Cost Effective Flow Peptide Synthesis: Metamorphosis of HPLC

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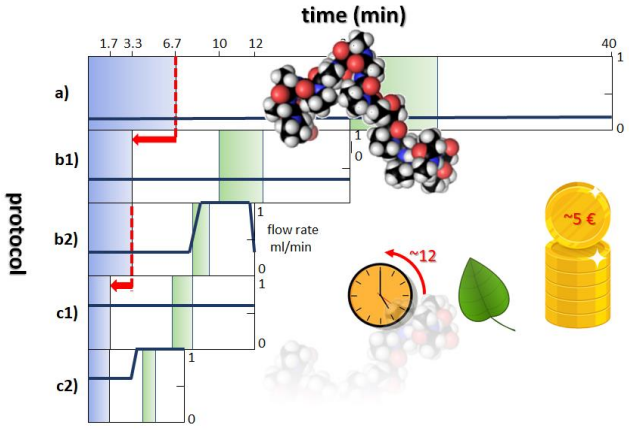
Abstract

We present the further development and fine-tuning of an efficient, economic (<3 molar excess) and environmentally friendly (6 ml organic waste/cycle) procedure for peptide synthesis, using fast amino acid coupling cycles (1.7 min/cycle). The designed setup can assist the synthesis of highly pure (>80%) raw materials even for long (up to 30 aa.) and/or difficult sequences. The significant reduction of the coupling time by using the effective PyAOP and DIC/HOBt coupling reagents was achieved and virtually racemization free (L-His/D-His <1-5 %) peptides can now be synthesized, even at high column temperate: T = 70 °C. The purity of the product and the efficacy of the synthesis were evaluated using different solid phase supports and protocols. We successfully completed the synthesis of "difficult sequences" (*e.g.* Insulin B-chain, Cecropin A(1-7)-Melittin (2-9) hybrid peptide) wasting only a fraction of the organic solvents compared to other methods, furthering peptide chemistry toward a greener approach.

Graphical Abstract



Keywords

flow chemistry, solid phase peptide synthesis, green chemistry approach, automated amino acid coupling, fast and robust polypeptide synthesis

Abbreviations:

Boc: tert-butyloxycarbonyl, **DIC**: *N,N'*-diisopropylcarbodiimide, **DIEA**: *N,N*-diisopropylethylamine, **DMF**: *N,N*-dimethylformamide, **EDT**: ethanedithiol, **FLP**: flow peptide chemistry **Fmoc**: fluorenylmethyloxycarbonyl, **HOAt**: 1-hydroxy-7-azabenzotriazole, **HOBt**: hydroxybenzotriazole, **HRMS**: high resolution mass spectrometry, **MW**: microwave, **NMP**: *N*-methylpyrrolidone, **Oxyma Pure**: ethyl cyano(hydroxyimino)acetate, **PyAOP**: (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, **PyBOP**: benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, **PS**: polystyrene, **SPPS**: solid phase peptide synthesis, **TFA**: trifluoroacetic acid, **TIS**: triisopropylsilane

Introduction

The ever expanding variety of the applications for peptides and peptidomimetics[1] as synthetic nanomaterials and pharmaceuticals provides a continuous boost of the automation of synthetic methods, including automated solid phase peptide synthesis (SPPS) and flow peptide chemistry (FLP). Early development of these methodologies was hindered by the fact that the semi and fully automated protocols are poorly compatible with one of the most-widely used, classical Boc-chemistry, as the highly acidic TFA used for deprotection rapidly corrodes valves and junctions of any apparatus. Also, the pressure and current applied in flow-reactors fragment the polystyrene (PS) solid support, producing debris that can cause blockade and various malfunctions.[2] Therefore, technical challenges dislodged the advance of flow chemistry in peptide-synthetic applications thus, the method remained inferior compared to - for example - microwave assisted peptide synthesizers. The appearance and wide-spread application of Fmoc-chemistry, using milder reagents,[3] and the design of polyethylenglycol (PEG) based resins, such as TentaGel® or ChemMatrix®, which show more robustness and better pressure resistance opened a new era for FLP.[4] The main advantages of SPPS are the highly efficient transfer of the solvent, activated coupling materials and excess of the unreacted reagents and thus, environmentally more friendly compared to batch processes.[5] By using of continuous flow as well as raised temperature and pressure, chemical reactions (*i.e.* amino acid coupling and Fmoc deprotection) are forced in the resin loaded reactor enhancing the chemical reaction intensification.[5a,6]

However, FLP methods need to become even faster, more efficient, robust and economic to produce α-, β-, and chimera peptides both for research and development purposes. Mándity et al. constructed first an HPLC based synthesizer of yet tested efficacy primarily for coupling β-amino acid residues, using 1.5-3 equivalent of reagent excess, at 70 °C, and 60 bar pressure,[5b] and the instrumentation and protocols have since been under continuous development.[6b,7] Mijalis et al. developed an impressively fast technique, reaching a breathtaking coupling and deprotection rate of 40 s/cycle, but at very cost as using 20 molar equivalent reagent excess at high temperature (90 °C).[8] Our aim was to combine the advantages of the available approaches and further their optimization by:

i. using a commercially available HPLC based continuous-flow peptide synthesizer,
ii. increasing the purity of the cleavage product,

iii. drastically reducing the solvent consumption,

iv. accelerating the coupling rate to reduce coupling time to <2 min!

We have tested cost saving coupling reagents (*e.g.* DIC), and probed the performance of our methods to produce difficult sequences, chosen either from the literature or designed and predicted to be challenging by the PeptideCompanion software.[9]

The main goal of this study was to improve the performance of a cost-effective HPLC –based flow reactor dedicated to peptide synthesis and to conduct a comparative analysis with respect to the available alternatives, *e.g.* microwave assisted SPPS.[10] We optimized several aspects of a broad range and versatile peptide synthesizer, including chemical flux and surplus molar equivalence and cycle time, and investigated their effect on cleavage product purity, running costs and maintenance, *etc.* with all their associated environmental impacts. Here we report optimization of protocols (**Figure 1**) with improved sustainability and cost-effectiveness allowing routine overnight synthesis of even long and challenging peptides sequences.

