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MINIREVIEW ARTICLE

Microreactors for peptide synthesis: looking through the eyes of twenty first century !!!

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Abstract The twenty first century has witnessed several advances in synthetic chemistry, among them microreactors. It is expected that these devices will have a considerable impact on synthetic organic chemistry since they offer a wide range of applications in various fields. Perhaps the synthesis of peptides deserves mention in this regard as these molecules are emerging as therapeutics and offer several advantages over the so-called small molecules. This minireview does not aim to address microreactors in detail, but explains various peptide synthesis methods that involve microfluidic techniques, highlighting the need for further improvement and expansion of microdevices/microreactors.

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Introduction to microreactors: the chemist's round-bottomed flask!

Many products are now produced by chemical means. In this regard, the application of chemistry extends from the manufacture of life-saving drugs to all essential modern-day commodities of society. Apart from food supply, essential items like plastics, polymers, paints, pigments, dyes, and other man-made materials depend heavily on synthesized products.

Given the relevance of chemistry, we concede that improvements are needed in current working practices in order to minimize the depletion of natural resources. Thus to meet the ever-increasing demands of productivity and efficiency, alternative synthetic approaches are required. Also, considerations such as rocketing prices, scale-up issues, reduced reproducibility, and wastage of manpower support the need to develop novel synthetic approaches. Furthermore, to survive in a competitive market, the pharma industry must adapt and innovate. The pressure to develop novel therapeutics is high and these products will come about only from new technologies.

As the global emphasis toward achieving higher safety standards and more sustainable practices unfolds, it is becoming necessary to re-evaluate how chemical synthesis is conducted (www.suschem.org). It is also important to note the reliability and timeliness of preparative processes. There is, therefore, a need for new chemical processing techniques that can perform industrially relevant chemical syntheses faster and in a directly scalable fashion. Consequently, these techniques should involve greater levels of

automation to facilitate continuous scale-up, whilst being coupled to integrated diagnostic methods that ensure the highest standards of quality control. In the wake of the above, microreactor flow technologies offer several potential advantages for chemical production and hold great promise for the future.

Microstructured reactors, otherwise known as microreactors, flow reactors, or microfluidic reactors, have many advantages over conventional laboratory techniques (Fletcher et al. 2002; Hessel et al. 2005a, b, c; Watts and Haswell 2005; Watts and Wiles 2007; Geyer et al. 2006). Several examples of the integration of flow reactors into pilot plants or even into large-scale production lines have been reported (Schwalbe et al. 2002). The use of microreactors allows the controlled mixing of reagents; and the length of the reaction with a substrate depends on the length of the microchannel. Furthermore, these devices allow new methods for successful chemical transformations that result in high space–time yields since they have the capacity to control thermal or concentration gradients. Although hazardous intermediates are produced in situ, the amount generated is low and hence safe. When integrated into suitable analytical devices, microreactors provide rapid feedback for optimization purposes. Not only can liquids be synthesized using these instruments but also solids.

Although microfluidic reactors are efficient, they have been largely ignored by many organic chemists. This can be attributed to the considerable cost of building and maintaining the reactors, and also, the success of the current approaches used to synthesize molecules of interest. However, because of increasing demands for synthetic products in day-to-day life, a change in how organic reactions are achieved is desirable. Microreactors offer a solution because they generate less waste than traditional methods and also confer extraordinary control over the reaction. In their early days, flow reactions in a microreactor were costly; however, inexpensive, simple-to-build, and easy-to-modify reactors are now available for organic reactions. The advantages of performing chemical reactions in microstructured devices have been widely demonstrated (Gavriilidis et al. 2002; Ehrfeld et al. 1999; Hessel and Lowe 2002; Jensen 2001; Schubert et al. 2001; De Mello and Wootton 2002).

History of microreactors: down memory lane

In 1986, a patent from Germany described the features of a microstructure reactor for the purpose of chemical reactions (Lohder and Bergann 1986). Of note, this patent contains with all the manufacturing processes under the then economic circumstances but was not implemented for various reasons. By the end of 1989, Forschungszentrum Karlsruhe (Germany) had built the first microheat exchanger and described its potential for microchemical reactions (Schubert et al. 1989). This type of work was started in 1993 at the Pacific Northwest National Laboratory (PNNL, USA) (Wegeng et al. 1996). Later in 1995, at a workshop held at Mainz, Germany, the use of microstructured reactors for chemical and biological reactions was discussed, marking the starting point for the worldwide development of flow reactors (Ehrfeld 1995).

Definition of microstructured reactors

Microfluidic devices in general have 3D structures, the inner dimensions of which are usually between ten and a hundred micrometers (Ehrfeld et al. 1999; Wegeng et al. 1996). Hence the salient feature of these devices is the high surface-area-to-volume ratio when compared to conventional chemical reactors. The latter have a surface area of about 100 m^2m^{-3}, while that of microstructured reactors lies between 10,000 and 50,000 m^2m^{-3} (Jnckel 1996). Because microreactors have high heat-exchange efficiency, reaction mixtures are rapidly heated and cooled, and hence the reactions under isothermal conditions can be performed with precise residence time (Schubert et al. 1998; Lerou et al. 1996; AlTpTe et al. 2000).

When reactions in microdevices are carried out in small volumes, process parameters such as pressure, temperature, residence time, and flow rate are more easily controlled. Moreover, the likelihood of hazard caused by strong exothermic or explosive reactions can be largely reduced (Veser et al. 2000; Veser 2001). Furthermore, microreactors offer increased safety when reactions involve toxic substances or high operating pressures (Lowe et al. 2000; Ehrfeld et al. 2000a, b, c). Many other characteristics of microflow chemistry have been comprehensively reviewed (Thayer 2005; Ehrfeld et al. 2000a, b, c; Jahnisch et al. 2004; Pennemann et al. 2004; Kiwi-Minsker and Renken 2005; Hessel et al. 2005a, b, c; Kolb and Hessel 2004; Kockmann et al. 2006).

Microreactor structure: fabrication, micromixing, and process intensification

Microstructured reactors have a series of small channels measuring 10–1000 μm in length/in diameter that are joined in various geometries, thus allowing the spatial and temporal manipulation of minute amounts of fluids/reagents. In these reactors, the fluid behavior is achieved by nonconvective laminar flow, in which mixing is allowed only by diffusion. Microreactors look like a deck of playing cards or a tiny dining plate. They contain auxiliary pumps and fluid ports that control factors like the addition of reagents, mixing of substances, reaction time, separation,

and analysis. Each channel is connected by T- or Y-shaped junctions; however, more shapes of channels and configurations can also be achieved (Ehrfeld et al. 2000a, b, c).

