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Facilitated Peptide Synthesis Using Continuous-Flow Conditions and a Polymeric Membrane Solid Support

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ABSTRACT

FACILITATED PEPTIDE SYNTHESIS USING CONTINUOUS-FLOW CONDITIONS AND A POLYMERIC MEMBRANE SOLID SUPPORT

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The specific objectives of this work were the synthesis of high-quality peptide products with speed, simplicity, convenience, the potential for new applications, and the reduction of overall synthesis cost. It was decided that these goals could be achieved through the use of the Fmoc-protecting group strategy on a continuous flow instrument utilizing a novel solid support. Further improvements in the overall synthetic process were achieved with real-time feedback monitoring and advances in the chemistry used for anchoring the Fmoc-amino acid to and cleaving the peptide from the solid support. Peptides were assembled using Fmoc-pentafluorophenyl esters on a continuous-flow Waters 9050 PepSynthesizer. The polypropylene membrane used in this study has many attractive properties that make it well-suited as a solid support for peptide synthesis. These include chemical and mechanical stability, controlled porosity, high internal surface area to weight ratio, and ease of handling. The membrane was derivatized with primary amino groups and a Fmoc-amino acid-linker was added by the instrument to provide a quick, racemization-free attachment to the support. The acylation step in each cycle was continued until the monitor determined that the coupling was complete. The final peptide product was then deprotected and cleaved from the membrane in less than 15 min using a methanesulfonic acid-based reagent. The crude products from these syntheses were comparable in yield and purity to those

obtained from conventional particulate supports (polydimethylacrylamide on kieselguhr or polystyrene). The possibility of leaving the side chain deprotected peptide covalently attached to the membrane enables construction of novel devices for affinity purification of biomolecules, development of diagnostic devices, and new methods of epitope mapping.

MEMBRANE SOLID SUPPORT

The solid support used in this study consists of a polypropylene membrane which has been coated with amino functionality. The small pore size (0.2 microns) provides a very large surface area (x m^2/cm^2) on which to perform peptide synthesis. The membrane has been laminated to a non-woven polypropylene mesh which provides the path for the flow of solvents and reagents. This composite is then treated to coat it with amino functionality to a loading of 300 - 500 nmol amine/cm^2. The amino-membrane is slit into 1 to 1.25" widths and wound around a solid polyethylene core to form a compact membrane roll with a total of 0.2 mmol of accessable amine. This roll is inserted into a holder (see Figure 1) that directs the flow of solvents and reagents past all surfaces of the membrane. This design results in very efficient continuous washing of the membrane leading to shorter wash times with little wasted solvent.

INTRODUCTION

The objective of an automated peptide synthesizer is to produce the purest peptide in a reasonable amount of time with little waste of reagents and solvents. This can be achieved by using efficient chemistries and solid supports, instrument designs that minimize wash volumes, and methods of bringing the amino acid coupling reactions to completion. To obtain a relatively pure peptide product, it is important for the coupling reaction to be very efficient. This is especially true for longer peptides which can become lost in a mixture of by-products (mainly deletion sequences) if the stepwise coupling efficiencies are not very close to 100 percent.

This work is the result of our continuing efforts to improve the automated synthesis of peptides. We have employed several new materials and methods to simplify the process of peptide synthesis as well as to produce better peptide products. In particular, we have achieved this by using a new membrane solid support, linkers that provide a racemization-free attachment of the first amino acid to the solid support, real-time feedback monitoring of the coupling reaction, and rapid cleavage and deprotection of the final product.

