Continuous flow brings SPPS to a new level

Vapourtec's chief scientific officer **Manuel Nuño** talks about the journey of peptide synthesis in flow

Over the last decade, the focus of the pharmaceutical industry has been steadily shifting from small molecules to treatments based on peptides. In 2014, sales of peptide-based therapeutics exceeded $1 trillion worldwide.[1] Between 2015 and 2019, 15 out of the 208 drugs approved by the FDA were peptides.[2]

For peptide-based drug development, it is crucial to be able both to synthesise peptide libraries and to optimise the synthesis rapidly before scaling to gram quantities for pre-clinical trials.

In early 2017, New Path Molecular Research challenged Vapourtec to adapt its platform for library synthesis to facilitate automated continuous solid-phase peptide synthesis (SPPS) flow. This kick-started Vapourtec's journey into addressing the key issues associated with peptide synthesis in flow.

SPPS is the most convenient chemical route to synthesise a peptide quickly. It is based on a repetitive sequence of deprotection and coupling steps quickly. As Figure 1 shows, a Fmoc protecting group is removed before a new Fmoc-protected amino acid is coupled to it.[3]

The first continuous flow peptide synthesisers (CF-SPPS) were developed in the 1980, but batch synthesisers remained the preferred choice and dominated the market into the early 2000s.[4] Over the last decade, interest in CF-SPPS has grown again after excellent work published by many research group, including the work in fast peptide synthesis by the Pentelute lab at MIT.[5-8]

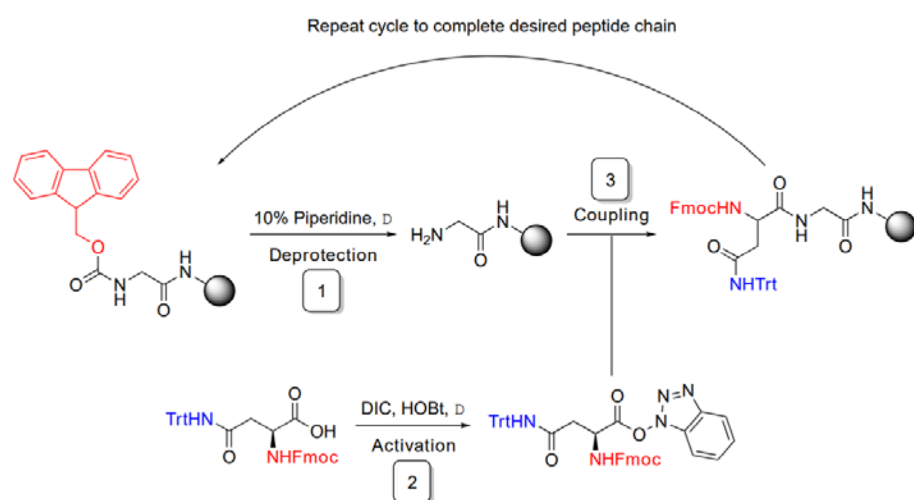


Figure 1 - Chemical reactions in SPPS

Currently, flow platforms offer an extraordinary level of automation and in-line data, which is inherently difficult to obtain with a batch system. In flow, solid reagents are typically handled by using a fixed bed reactor. This simple set-up works well with solid catalysts, for example, as there is no volume change.

SPPS on the R-Series

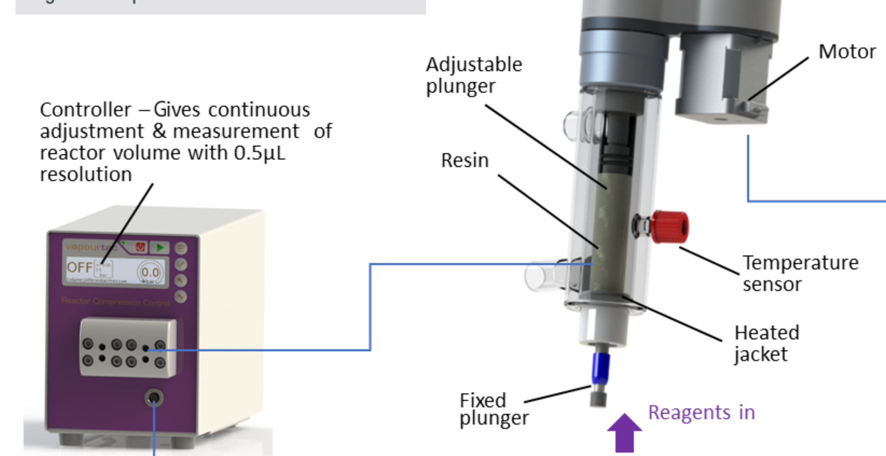
To tackle CF-SPPS we built on our knowledge of how to do automated library synthesis. Our R-Series can perform automated library synthesis of compounds. Reagents are automatically loaded in the flow network via sample loops. For CF-SPPS, we would just need to prepare in vials as many different Fmoc-protected amino acids as the sequence contains.

