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## Rapid Flow-Based Peptide Synthesis

Mark D. Simon<sup>a</sup>, Patrick L. Heider<sup>b</sup>, Andrea Adamo<sup>b</sup>, Alexander A. Vinogradov<sup>a</sup>, Surin K. Mong<sup>a</sup>, Xiyuan Li<sup>a</sup>, Tatiana Berger<sup>a</sup>, Rocco L. Policarpo<sup>a</sup>, Chi Zhang<sup>a</sup>, Yekui Zou<sup>a</sup>, Xiaoli Liao<sup>a</sup>, Alexander M. Spokoiny<sup>a</sup>, Prof Klavs F. Jensen<sup>b</sup>, and Prof Bradley L. Pentelute<sup>a</sup>

Bradley L. Pentelute: blp@mit.edu

<sup>a</sup>Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, United States

<sup>b</sup>Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, United States

### Abstract

A flow-based solid phase peptide synthesis methodology that enables the incorporation of an amino acid residue every 1.8 minutes under automatic control, or every three minutes under manual control, is described. This is accomplished by passing a stream of reagent through a heat exchanger, into a low volume, low backpressure reaction vessel, and through a UV detector. These features enable the continuous delivery of heated solvents and reagents to the solid support at high flow rate, maintaining a maximal concentration of reagents in the reaction vessel, quickly exchanging reagents, and eliminating the need to rapidly heat reagents after they have been added to the vessel. The UV detector enables continuous monitoring of the process. To demonstrate the broad applicability and reliability of this method, it was employed in the total synthesis of a small protein, as well as dozens of peptides. The quality of the material obtained with this method is comparable to traditional batch methods, and, in all cases, the desired material was readily purifiable via RP-HPLC. The application of this method to the synthesis of the 113 residue *B. amyloliquefaciens* RNase and the 130 residue pE59 DARPin is described in the accompanying manuscript.

### Keywords

Flow Chemistry; Native Chemical Ligation; Peptides; Rapid Synthesis; Solid-phase synthesis

### Introduction

The total chemical synthesis of peptides has been of great interest for over a century.<sup>[1]</sup> Chemical synthesis of peptides and proteins enables incorporation of non-proteogenic functionalities, without restriction on their location or number.<sup>[2,3]</sup> With the introduction of solid phase peptide synthesis by Merrifield in 1963, the total synthesis of short peptides became routine.<sup>[4]</sup> Subsequent advances extended the ability to assemble long

Correspondence to: Bradley L. Pentelute, blp@mit.edu.

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polypeptides,<sup>[5-7]</sup> and the advent of native chemical ligation enabled the preparation of polypeptides of theoretically unlimited length from smaller fragments.<sup>[8]</sup>

Despite these advances, the time required to assemble polypeptides, either as final targets or as fragments of a larger molecule, is a major limitation on studies employing such synthetic material. Most peptides are synthesized with Fmoc protocols, rather than faster and higher yielding Boc procedures, because highly toxic hydrogen fluoride is not required, and only small amounts of costly trifluoroacetic acid are used.<sup>[7]</sup> Standard Fmoc solid phase peptide synthesis methods require 60 to 100 minutes to incorporate each amino acid residue,<sup>[9,10]</sup> and some specialized procedures use complex microwave systems to reduce this to about 20 minutes per residue.<sup>[11,12]</sup> This manuscript describes the development of a flow platform that incorporates an amino acid residue every three minutes under manual control or 1.8 minutes under automatic control, without microwave irradiation.

## Results and Discussion

### Apparatus Design

To perform such rapid synthesis, we began with an analysis of existing kinetic data. It is known that at room temperature amide bond formation is 99.9% complete in less than 2 minutes with 0.5 M amino acid solutions and HATU activation,<sup>[13]</sup> and removal of N-terminal Fmoc protection is effected in 4–6 minutes with 20% (v/v) piperidine in DMF.<sup>[10,14]</sup> Our own model studies corroborated these data, showing a reaction half-life of  $4.6 \pm 0.6$  seconds for the formation of an amide bond between 0.3 M activated Fmoc-Leucine-COOH and resin bound phenylalanine at room temperature (See SI). Standard procedures allow these steps to proceed for much longer in an effort to improve the quality of difficult sequences, although this strategy is often of marginal utility.<sup>[15]</sup>

Assuming the reaction rate for these processes doubles for every 10 °C increase in temperature, at 60 °C amide bond formation should be complete in less than 10 seconds and Fmoc removal in less than 20 seconds. Based on this, we believed that robust Fmoc based peptide synthesis could be carried out in substantially less than five minutes per residue at 60 °C, using conventional heaters.

To study peptide synthesis on these time scales, we sought to build a device capable of delivering preheated reagents and solvents to a synthesis vessel, continuously monitoring the process, and rapidly switching between reagents. The second criteria pushed us to revisit continuous flow peptide synthesis, as several previous systems were able to effectively track the progress of peptide synthesis by monitoring the UV absorbance of the reaction.<sup>[16,17]</sup> Furthermore, with a flow-based system, solvents and reagents can be rapidly preheated by pumping them through a high efficiency heat exchanger with low residence time.<sup>[18]</sup> This arrangement eliminates the time required to heat reagents after they are delivered to the synthesis vessel and prevents degradation from prolonged storage of reagents at elevated temperature.<sup>[19]</sup>

Previous flow-based peptide synthesis systems had two major drawbacks, however. First, washes were very slow. All of these systems used a long, packed column containing the

solid support, similar to an HPLC column. As with equilibrating an HPLC column, several column volumes of solvent were required to effectively wash them. At low flow rate, this required tens of minutes to hours. As flow rate increased, the solid support collapsed and backpressure rose rapidly. Eventually, the support extruded through the frits used to confine it.<sup>[16]</sup> Although several solutions to this problem were proposed, none were ultimately successful, and commercial variants of these synthesizers are not currently available.<sup>[16,17,20,21]</sup> Second, these systems recirculated low concentration reagents rather than continuously replenishing high concentration reagents. This conserved activated amino acids, but resulted in slow amide bond formation. To overcome these problems, we designed a low volume, low pressure reaction vessel. This vessel reduced the volume of wash solvent required and enabled delivery of solvent and reagent at high flow rates, allowing reagents to be maintained at maximal concentration and removed rapidly.

To deliver the reagents required for peptide synthesis without a complex fluidic manifold, the apparatus shown in Figure 1a was developed. An HPLC pump was used to deliver either DMF or 50% (v/v) piperidine in DMF for the common washing and deblocking steps, and a syringe pump was used to deliver coupling reagents. The HPLC pump solvent was selected with a manually actuated 3-way valve, and the HPLC pump outlet was attached to the reaction vessel via a luer-lock quick connect. For the coupling step, the quick connect was manually moved to a syringe pump that delivered a solution of activated amino acid. The effluent was passed through a UV detector to continuously monitor the absorbance at 304 nm, a region where Fmoc amino acids and the dibenzofulvene-piperidine deprotection adduct absorb strongly.<sup>[17]</sup>

The first generation reaction vessel, shown in Figure 1b, was designed to be simple and easy to construct, while still giving low back pressure. A ¼" (6.4 mm) inner diameter × 3" (76 mm) long perfluoroalkoxy tube with Swagelok reducing unions as the inlet and outlet was used. A frit was positioned in the outlet using a short piece of tubing with a ¼" outer diameter. Installation of the outlet fitting and concurrent compression of the ferrule and tube sealed the frit in place. The total volume of the vessel was ≈2.5 mL. This vessel was assembled without machine or glass shop support, and allowed us to conduct model studies with up to 100 mg of resin.

