FAST FLOW PEPTIDE SYNTHESIS – THE ROUTE TO DIFFICULT PEPTIDES AND SCALE UP

PEER REVIEWED

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ABSTRACT

In recent years, fast flow solid-phase peptide synthesis (FF-SPPS) has been embraced by industry as way to quickly synthesise new peptide-based drugs for the discovery space. By applying uniform heating and inhibiting the movement of resin beads, high reaction efficiency is achieved, while also preventing β-sheet structure formation, thereby improving solubility of peptides as they are synthesised.
Using the FF-SPPS approach, protocols have been developed that work for both long peptides and for sequences where the peptides aggregate. These protocols achieve maximum cycle efficiency by flowing reagents in a single pass through the resin.
By way of example the suitability of FF-SPPS is illustrated by the synthesis of a 77-mer α-helical coiled coil peptide, with a synthesis time of under 20 hours and achieving a crude purity of ~62 %.
A further example is discussed for the linear scale up of a GLP-1 analogue where the use of FF-SPPS, results in a 300X scalability factor without further optimisation. The synthesis time for this GLP-1 analogue at 15 mmol scale is 15 hours with and crude purity of ~80 %.

KEYWORDS:
- Flow chemistry
- GLP-1
- Semaglutide
- Oligonucleotides & Peptides
- Peer Reviewed

INTRODUCTION

Largely, thanks to the recent success demonstrated by peptide drugs, such as Semaglutide and Tirzepatide, in the treatment of type-II diabetes and weight loss, there has been a significant shift in the pharmaceutical landscape towards peptides-based therapeutics. resulting from the improvement in the pharmacokinetic properties of peptide drugs, the peptide market is growing almost twice as fast as the "small molecule" market (1). There is now an increasing need to be able to quickly produce hundreds of grams of target peptides to satisfy the needs of pre-clinical and clinical trials. At this stage of the research cycle, time is of the essence, as short synthetic times will help maximising the life of the patent at the commercial stage of the drug.

For the synthesis of peptides in the discovery and pre-clinical development stages, scientists often rely on SPPS as a convenient route for the synthesis. Traditional batch technologies often give poor performance in situations where:

- Sequences exhibit aggregation events
- The synthesis of long peptides is necessary
- Rapid scale up of the synthesis is required

In these situations, existing batch processes are either too slow or do not yield the target compound, unless special reagents e.g. pseudo-prolines or dipeptides are used (2).

In recent years, academics as well as blue-chip pharma companies have embraced FF-SPPS in their early discovery labs as a way of synthesising complex peptides (3, 5). The reasons for such a move is based on the advantages continuous flow offers over existing batch technologies:

1. By using a variable bed flow reactor (VBFR), the resin bead movement is eliminated, and the total reactor volume minimised throughout the whole synthesis. This ensures a unique interaction between the reagents and the static solid support; back mixing is eliminated, and reaction by-products are continuously removed from the resin.

High reaction efficiency is achieved, but more importantly, by constraining both the resin and the direction of the reagent flow, the target peptide is preferred even at sub stoichiometric conditions. Solvent usage is also minimised, usage as low as 60 ml/mmol per cycle, has been demonstrated regardless of the scale.

2. The second advantage is the uniformity of heating. Heating has two important benefits:

- As the resin temperature remains constant, it prevents β-sheet structures that can lead to aggregation events
- It increases reaction kinetics which can be particularly beneficial for sterically difficult couplings.

However, not all heating is the same. Experience supporting continuous flow applications over the last 20 years has taught that uniform and constant heating is the key to reproducibility. Hot spots or uncontrolled temperature spikes will cause racemisation and can even lead to loss of the linker, particularly with chlorotrityl type resins.

3. In addition to the chemical advantages, with FF-SPPS we can access real-time in-line data never seen before at that level of detail: Reactor volume change (6), which can help detect aggregation events, and quantitative UV spectroscopy (6)–(8), eliminating

the need to take samples of the resin and do cleavages as the reaction progresses.
This article explores the synthesis of long peptides and the route to scale up FF-SPPS offers.

