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Research Paper

Commercial manufacturing of current good manufacturing practice peptides spanning the gamut from neoantigen to commercial large-scale products

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ABSTRACT

Peptide therapeutics represents a significant and growing area for manufacturing companies utilizing both chemical and recombinant methods. Approvals for orally administered peptides such as Linzess® (linaclotide), Trulance® (plecanatide), and most recently Rybelsus® (semaglutide) are pushing manufacturing requirements to quantities routinely exceeding 100 kg and potentially metric ton quantities especially for conditions such as type II diabetes where a worldwide epidemic creates the need for truly huge quantities. Additionally, personalized medicine has created a need for rapid synthesis of multiple peptides manufactured and released under current good manufacturing practices standards in the 50 mg range with a speedy delivery (< 4 weeks). These two aspects of peptide manufacturing represent the gamut, which companies must span in order to meet all of their potential customer requirements. The purpose of this review is to cover some of the newer aspects in manufacturing required to meet the demand that these two extremes represent.

1. Introduction

Synthetic peptide drugs became feasible in the mid-1950s with the ground breaking work of du Vigneaud with the sequencing and synthesis of oxytocin (1). Since oxytocin entered the market in 1962, the peptide pharmaceutical demand has grown exponentially over the past 7 decades (2). With more than 70 peptide active pharmaceutical ingredients (APIs) approved and marketed around the world, the manufacturing required to support these products has been built up either among the pharmaceutical company innovators or through contract development and manufacturing organizations (CDMOs).

Many of the earliest peptide APIs produced were made using classical solution phase methods for synthesis. This methodology was cumbersome and slow, requiring extremely long lead times for manufacturing. The primary manner by which a product could be accelerated was to develop a stockpile of the key fragments of the molecule of interest, which would facilitate a somewhat quicker production time for the API.

With the development of solid-phase peptide synthesis (SPPS), Bruce Merrifield completely revolutionized the way in which peptides could be produced with speed and ease (3). SPPS is probably the single greatest innovation to speed the development of peptide pharmaceuticals and has now been applied to synthesis of oligonucleotide drugs as well as complex carbohydrate molecules. One of the major limitations with the early Boc-chemistry processes was the resin cleavage and deprotection step requiring anhydrous HF. HF is a very dangerous reagent, which necessitates a special apparatus for safe handling. This limited both the scale and speed of the final cleavage step creating a bottleneck for large-scale commercial application beyond quantities < 10 kg.

Chemistry improvements beginning in the mid-1980s saw a fundamental shift from Boc-Bzl-based chemistry procedures to those employing Fmoc-tBu strategies (4). The Fmoc deprotection with a base such as piperidine was safer than the corresponding Boc cleavage with highly concentrated trifluoroacetic acid (TFA). Similarly, the final cleavage step with TFA is also intrinsically safer than HF and ultimately requires no special apparatus and can be carried out and scaled up in standard laboratory and production equipment and glassware.

Abbreviations: API, active pharmaceutical ingredient; Boc, t-butyloxycarbonyl; Bzl, benzyl; cGMP, current good manufacturing practices; COA, certificate of analysis; 2-CTC, 2 chlorotrityl chloride; dH_2O, distilled water; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DODT, 3,6-dioxa-1,8-octanedithiol; EDT, 1,2 ethanedithiol; FDA, Food and Drug Administration; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBT, 1-hydroxy-benzotriazole; HF, hydrogen fluoride; HPLC, high performance liquid chromatography; IpOH, isopropanol; IQ–OQ–PQ, installation qualification, operation qualification, performance qualification; LC–MS, liquid chromatography mass spectroscopy; MeCN, acetonitrile; Oxyma, Ethyl cyanohydroxyiminoacetate; Pbf, 2,2,4,6,7-pentamethyldlhydrobenzofuran-5-sulfonyl; RP-HPLC, reversed phase high performance liquid chromatography; SPPS, solid-phase peptide synthesis; tBu, tert-butyl; TFA, trifluoroacetic acid; Trt, triphenylmethyl; UPLC, ultra performance liquid chromatography; Z, benzyloxycarbonyl;.

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Coinciding with the development of Fmoc-tBu production, development of the syncytium blocking HIV peptide Fuzeon (enfurvirtide) by Trimeris and Roche, and the daily large dosing regimen (5) completely transformed the supply chain for the Fmoc-building blocks and solid-phase resins needed to produce potential commercial quantities of up to metric tons of API per year. This program and other large-scale programs such as bivalirudin led to the industrialization and regulation of Fmoc-protected amino acid derivatives, which has led to a significant improvement in the quality of the 20 standard building blocks (6). The Fmoc-protected natural proteinogenic amino acid derivatives have dropped in price by nearly a factor of 10 while quality has been vastly increased (7). Currently, we see a shift to very high purity Fmoc amino acid derivatives with very high enantiomeric purity and extremely low levels of other impurities to meet FDA expectations for both a new drug application (NDA) and abbreviated new drug application (ANDA) peptides. As a result of the greatly improved quality and reduced derivative pricing scenario, longer and more complex peptides entered development as they were considered as commercially viable.

It is the purpose of this review to discuss the two widely divergent aspects of peptide manufacturing. The development of personalized medicine has created a need to get multiple peptide neoantigen peptides in a very quick time frame that is still suitable for human use. The second aspect will cover the current status of commercial peptide manufacturing using solid-phase, solution-phase, and hybrid strategies (combinations of solid-phase synthesized fragments coupled together with solution methods).

2. Personalized medicine peptide manufacturing

Cancer is one of the leading causes of death in the developed world, and has historically been treated with surgery, radiation therapy, chemotherapy, and hormone therapy that try to remove or to kill cancer cells (8). More recently, therapies have focused on harnessing the immune system to fight cancer by developing a new class of immunotherapy known as checkpoint inhibitors. These molecules allow the immune system to reengage and attack these cells by preventing the signaling from cancer cells that suppress immune responses. In cancers where checkpoint inhibitors are not effective, neoantigen-targeted therapies may precisely direct the immune system to improve patient outcomes across both checkpoint-responsive as well as unresponsive disease. Genetic mutations, which result in aberrant proteins encoded by these changes, can lead to uncontrolled division and proliferation of the abnormal cells. By breaking the mutated proteins into a series of smaller peptides (neoantigens), they can be used to generate immune signals on the surface of cancer cells (9). The immune system can be primed to see these neoantigens as foreign, and mount an immune response against the abnormal cancer cells containing these exposed neoantigen epitopes cells just like it was a virus or bacteria. Neoantigens are different for every patient. Thus, production of a cocktail of unique neoantigens to make a cancer vaccine will be different for every patient leading to creation of personalized medicine (10).