Microchemical systems can be made from a wide range of materials, including polymers (Anzenbacher and Palacios 2009; Xia and Whitesides 1998; Lee et al. 2003; Willis et al. 2007; Grover et al. 2008; Yoon et al. 2008), glass (Fletcher et al. 2002; Chow 2002; Kikutani et al. 2002), silicon (Jensen 2001, 2006), ceramic (Knitter et al. 2001), and steel (Hessel et al. 2004; Ehrfeld et al. 2000a, b, c; Pennemann et al. 2004; Schwalbe et al. 2004). The selection of construction materials depends on the chemistry to be performed, the operating conditions, and ease of fabrication.

Silicon is the substrate most commonly used for the production of microreactors. Upon oxidation, silicon behaves in a similar manner to glass, and it is chemically inert to most reagents and solvents. However, in contrast to glass, silicon shows high thermal conductivity, thus allowing the development of microdevices with exceptional heat-transfer capacity. In this context, microreactors made out of silicon are generally used for exothermal reactions and also for those requiring extremely high or low temperatures (Ratner et al. 2005). The main drawback associated with this material is that it shows low durability and poor release properties (Becker and Gartner 2000). Hence an alternative method called LIGA [*Lithographie* (lithography), *Gal*V*anoformung* (electroplating), *Abformung* (molding)] process came about (Ehrfeld and Lehr 1995). On the other hand, glass has been widely used by synthetic chemists because of its chemically inert nature and its transparency for visual inspection of a reaction. Of all the materials mentioned, glass has been used the most successfully for building microreactors and has the added advantage that fabrication procedures are well established, photolithography is an example (Watts and Haswell 2005; McCreedy 2000, 2001; Watts et al. 2001; Fletcher et al. 1999), among many others. For instance, the AFRICA microreactor system can be integrated with glass reactors of different sizes (250, 60, and 1.0 mL) (Syrris Ltd.).

After the selection of a substrate, the replication of the pattern into the device material can be achieved by several indirect methods, including injection molding (McCormick et al. 1997), hot embossing (Martynova et al. 1997), elastomeric stamps (McDonald et al. 2000). While poly(dimethylsiloxane) (PDMS) is the material most commonly used to build microreactors, it has the drawback that it swells in most organic solvents (Chow 2002). To address this limitation, another material, perfluorinated elastomer, has been developed. This material has the advantage that it is compatible with methanol, dichloromethane, and toluene (Rolland et al. 2004).

After building the system, it has to be connected to a macroscopic instrument, for which a number of methods are available, including fluid wells incorporated directly into the device, ferrule-type connectors, HPLC fittings, and larger-scale barbed connectors for laboratory tubing. More examples are available in a review by Fredrickson and Fan (2004). Importantly, two classes of pumping techniques, namely hydrodynamic flow and electrokinetic flow, can be used to control fluids in microreactors (McDonald et al. 2000).

Hydrodynamic flow, which is also called pressure-driven flow, is achieved as a result of the difference in pressure between the inlet and outlet of the channel. In general, the inlet is given positive pressure whereas the outlet is open to atmospheric pressure. Hydrodynamic flow has the advantage that it can be used with any liquid and any device, regardless of the material from which it is made. However, the dispersion in flow rates leads to a distribution of residence times that results in poor yield and selectivity of the products (McDonald et al. 2000).

In electrokinetic flow, the potential is applied between the inlet and outlet of the channel. Electrokinetic flow operates in two ways (Fredrickson and Fan 2004; Li 2004); in the first, ions move in solution toward the electrode of opposite charge, while in the second, electroosmotic flow (EOF) arises from the electrical double layer phenomenon that forms on channels with charged surfaces. In contrast to hydrodynamic flow, here the dispersion of reagents is greatly reduced. Also, this method of "pumping" has some advantages over the former as it does not involve moving parts and can be readily miniaturized and controlled by a computer. The mixing of reagents inside the microreactors takes place by diffusion and convection (Hessel et al. 2005a, b, c). Since the devices are minute, diffusion occurs to a greater extent and mixing occurs in milliseconds.

Figure 1 depicts some of the miniaturized devices. In most cases, hydrodynamic flow is used with an internal volume ranging from nL to mL. A wide variety of chemical transformations have been achieved using these instruments, namely reactions like Aldol, Azo-coupling, Diels–Alder, enamine, Hantzsch, Michael-addition, Suzuki-coupling, among many others. Importantly, microstructured devices give better results than those obtained with batch reactions. This apart, many other reactions like chlorination, fluorination, hydrogenation, nitration, oxidation, addition, elimination, nucelophilic and electrophilic substitutions, cycloadditions, and radical-induced polymerization have also been performed. These processes are not explained in depth as they are beyond the scope of this review. Detailed information can be obtained from Ehrfeld et al. (2000a, b, c) and Hessel and Lowe (2002).

Flow reaction technology allows not only the synthesis of small amounts of material but also large-scale

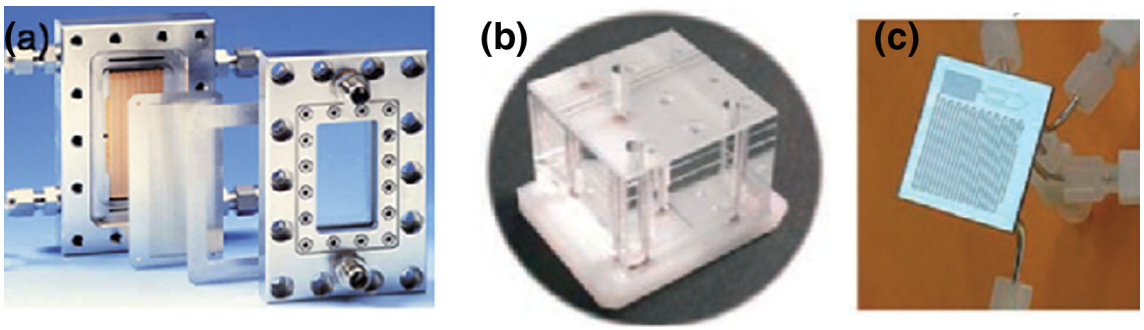


Fig. 1 Selected microreactors; **a** Stainless steel microreactor system (Jahnisch et al. 2004). **b** Glass microreactor made by Haswell (Watts and Haswell 2005). **c** Silicon-based microreactor (Ratner et al. 2005). Reproduced with permission

production by scale-out or numbering-up. While current production technology is based on the scale-up of bench-optimised processes, firstly by construction of a pilot plant, followed by a final increase in scale to achieve mass production, microreaction technology bases itself on the replication of successful reaction units. This approach not only removes the problems associated with any changes in surface-to-volume ratio (and hence the thermal and mass transportation properties of the reaction) that are experienced as a reactor is scaled-up but enables process flexibility (as reactors are no longer configured for a single operation) (Ehrfeld 2000a, b, c).

