



Combining solid phase synthesis and chromatographic purification for efficient peptide manufacture

KEYWORDS: Polystyrene resins, peptide synthesis, peptide purification, solid phase peptide synthesis, oligos-peptides.

ABSTRACT

In recent years, peptides have gained an important position in the drug arena for a variety of treatments. Solid-phase peptide synthesis (SPPS) continues to have a significant impact in both R&D as well as in production of commercial peptides. The combination of SPPS with reverse-phase (RP) chromatographic separations allowed for the peptides to be purified to levels suitable for drug usage. Thanks to their chemical and physical stability to high pressure, chemicals, temperature and oxidising reagents, polystyrene / divinyl benzene (PS/DVB) resins are the main solid support for peptide synthesis as well as being suitable for the chromatographic purification of the peptides by RP and ion exchange (IEX). Depending on the application, the optimal cross-linking changes, as do the optimal particle size, the uniformity in particle size distribution and the porosity of the resin. The characteristics and applications of the PS/DVB resins are explored in this paper for the synthesis and purification of peptides of commercial interest.

INTRODUCTION

Peptides are becoming an important class of Active Pharmaceutical Ingredients (APIs). When the Nobel Laureate Vincent Du Vigneaud reported in 1954 (1), the first synthesis of a peptide with biological activity, oxytocin, few analysts had predicted that today peptides would constitute 5-10% of the new chemical entities accepted by the FDA each year (2). The excellent properties of peptides, such as high activity and specificity, low toxicity, low accumulation in tissues, minimization of drug-drug interactions, biological and chemical diversity are sufficient to justify the importance of peptides in the drug arena.

Two factors proved crucial in the materialization of peptides as drugs. First, it was the development of the solid-phase peptide synthesis (SPPS) methodology by the Nobel Laureate R. Bruce Merrifield (3). SPPS technology signified a revolution in the field due to the possibility of synthesizing peptides up to 50 residues with low level of impurities and high yields. Although the implementation of the SPPS techniques in the pharmaceutical industry opened new avenues for the production of peptides as drugs, this was not self sufficient. The SPPS was able to render in both modes, research and production (multi Kgs scale), complex peptides with good to excellent purity, but not pure enough for safe human consumption (4). Furthermore, due to the intrinsic structural characteristic of the peptides,

impurities very often have a structure similar to the target peptide. Thus, some impurities contain one amino acid less (deletion sequences) or more (double hit / incorporation) than the desired peptide, epimers (racemization of one amino acid), β -peptides (isomerization of Asp- or Ser-containing peptides), or modified residues (alkylation of Trp, Tyr; oxidation of Met, Cys, Trp). The presence of these impurities increases with the size of the peptide, making the purification of large peptides more demanding, compounded by the greater proportionate resemblance of the impurities to the target peptide. The problem is yet further magnified when the SPPS approach is used, because intermediates are not isolated and therefore, they are not removed prior to final purification.

The definitive development necessary for peptides to be considered feasible APIs was the development of high-performance liquid chromatography in reversed phase mode (RP-HPLC). The roots of RP-HPLC systems can be found in the pioneering work of Jim Waters in Massachusetts (5), coincidentally around the same time as Merrifield's development of SPPS in the late 50's and early 60's. In the chromatographic context, Jean Rivier introduced RP-HPLC to the peptide world (6). His 1989 seminal paper, wherein he described the preparative solid-phase synthesis and subsequent purification by RP-HPLC of an analogue of LH-RH, could be considered the proof of concept for the use of peptides as APIs (7).

An important similarity other than their periods of development can be found between SPPS and RP-HPLC, as both rely on a solid support. In SPPS, the solid support acts as a protecting group for one end of the peptide, allowing for the simple elongation of the peptide chain. In RP-HPLC, the different components of the crude peptide produced by SPPS interact with varying strength with the solid phase on the basis of differing hydrophobicities leading to their separation. In both techniques, the solid support is key for good performance of either the synthesis or the purification. Although solid supports formed from different polymers could be used for both techniques, polystyrene (PS) cross-linked with divinylbenzene (DVB) has proven optimal for both synthesis and purification.

In this paper, PS/DVB resins have been used for the SPPS of peptides (e.g. bivalirudin) as well as for the RP-HPLC purification of the crude peptide following the synthesis. The resin characteristics for both applications are discussed alongside the peptide synthesis and purification details.

