Building parity between brand and generic peptide products: Regulatory and scientific considerations for quality of synthetic peptides

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Abstract

Peptides are a fast growing segment in the pharmaceutical industry. Consequently, the industry and regulatory agencies are increasing their focus on the regulatory path and quality considerations for peptide development and manufacturing. Although most peptides are synthetic, manufactured by solid phase synthesis, nevertheless they are complex molecules with challenging quality and regulatory aspects. This paper provides a structured overview of relevant quality issues for chemically synthesized peptides used as active pharmaceutical ingredients (API) in drug products. It addresses the unique characteristics of peptides pertaining to structural and physicochemical characterization, manufacturing and in process controls, impurities and aggregates arising from manufacturing and storage, along with their potential impact on safety (including immunogenicity) and efficacy of the peptide drug products.

23	
24	Contents
25	1. Introduction3
26	2. Regulatory context for peptide drug review and approval3
27	3. Scientific considerations for quality of synthetic peptides9
28	3.1. Characterization of peptide structure10
29	3.2. Control of peptide synthesis14
30	3.2.1. Control of starting materials16
31	3.2.2. In process control strategies for solid phase peptide synthesis18
32	3.2.3. In process control strategies for solution phase peptide synthesis and
33	ligation26
34	3.3. Strategies to control the quality of peptide drug products29
35	3.3.1. Sequence homology30
36	3.3.2. Impurities including aggregates31
37	3.3.3. Excipients used in the formulation40
38	3.3.4. Leachables from container closure system42
39	3.3.5. Peptide modification42
40	4. Conclusions43
41	
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43	peptide synthesis

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46 **1. Introduction**

47 The US peptide drug market is currently estimated at US\$15 billion annually and a recent
48 market study predicts that the global peptide therapeutics market will reach \$23.7 billion by
49 2020 [1]. Owing to the expanded attention on peptide pharmaceutical discovery and
50 development, many new peptide drugs have been approved for the US market in recent years.
51 Moreover, the recent industry shift towards generic versions of peptides intensified the
52 demands for timely evaluation of generic peptide drug product submissions and application of
53 same quality standards to both brand-name and generic drug products. Although advances in
54 the development of the peptide therapeutics have been made, numerous scientific and
55 regulatory challenges remain. This review discusses the critical synthetic peptide quality
56 considerations in the context of the relevant regulatory and scientific challenges.

57

58 **2. Regulatory context for peptide drug review and approval**

59 In terms of chemical complexity, peptides fill a niche between typical small molecules and large
60 proteins. As a result, peptides have raised a series of regulatory controversies. Depending on
61 the structure, properties and manufacturing, peptides/protein drugs have been regulated as
62 conventional chemical entity drugs in some cases, and as biologics in other cases (e.g.,
63 historically, some proteins have been approved as drugs under section 505 of the Food, Drugs
64 and Cosmetic (FD&C) Act through a New Drug Application (NDA), whereas others have been
65 licensed as biological products (BLA) under section 351 of the Public Health Service (PHS) Act).

The definition of biological product was changed to include “protein (except any chemically synthesized polypeptide)” with the passage of the Biologics Price Competition and Innovation Act. Moreover, on or after March 23, 2020, a protein approved under section 505 of the Federal Food, Drug, and Cosmetic Act, will be deemed to be a license for the biological product under section 351 of the Public Health Service Act (PHS Act) (42 U.S.C. 262) [2].

Because the distinctions among peptide, polypeptide, and protein have not been very well defined, the scientific borderline between protein and peptide products has, in many cases, not been clear. Although the FDA guidance on biosimilars provides clear regulatory definitions for protein (“any alpha amino acid polymer with a specific defined sequence that is greater than 40 amino acids in size”) and for chemically synthesized polypeptide (“any alpha amino acid polymer that is made entirely by chemical synthesis, and is less than 100 amino acids in size”) [3], the use of the term “peptide” varies in the scientific literature outside the current regulatory framework. The above regulatory definitions delineate that alpha amino acid polymers with specific defined sequences up to 40 amino acids in size and regardless of their production method (synthetic or of recombinant DNA origin) are considered peptides.

To date, there are over 100 peptide pharmaceuticals on the market in US, Europe, and Japan [4]. Table 1 presents some of the peptides that FDA approved over the years. Notably, many of the FDA approved peptides are synthetic, obtained through solution or solid phase chemical synthesis. It is also remarkable that a considerable segment of the market is allocated to the generic versions of these peptides.

88 Table 1. Peptide drugs approved by FDA [5]

<i>US Approval Date</i>	<i>Generic Name</i>	<i>Brand Name</i>	<i>Indication</i>	<i>Manufacture Method</i>	<i>No. of AA</i>
prior to 1982	Cosyntropin	Cortrosyn	Diagnostic: screening of patients presumed to have adrenocortical insufficiency	synthetic	24
prior to 1982	Oxytocin	Pitocin	Antepartum: initiation or improvement of uterine contractions Postpartum: produce uterine contractions during the third stage of labor and to control postpartum bleeding or hemorrhage	synthetic	9
prior to 1982	Corticotropin	H. P. Acthar Gel	Infantile spasms Multiple Sclerosis Rheumatic Disorders Collagen Diseases Dermatologic Diseases Allergic States Ophthalmic Diseases Respiratory Diseases Edematous State	extracted from porcine pituitary glands	39
prior to 1982	Desmopressin	DDAVP	Hemophilia A von Willebrand's Disease (Type I) Diabetes Insipidus	synthetic	9
1985 (discontinued, next approved in 1989)	Leuprolide	Lupron	Palliative treatment of advanced prostatic cancer	synthetic	9
1988	Octreotide	Sandostatin	Acromegaly Carcinoid Tumors Vasoactive Intestinal Peptide Tumors (VIPomas)	synthetic	8

1990 (discontinued)	Sermorelin	Geref, Sermorelin	Diagnostic: evaluation of pituitary function and also for increasing growth in children	synthetic	29
1995 (recombinant approved in 2005)	Calcitonin (Salmon)	Miacalcin, Fortical (recombinant)	Paget's Disease Of Bone Hypercalcemia Postmenopausal Osteoporosis	synthetic/ recombinant (Fortical)	32
1998	Eptifibatide	Integrilin, Eptifibatide	Acute Coronary Syndrome (ACS) Percutaneous Coronary Intervention (PCI)	synthetic	6
1998 (synthetic approved in 2015)	Glucagon	Glucagen (recombinant), Glucagon	Severe Hypoglycemia Diagnostic: temporarily inhibits movement of the gastrointestinal tract during radiologic examinations	recombinant (Glucagen)/ synthetic	29
2000	Bivalirudin	Angiomax	Percutaneous Transluminal Coronary Angioplasty (PTCA) Percutaneous Coronary Intervention (PCI)	synthetic	20
2000	Triptorelin	Trelstar	Palliative treatment of advanced prostate cancer	synthetic	10
2001	Nesiritide	Natrecor	Acutely decompensated heart failure	recombinant	32
2003	Enfuvirtide	Fuzeon	HIV-1 infection	synthetic	36
2003 (discontinued)	Abarelix	Plenaxis	Advanced symptomatic prostate cancer	synthetic	10
2004	Ziconotide	Prialt	Management of severe chronic pain	synthetic	25
2004	Secretin	Chirhostim	Diagnostic: secretin stimulation testing	synthetic	27
2005	Exenatide	Byetta	Type 2 Diabetes Mellitus	synthetic	39
2007	Pramlintide	Symlin	Type 1 or type 2 diabetes	synthetic	37

