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High-Efficiency Solid Phase Peptide Synthesis (*HE*-SPPS)

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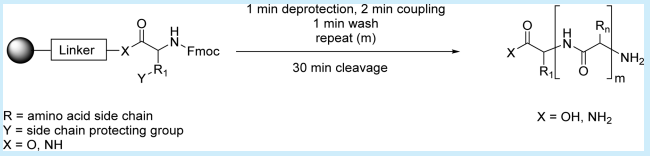
Supporting Information

ABSTRACT: A series of improvements to the standard solid phase peptide synthesis (SPPS) process allowing for significant gains in product purity along with only a 4 min standard cycle time and a 90% reduction in total waste produced is reported. For example, syntheses of the well-known $^{65-74}$acyl carrier protein (ACP) and $^{1-42}\beta$-amyloid peptides were accomplished with 93 and 72% purity (UPLC−MS) in only 44 and 229 min, respectively.



Solid phase peptide synthesis (SPPS) has proven invaluable for the successful construction of a diverse array of natural and modified peptide sequences.[1] Fmoc chemistry is the dominant method in use, which features the base labile α-amino Fmoc protecting group along with acid labile side chain protection and peptide-resin linkage.[2]

Although SPPS is instrumental for peptide production, it is not without challenges. The process is complicated by the well documented occurrence of aggregation thought to originate primarily from intermolecular hydrogen bonding.[3] This creates inaccessibility of the reactive end of the chain thereby leading to more difficult deblocking or acylation steps. Additionally, steric effects can lower reaction rates when the terminal amine is secondary (i.e., proline, N-substituted amino acids) or when bulky side chain protecting groups are present. An incomplete reaction at any step in the synthesis leads to deletion sequences, which can be extremely hard to separate from the desired target sequence. At the same time incomplete removal of previously used peptide reagents can lead to impurities such as substitutions and addition sequences by undesirably participating in a subsequent step. As a result SPPS is typically performed with long reaction times, large excesses of reagents, and many repetitive washing steps between each step.[4] For example, a 100 mg scale production of a peptide 20 amino acids in length can take 24 h to complete and produce several liters of chemical waste. Efforts to make SPPS maximally efficient therefore must address the efficiency of both reaction steps in each cycle and the overall washing process.

Our goal was to develop a high-efficiency (HE)-SPPS method that could be applied to any sequence with the goal of dramatically increasing the efficiency of the SPPS process. Microwave (MW) irradiation has been previously applied to SPPS and we explored this tool for the deprotection, coupling, and resin cleavage steps.[5−8] We identified a group of 6 known peptides from 10 to 42 amino acid length (Table 1) that together contain a range of synthesis challenges and common side reactions. Therefore, any method improvements that would hold constant across all 6 of these peptides would have strong support for general use in SPPS. In order to fully resolve any impurities that may be present, we utilized ultra-high-performance liquid chromatography (UPLC) with extended gradient times coupled to a mass spectrometer.

The selected peptides were first synthesized using conventional room temperature methods at a 0.1 mmol scale in order to establish a baseline purity level.[9] Common polystyrene resins were used for each synthesis.[10] The one exception was $^{1-42}\beta$-amyloid for which a 0.16 mmol/g loading PAL-PEG-PS resin was used because of the longer peptide length. The use of microwave for the TFA cleavage step in Fmoc SPPS demonstrated the ability to shorten this step to 30 min at 38 °C as confirmed by our results. Therefore, for all subsequent experiments the MW cleavage method was used in order to save time.

Even with long synthesis times, relatively low purity was obtained for the JR 10-mer, ABRF 1992, thymosin, and $^{1-42}\beta$-amyloid peptides (Table 1, entry 3−5, 9−10, 14). The ABC 20-mer was prepared in good purity using the standard conventional times (Table 1, entry 6, 7) but failed to give any target using shorter reaction times (Table 1, entry 8). For the thymosin peptide the purity increased from 37 to 47% (Table 1, entry 11) with the combined use of the more aggressive HCTU activation[11] and switching to a hydrophilic resin (0.52 mmol/g loading Rink Amide ChemMatrix) under standard long reaction times. Attempts to reduce the deprotection and coupling times under the same conditions, even with the added benefit of 10 equiv of the coupling reagents failed to improve the crude purity from 36% (Table 1, entry 12).