Advantages

Flow process technology offers many advantages over conventional synthesis. A brief list includes the following: (1) the duration of the reaction is short because of improved mixing and heating; (2) the yield and purity obtained are high; (3) reactions can be carried out up to 300 bar and with solvent superheating up to 300 °C; (4) cold reaction zones up to −120 °C; (5) multi-stage temperature zones can be adopted to increase the sensitivity; (6) real-time analysis and optimization, incorporated DoE (less waste and more automation); (7) scalability; (8) reactions involving toxic or explosive materials, which would be difficult in conventional synthesis, can be performed more safely; (9) purification can be performed using less expensive equipment; and (10) provides a versatile platform from which to build integrated multi-step transformations, delivering more advanced chemical architectures.

Disadvantages

In spite of the many advantages of microreactors listed above, these devices cause several inherent difficulties when multi-step synthesis is performed. These difficulties include the following: (1) the kinetics of the different reaction steps (integrating reactions in sequence with different reaction times) need to be compensated for; (2) the solvent has to be changed in each step, thus requiring a new protocol each time; (3) intermediate purification is required; (4) the addition of extra downstream flows cause dilution effects; and (5) each stage of the reaction has to be monitored and controlled.

In spite of these obstacles, microstructured reactors are still practical as process engineering tools as they provide information in a short period and in a safe manner. This information can later be transferred to the pilot and production scale. In this context, in the near future, microreactors will provide chemists with an alternative tool for the development/scaling/improvement of products. Far from being a comprehensive review, here we present the potential of flow reactions for various kinds of transformations. Also, to the best of our knowledge, this is the first review to report the combination of different kinds of techniques used for peptide synthesis in flow reactors.

Peptide synthesis in microreactors: toward better technology

The following pages are devoted to discussing the potential application of microstructured reactors for the synthesis of peptides. Here we wish to draw attention to the methods/systems/reagents used for the synthesis of various kinds of peptides (analogues).

Peptide synthesis is generally achieved by two methods. The first is via classical solution-phase, in which the two amino acids (with certain protections) are mixed in the presence of a suitable activator/base in liquid phase. Although this chemistry is cost effective, it takes several days to construct a small peptide because longer reaction times and extensive purification steps are required. As an alternative, in 1963, considered to be the 'birth of the new generation of peptide synthesis' because Merrifield (1963) discovered the solid-phase peptide synthesis (SPPS). This

technique is based on the reaction of an *N*-protected amino acid with an insoluble support, usually a polymer, through usually the *C*-carboxylic function. The reaction is extended by the removal of *N*-protection and the addition of second protected amino acid. The excess reagents used are removed by simple washing and filtration. Although SPPS is more advantageous, it has several drawbacks, including the requirement of expensive resin, and additional steps like attachment of the first amino acid to the resin and its final removal. In this regard, microreactors could overcome some of these shortcomings.

Synthesis of β-peptides (derivatives) in a miniaturized system

Watts et al. (2002a, b) demonstrated the synthesis of β-peptides using a borosilicate glass microreactor by EOF mobilization of reagents. They made use of a photolithographic technique and wet etching to create channels in the reactor. In brief, the surface of a borosilicate glass was deposited with a thin layer of chromium, which controlled the extent of undercutting during wet etching. This was followed by the spin coating of positive photoresist and the transfer of interconnecting channels to the photoresist layer by means of photolithography. After evaporating the plate to remove volatiles, the channels were chemically etched using a mixture of 1 % HF and 5 % NH_4F in water at 65 °C. Microporous silica frits (Christensen et al. 1998) were used in the channels in order to prevent hydrodynamic flow.

In the first example, they targeted the synthesis of a dipeptide **3** within the miniaturized system at room temperature. This involved the priming of the channels with DMF before performing the reaction to ensure that they were free from air and moisture. The reagents (at 0.1 M concentration) used in the reaction were Fmoc-β-alanine (**1**), EDCI {[1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide} and Dmab-protected β-alanine (**2**) {Dmab: 4-[*N*-(1-(4,4-Dimethyl-2,6-diococyclohexylidene)-3-methylbutyl)-amino]benzyl}, which were placed, respectively, in the reservoirs A, B, and C, whereas reservoir D was used to collect the products (Fig. 2). An external voltage was applied to induce EOF of the reagents, and the reaction, which took 20 min to complete, was monitored by HPLC. They observed that only ca. 10 % conversion to **3** was achieved when stoichiometric amounts and a voltage of 700 V were used. When 2 equiv. of EDCI was used, the conversion rate increased to ca. 20 %. However, when a stopped-flow technique was used, there was a further increase in the conversion, reaching approximately 50 %. The authors considered that the conversion of the reactants is highly dependent on the number of moles of EDCI used. It was found that EDCI was not soluble beyond a concentration of 0.2 M. Hence, DCC (dicyclohexylcarbodiimide) as an alternative carbodiimide coupling reagent was adopted. The use of 0.5 M DCC in DMF yielded ca. 93 % conversion.

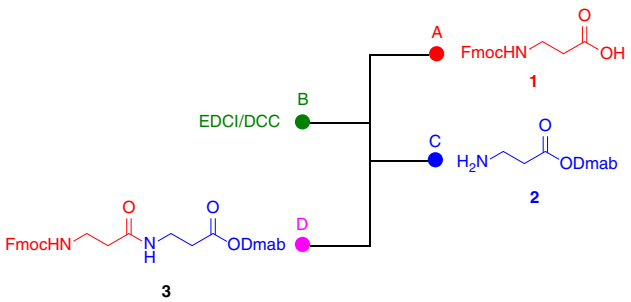


Fig. 2 Carbodiimide-mediated synthesis of β-dipeptide in flow

Using a microreactor, the same group demonstrated other methods of peptide synthesis involving a preactivated amino acid derivative like pentafluorophenyl (PFP) ester. Here, Fmoc-β-alanine-PFP (**4**) and amine **2** were placed in reservoirs A and B, respectively, and the product was collected in C reservoir (Scheme 1) quantitatively in 20 min. The authors claimed that this reaction results in significant yield when compared to the classical solution-phase method.