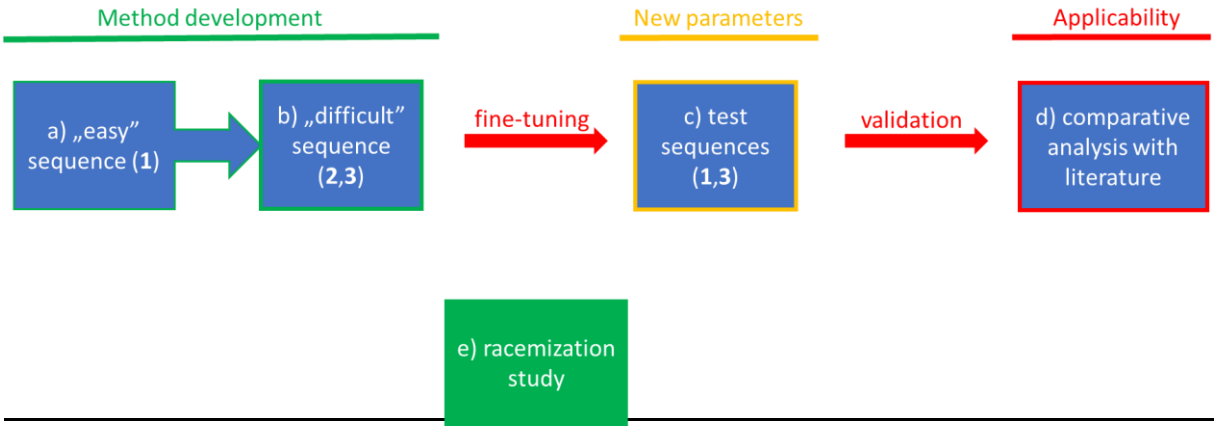


Figure 1. *Our stepwise optimization path concerning the fine-tuning of the apparatus and protocol*

Results and Discussion

Getting started

First, a 10 amino acid long, easy-to-synthesize polypeptide, H-IFDPETGTWI-NH_2 (**1**) [derived from Honda *et al.*[11]] was chosen, in which none of the amino acids has difficulty values higher than 1.2 (for more details see supplementary information). This peptide was prepared using 3 equiv. (calculated to resin capacity) of common coupling reagents such as, DIC/HOBt, DIC/Oxyma-Pure or PyBOP/DIEA, a flow rate of 0.15 ml/min at T= 70 °C and p= 75

bar (see **Table 2, Figure S1**) utilizing a coupling and Fmoc cleavage step (2 × 6.7 min) plus repeated resin-washing steps (2 × 13.3 min) (protocol **a**). The total time for coupling and deprotection was ~40 minutes/residue. The purity of the cleavage products were almost the same (>95%) (**Figure 2)**.

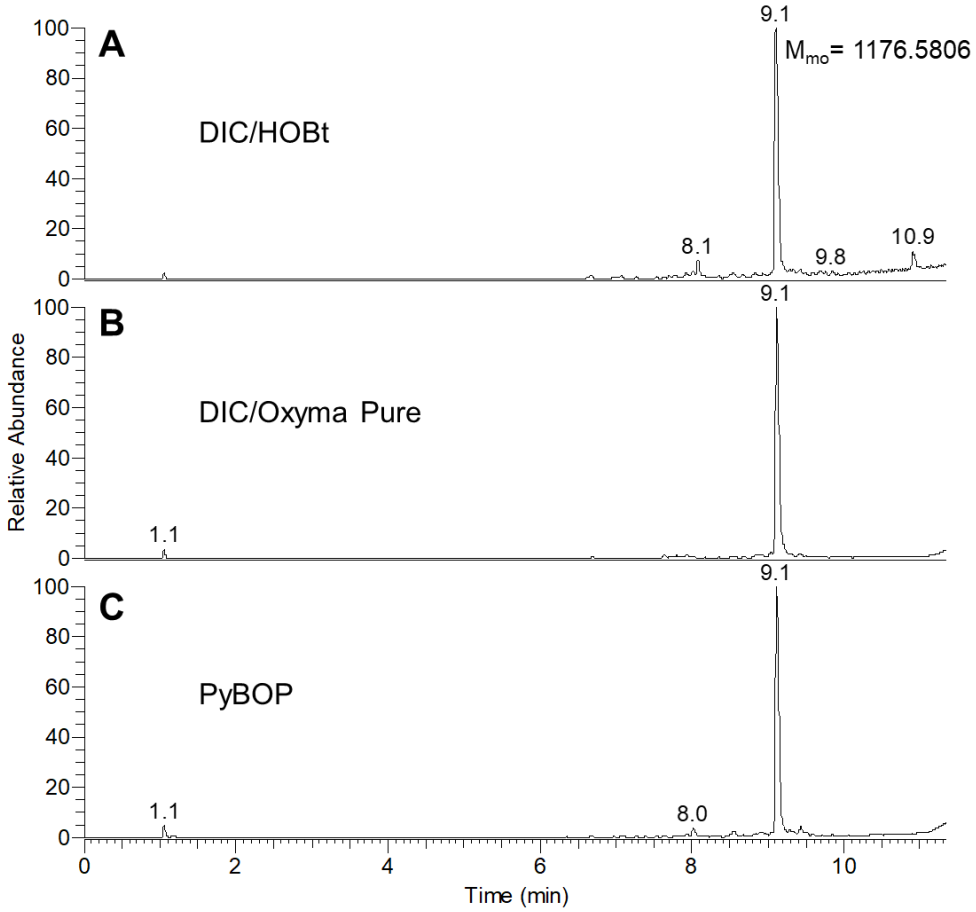


Figure 2. Purity of the cleavage product as the function of the applied coupling reagents. *Peptide* ***1*** *(*IFDPETGTWI*) was synthesized on TG RAM resin (c=0.24 mmol/g) using the following conditions: T = 70 °C, flow rate = 0.15 ml/min, p = 70-80 bar. Three equivalent (calculated to the capacity of the resin) coupling reagents were used (**A**: DIC/HOBt, **B**: DIC/Oxyma Pure, **C**: PyBOP).*