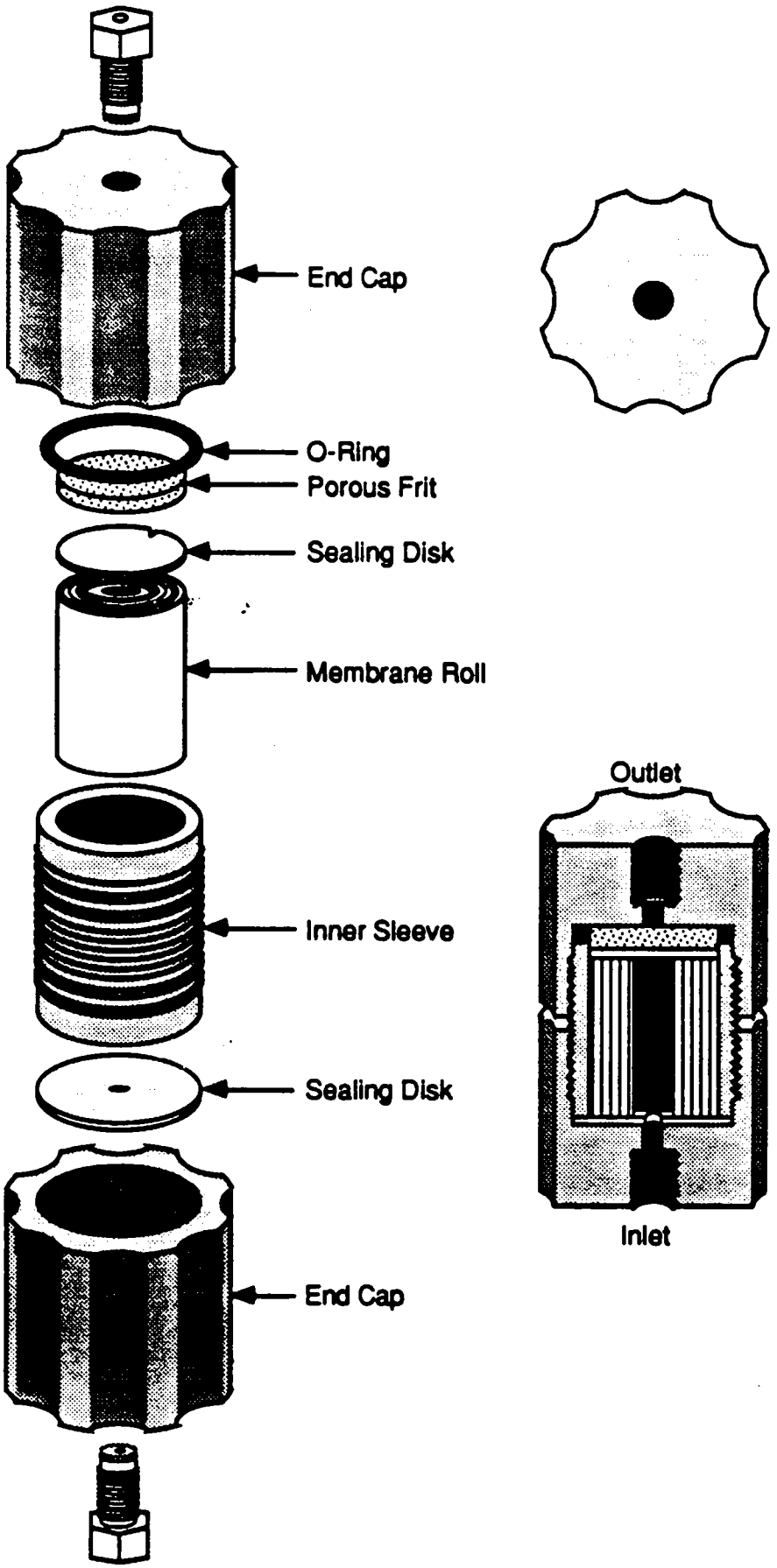


Figure 1: Membrane Cartridge

Benefits of Membrane-Based Synthesis

A membrane solid support has certain advantages over particulate solid supports (resins). The polypropylene membrane used in this study was chosen for evaluation as a peptide synthesis support because of its attractive physical and chemical properties. These include mechanical stability, good flow characteristics in a continuous flow system, controlled porosity, and high internal surface area to weight ratio. In contrast to conventional beaded supports, a sheet of porous contiguous polymer lends itself more easily to rapid simultaneous synthesis of large numbers of peptides, miniturization of automated devices, and novel reactor devices. Chemically, this membrane support allows for sufficient functionalization of the surface for synthesis and cleavage of the final peptide product as well as the possibility of leaving the peptide covalently attached to the support in order to allow for affinity purification of biomolecules, new methods of epitope mapping, diagnostic testing, and covalent sequence analysis.

In terms of general practicality, the membrane support is very easy to use and does not require complicated column packing procedures. The compact nature of the membrane support and the design of the flow path allows for very efficient washing that is not possible with many particulate supports. These features lead to a decrease in the amount of solvents consumed and an increase in coupling efficiency (due to a higher effective concentration of the

coupling reagents). Table 1 indicates the solvent and time savings that are possible when using the membrane support as compared to a kieselguhr resin on the 9050 Peptide Synthesizer.