Microwave irradiation was then applied to both the deprotection and coupling steps using previously reported conditions.[12−14] For activation we chose to use the well-known carbodiimide (DIC) based techniques with additives such as HOBt, HOAt, and Oxyma. For the present study we used Oxyma[15] as our additive of choice, as hydroxybenzotriazole derivatives such as HOBt and HOAt have been classified as Class 1c explosives.[16] Often, the use of DIC/(HOBt or Oxyma) activation is associated with an initial preactivation

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Table 1. Baseline Conventional Synthesis Results

entry	peptide[a]	deprotection time (min)	activation	coupling time (min)	UPLC purity (%)	crude yield[f] (%)
1	$^{65-74}$ACP	5 + 10	DIC/Oxyma	60	38 (39[e])	94 (96[e])
2	$^{65-74}$ACP[b]	5 + 10	HBTU/DIEA	30	90	24
3	JR 10-mer	5 + 10	DIC/Oxyma	60	42 (42[e])	80 (80[e])
4	JR 10-mer[c]	0.5 + 3	HCTU/DIEA	5	35	58
5	ABRF 1992	5 + 10	DIC/Oxyma	60	56	82
6	ABC 20-mer	5 + 10	DIC/Oxyma	60	70 (70[e])	94 (96[e])
7	ABC 20-mer	5 + 10	HBTU/DIEA	30	68 (70[e])	98 (94[e])
8	ABC 20-mer	0.5 + 3	DIC/Oxyma	5	<5	–
9	thymosin	5 + 10	DIC/Oxyma	60	35 (37[e])	79 (81[e])
10	thymosin	5 + 10	HCTU/DIEA	30	34	70
11	thymosin[c]	5 + 10	HCTU/DIEA	30	47	65
12	thymosin[c]	0.5 + 3	HCTU/DIEA[d]	5	36	56
13	thymosin	0.5 + 3	HCTU/DIEA	5	25	68
14	$^{1-42}\beta$-amyloid	5 + 10	DIC/Oxyma	60	56	85
15	$^{1-42}$ β-amyloid	0.5 + 3	HCTU/DIEA	5	14	72

[a]Peptide Sequences: $^{65-74}$ACP: VQAAIDYING, JR 10-mer: WFTTLISTIM-NH_2, ABRF 1992: GVRGDKGNPGWPGAPY, ABC 20-mer: VYWTSPFMKLIHEQCNRADG-NH_2, thymosin: SDAAVDTSSEITTKDLKEKKEVVEEAEN-NH_2,$^{1-42}\beta$-Amyloid: DAEFRHDSGYEVHHQKLV-FFAEDVGSNKGAIIGLMVGGVVIA-NH_2. [b]Resin = 0.44 mmol/g of Gly-Wang ChemMatrix. [c]Resin = 0.52 mmol/g of Rink Amide ChemMatrix. [d]10 equiv used. [e]MW resin cleavage method. [f]Crude yield corresponds to lyophilized product without any purification.

step because of formation of the initial *O*-acylisourea proceeding faster in a nonpolar solvent such as DCM, while the subsequent acylation step proceeds faster in a more polar medium such as DMF or NMP. We felt that microwave irradiation would allow both processes to occur rapidly in a single solvent, thereby simplifying the overall process.

Initial attempts to use microwave irradiation proved encouraging, as shown in Table 2. Each of the six peptides investigated were synthesized in the same or higher purity than the optimized conventional conditions with an average cycle time of around 30 min. This is in agreement with previous reports that show microwave irradiation can allow for not only fast deprotection and coupling reactions, but in many cases also higher purity synthesis results.[14] We then began a series of sequential optimization steps to achieve our goal of increasing the efficiency of SPPS. First, we focused on optimizing the MW conditions used for the deprotection and coupling steps with the goal of performing both steps as rapidly as possible. We recognized that significant time could be saved by performing the deprotection step in a single stage as opposed to the 2-stage deprotection used as standard practice. Additionally, we also reasoned that both the deprotection and coupling steps could be performed significantly faster by more aggressive application of microwave power while also maintaining a higher temperature. This approach requires precise and rapid temperature feedback, as aggressive temperature ramping could lead to undesirable temperature overshooting.[17] We used an internal fiber optic probe (Figure S4, Supporting Information) during MW irradiation and found that we could reproducibly reach a control temperature of 90 °C with maximum temperature of 92 °C in only 20 s during both the deprotection and coupling steps. Through optimization we showed that equal or higher purity could be obtained for each peptide in the set using a single 1 min deprotection and 2 min coupling.

