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# Peptide Bond Formations through Flow Chemistry

Nisar Ahmed\*

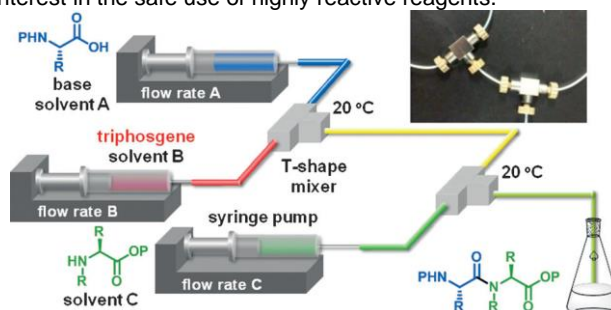
Dedicated to my parents (Riaz Ahmed and Muneeran Bibi), daughter (Liyana Ahmed) and wife (Bahareh Shirinfar)

Peptides and proteins play important roles in body functions<sup>1,2</sup> and are used exclusively in drug discoveries, having advantages because of their high biological activity, high specificity, and low toxicity. For peptide synthesis, researchers mostly use the solid-phase peptide synthesis (SPPS)<sup>3</sup> with modern modifications.<sup>4</sup> However, scientists failed to overcome two main factors; the concentration and time required for peptide coupling. The flow-based technology may help in the rapid production of peptides due to having advantages over batch reactions<sup>5–7</sup> in terms of productivity, heat and mixing efficiency, safety, and reproducibility. Herein, we discussed both solution and solid phase synthesis of peptides in flow.<sup>8–12</sup>

Baxendale and Ley et al. reported<sup>8a</sup> the synthesis of di- and tripeptides in a simple flow process, comprising various packed columns holding immobilized reagents, scavengers, and catalysts. The authors made use of a readily available Syrris AFRICA® micro or mesofluidic pumping method. This approach led to the preparation of a small library of Boc-, Fmoc-, and Cbz- peptides. The same group also reported<sup>8b</sup> an efficient and sequence-specific stop flow strategy for the synthesis of  $\alpha$ -peptides using natural and unnatural amino acids activated as *N*-carboxyanhydrides with traceless protection groups. They claimed high yields and purities via using a lower reagent excess that runs under a dedicated software control. In contrast to  $\alpha$ -peptides,  $\beta$ -peptides have also gained much attention because few residues form secondary structures and they show a high metabolic stability. However, the secondary structures create problems in synthesis. Fogel et al. reported the first synthesis of  $\beta$ -peptides using a silicon continuous flow microreactor.<sup>8c</sup> They made use of acid fluorides as activated forms of the amino acids. They claimed that this synthesis was the first demonstration (i) to achieve tertbutyloxycarbonyl (Boc)- and 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acid coupling in only 1–5 min at a high temperature (120 °C); (ii) to use  $\beta^2$ - and  $\beta^3$ -homoamino acid fluorides for  $\beta$ -peptide synthesis; and (iii) show the significance of a C<sub>10</sub>H<sub>4</sub>F<sub>17</sub>-substituted benzylic ester protecting group in a solution-phase peptide coupling.

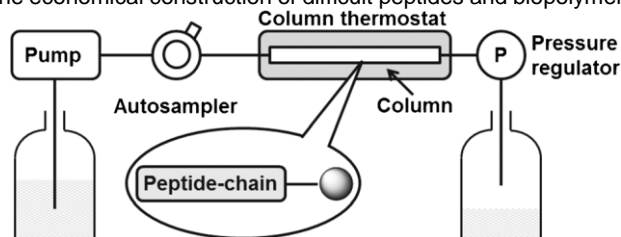
Flash chemistry works well under vigorous conditions.<sup>9</sup> Fuse et al. used a microflow reactor in the solution-based peptide bond formations with more racemizable amino acids by using solid triphosgene.<sup>9c</sup> They reduced the residence time of the reactive species to less than one second so that the reaction could be completed with an amine in 4.3 seconds at 20 °C and produced a good yield. Their microflow reactor was divided into three parts (See Figure 1), The first part, A, contained carboxylic acids with a base and the second part, B, with triphosgene, and both were connected through a T-shaped mixture where, most

probably, the formation of phosgene and further formation of the corresponding acid chloride took place under 20 °C, which subsequently reacted with amine of Part C under the water bath (20 °C) to form a peptide bond with an excellent yield without severe epimerization. This highly efficient amide bond formation, based on the concept of rapid and strong activation of carboxylic acids, could not be achieved under the conventional batch conditions because controlling the temperature and time precisely is difficult. This concept of microflow synthesis would develop an interest in the safe use of highly reactive reagents.



