Protein and Peptide Biopharmaceuticals: An Overview

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Abstract: Bioactive proteins and peptides are recognised as novel therapeutic molecules with varying biological properties for potential medical applications. Development of protein and peptide-based therapeutic products for human use is growing steadily as they continue to receive an increasing rate of approval by the United States Food and Drugs Administration (US FDA). In this short review, we describe the current status and methodologies involved in the synthesis of protein and peptide biopharmaceuticals with an emphasis on the drivers and restraints to their exploitation in the therapeutic products sector.

Keywords: Bioactive peptides, bioactive proteins, therapeutics, biopharmaceuticals, bioprocessing.

INTRODUCTION

PROTEIN- AND PEPTIDE-BASED DRUGS

Since the production and successful entry of the first recombinant protein insulin into the market, several protein-based pharmaceutical products have been developed and approved for human use. The past three decades have seen a rise in regulatory approval and sales of protein and peptide therapeutics. For example, a synthetic nonapeptide called LupronTM (or leuprolide acetate, Abbott Laboratories) introduced for the possible treatment of central precocious puberty and advanced prostate cancer in children and in men respectively earned US\$ 2.3 billion in 2011 alone [1]. The year 2012 saw the highest approval of peptide drugs, and based on worldwide sales, seven out of the top ten therapeutics were protein-derived products and achieved a sale of around \$52.05 billion, a value which accounts for ~73% of total sales made by the top ten drugs [2]. In 2015, five out of the top fifteen drugs were protein/peptide-derived, and accounted for about 25% of total sales from those 15 drugs [3]. Protein-based drugs reached a global market value of \$174.7 billion in 2015 at a compound annual growth rate (CAGR) of 7.3%, and it is estimated to hit about \$248.7 billion in 2020 [4]. Protein-and peptide-based drugs have therefore been among the most widely sold therapeutic products in the past few years. Recent developments in biotechnology have

improved the production of several biomolecules for therapeutic and diagnostic applications. For instance, more than 130 protein-based therapeutics [5, 6] and about 140 peptide therapeutics [7] have either been granted approval for clinical use by the FDA or are under clinical evaluation, while over 500 are in pre-clinical development [8].

Current human therapeutics is categorized into two classes: small and large molecule on the basis of their molecular weights. However, the valuable contributions and huge success of biological proteins (hormones and monoclonal antibodies) continue to heighten the interest of pharmaceutical manufacturers [9, 10]. Reasons such as wide spectrum of action as well as high efficacy, safety, and selectivity to targets, among others, account for the increased acceptance of therapeutic proteins and peptides [11]. However, challenges such as poor *in vivo* stability of proteins and peptides, poor oral bioavailability and low membrane permeability continue to affect the full exploitation of this class of therapeutics. The present report gives an overview on the key production techniques as well as drivers and restraints to the exploitation of protein and peptide therapeutics.

PRODUCTION OF PEPTIDE BIOPHARMACEUTICAL

The intrinsic abilities of peptides to enhance or block signals in the human body have been harnessed to treat metabolic, cardiovascular, and neurodegenerative disorders. Central to this undertaking is the production aspects which must be done in a cost effective manner without compromis-

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ing the biological activity of the peptide [12]. This has stimulated research interest in both genetic expression, as well as enzymatic and chemical synthesis techniques for producing proteins and peptides to fulfil growing needs [1, 7, 13]. Traditionally, there are three major approaches for the production of therapeutic peptides (a) expression in host cells using recombinant technologies, (b) chemical or chemo-enzymatic synthesis, (c) enzymatic degradation or fermentation of proteins [14–16]. An appropriate technology is often selected based on factors such as physicochemical and biological properties of the desired peptides, scale of production, and cost.

Expression in Host Cells

Tools and techniques in biotechnology provide a significant platform for the development of heterologous hosts used in low cost production of proteins and peptides. The first recombinant human drug, insulin, was synthesised using recombinant *E. coli* and is marketed for the treatment of diabetes. Since the approval of insulin by the US FDA, many other prevailing peptide biopharmaceuticals have been approved or are in development utilising recombinant technology for production. For example, hepatitis vaccine, human serum albumin, desirudin, inflectra, ecallantide, virus-like particles and teriparatide are all produced by microbial expression systems.