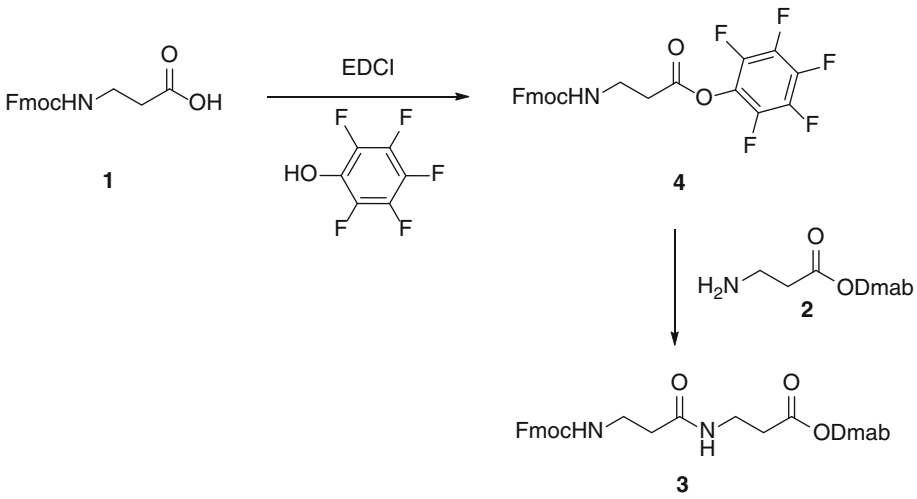
Also, the effect of substituting Boc **5** for Fmoc **1** was studied in a flow reactor using Boc-β-alanine-PFP (**6**) and amine **2** via EDCI/DMAP (DMAP: dimethylaminopyridine) coupling. This approach gave quantitative conversion to dipeptide **7** (Scheme 2).

A number of dipeptide analogues have been prepared following the same strategy, as outlined in Table 1.

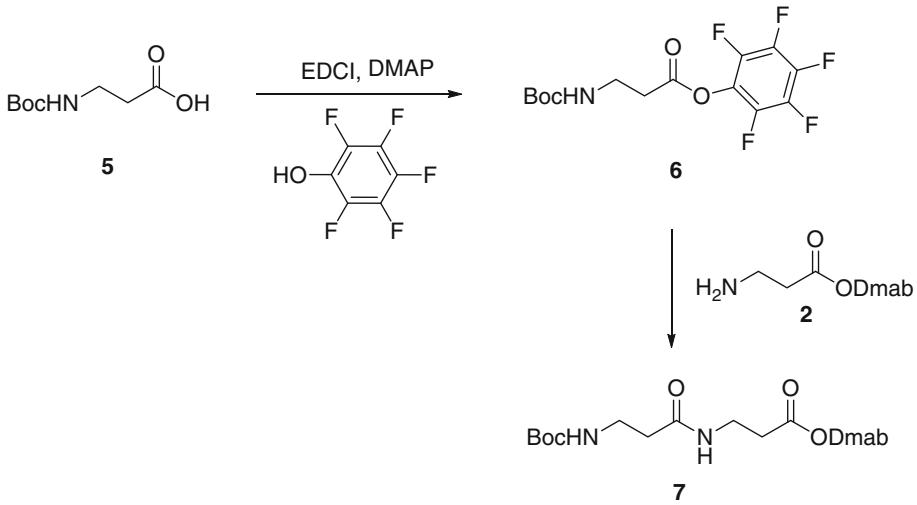
Having established the protocol for the synthesis of dipeptides in a microreactor, further extension of the method to longer peptides was addressed. For this, removal of the Fmoc/Boc is essential. For Fmoc, the commonly used deprotecting agent is 20 % piperidine in DMF. This method is advantageous since piperidine reacts with dibenzofulvene to form a piperidine adduct which is soluble in DMF. This reaction is beneficial for microreactions since the precipitation or polymerization of the dibenzofulvene that would otherwise block channels in the reactors is avoided. An experiment using 10 equiv. of piperidine revealed that none of the desired dipeptide formed. It was thought that excess piperidine reacted with the PFP ester. In order to prove this, a batch reaction was conducted between Boc-β-alanine-PFP (**6**) and piperidine. The product of the reaction was an amide **8** (Scheme 3), which was formed by the nucleophilic attack of the base on the ester.

For this reason, an alternative choice was the use of 1 equiv. of 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) (Wade et al. 1991; Kates et al. 1996). When DBU was employed in continuous flow, only 25 % of product **7** was

Scheme 1 Peptide synthesis using PFP ester of amino acid



Scheme 2 Peptide synthesis using Boc-protected PFP ester



obtained. By altering the voltages, a conversion of 96 % was achieved. This finding illustrates that deprotection can be carried out using only 1 equiv. of DBU in microreactors, in contrast to the 2 % used in SPPS (Wade et al. 1991; Kates et al. 1996).

On the other hand, removal of the Dmab group in a flow reactor was achieved by means of hydrazine (Chan et al. 1995). An equimolar solution of Fmoc-β-alanine-Dmab (**9**) and hydrazine were reacted in a microfluidic condition to obtain carboxylic acid **1** in quantitative yield (Scheme 4). Of note, the microreaction of **9** and only 1 equiv. of hydrazine was sufficient to cleave the ester whereas a 2 % solution was required in SPPS (Chan et al. 1995), thereby suggesting that microstructured reactions are more molecule-efficient.

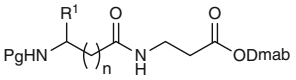
Taking the advantage of formation of peptide bonds and selective removal of protecting groups under flow conditions, the method was extended to the chain elongation of peptides. Here, first the dipeptide was synthesized from **4** and **2**, which were placed in reservoirs A and B, respectively. Reservoir C was filled with DBU, and a second equiv. of **4** was added to reservoir D. The products of the reaction were collected in reservoir E (Fig. 3). An overall 30 % conversion was observed for tripeptide **10**.

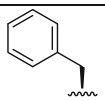
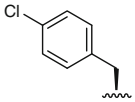
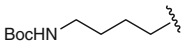
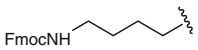
Immobilized reagents/scavengers/catalysts enabled peptide synthesis under flow conditions

An alternative method to synthesize di- and tripeptides in a simple flow process comprising various packed columns holding immobilized reagents/scavengers/catalysts was reported (Baxendale et al. 2006). This approach led to the preparation of a small library of Boc-/Fmoc-/Cbz (or Z)-peptides. Those authors made use of a readily available Syrris AFRICA® micro or mesofluidic pumping method; however, any HPLC grade pump could be used to start the flow.

The synthesis of peptides containing Boc- and Z-protecting groups was started using a glass Omnifit® column

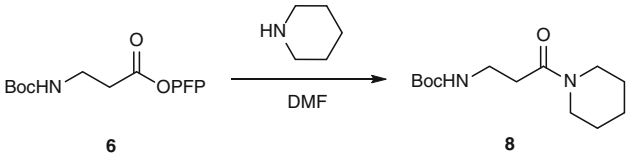
Table 1 Demonstration of the synthesis of a selection of peptides



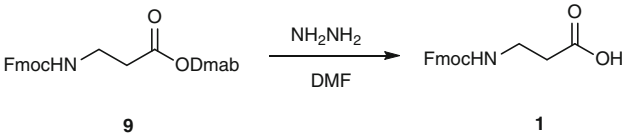
Pg	n	R^1	Yield (%)	Time (min)
Fmoc	1		>35[a]	20
Fmoc	1		36	20
Fmoc	0		100	20
Boc	0		100	20