Subsequently, a more difficult sequence (**2**: EEEAV**R**LYIQWLK, a fragment of Trp-cage type miniprotein contains a problematic arginine in the middle) was synthesized.[12] Couplings were completed in DMF at 70 °C and 70-80 bar, using 3 equivalent of amino acids and reagents (DIC/HOBt) on a TG RAM resin (*c*= 0.24 mmol/g) at a flow rate of 0.15 ml/min. Beside the main product (65%), the characteristic side product (~33%) of the Arg deficient peptide (**Figure 3/A**) was obtained. This was achieved by applying reagent recycling (entry B, **Table 1)**, which is a common concept in flow chemistry, since it allows for augmenting the

contact time between the solid support and the soluble reagents, supporting enhanced conversion and coupling efficacy.[5b,13]

Table 1. *The applied synthetic conditions to produce the 13-mer* ***2*** (EEEAV**R**LYIQWLK)

protocol	**operation mode**	**flow rate** (ml/min)	**concentration of Arg^6** (M)	**residence time** (min)	**solvent of the used reagent**	**purity of the crude peptide**	**total synthesis time** (h)
A	straight	0.15	0.1	6.7; 10	DMF	65%	9.8
B	recycling	0.15	0.1	2 x 6.7	DMF	<20%	25
C	straight	0.15 & 0.05^{Arg} & $0.1^{Val\text{-}Glu}$	0.2	6.7; 10	NMP, DMF	80%	9.8
D	straight	0.15 & 0.05^{Arg} & $0.1^{Val\text{-}Glu}$	0.2	6.7; 10	NMP	81%	9.8

However, we found recycling unrewarding, as the purity of the cleaved crude product was unsatisfactory (20%) (**Figure 3/B**). Thus we altered the protocol as follows: no recycling, increased residence time and excluded repeated washing steps. Reagent residence time on the resin was increased by decreasing the flow rate of Arg^6 from 0.15 ml/min to 0.05 ml/min and using higher concentration (c = 0.2 M) of Arg. This modification led to a purer product (80%) (**Figure 3/C**), in a more environmentally friendly process. In addition, the thermostable NMP as a co-solvent was probed,[10,14] which also clearly improved the purity of the raw product (**Figure 3/C, D**).

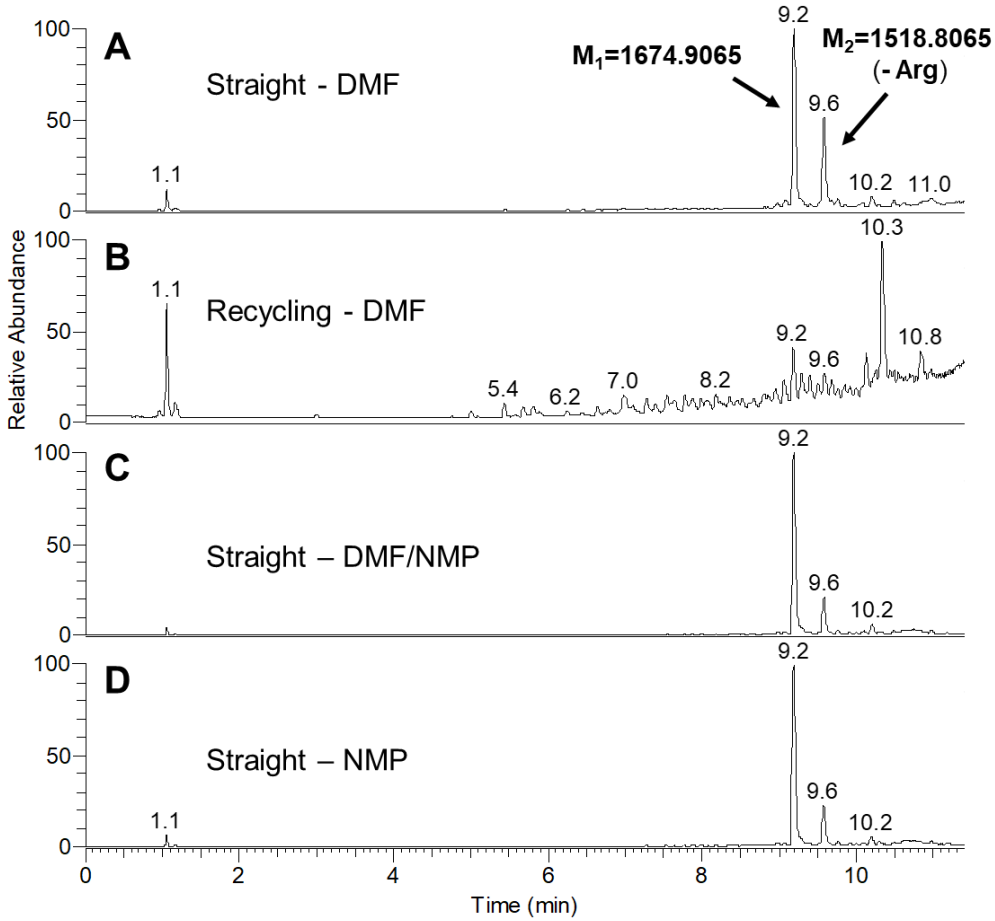


Figure 3. *LC-MS analysis of the raw products of* ***2*** *by either using recycling or straight synthesis without and with NMP. (For more parameters on the synthesis see* ***Table 1****.)*

Enhancing coupling efficacy

Active ester formation is the key step to consider during peptidic bond formation. Both DIC/HOBt and PyBOP form the same active ester, but with a different conversion efficacy. The mechanism of active ester formation was described exhaustively in the literature.[15] Most recently, based on NMR analysis quantitative data were obtained and a kinetic model of the coupling mechanism was constructed including the negative impact of the most significant side reaction, namely hydrolysis.[16] Our finding was in line with the previous observations of Albericio *et al*. denoting that if larger molar excess and stirring was applied, conversion up to 75 % could be obtained even faster (2 min) for PyBOP.[15a] Indeed, we found that both PyAOP and HATU results in a rapid amino acid active ester formation for **1** (**Table 2**: #10). Probably flow conditions (*T*= 70 °C, *p*= 80 bar) further accelerate the very same reaction. As the general rule of thumb, every 10 °C increase of temperature doubles the

reaction rate coefficient, especially if high pressure is applied simultaneously for a condensation reaction.

Due to our optimization efforts the initial reaction time (6.7 min/coupling) was successfully reduced to 1.7 min/coupling. Using PyAOP the flow rate was systematically increased (**Table 2** /entries #7-9) as the active ester formation is fast, without compromising the coupling efficacy. This concludes in a net reduction of cycle time (**Table 2**: #7, 8, 9→ 40, 20, 12 min/cycle) and increase of the overall cycling rate, with keeping the purity of the raw product as high as >95 %. Using the cost effective DIC/HOBt combination, reaction time could be reduced, coupling became more effective, especially when both DIC molar excess and temperature were increased (**Table 2**).

