vessel parallel hydrofluoric acid (HF) cleavage apparatus was developed *(23)*. Later it was found that the application of gaseous HF works well for the parallel cleavage of peptides synthesized in both tea-bags and microtiter plates *(24,25)*. Chambers for the cleavage of 1728 products from resin in 18 microtiter plates were constructed *(26)*.

A similar approach utilizing cellulose paper *(27,28)* (originally developed for DNA synthesis *[29]*), was later modified by using a printer to label individual pieces of synthetic support *(30)*. Cotton, as the most pure form of cellulose, was found to be a convenient support for parallel synthesis as well *(31–34)*, and was used for the synthesis of combinatorial libraries with guaranteed uniform representation of each structure *(35)*.

Cellulose paper is ideal for the so-called SPOT synthesis *(36–38)* (for an extensive review of SPOT techniques *see* **refs.** *39,40*). This technique is based on the concept of inclusion volume coupling *(41)*. Reagents are spotted onto the porous material (cellulose paper, cotton) and coupling occurs utilizing only the volume of reagent soaked into the pores of the support. No external volume is needed for the completion of the reaction. Common steps (washing, deprotection) are performed by immersion or flooding the support with the appropriate solvent. The process was later automated; a synthesizer using this technology can be purchased from Intavis AG, formerly Abimed (http://www.intavis.com/

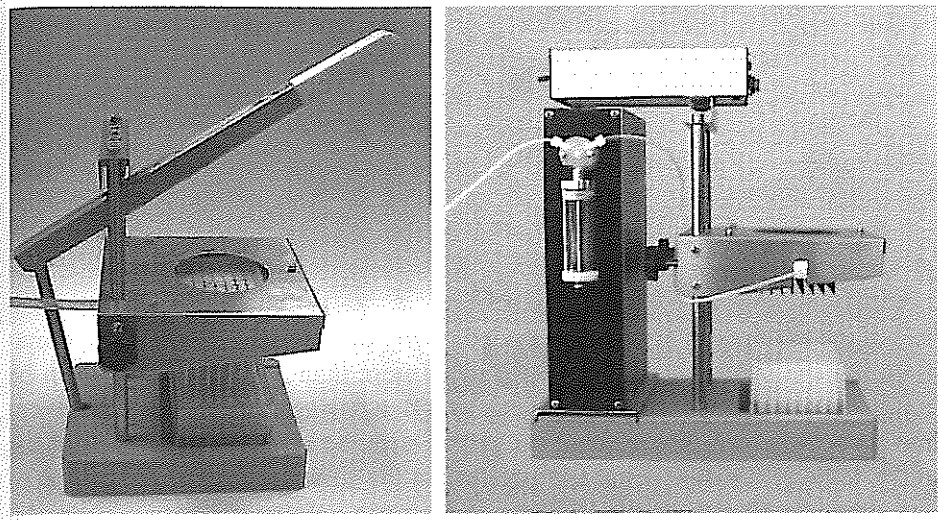

Fig. 1. Aspiration (left) and delivery (right) station for 96-well plate-based synthesis employing surface suction concept. Pushing down spring-supported handle lowers the array of flat-end needles against the surface of liquid in the wells of microtiter plate. (Pictures courtesy of Viktor Krchnak.)

autospot_s.html). The success of Jerini (http://www.jerini.de) as a provider of high-throughput peptide synthesis products is also based on SPOT synthesis.

Another very efficient parallel synthetic technique utilizes the principle of surface suction. In this case liquid is removed from the reaction mixture after the solid particle is sedimented by aspiration. However, immersion of a needle directly into the liquid could disturb the system and remove sedimented particles together with the bulk of the liquid. To prevent this problem, aspiration (suction) is applied through a needle before it touches the liquid surface and the needle is then slowly lowered against the liquid surface. This allows the liquid to be "shaved" from the surface without disturbing the bulk of the liquid. Therefore, the needle can go very close to the layer of sedimented particles without removing them from the mixture. Obviously, this method requires that the solid phase sediments in the washing step. This is relatively easily achievable, because a dense solvent can always be replaced with a less dense one by evaporation and replacement. Simple dilution with solvents such as *tert*-butyl methyl ether will also achieve sedimentation of solid particles ***(42)***. Aspiration and delivery stations for 96-well microtiter plates (**Fig. 1**) are commercially available (http://www.torviq.com), as well as software to drive the syringe pump (http://www.promptpublishing.com/software.html).

The first commercial manual multiple synthesizer, RaMPS, was marketed by E. I. duPont de Nemours. "Semiautomation" in this instrument consists of the simultaneous evacuation of reactors *(43)*. This synthesizer, however, is no longer available. For laboratories that are eager to start the parallel synthesis of peptides with minimal investment, synthesis in plastic fritted syringes can be highly recommended *(44–49)*. The only equipment needed is plastic disposable syringes with tightly fitting frit material at the bottom, commercially available from several sources; (*see*, for example, http://www.5z.com/csps or http://www.torviq.com) and a suitable shaker (rotary, linear, etc.). The repeated use of the syringes is not recommended owing to the possibility of contamination. For the synthesis of longer peptides (more than 30 mer) it may even be advisable to replace the syringe with a new one during the synthesis to prevent mechanical failure. The syringe is charged with the appropriate amount of the resin, taking into an account the swelling in the solvents used in the course of the synthesis as well as the increase of the resin volume in the synthesis. All steps are simply performed by aspirating the appropriate reagent through the needle, stoppering the needle by sticking it into a rubber stopper, and placing the syringe on the shaker. Every syringe is appropriately labeled and no particular arrangement is therefore necessary. If the syringe is equipped with the bar code, than the management software simplifies the work of the chemist tremendously (http://www.promptpublishing.com/software.html). Performing the synthesis in the syringe has one additional advantage—the possibility of monitoring resin swelling, which is very indicative to the internal aggregation of the growing peptide chains and accompanying problems in coupling and deprotection *(44–53)*. Syringes allowing utilization of extreme conditions are constructed from Teflon® (http://www.torviq.com).