RESULTS AND DISCUSSION

Characteristics of resins for HPLC and SPPS applications

Typically, the resins for SPPS applications are non-porous beads made of PS cross-linked with 1% DVB. This formulation allows for the solid support to undergo important swelling in organic solvents and expose all the functional groups grafted on the polymeric backbone, readily available for the peptide synthesis. PuroSynth® PuroSynth™ resins for peptide synthesis are manufactured with a particle size range of 75–150 microns, at large scale using a proprietary “can jetting” technology (8). The technology allows for the production of resin beads in a very narrow particle size distribution without the need for screening, which is generally required for traditional suspension polymerisation. Following the jetting process, the beads undergo chemical transformations to obtain all main functional groups needed for peptide synthesis.

By comparison, the resins designed for chromatographic applications contain a higher proportion of cross-linking agent (DVB), which minimises swelling in aqueous and organic solvents in order to ensure stable back pressure in the column in both high- and low-pressure chromatography. Chromatographic resins require a controlled porosity to ensure good separation of molecules (peaks) and very tight particle size distribution that increases the number of theoretical plates per metre (N/L) and therefore improves resolution. The polymerisation of the chromatographic resins is carried out using a specific technology named “seeding”, which allows for uniform bead formation up to 70 microns in diameter. The uniformity of beads is measured by Uniformity Coefficient (UC), with a UC of 1 representing monosized beads. In the case of chromatographic resins, it is possible using seeding to obtain UC values < 1.15, which is very close to monodispersity. Comparatively, suspension polymerisation generally delivers a UC of >1.5. The “seeding” process consists of the swelling of PS oligomers in a controlled environment, in the presence of DVB and inert solvent to produce beads with a uniform particle size distribution and pre-defined porosity. Combining the hydrophobic characteristics of the polymer backbone with the controlled porosity and the high chemical and mechanical stability, the Chromalite® DVB-based beads represent a suitable choice for the purification of peptides and oligonucleotides alongside other small molecules. Moreover, the synthetic Chromalite 15AD2 resins tested here are chemically more stable than the traditional silica RP-HPLC particles, especially at the extreme pH values needed for column cleaning and specific purification processes.

A comparison of the resins used for SPPS and for chromatographic applications is shown in Table 1.

Application	Resin brand	Resin description	Particle size (µm)	Average pore diameter (Å)	Surface area (m ² /g)
SPPS	PuroSynth	PS with 1% DVB	75-150	N/A	N/A
Chromatography	Chromalite 15AD2	50% DVB	13.5-16.5	300-700	>400
Chromatography	Chromalite 15AD2	80% DVB	13.5-16.5	300-700	>400

Table 1. Characteristics of the PuroSynth SPPS beads and the Chromalite chromatographic beads.

RESIN MORPHOLOGY

The “jetting” and “seeding” technologies enable the production of beads with a consistent and reproducible distribution of their particle sizes. Figure 1 shows scanning electron microscope (SEM) images for one PuroSynth resin and one Chromalite resin with different particle size ranges. The uniformity of the beads is highlighted as a positive, especially for a chromatographic resin.

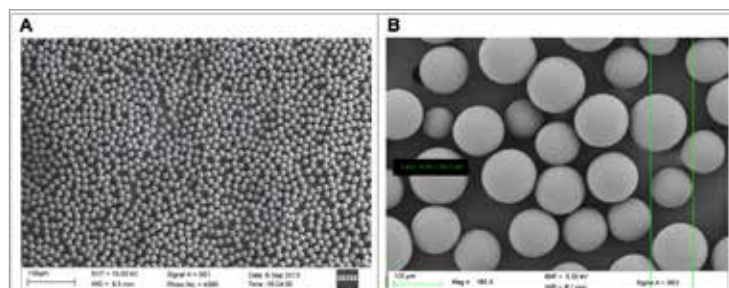


Figure 1. SEM images of A. Chromalite® 15AD2 (13.5–16.5 µm), B. PuroSynth resin (75–150 µm, measured 88.6 µm). The dried resin beads were coated with a thin layer of gold using a Quorum Q150 RES sputter coater (Quorum Technologies Ltd, East Sussex, UK) and photographed using a scanning electron microscope (Zeiss ultra plus FE-SEM, Jena, Germany).

For research purposes, there is an interesting possibility of recycling the SPPS resin, mainly when 2-chlorotrityl chloride (CTC) resin is used. In this regard, it is important to analyse the morphology of the resin after the overall synthetic process, including the peptide precipitation step with the appropriate ether. Of note, cyclopentyl methyl ether (CPME) has proven to be an optimum ether for peptide precipitation purposes, the resin’s morphological characteristic remaining intact after the treatment with this ether (Figure 2). On the other hand, 2-methyltetrahydrofuran (2-MeTHF) has mildly altered the resin (9).

