# Next Generation Microwave SPPS – 4 minute Cycle Times, Scalable, and 90% Waste Reduction

Jonathan Collins, Sandeep Singh, Keith Porter, Grace Vanier, Alicia Douglas, Eric Williamson,
Joseph Lambert, David Herman • CEM Corporation, Life Science Division, Matthews, NC, 28104 USA www.cem.com

## Introduction

The development of SPPS was a major breakthrough for simplifying the production of peptides and allowing for automation of the process[1]. Since that time the technique has extensively developed with the improvements in chemical protecting groups, newer activation methods, and the introduction of chemical ligation as examples. More recently, the use of microwave energy has proven to be an effective tool for decreasing reaction times, and in many cases increasing peptide product purity. However, even with all of these improvements, the synthesis time for peptides is still a bottleneck, and the process generates substantial chemical waste. Additionally, the development of UPLC has shortened analysis methods, which makes the potential value of shortening SPPS more profitable. We therefore focused on developing a method for SPPS that eliminates the synthesis bottleneck, drastically reduces the chemical waste, and is scalable.

A new process for SPPS is presented that allows for cycle times of only 4 minutes, along with up to a 90% reduction in total chemical waste compared to existing methods using a new Liberty Blue peptide synthesizer. The effectiveness of this new method was verified against the selected set of peptides shown in Table 1. These peptides as a group encompass a full range of common synthesis difficulties and demonstrate the robustness of these new methods.

## Materials and Methods

All peptides in Table 1 were synthesized conventionally (Table 2) and with standard microwave synthesis conditions (Table 3) using a CEM Liberty 1 and Liberty 12 systems. The conventional results were obtained without the use of microwaves for either the deprotection or coupling steps. The $^{65\text{-}74}$ACP, ABRF 1992, and $^{1\text{-}42}\beta$-Amyloid peptides in table 4 were synthesized with Liberty Blue peptide synthesizer. Cleavage was performed in all cases with TFA/TIS/$H_20$ /DODT for 30 min at 38°C using microwave irradiation. Analysis was performed with a Waters Aquity UPLC system using a 3100 Mass Detector system. Rink Amide MBHA PS resin (0.60 mmol/g) was used for the synthesis of Thymosin, JR 10mer, and ABC 20mer peptides, Fmoc-Gly-Wang PS resin (0.79 mmol/g) was used for the $^{65\text{-}74}$ACP synthesis, Fmoc-Tyr(tBu)-Wang PS (0.88 mmol/g) was used for the ABRF 1992 synthesis, and PAL-PEG-PS resin (0.16mmol/g) was used for the $^{1\text{-}42}\beta$-amyloid synthesis.

### Table 1. Selected Set of Peptides

| # | Peptide | Sequence | Difficulty | Synthesis Challenges |
|---|---|---|---|---|
| 1 | $^{65\text{-}74}$ACP | VQAAIDYING | Medium | A |
| 2 | JR 10-mer | WFTTLISTIM-$NH_2$ | High | B |
| 3 | ABRF 1992 | GVRGDKGNPGWPGAPY | Medium | C |
| 4 | ABC | VYWTSPFMKLIHEQCNRADG-$NH_2$ | Medium | D, E |
| 5 | Thymosin | SDAAVDTSSEITTKDLKEKKEVVEEAEN-$NH_2$ | High | F |
| 6 | $^{1\text{-}42}\beta$-Amyloid | DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA-$NH_2$ | High | A, E |

A = Aggregation; B = Difficult Deprotection; C= δ Lactam Formation; D = Aspartimide Formation; E = Epimerization (Cys, His); F = β-branched side chains

## Results and Discussion

The selected peptides were first synthesized under conventional room temperature conditions in order to establish a baseline purity level that is obtainable. Attempts were made to improve the results of the Thymosin peptide through the use of the stronger activation chemistry HCTU/DIEA as well as use of the hydrophilic ChemMatrix resin with shorter reaction times. For most peptides relatively low purity was obtained indicating expected synthesis difficulties.

### Table 2. Conventional Synthesis Results

| Peptide | Deprotection | Coupling | | Analysis | |
|---|---|---|---|---|---|
| | | Reagent | Time (min) | Purity | Crude Yield |
| $^{65\text{-}74}$ACP | 5, 10 | DIC/Oxyma | 60 | 38 | 94 |
| $^{65\text{-}74}$ACP[a] | 5, 10 | HBTU/DIEA | 30 | 90 | 24 |
| JR 10-mer | 5, 10 | DIC/Oxyma | 60 | 42 | 80 |
| ABRF 1992 | 5, 10 | DIC/Oxyma | 60 | 56 | 82 |
| ABC 20-mer | 5, 10 | DIC/Oxyma | 60 | 70 | 89 |
| ABC 20-mer | 5, 10 | HBTU/DIEA | 30 | 70 | 94 |
| Thymosin | 5, 10 | DIC/Oxyma | 60 | 35 | 79 |
| Thymosin | 5, 10 | HCTU/DIEA | 30 | 34 | 70 |
| Thymosin | 5, 10 | HCTU/DIEA | 5 | 25 | 68 |
| Thymosin[b] | 5, 10 | HCTU/DIEA[c] | 5 | 39 | 56 |

