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Critical parameters
for solid-phase manufacturing of therapeutic peptides

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ABSTRACT *Solid-phase peptide synthesis (SPPS) represents the standard for cGMP-peptide manufacturing. Recent improvements in chemistry & technology are highlighted. In the field of chemistry, scope and limitations for the application of innovative building blocks, e.g., pseudo-proline dipeptides, and the new coupling reagent COMU are summarized. On the technology side, the inverted filter centrifuge as a device to support large-scale final cleavage of the peptide from the resin is described. Both, additional innovative engineering solutions and progress in chemistry are thought to further increase productivity for the manufacturing of therapeutic peptides following a solid-phase process.*

INTRODUCTION – THE SOLID PHASE APPROACH IN THE CONTEXT OF OTHER METHODS FOR PEPTIDE SYNTHESIS

Historically, peptides were assembled in solution, and for the experienced chemist, the development of a synthetic scheme for peptides > 10 amino acids required several months of intensive work. The sheer number of synthetic steps in solution and the subsequent work-up including purification, still results in a time-consuming effort even for routine production. Thus, standard manufacturing can be associated with high costs. Although the use of enzymes and recombinant approaches are alternatives for the production of peptides, none of these methods has commonly been used for routine development. In the case of smaller peptides, enzymatic synthesis has been successfully performed at industrial scale for only a limited number of products, mostly applied as a nutritional supplement (1). The sweetener Aspartame, a dipeptide manufactured at > 10 000 tons/year, is a prominent example. Regarding recombinant production, the development of a suitable expression system for peptides is expensive, and the presence of protease-activity may constitute a problem during work-up. In particular, the restriction to use only the amino acids able to be incorporated by the organism of choice further limits this approach. Therefore, the preferred production method for most of the more recently developed therapeutic peptides was established on the basis of solid phase peptide synthesis (SPPS) (2).

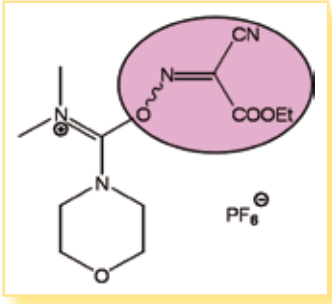


Figure 1. Structure of the coupling reagent COMU (Oxyma as part of the structure in colour).

THE GROWING IMPORTANCE OF STARTING MATERIAL QUALITY

Improvements in peptide chemistry were accompanied by a request for higher quality, including the challenge for characterization of minor impurities. As a consequence, the choice of starting materials becomes an important factor to achieve the required final purity and to avoid extra efforts for down-stream processing. In contrast to amino acid derivatives, it is not so easy to characterize another important component needed for SPPS – the solid support. Although progress in the visualization of nanostructures and solid-state NMR methods is still on-going, the critical parameters for a given resin cannot yet be adequately identified. Thus, for this moment, the resin characteristics, to qualify a particular support for the synthesis of a long peptide, are unknown. In the light of the trend to longer and more complex peptides, even marginal impurities in starting materials, or side reactions as a consequence of contaminated reagents or solvents, have to be eliminated due to the fact that purification of intermediates is not possible during standard SPPS. Minute impurities can accumulate and may not be readily removed, although detected by modern analytical techniques. Unfortunately, the development of preparative purification technologies has not been able to keep pace with improvements in analytical methods.

PROGRESS IN PEPTIDE CHEMISTRY

Although already introduced by Bruce Merrifield in 1963 (3), SPPS was only considered in the last two decades as a viable option to address large-scale synthesis. While a variety of solid supports were developed, the classical polystyrene resin is still applied nowadays, if up-scaling is required. Similarly, protecting group combinations used for large scale production have not yet been significantly improved, and Fmoc-based strategies are still preferred due to the handling problems related to the hazardous HF applied for the alternative Boc-method. On the other hand, a large variety of new coupling reagents were discovered, and the recently introduced reagent COMU seems to offer some advantages (4) (Figure 1).

Following the trend to use safer chemicals, the benzotriazole moiety of TBTU or similarly dangerous (explosive) uronium salt structures could be replaced by a molecule called Oxyma (ethyl 2-cyano-2-(hydroxylimino) acetate) (Figure 1). Calorimetric studies showed a much slower and more controlled decomposition of COMU. The other part of the molecule, the morpholino-unit of COMU, acts as a proton scavenger and/or hydrogen bond acceptor, thus, reducing the amount of base required for activation. Since base-catalysed side reactions like aspartimide formation and epimerisation still represent persistent problems in peptide chemistry, COMU is believed to improve the quality of the SPPS process.

