ALTERNATIVE STRATEGIES WHICH HELP IMPROVE SUSTAINABILITY AND "GREEN" PEPTIDE MANUFACTURING

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ABSTRACT Peptide production has evolved over the past 50 years into a mature industry of both contract development and pharmaceutical manufacturers. Key to this evolution were improvements to the solid-phase production methods which utilized Fmoc-tBu protection strategies. Inherent to peptide synthesis are principal issues which result in a large impact on the green and sustainability factors for this industry. Some of these include: regular use of both transient and semi-permanent protecting groups which diminish the atom efficiency, generation of large amounts of hazardous chemical waste, and energy inefficient processes such as lyophilization. This short review describes some of the initiatives in our industry such as "greener" solvents, recycling key solvents, and alternatives to lyophilization such as precipitation, crystallization or spray drying.

KEYWORDS: Peptide production, green chemistry, sustainable production, Solvent Recycling, peptide isolation, improved environmental impact, oligonucleotides.

INTRODUCTION

Peptide pharmaceuticals represent a unique group of products which are larger than small molecule drugs yet smaller than most biological drugs. With exquisite selectivity, lower off-target effects and ease of production, peptides are a very attractive modality for development. Peptides were rarely developed by large pharmaceutical companies in the 20th century. Now, they represent worldwide sales of >$20 billion and green chemistry initiatives are needed to ensure the long-term sustainability of these products (1). With more than 70 approved peptide drugs and several hundred in clinical development (2), commercial peptide sales are projected to surpass $60 billion by 2026.

The first synthetic peptide drug, oxytocin, entered the clinic in 1962. The original oxytocin process was a liquid-phase assembly (LPPS) which was both time-consuming and cumbersome for the nine-residue peptide (3). Solid-phase peptide synthesis (SPPS) developed by Merrifield in 1963 (4) and its subsequent refinement throughout the following five decades has been essential for the growth and success of peptide therapeutics. The solid-phase principle immobilizes the C-terminal amino acid to a solid-support (typically divinylbenzene-crosslinked polystyrene beads) which eliminates its reactivity and facilitates chain assembly. The desired sequence is built up in a vectoral manner on the solid support which is facilitated by washing away the impurities while the desired product is retained. Intrinsic to SPPS is the use of large amounts of organic solvents to wash away the impurities as well as perform the key deprotection and coupling steps. Furthermore, cleavage and downstream processing steps such as purification, salt exchange and isolation generate large volumes of hazardous chemical waste and/or consume large amounts of energy.

As we are early in the 21st century, peptide manufacturing improvements are necessary to make our industry more sustainable (5). This short review will focus on several of the initiatives which we have recently employed at AmbioPharm to address the sustainability of our operation which are likely being adopted at other CDMOs and pharmaceutical manufacturers around the world.

SOLVENT INITIATIVES

The standard solvents used in nearly all commercial peptide processes are dimethyl formamide (DMF), N-methyl pyrrolidinone (NMP), dichloromethane (DCM), methyl t-butyl ether (MTBE), acetonitrile (MeCN), methanol (MeOH) and isopropanol (IpOH). In some regions, DCM has been completely phased out due to environmental regulations. The other solvents all lead to high volume hazardous waste streams which ultimately affect the long-term sustainability of the process (1). The principal solvents DMF and NMP are considered reprotoxic and may have future limitations due to the REACH program (6). Thus, alternatives to these key solvents in SPPS and LPPS are needed as well.

Greener alternative solvents such as N-butyl pyrrolidinone (NBP), 2-methyltetrahydrofuran (2-Me-THF) and γ-valerolactone have been recently studied for applications in SPPS and LPPS (7,8). In fact, 2-Me-THF has found even greater utility with the LPPS technologies being developed at companies such as Jitsobu and GAAP Technologies (9 - 11). In these approaches, a C-terminally positioned protecting group is utilized like the solid-phase resin bead in SPPS. This protecting group is designed with properties which limits its solubility in an aqueous environment. Thus, the major steps of the synthesis are carried in water-miscible solvents such as 2-Me-THF. Following each coupling step, the product is precipitated into water to wash away reactants prior to proceeding to the next step which returns to 2-Me-THF.