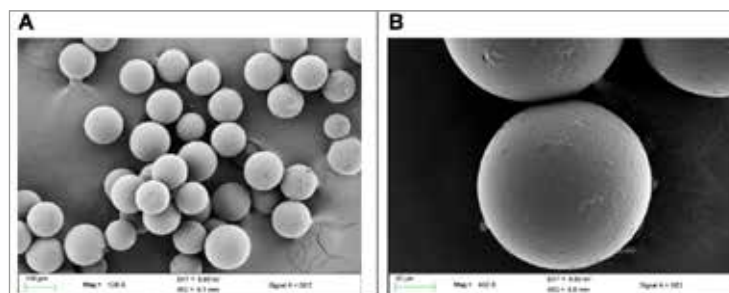


Figure 2. SEM images of various PuroSynth resins after precipitation with CPME. See legend of Figure 1 for experimental details.

Following the global deprotection and peptide cleavage, the generated PuroSynth 2-chlorotrityl alcohol (CTA) resin can be easily activated using thionyl chloride to regenerate the CTC functionality suitable for reuse in the SPPS process, especially at laboratory scale.

SWELLING

An important difference between the two groups of resins

is the swelling in aqueous and organic solvents. While the Chromalite 15AD2 resins for RP-HPLC should be compatible with most solvents used in chromatography, with minimal swelling, the swelling of the SPPS resin in organic solvents is a crucial

prerequisite to a successful peptide synthesis. PuroSynth resin showed great swelling capacity versus a commercial resin (Figure 3). In all tested solvents: methylene chloride (DCM), N,N-dimethylformamide (DMF), acetonitrile (ACN), and the green solvents 2-MeTHF and γ -valerolactone (GVL), the swelling of the PuroSynth resin was comparable to or, in some cases, greater than the commercial resin.

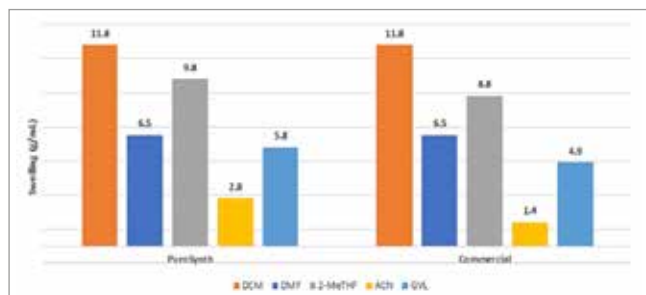


Figure 3. Swelling profile of chloromethyl (CM) resin: PuroSynth versus commercial. Experimental: 200 mg of resin swelled in 10 mL of solvent.

SOLID PHASE PEPTIDE SYNTHESIS (SPPS)

In this work we also looked at the synthesis and purification of several peptides of therapeutic interest. Bivalirudin (Angiox®, Angiomax®) is a direct thrombin inhibitor that acts as anticoagulant used in patients undergoing angioplasty. It is a 20-mer C-acid peptide (H-DPhe-Pro-Arg-Pro-Gly-Gly-Gly-Gly-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH) manufactured via SPPS. Using PuroSynth Wang resin (0.64 mmol/g), a non-optimized manual SPPS was carried out resulting in a crude peptide with a purity of > 90% as determined by RP-HPLC. The main impurity is the des-Arg peptide, which is due to the formation of the δ -lactam and is mostly unrelated to the solid support used. After the global deprotection, bivalirudin was precipitated in various ethers: diethyl ether (DEE), diisopropyl ether, and the green ethers, cyclopentyl methyl ether (CPME) and 2-methyl tetrahydrofuran (2-MeTHF). Identical purity profiles were obtained in each ether precipitation as measured using a traditional silica RP-HPLC column (Figure 4). In this case, the different ethers used for the precipitation of bivalirudin didn't influence the purity of the peptide, offering the possibility to choose a green ether and reduce the environmental impact of such process.