[a]Resin = 0.44mol/g Gly-Wang ChemMatrix; [b]Resin = 0.52 mmol/g Rink Amide ChemMatrix; [c]10 equivalents used

We then investigated the synthesis of this same series of peptides using existing microwave peptide synthesis technology. DIC/Oxyma was chosen as the activation method of choice as it avoids the explosive properties of benzotriazole reagents[2], is inexpensive, and stable in solution[3]. As shown in Table 3, the microwave approach allowed for rapid reaction times of all peptides. Additionally, a substantial increase in purity was observed for the JR 10-mer, Thymosin, and β-amyloid peptides compared to the conventional synthesis approach used, while the $^{65\text{-}74}$ACP and ABC peptides were synthesized at equivalent purity levels.

The coupling step for Fmoc-His(Trt)-OH and and Fmoc-Cys(Trt)-OH was performed at a maximum temperature of 50°C using a 6-minute total method to limit undesirable epimerization which can occur. However, once incorporated onto a peptide chain, these amino acids are inert to epimerization even at very high temperatures[4]. This allows one to use more moderate temperatures during the coupling steps of these sensitive amino acids, but more aggressive conditions for others, without additional risk of epimerization. The coupling time of Fmoc-Arg(Pbf)-OH was also longer to minimize γ-lactam formation which can be accelerated at higher temperatures.

### Table 3. Standard Microwave Synthesis Results

| Peptide | Deprotection | | Coupling | Analysis | |
|---|---|---|---|---|---|
| | Reagent[a] | Time (min)[b] | Time (min)[b,c] | Crude Purity | Crude Yield |
| $^{65\text{-}74}$ACP | A | 0.5, 3 | 5 | 91 | 99 |
| JR 10-mer | A | 0.5, 3 | 5 | 60 | 74 |
| ABRF 1992 | B | 0.5, 3 | 5 | 79 | 96 |
| ABC 20-mer | A | 0.5, 3 | 5 | 71 | 91 |
| Thymosin | A | 0.5, 3 | 5 | 58 | 87 |
| $^{1\text{-}42}\beta$-Amyloid | A | 0.5, 3 | 5 | 60 | 87 |

[a]A = 20% Piperidine w/ 0.1M Oxyma, B = 10% Piperazine; [b]Max Temp. = 75 °C; [c]AA/DIC/Oxyma 1:1:1 w/ 5-equivalents

The same series of peptides were then synthesized with extremely rapid microwave reaction conditions up to 90 °C, washing, and mixing. A new piperazine based deprotection cocktail was used that allowed for rapid deblocking and elimination of piperidine. Additionally, the solvent used for all reactions and washing steps was able to be significantly reduced resulting in a substantial waste reduction. In all cases the crude purity matched or exceeded the standard microwave results. The use of microwave was then tested on a new 2.5 L automated synthesizer up to 100mmol scale which showed rapid heating rates and a significant reduction in synthesis time (see poster # 335).

### Table 4. Optimized Methods

| Peptide | Deprotection[a] | Coupling[d] | RV Waste | Analysis | |
|---|---|---|---|---|---|
| | Time (min)[b] | Time (min)[b] | mL | Crude Purity | Crude Yield |
| $^{65\text{-}74}$ACP | 1[c] | 2 | 136 | 92 | 98 |
| JR 10-mer | 1 | 2 | 128 | 67 | 72 |
| ABRF 1992 | 1 | 2 | 188 | 77 | 97 |
| ABC 20-mer | 1[c] | 2 | 248 | 73 | 95 |
| Thymosin | 1 | 2 | 344 | 64 | 95 |
| $^{1\text{-}42}\beta$-Amyloid | 1 | 2 | 1253 | 65 | 87 |

[a]Deprotection: 10% Piperazine; [b]Maximum Temp. = 90°C; [c]with 0.1M Oxyma; [d]AA/DIC/Oxyma 1:1:1 w/ 5-equivalents;

## Conclusions and Future Directions

Extremely rapid and efficient methods have been developed for the synthesis and cleavage of peptides. **These methods allow for cycle times as low as 4 minutes with a significant reduction in overall chemical waste. For example, the $^{65\text{-}74}$ACP, ABRF 1992, and $^{1\text{-}42}\beta$-Amyloid peptides were synthesized in 38 minutes, 101 minutes, and 247 minutes respectively.** The methods utilize inexpensive resins, eliminate the need for piperidine, and use a low cost DIC/Oxyma based activation scheme with standard reagent excesses. This new process for SPPS should have a major impact on synthesis of difficult peptides, high throughput peptide production, and larger scale peptide production, as it offers improvements in speed, chemical usage, and synthesis efficiency.

## References

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