MATERIAL AND METHODS

All reagents were obtained from commercial suppliers:

Fluorochem: N α-9-fluorenylmethoxycarbonyl (Fmoc) amino acids, ethyl(hydroxyimino) cyanoacetate (Oxyma), diisopropyl carbodiimide (DIC), triisopropylsilane (TIPS)

Rathburn chemicals: piperidine, dimethylformamide (DMF)

Sigma Aldrich: trifluoroacetic, formic acid diethyl ether and acetonitrile

Final cleavage and analysis - After the synthesis of the peptide, the resin is washed by pumping DCM and then dried. The peptide was then cleaved in flow at 50 °C with 20 min residence time (Rt) for full deprotection of side protective groups. The peptide was collected and precipitated in cold ether.

Crude samples were then dissolved in Acetonitrile:H_2O (1:1 v/v) and analysed by HPLC. {Agilent 1220; Eclipse XDB-C18 5 µm column (4.6 mm x 150 mm, flow rate = 2 ml/min) heated at 40 °C}. The following solvent was used: Solvent A, Water containing 0.1 % TFA; Solvent B, acetonitrile containing 0.1 % TFA. The column was eluted using a linear gradient from 5 % to 60 % solvent B over 20 min. Mass analysis was carried out by ESI-MS (Advion expression LCMS).

EXPERIMENTAL

Stock solutions of amino acids were prepared to 0.3 M in DMF, containing Oxyma at 0.45 M. Deprotections were carried using 20 % piperidine solutions (in % v/v) in DMF with 2 % formic acid (in % v/v) as buffer to prevent aspartamide formation (9).

Reaction kinetics were enhanced by working at 80 °C, except for Fmoc-His(Trt)-OH and Fmoc-Arg(Pbf)-OH, in which a lower temperature protocol was used to prevent epimerisation and cyclization of the reagents. The optimisation reactions at 50 µmol scale were performed on a Peptide-Explorer™, a lab scale continuous flow platform with a scale range of 50 µmol to 1 mmol. The Peptide-Explorer is a fully automated system that delivers amino acid solution, piperidine solution, DIC solution and HFIP solution for side chain deprotection. In this platform a complete deprotection-coupling cycle time is 7 min.

For the pilot scale synthesis, the same reaction conditions were transposed to the PS-30™ Pilot scale synthesiser, which has a scale range of 2- 30 mmol, with a cycle time of 25 minutes. It is important to note that scale up was achieved by using reactors with same mixing and heating characteristics while maintaining the key reaction parameters constant: stoichiometric ratio, residence time and temperature.

Figure 1 shows the basic flow schematic which both platforms are based on. The main difference between lab and pilot scale are the size of reactors and the pump flowrate capabilities.

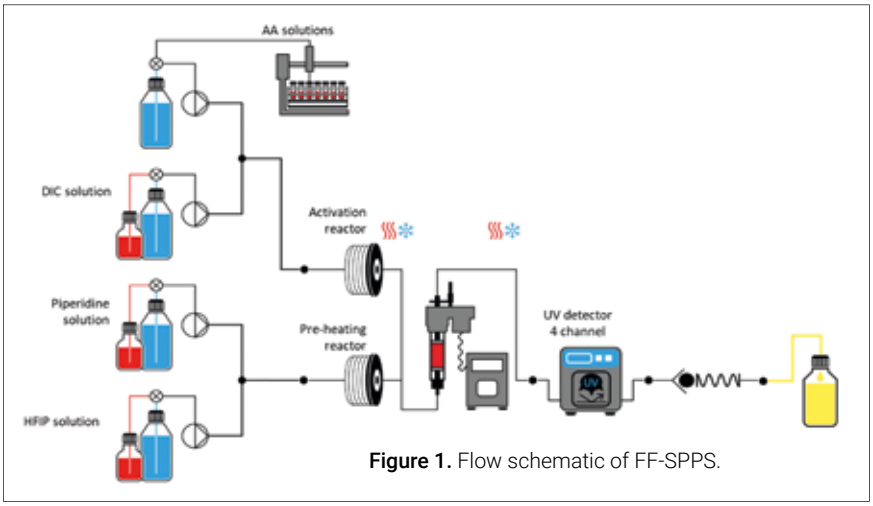

Figure 1. Flow schematic of FF-SPPS.