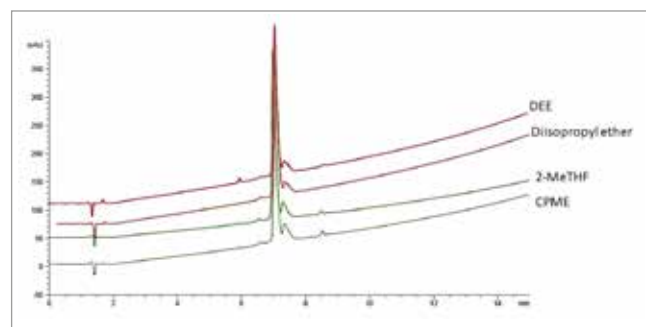


Figure 4. Bivalirudin 20 mer peptide synthesized using PuroSynth Wang resin (0.64 mmol/g). Green vs. traditional ethers used for precipitation. HPLC operating conditions: Mobile phase A: 0.1% trifluoroacetic acid (TFA) in H_2O ; mobile phase B: 0.1% TFA in ACN; 5–95% gradient elution in 15 min, $\lambda = 220$ nm. Commercial column: Phenomenex Luna C₁₈ (3.6 μ m, 4.6 $\times$ 150 mm) column. Flow rate 1 mL/min.

Global deprotection, where the unprotected peptide is liberated from the resin with concomitant removal of the side-chain protecting groups, is carried out with

high concentration of TFA in the presence of scavengers to quench the formed carbocations coming from the cleaved protecting groups at room temperature (rt). The cleavage yield depends on the C-terminal amino acid, the peptide sequence (structure and size), and most importantly the solid support. Generally, yields of peptides cleaved from Rink-amide resin and, to a lesser extent, from the Wang resin are far from quantitative. In this regard, we have investigated the possibility of increasing slightly the temperature of the cleavage up to 40 °C. Results shown in Table 2 indicated that the yields have increased, while maintaining the purity of the final product (results not shown). The peptides considered in this study were: Leu-enkephalin, a 5-mer peptide (H-Tyr-Gly-Gly-Phe-Leu- NH_2), and Thymosin, a complex 28-mer 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).

Peptide	Conditions		
	1h rt	2h rt	2 h 40°C
Leu-Enkephalin 5 mer peptide	63.3	91.0	95.1
Thymosin 28 mer peptide	35.2	57.1	60.6

Cleavage cocktail: TFA-Trisopropylsilane- H_2O (95:2.5:2.5)

Table 2. Heated vs rt cleavage of Rink Amide AM resin (0.70 mmol/g)

CHROMATOGRAPHIC SEPARATION OF PEPTIDES

Chromatographic separation of various peptides was investigated by means of RP-HPLC. The Chromalite 15AD2 resins with an average particle size of 15 μ m and varying DVB content were packed in 4.6 $\times$ 150 mm HPLC columns and compared with a silica C18 column and a synthetic resin from a commercial supplier. The peptides for this experimental work were selected to cover a large range of amino acids comprising various chemical structures.

The number of amino acids were 5 for Leu-enkephalin pentapeptide and 16 for the ABRF1992 peptide (H-Gly-Val-Arg-Gly-Asp-Lys-Gly-Asn-Pro-Gly-Trp-Pro-Gly-Ala-Pro-Tyr- NH_2) with both peptides being produced at laboratory scale by SPPS using PuroSynth resins.

For each peptide, the purity profile was compared among the four RP-HPLC columns with similar elution profile as shown in figures 5 and 6 and table 3.

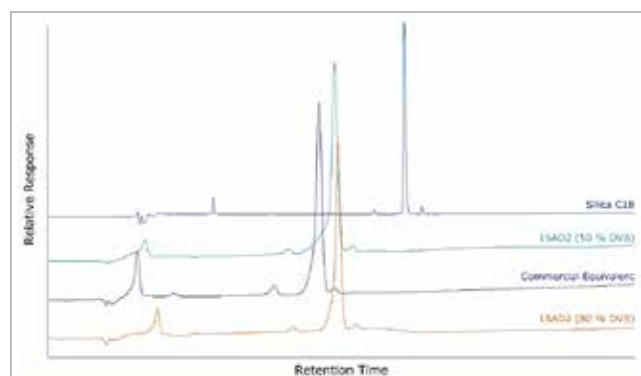


Figure 5. Leu-enkephalin pentapeptide chromatographic profile using chromatographic columns: A. Commercial Silica C18 (5 μ m); B. Purolite #1 (15AD2 (50 % DVB, 15 μ m)); C. Commercial PS/DVB (15 μ m) D. Purolite #2 (15AD2 (80 % DVB, 15 μ m)). HPLC operating conditions: Mobile phase A: 0.1% TFA in H_2O ; mobile phase B: 0.1% TFA in ACN; 5–95% gradient elution in 45 min, $\lambda = 220$ nm. Flow rate 1 mL/min.