All the reactions were completed in 20 min. The rate of the reaction in a microreactor was faster compared to that in a bulk reaction and it was postulated that enhancement in the rate was due to electrochemical phenomenon caused by the electric field in which the reaction was performed

[a] A greater yield was obtained using flow than conventional synthesis (35 %)



Scheme 3 Nucleophilic attack of piperidine on PFP ester



Scheme 4 Deprotection of Dmab using hydrazine

packed with polymer supported (PS) HOBt (1-hydroxybenzotriazole). This column was pumped with Boc- or Z-protected amino acid, DIPEA (diisopropylethylamine) and PyBroP® (bromo-tris-pyrrolidino-phosphonium hexafluorophosphate) (Coste et al. 1991) in DMF. This procedure not only led to the sequestration of activated amino acid by PS-HOBt, thus allowing the substrate for later use by means of "catching", but also directed the by-products formed to the waste. After several DMF washes, the column was connected to PS-dimethylaminopyridine (PS-DMAP) and PS-sulfonic acid resin (MP-SO_3H), which were linked in series DMAP-HOBt-SO_3H (Scheme 5). HCl salt of next *C*-terminal protected amino acid (in DMF) was then added and passed through all three

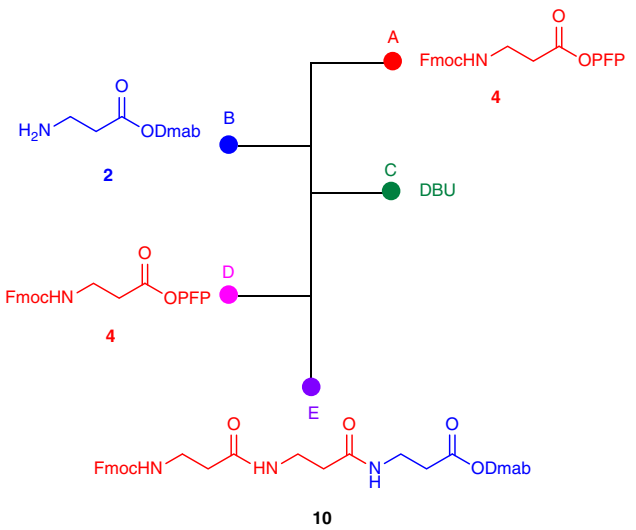


Fig. 3 Tripeptide synthesis in microreactor

columns. PS-DMAP served to release the amine from its salt, which reacted with the HOBt-activated Boc- or Z-amino acid, whereas MP-SO_3H was used to scavenge any unreacted amine. Table 2 shows the results of the synthesis of Boc- and Z-containing dipeptides under a microflow system.

Fmoc-containing peptides were constructed using a similar protocol. Initially, the presence of DIPEA degraded Fmoc resulted in lower purity (<80 %). To overcome this, PS-IIDQ (2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline) (Valeur and Bradley 2005) was used to obtain an anhydride of the Fmoc-amino acid and then allowing through PS-HOBt column (Scheme 6). The remaining steps, as explained before, were the same.

The same method was extended for tripeptide synthesis, which involves an additional deprotection step (Scheme 7). Here, two PS-HOBt columns, named as $HOBt^1$ and $HOBt^2$, were used to activate Z–amino acids. These columns were followed by PS-DMAP and MP-SO_3H in a series. Then the HCl salt of the amine was passed through these columns and directed toward a flow hydrogenerator (H-Cube) to deprotect the Z group of the dipeptide. Final coupling was done by again pumping the deprotected dipeptide through the columns i.e., PS-$HOBt^2$ and MP-SO_3H, and the tripeptide in a single diastereoisomer was collected in DMF (yield 59 %).

β-Peptide synthesis in miniaturized chemical reactors using fluorine-activated amino acids

Parallel to α-peptides, β-peptides have also gained much attention because of their properties, including the fact that they require fewer residues for the formation of secondary structures and they show high metabolic stability. However, the synthesis of these molecules poses few problems

Scheme 5 Schematic of the synthesis of Boc-/Z-dipeptides using immobilization chemistry

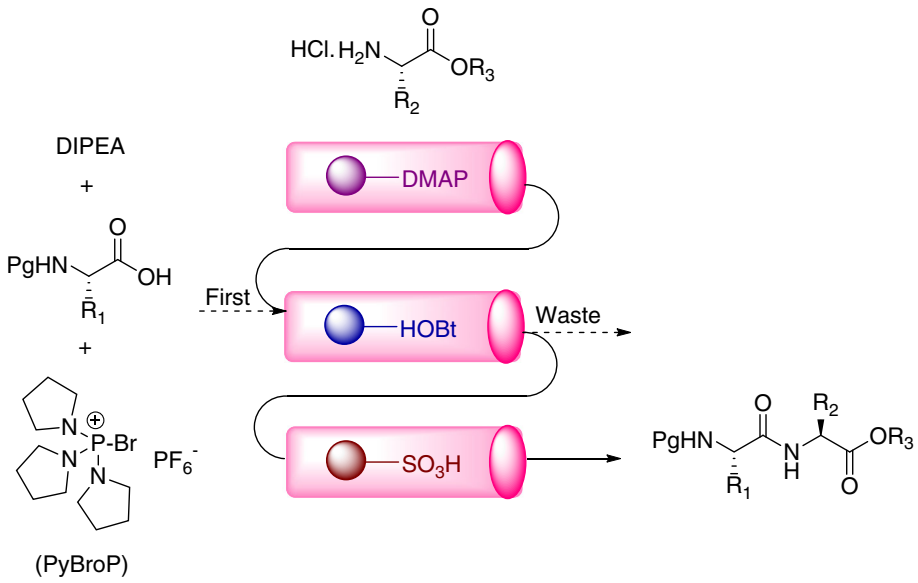


Table 2 Various Boc- and Z-containing dipeptides prepared by an immobilized-flow-assisted reaction

Protected dipeptides	Yield (%)
Boc-Ala-Phe-OEt	80
Boc-Ala-Gly-OEt	81
Boc-Ala-Val-OMe	83
Boc-Ala-Pro-OMe[a]	66
Z-Phe-Val-OMe	79
Z-Phe-Gly-OEt	76
Z-Ala-Phe-OEt	75
Z-Ala-Gly-OEt	78
Z-Ala-Pro-OMe[a]	61

Boc-containing compounds gave the highest yields

[a] Proline-derived peptides gave moderate yields, possibly due to steric hindrance exhibited by the secondary amine

because of the generation of secondary structures. Although β-peptides have been synthesized in both solution (Cheng et al. 2001) and solid (Seebach et al. 1996) phases, satisfactory results have not yet been achieved. In this context, there is a need for alternative tools. Taking advantage of this, (Flogel et al. 2006) undertook the first synthesis of β-peptides using a silicon continuous flow microreactor. They have also claimed that this synthesis was the first (a) to achieve Boc and Fmoc amino acid coupling in only 1–5 min at high temperature (120 °C); (b) to use β^2- and β^3-homoamino acid fluorides for β-peptide synthesis; and (c) show the relevance of a $C_{10}H_4F_{17}$-substituted benzylic ester protecting group in solution-phase coupling.

