OTHMAN AL MUSAIMI[1,2], SIMONA SERBAN[3], YAHYA E. JAD[1,2], ZHICHAO MA[3], ASHISH KUMAR[1,2], CHENG JI[3], BEATRIZ G. DE LA TORRE[2], ALESSANDRA BASSO[3]*, FERNANDO ALBERICIO[1,4]*

*Corresponding authors

1. Peptide Science Laboratory, School of Chemistry and Physics, University of KwaZulu-Natal, Durban, South Africa

2. KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

3. Purolite, Llantrisant, United Kingdom

4. CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine and Department of Organic Chemistry, University of Barcelona, Barcelona, Spain

Jetting manufacturing of resins for solid-phase peptide synthesis

KEYWORDS: SPPS, resins, swelling, loading, styrene/DVB, peptide synthesis.

ABSTRACT

Peptides are an important class of Active Pharmaceutical Ingredients. Indeed, it is now feasible to synthesize peptides of up to forty amino acids in a multi-kilogram scale. This achievement has been made thanks to the implementation of the solid-phase synthesis methodology described by the Nobel Laureate R. Bruce Merrifield in 1963. Successful solid-phase peptide synthesis depends directly on the quality of the polymeric support, namely the resin. Here we describe new polystyrene in-house resins produced by 'Jetting' technology. The excellent properties (swelling, loading, particle size distribution, morphological structure) of these resins assure the quality of the final peptide.

INTRODUCTION

The pharmaceutical business is unique in the industrial arena as it is completely transdisciplinary. The process of taking an idea to the market requires the efforts of hundreds if not thousands of professionals from numerous disciplines. Importantly, even the customers (patients) play a key role in the clinical trials during the drug development phase.
It takes between 10 and 15 years for a new drug to reach the market, a period that far exceeds that required for products manufactured by other sectors. The investment required for getting a new drug onto the market is also a differential trait (US $1 billion). Together, all these factors make the pharmaceutical industry one of the most intriguing, and more so when you consider that at some point in our lives we will all become customers of this sector.

Briefly, Active Pharmaceutical Ingredients (APIs) can be divided into two groups, namely New Chemical Entities (NCEs) and Biologics, which can be differentiated by their production method. While NCEs are produced by chemical synthesis, the latter are manufactured using biotechnology. However, among NCEs there is a special class, the TIDES, which share a number of features with Biologics. TIDES, which group peptides and oligonucleotides, are chemically synthesized, but have their roots in the biological world.
Fifty years ago, it was unthinkable to consider chemically synthesized peptides, small proteins, and oligonucleotides as forming part of the drug arsenal. The synthetic methodology available at that time did not allow the production of these compounds in multi-kilogram scales with the purity required by the corresponding drug agencies.
This paradigm started to change in the 1960s when R. Bruce Merrifield described the solid-phase peptide synthesis (SPPS) methodology. Merrifield discovered that anchoring the growing peptide chain to an insoluble polymeric support enhances the synthetic process. Thus, the completion of the reaction is driven using an excess of reagents, which can be easily removed by simple filtration and washing steps.
At the end of the peptide elongation step, a straightforward treatment releases the peptide from the resin, with the concomitant removal of the side-chain protecting groups (1).
The adoption of the solid-phase methodology by peptide-based Contract Manufacturing Organizations (CMO), without doubt fuelled the arrival of peptides onto the market. It is now possible to produce peptides comprising from 10 to 40 amino acids in multi-kilogram scales for the drug market. Indeed, this has been done for many years for the so-called small molecules (< 500-700 Daltons). As a result, almost 100 peptides are already on the market, and more than 300 peptides are being evaluated in clinical trials (2). This pipeline ensures a dramatic increase in the number of peptide-based drugs on the market in the coming years. Very importantly, peptides are resulting beneficial for addressing all therapeutic targets.
Although SPPS involves protected amino acids, coupling reagents, solvents, and other reagents, the resin is the key for success. In the literature, a great variety of resins and other materials have been reported for SPPS. However, for peptide production, polystyrene resins are broadly accepted as the resin of choice. These solid supports are mechanically stable, swell well in the solvents used for peptide synthesis, are compatible with moderate-to-high temperatures, and can be produced in multi-kilogram batches, thereby assuring the different synthetic procedures involved in the production of peptide-based APIs.
Taking advantage of previous experience in the production of functionalized styrene resins, a new platform of polystyrene-based resins manufactured using 'Jetting' technology called Purosynth™, were developed for peptide synthesis. Here we evaluated the capacity of 2-chlorotritylchloride (CTC) and Wang resins- used for the synthesis of C-carboxylic acid terminal peptides- and of 4-methylbenzhydrylamine (MBHA) and aminomethyl (AM) resins for the incorporation of a broad range of handles.