Table 1: Waste and Time Savings Using Membrane Supports

		Kieselguhr	Membrane
Waste generated per cycle	**DMF**	116 mL	56 mL
	PIP	35 mL	20 mL
Final AA concentration		0.05 M	0.09 M
Time per cycle		60 min	34 min

Typical savings for the synthesis of a 25-mer:

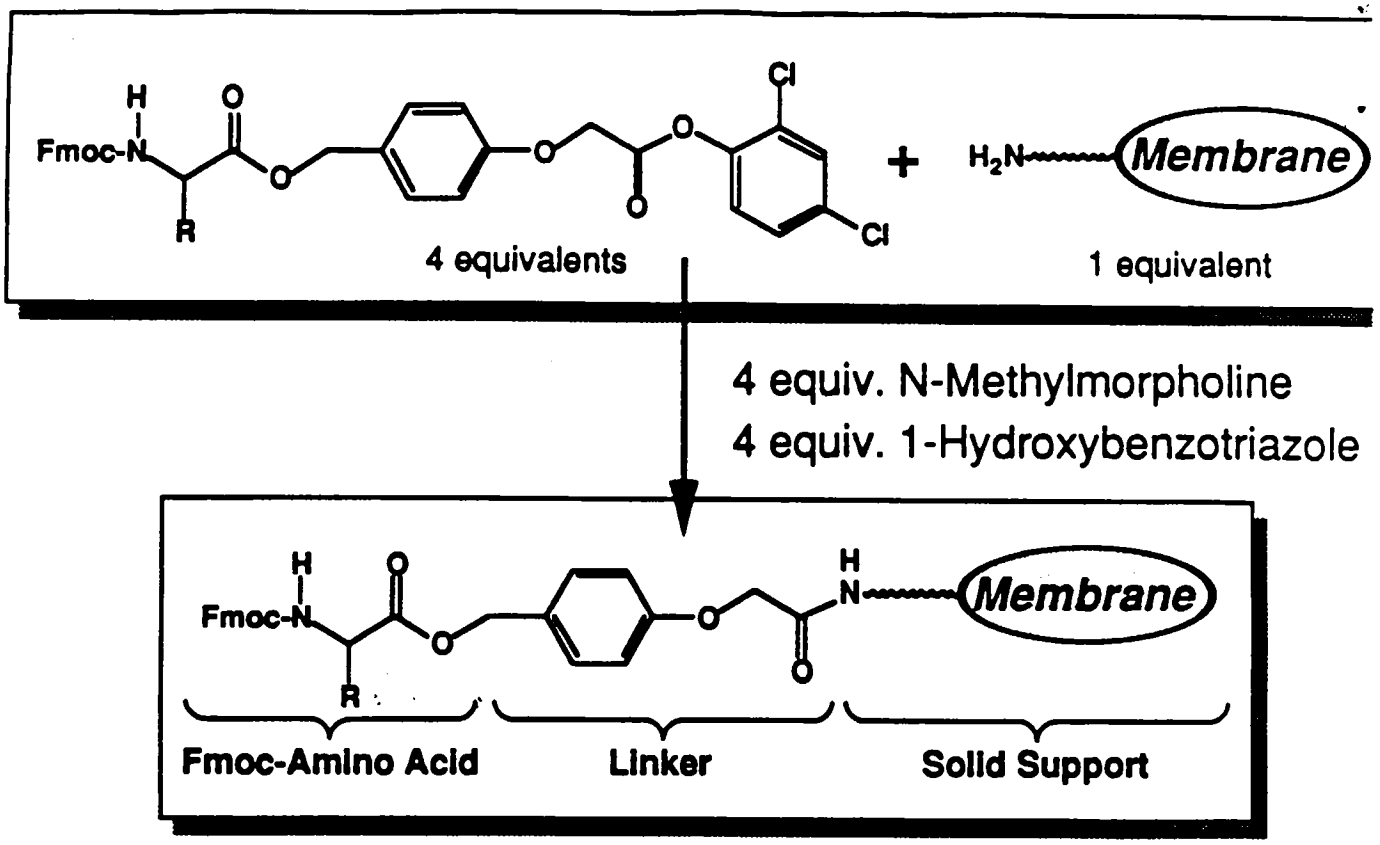
DMF 1.5 L
PIP 0.4 L
Time 11 h

Racemization Free Attachment of Fmoc-Amino Acids to Synthesis Support*

When the Fmoc protecting group strategy is used for peptide synthesis, the C-terminal amino acid is generally attached by chemical activation of the carboxyl group and its subsequent esterification to 4-alkoxybenzyl alcohol functionalized polymers. In order to achieve appropriate levels of functionalization by such a process, it is necessary to utilize and acylation catalyst such as 4-dimethylaminopyridine (DMAP). Such methods suffer from disadvantages attributable to the ability of DMAP to promote racemization and produce anchored dipeptide by-products.