To verify the feasibility of Fmoc SPPS with this system, we synthesized the model peptide Fmoc-ALFALFA-CONH<sub>2</sub> on a 0.1 mmol scale (100 mg of resin). Based on an initial estimate, we chose to start with a 2 minute DMF wash at 10 mL/min, a 2 minute Fmoc deprotection at 6 mL/min, another 2 minute DMF wash, and a 6 minute room temperature coupling with 2 mmol of activated amino acid delivered at 1 mL/min. This sequence yielded highly pure material, enabling peptide synthesis in 12 minutes per residue. To achieve a maximal concentration of activated amino acid and rate of amide bond formation, coupling solutions were prepared by dissolving amino acids in one equivalent of 0.4 M HBTU in DMF. The activating base was added immediately before use, giving a final concentration of activated amino acid of about 0.3 M. This concentration of coupling reagent was used for all experiments, including those with HATU activation (Figure 3a).

Based on our initial investigations and prior reports, we decided to carry out all subsequent studies at 60 °C to minimize the cycle time without significantly increasing formation of side products.<sup>[22,23]</sup> To consistently and quickly bring reagents to 60 °C, a heat exchanger was placed between the synthesis vessel and the luer-lock quick connect. A 5' (1.6 m) coil of open tubing with a 1/16" (1.59 mm) outer diameter and 0.030" (0.76 mm) inner diameter was used. This preheat loop was immersed with the reaction vessel in a water bath maintained at 60 °C and effectively increased the temperature of solvent from 18 °C (RT) to 59 °C, as measured by a thermocouple inserted into the outlet of the loop. PFA tubing was effective at flow rates up to 20 mL/min; stainless steel was used for all experiments at higher flow rates.

### Determination of Minimum Cycle Time and Model Studies

To determine minimal cycle times, each step of peptide synthesis was studied. First, the time required to wash amino acid solution out of the reaction vessel was investigated as a function of flow-rate via the UV absorbance of the effluent. At 1 mL/min, about 16 minutes (16 mL) were required to remove 99% of the amino acid precursor. As flow rate increased, however, the amount of solvent required decreased, with only 1 minute (10 mL) required at 10 mL/min. To guarantee an effective wash, 20 mL of DMF were used. These 20 mL were delivered over two minutes at 10 mL/min, as the first generation reaction vessel could not reliably accommodate higher flow rates. Analysis of the crude peptides did not show double incorporation of amino acids, which would result from an inadequate wash. Increasing the wash volume did not improve the crude peptide quality.

We then investigated the rate of Fmoc removal by monitoring formation of the UV active dibenzofulvene-piperidine adduct. We chose to use 50% (v/v) piperidine in DMF over the more common 20% (v/v) because our preliminary work indicated it removes Fmoc more rapidly. To decouple the time spent removing the Fmoc protecting group and the time required to wash the byproduct from the resin, deprotection reagent was delivered briefly, the resin was washed, and more deprotection reagent was delivered. A second UV absorbance peak indicated the formation of additional dibenzofulvene-piperidine, and an incomplete initial deprotection. The initial deprotection was performed at 10 mL/min for 60, 30, 15, or 6 seconds. A second peak was observed after a 6 second deprotection, but not a 15, 30, or 60 second deprotection. Fmoc removal is reported to be sequence dependent,<sup>[8]</sup> so, to ensure robust deblocking, the deprotection reagent was delivered for 20 seconds at 10 mL/min.

With wash and deblocking conditions, the time required for robust amide bond formation was determined by synthesizing two model peptides, LYRAG-CONH<sub>2</sub> and Fmoc-ALF-CONH<sub>2</sub>. Each peptide was synthesized five times, and, for each synthesis, 0.3 M amino acid solutions were coupled for a nominal time of 90, 45, 30, 15, or 7 seconds (Figure 2). For syntheses with 90, 45, and 30 second couplings, 2 mmol of each amino acid were used. Since the syringe pump could not infuse 6 mL (2 mmol, 0.3 M) of amino acid solution in less than 30 seconds, for the synthesis with 15 second couplings, 3 mL (1 mmol) was used, and 1.2 mL (0.4 mmol) was used for the synthesis with 7 second couplings. For Fmoc-ALF-CONH<sub>2</sub>, we found no significant difference in the quality of the crude product as a function

of coupling time. For LYRAG-CONH<sub>2</sub> we observed a significant increase in the Arg deletion peptide when all residues were coupled for 7 seconds. Based on these results, a 30 second coupling time was selected. The final synthetic timeline used with the first generation reaction vessel is shown in Figure 2c.

To further explore our approach under a variety of synthetic conditions, we studied the synthesis of ACP(65-74). This peptide serves as a model to validate new peptide synthesis protocols, as it is considered difficult to prepare.<sup>[13,24-26]</sup> The main synthetic impurity is a chromatographically resolved Val deletion. The LCMS data for the synthesis of ACP(65-74) with our methodology, as well as two controls, is shown in Figure 3. Using our protocol and the HATU coupling agent, a minor Val deletion product was observed. When using HBTU, more Val deletion was observed; this is consistent with prior reports.<sup>[13,24]</sup> ACP(65-74) synthesized with our protocol, but at room temperature, showed large Val and Gln deletions, confirming that reaction temperature is important. No major differences between the product composition from this flow-based room temperature synthesis and an analogous room temperature batch synthesis following the same synthetic timeline were observed.

Next, two additional “difficult” peptides were prepared: a conotoxin variant and a fragment of the HIV-1 protease.<sup>[13]</sup> The initial syntheses of these peptides yielded several products of equal molecular mass, which were determined to result from racemization of cysteine during activation. We therefore carried out model studies using the peptide GCF, and found several conditions that produced less than 1% diastereomer and maintained the same cycle time (See SI, Figure S5). This level of racemization is consistent with literature for Fmoc protocols,<sup>[27]</sup> and the identified conditions were also used to couple racemization prone histidine and tryptophan when used in the syntheses of Barnase and the DARPin (see accompanying manuscript). Using modified activation conditions for Cys, the peptides shown in Figure 4 were prepared on a 0.1 mmol scale using the first generation synthesis vessel and cycle.

To demonstrate the suitability of the reported flow-based synthesis method for ligation based protein synthesis, it was applied to the synthesis of a 58 residue tri-helical protein based on the Z domain of protein A (referred to as the affibody). The synthetic strategy uses peptide-hydrazides as thioester precursors. Peptide hydrazides can be oxidized with NaNO<sub>2</sub> to form a C-terminal acyl-azide, which will react with a thiol to form a peptide thioester suitable for use in native chemical ligation.<sup>[8,28]</sup> The LCMS data for the crude synthetic peptides are shown in Figure 5. We purified each peptide, synthesized the affibody according to the strategy in Figure 5a, and isolated highly pure, full-length affibody after purification (Figure 5e). The fragments were produced and cleaved from the resin in one day. In contrast, production of similar fragments with optimized Boc in situ neutralization protocols required more than three days, and yielded crude peptides of similar quality (Figure S7).