Plastic syringes can be arranged in various types of "blocks." One of the classical designs is shown in **Fig. 2**. This instrument was designed by Krchnak and Vagner *(48)* and is constructed from Teflon®, polypropylene, glass, and stainless steel. Reactors equipped with plastic frits are either stoppered on both ends by flexibly mounted multistoppers (for coupling) or the whole block of reactors is placed on a suction table and all reactors are washed simultaneously. Individual spring-supported stoppers arranged to match the grid of syringes allows individual stoppers to be pushed onto the openings with the same force, thus compensating for any irregularities in the syringe array. After placement of this "multiblock" onto the lower multistopper, coupling reagents are added, the upper multistopper is attached, and the assembly is placed onto a shaker, or it is shaken only occasionally by hand. Each reactor can hold up to 2 mL of swollen resin, i.e., about 0.4 g of resin. Reactions can be performed in an ultrasonic bath *(47)*. The multiblock design is optimal for the noninvasive continu-

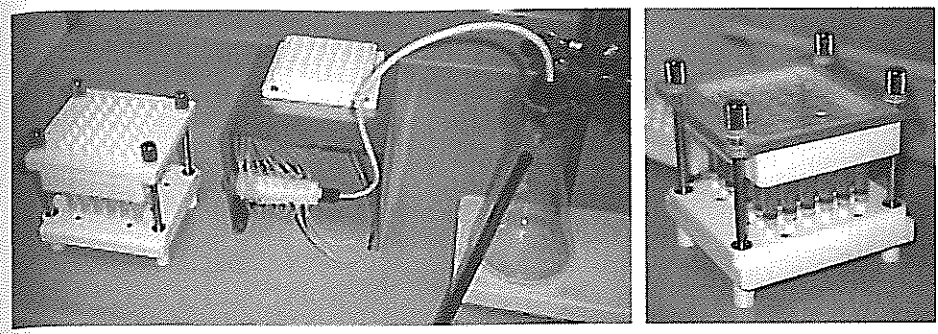

Fig. 2. Multiblock. Arrangement of 42 syringes with multistoppers and washing station. Left—Multiblock with upper and lower multistopper attached to it for coupling reaction next to the washing station. Right—Multiblock with cover used for resin distribution. (Photo by ML.)

ous monitoring of amide bond formation *(54)*. Unique features of this block are the capability of randomization and the synthesis of one-bead-one-compound libraries. For this purpose, the resin is combined in the common area above the syringe tops by simple inversion of the block. After shaking and inversion, sedimentation uniformly distributes the resin. Detailed instructions for use of this block are available on the internet (http://www.5z.com/csps/comer/c_synth/manual.html).

Another unique application of synthesis in plastic syringes is the concept of "domino blocks" *(55)*. In this case, the syringes are used individually for the coupling step, but for common operations they are attached to the block, which is connected via a vacuum source to common reagents and solvents (*see* **Fig. 3**). Blocks are placed on the shaker platform and syringes are evacuated. After switching the selector valves into a proper position, liquid is aspirated into all syringes simultaneously and syringe contents are shaken before repetition of the process. This arrangement eliminates tedious "plunger pushing" and increases the productivity of syringe synthesis many times (http://www.torviq.com).

Ontogen has developed OntoBLOCK, a system that contains 96 reaction vessels and is capable of a wide array of organic syntheses. In combination with a pipetting system operating on multiple blocks, an in-house combinatorial chemistry automation system can produce 1000 to 2000 peptides or small organic molecules per day *(56)*. Bohdan Automation Inc. (now acquired by Mettler Toledo) developed a similar reaction block (http://www.bohdan.com/miniblock.htm) that utilizes the simultaneous pinching of flexible tubing as a mechanism for the closing of vessels (*see* also **ref. *57***).