Manufacturing neoantigen peptides comes with serious constraints with respect to the time required to quickly get them into cancer patients who may only have a short therapeutic window for treatment (11). Typically, these projects come as a series of 25–40 unique peptides with a time commitment of 4–5 weeks including quality control (QC) release. The quantities (~50 to 60 mg) required are usually much smaller than standard GMP peptides involved in clinical trials. Thus, the type of equipment involved in this type of peptide manufacturing is geared more toward automated and simultaneous parallel methods for synthesis, purification, and analysis.

All of the automated multiple peptide synthesizers, which are available for rapid neoantigen synthesis, utilize Fmoc-tBu chemistry. Some of the more popular synthesizers include: Symphony X®, Prelude X® (Gyros Protein Technologies) (Figure 1A), Liberty Blue® (CEM) (Figure 1B), CS136M® (CSBio Inc.) (Figure 1C) and Tetras® (Advanced Chem Tech) (Figure 1 D). These machines allow for the rapid synthesis from six (Prelude-X, CS136M), to twelve and twenty-four (Liberty Blue®, Symphony X®), or thirty-one (Tetras®) individual peptides. Installation of this equipment has standard IQ–OQ offered from the vendors to provide proper operation and documentation for qualification. Additionally, the vendors of these instruments offer services such that they can be requalified by field engineers on an annual basis facilitating GMP compliance. Most of these synthesizers utilize software, which is 21 CFR part 11 compliant.

Speeding up the cycle time on these automated machines is key to accelerating the assembly. Several of synthesizers utilize different forms of heating. These can include microwave (12), infrared (13), and jacketed reaction vessels. The reaction chemistry has been optimized using a high excess of activated amino acid relative to the resin-bound amine component. This helps drive the reaction quickly to completion. Over the past decade, optimization of the cycles with these heat-accelerated protocols has been sorted out. Many of the issues with side reactions were also solved during this same period by using lower temperature coupling for amino acids such as Arg(Pbf), His(Trt), and Cys(Trt). These improvements have been implemented as standard cycles (14). In fact, some of the cycles for the microwave machine are as short as 4 min per amino acid at very small scale (50 μmol).

Coupling chemistries vary from machine to machine and are capable of being selectively determined by the chemist. The higher temperature couplings seem to favor carbodiimide-mediated active esters using HOBT or Oxyma® (15). Use of high temperature couplings with base-mediated chemistries such as HBTU often leads to more side reactions and in particular racemization. Base-mediated coupling chemistry works better at ambient temperature (~22°C). Choice of the base is usually selected by the chemist, but in most cases, it is either DIEA or *N*-methylmorpholine. Depending on the chemistry and temperature used, this has an impact on the cycle speed. Ambient temperature HBTU chemistry usually can have cycle times of about 30–45 min per cycle. Ambient temperature in situ carbodiimide-mediated active ester chemistry usually has cycle times of about 2–3 h. Microwave techniques usually have cycle times of <10 min.

Critical to the CEM microwave machines which have heat-accelerated chemistry options, are real-time temperature monitoring and feedback control (14). This control allows the internal reaction vessel solution temperature to be carefully monitored and adjusted to minimize side reactions such as epimerization, which can occur when it is not controlled. This improvement was critical to bringing these machines in line for providing high-quality crude products with acceptable levels of these process-related impurities (16).

An additional improvement on several of the Protein Technology machines allows for UV-based real-time Fmoc removal monitoring (17). This improvement allows the user to set parameters for Fmoc removal before starting the next coupling. This helps ensure that a sluggish Fmoc removal gets additional time or fresh reagent helping to improve the crude product quality.

At the conclusion of synthesis, cleavage of the solid-phase bound products is required to get the crude deprotected peptide. This cleavage step is typically an acidolytic treatment (usually trifluoroacetic acid) for a set time period with a variety of cationic scavengers depending on the side chain-protecting groups present on the resin-bound peptide. Common scavengers, recipes, and cleavage times are summarized in Figure 2 (18,19). Cleavage can be performed as a final step on the Protein Technology machines, which have a cleavage module, which helps automate this cumbersome task. CEM also offers a user-programmed heat-accelerated platform (Razor®) to speed up the cleavage step for up to 12 peptides in parallel (Figure 1E). This apparatus can help reduce cleavage times to 30 min even for Arg-rich peptides. Otherwise, the resin-bound peptide can be individually processed manually in small capped vessels

Figure 1. Assorted Automated Synthesizers and cleavage equipment used for Neo-antigen manufacturing. (A) Gyros-Protein Technologies Symphony X. (B) CEM Liberty Blue HT-12. (C) CSBio 136 M. (D) Advanced Chem Tech Tetras. (E) CEM Razor. (F) Multistir plate mixer.

on a multistirrer hotplate (Figure 1F). The crude cleavage mixture can be subsequently filtered to remove the spent resin beads, which is also accomplished by both the PTI and CEM products. Once the filtered TFA cleavage mixture is obtained, it is precipitated with cold diethyl ether. The solid product can be isolated either by filtration or centrifugation. Precipitation into disposable polypropylene centrifuge tubes (50 mL), either on the synthesizer or from the cleavage apparatus, is very convenient and speeds up processing and washing of the deprotected products. The crude peptides can be subsequently dried to remove volatile organic solvents to get a constant weight. This is an important step especially when considering GMP documentation to accurately record yields.

Once a set of peptides has been synthesized and the crude products have been isolated, the next step in the process is purification. The major

