MODERN PERSPECTIVES ON PEPTIDE SYNTHESIS
INTRODUCTION

WHITEPAPER

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The complexity of synthetic peptide products, whether as reagents used in research or as therapeutic APIs, is increasing. As researchers understand more about the biochemical systems which are targeted, and invent ever more sophisticated products, manufacturing methods must keep pace. We look in this article at a number of challenging product classes, the specific problems they present, and potential solutions:

- Long peptides
- Multi-peptide cocktails
- Site-specifically modified products.

BIG IS BEAUTIFUL

The length of peptides which can reasonably be synthesized by solid phase synthesis has been the topic of some debate. On one hand, the capacity of a solid phase reactor is limited by the level of chemical functionalization and swelling properties of the resin. The industry has tackled this by developing new solid supports which enable higher levels of functionalization so that more peptide is obtained from every kilo of resin used.

In tandem with functionalization, however, is the effectiveness of the coupling chemistry itself. High coupling and deprotection efficiency is required and to maximize chemical yield, and with the repeat nature of peptide synthesis chemistry, even 99% efficiency over 40 coupling and deprotection steps required to synthesize a 20mer results in a chemical yield of 82%, and of a 50mer is 37%. With the ever increasing interest in long peptides, by the time the calculation is extrapolated to a 100mer, the yield is 13%, and so even highly efficient chemistry gives diminishing returns. This has been tackled with inventive ways of intervening in the chemical synthesis, identifying problem areas, and implementing solutions.

Even once synthesis efficiency is improved to an acceptable level, purification of long peptides remains a real challenge, whether for research grade peptides, and even more so for GMP peptides. Almac has, for many years now, developed synthesis protocols of chemokines, peptides around 70 amino acids in length. In addition to simply the length of the peptide chain being a challenge for synthesis, chemokines contain two pairs of cysteines, which must be correctly oxidised to form a defined disulfide bridge pattern.

Typically, two purification methods are required, before and after the oxidation step, but methods are now reasonably standardized such that protocols sit broadly within the comfort zone of the peptide chemist. The primary steps comprise well understood solid phase synthesis, cleavage and deprotection, and reverse phase HPLC purification, and such methods have been applied to several GMP campaigns on different chemokine products.

More recently, Almac have stretched horizons by synthesizing full length histone H3 proteins. These proteins are 135 amino acids in length, and are involved with the growing field of epigenetics – changes in the regulation of gene expression without modification to the underlying DNA sequence. There are very many modifications to the backbone sequence of the protein, such as Lys methylation or acetylation, Arg methylation, Ser phosphorylation which are straightforwardly incorporated by chemical synthesis, but precluded by the ability to synthesize the full length sequence.

In order to successfully synthesize full length H3, and incorporate different modifications, we have had to step out of the normal comfort zone of the peptide chemist:

- Synthesis protocols for long peptides were largely successful, but specific difficulties had to be addressed to improve overall synthesis yield, and “synthesize out” close-running impurities.
- Traditional HPLC purification alone was not successful in obtaining the desired purity, and alternative methods such as ion exchange and size exclusion chromatography had to be explored in tandem with HPLC.
- Monitoring the effectiveness of the synthesis and purification process was not straightforward by LCMS due to the highly charged nature of the proteins, and so traditional protein analysis methods, such as SDS-PAGE had to be employed.

In the end, thinking of the product as a peptide (the chemist) and proteins (the biologist) enabled us to identify problems in the manufacture process, devise solutions, and finally prove the methodology in the production of 10 proteins for research use. For an example, see Figure 1.
PEPTIDE COCKTAILS

To circumvent the need to synthesis a single long target peptide, one approach to take is to synthesis multiple shorter peptides. This philosophy of defining a “peptide cocktail" is particularly well employed in peptide vaccines. A particular protein may be implicated in the propagation of a virus, and hence viewed as an appropriate candidate for a vaccine. Rather than synthesizing the full length protein, shorter antigenic fragments (i.e. peptides which evokes the production of antibodies) may be identified, covering all, or a subset of the entire protein length, and the vaccine product becomes a “cocktail" of shorter peptides. Alternatively, the cocktail is derived from antigens from different target proteins identified to cover different population subsets.

Defining a therapeutic product as a mix of peptides itself creates issues with process development, manufacture and regulatory management. In the classic model of a product consisting a single API, development and manufacture is focused and contained. On the other hand, where multiple APIs have to be manufactured, whilst in many ways they are treated as single entities, and in fact are released as separate drug substances, at some point the products are combined, increasing the level of complexity in product formulation, and analysis.

Certain steps can be taken to mitigate potential technical risk of using the peptide cocktail approach. In particular, at Almac, we advocate investing time in pre-formulation development. Here, we examine characteristics of the individual peptides, and establish how they compare in terms of solubility at different pH. This study can give some early warnings as to how the peptides will behave in a formulation which has to fit all products. Additionally, indicative stability studies of the individual peptides, and of the multi-peptide cocktail, help flag quickly if any problems exist during product storage.

