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Solid-phase peptide synthesis:
recent advances through adoption of continuous flow

KEYWORDS: CF-SPPS, flow chemistry, VBFR, JR10, inline analysis, oligos-peptides.

ABSTRACT

Two of the major challenges on solid-phase peptide synthesis are the difficulties in monitoring reactions in real time and the need to accommodate volume changes of the resin beads as the peptide elongates. The lack of accurate inline data to monitor reactions and the physical limitation of reactor vessels often slow down the discovery of new peptides. Vapourtec has developed a new "intelligent" packed bed reactor that accommodates volume changes whilst providing valuable insight into the reaction kinetics. By monitoring and controlling the packed density of the resin beads, channelling of reagents is minimised; reducing the need for excessive reagent and wash volumes. This accurate control of the reactor volume also allows evaluation in real-time when aggregation events occur.

INTRODUCTION

Peptides cover a niche section in medicinal therapy. By definition, a peptide consists of between 2 and 50 amino acids, it is smaller than a protein. As a drug, it falls between small molecules and proteins. The application of peptides in the pharmaceutical industry has been growing in recent years. Peptides possesses high selectivity and activity against biological targets and low toxicity (1, 2), making them an attractive type of drug.

By 2018, more than 60 peptide drugs had been approved in the US, Europe and Japan; 28 of which are non-insulin peptide drugs released post 2000. In addition to approved peptides, 260 have been tested in clinical trials and over 150 are currently in active clinical development (1).

Since 1963, when R. B. Merrifield published the first synthesis of a peptide supported in solid phase (3) the use of this technology spread fast thanks to the "simplicity" and speed of this technique (4), compared to homogeneous phase peptide synthesis. Figure 1 shows a cycle overview of Solid-phase peptide synthesis (SPPS).

Due to the repetitive sequence of reactions, automating peptide synthesis was a "simple" task that companies managed to do in batch. Batch peptide synthesisers then flourished on the side of this technology, as they were considered the best approach for building difficult peptide sequences.

Although SPPS moved forward since the early 60's, some problems that Merrifield published in his first work still remain. Racemisation, side reactions, peptide aggregation and solubility issues can all contribute to making it challenging to evaluate synthesis problems in 'difficult' peptides. Difficulty in monitoring in real time adds an extra layer of complication when elucidating which synthetic step went wrong.

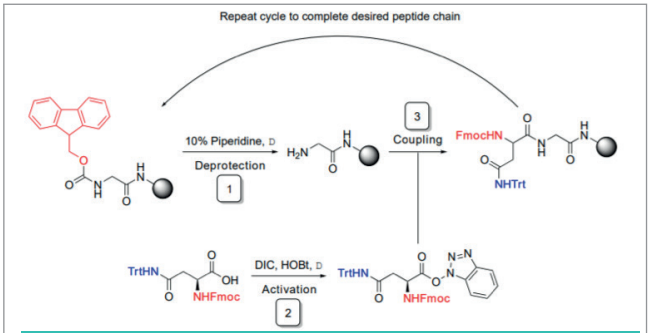

Figure 1. Brief overview of the chemical reactions in SPPS (5).

Continuous flow was evaluated in the 70s and 80s for SPPS (6, 9), but the superiority at that time of batch systems, overshadowed continuous flow applications.

The advantages of SPPS in continuous flow, rather than in batch, are of relevant importance for an efficient synthesis (10). For a continuous flow system, resin beads are usually packed in a column reactor. This arrangement of the resin spheres, more compacted than in a batch reactor, requires less solvent to wash any excess reagent off the reactor (10, 11). It is also worth noting the same implications on reagent needed per reaction; as the plug of active amino acid flows through the packed resin, each active site on the resin will be exposed to a more concentrated solution than in a batch type reactor containing the same quantity of resin. In addition to this, the possibility to connect an inline detector (i.e. FTIR or UV-Vis), gives a more accurate insight than the analysis of aliquots in a batch process.

With the development of continuous flow systems in the last decade, several researchers successfully translated SPPS into flow (10, 12–16).

For instance, Christopher Gordon's team showed how a continuous flow SPPS could be built simply from a repurposed HPLC, achieving high purities (13).

Huimin Ma's team developed a novel microchip reactor concept, that could accommodate resin beads for continuous flow SPPS (16). Six different peptides (between 4 and 6 mer) were synthesised simultaneously, yielding purities between 68 – 80 % (16).

At MIT, Bradley L. Pentelute took a different approach, with his main interest focussing on achieving fast couplings (14, 15, 17, 18), reducing the synthesis cycle down to 40 seconds per coupled amino acid and using SPPS to synthesise peptides of greater the 100 mers. This has not been possible with batch reactors.