### Acceleration and Scale Up

After the completion of these model studies, we sought to increase the synthetic scale and decrease the cycle time of this system. All attempts to increase the flow rate or add more resin to the first generation reaction vessel were thwarted by rapidly increasing backpressure. A high pressure stainless steel reaction vessel was constructed to study the

effect of simply providing more pressure to maintain a high flow rate. This resulted in extrusion of the resin through the frit, as has been previously observed.<sup>[16]</sup>

Therefore, the second generation synthesis vessel shown in Figure 6 was constructed. The diameter is twice that of the first generation and volume limiting inserts restrict the volume to 2 mL, about the same as the first generation. Increasing the diameter of the vessel drastically reduced the backpressure, and maintaining a comparable volume allowed the same volumes of solvents and reagents to be used. This vessel accommodated up to 200 mg of resin, and flow rates up to 60 mL/min. More resin should not be used, because the resin swells as the peptide is elongated and the volume limiting inserts restrict the swollen volume to 2mL. Long peptides may result in reactor failure if more than 200 mg of resin is used. Higher flow rates were not tested, but the observed backpressure indicates they are accessible.

With this new reaction vessel in hand, cycle time was reduced by washing at a higher flow rate. As expected, the required wash time continued to decrease with increasing flow rate, with 99% of the amino acid removed in 36 seconds at 20 mL/min and 20 seconds at 40 mL/min. To allow operators adequate time to prepare each subsequent amino acid, one minute washes at 20 mL/min were used. After the instrument became available for general laboratory use, users occasionally successfully ran the system with 30 second washes at 40 mL/min.

To verify that the performance of the second generation synthetic protocol and vessel, using faster cycles and fewer equivalents of amino acids, was comparable to that of the first generation synthetic procedure, the syntheses of ACP(65-74), the conotoxin, the HIV-1 protease fragment, and the affibody fragments were repeated on 200mg of resin with the synthetic timeline in Figure 6d. The peptides were of comparable crude quality (Figures S9-S11), and this reactor was made available for general use.

Dozens of unique peptides were made for diverse applications, and almost all were of high crude purity. Several representative case studies are included here and in the supporting information (Figures 7, S12-S14). This method is sufficiently robust that all of these peptides were synthesized without UV monitoring of the reactor effluent. Figure 7 shows a library of model cysteine containing peptides, including some of low crude purity. The supporting information shows additional, longer analogues (Figure S13), a library of 10 model glutathione-like peptides prepared in a single day (Figure S12), and two biotinylated protease recognition sites (Figure S14). All case studies in the SI are of high crude purity. In all cases, peptides were produced on a 0.2 mmol scale, the major peak is the desired product, and crude material was successfully purified in one preparative RP-HPLC step.

This method was also used to produce peptide fragments of two additional proteins, the 113 residue *B. a.* RNase and the 130 residue pE59 DARPin. Following rapid optimization of the synthetic protocol, the full length proteins were obtained. For a detailed account of the synthesis of these targets, including effective and ineffective methods of suppressing aspartamide formation, deletions, methionine oxidation, and premature termination of the peptide chain, see the accompanying manuscript.



## Automation

Finally, we believed our rapid synthesis method may be difficult to automate, and, therefore, sought to demonstrate automation. To this end, the instrument in Figure 8a was constructed as a proof of principle. Two HPLC pumps delivered reagents; one delivered piperidine or solutions of amino acids and HBTU in DMF and the other delivered DIEA to activate the amino acids. A static mixer was used to ensure effective mixing of DIEA and the amino acid solutions, and the valve positions and pump flow rates were controlled by an Arduino microcontroller. Amino acids were stored as 0.4 M solutions with equimolar HBTU in DMF, and were stable for weeks without the activating base if stored in sealed vessels. This system used the heat exchanger, second generation synthesis vessel, and UV detection described above without modification. The SI contains details of the construction of this system.

The automated cycle time was not limited by the rate at which a user could complete manual tasks or a syringe pump could infuse, so the timeline was substantially accelerated (Figure 8b). Two 45 second washes at 50 mL/min (the maximum available), a seven second coupling, and a ten second deprotection resulted in the incorporation of an amino acid residue every 107 seconds (1.8 minutes). The coupling time and deprotection time represent such a small fraction of the total time they were not optimized. Using these cycles, ALFALFA was produced in 12.5 minutes, ACP(65-74) was produced in 17.8 minutes, and (ALF)<sub>7</sub>, a model 21-mer synthesized to demonstrate that the system is mechanically robust, was produced in 37.5 minutes. The crude quality of (ALF)<sub>7</sub> was nearly identical to a synthesis using the manual second generation synthesis protocol (Figure S15).

## Conclusion

It is clear that the reported methods generate high quality peptides extremely rapidly, on scales relevant to research. In all cases, the desired product was obtained and readily isolable by RP-HPLC. Based on published syntheses of ACP(65-74), the conotoxin, and the HIV protease fragment,<sup>[13]</sup> our experiments with manual Boc syntheses of the affibody fragments, and the synthesis of ACP(65-74) at room temperature in batch and under flow, we believe that crude material obtained with our reported methods is nearly identical to material obtained from traditional batch processes.

Extensive work has shown that SPPS chemistry can be fast and efficient at elevated temperatures.<sup>[12]</sup> The reported system further accelerates SPPS chemistry, far beyond what is currently possible with microwave assisted or other rapid peptide synthesizers, by leveraging a flow based approach. In addition to constantly supplying high concentration reagents, the flow-based platform overcomes a number of significant obstacles that hinder standard and microwave-assisted approaches. First, the completely sealed reaction vessel and heat exchanger can be immersed in a temperature controlled bath which allows solvents and reagents to be heated in a consistent and controlled manner immediately before reaching the resin bed. Rapid preheating is crucial to avoid thermal degradation of reagents<sup>[19]</sup> while quickly reaching the desired temperature, but this is extremely difficult in a batch system. Second, the flow platform can be scaled without increasing the cycle times. As demonstrated in the transition from the first to second generation reaction vessels, increasing the diameter and flow rate effectively increases the maximum scale, without slowing the synthesis. Third,



stirring is not required to effect adequate mass transfer, eliminating failure-prone moving parts and facilitating scale up. Fourth, high quality peptides can be obtained quickly without double coupling, double deprotection, or colorimetric tests of coupling efficiency. During our studies with ACP(65-74), we observed no decrease in the Val deletion peptide after double coupling Val and double deprotecting the preceding Gln, and these results are consistent with our experience optimizing the fragments of *B. a.* RNase and the DARPin. Such additional steps are often employed in batch mode syntheses, hindering synthetic progress. Finally, automation of this system enables faster cycle times, in contrast to the often slow automation of batch synthesis.<sup>[13]</sup>

For the first time, the reported platform enables the efficient, rapid Fmoc synthesis of polypeptides and provides a reproducible and systematic study of flow-based Fmoc SPPS chemistry at elevated temperature. To prove the synthetic utility of this system, we produced dozens of peptides suitable for various applications, including three fragments of an affibody that were successfully ligated to produce a full length protein.