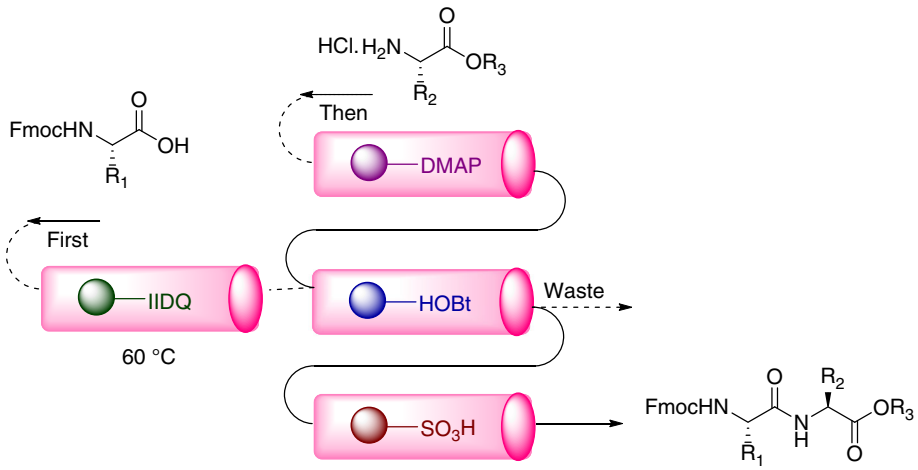
Here they made use of an acid fluoride form of amino acid since these molecules are readily available, strong acylating agents, and produce NH_4F as a soluble by-product (by reacting with the base, Et_3N). First, Fmoc-β^3hPhe-F (**11**) was coupled with H-β^3hLys(2-ClZ)-OBn (**12**) under flow reactor conditions (Scheme 8). The reaction was conducted at a range of temperatures (25, 60, and 90 °C) and times (1, 2, 5, and 10 min). Product **13** was achieved in maximum yield after 3 min at 90 °C. In a similar fashion, Boc-amino acid fluoride was also used to obtain a dipeptide.

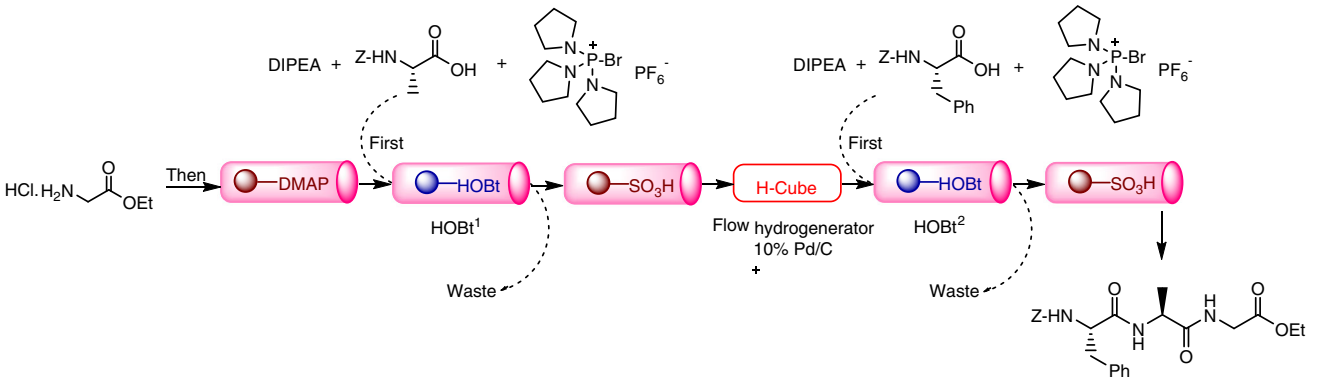
Having synthesized dipeptides, next aim of the authors was to obtain tetrapeptides (Scheme 9). The synthesis started with the introduction of a fluorous benzyl group onto the first amino acid by means of fluorous solid-phase extraction (FSPE), with the aim to enhance the purification. Dipeptide **14** resulted in 91 % yield after a 3-min reaction at 90 °C. Tripeptide **16** was formed in 93 % yield at 120 °C and with a residence time of 5 min. This was followed by tetrapeptide bond formation **18**, which was achieved at 120 °C and with a reaction time of just 1.5 min (Yield: 81 %). Free peptide **19** was released by the removal of OBn group and fluorous tag.

Peptide-macrocycle construction in microfluidics

Peptide macrocycles are attracting considerable attention (Jebrail et al. 2010 and references cited therein) as their properties render them stable against peptidases and therefore biologically active. In this context, (Jebrail et al. 2010) devised a new automated synthesis called 'digital microfluidics', in which nL to μL of samples can be used under the influence of electrical potential. Digital mirofluidics involve several reagent reservoirs and actuation electrodes, the latter used for dispensing, merging, and mixing droplets of reagents and products. They carried out a three-component reaction that contained amino acid **20**, aziridine aldehyde **21**, isocyanide **22,** which resulted in **23**, which in turn upon treatment with PhCOSH gave **24** (Scheme 10).

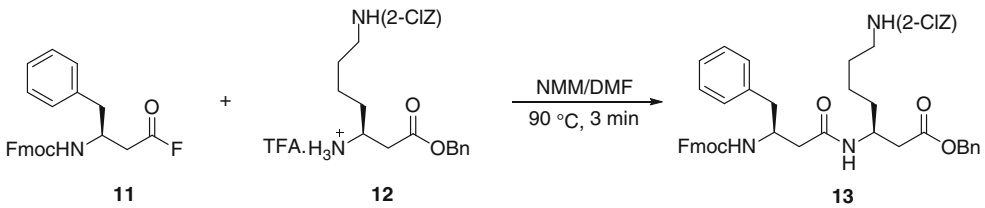
Scheme 6 Synthesis of Fmoc-containing dipeptides enabled by immobilized reagents





