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# The synthesis of peptides using micro reactors†

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**We have demonstrated the first application of multi-step synthesis within a micro reactor and have shown that peptides may be prepared in quantitative yield in a period of 20 min, compared with batch reactions where only moderate yields (40–50%) were obtained in a 24 h period.**

During the past ten years, there has been a rapid growth in the development of micro-Total Analytical Systems (μ-TAS)[1–3] which exploit electroosmotic flow (EOF).[4] The development of micro reactor devices for chemical synthesis based on complementary technology is less common. However, recent research has shown that Suzuki[5] and Wittig[6] reactions may be performed using micro reactor systems.[7]

Peptides have been commonly prepared *via* solid supported techniques since its introduction by Merrifield in 1963.[8] Solid phase peptide synthesis is based on the addition of a protected amino acid residue to an insoluble polymeric support. The acid-labile Boc group[9] and base-labile Fmoc group[10] have been commonly used for *N*-protection. After removal of the protecting group the next protected amino acid may be added using either a coupling reagent or a pre-activated amino acid derivative. If this dipeptide is the desired product, it may be cleaved from the polymer support using various reagents, one of the more common methods being treatment with 25–80% HF.[11] If a longer peptide is required additional amino acids can be added by repeating further coupling reactions.

Solid phase peptide synthesis has the disadvantage that a fairly expensive polymer support is required. In addition, extra steps are added to the synthesis as a result of initially linking the amino acid to the support and finally having to remove the peptide from the polymer. In this paper a micro reactor has been used to prepare peptides using solution phase chemistry in an attempt to overcome some of the current problems associated with such syntheses.

The micro reactor devices used in this work were prepared using standard procedures developed at Hull.[12] A schematic of a typical micro reactor produced using such fabrication techniques is shown in the graphical abstract.† Microporous silica frits[13] were placed in the channels to prevent hydrodynamic flow occurring.

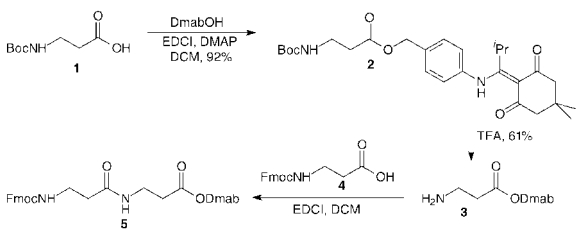
In the first instance a one-step reaction was considered in which an *N*-protected β-amino acid was reacted with an *O*-protected β-amino acid, to prepare the protected β-dipeptide. To enable the methodology to be applicable to the synthesis of more complex peptides, the use of orthogonal protecting groups was clearly required. After careful consideration, the base-labile Fmoc protecting group[10] was selected for *N*-protection while the Dmab ester[14] was chosen for protection of the carboxylic acid. Importantly, both protecting groups may be removed under mild conditions, since electroosmotic flow is retarded if the pH of the reaction is outside the range 3–10.

† Electronic supplementary information (ESI) available: schematic of a borosilicate glass micro reactor. See http://www.rsc.org/suppdata/cc/b1/b102125g/

Commercially available Boc-β-alanine **1** was protected as the Dmab ester using an EDCI [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride] and DMAP coupling reaction, to give the ester **2** in 92% yield in a bulk reaction (Scheme 1). Treatment of **2** with trifluoroacetic acid furnished the desired amine **3** in 61% yield, which was subsequently reacted with Fmoc-β-alanine **4** *via* a carbodiimide coupling reaction, to give a synthetic sample of dipeptide **5**.