Table 2. *Coupling efficacy of* ***1*** (#1-10) *and* ***3*** (#11-15) *as the function of the coupling conditions* [a]

#	**model polypeptide**	**coupling reagent applied: type** (equiv.)	**protocol**[a]	**column temperature** (°C)	**total synthesis time** (h)	**purity**[b] (%)	**yield**[c] (%)
1	**1** (10 mer)	DIC / HOBt (1.5/1.5)	**a**	70	7	73	47
2		DIC / HOBt (3/3)	**a**	70	7	80	54
3		DIC / Oxyma (3/3)	**a**	70	7	82	76
4		DIC / HOBt (6/3)	**b1**$^{80°C}$	80	3.5	93	84
5		PyBOP / DIEA (3/6)	**a**	70	7	93	57
6		PyBOP / DIEA (3/6)	**b1**	70	3.5	83	65
7		PyAOP / DIEA (3/6)	**a**	70	7	95	64
8		PyAOP / DIEA (3/6)	**b1**	70	3.5	95	61
9		PyAOP / DIEA (3/6)	**c1**	70	2.1	95	63
10		HATU / DIEA (3/6)	**c1**	70	2.1	94	57
11	**3** (14 mer)	DIC / HOBt (3/3)	**a**	70	9.7	~50*	89
12		PyBOP / DIEA (3/6)	**b1**	70	4.8	68	86
13		DIC / HOBt (6/3)	**b1**$^{80°C}$	80	4.8	83	79
14		HATU / DIEA (3/6)	**c1**	70	2.9	83	73
15		PyAOP / DIEA (3/6)	**c1**	70	2.9	82	98

1: IFDPETGTWI and **3**: KR**LFK**KLLFSLRKY were synthesized on TG RAM resin (c=0.24 mmol/g) using p = 70-80 bar

[a] see **Figure 4** for details

[b] purity of the crude peptide (HPLC analysis, peak area % using 220 nm UV-absorbance)

[c] calculated from theoretical yield using 150 mg resin (c=0.24 mmol/g): **1**: 42.4 mg, **3**: 66,2 mg

*purity couldn't be determined properly

To test the effectiveness of our emerging strategy a difficult sequence, **3** (KR**LFK**KLLFSLRKY, encompasses residues scored higher than 1.2 by PeptideCompanion, -**LFK-**[17]) was synthesized using both 3 and 6 molar excess of DIC as well as PyAOP reagents (**Table 2:** #11-15). The increased DIC ratio (DIC/HOBt: 6/3 molar excess) made coupling even faster and produced a more homogenous product (purity >83 %, **Table 3:** #13). Using more expensive reagents (PyBOP and HATU **Table 2:** #14,15) the purity of the product did not change significantly, however the yield was higher in the case of PyAOP (**Table 2**, #15, **Figure S2**).

To further shrink synthesis time, we modified the HPLC module based setup. As flow rate can routinely be adjusted during HPLC purification, the same can be applied during peptide synthesis. First, protocol **b1**$^{80°C}$ was modified by changing the flow rate of washing (increasing from 0.3 ml/min to 1 ml/min at 9 min) and of Fmoc deprotection (**Figure 4**, **b2**). Furthermore, protocol **c1** was altered (by implementing a flow rate gradient from 0.6 to 1 ml/min at 3 min) to get protocol **c2** (**Figure 4**, **c2**).

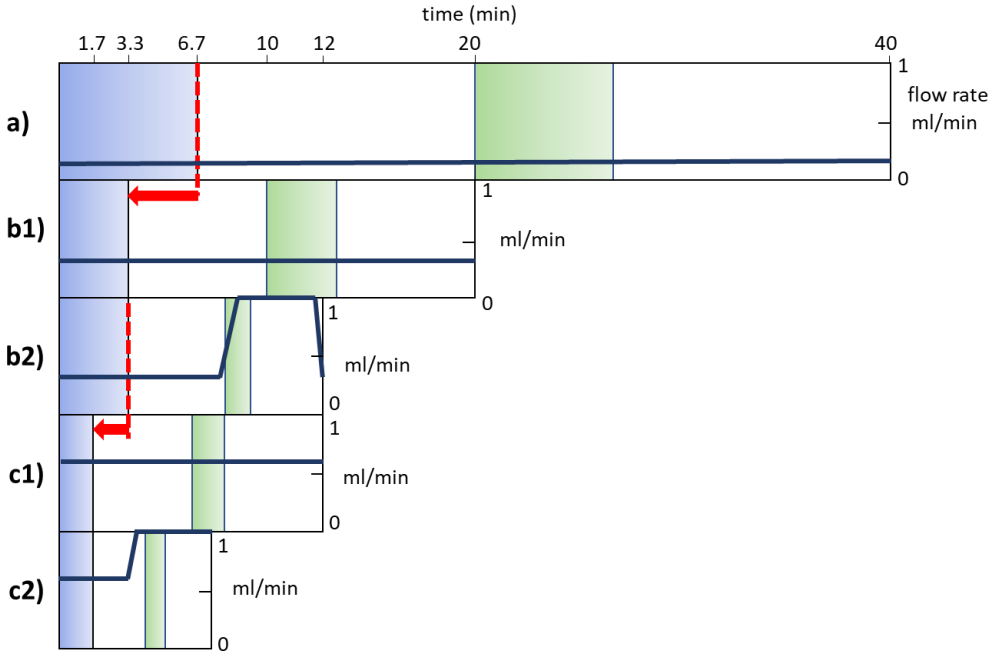


Figure 4. *Synthesis protocol step by step improved giving rise to faster polypeptide synthesis. Coupling (blue box), deprotection (green box) and washing (white box) steps are organized within a "cycle" and repeated as many times and requested by the primary sequence. Cycle time gets gradually shorter from protocol **a** to **c2:** 40.0 to 6.7 min. Flow rate changes as indicated (blue line). The red arrows highlight the reduced coupling time.*

Interestingly, using PyAOP and HATU coupling reagents and protocol **c2** of the shortest cycle time resulted in a high purity crude product. On the contrary, the same **c2** protocol failed with reagent DIC/HOBt. This can be explained with the higher reactivity of PyAOP (and HATU) compared to HOBt: the favorable neighboring effect of the azabenzotriazol group.[18] Altogether, applying PyAOP/DIEA supplies sufficient crude material purity both for **1** (>92 %) and **3** (>82 %) (**Table 3**).