2007	Lanreotide	Somatuline Depot	Acromegaly Gastroenteropancreatic Neuroendocrine Tumors	synthetic	8
2008 (2002 approved drug discontinued)	Teriparatide	Forteo	Treatment of Postmenopausal Women with Osteoporosis at High Risk for Fracture Increase of Bone Mass in Men with Primary or Hypogonadal Osteoporosis at High Risk for Fracture Treatment of Men and Women with Glucocorticoid-Induced Osteoporosis at High Risk for Fracture	recombinant	34
2008	Degarelix	Firmagon	Treatment of advanced prostate cancer	synthetic	10
2009	Romidepsin	Istodax	Cutaneous T-cell lymphoma (CTCL) Peripheral T-cell lymphoma (PTCL)	synthetic	5
2010	Liraglutide	Victoza	Improve glycemic control in adults with type 2 diabetes mellitus.	recombinant	37
2012 (discontinued)	Peginesatide	Omontys	Treatment of anemia due to chronic kidney disease (CKD) in adult patients on dialysis	synthetic	21
2012 (discontinued)	Lucinactant	Surfaxin	Prevention of respiratory distress syndrome (RDS)	synthetic	21
2012	Linaclotide	Linzess	Irritable Bowel Syndrome with Constipation (IBS-C) Chronic Idiopathic Constipation (CIC)	synthetic	14
2012	Teduglutide	Gattex	Short Bowel Syndrome (SBS)	recombinant	33
2014	Vasopressin	Vasostriect	Increase blood pressure	synthetic	9

			in adults with vasodilatory shock		
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90 Although many peptide drugs have been approved in the past decades and many more are
 91 currently in review, specific regulatory challenges remain especially related to establishing
 92 quality standards of both brand and generic peptide drugs. Moreover, there is a lack of official
 93 guidelines for peptide drugs, and current FDA and ICH guidelines regarding testing and quality of
 94 pharmaceutical products specifically exclude peptides.

95 In the context of peptides excluded from existing guidelines, one should begin with the end in
 96 mind when designing a peptide product [6]. Patient-related factors including route of
 97 administration, length of treatment, and patient characteristics should guide the design of the
 98 product and its container-closure/delivery system. Product-related factors including peptide
 99 modification and degradation resulting from the manufacture or storage conditions,
 100 formulation, leachables from the container/closure system, can all potentially affect the safety
 101 (including immunogenicity) and efficacy of peptide drugs, and are taken into consideration in
 102 the early stages of therapeutic peptide drug product development.

103 Furthermore, the manufacturer should demonstrate an understanding of the peptide drug
 104 product and its manufacturing process. In the planning stage, the quality target product profile
 105 (QTPP) of the peptide product is established having the end user, the patient, in mind. As
 106 described in ICH Q8(R2), potential peptide drug product critical quality attributes (CQAs)
 107 derived from the QTPP and/or prior knowledge are used to guide the product and process
 108 development. Several tools among which include standard failure modes effects analysis
 109 (FMEA), can be used to perform the risk analysis of the peptide drug manufacturing process. In

the manufacturing step, the critical material attributes (CMAs) and critical process parameters (CPPs) are identified and controlled as part of the control strategy. Process understanding and control can be achieved by challenging the ranges of the CPPs and defining the design of experiments (DOE) space.

Once these steps have been made by the manufacturers, it is the responsibility of the regulators to assess the quality with respect to its impact on the safety and efficacy of a peptide drug by taking a step-wise risk analysis approach. This approach includes: 1) understanding the complexity of the peptide, and its clinical use; 2) evaluating the process- and product-related factors that may impact safety and efficacy of the proposed peptide product; and 3) determining whether additional studies (*in vitro* or *in vivo*) are needed to address any residual uncertainty related to the safety of the peptide product. Importantly, this is not a “one size fits all” approach, but rather a case-by-case analysis approach depending on the factors listed above which are characteristic to each different peptide.

3. Scientific considerations for quality of synthetic peptides

The peptide drug development scientists have the challenge to identify all the factors that can impact the safety and efficacy of the product; on the other hand, the regulators are considering these factors when performing the risk assessment for a new peptide drug. The general principles for development of a peptide drug should include the following steps: 1) evaluate how the product should be characterized including types and amounts of impurities and/or aggregates present in the product at release, shelf life, and under stress conditions, as well as *in vivo* studies, if needed; 2) establish the nature of the risk depending on the type of the peptide,

indication, administration route, as well as types and amounts of impurities and/or aggregates that may impact quality, safety, and efficacy of the product; 3) evaluate appropriateness of adopted control strategies.

Critical aspects of the risk analysis that could be considered for evaluation of the quality and its impact on safety (including immunogenicity) and efficacy of synthetic peptide drugs (as illustrated in Figure 1) are discussed below from the perspectives of product (i.e. peptide structure, formulation factors including excipients, compatibility with container closure system, stability) and process (i.e. control of synthetic peptide manufacturing and peptide-related impurities).

Figure 1. Ishikawa diagram showing the potential risk factors that may impact quality of peptide drug products (bioassay being one way to assess the higher order structure risk factor)

3.1. Characterization of peptide structure

A vast array of analytical methods can be used to capture the complexity of the peptide structure. Like for other molecules, elucidation of chemical composition is at the base of establishing the identity of the peptide; it includes elucidation of the α -amino acids and their alignment in the peptide chain, which constitutes the so-called primary structure/sequence. In general, the primary structure has all the information needed for the peptide to adopt the appropriate secondary conformation, directly responsible for peptide safety and efficacy. Analysis of the primary structure of a peptide is, in fact, elucidation of its amino acids sequence

using chromatography which can be achieved employing simple amino acid analysis, in conjunction with sequencing methods such as N-terminal residue identification by Edman degradation, or peptide mapping coupled with mass spectrometry [7, 8]. The combination of Edman sequencing and tandem mass spectrometry is an efficient approach for elucidation of primary structure of complex peptides having disulfide bonds (such as calcitonin salmon) which may not be identified from amino acid analysis and sequencing [9].

Peptides emerging from various new drug discovery programs typically have a clearly defined amino acid sequence that can be generated through chemical solid phase or liquid phase peptide synthesis. Therefore, in case of new peptides, peptide characterization techniques are used to confirm amino acid sequence in the synthesized peptide, and/or to identify chemical and structural elements that are critical for peptide bioactivity. Likewise, generic peptides undergo extensive characterization with respect to primary and secondary structure, oligomer and aggregation states, and biological activity, as these should be the same as in the reference listed drug (RLD). For generic peptide drugs, the primary structure and the position of disulfide bond are critical determinants of the peptide drug substance sameness as compared to the RLD.

In addition, although most generics have formulations that are qualitatively and quantitatively the same as the RLD, thereby presenting low risk, in some instances where there are differences in formulations, the potential of the new excipients to affect peptide conformation or interact with the peptide needs to be considered in the generic drug development stage. These new excipients could lead to altered stability profile including formation of dimers and higher order aggregates, which may impact safety (including immunogenicity) and efficacy of the peptide drug product.

Peptides that contain less than 40 amino acids generally exhibit high conformational flexibility, which is characterized predominantly by random coil and some degree of secondary structures (e.g., alpha-helix, and/or beta-sheet). The structural ordering may have significant implications for the biological activity of some peptides; therefore, elucidation of secondary structure should be considered part of the peptide structure characterization. Commonly used methods to elucidate peptide secondary structure include circular dichroism (CD), Fourier Transform Infra-Red (FTIR), Raman spectroscopy, fluorescence spectroscopy, Nuclear Magnetic Resonance (NMR) and X-ray crystallography. Far-UV CD (170-250 nm) can be utilized to analyze α -helical structure, whereas near-UV CD (250-320 nm) is sensitive to environmental changes around aromatic amino acids [10, 11]. FTIR can be used to estimate the β -sheet formation and further distinguish between parallel and antiparallel forms, as well as aggregates [12, 13]. Raman spectroscopy is less affected by water absorption, and can be used to explore local interactions (such as those involving Cys or Tyr groups) in addition to the peptide conformation. Fluorescence spectroscopy may be used along with fluorophores such as Thioflavin T dye to investigate the presence of populations of unfolded and/or aggregated peptide species. Furthermore, NMR and X-ray crystallography are the only methods that allow characterization of the full molecular structure of the peptide by providing information about the relative position of all atoms (and thus all amino acids) within the peptide chain. Two-dimensional NMR methods such as NOESY and TOCSY are commonly used to identify the fingerprint peptide structure and potential differences in the peptide structure caused by environmental changes (such as new excipients), and therefore, can be employed by applicants to demonstrate sameness of higher order structure between the generic peptide and its RLD [14-16].