**Figure 1.** Microflow synthesis of peptides based on the concept of rapid and strong activation of carboxylic acids using triphosgene. Reproduced from ref. 9, Wiley Publishing Group.

SPPS required high amino acid equivalents for coupling. However, Fulop et al. reported<sup>10</sup> SPPS at low equivalents of amino acids by using a continuous flow (CF) reactor. They synthesized peptides in an automated way with high purity and yields from micrograms to grams by using amino acids up to 1.5 equivalents with low solvent consumption. A systematic representation of the setup used for SPPS is given in Figure 2. This has an HPLC-type pump, autosampler, column, thermostat, and pressure regulator. This methodology was used effectively for the synthesis of  $\beta$ -peptide foldamers with alicyclic side-chains, trans-ACHC residues, and exotic and expensive artificial amino acids sequencing. However, this methodology can be utilized for the economical construction of difficult peptides and biopolymers.

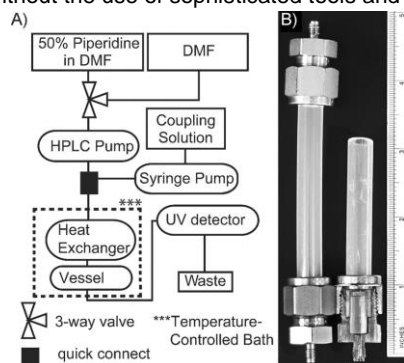


**Figure 2.** Schematic representation of the constructed CF reactor. Reproduced from ref. 10, Wiley Publishing Group.

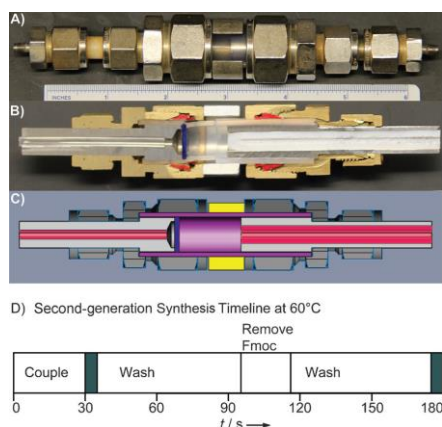
Most peptide syntheses proceed with Fmoc-protocols and take longer time for incorporation of each amino acid residue due to prolonged washing. Pentelute et al.<sup>11</sup> introduced the flow method to reduce the time required for the incorporation of amino acids for manual (3 min) and automated (1.8 min) peptides and protein synthesis by using a heat exchanger. They prepared a low volume and pressure vessel to reduce the wash solvent volume and the fast delivery of the solvent and the reagent so that the

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concentration remained at the maximal point and could be removed quickly. The apparatus used for peptide synthesis is mentioned in Figure 3A. First, they prepared the simple reaction vessel (Figure 3B), which allowed conducting model studies with up to 100 mg of resin and longer time was required along with the side products as well. To overcome these drawbacks, they modified the reaction vessel to conduct synthesis at 60 °C through the placement of a heat exchanger in the vessel (Figure 4). This vessel works up to 200 mg of resin and has flow rates up to 60 ml/min. However, they applied this synthetic mechanism in an automated way and synthesized a model 21-residue peptide. This is a rapid, highly efficient peptide synthetic way to generate high-quality peptides and can be easily constructed with a low cost. This method provides a guide for peptide and protein synthesis without the use of sophisticated tools and reagents.