Solid-phase peptide synthesis (SPPS) represents the standard for cGMP-peptide manufacturing

Another area of intensive investigations relates to the inherent property of peptides to adopt secondary structures. In particular, the formation of β-sheets, leading either to intra- or intermolecular association of peptide chains, constitutes not only a problem for solubilisation and purification as discussed earlier (5), but also for synthesis. Although additives were proposed to inhibit clustering of strands, for longer peptide sequences with a strong tendency to aggregate, they have proven insufficient. As an alternative, acid labile backbone protecting groups were applied following the concept to disrupt the chain association by introduction of steric crowding, and concurrently, to prevent the H-donor effect of the amide moiety. However, the introduction of a modified derivative may require more elaborate conditions for attachment and further processing. For Asp-Gly containing sequences, the benefits outweigh the risks, and modified dipeptides carrying amide protection are applied to inhibit aspartimide formation and aggregation (6).

A further increase in productivity for the assembly of therapeutic peptides is foreseen due to the steady advancements in the quality of starting materials, the development of synthetic peptide chemistry, and the use of continuous production technologies

Another means to disturb ordered structures of the β-type was demonstrated for the so called iso-peptide modification (7, 8). In this case, the inherent property of Ser/Thr side chain esters to rearrange to the native backbone, once their amino function is liberated, can be exploited (Figure 2).

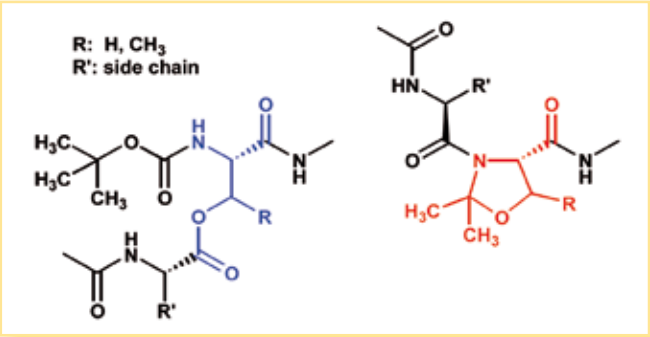


Figure 2. Iso-peptide (blue) and pseudo-proline (red) as incorporated into the peptide chain during SPPS.

The rearrangement to the native form is triggered by raising the pH. The disadvantage of this solution resides in the problem, that for longer peptides often harsher conditions for protecting group removal are required, which may not be compatible with the stability of the ester functionality intrinsic to the iso-peptide.

Interestingly, nature has devised a method to control secondary structure formation by incorporating proline residues. On the basis of this observation, dipeptide building blocks were designed for Ser and Thr, which mimic the five-ring structure of proline (9) (Figure 2). Upon acidic treatment, the cyclic structure is cleaved and the native side-chain functionality is regenerated. Incorporation of one pseudo-proline dipeptide unit within a stretch of about 10 – 15 amino acids is sufficient to inhibit aggregation phenomena due to their medium range conformational impact. Interestingly, neither the incorporation of pseudo-prolines, nor the coupling to these building blocks is faced with problems. In addition, pseudo-prolines are now available in high quality and even large-scale manufacturing has been realized.

These continuous improvements applicable to classical stepwise SPPS paved the way for routine production of larger peptides. As a consequence, even synthetic proteins in the range of 60 – 80 amino acids can now be assembled in an outstanding purity using a stepwise approach. Moreover, ligation technologies, relying on fragments obtained by SPPS, opened up the access to proteins of the size of 100-200 amino acids (10, 11). Nevertheless, so far, ligation reactions have not been employed for large-scale production. Most likely, patent protection of these technologies, the missing awareness of the reaction's efficiency, and the lack of experience to use traditional medicinal chemistry approaches on macromolecules have limited a widespread industrial application.

IMPROVEMENTS IN TECHNOLOGY

Nowadays, peptides are regarded as a viable option to address larger indications like type II diabetes and obesity. These more chronic diseases, affecting an increasing number of people around the globe, triggered the interest to secure large-scale production of therapeutic peptides. Thus, manufacturers had to consider how to produce at reasonable costs large amounts of these pharmaceuticals. Although micro-reactor technology has evolved to an extent that off-the-shelf solutions are available, so far, this technology or other continuous methods have not been applied for peptides. The recent application of ball milling opens up opportunities related to cost-effective manipulations on amino acids and even for the synthesis of short peptides (12). Simply applying this technique to an activated amino acid, mixed together with an amine component in the solid state, the efficient production of various dipeptides in good quality and yield has been demonstrated. Since the process is continuous and does not require any solvents, in principle, it qualifies for efficient large-scale production of derivatives and small peptides.