An attractive alternative utilizes novel 2,4-dichlorophenyl-N^{α}-Fmoc-aminoacyl-4-oxymethylphenoxyacetates (Scheme 1) to directly acylate amine-functionalized polymers. These derivatives incorporate the acid labile 4-oxymethylphenoxyacetyl linkage. All of the commonly used Fmoc-amino acid-linker derivatives have been prepared. The kinetics of attachment of these linkers to the amino membrane has been optimized for automated coupling. As can be seen in Table 2, all of the linkers can be attached to the membrane in less than 30 min.

*Bernatowicz, M. S.; Kearney, T.; Neves, R. S.; Köster, H.; *Tet. Lett.*, 1989, in press.


Fmoc-N
H
O
R
Cl
Cl
4 equivalents
+
H_2N
Membrane
1 equivalent
4 equiv. N-Methylmorpholine
4 equiv. 1-Hydroxybenzotriazole
Fmoc-N
H
N
Membrane
Fmoc-Amino Acid
Linker
Solid Support

Scheme 1

Table 2: Coupling Rates of Amino Acid-Linkers to a Membrane Support

Amino Acid	Side Chain	Rate (min^{-1})	t1/2 (min)	t99.9% (min)
Ala		0.68	1.0	10
Arg	Mtr	0.32	2.2	21
Asn	Mbh	0.51	1.4	14
Asp	OtBu	0.61	1.1	11
Cys	Acm	0.73	0.96	9.5
	tBu	0.43	1.6	16
	Trt	0.47	1.5	15
Gln	Mbh	0.85	0.82	8.2
Glu	OtBu	0.48	1.4	14
Gly		0.74	0.94	9.3
His	Boc	0.28	2.5	24
Ile		0.73	0.95	9.5
Leu		0.99	0.70	7.0
Lys	Boc	0.96	0.72	7.2
Met		1.0	0.67	6.7
Phe		0.77	0.90	9.0
Pro		0.67	1.0	10
Ser	tBu	0.71	0.98	9.8
Thr	tBu	0.45	1.5	15
Trp		0.55	1.3	13
Tyr	tBu	0.62	1.1	11
Val		0.67	1.0	10

FEEDBACK MONITORING

Often there are specific couplings or regions within a peptide where couplings are difficult and do not go to completion under standard conditions. One solution to achieve maximal coupling efficiency would be to assume every acylation reaction is difficult and take every measure to achieve nearly 100% coupling. This might include extended coupling times, double or triple coupling, and coupling under varied conditions. The problem with this approach is that it would greatly extend the time of the synthesis, waste a great deal of expensive reagents and solvents, and generate unnecessary waste. Also extending the coupling times at every cycle is not always beneficial since this allows more time for undesired side reactions to occur. A balance must be achieved in the synthesis so that the coupling reaction is maximized while side reactions are minimized. Therefore, it would be most useful to monitor the acylation reaction in real time so the synthesis can be continued as soon as the desired coupling efficiency is achieved.

Most monitoring techniques look at the amount of free amine remaining on the solid support. Many of these techniques either require removal and destruction of small amounts of solid support, are performed after the acylation step is complete and require a significant increase in solvent consumption and cycle time, or are not sensitive in the region where the coupling is approaching completion.

A monitoring technique that can determine when the coupling reaction is complete in real time, is easy to implement, and is most sensitive in the region where the