The majority of the recombinant proteins produced by using mammalian (Chinese Hamster Ovary cells) and microbial expression systems (*Escherichia coli* and *Saccharomyces cerevisiae*) with more than 50% of biopharmaceuticals being produced by microbial factories [17–19]. Mammalian systems often require post-translational modifications for precise target interactions, whereas microbial expression systems are preferred for higher expression yields [20–24]. The use of *E. coli* as biological platforms for controlled polypeptides production by recombinant technology is a comparatively inexpensive method. The whole process can be carried out by using microbial cell factories through relatively simple techniques and instrumentations. Moreover, extracellular protein production enables ease of subsequent purification. Also, a few microbial cell species are recognized as eukaryal models. These are often amply studied and deposited in ‘-omic’ (genomics, proteomics and metabolomics) databases thus allowing high-throughput screening and significant engineering of these organisms to improve their host-expression properties [25–28]. Microbial cells expression systems also allow post-translational modifications and subunit fabrication to include cyclo-addition, formation of disulphide bond, signal peptide processing, proteolytic stability and glycosylation of proteins [19, 29–31]. Inability to incorporate unnatural amino acids is the major drawback of biological expression system but other techniques such as chemical conjugation methods can be used to overcome this challenge. Also, microbial systems require extensive management and quality control, and in some cases, downstream purification can be complex.

Chemical Synthesis

Since the invention of solid phase synthesis by Robert Bruce Merrifield in 1963, chemical synthesis has become

very popular for the production of a wide range of drugs including peptide drugs [32, 33]. The unnatural incorporated components approach has some limitations too: low purified yield of final product due to reduction in efficiency of coupling steps. In the 1980s, fragment condensation was widely used for peptide synthesis, although it presented some reaction difficulties and high rate of racemization. Since then, different chemical strategies including pseudo-peptides and peptidomimetics have been developed to overcome drawbacks to peptide drugs [34, 35]. Two major chemical synthesis approaches: solution phase peptide synthesis (SPS) and solid phase peptide synthesis (SPPS) [33] are predominantly used for peptide drugs synthesis.

Solution-Phase Peptide Synthesis (SPS)

In 1953, solution phase peptide synthesis was first introduced by du Vigneud for the synthesis of a peptide amide with the hormonal activity of oxytocin [33, 36]. For long chain peptides synthesis, short peptide fragments are synthesized and then coupled together by fragment condensation at commercial scale. The chief advantage of SPS is that inexpensive reagents and building blocks are used [37]. Moreover, the final desired product can be obtained in maximum purified form by introducing intermediate purification approaches [38, 39]. Nanopeptide neuromodulating hormones (oxytocin), gastric acid secretion stimulator in the stomach (porcine peptide) and calcitonin are some examples of solution phase synthesized biomolecules used in clinical practices [32, 36]. Overall, SPS is an inexpensive, high-throughput and relatively simple method. However, the need for intermediate reaction modification, extended synthetic reaction scheme, prolonged synthesis time and the laborious work of purification are its main limitations [33].

Solid Phase Peptide Synthesis (SPPS)

SPPS was introduced about ten year after the development of SPS through the pioneering work of Merrifield [40]. SPPS is carried out by anchoring the peptide being synthesized on a resin support. An amino acid with a protecting group on the reactive α amino group is attached to the resin at the C-terminal. [41]. The protecting group is removed; then the amino acid to be attached is activated at the C-terminal with a good leaving group and coupled to the resin bound amino acid. The successive cycles of deprotection, wash, and coupling are repeated until the desired peptide sequence is attained. Protecting groups such as tert-butyloxycarbonyl (Boc) and 9-fluorenylmethyloxycarbonyl (Fmoc) are often used for the protection of α side chain and eliminated by treatment with acid and base respectively [33, 42–44]. The number and types of resins that can be used in SPPS also continue to grow [40, 44, 45]. In recent time, microwave assisted SPPS has been developed to enhance the synthesis process for long chain peptides and to attain high yields. The major industrial scale limitations for SPPS are heavy instrumentations and expensive resins utilization [32, 45]. Exanatide, degarelix, pramlintide, and ziconotide peptide therapeutics were all synthesized by this method and have been approved by FDA [33, 46, 47].