The main drawback of utilising standard packed columns with resin beads is the limitation of accommodate volume

changes. For instance, when synthesising a 30 mer peptide the reactor needs to more than double in volume (19), so the extra mass is accommodated within the reactor.

Resins are made of a polymer matrix, that swell when in contact with solvent, key for reagents to flow through their micropores. Excessive compression of the resin beads will reduce yield on coupling reactions (11).

When a peptide is being built, each coupling reaction will add mass and volume to the reactor, up to the point where it further compresses the resin matrix, creating high backpressure. Currently there are two approaches to this problem; a) starting with a headspace in the reactor, so the resin can grow or b) start with a packed reactor and work with increasingly high backpressures.

Neither approach is an actual solution to the problem. These options do not provide a constant packing density of resin beads throughout the experiments. This has the detrimental effect of channelling the reagents between these beads.

NEW REACTOR CONCEPT – A PACKED BED REACTOR WITH A BED HAVING VARIABLE INTERNAL VOLUME?

In order to advance continuous flow SPPS, this technical limitation must be resolved. The only way to control how the resin beads are packed is to create a reactor that could change its internal volume.

Since early 2017, Vapourtec has worked towards a solution for this problem. A new reactor concept, which could measure and control the packing density of the resin in the reactor was needed. The reactor should have a movable plunger that can adjust the internal volume with high precision. In addition to this a simple and reliable method to determine packing density was developed.

Figure 2 shows a schematic of the Vapourtec Variable Bed Flow Reactor (VBFR) with its reactor controller unit allowing for the adjustment of the reactor's volume to deliver a constant packing density, with a resolution of ±0.5 µl.

MATERIALS AND METHODS

All reagents were obtained from commercial suppliers:

Fluorochem: (Fmoc-amino acids, 1- hydroxybenzotriazole hydrate (HOBt·H2O), diisopropyl carbodiimide (DIC), triisopropylsilane (TIPS) and Fmoc-glycine Rink amide AM resin (0.3–0.8 mmol/g)). *Sigma-Aldrich*: piperidine, trifluoroacetic acid (TFA), DMF, diethyl ether and acetonitrile). N a-9-fluorenylmethoxycarbonyl (Fmoc) amino acids.

INSTRUMENT AND REACTOR SETUP

The continuous flow SPPS was configured using an existing Vapourtec RS-400 configuration. This compromises two R2C+ pump modules, one R4 reactor module and one autosampler unit to feed different amino acids from sample loops. An inline UV detector was connected to the reactor's output. In order to maintain a constant post-reactor pressure, a SF-10 pump was set as dynamic BPR. Figure 3 shows the system diagram that was set for the experimental work.

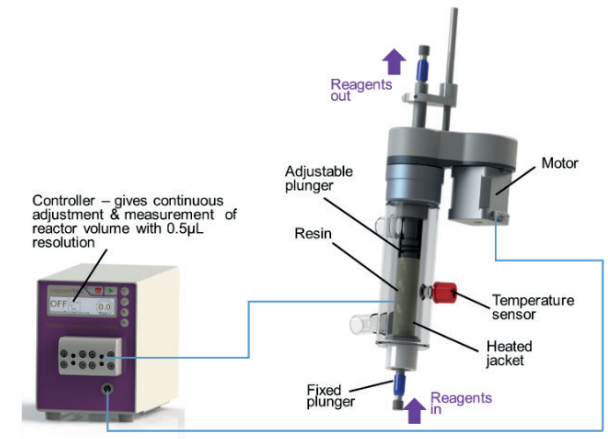

Figure 2. Schematic diagram of the Vapourtec VBFR and the reactor controller.

The VBFR has a number of different size reactors; for this work, a small reactor was chosen, as it allows for accommodation of 100-400 mg of resin beads.

In order to minimise cross contamination between deprotection and coupling reactions, an approach utilising two separate channel streams was adopted. Deprotection reagents would have a dedicated pump and heating reactor. Activator and amino acid would be loaded through sample loops, react to form an active ester through a tube reactor, and then passed through the VBFR for peptide chain elongation. In order to minimise space, a dual-core reactor was used, so both deprotecting and coupling streams were held on the same reactor.

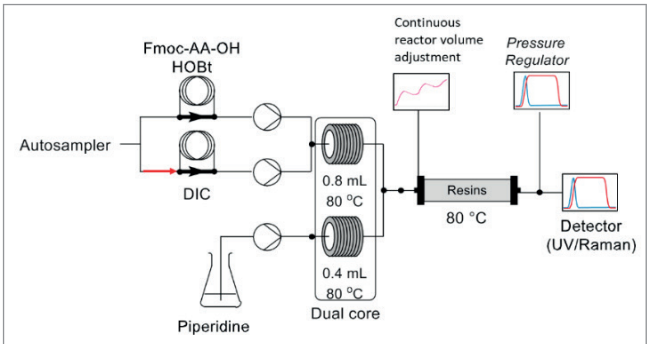

Figure 3. Diagram of reactor set up. Pumps A and B were set for the coupling reaction and pump D was set for the deprotection reaction.