By reanalyzing flow-based SPPS<sup>[16,17,20,21]</sup> and carefully designing a new system, we were able to overcome two longstanding challenges preventing rapid peptide synthesis. First, we were able to reduce the wash time from several minutes to one minute or less by minimizing the volume of the system. Although it is often not discussed, washing the resin requires a significant investment of time and solvent in most SPPS systems. With the reported system, wash times and solvent usage are significantly less than previous systems, but washing remains a key challenge in further accelerating the cycle.

Second, we eliminated the extremely high capital and maintenance costs of a microwave heating system by employing a simple, effective heat exchanger in a water bath. As a UV detector is not essential, our system can be assembled for about \$1000 with used pumps. Furthermore, all components of the system can be serviced by a non-expert, drastically reducing instrument downtime. We believe lowering the cost and complexity of rapid peptide synthesis is a major step towards its general adoption by chemists. Similarly, the reported system uses the cheapest, most common peptide synthesis chemistry. All reagents are commercially available, and no extraordinary hazards are associated with chain assembly or cleavage.

This platform was used to produce dozens of peptides, and, in an accompanying manuscript, we report the total synthesis of the 113 residue *B. a.* RNase and a 130 residue DARPin. Importantly, during the synthesis of the fragments of these proteins, most of the major side reactions in peptide synthesis were encountered. The accompanying manuscript provides an extensive description of procedures to overcome these side reactions, and constitutes a detailed tutorial for sequence specific optimization of long and complex peptides using our flow-based synthesizer.

In conclusion, we have developed a rapid, highly robust peptide synthesis platform. The system can be easily and cheaply assembled, then leveraged to generate high quality peptides. Ultimately, this and our subsequent work provide a guide for chemists inexperienced in

peptide and protein synthesis to quickly and independently carry out total syntheses of these complex biomolecules without the need for sophisticated tools, reagents, or equipment.

## Experimental Section

Extensive documentation of each synthesis and apparatus can be found in the supplementary information freely available from the ChemBioChem web page.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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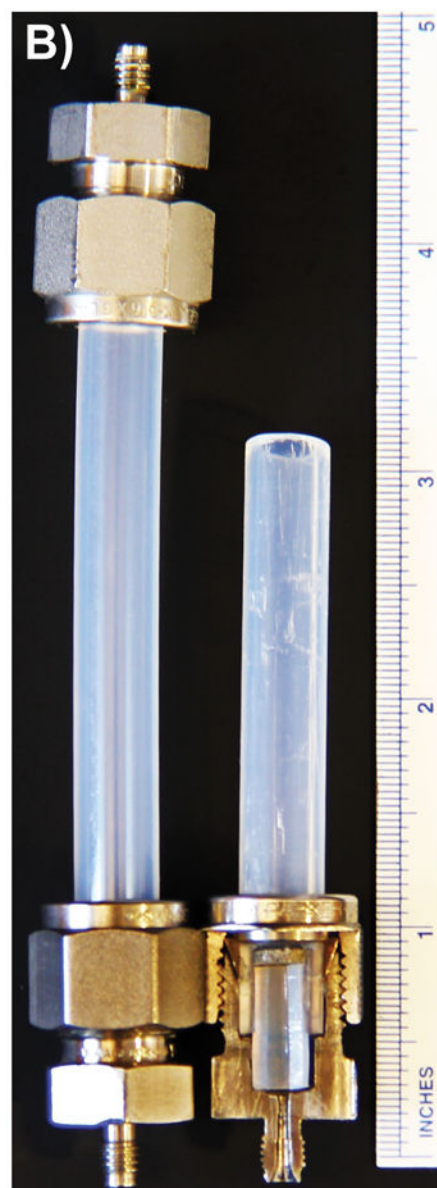
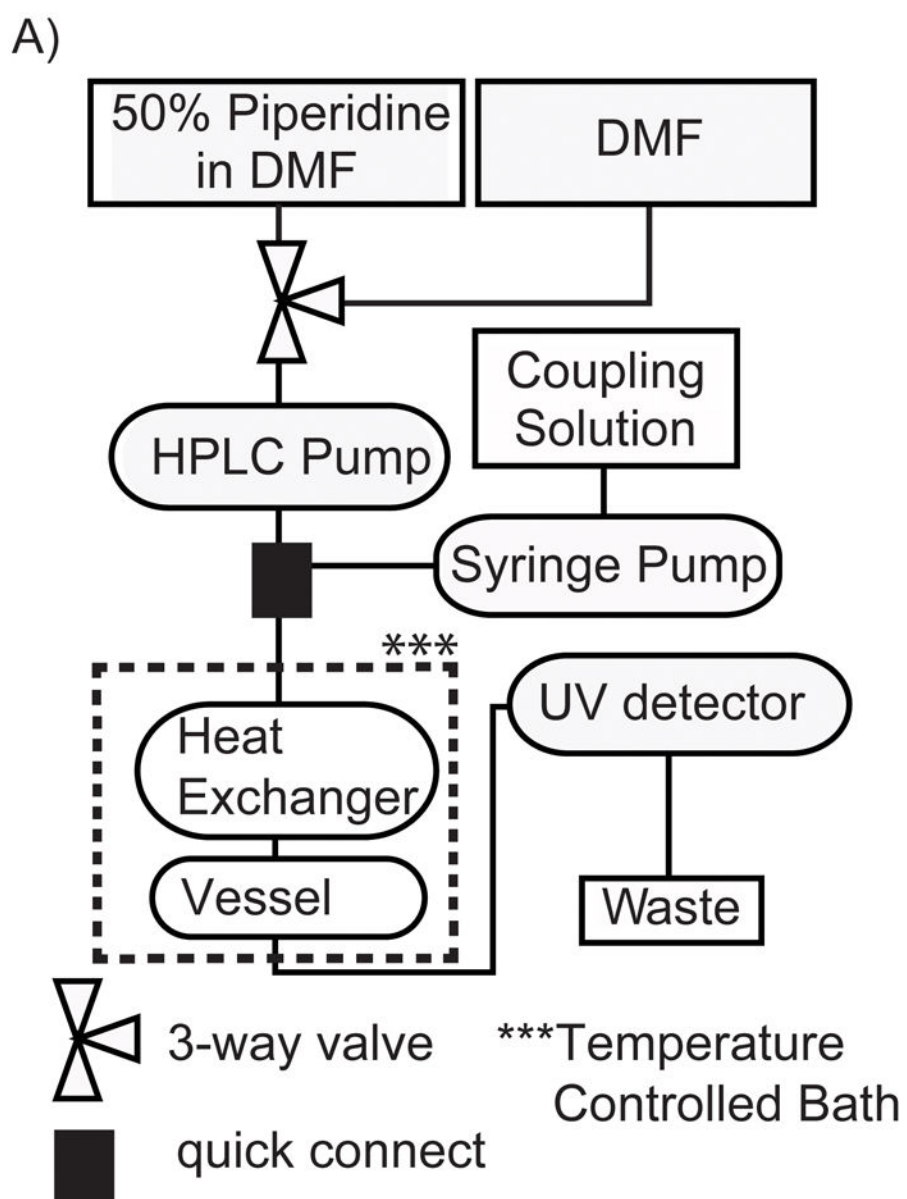
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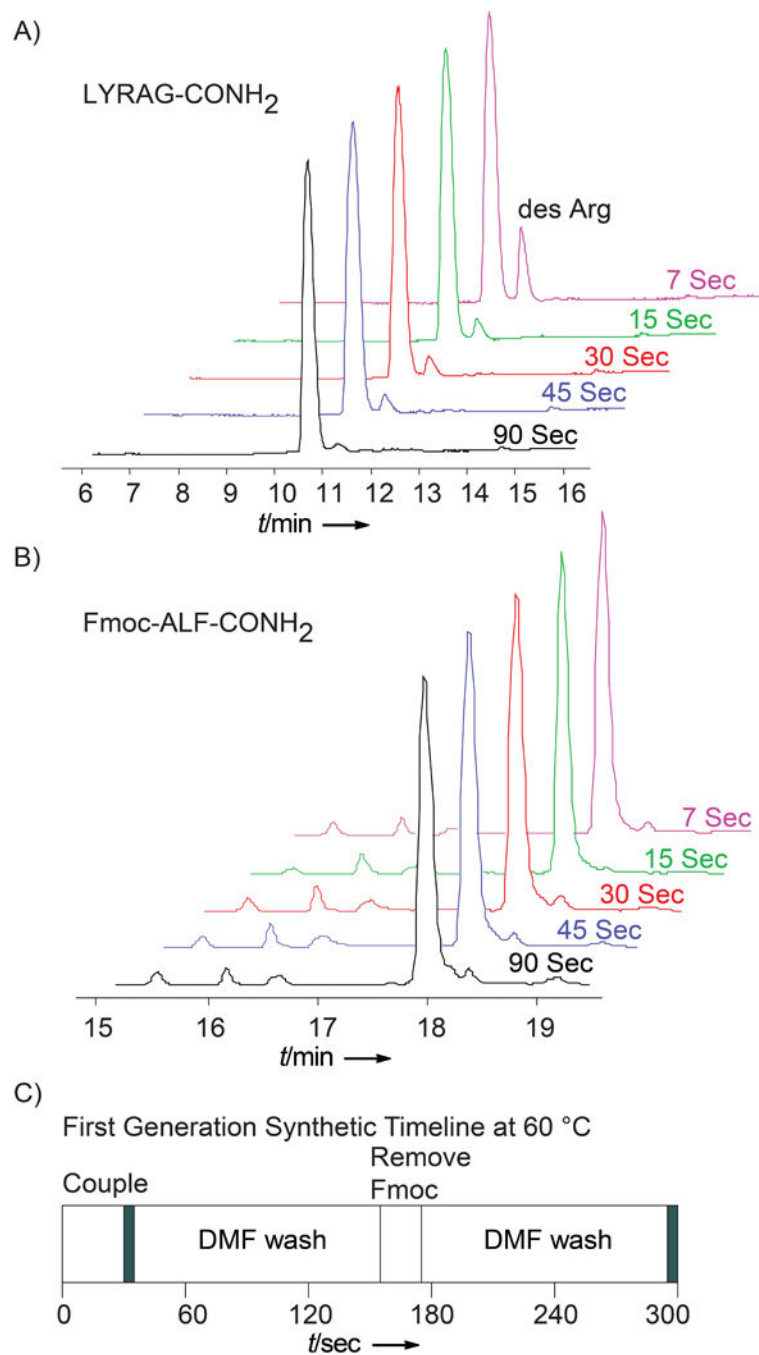
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## Abbreviations