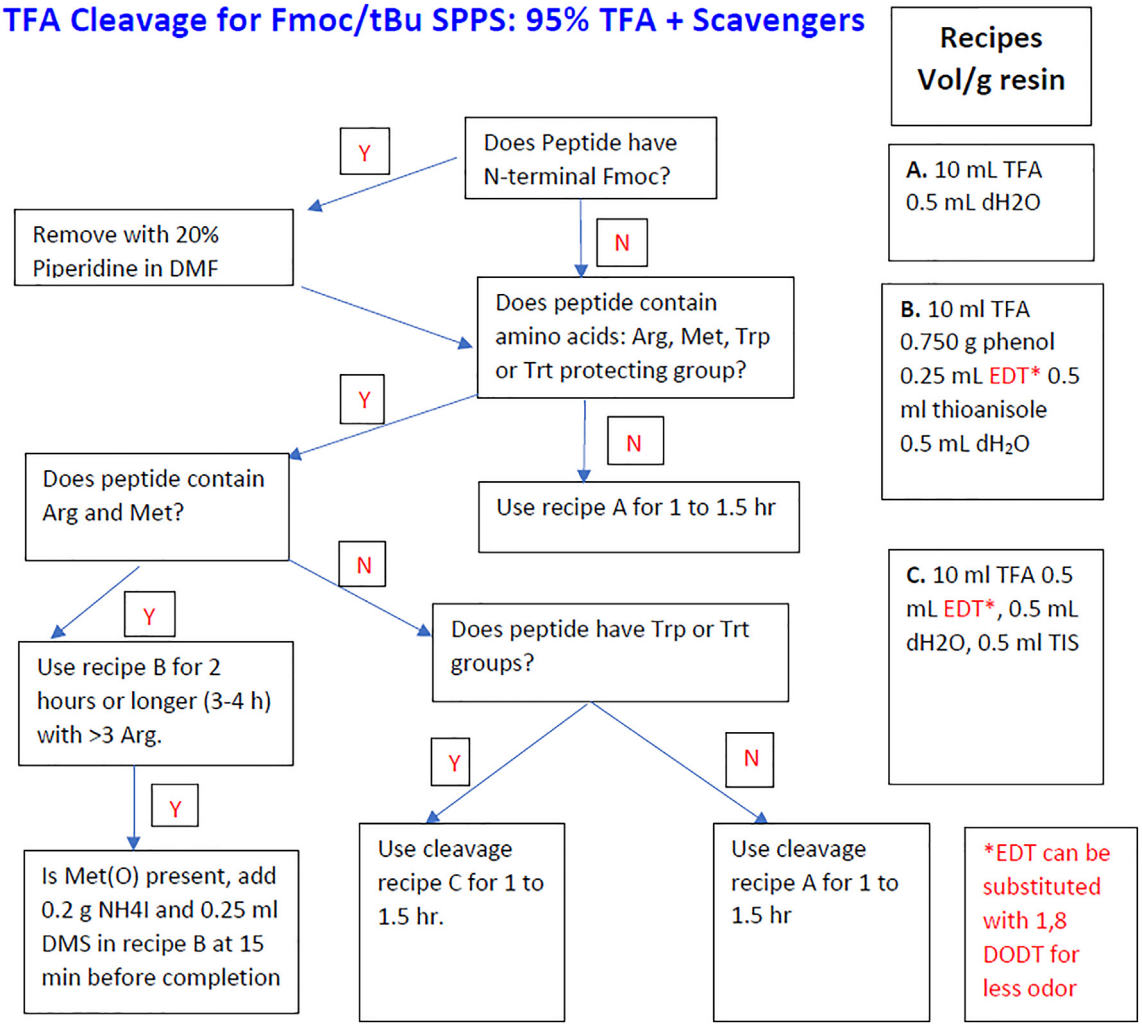


Figure 2. Fmoc-tBu cleavage flow diagram with standardized recipes.

technique for purifying the majority of peptides either for neoantigens or commercial peptide APIs is using reversed-phase high-pressure liquid chromatography (RP-HPLC). Prior to moving into purification, typically either an analytical LC-MS or a standard RP-HPLC run using an autosampler is performed, which provides data on the relative elution concentration of organic modifier (typically MeCN). When LC-MS is used, critical mass spectral data that the correct peptide is present in the crude product for each peptide is also generated.

Once these data have been collected, RP-HPLC for purification of neoantigen peptides takes place on an automated semipreparative HPLC system typically using C18 RP-HPLC media (5 μ, 100 Å) packed into 2.5 cm × 50 cm columns. Standard linear gradients with water vs. acetonitrile containing 0.05% TFA are easily accommodated on this type of instrument. Use of the proper buffer counterion (TFA) is critical for use of the mass spec feature of this equipment. Keeping the TFA concentration below 0.1% is critical to minimizing ionization suppression, which can occur with higher concentrations. This entire process can be automated by using an autosampler to inject each peptide sample followed by a purification gradient, column wash step, and re-equilibration for the next sample. The HPLC system usually has either a UV or diode-array detector as well as an inline line splitter for mass analysis (Figure 3A). The system can be programmed to actually collect the peptide sample containing only the desired mass range into a smart fraction collector. This fraction collector interfaces with the HPLC software and provides an output report, indicating the fractions for each product and those that have the correct mass (Figure 3B). This system quickly allows the operator to select the appropriate fractions for analysis using either UPLC or HPLC. The real benefit of UPLC-based analysis is faster processing time relative to standard analytical HPLC methods. The desired fractions can be quickly analyzed by using an autosampler coupled with the UPLC or HPLC.

Final isolation of the product remains the most cumbersome and time-consuming part of the neoantigen production process. After analyzing single fractions, those which meet the desired purity (>90%) are placed into a properly cleaned lyophilizer bottle with an isolation filter to help prevent cross contamination, shell-frozen, and placed upon a manifold lyophilizer to freeze dry the peptides. Depending upon the volume of the combined fractions, freeze-drying can take between 12 and 48 h to complete. Following completion of freeze-drying, the peptides are removed and packaged in a BSC (Bio Safety Cabinet) suitable cleanroom environment in qualified sterile glass vials with proper closures.

The products proceed to quality control for identity testing by mass spectroscopy, purity testing by HPLC or UPLC, and endotoxin testing. Typical testing takes place in an accelerated environment so as to meet the tight delivery deadline of 4 weeks from initiation of synthesis to limited GMP release with a certificate of analysis.

A generic robust UPLC TFA buffer vs. MeCN method is typically used to analyze each peptide for purity. Main peak purity assessment can be used to