Scheme 7 Synthesis of a tripeptide enabled by immobilized-flow-based chemistry

Scheme 8 Fmoc-β-dipeptide synthesis using fluorine-activated amino acid

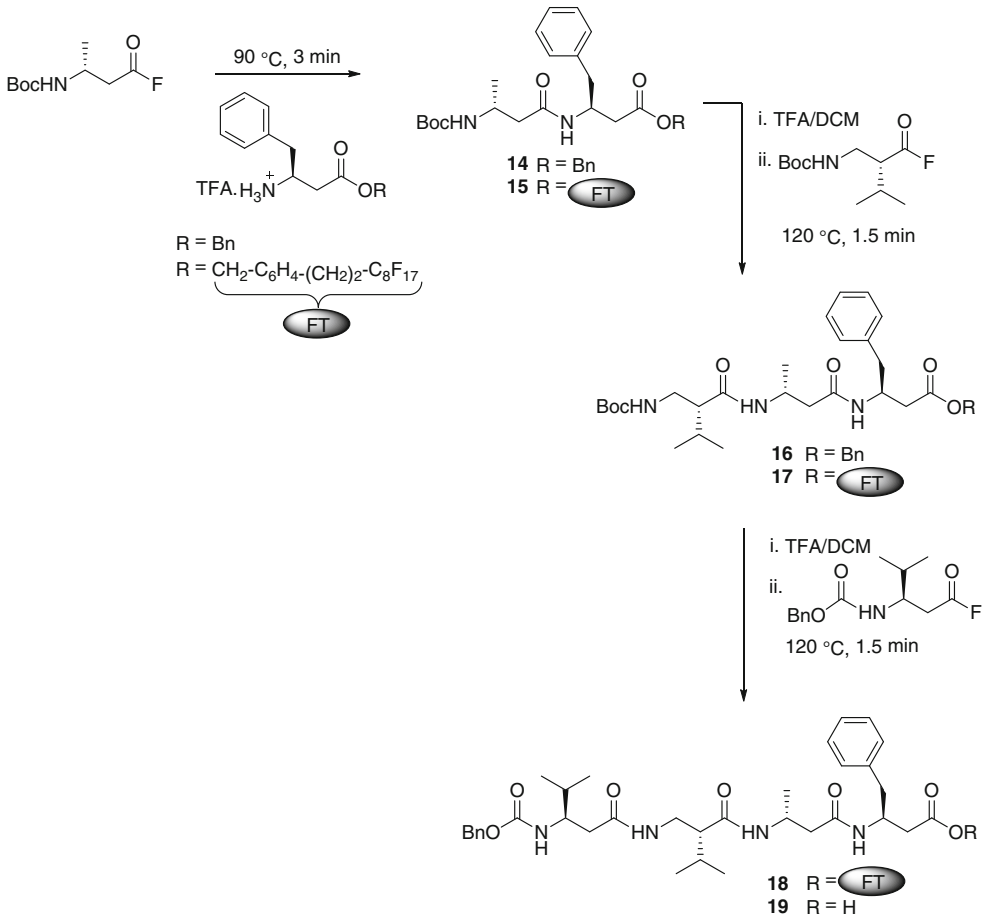


Furthermore, those authors extended this application to macrocyclization. A nine-membered cycle **26** was synthesized (Scheme 11) staring from prolyl-leucine (**25**) using the three-component method, as discussed above. This technique offers fast and automated synthesis of libraries of analogues that may have applications in drug discovery and high-throughput screening.

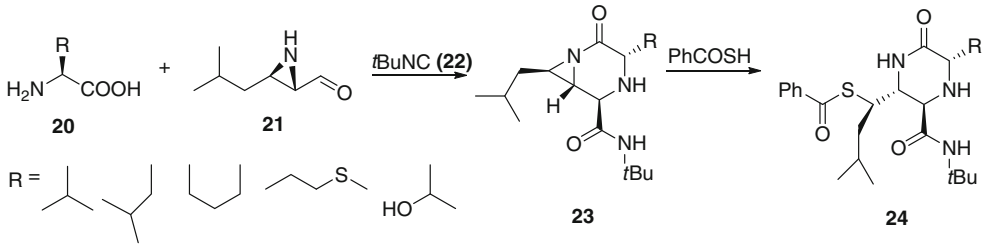
Microflow peptide synthesis facilitated by highly active species

Species regarded as 'highly active' are of interest to organic chemists since they react rapidly with sterically and electronically hindered reactants. In this regard, (Fuse et al. 2014) reported an efficient method for the formation of peptide bonds through rapid and strong activation of carboxylic acids (0.5 s). They used less toxic and inexpensive solid triphosgene for the activation of the carboxylic group of easily racemizable amino acids and found that racemization is as low as 3 % in microflow synthesis. The reaction between active species and less nucleophilic amines, which included *N*-methyl amino acids, was so rapid (4.5 s) that it yielded excellent results. The setup included two T-shape mixers with Teflon® tubing, and the samples dissolved in suitable solvents were pumped through these mixers. Experimentally, authors have identified that a symmetric, highly electrophilic anhydride is the actual active species formed, which was generated in just 0.5 s.

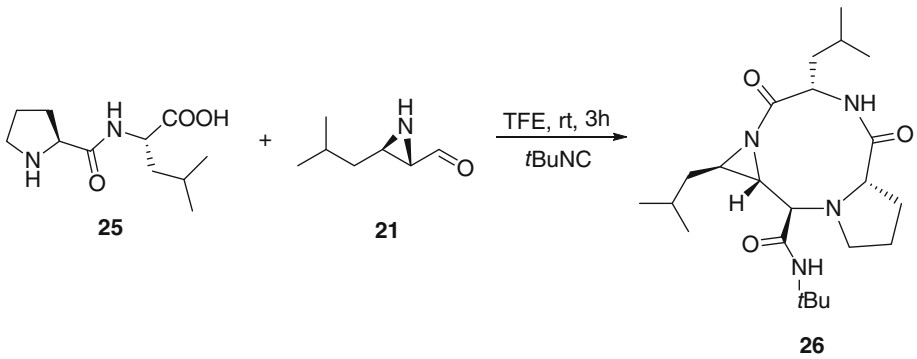
Scheme 9 Synthesis of a tetrapeptide under microfluidic conditions



Scheme 10 Peptide-based macrocycles in digital microfluidics



Scheme 11 Macrocyclization in microfluidics



Initially, substrates **27** and **28** were chosen because they contain α-substituted bulky functions (Scheme 12). The best results were obtained for dipeptide **29** (92 %) when 2.5, 1.0, and 3.0 equiv. of **27**, **28**, and DIPEA were used, respectively. In order to look at the scope and limitations of this method, several substrates were tested (Table 3).