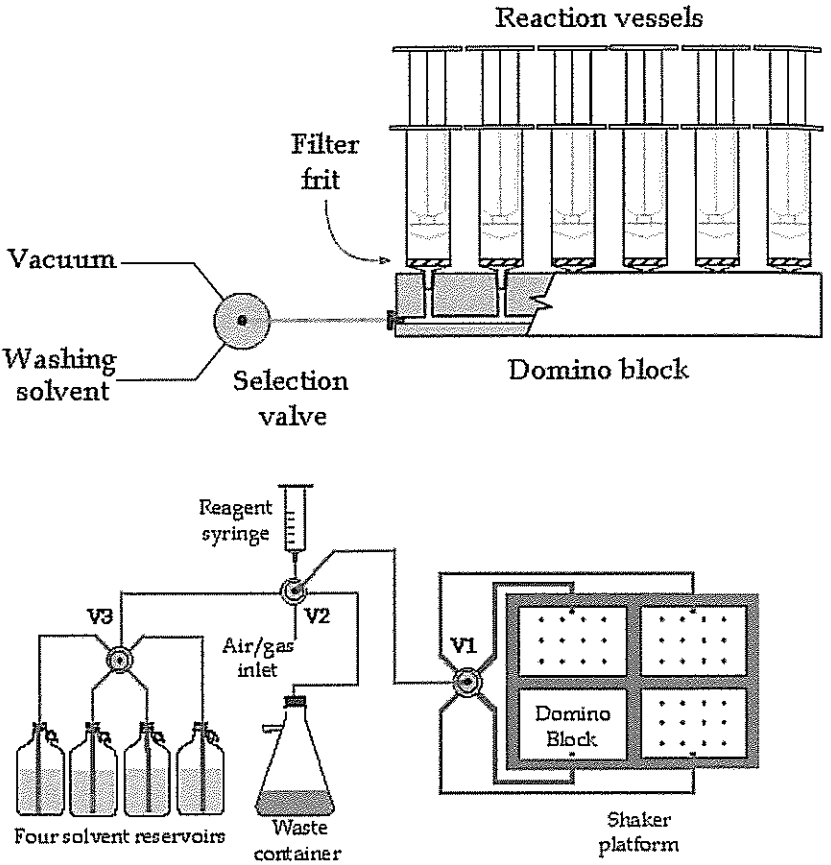

Fig. 3. Scheme of operation of "domino blocks." The block is either attached to vacuum (for emptying) or to source of liquid (for charging). Small amount of air or inert gas is introduced through V2 for creation of bubble in the syringe, allowing efficient mixing.

2.2. Synthesis Automation

Solid phase synthesis lends itself easily to automation. This fact was readily apparent to Merrifield, who built the first automated synthesizer *(8,58,59)*. Merrifield's pioneering effort was soon followed by other laboratories *(60–87)* and several synthesizers appeared on the market. The development of the Fmoc synthetic strategy *(88,89)* allowed for the substantial simplification of automatic synthesizers, since handling of the very "unfriendly" reagent, hydrofluoric acid, was no longer necessary. Cambridge Research Biochemicals was the first to introduce a Fmoc-based synthesizer, PEPSYNthesizer, to the market. This machine was capable of only one step of the synthesis, but it applied the

revolutionary principle of monitoring the reaction by following the UV absorbance (decrease during coupling, increase during deprotection) of the reaction solution recirculated through the column packed with solid support *(90)*. (Actually, Sheppard defined as truly automated only systems utilizing feedback control *[65]*). The completely automatic Pharmacia-LKB synthesizer Biolynx 4170 later used a similar concept. Curiously, none of these machines is available today.

Table 2 summarizes contemporary synthesizers and companies manufacturing them and, for historical reasons, also includes some discontinued products. As you can see, synthesizers with scales covering milligram to kilogram capacity are available with automation ranging from performing only single steps without human interference up to machines incorporating feedback control based on several monitoring concepts. Very soon, however, it became apparent that serial synthesis alone would not satisfy the demand for the large-scale supplies of peptides.

In order to accelerate the synthesis of large single-compound arrays, much effort was devoted to the design of high-throughput organic synthesizers (*see* e.g., **ref.** *91*). Numerous companies have recently started to develop and market instruments capable of automated parallel solid phase synthesis. As examples we can mention Nautilus, (http://www.argotech.com/products/other_products/nautilus.html), and Trident, (http://www.argotech.com/products/other_products/trident/trident.html) of Argonaut Technologies *(92)*, Myriad of Mettler Toledo, (http://www.bohdan.com/mcs.htm) and SOPHAS of Zinsser Analytic *(93)*, (http://zinsser-analytic.com). Even though these instruments are very sophisticated and can utilize a variety of reaction conditions, they are usually not particularly suitable for the automated repetitive multi-step synthesis of peptides (undeniably, peptides can be synthesized using these machines, but why should one use a Mercedes to go visit the neighbor, when a 5-min walk would do?). Most of these instruments are based on solid phase synthesis technology and use commercially available pipetting robots for the delivery of reagents and wash solutions to synthetic compartments. The capacity of these synthesizers ranges from 12 to 384 compounds that can be synthesized in one run. However, we will concentrate on the description of machines more or less designed for synthesis of peptides.

2.2.1. Synthesis Automation Based on Filtration

Single-vessel synthesizers were almost exclusively based on filtration. Much effort was spent on the design of an optimal reaction vessel that would accommodate various amounts of solid support. Vega (Tuscon, AZ; later Protein Technologies/Rainin), for example, adopted Merrifield's original design of a reaction vessel, and shaking by inverting the vessel, in its first synthesizer. That machine, however, performed only the washing and deprotection steps and all amino acid