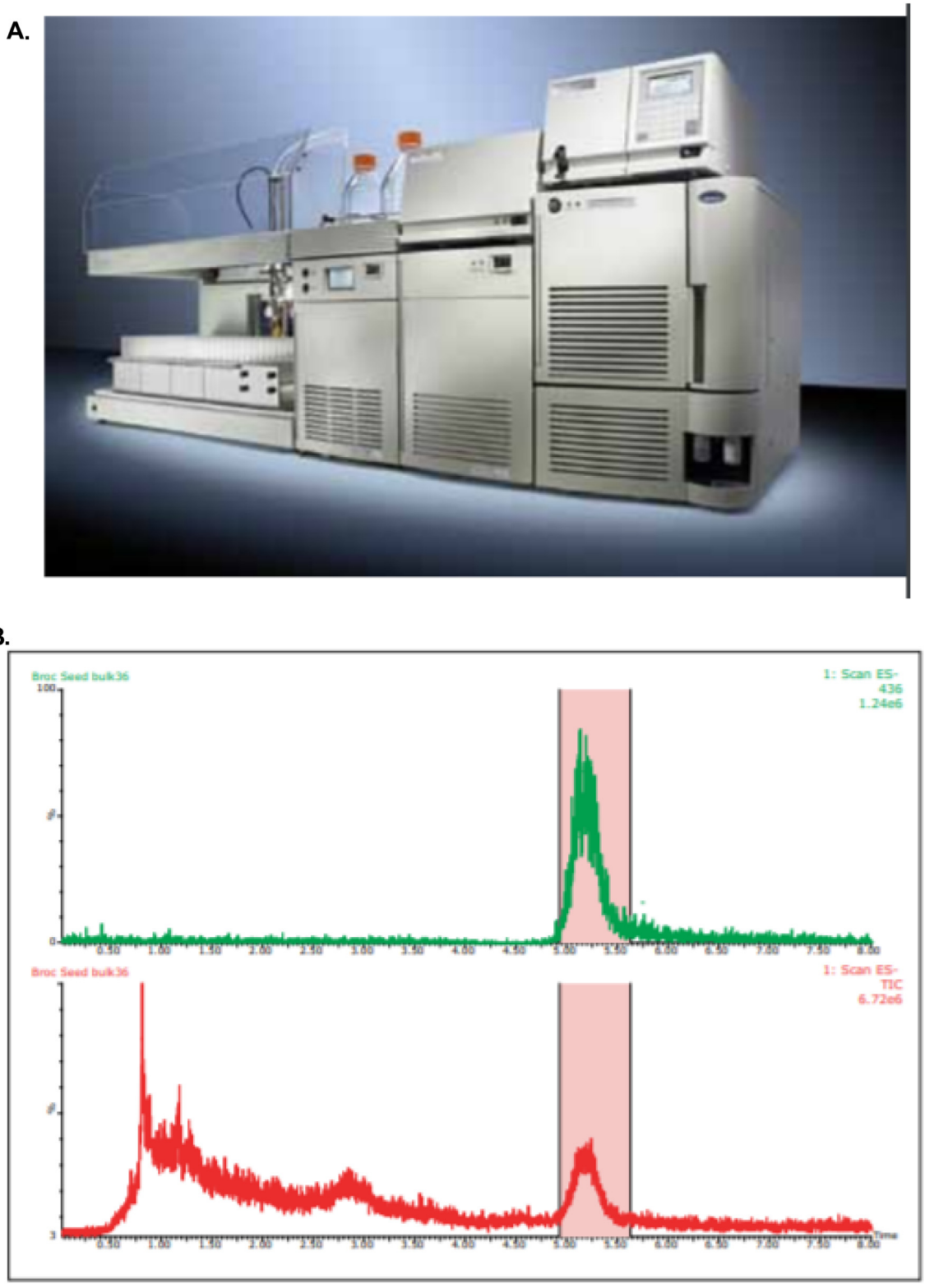


Figure 3. (A) Automated LC-MS semipreparative HPLC set-up for purification of peptides with smart fraction by mass. (Shown is system by Waters.) (B) Collection chromatogram based upon mass-based fractionation.

show the specificity of the method to achieve an accurate overall purity of the peptide, if needed. Typically, the peptides are stored at NMT −15°C. Stability studies are not needed based on history data of peptides and short storage time of less 60 days.

Of course, all the equipment and batch records must follow normal cGMPs. However, the batch records look considerably different when using an auto-synthesizer vs. the standard peptide batch record. All the equipment (including the auto-synthesizer) have to be fully qualified (IQ/OQ/PQ) and calibrated. The programming and verification processes have to be well-followed and documented. The validation activities show that the auto-synthesizer if setup correctly yields the correct peptide with acceptable/suitable purity.

One of the most important aspects that has emerged for the production of neoantigen-personalized medicine peptide products is that the process of making the products rather than the individual products themselves is what is validated (20). Since each product is made only once and in a small quantity, numerous peptides are made by using the same process. Thus, the products are **not** subjected to some of the more lengthy tasks such as stability (24 months), impurity limits, and identification of related substances/impurities as are appropriate for standard ICH guidelines for standard peptide APIs. As a final step in the process, the final drug product must be formulated, filled, and sterilized under sterile conditions to meet sterility requirements.

3. Large-scale commercial manufacturing

Large-scale commercial manufacturing of peptides in quantities >100 kg began with Fuzeon (enfurvirtide) and Hirulog (bivalirudin). The dawn of oral peptide delivery has arrived, which will require even larger quantities due to the higher dosing regimens resulting from the lower adsorption rates. Starting with Linzess® (linaclotide) and Trulance® (Plecanatide), CDMO manufacturing began to produce peptides in a cost-effective manner, which helped to propel oral peptide delivery as being viable. Even though the site of action for these peptides is actually in the gastrointestinal tract, the peptides have to successfully transit the early part of the digestive organs in order to stimulate the GPCR receptors in the large intestine to facilitate relief from constipation (21). More recently, Rybelsus® (semaglutide), an orally delivered glucagon-like peptide (GLP) analog (22), for treatment of type II diabetes, also known as metabolic disease, was recently approved in both the United States and Europe (23,24). Semaglutide was produced in early discovery by synthetic methods (25) and later a commercial production by a recombinant combined with a synthetic modification step for attachment of the N-terminal dipeptide and the side chain albumin binding motif was developed (26).

With patent expiration approaching for large quantity, highly successful injectables such as Victoza® liraglutide, synthetic processes have been developed to compete with this recombinantly produced synthetically modified product. These processes require highly efficient and robust manufacturing processes to truly be competitive with the reference list drug (RLD). Large-scale peptide manufacturing will be the second topic covered in this manufacturing overview. Other comprehensive and detailed reviews regarding commercial peptide production have been written earlier (27); this overview is to give insight into commercial production of quantities meeting demands of >100 kg/annum.

4. Commercial manufacturing of >100 kg peptide APIs

High-quality raw materials from QA-audited companies start the process. These amino acid derivatives, resins, and solvents must be internally quality controlled and released for use by QA. Of critical importance is the control of enantiomeric impurities in the amino acids. If care is not taken to use high-quality amino acid derivatives, downstream processing to remove the diastereomers is extremely difficult if not impossible. The regulatory agencies expect tight controls (specifications) of the amino acid derivatives to ensure high-quality peptides. Significant improvements in the activation chemistry, which minimize racemization of the amino acid during the chain elongation steps, are another essential advancement to also eliminate the formation of diastereomers during synthesis. Discussion of this topic has been thoroughly covered in other excellent reviews and is beyond scope of this article (28).