Nonetheless, given that many of these peptides exhibit significant conformational flexibility, their higher order structure is dictated by thermodynamics, as opposed to a kinetic folding phenomenon. Therefore, for generic peptides that have the same amino acid sequence and are formulated qualitatively and quantitatively the same as the RLD, it can be generally inferred that they have the same higher order structure as the RLD.

Characterization of the peptides by various methods, including but not limited to the ones listed above, supports elucidation of peptide structure; given that each method can only capture specific aspects of higher order structure, using orthogonal characterization methods is critical to ensure the correctly elucidated peptide secondary structure.

Furthermore, in addition to the methods that analyze peptide primary and secondary structures as illustrated above, an *in vitro* bioassay providing information on peptide structure-activity relationship is considered an essential aspect of efforts undertaken for characterization of higher order structure and biological activity for complex peptides (e.g. chromogenic human thrombin inhibition assay for bivalirudin). Such an assay was used in the past to measure the potency of synthetic peptides. Assessment of the biological activity (which can be done by animal-based or cell-based assays, biochemical assays, or immune responses assays) is particularly important in cases where the synthetic peptide is used as a drug substance for a generic drug, if an additional confirmatory test is needed for demonstrating sameness between the proposed generic and the RLD products.

3.2. Control of peptide synthesis

Quality should be designed and build into the peptide and is governed by the process used to manufacture that peptide. There are three stages in the development of a process used to manufacture a peptide: 1) process design, 2) process qualification, and 3) continued process verification [17]. During manufacturing process design, every unit operation is carefully evaluated starting with the raw materials and continuing with in-process controls; here, the CMAs and CPPs are identified as factors likely to have the greatest impact on the peptide quality (i.e., CQAs). At this stage, data analysis to establish appropriate ranges of operating, including a design space, and identification and control of important variables is achieved. A feedback loop for corrective and preventing actions (CAPA) is in place depending on the criticality of individual process steps and the CPPs that control them. Risk assessment, with calculation of the risk priority number for each failure mode, is employed to determine the functional relationships that link the CMAs and unit operations CPPs to peptide drug product CQAs. During process qualification, the process designed as described above is validated by manufacturing the commercial batches. Finally, through continuous process verification, the process is maintained in a state of control during commercial manufacture. Next, in this section, we discuss important CMAs and CPPs monitored during the manufacture of a synthetic peptide by solid phase peptide synthesis (SPPS). A non-exhaustive list of CMAs and CPPs, as well as potential in process tests are presented in the SPPS chart below (see Figure 2).

Figure 2. SPPS flow chart including CMAs and CPPs and potential in process tests

3.2.1. Control of starting materials

An increased regulatory emphasis has been placed lately on the control of starting materials used in the manufacture of synthetic peptides [18]. Starting materials include resins used as solid support, amino acids and protected amino acids derivatives, peptide fragments, reagents used in coupling, deprotection and cleavage steps of the synthesis, and solvents.

Resins: The first step of SPPS involves anchoring the first amino acid (through formation of an ester bond) onto a solid support matrix such as polystyrene (PS), polyacrylamide, PS functionalized with polyethylene glycol (PEG), or cross-linked PEG. Many such resins (chloro, bromo – e.g., trityl chloride; amino, hydrazine – e.g., Rink-amide; hydroxyl - e.g., Wang; carboxy; aldehyde; enol; thiol; carbonate; silicon; or alkenylcarbonyl functionalized resins, to name a few) are commercially available with a linker that protects the peptide C-terminus from side reactions [19]. Other custom functionalizations or modifications of the resin with a linker are possible, and should be confirmed by the manufacturer by simple “on bead” color tests; in addition, the stability of the linker should be tested under identified acid and base conditions employed in the SPPS. The selection of the resin itself affects the outcome of the SPPS. The resin should have an appropriate particle size that allows ease of handling and removal of solvents. Moreover, the resin should be inert, but have good swelling in the solvents used during SPPS. The extent of the amino acid initial loading onto the resin is determined by the resin swelling (mL/g) properties, as well as by its substitution ratio (mmol/g); an inefficient loading may lead to C-terminal deletion/truncated peptide impurities, and therefore, directly impact the purity, as well as the yield of the final peptide [20]. Notably, special attention is given to peptides containing Cys, His, Pro, Met and Trp residues at the C-terminus.

Esterification of Cys and His to Wang resin, for example, is problematic as these amino acids are prone to enantiomerization [21]. To avoid these side reactions, trityl-based resins (having a “bulky” linker) can be used instead, and are strongly recommended especially for peptides containing C-terminal Pro, Met, and Trp residues. The substitution of the resin can be easily determined after loading of the first amino acid by common color or UV tests.

Amino acids and amino acids derivatives: When evaluating the quality of the amino acids and amino acid derivatives, particular interest is given to their chiral properties and impurities.

Impurities in raw materials including (i) free amino acids or amino acid derivatives, (ii) amino acid contaminants, i.e. amino acids other than the desired one, (iii) incorrect enantiomers, i.e. D-isomer [22], (iv) dipeptides or oligopeptides and (v) β -alanine containing contaminants [23] have the potential to be carried-over into the final peptide drug substance, and therefore, should be tightly controlled. Stringent limits for such impurities minimize the potential impact on the quality of the peptide drug substance; they can be set up based on prior knowledge acquired from manufacturing similar peptides, supported by process risk assessment.

Reagents and solvents: Generally, there is limited concern regarding the quality attributes of reagents and solvents used in the initial stages of the peptide manufacturing (e.g., dichloromethane, N,N-dimethylformamide solvents; HBTU, hydroxybenzotriazole (HOBt) coupling reagents) as they are unlikely incorporated in the final peptide drug substance. These materials are tested in house for vendor qualification, and at minimum, should be pure to avoid side reactions; solvents used during coupling stages in particular, have excellent solvating properties in order to reduce folding and aggregation of the growing peptide chain which can lead to incomplete coupling and deprotection. Nevertheless, solvents and reagents (e.g.,

acetonitrile, methanol, isopropanol, acetic acid, trifluoroacetic acid) typically used in the purification or precipitation steps of peptide synthesis can be carried over and should be quantified in the final peptide drug substance.

3.2.2. In process control strategies for solid phase peptide synthesis

For a peptide manufactured by SPPS methods, special attention is given to each step of the synthesis including initial coupling, synthesis cycle, final deprotection and cleavage, and in process controls. Appropriate in process quality control strategies are particularly important for SPPS in order to avoid amplification of peptide impurities from one synthesis step to the next. For example, for the SPPS of enfuvirtide, a 36 amino acids peptide, a 0.1% decrease in the efficiency causing a decrease of 0.3% in purity for each coupling step is amplified to an overall 9.9% decrease in purity of the crude peptide [24]. The control strategy developed during manufacturing process design stage refers to identification of potential side-reactions and impurities, and steps undertaken to minimize their levels.

Following the selection and control of the raw materials and attachment of the first amino acid, the peptide chain is built through a repeated cycle, involving first the deprotection of N- α -amino protecting group (Fmoc), and then activation and coupling of the next amino acid.