Table 2
Automated Peptide Synthesizers

Company	Model	Capacity	Scale (mmol)	Chemistry	Comment
Advanced ChemTech[a]	ACT 384	384			
Advanced ChemTech	Apogee	10	0.1–0.5	Boc/Fmoc	Fast cycles
Advanced ChemTech	Velocity 16	16		Boc/Fmoc	Fast cycles, flow washing
Advanced ChemTech	ACT 90	2	0.05–35	Boc/Fmoc	
Advanced ChemTech	Apex 396	96	0.015–2	Boc/Fmoc	Overpressure filtration, vortex mixing
Advanced ChemTech	ACT 400	1	100–1000	Boc/Fmoc	
Advanced ChemTech	Vanguard	96		Boc/Fmoc	Only one step
Applied Biosystems[b]	ABI 433A	1	0.005–1	Boc/Fmoc	
Applied Biosystems	Pioneer	32	0.025–0.1	Fmoc	Continuous flow
Argonaut[c]	Quest 210	10	0.05–0.5	Boc/Fmoc	Only one step, manual
CEM[d]	Odyssey	1	0.005–5	Boc/Fmoc	Microwave, 12 consecutive
Chemspeed[e]	PSW1100	80	0.005–5	Boc/Fmoc	On-line cleavage and workup
CRB	PEPSYNthesizer	2	0.2–1	Fmoc	Not in production
CS Bio Co.[f]	CS336	3	0.05–0.25	Boc/Fmoc	Up to 108 AA
CS Bio Co.	CS736	1	2.0–25.0	Boc/Fmoc	
CS Bio Co.	CS936S	1	5–100	Boc/Fmoc	Customizable
CS Bio Co.	CS936	1	5–500	Boc/Fmoc	Customizable, mobile
Dan-Process[g]		1		Fmoc	Flow-through, industrial scale
Gilson	AMS422			Fmoc	Not in production
Heidolph Instruments[h]		24			Heating/cooling, single step only
Intavis AG[i]	ResPep	6	0.025–0.2	Fmoc	Column reactors
Intavis AG	ResPep Microscale	24	0.002–0.005	Fmoc	Column reactors
Intavis AG	MultiPep	192	0.002–0.01	Fmoc	Preactivation, filter multititer plates
Intavis AG	AutoSpot	800	0.000003	Fmoc	Only spotting automated

Intavis AG	MultiPep Spot	800	0.000003	Fmoc	Automated spot synthesis
Milligen/Biosearch	Milligen 9050	3	0.2–1	Fmoc	Not in production
Peptide Scientific[n]	PSI500	1	0.1–1000	Boc/Fmoc	Monitoring feedback
Pharmacia-LKB	Biolynx 4170	3	0.2–1	Fmoc	Not in production
Protein Technologies[j]	Symphony	12	0.005–0.350	Boc/Fmoc	12 independent protocols possible
Protein Technologies	Symphony-Cascade	12	0.05	Boc/Fmoc	Randomization chamber
Protein Technologies	Sonata	1	0.5-50	Boc/Fmoc	
Protein Technologies	PS3	1	0.01–0.25	Boc/Fmoc	Cartridges
Shimadzu	PSSM-8	96	0.005–0.4	Fmoc	Not in production
Spyder Instruments[k]	Compas 242	24	0.01–0.05	Fmoc	Centrifuge, tea-bags, not in production
Spyder Instruments	Compas 768	768	0.002–0.005	Fmoc	Centrifuge, 8 plates
Technikrom[l]		1		Fmoc	Flow-through, industrial scale
Zinsser Analytic[m]	SMPS350	144	0.05	Fmoc	First commercial multiple synthesizer
Zinsser Analytic	Pepsy-System	864	0.002	Fmoc	35 synthesis pens

[a] http://www.peptide.com
[b] http://www.appliedbiosystems.com
[c] http://www.argotech.com
[d] http://www.cem.com
[e] http://www.chemspeed.com
[f] http://www.csbio.com
[g] http://www.dan-process.dk/peptid.htm
[h] http://www.heidolph.com
[i] http://www.intavis.com
[j] http://pti-instruments.com
[k] http://www.5z.com/spyder
[l] http://www.technikrom.com
[m] http://zinsser-analytic.com
[n] http://www.peptidescientific.com

derivatives had to be added manually. The V-shape vessel of the Beckman model 990 synthesizer was capable of accepting various scales of synthesis and became a workhorse of several laboratories. Nowadays, most popular reaction vessels are cylindrical or spherical, usually made of glass with a volume range from several milliliters to tens of liters (CS Bio CS936 or Advanced ChemTech ACT400). Mixing in single-channel synthesizers is achieved by stirring, inert gas bubbling, vortexing, shaking, or liquid recirculation. Synthesizers based on the flow-through principle where solutions are recirculated through the stationary bed are quite rare; however, even this principle is used in an industrial-scale synthesizer (Technikrom, Dan-Process). Birr has built a synthesizer in which filtration was enabled by centrifugation of the vessel with porous walls *(61)*.

Historically, the first commercially available automated multiple synthesizer, SMPS350, was brought to the market by Zinsser Analytic *(70,94)* in 1988. This machine was capable of the parallel synthesis of 144 peptides. Removal of the liquid from the solid phase slurry was performed by a needle equipped with stainless steel mesh that was consecutively immersed into each vessel. Obviously, this arrangement required washing of the needle after each immersion by backflushing with solvent and resulted in very long cycle times. The design of this synthesizer was "copied" by Advanced ChemTech and started a long array of synthesizers produced by this company.

One of the key issues 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 (e.g., in instruments from Applied Biosystems), or application of pressure from the top of the compartments (e.g., in instruments from Advanced ChemTech). 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. This is especially dangerous in the case of enclosed systems such as the Teflon synthetic blocks of Advanced ChemTech instruments in which visual inspection of synthetic progress is not possible.