At the present time, most commercial peptide processes utilize preparative RP-HPLC to purify the products and eliminate impurities to meet the ICH guidelines for impurity profile and residual solvents. Additionally, RP-HPLC is also used for the process of salt exchange

of the final product [12]. The process of RP-HPLC utilizes a gradient of decreasing polarity to elute the products which have been retained in the matrix (typically an alkyl chain bonded to silica) on the column via hydrophobic interactions. The gradient is created by slowly increasing the ratio of a water miscible solvent into an aqueous buffer. The peptide elutes once a critical concentration of organic modifier causes the desorption of the product from the stationary phase. RP-HPLC generates tremendous volumes of aqueous waste containing high levels of MeCN, IpOH and/or MeOH depending upon which solvents are used in the purification as well as in the cleaning of the column between runs.

Recently, mixed mode chromatography (MMC) applications have entered peptide purification technology (13). This strategy basically mixes a small percentage of an ion exchange resin (either cationic or anionic) into the RP-packing material. This application separates based upon hydrophobic interactions as well as ionic interactions. Processes which employ MMC-resins may enjoy the benefit of somewhat lower organic solvent usage to develop the column.

CDMO's such as AmbioPharm have recently begun to implement Green initiatives for peptide manufacturing. At its newly constructed Shanghai Campus, the investment into large-scale solvent recovery and recycling has now been established. Using proven technology of distillation and fractional distillation, the investment into the recycling key solvents such as DMF, MTBE and MeCN results in a much more sustainable process which will have longer term environmental benefits reducing the waste streams which come from production while simultaneously providing economic value by lowering solvent and waste disposal costs. Careful control of the solvent recycling process, proper analysis and documentation for trace impurity levels is monitored by GC to ensure suitability for use. Shown in Figure 1 is the equipment which handles this MeCN recovery process at AmbioPharm. (Figure 1)

Figure 1. MeCN solvent recycling unit at AmbioPharm Shanghai new campus. Membrane dehydration with column purification.

Figure 2. North Augusta Site Precipitation Crystallization Suite.

CHEMISTRY INITIATIVES

Boc-SPPS methods were difficult to scale-up due to the dangerous reagent usage of HF for the final cleavage step. Thus, Fmoc-tBu SPPS (14) became the production method of choice especially following the development of enfurvirtide by Roche where the key building blocks required were produced at ton scale, thereby lowering the cost. The lower cost for chemical synthesis now allowed for longer molecules to be produced. As the average length of peptides that are being developed steadily increases as well as the complexity of the molecules, Fmoc-SPPS methods have become the production method of choice for CDMOs. One of the major limitations from the chemistry perspective is a lack of atom efficiency for the Fmoc derivatives commonly used and this is without even considering the contributions for their production.

Often, synthesis of longer peptides is accompanied with coupling problems due to aggregation on the resin (15-17). Hybrid strategies which utilize a combination of SPPS and LPPS can often bypass these issues by combining the speed of SPPS to produce a series of fragments and a final assembly step in solution. This eliminates some of the aggregation issues and results in a higher quality crude product. This approach is particularly useful for making peptides >30 amino acids. In fact, the aforementioned enfurvirtide (36 residues) process utilized such a hybrid process to reach ton scale production (18). More recently, strategies to make synthetic versions of recombinant peptide generic APIs of liraglutide and teriparatide have also been reported (19-21).

SPPS syntheses require an excess of amino acids, coupling reagents

and tertiary base for nearly quantitative conversion at each step. Fmoc removal requires a large excess of (>20 equiv) piperidine or other suitable bases.

As mentioned earlier, these processes routinely use large volumes of solvents (typically >8ml/g of resin) to wash the resin after each step in the process. This results in increased process mass intensity (PMI: ratio of total mass of all input material over mass of isolated product) and much higher cost of goods than for small molecules. Sometimes in the tactics used for the syntheses of longer, as well as hydrophobic peptides including specialty resins (ChemMatrix or Tentagel with lower substitution levels), use of alternative solvents to minimize aggregation represent additional challenges to "greening" the process.

Amide bond formation still requires the use of less green chemicals such as carbodiimides with HOBT, 6-Cl-HOBT, uronium/aminium salts HATU, HBTU, TBTU and phosphonium salts PyBOP, PyAOP and PyClock (5). These all are excellent activators which offer low amino acid racemization as an added benefit. However, some of these reagents (HOBT and related compounds) may not be useful at elevated temperatures due to their explosive potential (22). Still, the major activation chemistry used in commercial production is diisopropyl carbodiimide with HOBT.