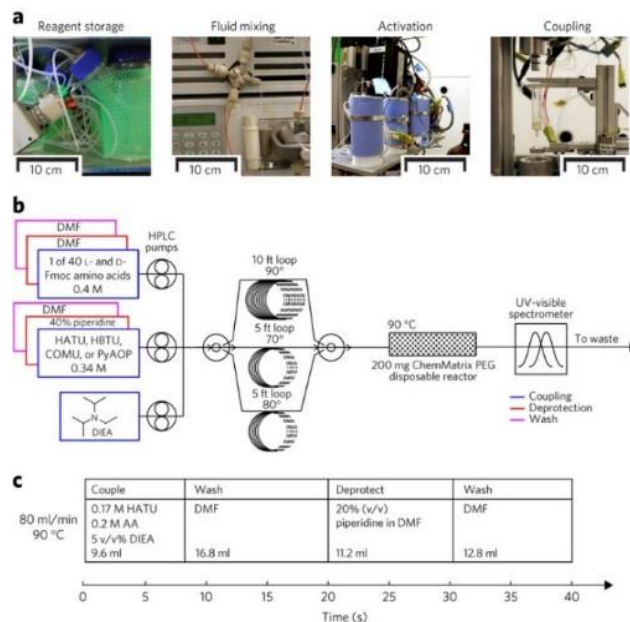
**Figure 3.** Flow platform for Fmoc SPPS. A) Schematic of the synthesizer. The reaction vessel can be placed in a temperature-controlled bath. B) The assembled reaction vessel (left) and a cutaway showing the downstream components (right). Reproduced from ref. 11, Wiley Publishing Group.



**Figure 4.** Second-generation reaction vessel. A) Assembled unit. B) Cutaway showing fittings (brass), frit (blue), and large ferrules (red). The image has been color enhanced, and the background objects have been removed. C) False-color drawing of the cutaway showing fittings (dark gray) and the frit (blue). D) The final synthetic timeline used with the second-generation reaction vessel; gray bars indicate the time required to move the quick connect. An amino acid residue is incorporated every 180 s. Reproduced from ref. 11, Wiley Publishing Group.

The chemical synthesis of peptides from protected and pre-activated amino acids monomers under high pressure takes a long time from minutes to hours per amide bond, and these approaches need modifications. Toward this, Pentelute et al. recently reported a fully automated flow-based approach for accelerated peptide synthesis.<sup>12</sup> This method is flexible with accelerated solid-phase chemical synthesis and reduces the time

for the amide bond formation of standard fluorenylmethoxycarbonyl (Fmoc) peptide synthesis to 7 seconds. The entire cycle for each amino acid addition is complete in 40 seconds with a control way, which makes this method prominent over the existing manual or automated methods. During synthesis heating, in-line mixing of reagents, peptide bond activations in a modular format with control pumping system, a high flux of wash solvent, efficiency measurements by UV, help in rapid peptide synthesis. They used an automated synthesizer with five modules (See Figure 5): the first part contained a mixing chamber for amino acids with coupling agents. Then the mixed solution electrically flowed over a preheated tubular reactor to form an active ester and subsequently, the activated amino acid flowed through the coupling module that contained resin at 90 °C, where amide bond formation was complete in 7 seconds. The UV absorption spectrometer was fixed as well to monitor the peptide bond formation in each cycle. This synthesizer gave a quantitative yield of peptides. This methodology would overcome the problems like sufficient control over temperature, activation, fluid handling, and in-line monitoring. However, this approach would limit the human effort needed for manual handling and delivery of lethal reagents by combining sophisticated automation with innovations from the peptide synthesis and the continuous-flow pharmaceutical industrial fields.



**Figure 5.** Automated flow peptide synthesis enables 7-s amide bond formation and complete solid-phase peptide synthesis cycles in 40 s. (a) Photographs of the automated-flow solid-phase synthesizer modules. (b) Process flow diagram. Amino acid, activating agent, and DIEA are merged together by three HPLC pumps. Multi-position valves control the selection of the amino acid and activating agent. Amino acids are activated in one of several heated flow paths, determined by a column selector valve, then flowed over a peptidyl resin housed in a 6-ml fritted syringe in a heated jacket. The effluent is passed through a UV-visible spectrometer to waste. (c) Cycle diagram showing the duration of each step, the solution composition during each step after mixing, and the total volume of reagent used at each step. HATU, *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide. Reproduced from ref. 12, Nature Publishing Group.

In the future, flow chemistry could play a key role in the development of the fast-automated synthesis of peptides and proteins through the quick activation of intermediates for coupling at low concentrations of reagents. Selected examples highlight

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the role of flow chemistry for the advancements of peptides bond formations.

### Acknowledgements

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Chemistry • Flow Reactors • Flash Chemistry

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