Table 2. Baseline MW Synthesis Results

entry	peptide	deprotection reagent[a]	UPLC purity (%)	crude yield[b] (%)
1	$^{65-74}$ACP	A	91	99
2	JR 10-mer	C	60	74
3	ABRF 1992	B	79	96
4	ABC 20-mer	A	71	91
5	thymosin	A	58	87
6	$^{1-42}\beta$-Amyloid	A	67	87

[a]A = 20% piperidine w/0.1 M Oxyma; B = 10% piperazine; C = 20% piperidine. [b]Crude yield corresponds to lyophilized product without any purification.

Next we focused on optimization of the washing that is required during SPPS. Since both DMF and NMP are used routinely for washing, we investigated both. It was noted that the resin maintained a residual temperature of 50 °C or higher immediately after draining each reaction. We reasoned that this could provide a benefit to the washing process as a higher diffusion was more likely to occur at elevated temperature. To our satisfaction we found that 3 washes of only 1, 2, and 3 mL with NMP and 2, 2, and 3 mL with DMF for standard polystyrene resins were required as confirmed by UPLC–MS analysis. The one exception to this was the low loaded PAL-PEG-PS resin used for $^{1-42}\beta$-amyloid, which required 4 × 4 mL washing with DMF after the deprotection step because of its higher mass from significantly lower substitution.

Postcoupling washes are typically done to prevent residual activated amino acid from undesirably coupling during the subsequent deblocking step. A closer analysis reveals inherent protection in the SPPS process against this occurring. First, the residual activated amino acid remaining after vessel draining will be very small compared to the large excess of deblocking base added. The deblocking base, as a secondary amine, should react rapidly with the residual activated amino acid present in a solution phase process providing complete destruction of the ester.[18,19] Initially almost all peptide chains should be blocked with Fmoc from the previous coupling reaction further limiting the ability of the activated ester to couple in a kinetically slower solid phase reaction. We therefore reasoned that washing could possibly be eliminated after the coupling step. This was indeed confirmed with every peptide tested in the data set and eliminated a major portion of the synthesis process and chemical waste.

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Piperidine can be difficult to obtain because of its classification as a controlled substance. As an alternative, piperazine has been previously investigated both conventionally[20] and in MW SPPS.[12] However, piperazine has an approximate maximum concentration in DMF or NMP of 6%. We reasoned that a new solvent system that increases piperazine concentration without negatively affecting solubility of the peptide-resin complex would be useful. We found that a mixture of EtOH and NMP (10:90) allowed dissolution of piperazine at 10%. Pleasingly this new mixture increased the effectiveness of Fmoc deblocking to levels that matched 20% piperidine.

We next focused on improving the coupling methods of cysteine and histidine because of their epimerization sensitivity and longer coupling time.[21] We investigated whether lack of a hindered amine present with the in situ carbodiimide approach would allow cysteine and histidine to be coupled under more aggressive temperature conditions using the ABC 20-mer peptide with known sensitivity to these side reactions.[12] Using the more aggressive two minute coupling method at 90 °C for cysteine led to only 1.04% D-Cys as shown in Table 3.

Table 3. Epimerization of Fmoc-Cys(Trt)-OH

entry	ramp (min)	hold (min)	max temp (°C)	activation	% D
1	NA	30	rt	HBTU/DIEA	1.38
2	NA	60	rt	DIC/Oxyma	0.34
3	2	4	50	DIC/Oxyma	1.14
4	0.5	1.5	90	HBTU/DIEA	16.7
5	0.5	1.5	90	DIC/Oxyma	1.04
6	0.5	1.5	90	DIC/HOBt	3.73
7	0.5	1.5	90	DIC/HOAt	2.01

Importantly, this shows that the in situ carbodiimide activation method is uniquely suitable for cysteine under elevated temperatures, as lack of a hindered amine limits epimerization from direct enolization. Since histidine displayed significant epimerization, we then attempted to utilize a recently introduced side chain protecting group, Mbom, that protects the π-nitrogen on the imidazole side chain.[22] An optimized 3 min coupling step at 80 °C led to only 1.43% D-His (Table 4, entry 5).