Deprotection control strategies: The removal of Fmoc protecting group is normally achieved through short exposure to a solution of 20-30% piperidine in DMF. The procedure is normally performed twice to ensure complete removal of the protection group, as incomplete removal can lead to deletion peptide-related impurities [25]. While in most cases, deprotection strategies are effective, for long peptides, incomplete deprotection can occur even at high concentration of piperidine and after repeated trials. In this case, it is advisable to increase the

time allocated to the deprotection step or to use a stronger base (e.g. DBU). For peptides containing Asp, a side reaction at this step is aspartimide formation (cyclization). This side reaction is catalyzed by either acid or base; therefore, it can also ensue at a later step, during cleavage with TFA. The aspartimide ring can further open, which leads to a series of peptide related impurities [26-28]. Addition of HOBt during deprotection reduces this side reaction [29]. Extra caution is also given to C-terminal Cys which is prone to sulfhydryl elimination following piperidine addition [30].

A washing step is introduced between deprotection and next coupling step to remove the piperidine reagent. Incomplete removal of piperidine may lead to premature deprotection of the next amino acid during coupling step, and generate insertion impurities. Therefore, in process tests to ensure complete removal of piperidine should be employed, including pH of washing solvent and Chloranil test (for piperidine secondary amine) [31].

Coupling control strategies: In the coupling step, the free carboxyl terminus of the incoming amino acid is transformed into an active electrophilic form by coupling reagents. This is probably the most important variable to be optimized, because the selection of the optimal activation agent allows efficient acylation of the amino function, thus increasing the coupling efficiency and limiting side reactions, such as racemization (loss of chiral integrity). In principle, racemization can occur in any step of the SPPS; however, the risk is higher during activation and coupling. Two mechanisms have been identified to be responsible for the loss of chiral integrity during coupling, and include direct enolization, and formation and epimerization of 5(4H) oxazolone [32-34]. Recent developments in SPPS have greatly resolved the racemization problem by introduction of auxiliary nucleophiles such as HOBt [35], HOAt [36] and Oxyma [37],

which form active esters with carboxyl and trap O-acylisourea before it can form oxazolone; the so-called “in situ” coupling reagents uranium/aminium salts (e.g., HBTU, TBTU [38]) and phosphonium salts (e.g., PyBOP [39], COMU [40]), which form OBt esters with the carboxyl in situ can also be used to improve coupling efficacy and limit racemization. Extra caution is still needed for certain amino acids that are prone to racemization (e.g. Hys, Cys, Asp [26, 41, 42]), as well as at elevated temperature [43], or excess of base [44] reaction conditions; in these circumstances, an “overactivated” carboxyl group can go through direct enolization or form oxazolone before reacting with amines. At this time, racemization impurities may not be readily detected and separated by HPLC, and they may cause aggregation of peptide product, as well as potentially have biological consequences [45-47]; it is therefore important to develop strategies to minimize this type of side reaction as early as the process design stage.

Another side reaction that is common during coupling is insertion (by a mechanism different from the one caused during deprotection with piperidine, as mentioned earlier), where an incoming amino acid first acylates an amide bond of the resin bond peptide, and then inserts itself into the peptide sequence during next round of coupling [48]. The insertion can be caused by an excess amount of Fmoc-protected amino acid used for driving the coupling reaction to completion, and is more likely to happen at locations on the peptide sequence where the amide bond is further away from bulky side chains that introduce steric hindrance [49].

For certain difficult-to-react amino acids, recoupling may be necessary to reach completion of the reaction; however, if recoupling is still not able to completely react all the free amines, a capping step is needed to prevent the generation of deletion sequences. Capping itself will

generate a truncated sequence; however, the truncated sequences are much easier to separate than the deletion sequences. The completion of coupling can be determined and controlled using suitable in process color tests such as Kaiser, trinitrobenzenesulfonate (TNBS), chloranil, or bromphenol blue which are able to detect the presence of residual amine (see Table 2 for details). Each of these tests has its limitations; therefore, two independent tests are recommended to monitor the completion of the coupling reaction. To avoid false results, it is also advisable to perform these tests with protected amino function as negative control, and deprotected amino function as positive control.

Moreover, a thorough washing after coupling avoids generation of insertion impurities as described here. In process tests such as pH measurement or UV absorption (when applicable; for example, in case of HOBt coupling agent) of the washing solution can effectively control the removal of excess reagents.

The deprotection-coupling cycle is repeated until the last amino acid is attached to the resin bound peptide.

Table 2. In process color tests

<i>Color Tests</i>	<i>Target</i>	<i>Purpose</i>	<i>Notes</i>
Kaiser Test (Ninhydrin test)	Primary amines	Negative result indicates complete coupling (99%-100%)	<ol style="list-style-type: none"> Also used to verify deprotection, however cannot indicate completeness (positive from 2-100% deprotection) Requires heating; prolonged heating and overheating can lead to false positive results due to deprotection [50].
TNBS Test	Primary amines	Same as Kaiser test	<ol style="list-style-type: none"> Equally sensitive as the Kaiser test Does not require heating

Acetaldehyde/ Chloranil Test	Primary and secondary amines	<ol style="list-style-type: none"> 1. Negative result indicates complete coupling (99%-100%) 2. After deprotection and washing, negative result indicates complete removal of piperidine. 	<ol style="list-style-type: none"> 1. Used instead of Kaiser test for secondary amines detection such as N-terminal Pro 2. Can also detect primary amine (with beads turning dark red instead of dark blue)
Bromophenol Blue Test	All types of amines	Same as Chloranil test	<ol style="list-style-type: none"> 1. Trace of acid can lead to false negative result. 2. Residual DIPEA or collidine from coupling can lead to false positive results [51].

367

368 *Cleavage control strategies:* During the cleavage step, the entire peptide chain, as well as the

369 side chains protecting groups of the amino acids residues, is cleaved simultaneously or

370 separately. Concentrated TFA (95%) cocktail with scavengers, such as water and

371 triisopropylsilane (TIS), is the most commonly used cleavage reagent in Fmoc SPPS. Scavengers

372 are added to protect sensitive side groups (especially the nucleophilic ones, such as Trp, Met,

373 Tyr and Cys) by converting the cations generated during the reaction into corresponding

374 hydrocarbons [37]. In particular, scavengers containing sulfur, such as EDT (ethanedithiol), DTE

375 (dithioerythritol), and thioanisole, are helpful to trap carbocations, and prevent oxidation and

376 disulfide bond formation for Met and Cys [52, 53]; whereas TIS can replace EDT to protect Arg

377 and Trp residues [54]. Failure to properly protect these sensitive amino acids may lead to

378 unwanted impurities, such as oxidation of Cys and Met which can further produce peptide

aggregation [55]. Possible side reactions caused by side chain reactivity at cleavage step are summarized in Table 3.

Table 3. Side chain reactions possible during cleavage

<i>Amino Acid</i>	<i>Side reaction</i>
Cys	Oxidation and formation of disulfide bond leading to aggregation
Met	Oxidation and formation of methionine sulfoxide
Trp	Oxidation and alkylation of indole ring [56]
His	Acylation of imidazole ring [57]
Tyr, Ser and Thr	Alkylation of hydroxyl groups [58, 59]

The duration of the cleavage step also affects the quality of final peptide; prolonged exposure of peptide to the strong acid (i.e., TFA) and high temperature conditions inevitably lead to side reactions as mentioned above, as well as incomplete removal of protection groups, and formation of adducts with scavengers [58, 60 - 62]. Therefore, cleavage time and temperature need to be optimized during development stage and strictly monitored in peptide production.