<b>Fmoc</b>	Fluorenylmethoxycarbonyl
<b>HATU</b>	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
<b>HBTU</b>	O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
<b>HPLC</b>	High Performance Liquid Chromatography
<b>NCL</b>	Native Chemical Ligation
<b>SPPS</b>	Solid Phase Peptide Synthesis

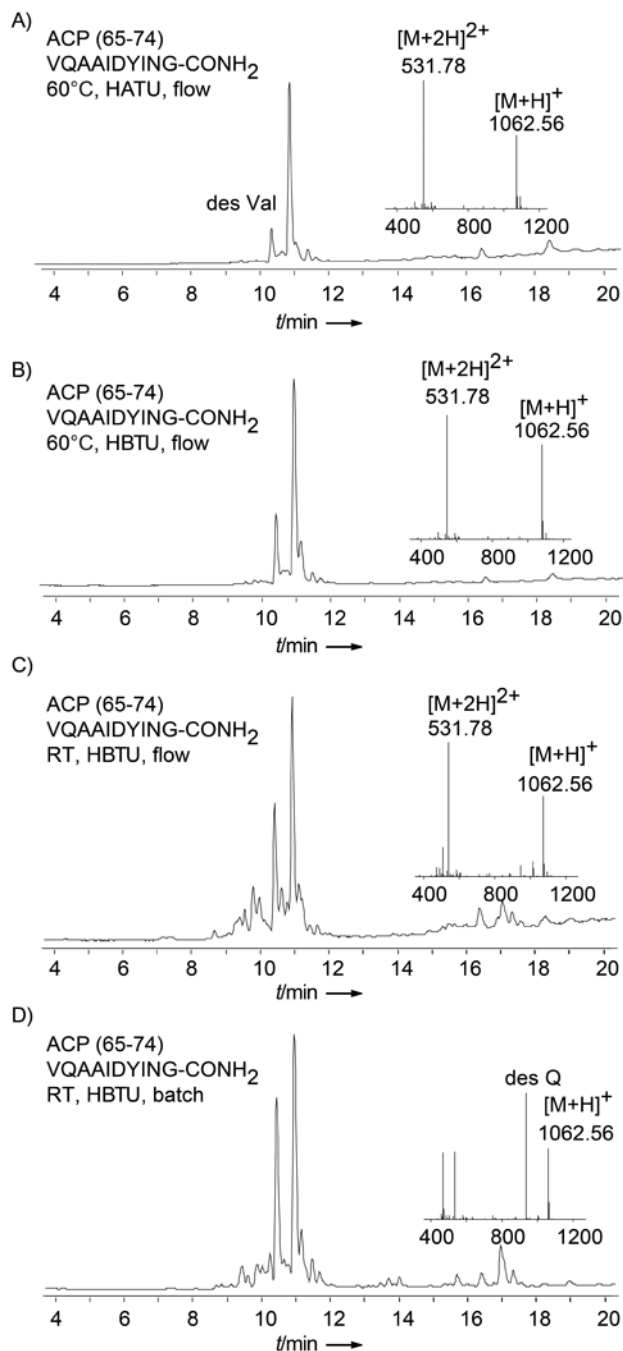


**Figure 1.** Flow platform for Fmoc SPPS. (A) Schematic of the synthesizer. The synthesis vessel can be placed in a temperature-controlled bath. (B) Photograph of the assembled reaction vessel, left, and a cutaway showing the down-stream components, right.