RESULTS AND DISCUSSION

Synthesis of long peptides

As example of the synthesis of a long peptide, a fibril-forming α-helical coiled-coil 77-mer peptide was chosen, based on FF03, an α-helical coiled-coil peptide with multiple applications (6, 10).

H_2N- KELKKEL EKLKKEL KELKKEL EKLKKEL KELKKEL EKLKKEL KELKKEL EKLKKEL KELKKEL EKLKKEL -$CONH_2$

A 100 µmol synthesis was completed within one day, the VBFR volume change showed no signs of aggregation, with an overall volume growth of 3.5 ml, approximately 7 times the initial volume of the reactor.

The overall purity was ~62 %, which translates to a cycle efficiency greater than 99.4 % after 77 cycles.

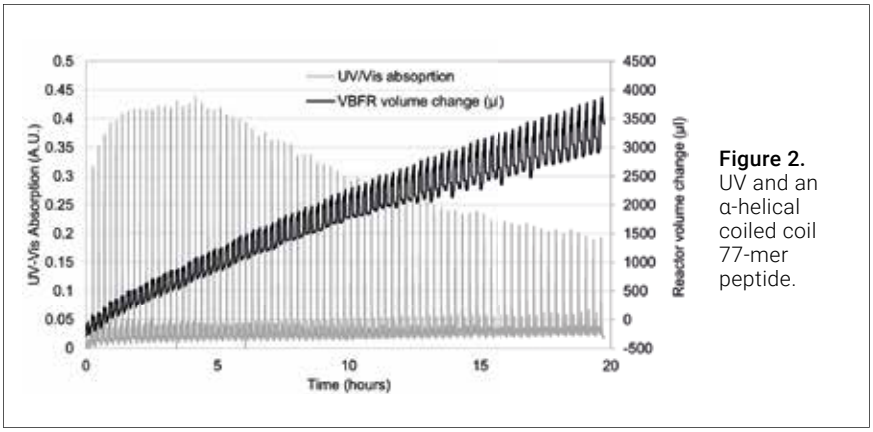

Figure 2. UV and an α-helical coiled coil 77-mer peptide.

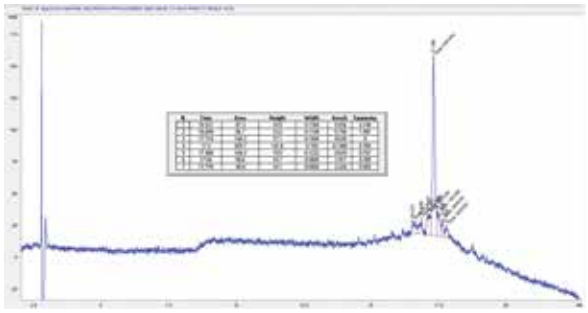

Figure 3. HPLC chromatogram of crude 77-mer peptide.

Scale up synthesis of GLP-1 analogues

Current batch technologies require weeks and many syntheses to scale up a peptide synthesis previously only optimised at lab scale.

By contrast large scale flow reactors have been developed that matched the performance of lab scale FF-SPPS systems. The goal has been to optimise a synthesis at 50 µmol and bring those reaction conditions and synthesise 30 mmol of peptide without further refinement.

As an example synthesis for this study, a GLP-1 analogue was used. The optimisation of this peptide at lab scale was previously reported, yielding a crude purity of ~80 % (11).

The optimised reaction conditions were directly scaled up 300 times to a 15 mmol synthesis, which was completed within 15 hours, yielding nearly identical crude purity to the lab scale system.