RESULTS AND DISCUSSION

Manufacturing

Commercial resins on the market for solid-phase peptide synthesis are manufactured by suspension polymerization, and then sieved to obtain the desired particle size range, with obvious decreased yields and an increase of costs and risk of polymer breakage.

One of the key features of the resins (incorporated in this study) is the use of a unique manufacturing process named 'Jetting'. 'Jetting' allows the production of uniform particle size, with a low uniformity coefficient and high productivity in a semi-continuous mode. This enables a more economic and environmentally friendly (reduced waste) process than classic batch emulsion polymerization and, in addition, ensures a narrow particle size distribution.

'Jetting' technology operates with two different processes, depending on the particle size that is required. The first Purolite jetting process – membrane or 'can' jetting, uses a membrane with very small, discrete orifices, through which the monomers are passed. This device is capable of producing uniform particle size chromatographic resins from ~15 to 250 microns (3). The second process, plate jetting, utilizes a plate with many precisely engineered holes, through which the monomers are passed. Uniform particle size chromatographic resins of ~150 to >1000 microns can be produced using this method.

The narrow particle size distribution obtained by 'Jetting' does not require further screening as in the case of other commercial products for SPPS. 'Jetting' technology allows the production of large batches (500+ kg) in a very short time. In SPPS, the uniformity of particle size offers great advantages in terms of uniform kinetics and swelling during synthesis, thereby ensuring better purity of the final peptide.

The result of this 'Jetting' process is a narrow Gaussian distribution of uniform spherical beads. Figure 1 shows an example of a particle size distribution of an in-house produced CTC resin.

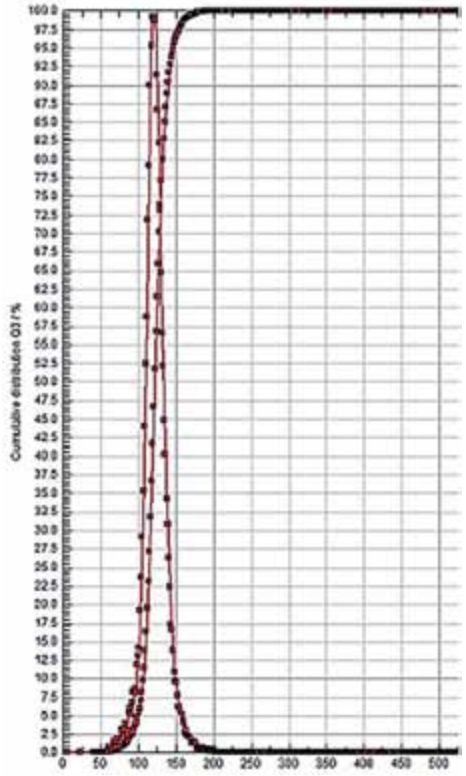

Figure 1. Particle size distribution for a batch of CTC resin; the graph was obtained using Qicpic Analyser (Sympatec), and it shows the cumulative particle size distribution versus the particle diameter.

Figure 2 shows an electron microscopy picture for different in-house polystyrene-based resin types. In both cases, it is important to highlight the narrow particle size distribution, which facilitates the excellent performance of these resins.