In this arrangement, the UV detector monitors the reaction solution as it exits the VBFR. This allows monitoring each step of the peptide synthesis. For example, when the resin is deprotected, the cleaved Fmoc group could be followed.

As the novel VBFR detects volume changes on the resin and accommodates its volume to deliver a constant packing density; the change in the reactor's volume is also recorded. As all the data generated by the system is recorded by Vapourtec's Flow Commander™ software, when using this change in volume in conjunction with the UV absorbance of the reactor's output, it provides a better insight into the reaction kinetics.

RESULTS

Synthesis of a peptide starts with loading the VBFR with preloaded resin. In the example described below, rink amide

AM resin preloaded with the first Fmoc-amino acid was used for the experimental work.

In order to speed the reaction kinetics, 80 °C was chosen as the reaction temperature, therefore the reactors were set at that temperature. Before the reaction starts, the loaded resin needs to be swollen of solvent. For this reason, we allowed 15 minutes for the resin to swell at reaction temperature while DMF was flowing through. This pre-swelling eliminates any effect of resin-solvent interaction from the observed change in volume.

For coupling, a 0.24 M solution of both amino acid and HOBt was reacted with a solution of 0.24 M of DIC, resulting in a final solution of 0.12 M of amino acid active ester.

For deprotection, a 10 % (v/v) solution of piperidine in DMF was used to cleave Fmoc group from the amino terminal.

Figure 4 shows how a peptide elongation cycle looks from Flow Commander's data. Two reactions are shown in this graph, *deprotection* (in orange), followed by the *coupling step* (blue).

Two sets of data are plotted; dotted line represents UV absorption (with its Y axis on the left hand side of the graph) and solid line represents to the VBFR volume change expressed in µl (with its Y axis on the right hand side of the graph).

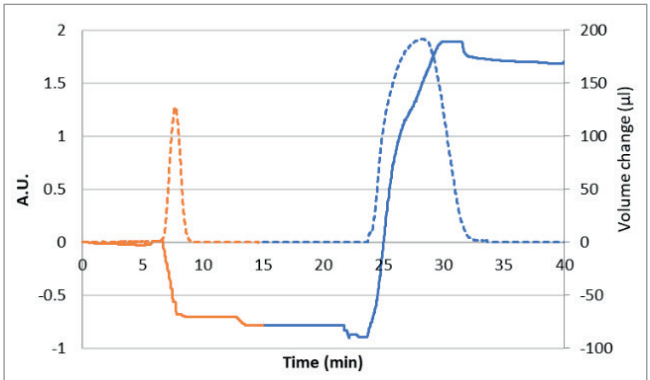

Figure 4. Deprotection and coupling cycle (dotted line: UV absorption; solid line: VBFR change in volume).

At minute 6, as piperidine starts cleaving the Fmoc group, the UV signal peaked whilst the reactor's volume started to decrease. Once all the Fmoc had been cleaved (circa minute 10), the VBFR had reduced in volume by 70 µl and the UV signal went back to baseline level. When the piperidine solution is then replaced at minute 13 by DMF (for the washing cycle), a small reduction in volume was recorded. This was due to the change in viscosity as 10 % piperidine solution was reverted to DMF.

When the amino acid active ester started to pass through the VBFR, there is a volume increment due to the amino acid being coupled to the resin-bound peptide. Typically, a 3-fold excess of amino acid is required to ensure high crude purity; once maximum growth has been reached, there is a plateau transition as excess 0.12 M solution of active ester is still passing through the VBFR. Once the reagent solution is replaced by DMF, the change in the solution's viscosity again reduced the reactor's volume slightly.

For an automated synthesis of a full peptide sequence, this cycle needs to be repeated, by simply selecting the next required amino acid for loading from Vapourtec's autosampler.

Several peptides have currently been reported using the Vapourtec VBFR as peptide synthesiser (5, 19).

E. Sletten et al. reported last year the advantages of working with the VBFR. Different peptides were synthesised; from a 7 mer sequence (AFLAFLA) to a 26 mer peptide (FF03) yielding high purities (19).

The VBFR was tested with a challenging peptide, JR10, known for its aggregation issues (15, 20-22). Thanks to the inline data, aggregation was detected occurring in the coupling on the leucine residue.