Large-scale peptide production utilizes the same solid-phase based manufacturing strategies used in the small-scale process except that the major processes are typically manually controlled or only using semi-automated strategies whereby the washing steps may be automated but the key steps of deprotection and coupling are carefully monitored. Monitoring of these steps is controlled using colorimetric tests such as the Kaiser

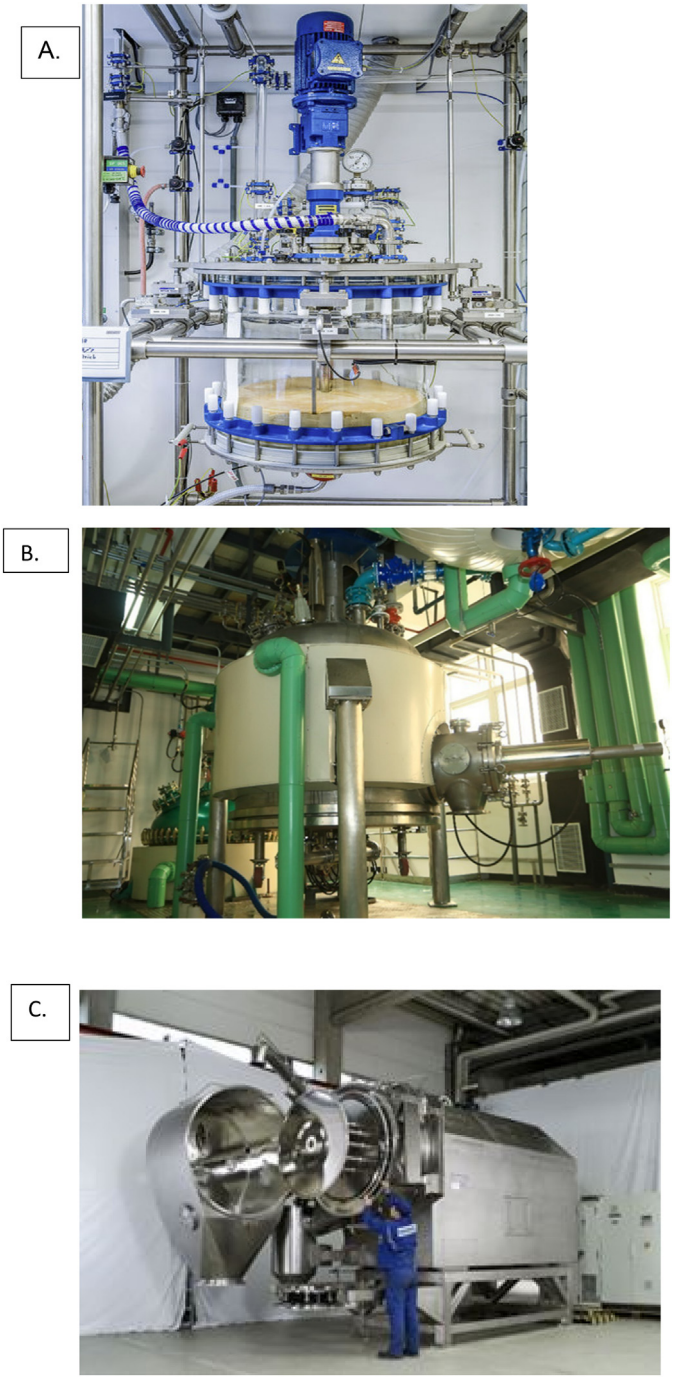


Figure 4. Commercial solid-phase Nutsche filter reactors. (A) 150 L reactor. (B) AmbioPharm designed 1000 L jacketed reactor type (C) an inverted centrifuge.

(29) or TNBS tests (30) for the presence of primary amines and secondary amines via chloranil testing (31,32). It is also possible to perform quantitative ninhydrin testing to get accurate values for each coupling step (33). The results of these in-process colorimetric tests inform the operator as to the success of a deblocking step as well as that of a coupling step. Results of these tests are used as part of the in-process synthesis records included in the BPR. Other in-process controls may include acidolytic microcleavage of small portions of the resin-bound peptide taken during the assembly phase with subsequent analysis by HPLC, UPLC, and mass spectroscopy. This data provides confidence in the quality of the product as it is being assembled on the solid phase.

Large-scale jacketed solid-phase reactors are typically used in commercial manufacturing (Figure 4). Most of this type of equipment is custom

produced based upon pharmaceutical filter dryers for manufacturers, which synthesize cGMP peptide APIs. The scale of this equipment can be from 300 L up to 5000 L. These reactors require a barrier at the bottom of the reactor to contain the resin-bound product and prevent it from passing into the waste. This containment is critical to facilitating resin washing steps. This barrier typically takes the form of a polypropylene membrane, which can be easily replaced if it becomes clogged during the synthesis. Efficient washing is critical to all solid-phase synthesis operations to remove the deprotection chemicals as well as the coupling chemicals and activated amino acid derivatives from the preceding cycle. Process development activities with proper design of experiments measuring the efficiency of the washing steps are critical to both the quality and the economics of the SPPS at commercial scale.

Additionally, alternative strategies, which employ techniques with fragment condensation either in solution or on a resin-bound peptide, are also employed. The latter method, which utilizes fragments coupled with solid-phase synthesis tactics, is often referred to as a hybrid method. This technique is often used to improve the quality of the crude peptide by coupling a small peptide fragment, which has been identified through process development activities to be a difficult part of a peptide to synthesize. By making a small protected peptide fragment, which is easier to produce in high quality and coupling this to the resin-bound peptide, it will by-pass a difficult coupling region of a peptide. This is particularly useful on longer peptides, which typically begin to have difficulties beyond synthesis of 12 residues (34) due to solvation issues of the peptide chain (35,36) as well as formation of secondary structures, which can form in the protected resin-bound peptide and impede synthesis progress from hydrogen bonding (37,38). Improvements to disrupt these inter- and intramolecular interactions by utilizing pseudoproline dipeptides have greatly facilitated the synthesis of longer peptides as an alternative to hybrid methods allowing for total linear SPPS strategies (39).

Hybrid Case Study: Manufacturing Enfuvirtide acetate salt Ac-Tyr-Thr-Ser-Leu-Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln^{16}-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Leu-Glu^{27}-Leu-Asp-Lys-Trp-Ala-Ser-Leu-Trp-Asn-Trp^{35}-Phe-NH_2 acetate salt.