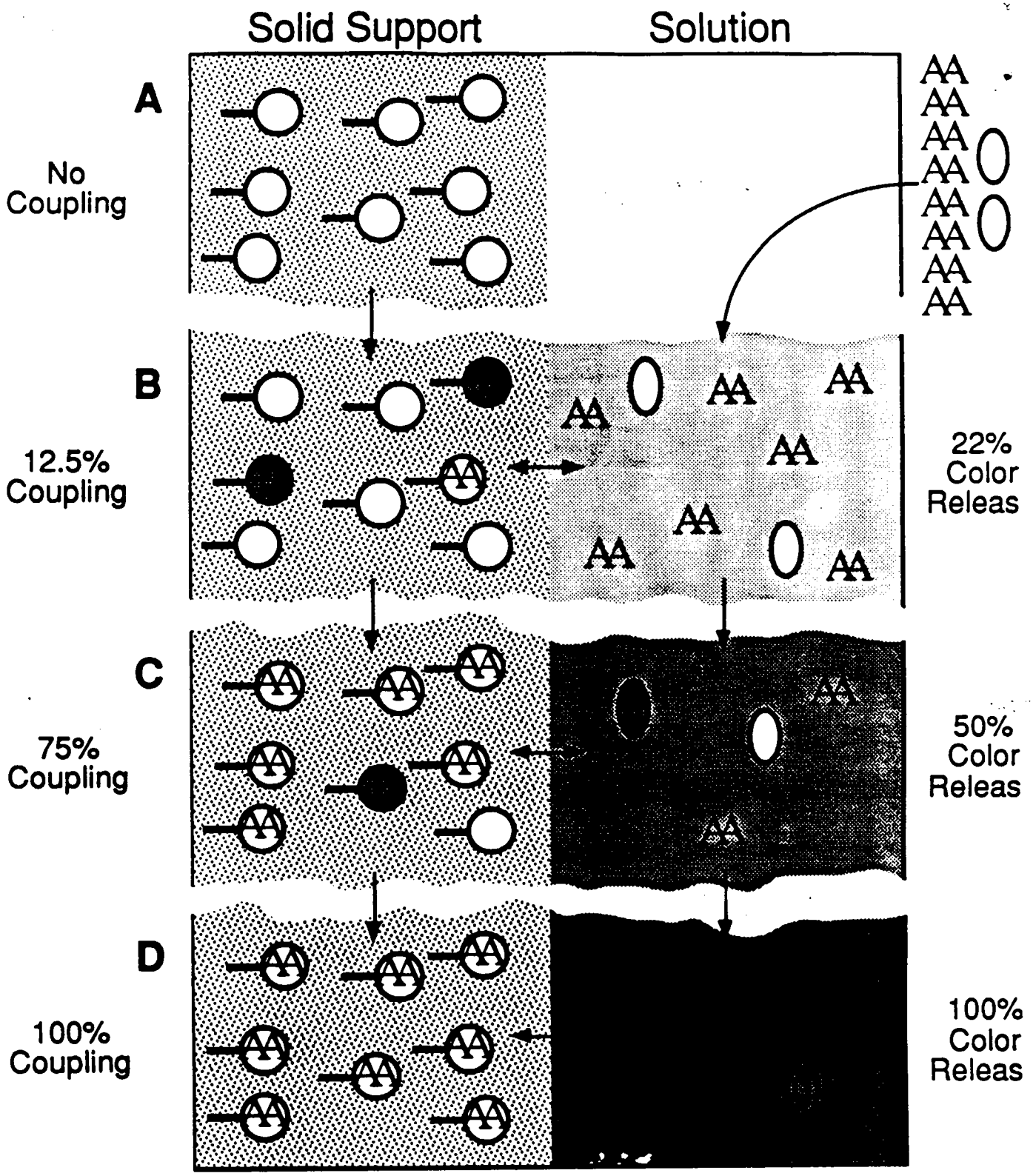
coupling reaction is approaching completion, would allow accurate automated feedback control over a synthesis. A technique that meets these requirements is called counterion distribution monitoring (CDM)*.

MONITORING METHOD*

The basic principle of the CDM method is the partitioning of an acidic dye between the basic free amino groups on the solid support and an added base in the coupling solution. Scheme 2 diagrams the basic principles of CDM in solid-phase peptide synthesis. Before the acylation reaction, the solid support is treated to remove the N-terminal blocking group on the amino acid or peptide so that the free amino group is available for reaction (open circles in Panel A). In this example an equivalent of an activated amino acid (with N^{α}- and appropriate side chain protection) is then added to the solution along with 0.25 equivalents each of base (open oval) and acidic dye (shaded circle). The acidic dye forms ion pairs with any basic sites and as the solution diffuses into the solid support, partitions between the amino groups on the support (shaded circles) and the base in solution (shaded ovals). Since there are initially more basic sites on the support than in solution, most of the dye binds to the support (Panel B). As the activated amino acid reacts with the available amino groups on the support (AA circles), these sites are no longer available for interaction with the dye and more dye is released into solution. In this example, when the coupling reaction is 75% complete (Panel C) then there is an equal number of support bound amines and bases in solution. Assuming that the interaction of the dye with the bound amino groups and the base is equal then half of the dye is on the support and half is in solution. As the coupling reaction proceeds to completion, all of the bound amino

groups have been reacted and all the dye is in the solution (Panel D).

Counterion distribution monitoring: a novel method for acylation monitoring in solid-phase peptide synthesis (1990), by S. C. Young, P. D. White, J. W. Davies, D. E. I. A. Owen, S. A. Salisbury, and E. J. Tremeer. **Biochemical Society Transactions, 18**, 1311-1312.