Having prepared dipeptide **5**, it represented a synthetic target for preparation using the micro reactor. Prior to synthesis, the micro reactor channels were primed with anhydrous DMF to remove any air and moisture from the channels and the microporous silica frits. A standard solution of Fmoc-β-alanine **4** (50 μl, 0.1 M) in anhydrous DMF was added to reservoir A, a solution of EDCI (50 μl, 0.1 M) was placed in reservoir B and a solution of amine **3** (50 μl, 0.1 M) was placed in reservoir C. Anhydrous DMF (40 μl) was placed in reservoir D, which was used to collect the products of the reaction. Platinum electrodes were placed in each of the reservoirs (A, B and C positive, D ground) and an external voltage was applied to the channels inducing electroosmotic flow of the reagents. The reactions were conducted at rt for a period of 20 min, in order to acquire sufficient volume of product to determine the yield of the reaction. Analysis was achieved by high performance liquid chromatography (Jupiter $C_{18}$ 10 μm, 4.6 × 250 mm, obtained from Phenomenex), mobile phase composition: 0.1% TFA in water and 0.1% TFA in acetonitrile, using a gradient system of 30% aqueous to 70% aqueous over 20 min, with a flow rate of 2.5 ml $min^{-1}$ at rt).

When stoichiometric quantities of the reagents were used only *ca*. 10% conversion to peptide **5** was achieved when a voltage of 700 V was applied to the reagents (A, B and C). However, by using two equivalents of EDCI (0.2 M solution) the yield of the reaction was increased to *ca*. 20%. By applying a stopped flow technique (2.5 sec injection length with flow stopped for 10 sec) the yield of the reaction was further increased to 50%. Since the yield of the reaction appeared to greatly depend on the number of equivalents of EDCI used, we wished to further investigate the effect of carbodiimide concentration on the reaction, however we found that EDCI was insoluble in DMF above 0.2 M concentrations. In further experiments DCC was used as the coupling reagent as it was considerably more soluble in DMF. Using 5 eq. of DCC (0.5 M solution in reservoir B) a 93% yield of dipeptide **5** was obtained using the optimised conditions described above.




**Scheme 1** Synthesis of standard dipeptide derivative.

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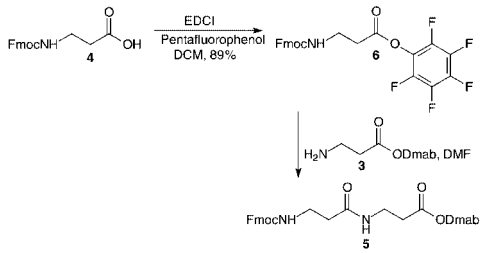
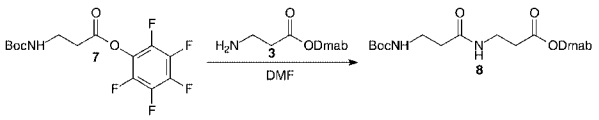
Another common method utilised in peptide bond formation involves the reaction of a pre-activated amino acid derivative, such as a pentafluorophenyl ester, with an amine.[15,16] Fmoc-β-alanine **4** was activated as the pentafluorophenyl ester **6** *via* an EDCI coupling reaction (Scheme 2). The pentafluorophenyl ester **6** was stable and could be stored indefinitely in the freezer. The ester **6** was subsequently reacted in bulk with amine **3** to produce dipeptide **5**.




**Scheme 2** Preparation and reaction of pentafluorophenyl esters of Fmoc protected amino acids.

Having prepared dipeptide **5** *via* the alternative pre-activated strategy, we wished to investigate if the reaction could be performed in a micro reactor. A standard solution of the pentafluorophenyl ester of Fmoc-β-alanine **6** (50 μl, 0.1 M) in anhydrous DMF was added to reservoir A, a solution of amine **3** (50 μl, 0.1 M) was placed in reservoir B and anhydrous DMF (40 μl) was placed in reservoir D, which was used to collect the products of the reaction. It was found that using continuous flow of both reagents, where the ester **6** was maintained at 700 V and the amine **3** was maintained at 600 V, dipeptide **5** was produced in quantitative yield in just 20 min. This represented a significant increase in yield compared with the traditional batch synthesis where only 40–50% conversions were obtained.