Post-cleavage work-up control strategies: Certain peptides, such as ziconotide, romidepsin and linacotide, need to undergo cyclization through formation of oxidative intramolecular disulfide bridges. Careful planning of the synthetic strategy for these peptides is essential, if the peptide is to be obtained in good yield and having the correct structure. To this regard, a regioselective strategy with orthogonal Cys protection groups can be employed for peptides with multiple

disulfide bonds [63], and oxidation conditions (temperature, duration) can be controlled to minimize formation of intermolecular disulfide bonds responsible for dimers and aggregates formation [64, 65]. In addition, controls such as the Ellman test or other suitable procedures are established to assess the extent of disulfide linkages formation in these peptides. Finally, selection of the peptide purification route takes into account the size, polarity and ionic character of the desired peptide and process-related impurities as mentioned throughout this section, including diastereomeric, deletion, truncated, deaminated, isomerized, and oxidized peptides, disulfide exchange products, oligomers and/or aggregates, by-products generated by incomplete deprotection of amino acid side chain protecting groups, and reagents and solvents used in the peptide synthesis. Most commonly used separation methods are reversed phase chromatography (RPC) and ion exchange chromatography (IEC). Hydrophobic interaction is at the basis of RPC separation principle; by using different buffer systems, organic gradients, as well as ion-pairing agents, peptide hydrophobicity and elution pattern can be manipulated to allow efficient separation of the target peptide from unwanted impurities. The pH of the mobile phase significantly influences the peptide retention; however, it can cause instability of the stationary phase or of the peptide itself. Ion-pairing agents are helpful for separating peptides with charge differences, but they may lead to impurities if not properly removed in desalting or ion-exchange stages [66, 67]. Furthermore, the organic solvents and gradients modify the polarity of the mobile phase, and thus, its eluting strength to separate peptides with different hydrophobicity [68]; however, they may also permanently change the peptide's higher order structure. Besides the mobile phase factors presented here,

416 solid phase parameters (surface bonding, size, pore diameter), temperature, as well as column
417 load, also affect peptide separation [69]. The design space of all these parameters should be
418 identified and controlled during purification for an effective and fine-tuned peptide separation.
419 On the other hand, IEC uses ionic charge interaction between the peptide and the
420 stationary/mobile phase for the separation. The interaction strength can be manipulated by
421 changing the pH of the mobile phase or the salt concentration. Subtle changes of pH
422 significantly affect the peptide retention by affecting the peptide overall charge depending on
423 the side chain groups pKs. At the same time, the pH requires tight control over the separation
424 course, as peptide sensitivity to pH may lead to deamidation [70]. With respect to salts, the
425 most commonly used ion displacer, NaCl, has minimum influence on peptide structure and
426 stability. Other anion/cation must be evaluated not only for their ability to adjust elution
427 strength and achieve selective separation, but also with respect to their potential toxicity [71].
428 Other process parameters that can affect peptide separation such as elution gradient (salt
429 concentration/ionic strength), stationary phase quality, peptide load, and temperature also
430 need to be controlled to ensure a high quality peptide separation.
431 Due to concerns for stability in aqueous solutions, many peptides are supplied as lyophilized
432 (freeze-dried) products. During lyophilization, the peptide in aqueous solution is first frozen
433 (freezing stage), ice is then removed through sublimation (primary drying stage), and any
434 residual water is finally eliminated through desorption from the solute phase (secondary drying
435 stage). Strict control of the lyophilization conditions (temperatures, pressures, rates, and
436 durations) at all stages and of the residual moisture in the lyophilized peptide is needed because
437 high amount (5-20%) of residual moisture reduces the glass transition temperature of the

product (Tg) and results in instability issues; consequently, residual moisture should be monitored and controlled throughout product shelf life.

Moreover, removal of bulk water creates instability by reducing the hydrophobic interactions that are critical for the higher order structure of the peptide. Variation in the freezing rate may also increase the damage of the peptide, which may result in increased levels of peptide aggregation. Aggregation also may occur when highly concentrated peptide solutions are frozen which favors peptide-peptide interactions. A high ionic strength and large pH shifts also cause degradation of peptides, and therefore should be avoided.

3.2.3. In process control strategies for solution phase peptide synthesis and ligation

Solution phase peptide synthesis (SPS) has a longer history than SPPS, the first dipeptide Gly-Gly being synthesized in 1901. Classical SPS is based on the coupling of single amino acids in solution; small peptides composed of several amino acids can be efficiently prepared by this method using orthogonally protected amino acids, that avoid unwanted side reactions and overcome synthetic difficulties [72, 73]. The core chemistry of SPS including deprotection, activation, and coupling are similar with SPPS; therefore, overview of the process chemistry, and the considerations on quality and impurities of the synthetic peptides, as presented above, are not repeated in this section.

The prime advantages of SPS are related to easiness to scale up, fewer side reactions, high purity of the final peptide, as well as convenient and flexible control methods applicable throughout the entire synthetic process [74, 75]. Oxytocin, triptorelin, and bivalirudin are examples of peptides synthesized by SPS [24, 76, 77]. Although SPS can be scaled up in an easy and inexpensive manner, its long reaction time remains a major disadvantage [78]. Another

major difference between SPS and SPPS is the need to employ purification in SPS after coupling and deprotection reactions. Because no solid support is used in SPS, extensive extraction, precipitation, and washing are needed to yield a relatively pure peptide intermediate. However, unlike the difficulties encountered with in process controls during SPPS, samples can be readily taken during SPS to quantitatively and qualitatively test the completion of reactions (using methods such as TLC, HPLC, etc.), and control the quality/purity of the peptide intermediates. Nevertheless, as the target peptide becomes longer, the number of needed solution-phase stages and isolations obviously increase, which considerably complicates the process [24]. To this end, fragment condensation method has been used for the synthesis of long peptides; specifically, short fragments of the required peptide are first synthesized, then coupled together to form the final long peptide. Several methods, as described next, had been developed for segment condensation and ligation.

One of the most commonly used ligation methods is azide procedure which gained popularity due to its ability to retain chiral integrity at the activated residue [79]. The low racemization rate can be explained by the reaction mechanism, which does not lead to oxazolone formation, unlike the typical activation reaction [80]. Another advantage, especially for long peptides, is the high specificity of the azide reaction requiring minimal side-chain protection (except for Lys and Cys) [81]. However, this method has a major disadvantage as it is characterized by a considerably low rate of coupling which favors the rearrangement reactions, leading to carbamide-peptide impurities which are difficult to separate [82]. A potential solution to this problem is to use diphenylphosphoryl azide (DPPA) reagent which forms acyl azide intermediates that facilitate peptide bond formation [83].

Another method used to ligate short peptide fragments to form long peptides is natural chemical ligation (NCL). NCL can be carried out either in solid phase or in solution phase, as the substrate does not affect the reaction itself. NCL is a highly chemoselective ligation of unprotected peptide fragments in aqueous solution; the reaction occurs between a C-terminal thioester and N-terminal Cys, with the formation of a disulfide bond, that can be then transferred to form a native peptide bond [84]. Moreover, the reaction provides the benefit of minimal racemization [85]. This method was further extended to non-Cys containing peptides where a more common amino acid, Ala, serves as a “ligation site” by conversion of Cys to Ala through desulfurization using H₂/metal reagents after the ligation [86].

Even though, in the past, many peptides (e.g., oxytocin, bivalirudin, leuprolide, etc.) synthesized by SPS are now mostly produced by SPPS due to reduced development time and synthetic cycle, solution phase ligation is still the most desirable method to synthesize long peptides (having around 40 amino acids). When the peptide length is around 40 amino acids, the yield of SPPS is extremely low due to significant increase in truncated impurities and aggregation [24].