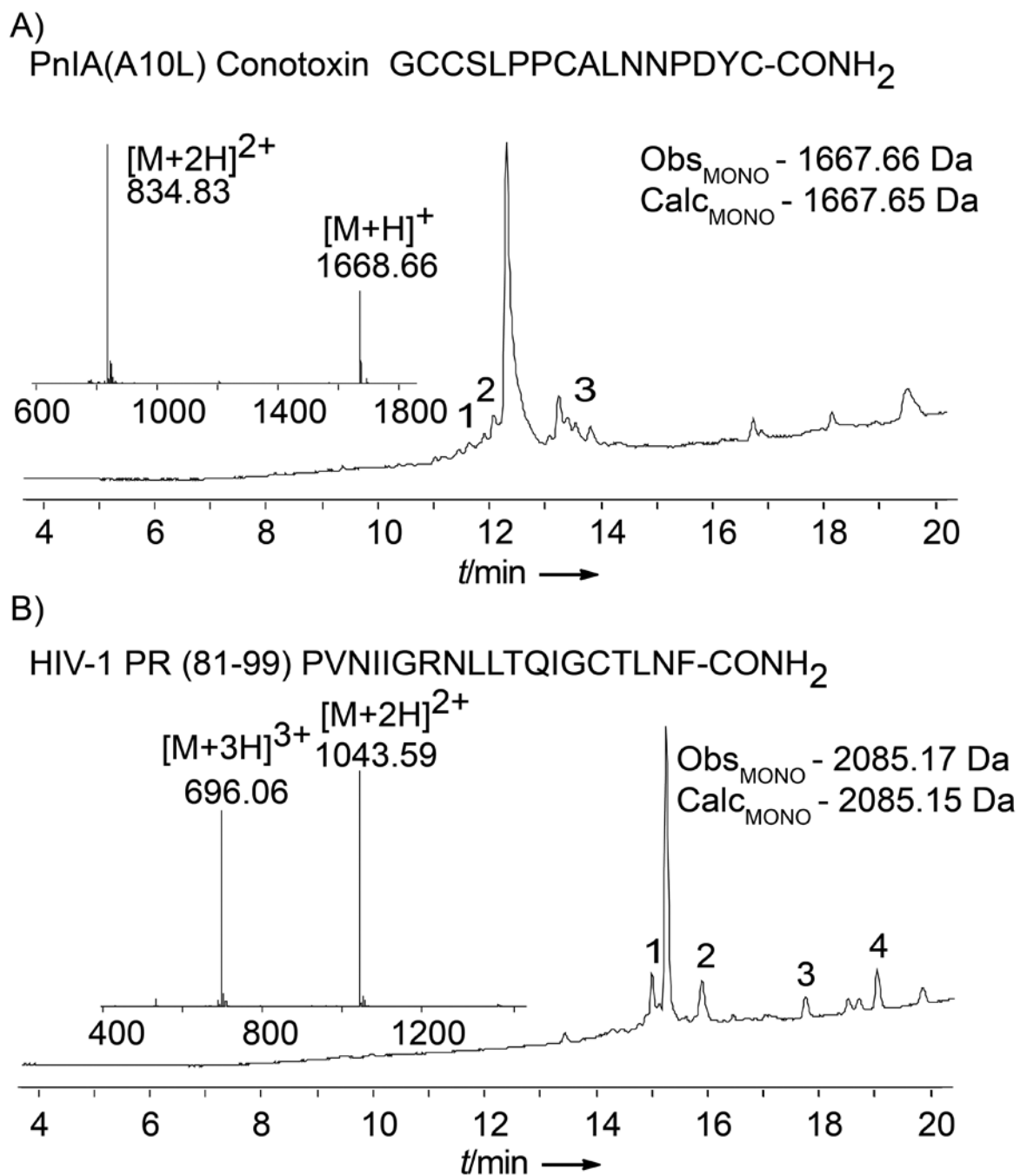
**Figure 2.** Systematic investigation of coupling time. (A) LC data for crude LYRAG-CONH<sub>2</sub> produced by coupling every amino acid for a nominal 90, 45, 30, 15, or 7 seconds. The amount of Arg deletion peptide was greatest for 7 second coupling. (B) LC data for the synthesis of Fmoc-ALF-CONH<sub>2</sub> under the same conditions, showing no change in peptide quality with reduction in coupling time. (C) The final synthetic timeline used with the first generation reaction vessel, where grey bars represent the time required to move the quick connect. An

amino acid residue is incorporated every 300 seconds. The total ion current is displayed in each chromatogram.

**Figure 3.**

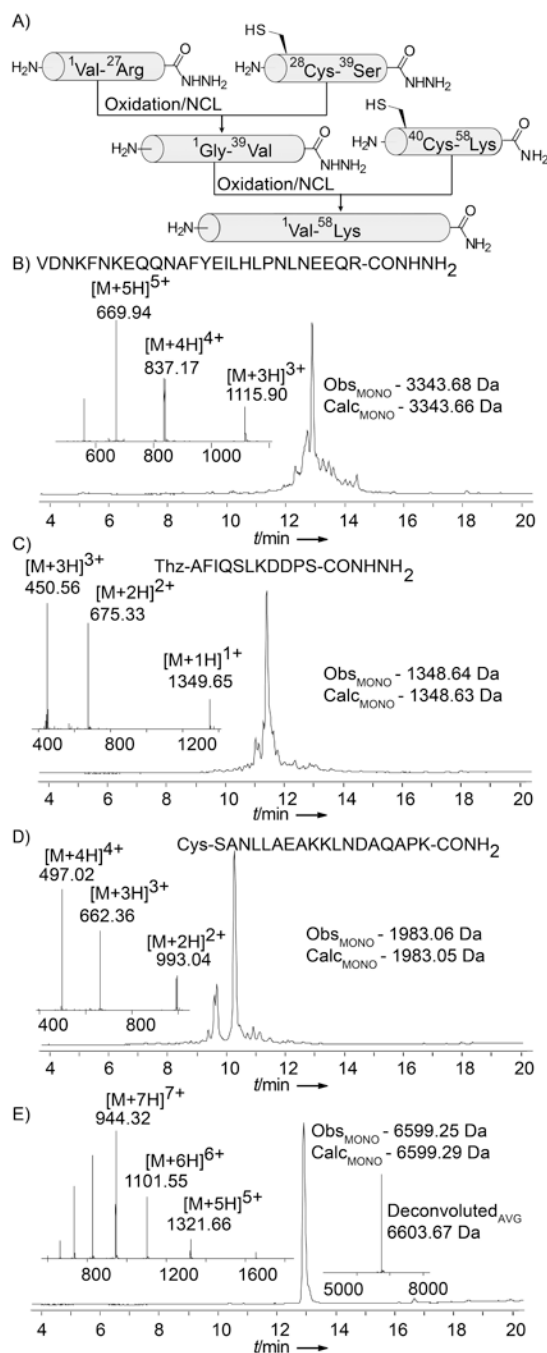
LCMS data for ACP(65-74) model studies. The synthetic timeline in Figure 2c was used. Crude LCMS chromatograms for ACP(65-74) synthesized at (A) 60 °C using HATU as an activator, (B) 60 °C using HBTU as an activator, (C) RT using HBTU as an activator, and (D) RT using a manual batch method following the same timeline. The total ion current is displayed in each chromatogram.