Chemspeed's PSW1100 offers synthesis in independent glass reactors (up to 80) of sizes from 13 mL to 100 mL. It is one of the most flexible synthesizers, allowing a variety of reaction conditions (preactivation, elevated temperature) and protocols and is capable of the automated cleavage of the final peptides, evaporation of the solution, and connecting to HPLC. **Figure 4** provides more details of this synthesizer.

Instead of building dedicated synthesizers, Tecan (http://www.tecan.com) decided (after the rather unsuccessful introduction of its own synthesizer) to

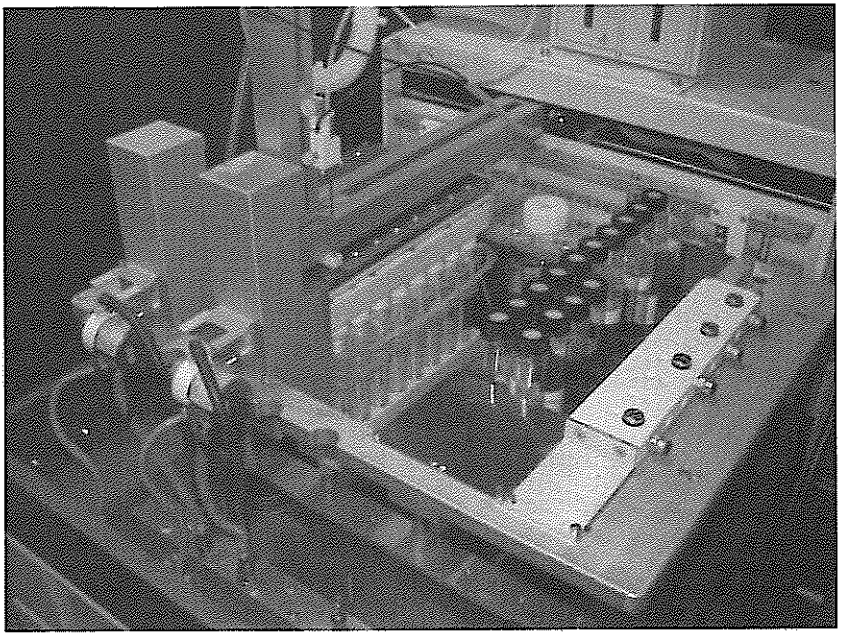

Fig. 4. Synthesis bed of Chemspeed's PSW1100. (Photo by ML.)

integrate the synthesis blocks of different producers (Robbins, Charybdis, H&P, Polyfiltronics, Bohdan, and others) with its own pipetting system. This flexible approach is definitely one to consider when building a dedicated peptide production facility.

The uniqueness of APLS1 ("Randomizer") is based on the fact that the 20 individual reaction compartments are connected into a larger continuous area. If there is a requirement to randomize (split and mix, or divide and recombine, in different author's terminology), the upper larger compartment is filled with the solvent, resin is pushed from the individual compartment into the common area by a flow of nitrogen, and the whole content is stirred. After sedimentation, the resin is uniformly distributed back into individual reaction chambers *(95)*. Continuous stirring was used in the design of two synthesizers capable of resin randomization *(85,96)*, one of which was commercialized but is now discontinued *(82)*.

Zuckermann (Chiron) has developed a combinatorial synthesizer also capable of randomization *(78,97,98)*. In this case, a Zymark robotic arm operated a syringe into which an aliquot of an "isopycnic" suspension of resin, after mixing all aliquots, was drawn and distributed into individual reaction compartments. One problem with this concept was fact that the swelling, and therefore

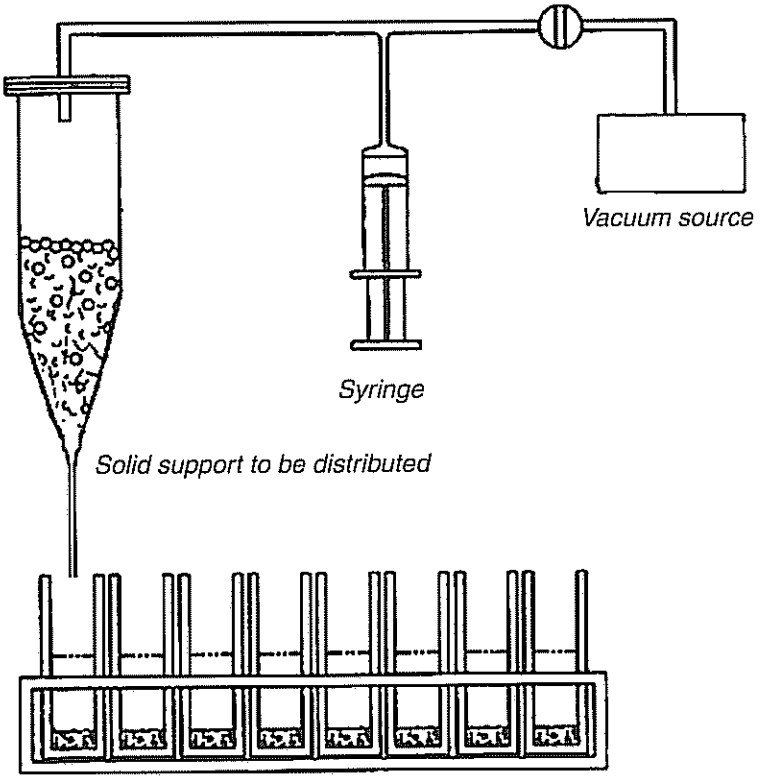

Fig. 5. Scheme of a tool for randomization by continuous bubbling.

the buoyancy, of the resin particles could change during the synthesis, the net result being uneven distribution of the aliquots.