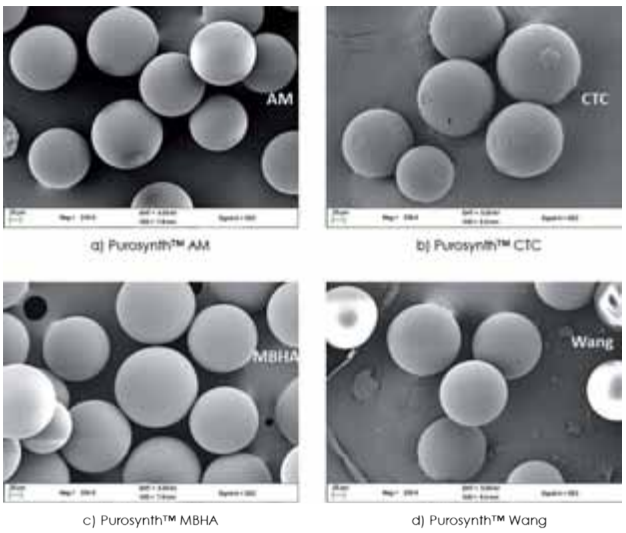

Figure 2. Scanning electron microscopic images for various in-house Purosynth™ resins; all in S grade: 75 -150 microns (100-200 mesh). Dry resin beads were coated with a thin layer of gold using a (Quorum Q150 RES sputter coater, UK), and photographed using a scanning electron microscope (Zeiss ultra plus FESEM, Germany).

All resins examined in this study (in-house and commercial) are cross-linked with 1% divinylbenzene (DVB), which is the optimal amount for swelling purposes (see the next section). Higher cross-linked resins, such as polystyrene with 2% DVB, result in a significant decrease in swelling.

SWELLING

Swelling is a key requirement for the good performance of a resin in SPPS. It is important to bear in mind that the reaction kinetics in SPPS is diffusion-dependent. Consequently, the resin with greatest swelling capacity will enhance the reaction yield and decrease the reaction time by facilitating the diffusion (4-9). In contrast, a reduction in swelling capacity, triggers undesired peptide aggregation pathways, thereby increasing coupling difficulties (5, 6).

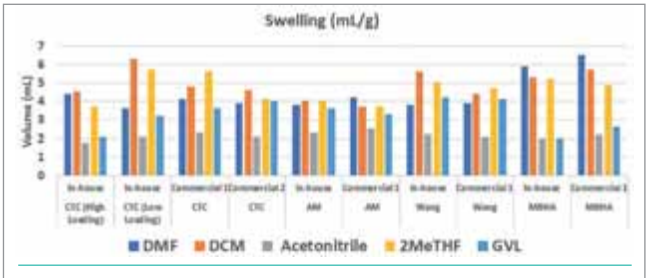

Figure 3. Swelling properties of the resins examined.

In this study, the following five solvents were used in a resin swelling test: *N,N*-dimethylformamide (DMF); dichloromethane (DCM); acetonitrile (ACN); 2-methyltetrahidrofurane (2-MeTHF); and γ-valerolactone (GVL). DCM and DMF are two of the most widely used solvents for SPPS while GVL and 2-MeTHF are reported for green SPPS (10-12). Finally, ACN, which is not optimum for SPPS because it does not facilitate resin swelling, can be used in certain cases.

As shown in Figure 3, the in-house resins show excellent swelling properties in all solvents, except ACN, and are also fully comparable with the commercial resins.