Scheme 2: Principles of CDM

The relationship between the percent coupling and color release is not linear. By adding small amounts of base relative to the total loading on the support most of the color is released near the completion of the coupling reaction (see Figure 2). For instance, if 1% base is added then 50% of the color would not be released from the support until 99% of the coupling had occurred (at this point there is an equal number of support-bound amines and bases in solution). Therefore, the greatest sensitivity with this method is in the region of interest where the coupling is approaching completion.

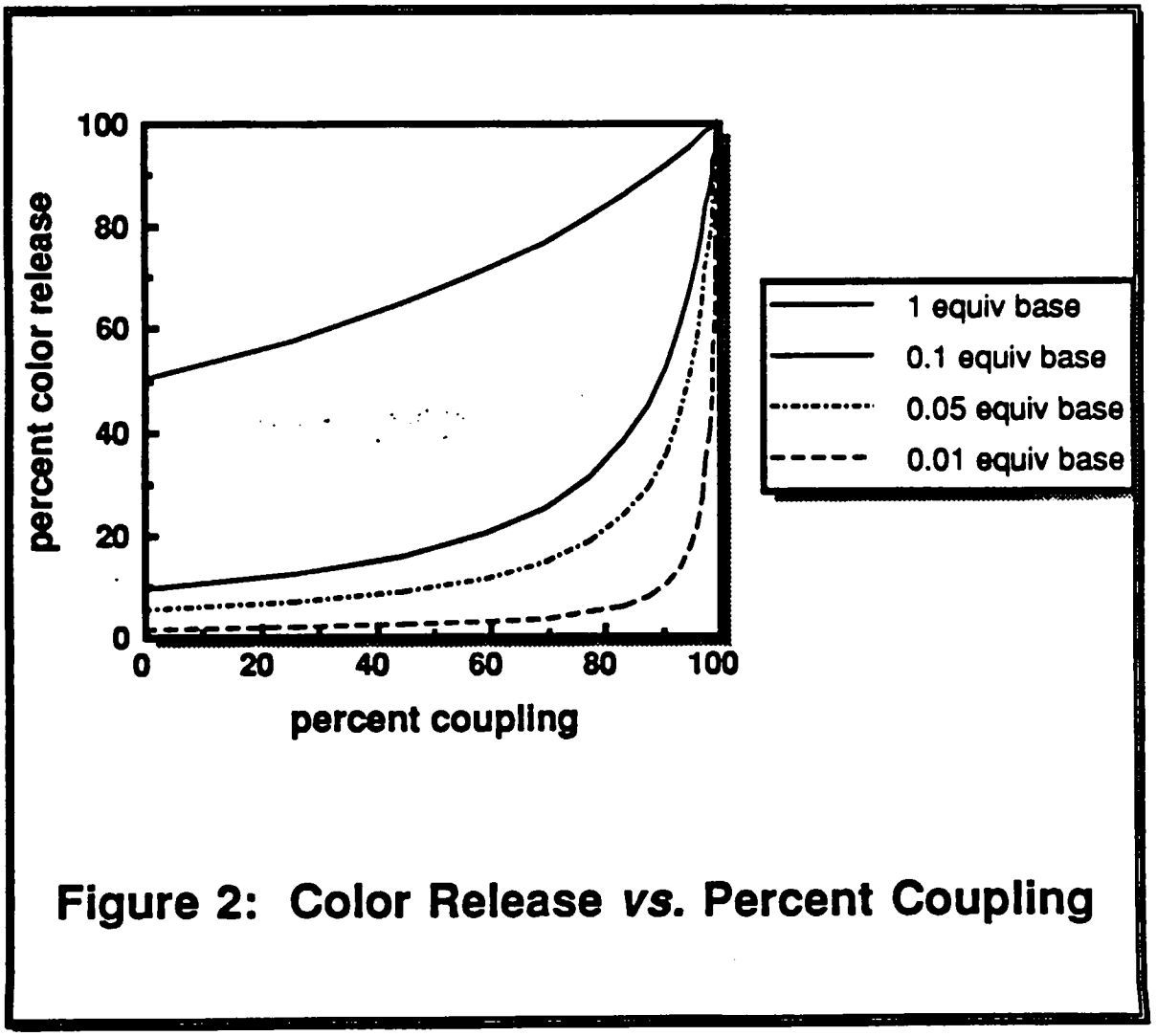


Figure 2: Color Release *vs.* Percent Coupling

Principles of Instrument Operation

The MilliGen/Biosearch 9050 Peptide Synthesizer is well suited for use with feedback monitoring. There is a continuous flow of reagents through the reactor column as well as both pre- and post-column monitoring of the effluent. In order to implement the CDM technique on the 9050 Peptide Synthesizer, the pre-column filter is changed to a wavelength which detects only the dye and none of the other reaction components and the software is modified to interpret the data from the pre-column detector. In addition, a dye and base are added to the amino acid dissolution reservoirs.