PEPTIDE MODIFICATIONS

Another dimension of peptide synthesis with increasing popularity in both the research and therapeutic fields, as product design becomes more sophisticated, is the ability to modify native peptide or protein sequences. Modifications are wide ranging, from the incorporation of unnatural building blocks to the post-translational modifications of the native sequence.

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**Figure 1: SDS-PAGE and MS of H3 S10p K14ac**

Sequence (H3 S10p K14ac)

Ser$^{10}$ modified with phosphate; Lys$^{4}$ modified with ε-acetate

ARTKQTARKS(p) TGGK(Ac)APRKQL ATKAARKSAP ATGGVKKPHR YRPGLVALRE IRYYQKSTEL LIRKPQQLRRL VREIAQDFKT DLRFQSSAVM ALQEACEAYL VGLFEDTNLC AIHAKRVTIM PKDIQLARRI RGERA

Required mass: 15394.9

Found mass: 15394.6

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<table>
<thead>
<tr>
<th>Single API</th>
<th>Peptide Cocktail</th>
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<tbody>
<tr>
<td>Single development and manufacture program</td>
<td>Parallel development and manufacture program. Different products may progress at different rates in development depending on difficulties encountered</td>
</tr>
<tr>
<td>Single purity method for analytical release of API</td>
<td>A single purity method is unlikely to be suitable for all products, requiring greater investment in analytical method development.</td>
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<tr>
<td>Drug product analytical method required to detect a single API</td>
<td>The drug product analytical method is required multiple APIs, which may have different sensitivities. Either very similar or very different elution profiles can be problematic</td>
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<tr>
<td>Product stability dependent on inherent nature of the peptide, and hold conditions</td>
<td>Product stability is influenced by the other APIs in addition to the inherent characteristics of individual APIs ad hold conditions.</td>
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into the amino acid back bone, or modification with hydrophobic groups or PEG to improve half-life, to conjugation with other bioactive species such as cytotoxics, or even antibodies. A significant challenge, then, is to devise chemistry which enables the required transformation to proceed selectively, and high yield to produce a clean, well-characterized product.

Almac have combined the benefits of chemistry and biology to demonstrate site-specific modification of peptides and proteins in a range of applications such as protein PEGylation, incorporation of imaging agents, and generation of drug antibody conjugates. With PEGylation an increasingly applied tool to extend product half-life, Almac have exemplified methodologies to produce both synthetic peptides and recombinant proteins. Random PEGylation results in multiple PEGylation at multiple sites in the protein, reducing the overall potency of the molecule. It has been demonstrated, that by controlling chemistry, a single incorporation of PEG at a single site on the protein, retains the overall potency of the molecule.

Using intein fusion technology, we have devised a method by which a C-terminal hydrazide functionality can be selectively incorporated to recombinant proteins (Figure 2). After purification, this can then be reacted with keto-functionalized PEG in good yield. This was applied to Interferon α2b, resulting in a PEGylated form which was more than double the potency of the commercially available form, ViraferonPEG®.

Analogous methodology has also been developed for synthetic peptides, and crucially, in labeling folded synthetic proteins. In the case of the chemokine, SDF-1α, a 67 amino acid protein with two disulfide bridges, we have introduced an unnatural building block near the C-terminus of the sequence. After purification, the unnatural building block is treated to “unmask” thiol functionality (Figure 3), which has been demonstrated to react with maleimide-functionalized PEG without disrupting the required disulfide bridge folding pattern.

Figure 2: C-terminal PEGylation of recombinant proteins

![Figure 2: C-terminal PEGylation of recombinant proteins](image)

Figure 3: Site-specific PEGylation of folded synthetic SDF-1α

![Figure 3: Site-specific PEGylation of folded synthetic SDF-1α](image)
CONCLUSIONS

In many ways peptide manufacturing methods are well established. As more sophisticated fundamental research and product design is performed by inventors, challenges remain to ensure that manufacturing methods keep pace with product development. The peptide synthesis CMO is required to adapt to the demands of sponsors by devising evermore sophisticated methodology, drawing on techniques from both chemistry and biology, and focusing attention equally on manufacturing methods and analysis.

ABOUT THE AUTHOR

Alastair Hay graduated from the University of Strathclyde in 1995 with a BSC in chemistry. He holds a PhD from the University of Edinburgh in synthetic organic chemistry, focused on novel methods of generating small molecule compound libraries.

In November 1998, he joined Zeneca Agrochemicals (Grangemouth) in the Process technology Department as a process development chemist. Here, he was responsible for developing a multi-stage process for a new cereal fungicide on the hundreds of tonnes a year scale. In 2002, he rejoined Bob Ramage in his peptide synthesis company, Albachem. At Almac, he initially worked on complementary methods for peptide purification alongside HPLC. Following the acquisition by Almac in 2004, he moved into operations, managing the custom synthesis team, helping the group expand as the business grew. Since 2010, Alastair has also managed the process development group for GMP manufacture.

REFERENCES


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