Standard peptide manufacturing still follows a batch-wise mode using stirred reactors equipped with a filter plate at the bottom. Alternative to this procedure, in the continuous-flow synthesizers, the resin is packed in a column and all solutions are circulated several times through the immobilized support. This method has the advantage that a large local excess of reagents drives the reactions more readily to completion. However, the expansion of the solid support in different solvents, and the unpredictable swelling behaviour during chain elongation in the course of SPPS, may affect the regular flow through the resin bed. So far, this technology has been applied only for small scale synthesis, and instrument companies are only starting now to develop the corresponding hardware applicable to larger scale.

In the view of ton-scale peptide synthesis, Rosenmund-filter dryers holding volumes of around 1 m^3, have been adapted to SPPS. In particular, having the possibility to work under controlled temperature, in an inert atmosphere and under pressure control favours the application of this device. Although, current market demands for peptides do not necessarily require this scale of production, reconfigured filter dryers can support large-scale SPPS. As a consequence, the assembly of the peptide is not considered the bottleneck for ton-scale synthesis.

However, the final operation in SPPS, the cleavage reaction, represents a critical step in the overall synthesis. In the case of large-scale applications, holding times and volumes have to be optimized in order not to compromise quality. Moreover, peptides tend to precipitate in a gel-like form, and, as a result, filterability is poor due to clogging of the filter cloth. Other potential problems relate to partial open handling in the standard batch procedure. Consequently, the unintended exposure of the product to air and to employees has to be minimized for large-scale production.

An inverted filter centrifuge from Heinkel (Germany) was installed at Bachem AG to solve the above mentioned issues. In Figure 3, the front part of the machine is shown. The machine part, actually housed in a separate room next to the handling area, is not visible on this picture. The principal operations of the inverted filter centrifuge are illustrated in Figure 4. The slurry containing the solid to be separated from the liquid stream, is delivered to the process chamber consisting of a rotating drum and a feed pipe (Figure 4a).

Usually the drum spins at a pre-set value chosen from the speed range of the machine employed. Product not retained by the filter cloth during initial application can be re-centrifuged by applying the mother liquid in a 2nd run from a dedicated tank foreseen for intermediate liquid storage. Once the filling step is completed, the washing cycle starts (Figure 4b). The washing liquid is introduced and controlled in the same way as the product slurry. Due to centrifugation, a homogeneous cake builds up, and each part of the solid peptide is washed and drained uniformly (Figure 4c).