The purification of Leu-enkephalin pentapeptide shows good results on the silica C18 column, with clear baseline separation of the main peak from the neighbouring impurities. Chromalite resins also show baseline separation of these peaks, with a slightly larger fronting shoulder present for the 50 % DVB resin. The commercial equivalent synthetic resin also shows reasonable separation, although the later-eluting peak may overlap slightly with the main elution peak. Notably, all three synthetic resins demonstrate a shorter retention time for the peptide than the silica C18 resin.

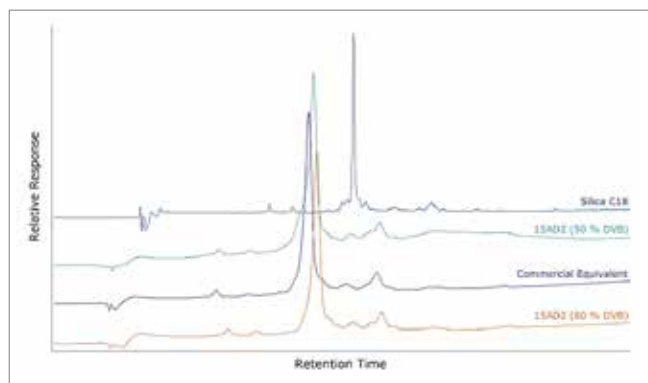


Figure 6. ABRF1992 peptide chromatographic profile using chromatographic columns: A. Commercial Silica C18 (5 μm); B. Purolite #1 (15AD2 (50 % DVB, 15 μm)); C. Commercial PS/DVB (15 μm) D. Purolite #2 (15AD2 (80 % DVB, 15 μm)). See legend of Figure 5 for chromatographic conditions.

The purification of ABRF 1992 peptide again shows reasonable results on the silica C18 column, this time with less clear separation of the main peak from the neighbouring impurities. Synthetic resins show less clear separation of peaks, with a lower definition of individual impurities neighbouring the main peak. The commercial equivalent synthetic resin shows comparable results to the Chromalite resins, separating some impurities better and some worse. Again, all three synthetic resins demonstrate a shorter retention time for the peptide than the silica C18 resin.

Resin	Retention Time Leu-enkephalin (min)	Retention Time ABRF1992 (min)
Silica C18	15.698	13.542
Chromalite 15AD2 (50 % DVB)	12.665	11.767
Commercial Equivalent	11.989	11.557
Chromalite 15AD2 (80 % DVB)	12.821	11.921

Table 3. Retention times of Leu-enkephalin and ABRF1992 on HPLC resins:

One feature not discussed thus far is the difference in particle size. The silica C18 resin has a particle size of 5 μm , whereas all three synthetic resins are 15 μm in diameter. Bearing this difference in mind, some degree of lost resolution is inevitable, and a significant gain can be made in the backpressure of the column. Indeed, the measured backpressure under the purification conditions for the silica C18 was 120 bar compared to 50 bar for the synthetic resins, which would theoretically enable a much higher flow rate and therefore productivity of the purification process. This gain in productivity is amplified by the faster elution of the peptide from the synthetic resins compared to the C18 silica which can lead to shorter process time and reduced solvent consumption.

CONCLUSIONS

'Jetting' technology developed by Purolite allows the production of PS/DVB resins with a low degree of cross-linking

for SPSS and a narrow particle size distribution favouring a consistent peptide synthesis and a more economical process. "Seeding" technology is used for the production of nearly uniform beads (UC <1.15) up to 70 μm in diameter and with a high degree of cross-linking for RP chromatographic purification of peptides. These new manufacturing technologies each result in advantageous performance characteristics over alternative production methods, due to a pronounced increase in particle size uniformity and the ability to tightly control porosity. This leads to improved chromatographic performance and more reproducible results. Applications of Purolite resins for synthesis and purification of pharmaceutically interesting peptides have shown the importance of the resins for the development of peptides as drugs.

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Dr. Simona Serban has joined Purolite U.K. in 2012 when the Life Sciences division was created. Her major contribution is in the development of the resins and corresponding applications for the LifeTech range of resins for enzyme immobilisation and immobilised enzymes, PuroSynth range of resins for solid phase peptide synthesis and the Chromalite range of resins for chromatographic applications