Universal randomization and distribution of small volumes of resin particles can easily be achieved by the application of a continuously bubbled-through delivery vessel *(57)*. This principle of operation is illustrated in **Fig. 5**. A defined volume of the slurry is aspirated into a V-shape vessel with an opening at the bottom. Continuous suction through the vessel prevents slurry leakage and provides mixing. The gas connection has a T-junction to a syringe pump, which draws a gas volume equivalent to the volume to be dispensed. In the next step, suction through the vessel is stopped and the volume of gas in the syringe is immediately discharged, expelling an aliquot of slurry from the vessel. Suction is reapplied and the syringe is prepared for the delivery of the next aliquot.

The SPOT synthesizer mentioned earlier avoids the clogging problem because the filtration support becomes the synthetic substrate *(36)*. The size of the individual "spots" on which the synthesis is performed determines the scale and throughput of the synthesis. Usually, only nanomolar amounts of peptides are

Fig. 6. PepSy of Zinsser Analytic. Left—View of the system; Right—Tray of barcoded synthetic pens. (Photo by ML.)

generated in quantities up to 800 peptides per batch (1.5 mm spot size). Even though the peptides can be cleaved from the support and processed separately, very often the biological assay (e.g., binding) can be performed with peptides attached to the synthetic support *(39,40)*.

The PepSy synthesizer, recently introduced to the market by Zinsser Analytic (http://www.zinsser-analytic.com), is shown in **Fig. 6**. In this synthesizer the solid support is sandwiched between two layers of porous material and reagents are delivered by the "synthetic pens." These pens are loaded with preactivated amino acid derivatives and the volume delivered to the support is defined by the time of contact of these pens with the porous cover of the synthetic chamber. The top of the pen is labeled with a two-dimensional bar code preventing misplacement of the reagent in the tray (positive recognition of the proper reagent by synthesizer is necessary before its use in synthesis). Even though this synthesizer is cleverly designed, it is not without problems. Only extremely small-scale synthesis can be performed because there is no means of mixing the support during the reaction, and the selection of the activated species is very limited—only preactivated derivatives can be used (e.g., fluorides). The recent modification of the synthesizer does not utilize resin embedded between first material, and therefore the pen is completely redesigned.

Synthesis in syringes also uses filtration as the separation principle. However, instead of total parallel processing, staggered partially parallel processing can also result in multiple synthesis. The synthesizer using this concept is probably closest to the concept of "robotic" synthesizers in the sense that it exactly mimics the process performed by the chemist handling the syringes manually *(86)*. There are four types of syringes in this synthesizer: (1) syringes waiting to be included in the synthesis, (2) syringes in synthesis, (3) syringes with all steps of synthesis completed, and (4) syringes with reagents. A robotic arm

equipped with a specialized syringe gripper picks the syringe waiting to be included in the process, aspirates into it the appropriate reagent, shakes it, and places it on the tumbler for reaction. After that it checks whether any syringes in the process need attention and, if not, processes a new syringe. If the preparation of activated amino acid is required, the arm picks the syringe with the reagent, measures an appropriate volume of protected amino acid solution into the activation cup, adds the activator, and aspirates the freshly created mixture into a syringe ready for coupling. If the particular syringe has undergone all steps of synthesis, it is placed into the outgoing bin, where it awaits final processing. In this way, all syringes in the synthesis are in a different stage of processing, but the robotic arm is always busy, processing as many syringes as it can incorporate into its schedule. Long and short peptides can be synthesized at the same time, utilizing different protocols and scales.

Besides performing multiple syntheses in parallel, the accelerating of reaction rates may also achieve the same result—production of multiple peptides in the same time period. CEM (http://www.cem.com) has employed microwave irradiation to shorten both coupling and deprotection times. Their synthesizer, Odyssey (**Fig. 7**), achieves one cycle of peptide synthesis (Fmoc-based) in less than 10 min and is theoretically capable of the synthesis of 12 peptides in a row. However, for each new peptide the reaction vessel has to be cleaned and new batch of resin has to be transferred into it. This step raises concerns with the authors of this review about the possibility of cross-contamination.

An alternative method, avoiding filtration completely, employs aspiration of the liquid from the surface ***(42,99)***. This technology was automated and a robotic station was built that can process up to 72 microtiter plates (6912 compounds) in one batch ***(100)***. A robotic arm moves microtiter plates into stations into which the delivery of reagents is performed by 96-channel distributors and solvent aspiration is achieved by lifting the plate against an array of needles attached to a vacuum source.

2.2.2. Synthesis Automation Based on Centrifugation

Centrifugation is a powerful technique allowing the parallel processing of an unlimited number of reaction compartments ***(101)***. The first centrifugal multiple peptide synthesizer, Compas 242 ***(76,102)***, utilized centrifugation for liquid removal from the functionalized cotton used as the solid support or from resin contained in polypropylene mesh bags ***(103)***. This system enabled the automation of "tea-bag" synthetic methodology. In principle, however, separation of solid and liquid phases was still accomplished by filtration.