***Table 3**: Coupling efficacy of **1** and **3** as function of the applied conditions*

#	peptide	coupling reagent	protocol [a]	***T*** (°C)	total synthesis time (h)	purity [b] (%)	yield [c] (%)
1	**1**	PyAOP/DIEA (3/6)	**c2**	70	1.4	92	62
2	**3**	PyAOP/DIEA (3/6)	**c2**	70	2.0	82	72

1: IFDPETGTWI and **3**: KR**LFK**KLLFSLRKY were synthesized on TG RAM resin (*c*= 0.24 mmol/g) using *p* = 70-80 bar
[a] see **Figure 3** for details
[b] purity of the crude peptide (HPLC analysis, peak area % using 220 nm UV-absorbance)
[c] calculated from the theoretical value (100%) using 150 mg resin (c=0.24 mmol/g), theoretical yield: **1**: 42.4 mg, **3**: 66.2 mg

The effect of resin type on the peptide purity and synthesis yield

The chemical composition and hydrodynamic properties of a resin *apriori* determine the success of the synthesis and thus, the efficacy of all couplings.[19] Polystyrene (PS) based resins are not suitable for flow chemistry as the applied temperature and pressure leads to fragmentation of the resin beads and small particles can create obstruction in the tubes and block the flow lines.[2] Polyethylene glycol (PEG) and PS block copolymer resins resist much better against higher pressure as well as temperature and perform better thanks to the special polarity and solvation propensities of the PEG chains. Purely PEG resins, such as ChemMatrix® are among the most efficient solid supports.[4d]

In our study three PS-PEG copolymer based resins and one PEG resin were probed. HypoGel® 200 and 400 are hydrophilic PS-PEG gel type resins which combines high capacities with good solvent compatibility. HypoGel® 200 consists of glycol spacers with *n*= 5, while HypoGel® 400 bears *n*= 10 ethylene glycol subunits. TentaGel® resin is a grafted copolymer consisting of a low crosslinked PS matrix on which 50-70 % PEG (w/w) is grafted. ChemMatrix® is a 100 % PEG resin that can improve the swelling properties in both polar and apolar solvents.

Both an easy (**1**) and a difficult (**3**) peptide were synthesized by using HATU/DIEA coupling reagents on 4 different solid supports. Cleavage products of **1** showed >90 % purity for all 4 resins (**Figure 5a**), while significant differences were detected when the more difficult sequence (**3**) was synthesized (**Figure 5b** and **5**). Synthesis on the ChemMatrix® resin resulted in the highest purity product (>80 %) for **3**, although TentaGel® resin gave relatively high purity cleavage product (74 %) as well. In contrast, the synthetic product on HypoGel®

200 and 400 resins exhibit a mixture of truncated sequences as well as uncompleted fragments. HypoGel® 400 resulted in a fewer number of side products compared to HypoGel® 200 (**Figure 6**). We found that the most frequent impurities were those where either Arg and/or Lys residues were deleted from the sequence (**Figure S3**).

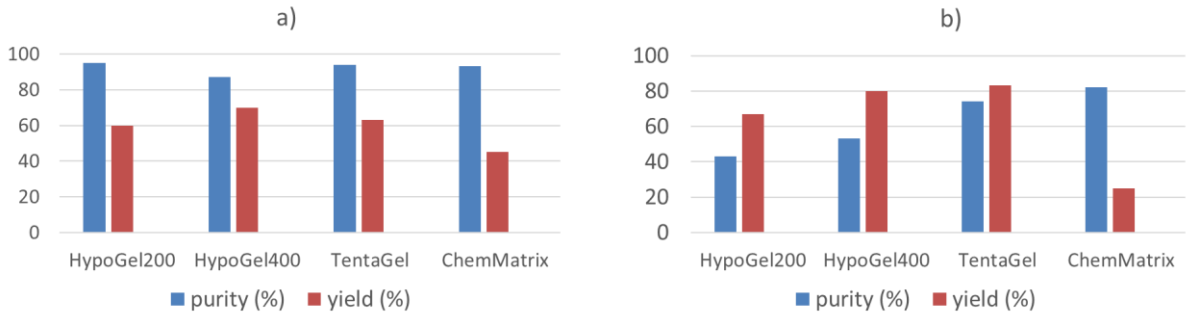


Figure 5. *Synthetically made crude product purity (%) and yield (%) of a)* ***1*** *and b)* ***3*** *as function of the different solid supports. Purity calculated according to the HPLC analysis, yield can be obtained from calculation using by resin capacity and volume (see Experimental)*

Unfortunately, the ChemMatrix® resin has resulted in a significantly lower yield (15.6 mg, yield 25 %) calculated to the resin capacity (75 mg, *c*= 0.51 mmol/g) (**Figure 6**). On the contrary, higher crude product yields were obtained when HypoGel® 200, 400 or TentaGel® were in use.

In conclusion, the PEG content of the solid support enhances the favorable synthetic properties of the resin, especially when a difficult sequence is synthesized both HypoGel® resins (200 & 400) performing poorly. Although the ChemMatrix® resin gave better synthetic profile, it resulted in only moderate yields. Therefore, from these commercially available resins, the TentaGel® is suggested here to be used, especially for demanding and more difficult peptide sequences.