The heart of the feedback monitoring system is establishing when the coupling reaction is complete (or has gone as far as it is going to). This is determined by looking at the pre-column detector signal and deciding when it is 'stable' (see Figure 3). Initially the detector signal will be oscillating until the coupling solution in the recycle loop is homogeneous. If the reaction is not complete, the dye will continue to be released from the resin and the signal will be rising. When the signal becomes 'stable' (or when the set maximum coupling time is reached), the percent coupling is calculated. If the percent coupling does not meet the desired minimum coupling efficiency, then the instrument will act depending on which monitoring mode is active.

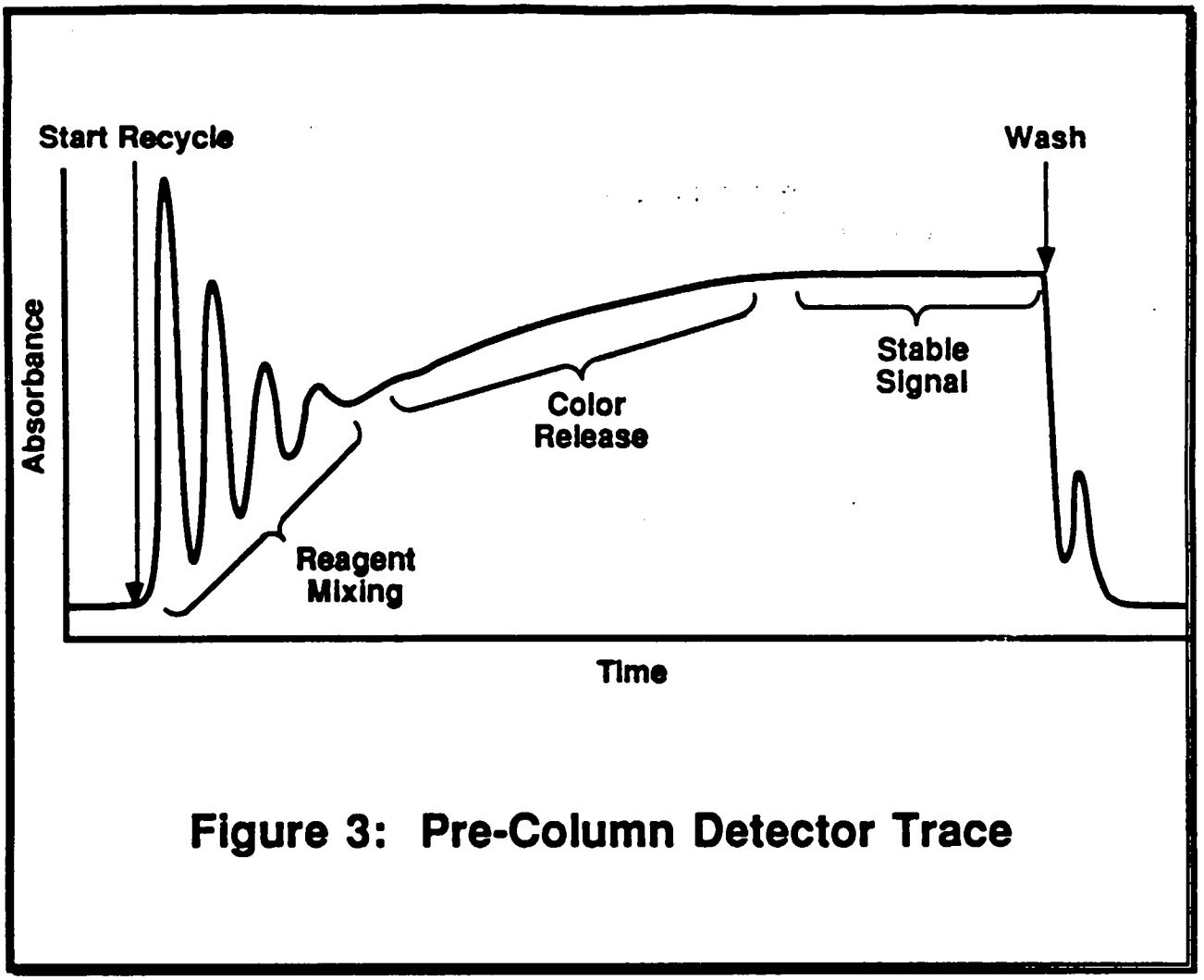


Figure 3: Pre-Column Detector Trace

There are three modes of operation that can select for operating the instrument.