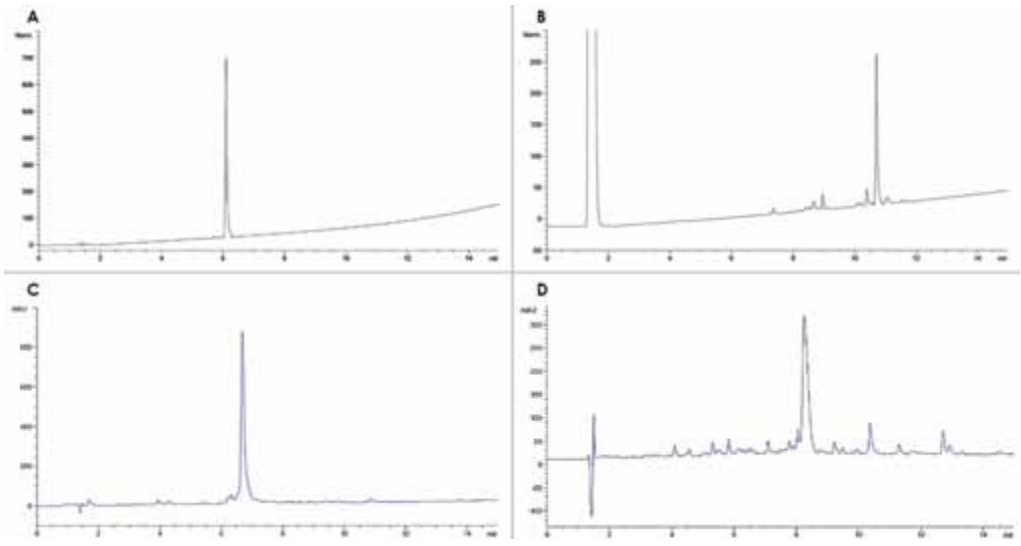

Figure 4. HPLC trace of: **A**. Leu-enkephalin on CTC-resin, linear gradient (5-95) % in 15 min. **B**. JR 10mer peptide on Rink-Amide-AM resin, linear gradient (15-60) % in 15 min. **C**. ABRF1992 synthesized on Rink-Amide-MBHA resin, linear gradient (10-60) % in 15 min. **D**. Thymosin synthesized on Rink-Amide-AM resin, linear gradient (15-25) % in 15 min. HPLC was performed on an Agilent 1100 system using a Phenomenex Luna C18 (3.6 µm, 4.6 × 150 mm) column, with a flow rate of 1.0 mL/min and UV detection at 220 nm. Buffer A: 0.1% trifluoroacetic acid (TFA) in H2O; buffer B: 0.1% TFA in CH3CN.

LOADING

Loading (capacity) is also considered a key parameter for the efficiency of the synthetic process, and the purity of the final peptide. High loading values will result in greater efficiency than low ones, because with the same amount of resin, more peptide will be obtained. In production, this is translated into the optimization of the whole process. On the other hand, high loading can facilitate interchain interactions, which is one of the main causes of the low performance associated with the two main SPPS reactions: removal of the protecting group, and coupling (5). Therefore, the control of loading is critical for the success of peptide production. It is widely accepted that for the synthesis of common peptides, loadings in the range of 0.5-1.0 mmol/g are optimal (13). On the other hand, for difficult peptides prone to inter/intrachain interactions, lower loading (around 0.1-0.3 mmol/g) is desirable.
The resins used in this study show optimal loading values.
It is important to highlight that the technology used for the preparation of CTC resins allows strict control of loading. Therefore, two loading values, of around 0.5 mmol/g and around 1.0 mmol/g, are available. Furthermore, CTC resins are prepared using a method that does not involve metals, thus implying less impact on the environment.

APPLICATION FOR PEPTIDE SYNTHESIS

The standard SPPS conditions used were: 9-fluorenylmetholoxycarbonyl (Fmoc)/*tert*-butyl (*t*Butyl) chemistry and *N,N'*-diisopropylcarbodiimide (DIC)-OxymaPure™ for coupling; and 20% piperidine/DMF for Fmoc removal. When AM and MBHA resins were used, either Wang or Rink linkers were incorporated using DIC-OxymaPure™ as a coupling method.
Four peptides were synthesized using the aforementioned resins namely: Leu-enkephalin pentapeptide "H-Tyr-Gly-Gly-Phe-Leu-OH"; Jung-Redemann decapeptide (JR) "H-Trp-Phe-Thr-Thr-Leu-Ile-Ser-Thr-Ile-Met-NH_2", which is N-terminal of a 10-mer peptide possessing a difficult sequence; a 16mer peptide, which was the challenging peptide proposed by the Peptide Synthesis Research Group of the Association of the Biomolecular Resource Facilities in 1992 (ABRF1992) "H-Gly-Val-Arg-Gly-Asp-Lys-Gly-Asn-Pro-Gly-Trp-Pro-Gly-Ala-ProTyr-XH; X = O, NH"; and Thymosin 28mer peptide "H-Ser-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-NH_2".
The synthesized peptides were evaluated with respect to purity by HPLC and the percentage recovered from the resin after the final global cleavage, followed by lyophilisation (Table 1, Figure 4).
Of note, a green ether (CPME) was also successfully used as a precipitation agent after the global deprotection step (14).