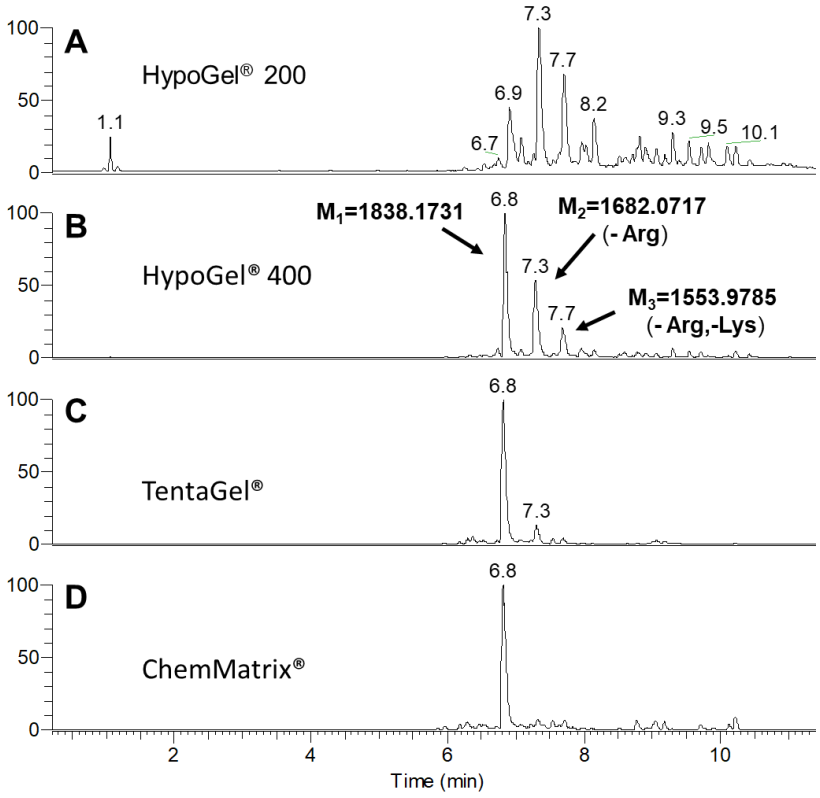


***Figure 6**. Quality control chromatogram of the cleavage products of polypeptide **3** synthesized on HypoGel® 200 (**A**), HypoGel® 400 (**B**), TentaGel® (**C**) and ChemMatrix® resin (**D**).*

Checking for Racemization

Both Cys and His residues are sensitive to racemization during coupling at higher temperature especially when bases are applied.[15b] We have monitored the degree of racemization by using HPLC for selected peptides. Racemization was followed in case of two *N*-acetylated tri- (**4** (F**H**L) and **5** (G**C**F)) and two *N*-acetylated hepta-peptides (**6** (Ac-V**H**NRTIG) and **7** (Ac-V**C**NRTIG)), all containing either His or Cys residues using either DIC/HOBt or PyAOP coupling reagents on TentaGel resin. The degree of racemization of the above 4 test peptides was quantitatively characterized by the *D*-His/*L*-His or *D*-Cys/*L*-Cys ratios (in %, according to HPLC analysis).

Table 5. *Degree of racemization of His and Cys residues during the synthesis of* ***4, 5, 6, 7*** *peptides.*

4 (FHL)				
#	**reagent**	**protocol**	***T*** (°C)	**D-Xxx / L-Xxx** (%)
1	DIC/HOBt	b2	80	<1
2	PyAOP/DIEA	c2	70	3
5 (GCF)				
1	DIC/HOBt	b2	80	<1
2	PyAOP/DIEA	c2	70	3
6 (Ac-VHNRTIG)				
1	DIC/HOBt	b2	80	1
2	PyAOP/DIEA	c2	70	5
7 (Ac-VCNRTIG)				
1	DIC/HOBt	b2	80	1
2	PyAOP/DIEA	c2	70	4

We found that when using PyAOP the diastereomeric ratio of the oligopeptides (**Table 5**) reached 5%, which was probably the consequence of the applied base (DIEA, 6 equivalent). On the contrary, when applying DIC/HOBt (6/3) racemization was significantly lower (1 %) for **6** (Ac-V**H**NRTIG) owing to the acidic character of the coupling reagent HOBt. The results also underline that the degree of epimerization of racemization sensitive His/Cys residues distant from the *C*-terminus is higher.

Additional peptide sequence

Finally, to test both the capacity and reliability of the current method, peptides of different length and difficulty (according to PeptideCompanion results, see supplementary material) were synthesized by using both PyAOP/DIEA and DIC/HOBt coupling reagents (**Table 6**). Polypeptides with sequences of elevated risk of on-resin aggregation (*e.g.* antimicrobial peptide CM15 (**8**), and miniprotein Tc6b (**9**))[14,17,20] were synthesized by using protocol **b2, c1** and **c2**, as these were all shown to perform well and efficiently. Our recommended protocol **c2**, which uses PyAOP/DIEA and a 6 min cycle-time is especially fast, though more expensive, than the others tested, but still remains at a lower cost Protocol **b2** requires longer time (12 min cycle time) and uses DIC/HOBt (6/3), clearly the least expensive set of reagents yet introduced. The results are summarized in **Table 6**.

Table 6. *Probing our method for longer and aggregation prone primary sequences.*

#	polypeptide code	primary sequence	purity [a](%) (and total synthesis time (h))		
			protocol **c1** [b]	protocol **b2** [c]	protocol **c2** [d]
1	**2**	EEEAVRLYIQWLK	**90** (2.7)	**82** (2.7)	**87** (1.7)
2	**8**	KWKLFKKIGAVLKVL	**89** (3.1)	**81** (3.1)	**82** (2.0)
3	**9**	NLYIQWLKEGGYSSGRPPPS	**93** (4.1)	**75** (4.1)	**79** (2.6)

[a] purity of the crude peptide by HPLC: analysis uses peak area % at 220 nm)
[b] protocol **b2**: DIC/HOBt (6/3), [c] protocol **c1:** PyAOP/DIEA (3/6), [d] protocol **c2**: PyAOP/DIEA (3/6)

Using a coupling cycle time of 6 min enables the total synthesis to be completed within a few hours. As typically 3 molar excess of coupling reagent and 6 ml/ cycle organic solvent was used, our method is definitely environmentally acceptable, cost effective and fast enough for routine laboratory practice. It's important to mention, that straightforward and dependable synthesis of 15-30 residue-long polypeptides composed of proteinogenic amino acids, obtaining 80-90 % crude product purity with an ignorable amount of racemization is in itself a significant result.