Over the past decade, some potentially new greener alternatives have started to appear such as Oxyma (ethyl cyano hydroxyimino acetate) used with carbodiimides and its uronium salt form COMU (23,24). The sustainability of these reagents has yet to be established. However, care must be taken with these reagents as there have been reports to HCN evolution during activation using these Oxyma (25). It should also be noted that COMU is unstable in DMF solutions which minimizes its effectiveness on automated synthesizer platforms (8).

For LPPS, the green aspects have improved over the past decade resulting from the technology being developed by Jitsobu and GAAP peptides using their proprietary C-terminal soluble anchoring tags (9-11). The ability to perform solution peptide chemistry with readily available Fmoc-tBu amino acids makes it economically advantageous. Furthermore, the ability to use more environmentally friendly solvents such as 2-MeTHF coupled with water precipitation enhances the sustainability of these processes which also eliminate reprotoxic solvents such as DMF. This technology will likely find its first commercial application to produce cosmetic peptides and perhaps later with shorter length peptide APIs.

Figure 2. Precipitation/Crystallization Isolation Suite in North Augusta Site (Panel A: Precipitation reactors with wall mounted centrifuge. Panel B: 50 L rotary evaporator and jacketed reactor).

GREEN ISOLATION INITIATIVES

For many of the early peptide APIs which were prepared using LPPS strategies, crystallization and precipitation techniques were established. In the cases where purification was required on the protected fragments, techniques such as counter current distribution were employed. Many of these peptides were short (<10 residues) and following a convergent synthesis from nearly pure crystalline fragments, the final product could be isolated without the need for RP-HPLC.

As peptides got longer, lyophilization isolation technology evolved to handle the large volumes resulting from the preparative RP-HPLC required to purify the products. Peptides are somewhat thermodynamically unstable and tend to degrade over time (26). Lyophilization helps minimize this by keeping the temperature low and reducing exposure to air while removing the solvents from the product (27). With peptide quantities in the intermediate range, from 5 kg to 100 kg, lyophilization, although not ideal, serves a valuable purpose. Even so, lyophilization requires a tremendous amount of energy, resulting in an overall unsustainable process.

Several peptide APIs currently in clinical development have anticipated annual requirements potentially exceeding metric tons with market approval. These products will still require RP-HPLC processing and this results in large volumes of material from which the product must be isolated. Crystallization, precipitation, and spray drying techniques potentially offer more sustainable alternatives to lyophilization. Thus, there is considerable interest into the final isolation step of the manufacturing process.

Crystallization of longer peptides is often very challenging and not viable for production. However, precipitation is ultimately more straightforward to accomplish due to the unique properties present from the amino acids found in the molecule. Development of a suitable process involves a series of trials which include optimizing variables such as selection of the water miscible solvent, pH,

salt concentrations, counter ion, peptide concentration, and temperature among others. Once the precipitation conditions have been fixed, scale up into jacketed reactors facilitates scaling the process, with isolation of the precipitate using centrifugation followed by the appropriate wash steps. The final isolated product is subsequently dried in vacuum tray dryer ovens to reduce moisture and residual solvents to the desired levels (12). The energy requirements and scalability make precipitation a more sustainable process than lyophilization. The particle size and density of the precipitate is usually much greater than that of a lyophilized product. Ambiopharm has established this technology both at its US headquarters as well as at its Shanghai subsidiary to facilitate these large commercial-scale peptide products. (Figure 2 NASC Precipitation Suite Photos)

Spray-drying has also begun to find application to the peptide industry (28). Decreases in the temperatures required to volatilize the solvents coming from the RP-HPLC purification step, spray dryers have operating ranges from 35° – 70° C. The time that the peptide experiences this temperature is very short leading to minimal degradation. As the process uses flammable solvents originating from the HPLC step, the system must be closed to minimize explosion potential. Commercial application to quantities greater than 300 kg make spray drying the most energy efficient of any of the isolation processes.

CONCLUDING REMARKS

Peptides have become a very important drug modality. The processes that have been developed need to be updated to make them more sustainable and environmentally friendly. Research into new methodology will be critical for this to be truly successful. Ambiopharm has begun the steps to optimize its environmental impact by developing both solvent recovery and recycling as well as precipitation isolation strategies. We are currently evaluating spray drying technology and expect that this will soon be another part of our green strategy. It is important to realize, however, that both precipitation and spray drying will impact of the density of the product and this must be optimized to minimize impact to the formulation step in preparation of the drug product.

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