The key feature of the alternative centrifugation synthetic technology is a new method for the separation of the solid support from reagent solutions, termed "tilted plate centrifugation," which uses centrifugation as a means of liquid

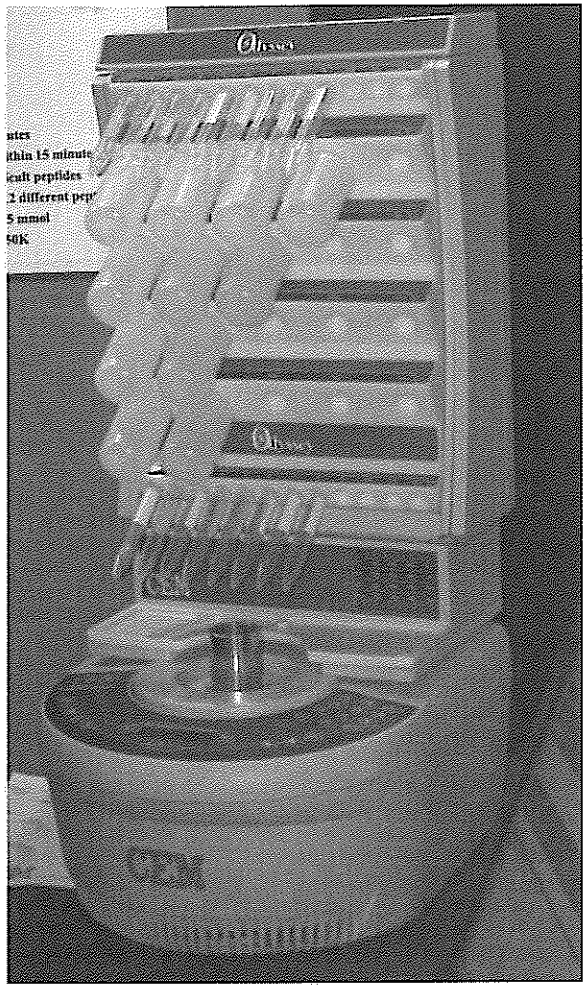

Fig. 7. CEMs Odyssey microwave synthesizer. (Photo by ML.)

removal in conjunction with the use of tilted microtiter plates as reaction vessels. The tilted plate centrifugation technology greatly improves the earlier centrifugation method by using the wells of microtiter plates as synthetic compartments, thus enabling the parallel synthesis of much larger compound arrays (e.g., 3072 compounds when eight 384-well plates are used). The plates are mounted on a centrifugal plate and tilted slightly down toward 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 (**Fig. 8**).

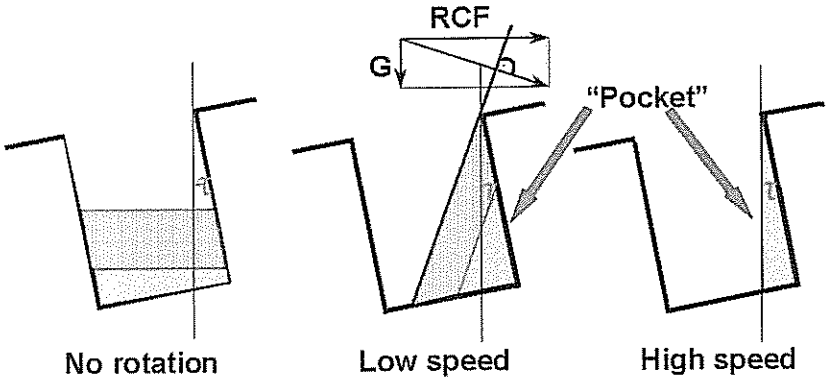

Fig. 8. Formation of the pocket in the well of a tilted plate during centrifugation (direction: left to right). The solid support (lower layer) is collected in the pocket, while the liquid (upper layer) is expelled from the well. The liquid surface angle is perpendicular to the resulting force vector of the relative centrifugal force (RCF) and gravity (G).

In order to ensure efficient liquid removal (i.e., no solution remaining in the wells after centrifugation), and at the same time 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. This can be achieved by adjusting the pocket size by using plates with varying well volumes and/or modifying the tilt angle, as well as the speed of rotation.

An essential feature of this approach is that well-to-well cross-contamination with reagent solution or resin is avoided by the fact that the plates are tilted, while the direction of centrifugation is horizontal. Consequently, any liquid or resin expelled from the wells is either captured in the inter-well space of the plate or collected on the wall of the centrifugal drum. HPLC/MS analysis of all products prepared on the microtiter plate proved the fact that cross-contamination is not an issue.

Tilted-plate synthesis technology ***(26,101,104,105)*** is applicable not only to solid phase synthesis, but also to fluorous synthesis ***(106)***, solid and liquid-liquid extraction, and in-plate washing. A production facility (http://www.illumina.com/prod_oligos.asp) with a capacity of more than 30 million oligonucleotides (20 mers) per year was built on the basis of tilted centrifugation ***(101,107)***.