For CF-SPPS to succeed, one of the key unsolved issues was how to handle the solid support. As the peptide is grown attached to the resin, there are significant volume changes that a fixed bed reactor simply cannot accommodate.

For example, synthesising a 30-mer peptide will double the initial resin volume. If the volume is not adjusted, the resin will compress so much that it loses structure and causes blockages. If this volume increment is not accounted for from the beginning, solvent voids will cause dilution and lack of packing density will allow reagents to channel through the reactor, reducing the overall efficiency of the process.

With a standard packed bed reactor, CF-SPPS was possible in our R-Series, but it required manual intervention from the users. As the peptide grew, to prevent issues, they needed to adjust the packed bed reactor every couple of cycles.

Figure 2 - Vapourtec VBFR & reactor controller



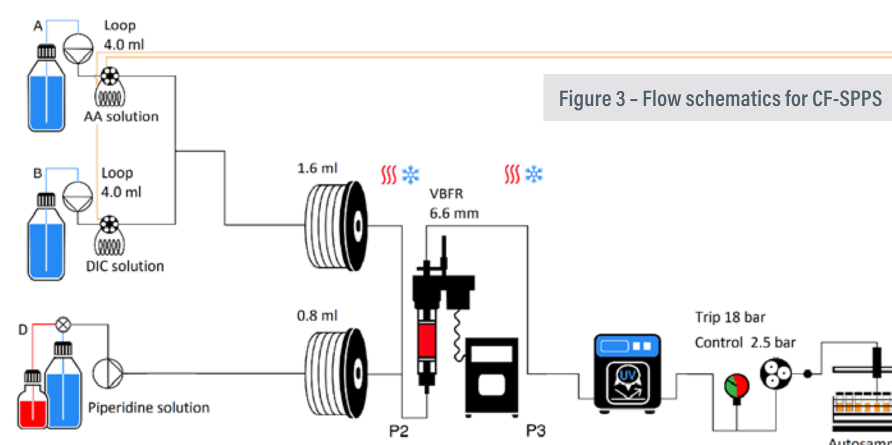


Figure 3 - Flow schematics for CF-SPPS

New concept in flow

To be able to handle internal volume changes automatically, we developed the Variable Bed Flow Reactor (VBFR), a new concept reactor which monitors and controls the packing density of the solid media. This ensures the resin is constantly packed, eliminating channelling of reagents and providing useful in-line data about the peptide growth.

With the handling of the resin solved and using our existing approach for library synthesis, we looked at the chemical mechanism of activating an amino acid and reacting it with the solid media. In the majority of batch reactors, activation and coupling happen at the same time, usually at moderate temperatures. This is the reason why batch synthesis usually relies on very active species for activators, such as HATU.

In flow, we separated both reactions by simply adding a reactor before the VBFR, as shown in Figure 3. This configuration ensures that all the amino acid is activated prior to entering the resin, leading to a more efficient use of reagents; only active amino ester would pass through the resin beads.

Thanks to this configuration, we evaluated and optimised both activation and coupling residence times. Our protocols are based on an isothermal activation time of 40-60 seconds, which gives a cycle time of ten minutes per coupled amino acid.

Flow chemistry systems can easily be integrated with inline analytical techniques (i.e. optical spectroscopy, Raman or even IR), which provides real-time information on how the reaction is progressing. By combining UV/Vis spectroscopy with the data generated by the VBFR, we accessed information never seen before.

By looking at these two datasets combined we can, for example, 'see' what is the performance of each deprotection step, in both UV and VBFR volume change. This real-time feedback can be used to monitor and take action to ensure couplings and deprotections are 100 % complete even when aggregation occurs or unnatural amino acids are used.

By the end of 2018, we successfully synthesised a variety of peptides between 10 and 40-mer at any scale between 100 mg to a couple of grams. To learn more from the experts, we collaborated with the Max Planck Institute at Germany. During our collaboration we explored more in detail the effect of different resins in different syntheses and even in the synthesis of carbohydrates via solid-phase synthesis.[9-10]

We also learned that aggregation is a sequence-dependent issue and that even with the best of the systems and reaction conditions, sometimes it just cannot be avoided. In these cases, we can use the real-time data to evaluate chemical alternatives to prevent such events.

This was the case with JR-10, a 10-mer sequence that completely aggregates during synthesis, yielding a low-quality crude peptide.[9] Thanks to the VBFR, we identified where, in the sequence, aggregation occurred, and evaluated the effect of using different conditions without the need to wait for an LC-MS.

Within a few days, several syntheses were completed using different solvents, low loading resins and even pseudo-prolines. With the successful method in place, JR-10 was obtained in high crude purity.

Next steps

Our next step in the journey was to minimise reagent consumption. Unnatural amino acids can be hundreds of times the price of natural amino acids. We optimised solvent and reagent usage by fine-tuning the coupling step. Now we can achieve efficient synthesis with as low as two equivalents of amino acid.