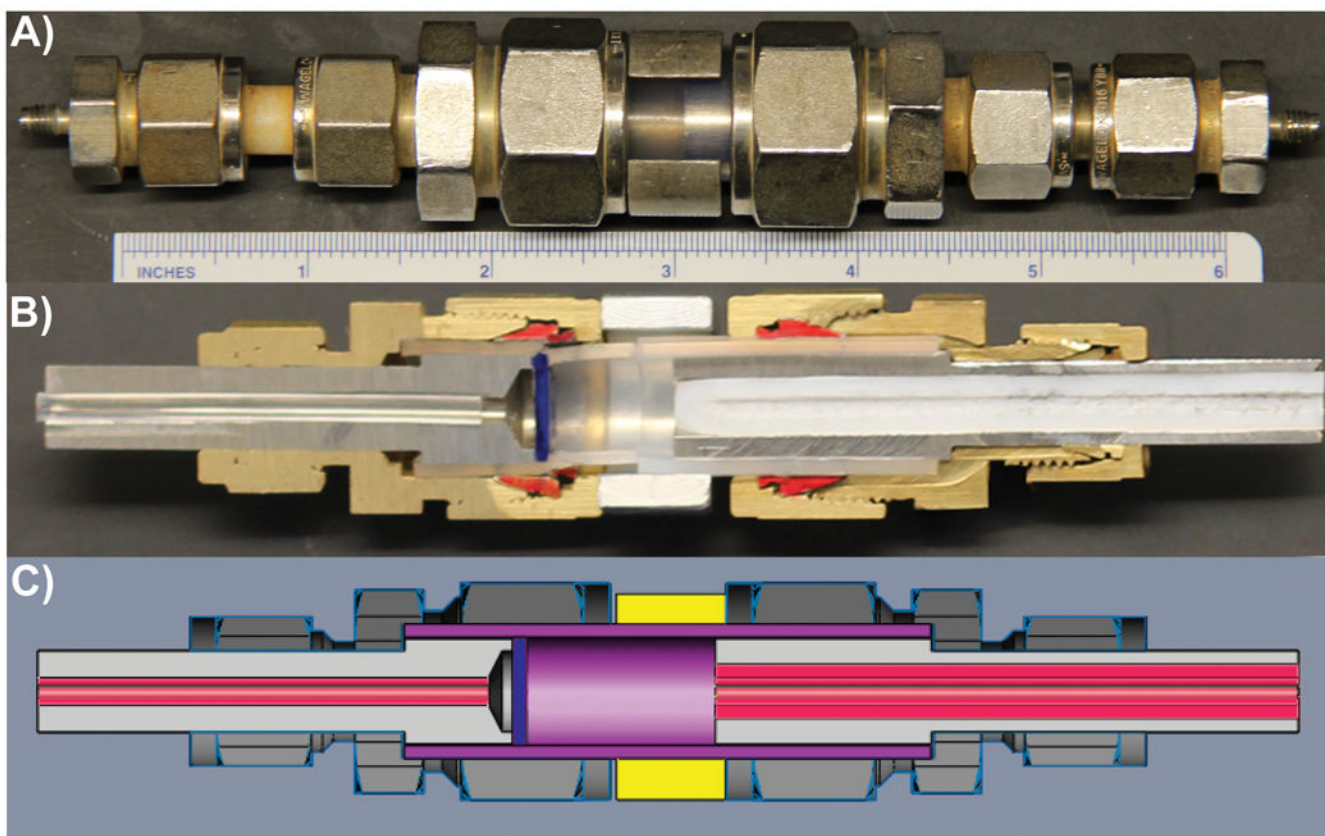


**Figure 4.**

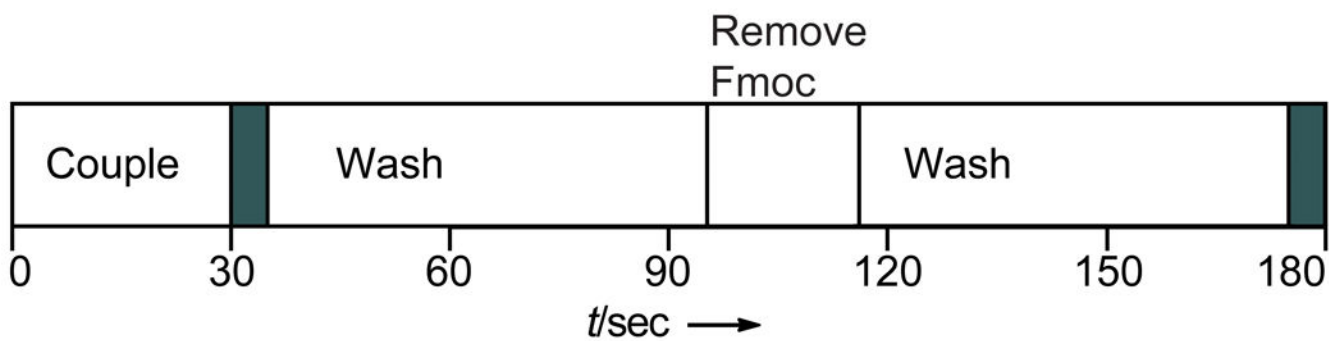
Synthesis of difficult peptides under flow. LCMS data for the crude peptides: (A) PnIA(A10L) (1 = Cys deletion, 2 = Cys deletion, 3 = incomplete side-chain protecting group removal) and (B) HIV-1 PR (81-99) (1 = peptide truncation at Arg and 2,3,4 = incomplete side-chain protecting group removal). The total ion current is displayed.



**Figure 5.** Synthesis of the affibody. (A) Synthetic scheme used to produce the affibody (B) Crude fragment [1-27]-CONHNH<sub>2</sub> (C) Crude fragment Thz-[28-39]-CONHNH<sub>2</sub> (D) Crude fragment Cys-[40-58]-CONH<sub>2</sub> (E) Purified full-length affibody. Listed observed and calculated masses are monoisotopic. The total ion current is displayed in each chromatogram.