As an example, and case study for the commercial peptide Fuzeon® (enfurvirtide), a hybrid approach is used to make hundreds of kilograms of this 36-residue peptide. The reported process utilizes three fragments each assembled on 2-CTC resin (40). The protected peptide fragments are released from the 2-CTC resin by treatment with 1% TFA in methylene chloride and immediately neutralized with pyridine. Care must be taken with 2-CTC resins as they are particularly prone to hydrolysis-related decomposition as well as premature cleavage of the peptide with coupling additives such as HOBT. The products are isolated by concentration and precipitation with diisopropyl ether and obtained in 85% yield at 90% purity. As shown above and in Figure 5, the split points are shown in different colors for each fragment. The N-terminal fragment is protected on its alpha amine with an acetyl group. To begin final assembly, fragment 3 (Fmoc protected peptide 27–35) is activated in solution and coupled to Phe-NH2 in DMF. The product is precipitated into water, isolated by filtration and carried forward to the next step following removal of the Fmoc group, the C-terminal peptide is coupled to the Fmoc-protected middle fragment (peptide 17–26) also in DMF. Following a similar isolation procedure used for the first fragment 27–36, the Fmoc is removed and the last step is coupling the acetylated N-terminal 1–16 fragment to the 17–36 amide intermediate. The resulting process has 106 chemical transformations and 7 isolation steps. The final product is isolated and treated with a TFA cocktail to remove the side chain-protecting groups. This process was developed at a time when there were not suitable resins for producing protected C-terminal amidated peptides. The crude product has an average crude purity of 75% and the final product is obtained in approximately 30% overall yield. Reduction in cost was afforded by utilizing a solvent recovery and recycling approach.

Classical solution-phase peptide synthesis or liquid-phase peptide synthesis was used for commercial peptide manufacturing prior to the development of super-acid sensitive resin tactics for making protected peptide fragments. In fact, the majority of the peptides produced require stockpiling of key intermediate fragments to help achieve more favorable delivery times. Many of the early approved peptide drugs such as the gonadotropin-hormone-related peptides (LH-RH antagonists) (leuprolide, goserelin, triptorelin, cetrorelix) were produced by classical processes. Part of the rationale for this development is the conventional size of these peptides of approximately 10 residues. Furthermore, the size of these peptides facilitates the development of crystallization and/or precipitation methods for isolation of the intermediates and sometimes the final product as well. Longer peptides such as calcitonin were also produced by classical methods by producing a series of fragments using a convergent approach to assemble them into the final product.

A typical convergent solution phase strategy is to produce protected peptide fragments of approximately 3–6 amino acid residues. Doing small-scale trials is critical in designing the optimal strategy. This development work is facilitated by using solid-phase-prepared protected fragments made on 2-CTC resin (41,42). This approach allows one to optimize a process as well as observe key parameters such as fragment solubility and racemization issues. Having nicely soluble fragments is absolutely paramount to the success of a solution-phase process. Ideally, in order to prevent racemization, the C-terminus of each of these fragments is either a Gly or Pro. When this is not possible, choosing residues which are less prone to racemization (Ala, Arg) as the C-terminus is advised. Assembly of the fragments to the final product must be carefully controlled. Analysis of the products has been accelerated by making substituted D amino acid analog sequences at key suspected racemization positions to facilitate identification and quantitation in spiking experiments with RP-UPLC equipment.

Conventional classical synthesis strategies typically employ a benzyl-based protecting group strategy for side chains and C-terminal acids allowing for final removal by hydrogenation. N-terminal protecting groups are usually Boc, which allows for removal using 4 N HCl in dioxane. *Z*-groups are also be cleaved by HCl treatment if no other labile benzyl-based side chain protection is used. Activation chemistry must be chosen carefully in order to minimize racemization. Use of preactivated amino acids can help bypass this issue in the assembly of fragments but may have a prohibitive cost. Mixed anhydride, DIC-HOBT, and azide chemistry are often employed for coupling the fragments together.

Depending on the protecting groups used, final deprotection of the peptide is often H_2/Pd for Z and benzyl groups (43). If acid labile groups are used, then deprotection can be accomplished with TFA-based cocktails if Boc and tBu have been used or with HBr in AcOH if Z and Benzyl have been used (43). Isolation of the crude product following cleavage usually takes place in an inverted filter centrifuge (Figure 4 C) in large-scale operations (44).

Recently, a series of hydrophobic tags with variable length alkyl moieties on an aromatic scaffold have been used to derivatize the C-terminal amino acid for solution-phase approaches (45–47). This tag acts in a similar manner as the resin in SPPS by eliminating reactions at this position and providing an optimal manner in which to precipitate the intermediates peptide as they are being assembled. The technology has been given the name molecular hiving. As this is a very new technology, commercial applications may invigorate more classical solution-phase approaches for peptide APIs.

5. Purification

By far, the most readily used method for purification of peptides is RP-HPLC (48). This is a necessity as the impurities generated during synthesis (process-related impurities) are very similar to the main product and use of a gradient of increasing organic solvent vs. a buffer aqueous solution helps to remove them. These include racemized species, deletions, insertions, and incompletely removed protecting groups. Many of these impurities can elute either very close to the main product peak or even coelute with it. Ultimately, the