Glucagon-like peptide 1 is a 30-mer peptide used for the treatment of Type ➢

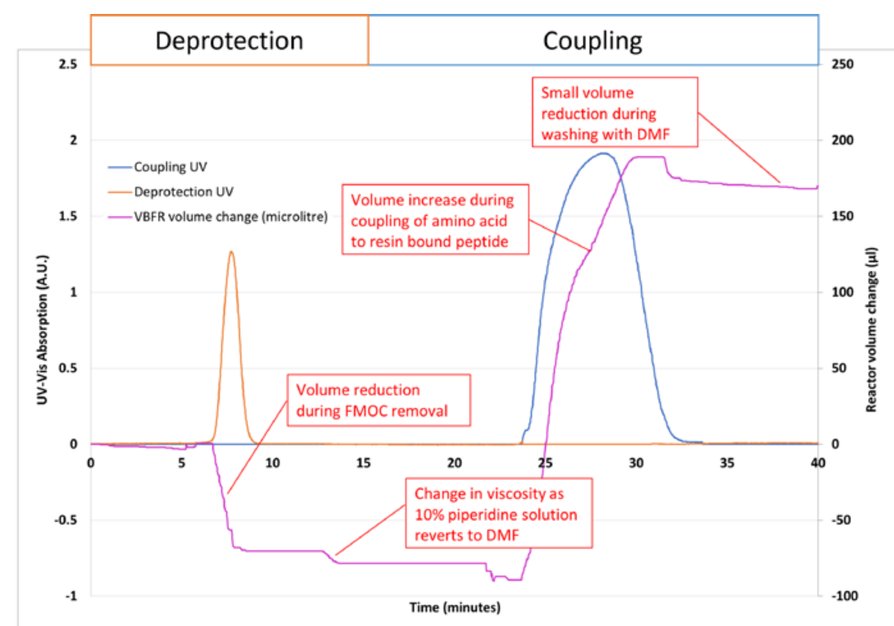


Figure 4 – Real-time data from deprotection & coupling cycle

II diabetes. Synthesising this peptide is a good benchmark to compare synthesisers' performances. GLP-1 possesses the main challenges in the playbook of SPPS: aggregation and racemisation.

We took the challenge and worked on this peptide using several resins, synthesising it with over 80% crude purity in under five hours.[11] We found that working with a different resin core could delay aggregation, but not solve it.

Side-chain modification is a strategy used in SPPS to modify physicochemical properties of peptides without reducing its biological activity. In the case of GLP-1, if we add a fatty acid chain in the middle of the peptide chain, it can improve its pharmacokinetic properties.[12]

This peptide is commercially sold under the name of semaglutide. Chemically, it involves a solvent switch and deprotection, before returning to the previous system solvent. Although sounding complex, in continuous flow it simply means the addition of a fourth pump. The whole peptide sequence was easily programmed to run in a fully automated way, and we obtained semaglutide with moderate crude purity.

Larger sequences

Synthesising larger sequences was next on the list. Proteins are chains of more than 50 amino acids. To test the robustness of our flow platform and protocols we chose a repetitive sequence, KELKKEL EKLKKEL. A 77-mer based on this sequence would pinpoint any inherited issue.

Synthesis was completed within 19 hours, yielding the desired peptide with a crude purity of 62%. We found these results both excellent and fascinating. To obtain 62% crude purity on a 77-mer, we achieved an average efficiency of 99.4%/cycl.

The last step of peptide synthesis is to isolate the peptide. To achieve this, the peptide needs to be cleaved off the resin, as well as removing its side protective groups.

Trifluoroacetic acid (TFA) is the main component of many cleavage cocktails. Peptide cleavage is done in batch, being a tedious and hazardous procedure. It takes between two and four hours and the chemist needs to handle mixtures of TFA at every step of the process.

We wanted to improve the safety aspect of it, so we translated this reaction into flow. Now, our chemists only need to load TFA on the system once, drastically minimising exposure to this acid.

In continuous flow, we can now perform cleavages at higher temperatures, which dramatically reduced the reaction time, as well as providing the same real-time high-quality data. In under 40 minutes, we can collect and precipitate the crude peptide in an automated way.[13]

Summary

The journey of SPPS has been a continuous evolution since the 1960s. For Vapourtec, the last four years of this journey has been a steep learning curve. We learned there are no two identical peptide syntheses, and it also reminded us we had to rely on good quality, real-time data to solve problems.

For a small team like ours, being able to pinpoint a specific reaction on a synthesis helped us to quickly identify errors and sequence-related issues. We are still developing CF-SPPS further. Thanks to the scalability of continuous flow, during 2022, we will be looking at synthesising larger amounts of peptides in our R-Series, as well as using the technology for other solid-phase synthesis. ●

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