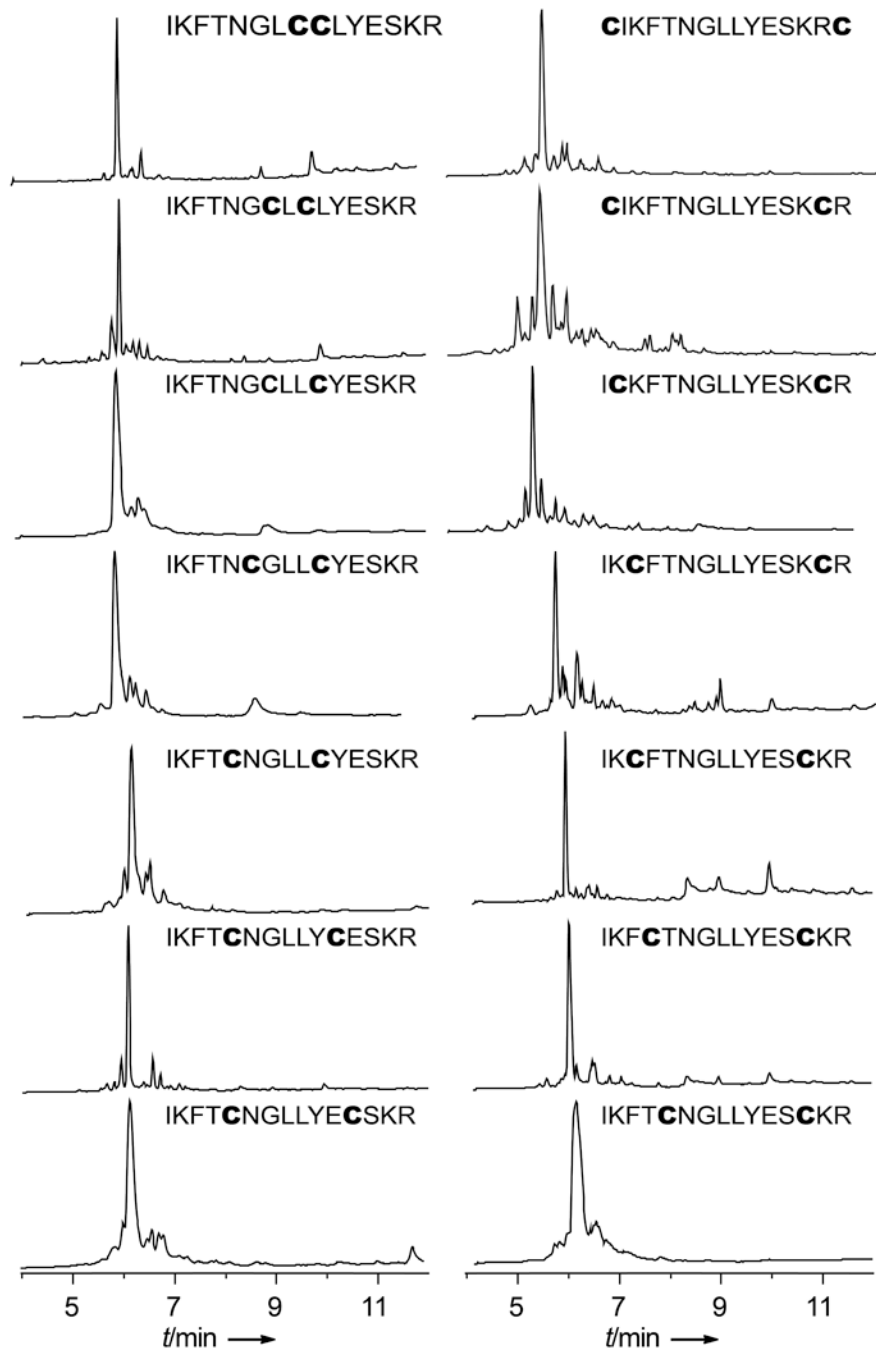


#### D) Second Generation Synthetic Timeline at 60°C

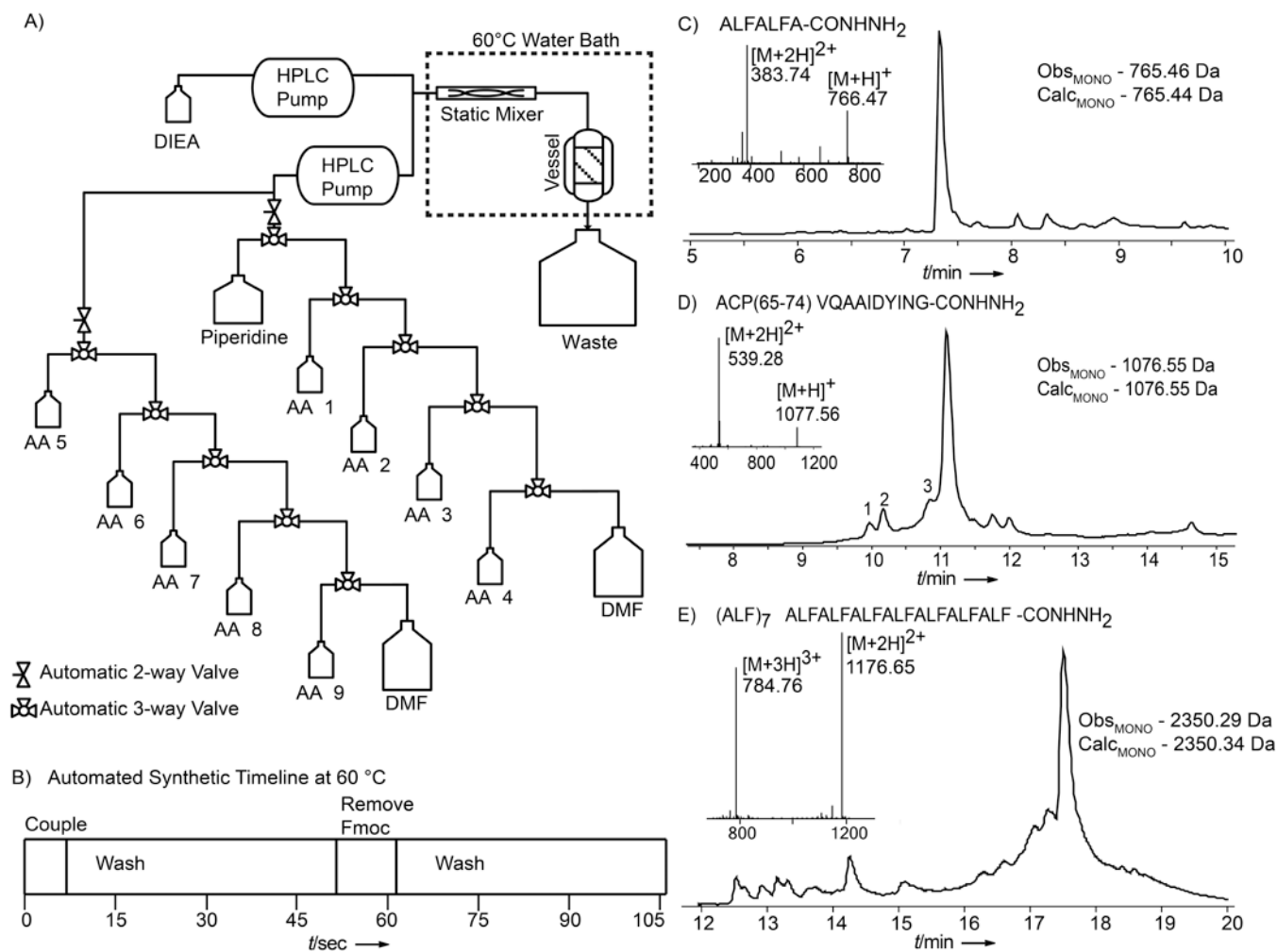


**Figure 6.**

Second generation reaction vessel (A) Assembled unit after hundreds of syntheses (B) Cutaway with non-wetted parts shown in brass, the frit in blue, and large ferrules in red. The image has been color enhanced and background objects removed. (C) False color drawing of the cutaway with non-wetted parts shown in dark gray and the frit in blue (D) The final synthetic timeline used with the second generation reaction vessel; grey bars represent the time required to move the quick connect. An amino acid residue is incorporated every 180 seconds.



**Figure 7.** LCMS traces of model 15-mers for cysteine macrocyclization, research synthesized in 45 minutes each. Cysteine is bolded and total ion chromatograms are shown. All peptides were produced as C-terminal amides, and, in all cases, the main peak was the desired mass (1772.9 Da obs and calculated monoisotopic).



**Figure 8.** Automated peptide synthesis platform. (A) Schematic of the device (B) The synthetic timeline used by this device to incorporate an amino acid residue every 107 seconds (C) ALFALFA assembled in 12 minutes (D) ACP(65-74) assembled in 18 minutes (1,2 = Ile deletion, 3 = hydrolysis of the C-terminus) (E) (ALF)<sub>7</sub> assembled in 37 minutes. Total ion chromatograms are shown.