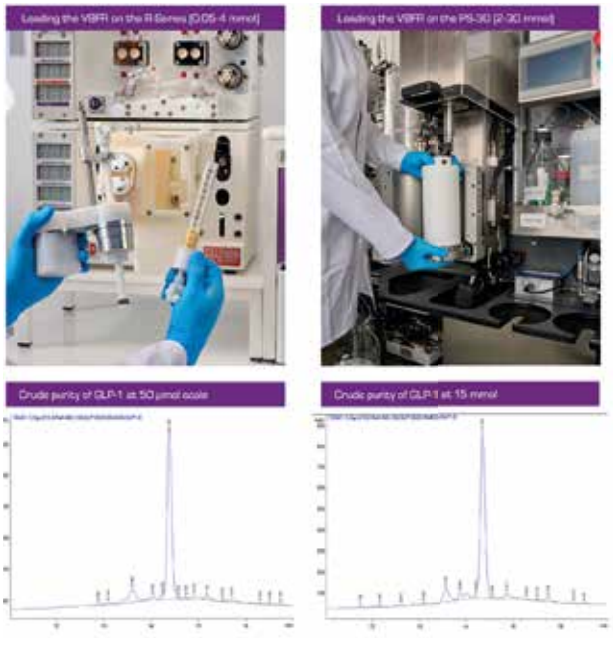

Figure 4. Crude purity of the GLP-1 analogues synthesised at 50 µmol (left) and 15 mmol (right).

When analysing the real-time data, the level of aggregation was also scalable, indicating it is a purely chemical phenomenon. When the VBFR volume change is normalised to the reaction scale, we can compare relative volume growth, showing the same level of aggregation, which starts after the 11th coupling, partially recovering but growth was compromised.

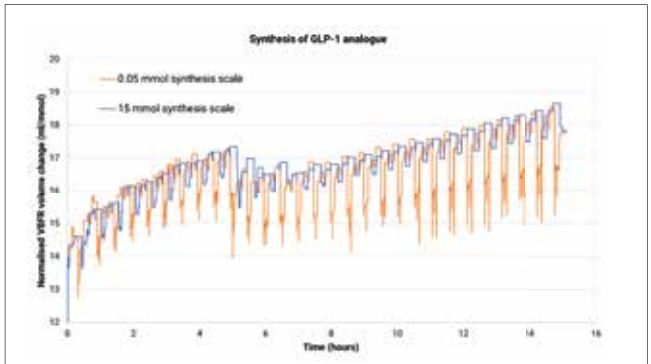

Figure 5. Normalised VBFR volume change at 50 µmol (orange) and 15 mmol synthesis scale (blue).

When the Fmoc peaks are integrated, the Fmoc area under the curve (Fmoc AUC) can be indicative of the cycle efficiency post aggregation.

When the Fmoc AUC are normalised to the synthesis scale, the performance of both syntheses can be compared, showing identical evolution of the Fmoc ACUs

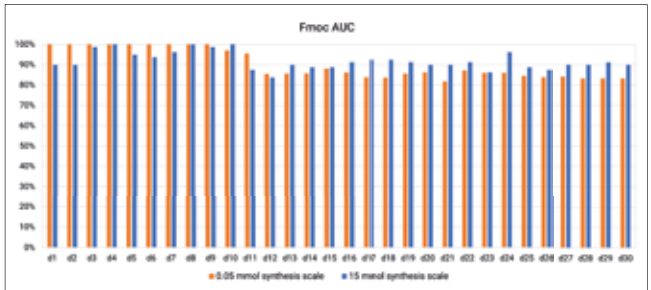

Figure 6. Fmoc AUC for 50 µmol (Orange) and 15 mmol synthesis scale (blue).

CONCLUSIONS

Fast flow peptide synthesis has proven multiple advantages in reaction time, purity and real-time analytics over any conventional batch technology at both lab and pilot scale.

With standard protocols, the advantages of FF-SPPS have been demonstrated in the synthesis of long peptides as well as difficult sequences. Platforms have been developed that can synthesise peptides from 50 µmol up to 30 mmol with the same protocol. As all the platforms use the same protocol, it eliminates the need to further refine reaction conditions as the process scales up.

Finally, the level of real-time data can not only be used to evaluate when aggregation occurs, but to quantify it in large scale synthesis making it a powerful tool for the scientist to evaluate different strategies in SPPS.