Comparison to other established methods

Our optimized protocols (**b2**: DIC/HOBt (6/3) and **c2:** PyAOP/DIEA) were compared to well established MW assisted SPPS methods, in case of the antimicrobial peptide CM15 (**8**).[14,17] Parameters and product properties of **b2** and **c2** protocols were compared to those described by Bacsa *et. al*. using commercial MW synthesizers (either a single-mode Discover SPS reactor from CEM Corp. (Matthews, NC) or a CEM Liberty Blue, **Table 7**).[14] We found that the **b2** and **c2** protocols operated as fast as the MW assisted reactors did. The purity of the crude peptide **8** obtained by **b2** and **c2** protocols were somewhat lower (82 and 81 %) than those of the MW assisted SPPS (91 and 94 %) (**Figure S4**). However, our methods use 75 % less organic solvent, namely 135 ml in total compared to 815 or 508 ml. Furthermore, approximately 40 % less reagents were used (3 instead of 5 molar equivalent).

Table 7. *Selected parameters of the synthesis of KWKLFKKIGAVLKVL*

#	coupling reagent	equiv.	*T* (°C)	coupling **time** (min)	total solvent **used** (ml)	**purity**[e] (%)
MW [a]	DIC / HOBt	3	86	10.0	815	91
MW [b]	DIC / Oxyma	5	90	2.0	508	94
FLP_ELTE(**b2**) [c]	DIC / HOBt	3	80	3.33	135	82
FLP_ELTE(**c2**) [d]	PyAOP / DIEA	3	70	1.67	135	81

[a] single-mode Discover SPS reactor from CEM Corp. according to Bacsa et al.[14]
[b] CEM Liberty Blue Automated Microwave Peptide Synthesizer
[c] our result by using protocol **b2**
[d] our result by using protocol **c2**
[e] purity of the crude peptide (HPLC analysis, peak area % using 220 nm UV-absorbance)

One of the ultimate tests of a polypeptide synthesis is the production of the 30 residue-long chain B of Insulin (**10**) (**Figure 7**). We carried out this task using our **c1** protocol, LC-MS analysis of the cleavage products revealed that the product is a mixture of a linear and a cyclic form of identical sequence, that latter formed by the intramolecular disulfide bridge formation of two Cys residues (**Figure 7**, **Figure S5**). The overall yield and purity were calculated by counting the two forms together.

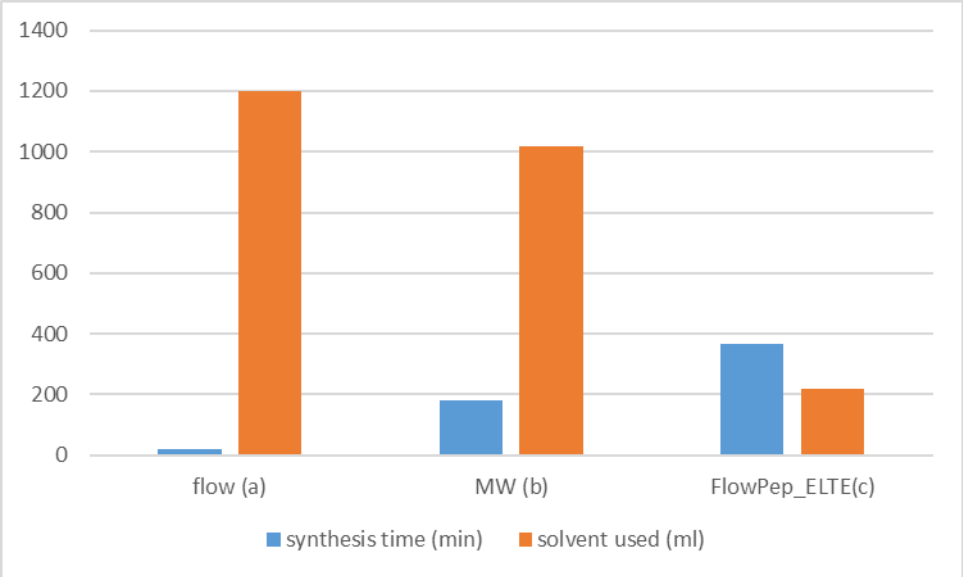
Our results (**Table 7**, **8**) clearly demonstrated the advantage of this method. The reduced solvent consumption is one of the most important requirements in organic synthesis. We were able to produce biologically active peptides of both equal or higher quality and quantity compared to other methods. Although the synthesis of the Insulin derivative was ten times longer than the protocol presented by Mijalis *et al.,* the required solvent volume was the quarter (**Table 8**, **Figure 7**) of their consumption.[8]

Table 8. *Selected parameters of the synthesis of Insulin chain B polypeptide* compared to other established methods.*

#	coupling reagent	equiv.	*T* (°C)	acylation time (min)	total time (min)	total solvent used (ml)	purity [e] (%)	yield [f] (%)
flow(MIT) [a]	HATU / DIEA	20	90	0.117	20	1200	54	48
MW(CEM) [b]	DIC / Oxyma	5	90	2	180	1016	62	58
FlowPep_ELTE(**c1**) [c]	PyAOP / DIEA	3	70	1.67	365	220	64	78

*H-FVNQH LCGSH LVEAL YLVCG ERGFF YTPKT-NH_2

[a] according to Mijalis et al.[8]
[b] CEM Liberty Blue Automated Microwave Peptide Synthesizer
[c] our result by using protocol **c1**
[e] purity of the crude peptide (HPLC analysis, peak area % using 220 nm UV-absorbance)
[f] calculated from theoretical (100%) yield



[a] according to Mijalis et al.[8],
[b] CEM Liberty Blue Automated Microwave Peptide Synthesizer,
[c] our result by using protocol **c2**,
[d] our result by using protocol **b2**

Figure 7. C*omparison of 4 methods that were used to produce the 30-residue long Insulin chain B peptide (*FVNQHLCGSHLVEALYLVCGERGFFYTPKT, Peptide **10***) in view of the organic solvent consumption and total synthesis time.*

Conclusion

Common, commercially available HPLC based peptide synthesizer was applied for solid phase peptide synthesis. The initial 40 min was effectively cut back to 6 min/cycle time, making it competitive to any currently available but significantly more expensive commercial synthesizer. With respect to the organic solvent consumption (6 ml/cycle) and waste, our setup is by far the best. High quality peptides can be synthesized in more environment friendly way. Furthermore, by using 3 molar excess of reagents even for the successful synthesis of such a long polypeptide sequence as the Insulin chain B, coupling efficacy of our method is superior to other available methods. In addition, as we use only a fraction (10-20 %) of organic solvent and reagents compared to others, our energy consumption is surprisingly low. Our explicit goal now is to move toward an even more user and environment friendly solution by replacing the current DMF solvent completely with MeTHF or MeCN and thus, resulting in an even greener alternative. In addition, our method can easily be tuned to work with non-proteinogenic amino acids[7e] or peptide conjugates, keeping the fully automated and environmentally friendly approach presented here. The apparatus is thus a flexible and modular setup, the Swiss army knife of green peptide flow chemistry.