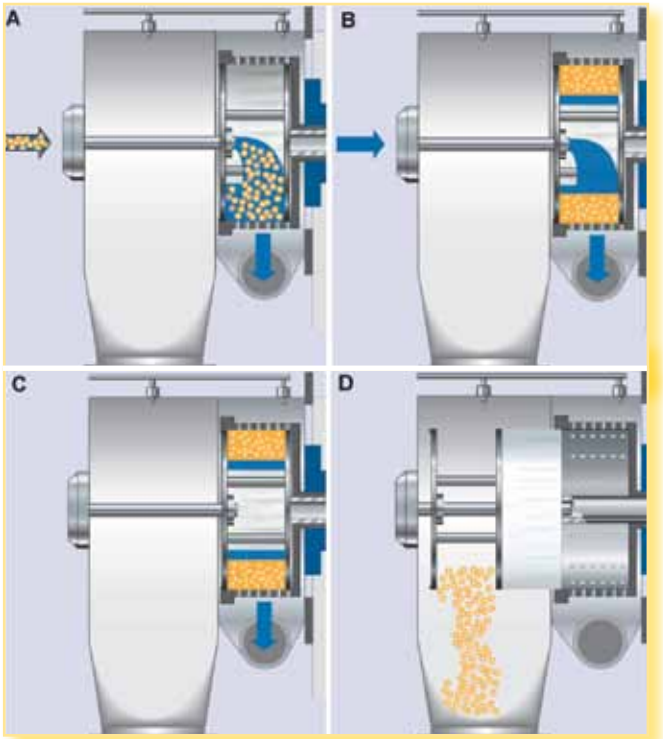
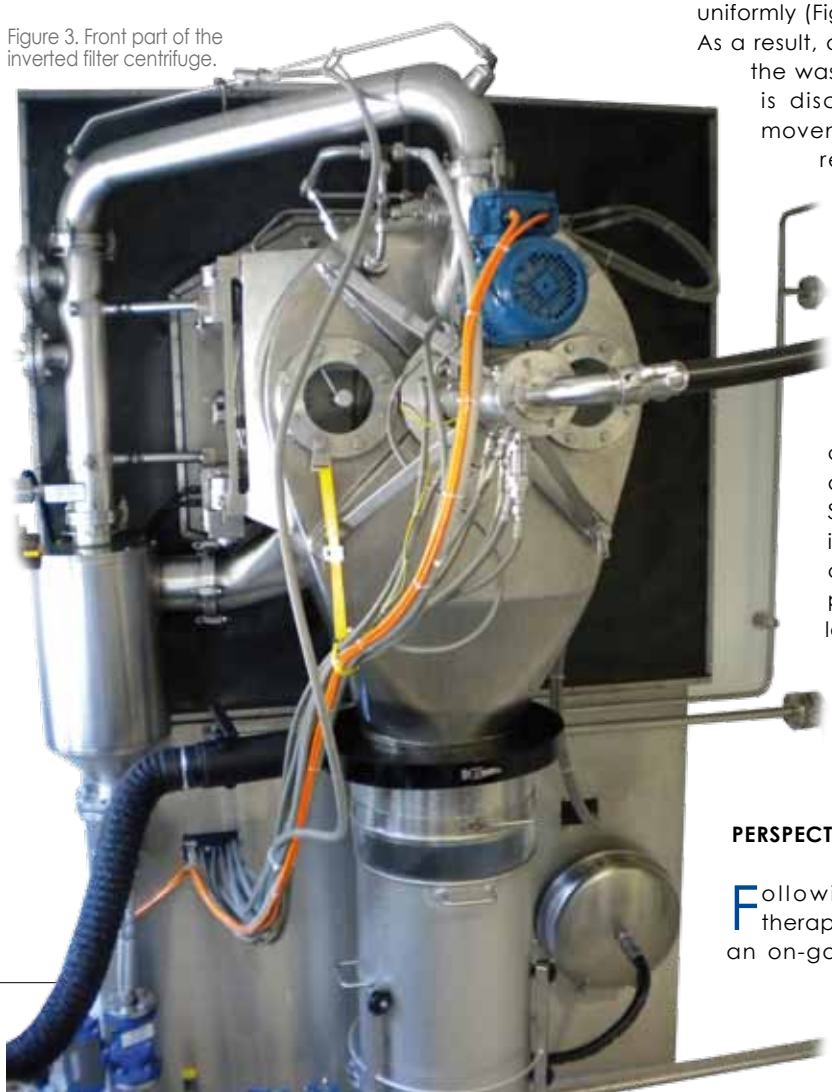


Figure 4. Principal steps of the inverted filter centrifuge a) Filling, b) Washing, c) Drying, d) Unloading.

Figure 3. Front part of the inverted filter centrifuge.



As a result, a consistent product quality is obtained. Once the washing steps are completed, the crude peptide is discharged from the centrifuge by the axial movement of the shaft (Figure 4d), while the bowl remains rotating at a low speed. Following termination of the centrifuge process, no manual emptying of the drum, and no opening of the process housing is required for discharge, since the product is bottled upon leaving the centrifuge. Even a validated cleaning protocol can be carried out under closed conditions applying the fully integrated automated cleaning system of the inverted filter centrifuge. As a consequence, product change-over is quick and easily completed.

So far, yields and purities, using the less work-intensive centrifugation method, have been comparable to the optimised batch procedure. Interestingly, the TFA-content was lower for all of the product batches processed by the inverted filter centrifuge, and, as a consequence, the crude peptide obtained from this alternative process is now better qualified for storage.

PERSPECTIVES FOR THE FUTURE

Following the trend towards more complex therapeutic peptides and larger quantities required, an on-going evolution of chemistry and production

technologies is expected. Due to the variability in the properties of peptides, in all likelihood, problems will have to be addressed by considering individual solutions rather than by a generic process. In addition, future developments in peptide chemistry will reduce the amount of impurities produced. The extensive progress in analytical techniques will encourage further advancements in synthetic techniques in order to achieve the ever increasing need for higher quality. On the technological side, there is still room for improvement, since the implementation of continuous processes for manufacturing including down-stream processing has not been fully realized. In summary, a further increase in productivity for the assembly of therapeutic peptides is foreseen due to the steady advancements in the quality of starting materials, the development of synthetic peptide chemistry, and the use of continuous production technologies. The more widespread application of peptides in clinical drug development and the progress in alternative delivery methods are regarded as the drivers for the therapeutic peptide market. As a consequence, solid phase manufacturing methodologies may be expected to continue their further evolution also in the future.

The more widespread application of peptides in clinical drug development and the progress in alternative delivery methods are regarded as the drivers for the therapeutic peptide market

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