REFERENCES AND NOTES

1. L. Otvos and J. D. Wade, "Big peptide drugs in a small molecule world ," Frontiers in Chemistry , vol. 11. 2023.
2. F. El Oualid et al., "Chemical Synthesis of Ubiquitin, Ubiquitin-Based Probes, and Diubiquitin," Angew. Chemie Int. Ed., vol. 49, no. 52, pp. 10149–10153, Dec. 2010, doi: https://doi.org/10.1002/anie.201005995.
3. B. L. Hartrampf, Nina; Saebi, Azin; Poskus, Mackenzie; Gates, Zachary P; Callahan, Alexander J; Cowfer, Amanda E; Hanna, Stephanie; Antilla, Sarah; Schissel, Carly K; Quartararo, Anthony J; Ye, Xiyun; Mijalis, Alexander J; Simon, Mark D; Loas, Andrei; Liu, Shun, "Synthesis of Proteins by Automated Flow Chemistry." 11-Feb-2020, doi: 10.26434/chemrxiv.11833503.v1.
4. B. L. Simon, Mark D; Heider, Patrick L; Adamo, Andrea; Vinogradov, Alexander A; Mong, Surin K; Li, Xiyuan; Berger, Tatiana; Policarpo, Rocco L; Zhang, Chi; Zou, Yekui; Liao, Xiaoli; Spokoyny, Alexander M; Jensen, Klavs F; Pentelute, "Rapid Flow-Based Peptide Synthesis," ChemBioChem, vol. 15, no. 5, pp. 713–720, Mar. 2014, doi: 10.1002/cbic.201300796.
5. Z. P. Gates and N. Hartrampf, "Flow-based SPPS for protein synthesis: A perspective," Pept. Sci., vol. 112, no. 6, p. e24198, Nov. 2020, doi: https://doi.org/10.1002/pep2.24198.
6. E. T. Sletten, M. Nuño, D. Guthrie, and P. H. Seeberger, "Real-time monitoring of solid-phase peptide synthesis using a variable bed flow reactor," Chem. Commun., 2019, doi: 10.1039/C9CC08421E.
7. N. Hartrampf et al., "Synthesis of proteins by automated flow chemistry," Science (80-.)., vol. 368, no. 6494, pp. 980 LP – 987, May 2020, doi: 10.1126/science.abb2491.
8. A. J. Mijalis et al., "A fully automated flow-based approach for accelerated peptide synthesis," Nat. Chem. Biol., vol. 13, p. 464, 2017.
9. T. Michels, R. Dölling, U. Haberkorn, and W. Mier, "Acid-Mediated Prevention of Aspartimide Formation in Solid Phase Peptide Synthesis," Org. Lett., vol. 14, no. 20, pp. 5218–5221, Oct. 2012, doi: 10.1021/ol3007925.
10. C. K. Thota, D. J. Mikolajczak, C. Roth, and B. Koksch, "Enhancing Antimicrobial Peptide Potency through Multivalent Presentation on Coiled-Coil Nanofibrils," ACS Med. Chem. Lett., vol. 12, no. 1, pp. 67–73, Jan. 2021, doi: 10.1021/acsmedchemlett.0c00425.
11. Vapourtec Ltd, "Application Note 69 - Automated CF-SPPS and evaluation of GLP-1 peptide," 2021.

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*Dr. **Manuel Nuño** completed his MChem at Universidad de Zaragoza, Spain and York University, UK moving to University of Bath, UK to undertake his PhD in photocatalysis under the supervision of Dr Richard J. Ball.*

Following completion of his PhD, Manuel advanced his process improvement skills during three years as process development chemist in food manufacturing industry working to improve the performance of large scale batch and flow chemical processes involving extraction, purification and crystallization of sugars. Early in 2019 Manuel moved to Vapourtec ltd, offering applications and chemistry support to customer and product development teams. Projects he has worked with include, photochemistry in flow, scale up of flow peptide synthesis, organometallic chemistry in flow and continuous electrochemical oxidation of natural products.