A high-throughput peptide synthesizer is shown in **Fig. 9**. Wash solutions and reagents common to all synthetic entities are automatically delivered through a multichannel distributor connected to a six-port selector valve. Building blocks and other specific reagents are individually delivered to their respective wells

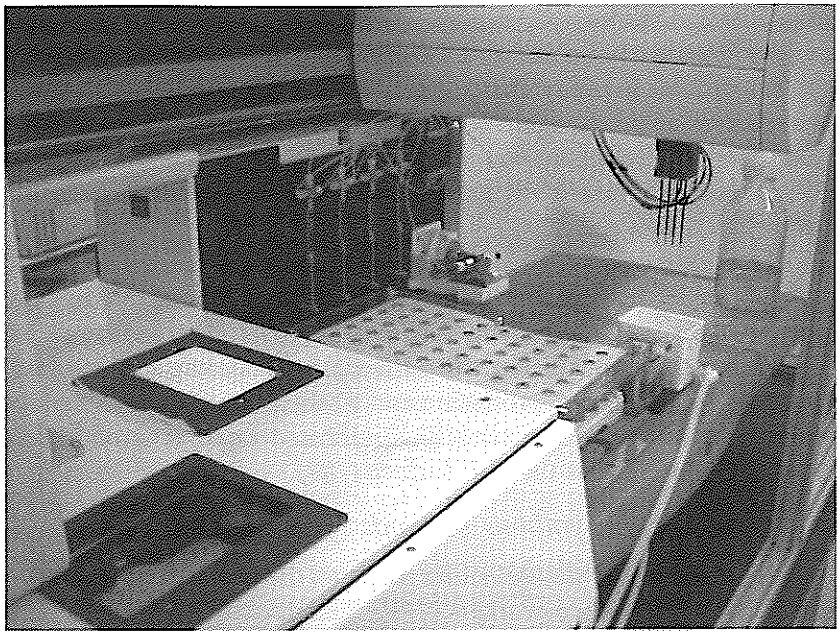

Fig. 9. View of the deck of peptide synthesizer utilizing tilted centrifugation principle. (Photo by ML.)

by an integrated pipetting machine. Synthesis is performed in 96-well polypropylene microtiter plates. A critical factor for successful performance of the peptide synthesis is the guaranteed retention of all solid support in the washing cycles using the centrifugation process. The synthesis of a 20 mer peptide requires up to 300 centrifugation cycles, therefore, the loss of even a small fraction of the support in every step would result in the complete loss of the product. We have shown that even after several hundred centrifugations the solid support was retained.

During the evaluation of the high-throughput synthesizer we realized that it would be extremely beneficial to have an automated synthesizer mimicking the performance of the large machine but on a much lower scale. This allowed us to optimize the protocols and respond very quickly to the requests of researchers requiring only a small number of peptides. We found the optimal number of compounds synthesized in one batch to be 24 to 48. This number of synthetic compartments, wells, can be placed on the perimeter of a small rotor with the diameter of 14 cm. The rotor is then placed in the drum of the centrifuge covered by the lid with an integrated array of nozzles connected through the solenoid valves with vessels containing the particular reagents. During one rotation of

the rotor, all synthetic compartments can be placed under the appropriate nozzle and the reagent can be delivered. Individual amino acids can be delivered through one opening in the synthesizer deck by a pipetting machine. The removal of the solvent is achieved again by centrifugation. This "pet synthesizer" can produce a similar throughput compared to other expensive commercial instruments, but owing to the simplicity of its concept can be built for a fraction of the cost.

Centrifugation was also used for automation of one incarnation of SPOT synthesis. In this case the synthesis is performed on a functionalized polypropylene disk of the size of a conventional compact disk. The surface properties of this material allow for the discrete delivery and therefore synthesis at 2500 locations. After coupling, deprotection and washing are realized by delivering appropriate solvents close to the disk center and rapid rotation efficiently "sweeps" the liquid across the disk surface ***(108)***.

2.2.3. Other Multiple Synthesis Methods

For completeness, we should not forget about the photolithographic method pioneered by Fodor and co-workers ***(109)***. This technology utilizes selective deprotection of photocleavable amino protecting groups on the surface of a glass chip and exposure of the whole surface to the activated amino acid. Only deprotected locations accept the new amino acid and after removal of activated species, another set of locations can be deprotected and coupling with another amino acid can be performed. A disadvantage of this technique is the necessity to repeat the process for each synthetic step as many times as the number of amino acids that need to be coupled in a particular step—for natural peptides, 20 times. A major advantage is the extremely high density of peptides created on the glass surface (tens of thousands). Because of the complicated process of creating lithographic masks, this process is not easily adaptable in the average laboratory, and is exclusively used by Affymetrix (https://www.affymetrix.com) (even though described for peptide synthesis, the technology is now used almost exclusively for DNA synthesis). The masks were later replaced by the use of a digital micromirror projector for the deprotection of selected locations ***(110)***.

Alternative technology based on the same strategy (repetitive selective deprotection followed by global coupling) uses photochemically generated reagent for the *in situ* deprotection of amino groups of growing peptide attached to the chip surface ***(111)***. "Peptide chips" created by this technique are available from Xeotron (http://www.xeotron.com).

Synthesis of peptides in solution is currently practiced in very few laboratories, or is applied in the case of large-scale peptide synthesis. Multiple solution

synthesis is even more rare. An automated system for solution phase synthesis was described by Japanese authors and applied to the synthesis of only short peptides *(112–114)*.

3. Conclusion

This chapter does not follow the usual format of this series; rather, it provides the reader with a general overview of techniques available for manual and automated high-throughput peptide synthesis. We were trying to be more specific in the description of techniques with which we are familiar, and that we believe are relevant for scientists in laboratories tasked to produce large numbers of peptides. In the case of automated synthesizers, we attempted to point out the potential problems that the user of a particular machine may face after the eventual purchase of the instrument—and that will definitely not be mentioned by the sales agent.

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