Aspartimide formation can accumulate during each deprotection step in Fmoc chemistry leading to α,β-aspartyl peptides and α- and β-piperidides (Scheme S1 (SI)). This base catalyzed side reaction typically occurs in Asp-X sequences [X = Gly, Asn, Ser, Thr] with Asp-Gly being the worst. The % D content of the three Asp residues in thymosin under optimized conditions was only 0.91%. However, the ABC 20-mer containing a C-terminal Asp-Gly segment had a % D Asp content of 4.94%. Adding 0.1 M Oxyma to the 10% piperazine cocktail reduced this to 3.57%, while use of the Fmoc-Asp(OtBu)-(Dmb)Gly-OH derivative led to only 0.36% D Asp (Table S1 (SI)). This demonstrates that aspartic acid is in general protected during the optimized deblocking conditions, but less aggressive deblocking conditions should be considered for the Asp-Gly case.

Table 4. Epimerization of Fmoc-His(Trt)-OH and Fmoc-His(π-Mbom)-H

entry	ramp (min)	hold (min)	max temp (°C)	activation	% D
1	NA	30	rt	HBTU/DIEA	1.79
2	NA	60	rt	DIC/Oxyma	1.42
3	2	4	50	DIC/Oxyma	3.24
4	0.5	1.5	90	HBTU/DIEA	25.6
5[a]	0.5	2.5	80	DIC/Oxyma	1.43
6	0.5	1.5	90	DIC/Oxyma	23.2
7[a]	0.5	1.5	90	DIC/Oxyma	2.96
8	0.5	1.5	90	DIC/HOBt	15.3
9	0.5	1.5	90	DIC/HOAt	18.9

[a]Fmoc-His(π-Mbom) used for entries 5 and 7.

Finally, in the synthesis of the ABRF 1992 peptide, we found that a single arginine coupling at 25 min room temperature followed by 2 min with MW irradiation at 75 °C was as efficient as the previously described double coupling method.[13] This allows for both a 50% reduction in the expensive Fmoc-Arg(Pbf)-OH derivative and a slight time savings. Taken together these optimizations along with fast automation allowed for significant improvements as shown in Table 5. The purity increased for all peptides with a standard cycle time of slightly longer than 4 min and a major waste reduction.

Table 5. Fully Optimized SPPS Methods

entry	peptide	UPLC purity (%)	crude yield[a] (%)	synthesis time (min)[b]	total waste (mL)
1	$^{65-74}$ACP	93	98	44	154
2	JR 10-mer	67	72	49	170
3	ABRF 1992	82	97	97	272
4	ABC 20-mer	73	95	127	340
5	thymosin	61	95	131	468
6	$^{1-42}\beta$-Amyloid	72	87	229	1019

[a]Crude yield corresponds to lyophilized product without any purification. [b]Does not include a 5 min resin swell time.

An optimized process for SPPS is presented that allows for complete cycle times of approximately 4 min along with a significant reduction (approximately 90%) in total chemical waste compared to traditional methods. Key features of HE-SPPS are newly developed microwave enhanced reaction conditions, a novel low-cost deblocking cocktail, low-cost in situ carbodiimide based activation, optimized washing, and ultrafast automation. This new method should have a significant impact on the assembly of difficult peptides, high throughput production, and larger scale peptide production as it offers improvements in synthesis efficiency, speed, and chemical usage.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedure, UPLC–MS chromatograms and data, and C.A.T. epimerization analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Authors are employees of CEM Corporation, which manufactures the peptide synthesizer used in this study.

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(9) Deprotection was performed with 7 mL of 20% piperidine in DMF with N_2 bubbling and repeated twice with fresh reagent for 5 and 10 min. For coupling, a 5-fold excess of reagents was used with common carbodiimide and aminium based activation methods in a 4 mL total volume using coupling times up to 60 min with N_2 bubbling. Five washes each with 7 mL of DMF and N_2 bubbling were performed between each reaction step.
(10) $^{65-74}$ACP: 0.79 mmol/g of Fmoc-Gly Wang PS. JR 10-mer, ABC 20-mer, thymosin: 0.60 mmol/g of Rink Amide MBHA PS. ABRF 1992: 0.88 mmol/g of Fmoc-Tyr(tBu)-Wang PS.
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