Chemo-Enzymatic Methods

With chemo-enzymatic approach, synthesis of peptides is carried out by combined biological (enzymatic) and chemical

techniques. The protein or peptide is often produced by chemical synthesis or recombinant expression, and coupled other functional compounds by the use of enzymes. The most widely used enzymes include hydrolases with acylation properties and reverse esterification or amidolytic properties often in aqueous-free solvents or ionic liquids. Lipases and esterases are example of enzymes with acylation properties [48-50]. Enzymes are often used for their stereo- and regio-selective properties. Liraglutide is an example of a chemo-enzymatically produced therapeutic peptide which was approved in 2010 by FDA for treatment of Type 2 diabetes [47, 51, 52].

***In Vitro* Enzyme Hydrolysis and Microbial Fermentation of Proteins**

The health benefits of food protein hydrolysates have also been extensively reviewed [37, 53-59]. By *in vitro* hydrolysis with an appropriate enzyme, almost all food proteins can yield a host of bioactive peptides with varying physiological properties such as immunomodulatory, antihypertensive, hypocholesterolemic and antithrombotic activities. These bioactivities have huge applications in pharmaceutical product development [60-63]. Inhibition of angiotensin converting enzyme (ACE) is the most widely investigated bioactive property of peptide – an observation that is justifiable considering that blood-pressure related and cardiovascular diseases are among the top killer diseases worldwide [64, 65]. Calmodulin binding peptides, dipeptidyl peptidase IV inhibition peptides, incretin modulatory peptides and other peptides with activity against metabolic syndrome are also gaining the attention of researchers [59, 66, 67].

Fermentation of proteins (particularly dairy goods) by using starter and non-starter bacteria also constitute a viable and industrially feasible approach for the production of bioactive peptides [56]. This relies on making use of the fermentation profile and proteolytic mechanism of food-grade bacteria such as lactic acid bacteria [68-70]. *In vitro* hydrolysis of proteins and fermentation through various microorganisms has been shown to be an effective route for obtaining peptides with various structural and physiological properties [71]. Various disorders such as cancer, hypertension, certain auto-immune syndromes and cardiovascular disorders have also been controlled by peptides or hydrolysates derived from this approach [72].

PEPTIDES AS BIOPHARMACEUTICALS: DRIVERS AND RESTRAINS

Peptides as Biopharmaceuticals: Drivers

Therapeutic proteins and peptides provide several advantages, the foremost being the provision of efficacy, selectivity and specificity over small organic molecules [35, 73], resulting in an appreciation of response by the drug manufacturing sector and in the biopharmaceuticals market [1, 74, 75]. This acceptance and positive outlook is as a result of the unique biological and physicochemical characteristics of proteins and peptides. Some of the drivers for proteins and peptides as therapeutic agents are captured in Table 1 and discussed below.

Safety and Low Bioaccumulation

Protein and peptide therapeutics are relatively safe because, whether engineered or synthesized, their composition and metabolism are often analogous to those of endogenous proteins. Further, it is worth noting that there is a high degree of structural similarity between a bioactive protein and its physiologically active peptide fragment.

Proteins and peptides are not “foreign” to *in vivo* systems and are classed as safe for *in vivo* applications, having predictable metabolic profiles [7, 37]. The large numbers of natural proteins and peptides and their important role in human physiology has been well documented. Peptides perform certain physiological actions in the capacities of hormones, chemokines and cytokines, thereby serving as important signal transducers in living body systems. There is also a minimal systemic risk during the degradation of proteins and/or peptides into amino acids. Peptides also accumulate less in body tissues due to their short half-life [76]. Due to the above reasons, the risk of unforeseen side-effects is unanticipated with peptide drugs. In fact, it is reported that side effects encountered from peptide drugs are often due to dosage or local reactions at injection sites [74].

