A green peptide synthesis protocol for improving peptide production: a new approach

L. Lastella[1], B. Biondi[2], I. Guryanov[3], F. Formaggio[1,2], M. Macis[3] and A. Ricci[3]

[1]Department of Chemistry, University of Padova, 35131 Padova, Italy [2]Institute of Biomolecular Chemistry, Padova Unit, CNR, 35131 Padova, Italy
[3]Fresenius Kabi iPSUM Srl, 45010 Villadose, Italy

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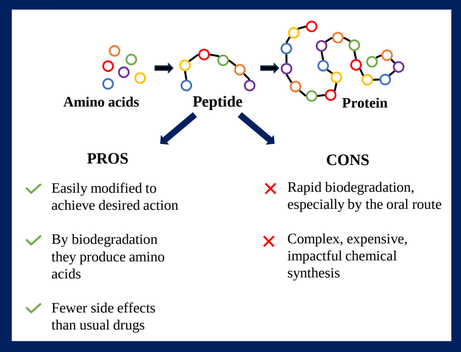
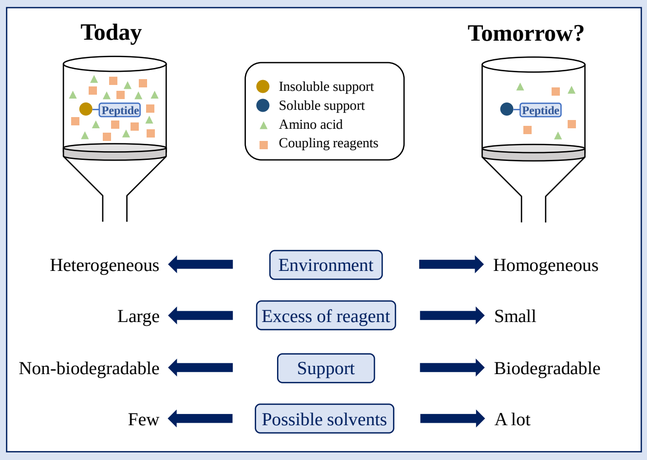
Introduction

Peptides play important roles in many fields, but especially in the pharmaceutical one, as they have numerous advantages. On both a laboratory and industrial scale, peptides are synthesised according to the Solid Phase Peptide Synthesis (SPPS) protocol, which involves the use of an insoluble solid support on which the peptide chain is grown. Despite the numerous advantages that make this protocol the most widely used methodology for peptide production, SPPS has some disadvantages: it is necessary both to use a large excess of reagents to obtain excellent yields and good purity of the final product, and to use a large quantity of organic solvent, especially in the washing steps.



New Scaffolds

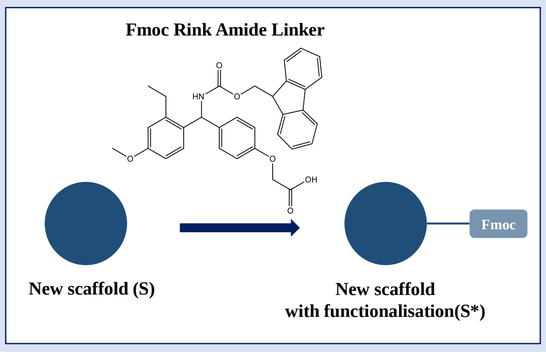
The main objective is to develop an alternative route for peptide synthesis.
The idea is to replace the common insoluble polystyrene substrates (resins) used for SPPS with soluble and biodegradable ones that allow the homogeneous phase to be used for peptide synthesis.

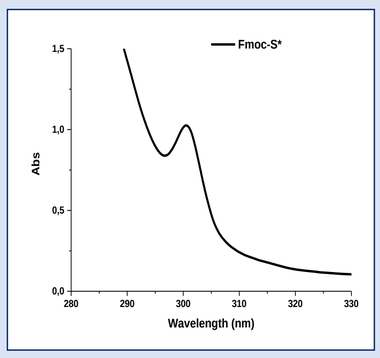


Heterogeneous phase synthesis has numerous advantages (such as the easiness with which excess reagents and by-products are removed by filtration) but also numerous disadvantages as mentioned above.