Entry	Peptide	In-house Resin	Recovery (%)
1	Leu-Enkephalin-COOH	CTC	79.2
2	JR-CONH2	Rink-AM	68.3
3	ABRF1992-$CONH_2$	Rink-AM	93.3
4	ABRF1992-$CONH_2$	Rink-MBHA	93.5
5	ABRF1992-COOH	Wang-MBHA	93.3
6	Thymosin-COOH	Rink-AM	74.8

Table 1. Peptides synthesized, resin used, and recovery.

CONCLUSION

Peptides are an important class of biomolecules that show a broad range of biological properties. From a chemical point of view, their structures can be considered complex, with molecular weights ranging from 1000 to 5000 Daltons. However, the development of SPPS by the Nobel Laureate R. Bruce Merrifield in 1963 has allowed the synthesis of these molecules in a multi-kilogram scale. This breakthrough has fuelled the increased numbers of peptide-based drugs approved by regulatory agencies (2).
The cornerstone of the SPPS methodology is the solid support, the resin. Here we report on a new platform of polystyrene-based resins developed by 'Jetting' technology. In this regard, CTC and Wang resins can be used directly for the synthesis of C-carboxylic acid terminal peptides, whereas MBHA and AM are ready to be immobilized with Wang or Rink Amide linkers.
The use of the 'Jetting' manufacturing process allows the production of uniform particle size, with a low uniformity coefficient and high productivity in a continuous mode.
The examined in-house resins show excellent swelling in the frequently used SPPS solvents. The unique technology allows the control of loading and adjustment to the requirements of each peptide. Thus, CTC resins can allow either high (~ 1.0 mmol/g) or low (~ 0.5 mmol/g) loadings.
The performance of the resins was evaluated in terms of purity and recovery of the final product. To this end, model and biological peptides of interest were prepared in a microwave-assisted peptide synthesizer (Liberty Blue, CEM) or in manual synthesis mode. In all cases, the examined resins provided excellent results.

ACKNOWLEDGEMENT

The work in the academic laboratories has been partially supported by the following: National Research Foundation (NRF) (CSUR Grant No: 105892 and Blue Sky's Research Programme # 110960) and the University of KwaZulu-Natal (South Africa); and the Spanish Ministry of Economy, Industry and Competitiveness (MINECO) (CTQ2015-67870-P) and the Generalitat de Catalunya (2017 SGR 1439) (Spain).

REFERENCES

1. R. B. Merrifield, *J. Am. Chem. Soc.*, 85, 2149–2154 (1963), 10.1021/ja00897a025.
2. O. Al Musaimi, D. Al Shaer, B. G. de la Torre, F. Albericio, *Pharmaceuticals (Basel)*, 11, (2018), 10.3390/ph11020042.
3. Kosvintsev, S. R. (2014). *U.S. Patent No. US 2014/0264984 A1*. Washington, DC: U.S. Patent and Trademark Office.
4. G. B. Fields, C. G. Field, *J. Am. Chem. Soc.* , 113, 4202-4207 (1991), 10.1021/ja00011a023.
5. K. C. Pugh, E. J. York, J. M. Stewart, *Int. J. Peptide Protein Res.*, 40, 208-213 (1992), 10.1111/j.1399-3011.1992.tb00293.x
6. V. Krchnak, Z. Flegelova, J. Vagner, *Int. J. Peptide Protein Res.*, 42, 450-454 (1993), 10.1111/j.1399-3011.1993.tb00153.x
7. J. P. Tam, Y.-A. Lu, *J. Am. Chem. Soc.*, 117, 12058-12063 (1995), 10.1021/ja00154a004.
8. J. M. Palomo, *RSC Adv.*, 4, 32658-32672 (2014), 10.1039/C4RA02458C.
9. D. C. Sherrington, *Chem. Commun.*, 2275-2286 (1998), 10.1039/A803757D.
10. Y. E. Jad, G. A. Acosta, S. N. Khattab, B. G. de la Torre, T. Govender, H. G. Kruger, A. El-Faham, F. Albericio, *Org. Biomol. Chem.*, 13, 2393-2398 (2015), 10.1039/c4ob02046d.
11. A. Kumar, Y. E. Jad, A. El-Faham, B. G. de la Torre, F. Albericio, *Tetrahedron Lett.*, 58, 2986-2988 (2017), 10.1016/j.tetlet.2017.06.058.
12. A. Kumar, Y. E. Jad, J. M. Collins, F. Albericio, B. de la Torre, *ACS Sustain. Chem. Eng.*, 6, 8034-8039 (2018), 10.1021/acssuschemeng.8b01531.
13. M. Meldal, *Methods Enzymol.*, 289, 83-104 (1997), 10.1016/S0076-6879(97)89045-3.
14. O. Al Musaimi, Y. E. Jad, A. Kumar, J. M. Collins, A. Basso, B. G. de la Torre, F. Albericio, *Curr. Opin. Green Sustain. Chem.*, 11, 99-103 (2018), 10.1016/j.cogsc.2018.06.017. ■