Increased Specificity to Targets

Therapeutic proteins and peptides also show a high bio-specificity to targets. This means minimal or absence of un-specific binding to undesired targets, and this leads to a high therapeutic index. For example, small molecular weight therapeutic proteins like monoclonal antibodies and hormones demonstrate specificity for binding to their target receptors even at picomolar concentrations [10, 77].

Diversity in Structure and Spectra of Activities

Protein and peptide drugs have shown potency in the treatment of a wide variety of medical conditions including cancers and tumours, metabolic, hormonal and immunological disorders, cardiovascular health, genetic disorders, infectious diseases, among others (see Figure 1). Additionally, some bioactive proteins and peptides, termed ‘multifunctional’ are able to exert more than one physiological activity [37, 58]. A case example is lactoferrin and its hydrolysate lactoferricin, produced in mammalian breast milk, which exerts over 17 different biological properties including antibacterial, antifungal, antiviral, antiparasitic, antitumour, anti-allergenic, anticancer, and immunomodulatory activities *in vivo* [78-80].

Peptides are also structurally diverse. Also, a combination of the 20 naturally occurring amino acids to form peptides gives an unlimited number of peptides (i.e., from dimers, trimers, tetramers, pentamers, and so on up to 20-mers). To form a 20-mer alone from the 20 naturally occurring amino acids will give $20^{20} = 1.05 \times 10^{26}$ possibilities! [7]. These present endless possibilities of therapeutic actions that are yet to be discovered and exploited.

Moderate Production Costs and Improved Synthetic Chemistry

The production of food-derived peptides with therapeutic properties is promoted on the grounds of its relatively low cost. These biologically active peptides can be produced

Figure 1. Target disease areas for FDA approved therapeutics peptides and proteins. Source [81] (Data is current as of 7 December 2016).

from cheap by-products such as skins, bones, cakes of food processing operations [37, 82, 83] while using proteolytic enzymes sourced from microbial organisms such as lactic acid bacteria [37, 84]. The use of bioinformatics tools has also been proposed as a means to further lower the cost of peptide bioprocessing by predicting peptide properties and rationally designing suitable purification strategies that will preserve the molecular integrity and activities of the peptides [12, 85, 86].

It is also worth mentioning that there have been several advances in synthetic chemistry such that several native or modified proteins and/or peptides can be synthesized with high purity and yield.

Peptides as Biopharmaceuticals: Restrains

Poor In Vivo Solubility and Stability

A number of weaknesses are observed with peptide-based drug candidates as opposed to small molecule chemical therapeutics [35, 87]. Generally, within plasma, peptides have low stability and poor solubility. Further, because the human body is equipped with about 569 proteases [88], peptides are easily degraded in the body, leading to short half-life and possible generation of degraded products which can interact with various targets and receptors, thus triggering undesirable immunogenic side effects [32, 89]. In some instances, peptides, such as antimicrobial peptides, are highly hydrophobic with relatively large hydrodynamic size and these two traits result in poor membrane permeability [90]. The poor plasma solubility also means peptides are cleared rapidly from the body.

However, a number of techniques have been reported to overcome the aforementioned challenges. Advances in synthesis and modification of therapeutic proteins and peptides have been shown to improve *in vivo* stability, minimize off target toxicity and enhance delivery efficiency to target organs [90-92]. For example, the conjugation of peptides and lipids has been shown to yield lipopeptide conjugates that have unique structural and biological properties. The conjugation offers the creation of novel therapeutic biomolecules that combines the biological properties of peptides and lipids

whiles yielding product with improved potency and selectivity. Lipidation of peptides also enhances bioavailability; increases the resistance of the peptide moiety against endopeptidase degradation; enhances plasma shelf life; and stimulate transport across cell membranes [93] Peptide lipidation has been suggested as a potent technique for converting peptides into drug leads [94].

PEGylation of proteins and peptides is another technique for improving peptide stability and half-life *in vivo* [95]. In one study, polyethylene glycol (PEG) was added to interferon to enhance the absorption time of PEG-interferon conjugate to reduce susceptibility to enzymatic action, renal clearance, and immunogenicity [35].