By exploiting synthesis protocols in a homogeneous environment, where reactivity is higher, it would be possible to reduce reagent excess and solvent volume. Furthermore, by using homogeneous phase synthesis, different types of organic solvents will be exploited.

The designed and synthesised scaffold (**S**) was then functionalised to allow subsequent synthesis of the peptide sequence.
Fmoc Rink Amide Linker was chosen to obtain the peptide with an amide at the C-terminal (**S***) but several other linkers can be used to achieve different functionalities on the C-terminal.



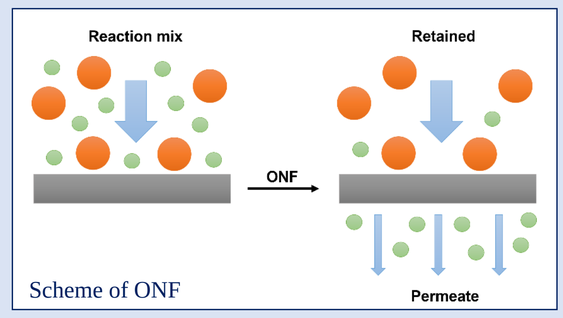


The UV absorption of the Fmoc group was used to confirm the presence of the linker on the scaffold.

The graph on the left shows the UV absorption spectrum of the soluble scaffold synthesised and functionalised with the linker (**S***).

Organic Nanofiltration

Crucial is the development of an organic nanofiltration (**ONF**) procedure that separates (i) the by-products and excess reagents present after the coupling reactions and (ii) the dibenzofulvene, generated during the Fmoc removal from the growth chain.



Scheme of ONF

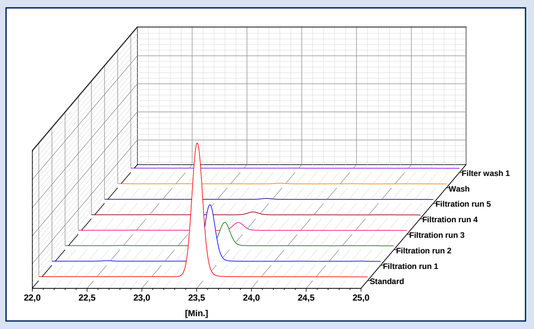
MetCell System

The nanofiltration system used is the MetCell System (Evonik Industries),
a system that uses membranes with a cutoff of 600Da.

The membranes identified are stable to various organic solvents of low polarity (alcohols, ethyl acetate, hexane, toluene).

Type of membranes	EtOH		ACN		DCM		DMF					
	Compatibility	Optimal flow rate	Compatibility	Optimal flow rate	Compatibility	Optimal flow rate	Compatibility	5% in DCM Optimal flow rate	10% in DCM Optimal flow rate	15% in DCM Optimal flow rate	20% in DCM Optimal flow rate	100% Optimal flow rate
Performance	✓	✓	✓	✓	✓	✓	✗	✓	✓	✓	✓	✗

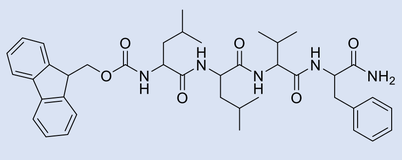
The best solvents are ACN and DCM, which allow rapid filtration with a flow rate of about 3mL/min. DMF (excluded anyway due to its high environmental impact) has been tested for comparison but is not compatible with the membranes used unless it is mixed with one of the other solvents (20% DMF in DCM). Alcohols, on the other hand, were excluded because they generate high back pressure during filtration and, being good nucleophiles, are avoided in peptide synthesis processes because they are competitive in peptide bond formation.



To use ONF, filtration conditions were first developed on a model system consisting of **S*** and Fmoc-Ala-OH 2mM. Numerous filtration cycles were performed and for each of them, an HPLC-MS analysis was conducted to detect the presence or absence of alanine in the filtrate and retained solution. From the results shown in the graph on the left, it can be concluded that four filtration cycles are sufficient to completely remove the low molecular weight components.