Scheme 12 Synthesis of dipeptides enabled by triphosgene in microflow

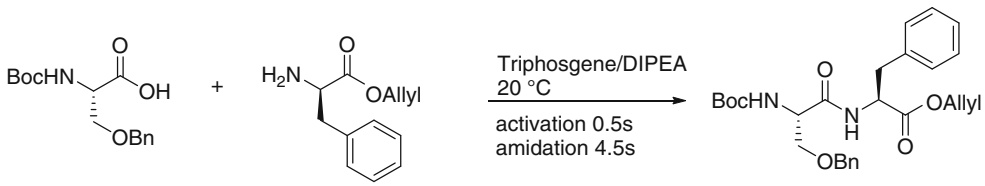
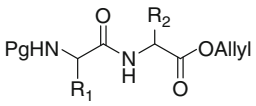
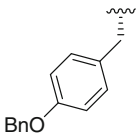
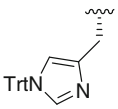
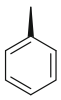
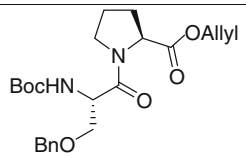
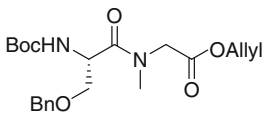
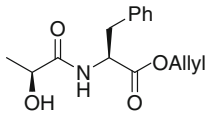


Table 3 Various dipeptides prepared under continuous flow conditions using highly active species



Pg	R_1	R_2	Yield (%)
Boc			92
Boc			quant.
Fmoc			92
Fmoc			94
Boc			97
Boc			89
			80
			74
			98

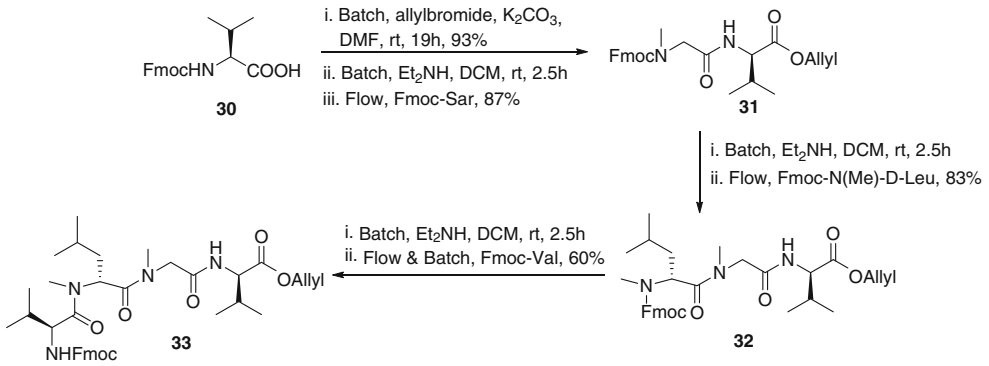
Subsequently, a tetrapeptide analogue **33** of a depsipeptidic natural product, auliride, was constructed in the microreactor by deprotection/washing/coupling/washing with the intermediate dipeptide **31** and tripeptide **32** (Scheme 13). For the sake of convenience, batch and flow conditions were maintained depending on the necessity.

Verification of racemization during peptide synthesis by microreactions

So far, we have discussed the synthesis of various kinds of peptides by different methods in flow reactors. However, another important issue, namely racemization while using α-amino acids, needs to be addressed. Watts et al. (2002a, b) worked on this concept by adopting low reagent concentrations and less reaction time during peptide synthesis within a microreactor. These authors performed the reaction of PFP ester of *R*-phenylbutyric acid **34** and *S*-α-methylbenzylamine **35** at 0.1 M concentration (Scheme 14). The desired product **36** was obtained in quantitative yield within 20 min with 4.2 ± 1.1 % racemization. When the concentration of the reactants was increased to 0.5 M, racemization increased to 7.8 ± 1.0 %. Next, the level of racemization was demonstrated using Boc-*D*-Ala **37a** and *S*-α-methylbenzylamine **35** via EDCI coupling to yield 61 % of the dipeptide **38a**. Similarly Boc-*L*-Ala **37b** was also converted to peptide **38b** with 84 % yield (Scheme 15). Under these flow conditions, 5.6 ± 0.8 % racemization occurred.

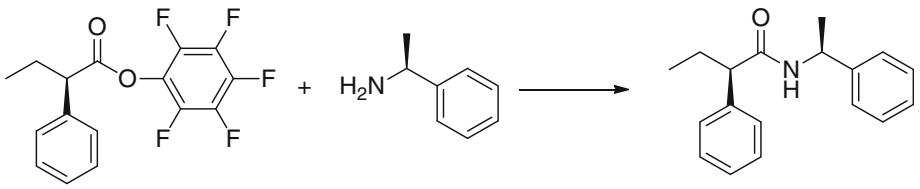
Summary and prospects

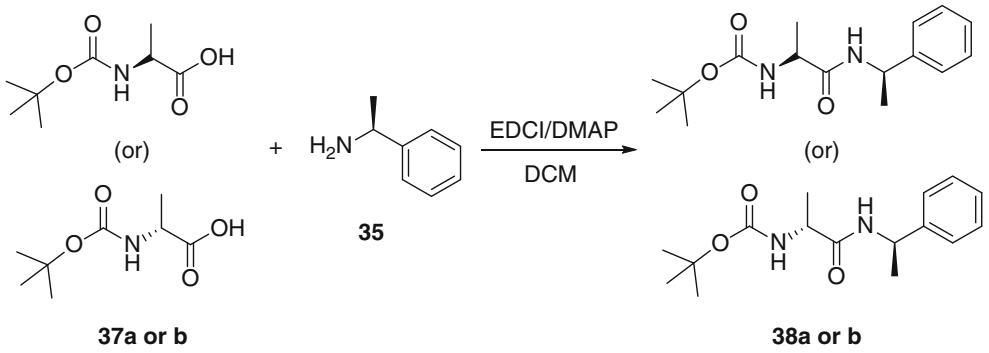
To date, an extraordinary variety of chemical transformations have been achieved in microreactors. Of these, several been adopted for industrial large-scale production purposes. These devices offer a number of advantages over other methods; they give excellent yields in a short period of time, they show greater effectiveness and acceptable selectivity, and they are environmental friendly, to mention but a few. Transformations carried out under microflow conditions have found applications mainly in the field of combinatorial chemistry and high-throughput screening for the generation of diverse compounds. The move from batch mode reactions into flow processing holds great potential for chemists. Although flow processing is progressing, it has not yet achieved the status that it truly deserves. In this regard,



Scheme 13 Assembly of a tetrapeptide in microreactor

Scheme 14 Active ester method of amide bond formation in flow reactor





Scheme 15 Microflow preparation of Ala-containing peptides and verification of the extent of racemization

synthetic chemists need to change their mind-set to embrace a new technology that offers greater productivity in less time. However, it is also true that flow chemistry will not bring an end to traditional batch reactions. In the long term, flow chemistry has the potential to bring about a revolution in synthetic chemistry, as did chromatography about six decades ago.

This minireview seeks to provide an overview of flow-enabled chemistry, particularly the synthesis of peptides (amide bond formation) since these molecules and their derivatives hold promise as therapeutics. This technology allows the preparation of small peptides in high yields in a short period, in a few instances in only a few seconds when using microstructured reactors. These initial breakthroughs deserve recognition as significant achievements that could be conveniently adopted by the scientific community in times that demand "large amounts in less time".

Conflict of interest Authors declare that they have no conflict of interest.

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