Therefore, the combination of SPPS and SPS ligation is the most effective strategy for synthesis of such long peptides. For example, the low yield (6-8%) and low purity (<30%) of the enfuvirtide, a 36 amino acids peptide synthesized by SPPS, can be significantly improved using a solution phase and solid phase hybrid process [87]. By coupling together short to medium length peptides (e.g., in case of enfuvirtide, three peptide segments of 9-16 amino acids length synthesized by SPPS), the hybrid method SPPS-SPS can effectively improve the overall yield to 30-40% and peptide purity to >75%. This approach has the advantage of greatly simplifying the product purification and avoiding the solubility problems common in traditional SPPS. Selection

of the fragments synthesized by solid phase and coupled in solution phase is directly related to their solubility, ease of synthesis, purity, and yield, and should be determined by experimental trials. With regard to solubility, selected segments should dissolve in a common solvent, and the ligation intermediates should be easily separated from the solvent through condensation. With regards to purity and yield, difficult synthetic sites and amino acids that are prone to epimerization should not be used as terminal segments, because epimerization impurities generated during ligation step are much more difficult to detect and separate as compared to those generated during SPPS. Therefore, quality control of the intermediate peptide segments becomes critical, and quantitative HPLC methods should be established to ensure segment purity according to the specifications.

3.3. Strategies to control the quality of peptide drug products

Quality is the underpinning of safety and efficacy of a peptide drug product. Particularly related to safety, the immunogenicity of peptide products presents concerns because it can have clinical consequences, including the generation of antibodies directed against the therapeutic peptide, loss of efficacy, neutralization of the human peptide counterpart, as well as other general immune system effects (including allergy and anaphylaxis) [88]. Changes in the quality of the peptides through modification and degradation resulting from the manufacture or storage conditions, residual solvents, formulations, leachables from the container/closure system, route of administration, length of treatment, and patient characteristics, all can potentially affect the safety, including immunogenicity of peptide

products [89]. Clearly, the potential for immunogenicity-driven adverse events is taken into consideration during the risk assessment of a given peptide drug. Evaluation of quality impact on safety is a multidisciplinary task, encompassing joint efforts of quality, non-clinical, and clinical experts. There are three categories of factors that potentially affect safety including immunogenicity, and therefore should be evaluated: patient, product, and treatment related. Consequently, the safety concerns of a peptide drug product can be mitigated by controlling its quality, meaning optimizing the manufacturing and purification processes, optimizing the drug formulation, and assuring proper storage conditions. We here focus on product related factors only: peptide origin, impurities (including degradation products such as aggregates), excipients, and leachables.

3.3.1. Sequence homology

In general, the peptide primary structure itself can be a strong determinant of immunogenic potential; the antigenic response to a therapeutic peptide is intrinsic to its amino acid sequence. All foreign peptides activate the immune system; therefore, the lower the degree of homology between the therapeutic peptide and its human counterpart, the higher its immunogenicity. The immune system is able to detect small differences between the structure of a synthetic peptide and its own [89]. For example, a peptide with only 50% homology to its human counterpart such as salmon calcitonin (for which 15 out of 32 amino acids correlate identically) will be recognized by the human immune system as a foreign peptide. As a result, its immunogenicity is common and antibodies occur in 40-77% of the patients. Although little can be done about reducing immunogenicity inherent to the peptide sequence, sequence analysis

during drug development can be used to predict the peptide immunogenicity to select candidates that have minimal potential to elicit immune response.

3.3.2. Impurities including aggregates

Safety concerns including peptide immunogenicity may be due not only to the peptide itself, but also to the impurities and contaminants that are arising from the manufacturing process and storage. Table 4 shows a compilation of peptide-related impurities resulting from synthesis or arising from degradation that may occur in peptide drug products.

Table 4. Process related impurities and degradants that may occur in peptide drug products [90]

Impurity	Mechanism
Denaturation	Modification of the peptide structure that changes its physical, chemical and biological properties; occurs in the presence of denaturing agents
Proteolysis	Upon exposure to harsh conditions extreme pH, high temperature or enzymes
Aggregation and precipitation	Association of hydrophobic amino acids residues together to form aggregates; precipitation occurs if on macroscopic scale
Deamination	Hydrolysis of the side chain amide linkage of an amino acid residue with formation of a free carboxylic acid
Oxidation and reduction	Oxidation induced by temperature, pH, metal ions and buffers during synthesis and/or storage
Disulfide exchange	Change in conformation due to reaction between disulfides within a peptide chain
Diastereoisomerisation (Racemization)	Alteration of L-amino acids to D, L mixtures, with formation of peptide bonds sensitive to proteolytic enzymes
B-elimination	Proceeds through a carbanion intermediate; susceptible residues under alkaline conditions are Cys, Lys, Phe, Ser, Thr
Deletion (incomplete coupling)	Incomplete coupling due to incomplete removal of the protecting group of the last coupled amino acid or insufficient activation of the incoming amino acid
Truncation	Missing one or more amino acid residues at either the N- or C-terminal end due to precipitation of resin beads during

	synthesis
Amino acid insertion	Insertion of an additional amino acid into the peptide sequence due to excess of Fmoc-protected amino acids and improper washing
Dimers	Self-association of hydrophobic amino acids residues in peptides to form aggregates
By-products generated by incomplete deprotection	Results in protecting groups covalently attached to peptide sequences

555

556 The first group of peptide-related impurities is process-related impurities present in peptides

557 manufactured by peptide synthesis, and discussed in the section 3.2 above.

558 The second group is the product-related impurities which are degradants of the peptide, and

559 may be generated regardless of the manufacturing process. The degradation of the peptide can

560 occur by two major pathways: chemical and physical instability, as presented next. Chemical

561 instability involves formation or cleavage of peptide covalent bonds through different

562 mechanisms including hydrolysis, oxidation, racemization, isomerization, deamidation, disulfide

563 exchange and β -elimination [91]. Physical instability involves changes in conformation/higher

564 order structure through surface adsorption, aggregation and/or precipitation, which may not

565 involve covalent bond modifications [92].

566 Chemical instability

567 *Hydrolysis*: Peptide hydrolysis refers to the breakage of an amide bond with a molecule of

568 water usually through the attack of nucleophiles [93], catalyzed by acid or base [94]. Hydrolysis

569 leads to peptide degradation through several pathways such as deamidation, cleavage of

570 peptide bond, and isomerization. Deamidation occurs when asparagine (Asn) and glutamine

571 (Gln) residues in peptides form aspartic acid (Asp) and glutamic acid (Glu) residues. The

mechanism of deamidation is pH and water dependent. Specifically, at $\text{pH} < 3$, Asn and Gln are directly hydrolyzed through acid catalyzed deamidation [95], whereas at $\text{pH} > 3$, deamidation of Asn occurs through cyclic imide formation, which is then attacked by either water or adjacent peptide bond nitrogen to form Asp or iso-Asp (isomerization) [42]. On the other hand, Gln is more stable at basic pH due to the difficulty of formation of a six-membered ring.

The rate of hydrolysis is affected by peptide primary sequence [96], and conformation [97, 98], as well as solution properties such as pH, temperature, ionic strength, and buffer ions [99]. By targeting these specific pathways, control strategies can be designed to improve hydrolytic stability of peptide.

Different peptides may have different optimum pH, therefore it is important to optimize and control pH appropriately (e.g., in solution, oxytocin is most stable at pH 4.5 [100], whereas calcitonin is most stable at pH around 3.3 [101]). Even for lyophilization products, pH should be controlled as it can vary widely during the lyophilization process due to changes in solubility of the buffer salt [102].

Moreover, the type of pH buffer and its concentration can also directly influence peptide hydrolytic stability. For example, citric acid stabilizes oxytocin through formation of complexes [103]; on the other hand, deamidation occurs at a faster rate in phosphate and bicarbonate buffers than in acetate, chloride and pyruvate buffers [104].