To allow full synthesis of the peptide, several approaches were evaluated to avoid aggregation. Increase solubility by changing solvent polarity, reduced resin loading and modification of the peptide orientation using pseudoprolines (21, 22).

A low loading combined with the use of pseudoprolines prevented aggregation and the peptide was fully synthesised yielding 71% purity (the main impurity was JR10 with a t-butyl protective group that failed to cleave).

Figure 5 shows the reactor's change in volume using standard conditions (DMF, high loading) and low loading combined with pseudoproline coupling (19).

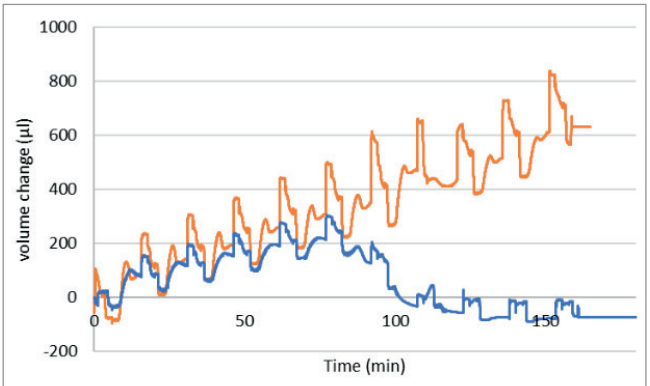

Figure 5. VBFR change in volume during optimisation of JR10 (Blue line: standard conditions; Orange line: low loading and use of pseudoprolines) (19).

CONCLUSIONS

SPPS has evolved since 1963, to include new types of resins, novel reagents to avoid racemisation and conditions to allow faster couplings/deprotections. Thanks to these advances, synthesising longer peptide sequences is now achievable. To further improve these syntheses.
Vapourtec has developed a new reactor that can adjust its internal volume to deliver a constant packing density of the resin. This "self-adjusting" packed bed reactor means the physical changes of the resin during reactions, in the form of swelling and shrinking, can now be accounted for. In practical terms, this better control of the reactor's volume provides a more uniform distribution of the resin beads within the reactor, reducing channelling and achieving better flow characteristics.

The acquired data can also be used to identify difficult reaction sequences and evaluate when aggregation occurs making it a powerful tool for the scientist to evaluate different strategies in SPPS.

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ABOUT THE AUTHORS

*Dr. **Manuel Nuño** completed his MRes and MChem at Universidad de Zaragoza, and York University, moving to University of Bath to undertake his PhD in photocatalysis under the supervision of Dr Richard J. Ball. Before joining Vapourtec in 2019 as research scientist, Manuel worked as process development chemist in food manufacturing industry during three years working on large industrial batch and flow processes.*

***Victoire Laude** is finishing her last year of chemical engineering Master's degree at the National Chemistry School in Clermont-Ferrand (France). In 2018, Victoire started to work with flow chemistry systems under the supervision of Christopher Gordon, focused in the development of Solid-Phase peptide synthesis in continuous flow. Victoire joined Vapourtec as intern chemist late in 2019.*

NEWS

CEPS AS GREEN ALTERNATIVE FOR SPPS

In a recent publication in Green Chemistry (Green Chem., 2019, 21, 6451-6467), PolyPeptide Laboratories (Malmö, SE) and EnzyPep (Geleen, NL) compared classical solid-phase peptide synthesis (SPPS) with chemo-enzymatic peptide synthesis (CEPS) using the antidiabetic drug exenatide as an example. Although comparative cost was the primary focus of the study, the data demonstrate dramatically the ecological benefits of using a CEPS approach. After initial studies to evaluate suitable fragment structures and reaction conditions, the authors opted for the fully unprotected crude exenatide fragments, exenatide(1-21)-hydroxymethyl-benzoyl-lysine and exenatide(22-39) amide. These were coupled enzymatically in the presence of omniligase-1, a broad specificity ligase. The crude product was purified to obtain >50 g exenatide API, demonstrating that CEPS can be used for large scale manufacturing of therapeutic peptides in economically as well as environmentally sustainable manner. Compared with data from a published commercial process, the yield was increased approximately two-fold, the cost of goods reduced by 70%. At the same time, the complete E factor (cEF) was reduced by over 85% and the total carbon intensity (CI) by over 90%. To a major extent, the reduction in the environmental footprint can be attributed to the reduction in the use of organic solvents and the higher overall yield. Since these highly significant improvements in the cost of manufacturing and ecological data were derived from an unoptimized process with no attempt undertaken to improve the carbon footprint of the fragment starting materials *per se* or to recycle solvents, the study gives an unambiguous insight into the potential environmental benefits to be reaped from CEPS technology.

NEWS