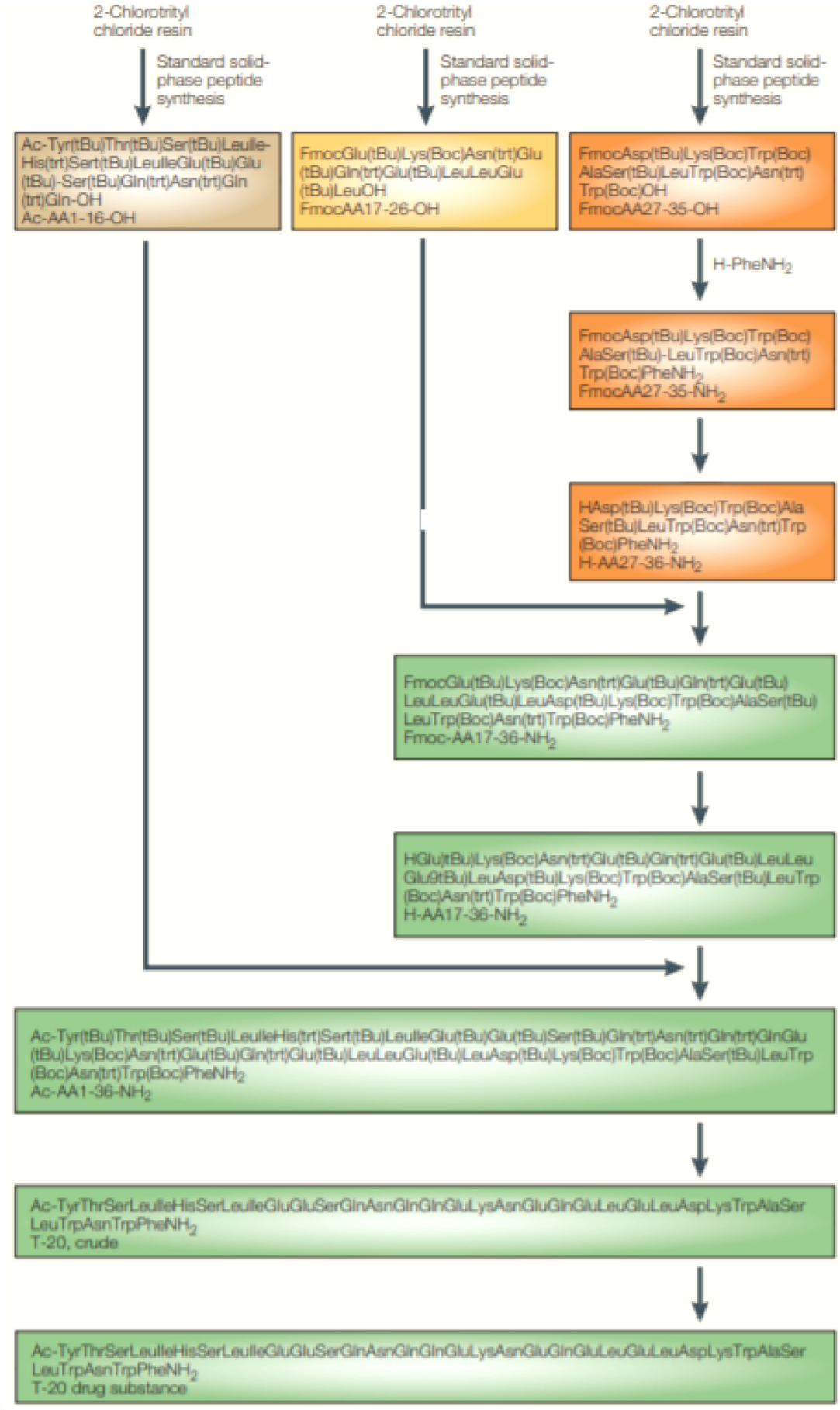


Figure 5. Hybrid Manufacturing Scheme for commercial production of enfurvirtide. Printed with permission from Nature Reviews.

purification process must be reliable and remove these process-related impurities to acceptable levels, which meet "Agency" expectations and current trends to try to meet ICH 3b guidelines. Degradation impurities (oxidation, aspartimide, pyroglutamate formation, aggregation species, etc.) must also be removed and controlled as well.

Typical media used for preparative RP-HPLC include C18, C8, or C4 alkyl groups bonded spherical silica (10 μ spherical particle size, 100 Å pore diameter), which has been end capped. End-capping helps prevent silanol groups acting like a cationic exchange resin causing peaks to broaden during elution. More recently, mixed mode media, which includes a mixture of cationic or anionic resins mixed with the reversed-phase media, have been gaining interest due to their improved resolving powers, which exploit both hydrophobicity and ionic charge (49). The media is packed in dynamic axial compression-based columns. The large-scale commercial columns used at most CDMOs are typically 60 cm. More recently, companies have started to utilize 100 cm diameter columns in preparation for the onset of these metric ton products such as liraglutide, which will soon be available as a generic with patent expiry in 2023. Commercial preparative HPLC systems generate tremendous amounts of aqueous waste due to high flow rates used to elute the product from the column during purification and salt exchange. In order to be environmentally responsible, it is highly recommended to have a solvent recovery/recycling plant on site to recycle the acetonitrile, methanol, or isopropanol solvents that are commonly used in RP-HPLC.

Ion exchange chromatography is also used in commercial production as alternative to separating peptides by their net charge. Either cationic or anionic resins work nicely and allow for easy application over a wide range of pHs for good separation (50). In the review from Andersson et al., (27) an example peptide described in the text listed cation exchange chromatography as a key step taking atosiban from crude cyclized product at 90% purity to ~96% using SP-Sepharose. It is also an effective way to remove free Peg from a peptide-peg conjugation reaction taking advantage of a peptide's charged state relative to the charge or lack thereof for the excess pegylating reagent (51).

6. Isolation

Lyophilization is still used as the primary method for isolation of the purified peptide following purification. Lyophilization is quite time-consuming and expensive in terms of the equipment and energy cost. Large-scale commercial lyophilizers with 800 L and 1000 L capacities are used for commercial product of batches of between 30 and 60 kg per loading. A carefully designed lyophilization cycle removes the organic solvents and water from either the purification or reconstitution buffer via sublimation and also removes residual buffer counter ions resulting in an amorphous fluffy powder (52). Design of the cycle is crucial to reducing the levels of residual solvents used in purification to meet USP 467 ICH Q3c guidelines (MeCN $<$410 ppm; MeOH $<$3000 ppm).

As scale levels now approach multi-hundred kilograms to even metric tons, alternatives to lyophilization become mandatory. Investment into crystallization or precipitation is another possible solution to the isolation of batches exceeding 100 kg. Peptides longer than 10 residues can be quite challenging to crystallize. The most common procedure for crystallization is to achieve a saturated solution of the peptide by allowing for the slow evaporation of solvent, which results in either precipitation or crystallization of the product. In a similar manner, supersaturation can also be achieved by cooling or addition of an antisolvent, which reduces the solubility of the product in solution. The rate at which the solution is cooled or the antisolvent is added directly influences peptide crystallization or precipitation, which can lead to either large crystals (slow super saturation) or small crystals (fast super saturation) (54).

Spray-drying has also seen a rising interest as an isolation process vs. lyophilization. Spray-drying is based on the transformation of an aqueous peptide solution into a powder by atomizing (transformation of the liquid feed stream into very tiny droplets of 10–500 μm diameter) the peptide solution through a nozzle into a chamber together with a heated gas in order to evaporate the liquid (53) (Figure 6A). By atomizing a liquid, a large surface area is created, which is dried much more rapidly and thoroughly. In order to avoid the decomposition of the peptide during this process, a special spray-drying technology has been adopted where a micro-mist spray drying process engineered by Fujisaki Electric is designed with a four-fluid nozzle that allows the drying temperature to be kept as low as 40°C. During the isolation process, no thermal degradation up to 170°C has been reported and the stability of the products is comparable for the old and new methods (54). Additionally, during spray-drying fine and dense particles are formed, which may have lower static properties than lyophilized products. For spray-drying vs. freeze-drying, the overall time comparison to lyophilization and the removal of acetonitrile is improved. The comparison (Figure 6B) shows the overall clear advantages compared to lyophilization. Spray-drying isolation can yield >2.5 kg/day capacity.