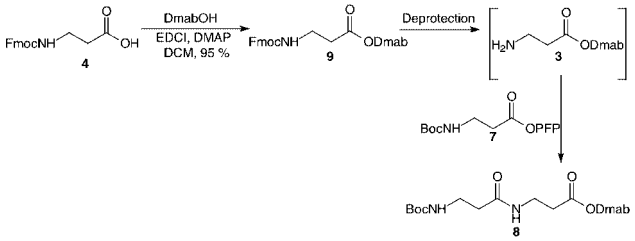
Similarly, the reaction between the pentafluorophenyl ester **7** of Boc-β-alanine and amine **3** was also investigated in the micro reactor (Scheme 3). In this case, when the reagents were mixed under a continuous flow regime, with both reagents maintained at 700 V, a quantitative yield of peptide **8** was observed. Importantly, this result demonstrates that both Boc and Fmoc protecting groups are suitable for use in the preparation of peptides using micro reactors.




**Scheme 3** Reaction of pentafluorophenyl esters of Boc-β-alanine.

Having successfully demonstrated that peptide bonds could be formed in micro reactors using two common methods, we wished to show that we could extend the methodology to the preparation of longer chain peptides. Consequently, we needed to be able to conduct deprotection reactions in the micro reactor and subsequently perform further peptide bond forming reactions. Fmoc-β-alanine **4** was converted into the Dmab ester **9**, in a bulk reaction, using standard conditions (Scheme 4). It was proposed to convert ester **9** into amine **3** by deprotection of the Fmoc group in the micro reactor and subsequently react the amine '*in situ*' with pentafluorophenyl ester **7**, to give the dipeptide **8**. Treatment of **9**, with 10 eq. of piperidine in DMF using the micro reactor, resulted in 60–70% deprotection over a 20 min period, to give amine **3**.[17]

Subsequently, a standard solution of the Dmab ester of Fmoc-β-alanine **9** (50 μl, 0.1 M) in anhydrous DMF was added to reservoir A, a solution of piperidine (50 μl, 1.0 M, 10 eq.) was placed in reservoir B and a solution of pentafluorophenyl ester **7** (50 μl, 0.1 M) was placed in reservoir C, in an attempt to prepare dipeptide **8** using this multi-step approach. Anhydrous DMF (40 μl) was placed in reservoir D, which was used to collect the products of the reaction. The HPLC of the reaction mixture showed that Fmoc deprotection had occurred, however




**Scheme 4** Multi-step peptide synthesis.

no peptide was evident. It was however found that the excess piperidine used in the reaction was reacting with the pentafluorophenyl ester **7** to give amide **10**.

As a result, an alternative method of Fmoc deprotection was required that would not cause the aforementioned problem. Using the micro reactor, the Dmab ester of Fmoc-β-alanine **9** was reacted with one equivalent of DBU to give the free amine **3** which was then reacted with the pentafluorophenyl ester of Boc-β-alanine **7**, in an attempt to form the dipeptide **8**.

In this case, when the reagents were mixed using continuous flow, with the reagents maintained at 700 V, product **8** was observed in typically 25% yield. By comparing the flows of each reagent at this stage we were able to optimise the reaction. The Dmab ester of Fmoc-β-alanine **9** was maintained at 750 V while reacted with DBU at 800 V. The deprotected amine was then reacted, using continuous flow, with the pentafluorophenyl ester of Boc-β-alanine **7** to give a conversion of 96%, based on the amount of Dmab ester **9** present at the end of the reaction.

Having shown that more complex peptides could be produced by removal of the *N*-protecting group we wished to determine if we could remove the Dmab protecting group using hydrazine. Hence, a solution of the Dmab ester of Fmoc-β-alanine **9** (50 μl, 0.1 M) in anhydrous DMF was added to reservoir A and a solution of hydrazine (50 μl, 0.1 M) was placed in reservoir B. Anhydrous DMF (40 μl) was placed in reservoir D, which was used to collect the products of the reaction. Using continuous flow of both reagents, maintained at 700 V, quantitative deprotection was observed to give carboxylic acid **4**.

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