Increased hydrolytic stability can also be achieved by using a cosolvent and/or a cosolute. As mentioned earlier, deamidation of Asp at neutral or basic pH involves the formation of a cyclic intermediate; it was found that a low dielectric solvent is able to destabilize the nucleophilic anion charged nitrogen needed to attack carbonyl and form the cyclic intermediate [105].

Thus, the deamidation and isomerization can be inhibited by reducing solution's dielectric strength using cosolvents and/or cosolutes such as PEG, PVP, glycerol, sucrose, and ethanol [106].

Oxidation: Some amino acids (Cys, Met, His, Tyr and Trp) are susceptible to be oxidized by oxygen radicals during peptide manufacturing, as well as during storage. For example, Cys and Met residues contain sulfur in their structure, which can be oxidized into sulfoxides, sulfones and/or form disulfide bonds. The oxidation of Cys in particular can be more complicated due to irreversible transformation of the original disulfide bond into mispaired disulfide bonds and scrambled disulfide bridges [107]. The oxidized species are more polar than the original amino acids residues which may cause further changes in the peptide conformation, or even lead to aggregation [108, 109]. On the other hand, His, Tyr and Trp are susceptible to oxidation due to their aromatic rings. Histidine's imidazole ring can be oxidized to yield asparagine/aspartate and 2-oxo-histidine (2-O-His) which may further trigger peptide aggregation [110], whereas oxidation of tyrosine may lead to several regio-isomers of hydroxy-indole, kynurenine, and N-formyl kynurenine, and may result in covalent aggregation through formation of bityrosine [111].

A thorough understanding of the factors that affect peptide oxidation, including exposure to light, harsh pH conditions, and the presence of metal ions, is the key to control oxidative impurities. Photo-oxidation is initiated when a compound absorbs a certain wavelength of light, which provides energy to raise the molecule to an excited state; the excited molecule can then transfer that energy to molecular oxygen, converting it to reactive singlet oxygen atoms (oxygen radicals) [73]. Photo-oxidation can oxidize certain amino acid residues such as Tyr and

616 His leading to covalent crosslinking and aggregation [112]; it can also degrade the peptide
617 backbone [113], or indirectly oxidize Met through neighboring amino acid residues [114, 115].
618 Alkaline pH was found to favor Cys oxidation because it helps deprotonate thiol groups making
619 them more nucleophilic [109]. Certain buffer systems can be used to control pH to neutral or
620 slightly acidic conditions to avert this degradation pathway.

621 Metal ions (Fe^{3+} , Ca^{2+} , Cu^{2+} , Mg^{2+} , or Zn^{2+}) are also known to catalyze oxidation by generating
622 highly reactive hydroxyl radicals if reacted with peroxide (through Fenton reaction) in a
623 concentration-dependent manner [113, 116]. Chelating agents such as
624 ethylenediaminetetraacetic acid (EDTA), desferal, diethylenetriaminepentaacetic acid (DTPA),
625 inositol hexaphosphate, ethylenediamine bis(o-hydroxyphenylacetic acid),
626 tris(hydroxymethyl)aminomethane (TRIS), citric acid, and tartaric acid are all commonly used
627 excipients for complexation of metal ions [117]. Sugars and polyols may also prevent metal-
628 catalyzed oxidation because of their complexation with the metal ions [118]. These ingredients
629 are added to prevent metal contamination from manufacturing process and containers.

630 More importantly, contaminations can also come from excipients [119] (e.g. PEG, Tween-80), or
631 from plastic or elastomeric materials used in primary packaging container–closure systems
632 [120]. To prevent peptide oxidation from these contaminations, antioxidants are used in the
633 formulations as chemical “sacrificial targets” that consume chemical species promoting
634 oxidation. Scavengers such as L-methionine and ascorbic acid are used for this purpose [121].

635 It is recommended that the above mentioned critical factors such as pH, metal ion
636 concentration, and contaminants levels in excipient and container leachable are well
637 controlled, and light protection is considered in the manufacturing of peptide product and

design of packaging. Moreover, when stabilizing excipients, such as chelating agent or antioxidants are used in the peptide formulation, their interaction with the peptide should be studied. That is because not all antioxidants are compatible with all peptides; for example, ascorbic acid promotes rather than inhibits oxidation of the Met residue of small model peptides in the presence of metal ions [122].

β -Elimination: The β -elimination reaction occurs when peptide is exposed to alkaline pH and elevated temperature [123]; it involves removal of HS⁻ or HO⁻ from Cys, Ser, and Thr respectively, with generating a C=C double bond between the α and β carbons and free thiols [83]. The free thiols generated from β -elimination also catalyze formation of disulfide bond exchange and reducible aggregates [124, 125]. Moreover, the double bond created by β -elimination is more electrophilic and susceptible to nucleophilic attack by either the amino group of Lys or the thiol group of Cys and formation of non-reducible covalent aggregates [126].

Effective control of pH and temperature during manufacturing and storage of peptide product is clearly critical to control β -elimination impurities. It was also reported that divalent metals can reduce β -elimination presumably through oxidization of free thiols [125].

As described in the previous sections of this review, control of peptide-related impurities and degradants is critical in the development and manufacturing stages of the peptide products. In general, the recommendations for reporting, identifying, and qualifying impurities for synthetic peptides differ from the recommendations provided in the guidelines cited for small molecules because synthetic peptide drug substance can have many closely related impurities.

Proposed thresholds for peptide-related impurities resulting from the synthesis or arising from degradation are usually established on a case-by-case basis taking into consideration the risk of

immunogenicity in the context of the clinical use of the peptide: for therapeutic use, as a vaccine, or for *in vivo* or *in vitro* diagnostics. However, ICH guidelines for impurities may be appropriate in case of small size peptides (i.e., peptides with less than 10 amino acids) that can be treated as small molecules; such peptides are highly pure and less likely to generate severe adverse immune events. For all other, especially when addressing safety/immunogenicity concerns of certain peptides, justification of the impurities levels (i.e., by clinical and toxicology data for innovator peptides, or by reference to available United States Pharmacopoeia (USP), European Pharmacopoeia (EP), British Pharmacopoeia (BP), Japanese Pharmacopoeia (JP) monographs, scientific literature, development and stability data, toxicology data, significant metabolite of drug substance, and/or RLD for generic peptides) may be more appropriate. Moreover, for peptides used as drug substance in a proposed generic drug product, a one-time study direct comparison of peptide-related impurity profiles between the proposed generic drug product and multiple batches of its RLD is needed to demonstrate comparably low levels of peptide-related impurities under long term storage and accelerated/stress conditions. When evaluating the quality of a generic peptide, it is expected that impurities will be different between a generic peptide and its RLD if the synthetic pathways employed are different (chemical synthesis vs. recombinant DNA technology vs. extraction from natural sources). To reduce the risks to patients, these specified impurities different from the RLD should be strictly controlled at low levels established based on prior knowledge and supported by risk assessment. In addition, comparative studies on non-clinical (e.g., *in silico* and *in vitro*) immunogenicity testing between the generic peptide product and the RLD may be needed to show that the impurities different from the RLD as present in the generic do not produce a

greater stimulation of innate immune activity as compared to the RLD. Since it is generally understood that peptide-Major Histocompatibility Complex (MHC) binding is a critical step leading to antibodies, *in silico* and *in vitro* approaches for determining peptide-MHC binding affinities can be used to identify the peptide sequences/impurities responsible for eliciting an immune response, and hence, inform the relative immunogenicity risk of these impurities different from the RLD [127-129]. This type of tool, in combination with other methods, is promising for ascertaining the risk of specified impurities unique to a synthetic peptide, and reducing residual uncertainty pertaining to safety/immunogenicity.