ABOUT THE AUTHORS

*In 2012, **Alessandra Basso** joined Purolite to set-up the Life Sciences division; since then more than 600 new products have been launched. In close connection with R&D and the global sales network of Purolite, Alessandra supports customers in developing applications, and expanding the scope of the business to the food, pharmaceutical and chemical industries. Alessandra is an author of more than 50 scientific papers in international journals.*

***Fernando Albericio** is Research Professor at the University of KwaZulu-Natal in South Africa and Full Professor at the University of Barcelona. His major research interests cover practically all aspects of peptide synthesis: new reactions, building blocks, coupling reagents, solid phase supports, protecting groups, and linkers, as well as synthesis of peptides and small molecules with therapeutic activities.*

NEW POWERFUL FREEZE DRYER OFFERS FULLY ASEPTIC OPERATION

SP Scientific announces the **LyoConstellationTM S10, S20 and S30** as the first in a new series of advanced freeze dryers. All are configured-to-order for faster delivery freeze-drying cycle development and small-scale aseptic production, such as production of stability batches or early phase clinical trial batches.

LyoConstellation S10 freeze dryer

LyoConstellation is a **Line of Sight™-** enabled freeze-drying system with very powerful refrigeration, delivering an expanded equipment capability and a large process space. This allows the development of highly aggressive cycles and the mapping of the furthest reaches of theoretical design space for a formulation. When handling high-value active pharmaceutical ingredient (API), and with a need to bring product to market as quickly and safely as possible, LyoConstellation can address early stage development under aseptic and controlled conditions. This means product behaves in the same manner once it is ready for scale-up and eventually ready for commercialization.

Offering a capacity of 9.8 ft2 (1m2) and precise shelf temperature control from -55°C to +65°C, the LyoConstellation S10 is designed, even when fully loaded, to hold 100mTorr vacuum and shelf temperatures of over +40°C enabling unmatched sublimation rates with high moisture applications with no evidence of choked flow. Isolator and cleanroom integratable, the LyoConstellation is capable of clean-in-place (CIP) and steam-in-place (SIP) for aseptic manufacturing.

The LyoConstellation series – including the S10, S20 and S30 offering shelf capacities from 9.8ft2 (1m2) to 33ft2 (3m2) – are all powerfully equipped as standard to provide very high capacity, broaden equipment capability and expand design space. Configurable with the latest technologies including SMART™ cycle optimization technology, ControLyo® Ice Nucleation Technology and LyoFlux® TDLAS (Tunable Diode Laser Absorption Spectroscopy) for vapor mass flow measurement and inference of critical data such as batch product temperature and batch Kv (vial heat transfer coefficient), the LyoConstellation is able to deliver an unmatched array of process analytical data. These technology options together with the LyoSTM software platform, for working in compliance with 21 CFR Part 11, are common to the full range of SP Scientific freeze dryers enabling the same technologies and equipment to be used for both R&D and production.

www.spscientific.com/LyoConstellationFreezeDryer/

NEWS