1) **Bypass**- This mode incorporates the features of the feedback monitoring system but will go on to the next cycle, even if the minimum coupling efficiency is not achieved.

2) **Recouple**- This mode will automatically recouple an amino acid if the minimum percent coupling is not achieved. The same sequence of amino acid vials is placed in two racks of the amino acid module. During a recouple the deblock step is omitted and the probe goes to the second rack to dissolve up the amino acid. If the minimum percent coupling is still not achieved, then the synthesis halts at the end of the cycle and the synthesis can be aborted, continued, or the last cycle can be repeated again (the amino acid vial would be replaced with a fresh one).

3) **User**- In this mode if the percent coupling is below the set minimum then the instrument halts at the end of the cycle and the same choices described for the Recouple mode are available.

Switching between monitoring modes during a single synthesis permits a great deal of versatility. For instance, the instrument could be run in the User mode while the operator is in the laboratory and switched to the Bypass or Recouple mode when the operator leaves for extended periods of time.

Calculation of Percent Coupling

The percent coupling is calculated using the formula:

$$\% \text{ Coupling} = 100 + \%\text{base} - \frac{\%\text{base}}{\text{Fraction Color Release}}$$

where

$$\%\text{base} = \frac{\textit{concentration} * \textit{density} * \text{dissolution volume}}{\textit{MW} * \text{scale}}$$

- The ***concentration***, ***density***, and ***MW*** are parameters of the base used.
- The **dissolution volume** is the number of mL delivered to the amino acid vial.
- The **scale** is the total loading of the solid support.

and

Fraction Color Release = \F(monitor AU * *calibration volume,calibration AU* * dissolution volume)

- The **monitor AU** is the absorbance value when the detector signal is determined to be stable.
- The **dissolution volume** is the number of mL delivered to the amino acid vial.
- The ***calibration volume*** and ***calibration AU*** are parameters from the calibration cycle.

Rapid Cleavage and Deprotection

A faster deprotection and cleavage method, in addition to added convenience and throughput, would reduce the exposure of the peptide product to the cleavage reagent and potentially decrease side reactions. The addition of 2.5% methanesulfonic acid (MSA) to standard TFA-based cleavage/deprotection reagents increases the rate of cleavage of methylphenoxyacetyl linkers and removal of protecting groups from the amino acid side chain by 60- to 80-fold (see Table 3). The more resistant side chain protecting groups found in Fmoc-based synthesis, most notably the methoxytrimethylphenylsulfonyl (Mtr) group sometimes used to protect the side chain of arginine, can be removed in minutes instead of hours. It must be noted, however, that extended storage of a peptide with any excess of a strong acid (i.e. MSA, TFA) leads to deamidation of C-terminal amides and Asn and Gln residues. Therefore, peptides to be stored for extended periods of time should either be left on the solid support or treated to remove residual components from the cleavage/deprotection treatment.

Table 3: Effect of Added MSA on Cleavage Rates

Amino Acid/ Peptide	Cleavage Reagent*	rate (min^{-1})	$t_{1/2}$ (min)	$t_{95\%}$ (min)
Val	P	0.016	45	190
	P+	0.88	0.79	3.4
Neurotensin 9-13	P	0.0076	91	390
	P+	0.64	1.1	4.7
GAWGL-OH	P	0.010	68	290
	P+	0.70	0.99	4.3
Prothrombin 1-9	P	0.0046	150	650
	P+	0.31	2.2	9.7

* *Reagent P:* 95% TFA
5% phenol

Reagent P+: 95% TFA
2.5% phenol
2.5% MSA

CONCLUSIONS

- Membrane solid supports are easily handled, lead to a reduction in solvent consumption and synthesis time, and are useful in a variety of applications.
- Racemization during the attachment of the first amino acid to the solid support can be eliminated by using 2,4-dichlorophenyl-Nα-Fmoc-amino acyl-4-oxymethylphenoxyacetates. These amino acid linkers react rapidly with the membrane support.
- The CDM method allows accurate real time feedback monitoring using a modified MilliGen/Biosearch 9050 Peptide Synthesizer. These modifications permit the use of several operational modes, including automated recoupling when a coupling efficiency is below the set minimum.
- The cleavage/deprotection step can be performed rapidly (in less than 15 min) by adding MSA to standard deprotection/cleavage cocktails.