There is also the technique of hydrocarbon stapling, where a peptide is “braced” or cross-linked using a hydrocarbon [96, 97]. This relatively new technique improves the pharmacological performance and target affinity of peptides, stabilizes structure (particularly α -helices) and helps promote resistance and enhanced cellular penetration of peptides [97, 98]. The technology of hydrocarbon peptide stapling to stabilize α -helical structures is being explored by some therapeutic manufacturers including Aileron (www.aileronrx.com).

Administrative Route and Delivery Challenges

The poor *in vivo* stability of peptides creates another challenge to effective delivery by a route that would ensure access to target sites and insure against peptide degradation. Peptide drugs are by and large administered by parenteral routes with 75% administered through injection [7].

Orally delivered proteins and peptides are prone to degradation by enteric enzymes and this can result in fragments with reduced or no biological properties. Also, the resulting fragments can trigger allergenic responses during intestinal absorption and this constitutes a clinical challenge in using bioactive peptides across different human populations [99]. Contrary to this clinical observation, peptides are used as possible ‘cures’ for allergies through the so-called specific allergen immunotherapy (SIT) [100-103]. SIT involves administration of allergen extracts in increasing doses over a period of time to induce allergen tolerance by sufferers

Table 1. Proteins and peptide pharmaceuticals: drivers and restrains.

DRIVERS	Safety, potency and high selectivity to targets	
	Tolerable ADME-Tox profile with low bioaccumulation	
	Metabolic and structural similitude to body signal transducers such as hormones and cytokines	
	Increased probability of regulatory approval for protein and peptide drugs	
	Unlimitedness of peptides and diversity of biological activities	Potential for treating a plethora of diseases of differing pathologies
		Opportunity for discovery of new potent peptides
		Exploitation of multifunctional proteins and peptides
	Production techniques that overcome technical challenges at lower cost	Improved synthetic chemistry for the efficient synthesis of difficult sequences
		Moderate production costs, i.e. <i>in vitro</i> via the action of microbial proteases and food proteins
		Advances in peptide conjugation and modification techniques to overcome challenges of stability, solubility, specificity, and membrane permeability
RESTRAINS	Poor <i>in vivo</i> stability due to susceptibility to hydrolysis	Low bioavailability
		Short half-life
		Fast elimination from body systems
		Difficulty to administer orally
	Poor solubility	Challenges in delivery to targets
		Low membrane permeability
		Possibility of aggregation
	Possibilities of undesired immunogenic responses	

Adapted from [7, 16, 37, 74, 90].

[101]. However, the use of whole allergen extracts often results in some side effects [102, 103]. To overcome this challenge, some authors have proposed the use of short synthetic peptides that represent the immunodominant T-cell epitopes of the allergen [104]. This approach is gaining research attention in its ability to control both allergenic and some autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), arthritis, allergic rhinitis, and asthmas, in clinical studies [101, 103, 105].

Further, novel peptide administration strategies such as transdermal [106], intranasal [107, 108], transbuccal [109, 110] and even oral [111, 112] routes are also gaining attention. The recognition of alternative ways of administration could enhance the significance of peptide drugs in different disease treatments beyond conventional treatment strategies [7]. Other techniques include peptide encapsulation and/or formulation strategies with coordination polymers and other polymeric particles such as dendrimers, liposomes, and polyelectrolyte microspheres [37].

FUTURE OUTLOOK

Biopharmaceutical enterprises have made significant progress in treating several diseases. For reasons of safety, biospecificity to targets, and the wide spectra of biological potency, protein and peptides biopharmaceuticals are gaining acceptance against small molecule contenders. In the opening of the decade, peptide drugs were grappling with chal-

lenges of poor stability and difficult delivery/administration routes. However, with recent innovative bioconjugates techniques and strategies, most of these challenges are being met. Peptide modification through amino acid substitution, conjugation with fatty acids and polymers such as PEG, and hydrocarbon stapling are among the techniques useful in managing challenges relating to potency, solubility, toxicity, specificity, formulation and skin penetration/or membrane permeability. The coming decade is expected to see more protein and peptide-based drugs on the biopharmaceuticals market.

CONFLICT OF INTEREST

Authors declare no conflict of interest

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