Physical instability

Aggregation: A special case of peptide impurities, aggregates, are physical degradation products that negatively impact the safety of the peptide drug products. It has been suggested that aggregates of therapeutic peptides/proteins that consist of minimum 10 to 20 epitopes at a repetitive spacing of approx. 100 Å and a molecular weight greater than 100 kDa can induce an immune response by efficiently eliciting T-cell help for antibody production [130, 131]. In addition to molecular weight of aggregates, their poor solubility is another detrimental factor, because insoluble aggregates are more immunogenic than the soluble aggregates and stimulate an immune response by alternate mechanisms [132].

The physical degradation (instability) is a process that starts with a change in the conformation (sometime referred to as denaturation), which leads to aggregation of peptide molecules, and eventually precipitation. The denaturation of peptide refers to the conformational change of a peptide structure in order to adapt to changes in environmental conditions including temperature [133-135], pH [136], ionic strength of the solution [137], excipients [138], etc.

Aggregates are a serious challenge in the development of peptide drugs as they may form regardless of the manufacture method, due to numerous stresses (e.g., temperature fluctuations, light, shaking, surfaces, pH adjustments, etc.) during manufacturing, processing, and shipping. They may also appear upon storage as a result of denaturation (through thermal, pH, dielectric constant, and/or ionic strength changes) or modification (through oxidation, deamidation, and primary sequence changes) of the peptide.

Furthermore, peptide aggregation can occur upon exposure to air-liquid or liquid-solid interfaces (e.g., during mixing, filling and shipping, or reconstitution of lyophilized products), or contact with chromatography columns, pumps, pipes, vessels, filters, etc. [139]. Also, solution contact with ice during (adventitious or deliberate) freezing can cause aggregation [135].

Peptide aggregates may also be induced by foreign particles generated during manufacturing process (e.g., stainless steel and other particles from filling pumps; rubber particles from tubing; glass particles generated during heating of containers for depyrogenation), or from container-closure system (e.g., silicone oil used as lubricant in prefilled syringes and coating material in glass syringes and cartridges). Some of these factors (e.g., temperature, pH, light) can be easily controlled during manufacturing and storage, but control is more challenging for others (e.g. mechanical stress, exposure to different interfaces [139-142]).

Knowing that a high content of aggregates can be correlated with increased safety risks including immunogenicity, in order to prevent any differences in the immunogenicity of the two products, it becomes necessary to evaluate and compare the aggregate profiles between a proposed generic peptide drug product and its RLD throughout product shelf-life, as well as in accelerated/stress conditions. This comparison can be done by using a combination of

orthogonal analytical methods to cover a wide range of aggregates sizes, such as size exclusion chromatography (SEC), analytical ultra-centrifugation (AUC), dynamic light scattering (DLS), and field flow fractionation (FFF). Demonstrating the comparability in peptide aggregate profiles through these analytical means in terms of aggregates sizes and levels can sufficiently address the safety/immunogenicity concerns due to aggregation.

3.3.3. Excipients used in the formulation

Excipients can affect the safety of peptide drug products, as it was reported that peptide interactions with preservatives may lead to changes in the peptide structure and result in increased immunogenicity, which possibly causes allergic reaction and even anaphylactic shocks. For example, interaction with preservatives (such as phenylethyl alcohol, chlorobutanol, benzalkonium chloride and benzyl alcohol) was described in literature as responsible for conformational change and aggregation in small proteins [143], as well as increasing immunogenicity and a number of anaphylactic shocks in calcitonin salmon peptide products [144]. Also, it was previously observed that peptide interaction with Tween 80 (polysorbate) micelles and exposure of its epitope on the micellar surface induced antibodies [145]. Based on the above, it can be concluded that formulation of a peptide drug product can alter propensity of the peptide to aggregate, deaminate, oxidize, etc.; therefore, it can enhance or diminish its immunogenic potential. An appropriate design of the peptide product formulation is not only important with respect to stabilization against physical and chemical degradation, but also critical to minimize peptide-excipient interactions that may affect the product safety/immunogenicity.

747 Use of so-called preferentially excluded excipients such as sugars and polyols, certain amino
748 acids, and even polymers can shield the hydrophobic structure of the peptide and reduce
749 peptide-peptide interactions, therefore diminishing its aggregation potential [146].
750 The proper selection of the buffer system and concentration is also critical for peptide stability;
751 it is therefore recommended to test different buffer systems during peptide drug development
752 stage to detect the one in which the peptide shows lower propensity to aggregate.
753 Moreover, peptide-excipient interactions and the effect of excipients on the peptide stability
754 should be assessed in real time and forced degradation studies. In this regard, a special case is
755 the one of generic versions of injectable peptide drug products, for which formulation is often
756 qualitatively and quantitatively the same as the RLD. The qualitative and quantitative
757 formulation sameness of a proposed generic peptide product and its RLD ensures comparable
758 peptide stability against the chemical and physical degradation, and eliminates the safety
759 concern due to the use of a different excipient. In formulations that are qualitatively and
760 quantitatively the same as the RLD, the interactions peptide-excipients are expected to be the
761 same as the RLD. However, when qualitatively and quantitatively the same formulations of a
762 proposed peptide generic injectable and its RLD differ in preservative, buffer, and antioxidant
763 (as this is an allowed difference in excipients of generic injectable products) [147], the effect of
764 the new excipient on the peptide stability is to be evaluated, by comparative real time and/or
765 forced degradation stability studies against the RLD. The possible interactions between the
766 peptide and the new excipient can be studied using an array of techniques including surface
767 plasmon resonance (SPR), nuclear magnetic resonance (NMR), differential scanning calorimetry
768 (DSC), and/or bioassay.

3.3.4. Leachables from the container closure system

Interaction of the drug product formulation with the rubber components of the container closure system may produce extractables and leachables. The safety concern over leachables was generated when it was shown that organic compounds leached from syringe uncoated chlorobutyl rubber plunger in contact with erythropoietin formulations (Eprex) containing polysorbate 80, may have been a factor contributing to increased immunogenicity [148]. Leachables from the rubber components of the container closure system may have a direct effect on the peptide immunogenicity by acting as adjuvants for peptides crosslinking and antibody induction [148]. Leachables must be studied at stability according to ICH and FDA stability guidance for drug products [149, 150].

3.3.5. Peptide modification

Chemical modification of peptides (e.g., with PEG) may affect their safety/immunogenicity. The reduced immunogenicity of the PEGylated peptides appears to be caused by the mechanical “shielding” of the antigenic epitopes of the peptide by PEG [89], which also reduces its recognition by the immune system. Moreover, PEG increases the apparent size of a peptide, therefore reducing its renal filtration and modifying biodistribution. The magnitude of these effects is directly influenced by the number, molecular weight, structure and location of the PEG chains attached to the peptide, as well as chemistry used to attach them to the peptide. Most useful for peptide modification are linear mini PEG and monomethoxy PEG (mPEG), which after activation with a functional group can be coupled to the peptide via Lys, Cys, His, Arg, Asp, Glu, Ser, Thr, Tyr N-terminus or C-terminus. Contrary to the general beliefs, a series of studies

report that PEGylation can elicit production of anti-PEG antibodies [151-153]. To address these concerns, the antigenicity of PEGylated peptides should be carefully assessed.

4. Conclusions

This paper summarizes the different aspects of quality of peptide drug products that are taken into consideration during development and manufacturing, as well as during regulatory assessment of a peptide drug. As there is no “formal” policy in relation to peptides, manufacturers and regulators are using a risk based approach to determine the risk of peptide quality to safety (including immunogenicity) and efficacy. If a potential risk is identified, a thorough analysis of product and process related factors must be performed to ensure the safety and efficacy of the peptide drug. The portfolio of investigated factors and analytical techniques appropriate for a given peptide must be based on knowledge of the complexity of the peptide structure and understanding of its manufacturing process, and needs to be determined on a case-by-case basis.

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815 do not necessarily reflect the views and policies of FDA.

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