Synthesis of a model peptide

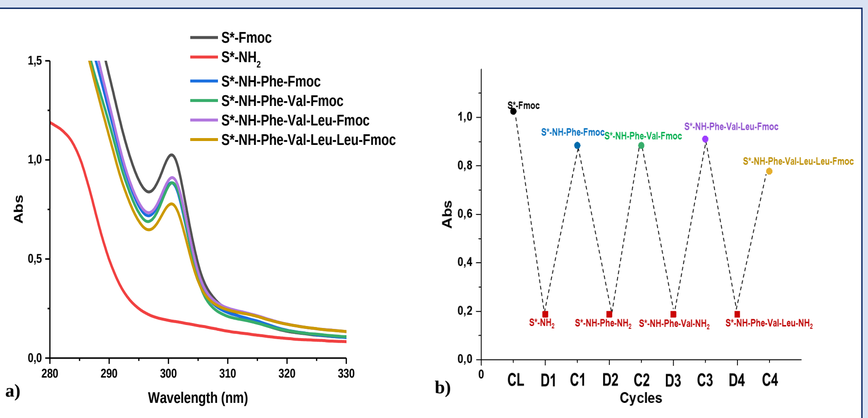
Fmoc-LLVF-NH_2



Fmoc deprotection → ONF → Coupling → ONF → (X nAA) → Cleavage → Purification

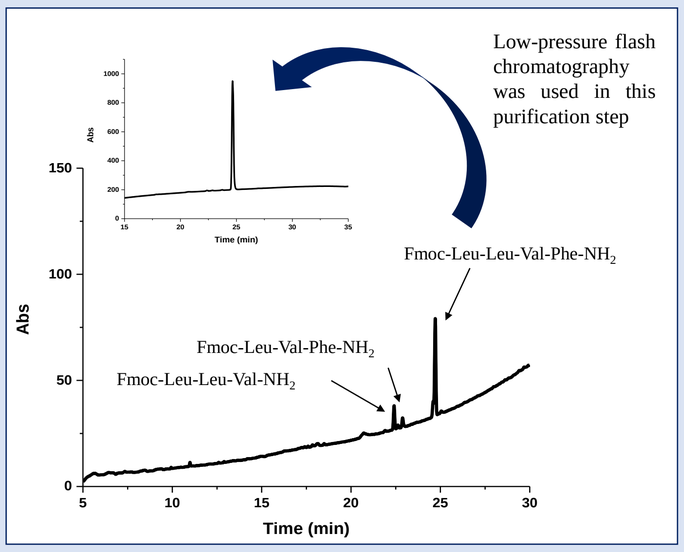
The model peptide **Fmoc-Leu-Leu-Val-Phe-NH_2** was synthesised according to the scheme on the left. The couplings were conducted with an excess of 3eq of amino acid, OxymaPure and DIC as coupling reagents. At the end of the reaction, excess reagents and by-products were removed by ONF. Fmoc removal was performed with 20% piperidine in the solvent used and also in this case an ONF step is necessary to remove the dibenzofulvene from the scaffold with the growing peptide.
The scaffold-peptide conjugate was then isolated in each step.

After each coupling, UV-Vis experiments were conducted on the isolated scaffold-peptide conjugate to verify the actual formation of the peptide bond between the chain present on the support and the amino acid. This was possible by detecting the presence of the Fmoc absorption band at λ=298nm. For comparison, the absorption spectrum of the scaffold-peptide conjugate isolated after ONF , after removal of the protective Fmoc group from the growing chain, was also recorded, which shows no UV absorption due to the presence of the free amine.



a) Overlay of absorption spectra of isolated species after coupling.
b) Graphical representation of the synthetic steps (C = coupling and D = Fmoc deprotection).

When the peptide has been completely synthesised, the chain was detached from the support. This product comprises the model peptide chain and the scaffold from which the chain was detached.



HPLC chromatogram shows the target model peptide and two by-products that confirm the results of the UV-Vis analysis.

Conclusions

- Soluble, biodegradable and non-toxic supports have been synthesised that can be used instead of common polystyrene resins.
- On these scaffolds, it was possible to synthesise a model peptide chain.
- The ONF procedure developed made it possible to separate the scaffold with the growing peptide from the mixture of by-products and excess reagents.
- Throughout the new process, we moved away from the use of DMF as a solvent.

However, due to solubility issues and some limitations of ONF, the developed scaffolds are still being studied.

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Please do not hesitate to contact me for questions and curiosities!!!

luana.lastella@phd.unipd.it