Experimental

The commercially available flow peptide apparatus (HPPS-4000, METALON Ltd., Hungary) consists of a conventional Jasco LC-4000 series HPLC system, except the PU-4180 HPLC pump, modified with an additional valve, allowing recirculation and regulation of solvent flow (*e.g*. cleavage mixture). ChromNAV2 software ensures the fully automated process. The autosampler injects the reagent solutions from a 2 ml sample vial, placed in the sample rack. PEEK chromatography column was used as a fixed bed reactor tube for the resin and DMF was used as solvent. 150 mg Fmoc-Rink amide TentaGel resin (0.24 mmol/g) was used unless mentioned otherwise. In addition, ChemMatrix (0.51 mmol/g, 75 mg), Hypogel200 (0.53 mmol/g, 112 mg) and Hypogel400 (0.52 mmol/g, 112 mg) resins were probed. The reagent solutions were injected on the resin-filled column. Solvent usage and residence times are summarized in **Table 9.** For Fmoc-deprotection the cleavage solution consisted of 20 V/V% piperidine in DMF, but for protocol **b2** and **c2** 40 V/V% piperidine solution was used. In the

vials 0.12 M protected amino acids with coupling reagents were dissolved in NMP. The activating agent (DIC or DIPEA) was added prompt before the coupling injected with the auto-sampler. During the synthesis the pressure varied between 70-90 bar, with the use of a backpressure regulator.

Protocols

a: the flow rate was set to 0.15 ml/min: T= 70 °C, cycle time= 40 min or in short: *Flow*: 0.15 ml/min {0-*t*-40 (min)}.

b1: (T= 70 °C, 80 °C) had also a constant flow rate set to 0.3 ml/min, T= 70 °C, with an overall cycle time of 20 min. In fact, **b1** run at two temperature: *T*= 70 °C, (**b1**) and T= 80 °C, or **b1**$^{80°C}$ both were summarized as: *Flow*: 0.3 ml/min {0.0-t-20 (min)}.

b2: was somewhat similarly to **b1**, except that the flow rate was changed (**Figure 4**). Initially, **b2** had a flow rate of 0.3 ml/min {0-*t*-8 (min)} which was changed gradually from 0.3 to 1.0 ml/min {8-*t*-9 (min)}. A gradient of such a slope is needed for protecting the resin from sudden pressure jump. The elevated flow rate remained until the end of the cycle and then returns to its initial value of 0.3 ml/min, requesting a cycle time for **b2** as 12 minutes in total. In summary **b2** goes as this: *Flow*: 0.3 ml/min {0-*t*-8 (min)}, *Flow*: 0.3→1.0 ml/min {8-*t*-9 (min)} *Flow*: 1 ml/min {9-*t*-11.5 (min)} and *Flow*: 1.0→0.3 ml/min {11.5-*t*-12 (min)}. (**Figure 4**)

c1 was programed as follows: *Flow*: 0,6 ml/min, {0-*t*-12.0 (min)}, *T*= 70 °C.

c2 works as follows: *Flow*: 0.6 ml/min {0-*t*-3 (min)}, *Flow*: 0.6→1.0 ml/min {3-*t*-3.5 (min)}, *Flow*: 1 ml/min {3.5-*t*-6.5 (min)} and *Flow*: 1.0→0.6 ml/min {6.5-*t*-7.0 (min)}, giving a total cycle time of 7 min.

Table 9. *Time program and the amount of solvent usage*

protocol	**coupling time** (min) [a] (amino acid / NMP)	**washing time #1** (min) [c] (DMF)	**Fmoc-deprotection time** (min) [b] (20-40% piperidine/DMF)	**washing time #2** (min) [c] (DMF)
a	6.70 (1.005 ml)	13.30 (1.995 ml)	6.70 (1.005 ml)	14.30 (2.145 ml)
b1$^{80°C}$	3.30 (1.00 ml)	6.70 (2.01 ml)	3.30 (0.99 ml)	6.70 (2.01 ml)
b2	3.30 (1.00 ml)	4.70 (1.41 ml)	1.40 (1.05 ml)	2.60 (1.55 ml)
c1	1.67 (1.002 ml)	3.33 (2.00 ml)	1.67 (1.00 ml)	5.33 (3.20 ml)
c2	1.67 (1.002 ml)	1.83 (1.20 ml)	1.00 (1.00 ml)	1.50 (1.40 ml)

[a] residence time of the activated amino acid on the resin
[b] residence time of the piperidine solution on the resin
[c] time of washing the resin from the excess of the reactants and side products, #1: after coupling step, #2: after Fmoc-deprotection step

After peptide synthesis the resin was washed with DCM, then dried in vacuum. Cleavage from the resin was accomplished with TFA in the presence of scavengers (H_2O, thioanisole, EDT, TIS, phenol : 5 ml, 250 µl, 250 µl, 125 µl, 60 µl, 250 mg) stirring for 3.5 hours. Then, the solution was filtered and freed from TFA *via* rotary vacuum evaporator. The peptide was washed with diethylether and dried in vacuum.

Cleavage products were analyzed by RP-HPLC on an analytical C-18 column (Phenomenex, Jupiter, 5µm, 250×4.6 mm, 100Å) using gradient elution, consisting of 0.1% TFA in water (*eluent A*) and 0.1% TFA in acetonitrile/water = 80/20 (v/v) (*eluent B*). The flow rate was 1 mL/min and the absorbance was detected at λ=220 nm. LC-MS analysis of the compounds was performed on a Thermo Scientific Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer connected directly to a Dionex 3000 UHPLC (Thermo Scientific, Bremen, Germany). The flow rate was 300 µL/min on a Supelco Ascentis C18 column (2.1x150 mm, 3 µm) using water/ acetonitrile mixtures of 0.1% acetic acid in water (A) and 0.1% acetic acid, in acetonitrile (B), with a gradient of 2% -> 100% B over 17 min and the column temperature was set to 40^{o}C. Data were analyzed by XcaliburTM program (Thermo Fisher Scientific).

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