7. Quality control and release

Commercial peptide drug substances have gone through a rigorous design campaign where the process has been continually improved and optimized to yield the product in good yield with high reproducibility. This aspect in the product lifecycle is demonstrated by manufacturing a series of three or more separate validation lots to demonstrate control of the process. This usually occurs around the timing of the phase IIb–III clinical trials. By this point, quality control specifications for the peptide have been established and the corresponding test methods validated. An ultra-high purity reference standard has been prepared and thoroughly characterized. Impurities have been determined using MS and spiking studies, and limits for these have been set. Product stability and forced degradation studies have been performed and the results assessed.

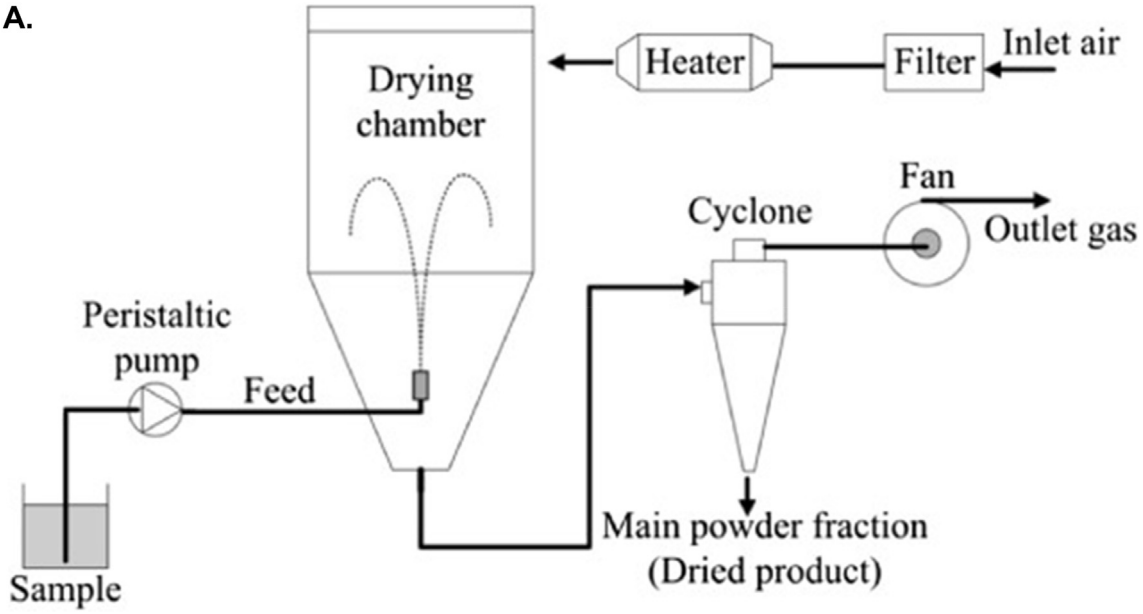
Typical specifications present on a certificate of analysis (COA) include purity (RP-HPLC or UPLC), several ID tests: standard and chiral amino acid analysis, MS, MS-MS sequencing, coelution, endotoxin, bioburden, residual solvents, counterion content, peptide content by nitrogen determination, and mass balance.

Final review of all of the batch production records, analytical data, and results is the responsibility of quality assurance (QA). This final step in the process results in the issuance of the COA, which confirms the quality and conformance of the product with all of the tests, which have been conducted. This document is signed and verified as accurate by QA. Issuance of the final signed COA completes the final requirements of cGMP manufacturing of the API drug substance.

8. Green initiatives

Peptide manufacturing, especially SPPS, generates enormous amounts of chemical waste due to all of the repetitive washing steps, which occur during every cycle of chain elongation (55). Furthermore, in order to achieve the desired peptide sequence, protecting groups must be employed on all reactive side chains of the amino acids (very low atom efficiency). The use of RP-HPLC also generates a huge amount of aqueous waste containing MeCN or other organic solvents. Lastly, lyophilization uses tremendous amount of energy, which is also financially and environmentally costly. Solvent recycling in both synthesis and purification for DMF and MeCN, respectively, is one step toward addressing the waste component. Use of green solvents such as 2-MeTHF and cyclopentyl methyl ether has recently been studied for synthesis (56). RP-HPLC purification strategies using ethanol instead of MeCN have also

A.



B.

+++ = good, ++ = medium , + = poor

Criterion	Spray-drying	Freeze-drying
Stability	+++	+++
Acetonitrile removal	+++	+
Counter-ion removal	+++	++
Static electricity (absence of)	+++	+
Powder density	+++	+
Particle size distribution	+++	+
Solubility	++(+)	++(+)
Investment cost	+++	+
Labour cost	++	+++
Maintenance	+++	+
Flexibility	+++	+

Spray drying vs. freeze drying: overall comparison

Figure 6. (A) Spray-drying schematic for peptide processing. (B) Table of advantages vs. lyophilization. Printed with permission from Chimica Oggi/Chemistry Today.

been reported (57). Replacement of lyophilization with spray-drying or crystallization is another improvement. The capital cost for utilizing green initiatives does impact the production cost, but over time will ultimately help our industry responsibly meet the needs to curtail energetically wasteful procedures and the accumulation of harmful chemicals with which future generations will have to deal.

9. Summary

Peptide manufacturing has matured as an industry over the past 5 decades. Improvements in the technology of synthesis, purification, and isolation continue to be driving the economic aspects for the continued growth of the industry (58). The incorporation of nonnatural derivatives favors development of chemical vs. recombinant processes for peptide manufacturing. With a robust global pipeline of approved products (>70) and more than 400 in clinical development (2), the next decade should be an exciting time for peptide manufacturing.

Author contributions

MWP wrote the article with input and direction provided by BZ and CB.

Declaration of competing interest

MWP, BZ, and CB are all employed as executive management at AmbioPharm Inc. AmbioPharm Inc. is a CDMO specializing in large-scale cGMP peptide APIs.

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Credit author statement

MWP prepared the manuscript. CB and BZ edited and provided technical summary details to relevant sections in the manuscript.

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