Review

Building better drugs: developing and regulating engineered therapeutic proteins

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Most native proteins do not make optimal drugs and thus a second- and third-generation of therapeutic proteins, which have been engineered to improve product attributes or to enhance process characteristics, are rapidly becoming the norm. There has been unprecedented progress, during the past decade, in the development of platform technologies that further these ends. Although the advantages of engineered therapeutic proteins are considerable, the alterations can affect the safety and efficacy of the drugs. We discuss both the key technological innovations with respect to engineered therapeutic proteins and advancements in the underlying basic science. The latter would permit the design of science-based criteria for the prediction and assessment of potential risks and the development of appropriate risk management plans. This in turn holds promise for more predictable criteria for the licensure of a class of products that are extremely challenging to develop but represent an increasingly important component of modern medical practice.

Therapeutic proteins

Although a reliable count of functionally distinct proteins in humans is lacking, estimates suggest that the number runs to at least several tens of thousands [1]. Abnormalities in one or more of these proteins can manifest as disease conditions. However, these molecules can also be developed as therapeutics for replacement therapy, augmenting an existing pathway, providing a novel function or targeting small molecule drugs (see [2] for a classification of therapeutic proteins). Therapeutic proteins represented 17% of new drugs approved by the USA Food and Drug Administration (FDA) in 2005 but increased to 32% by 2011 [3]. The influence of these medications on the practice of modern medicine is, however, deeper than these numbers alone, because protein drugs often address unmet medical needs, provide cures or permit the management of complex diseases, and improve quality of life. For example, the introduction of Factors VIII and IX as replacement therapy for hemophilia has significantly extended the life expectancy of patients and, equally importantly, created a profound improvement in quality of life. In the absence of treatment, recurring bouts of bleeding and inflammation occur in the joints resulting in a loss of joint cartilage and bone destruction eventually leading to deformity and crippling arthritis [4]. Intracranial hemorrhage is a less common complication of untreated hemophilia but has even more severe consequences with death rates exceeding 20% and even survivors experience long-term consequences such as seizures and neurological impairment [5]. Historically, these products were purified from animal and human sources but have almost completely been superseded by proteins manufactured by recombinant DNA technology.

The recombinant proteins pipeline now includes a new generation of therapeutics that involves engineering the protein (i.e., altering the genetic sequence) to achieve desirable therapeutic outcomes or manufacturing efficiencies [6]. Technological innovations and scientific progress have also presented manufacturers with the opportunity to develop so-called 'bio-betters' which are also the result of altered sequences.

Proteins are engineered for numerous reasons but involve three principal strategies: (i) genetically or chemically linking the therapeutic protein to another protein or polymer; (ii) the introduction of one or several mutations or deletions in the primary sequence of the therapeutic protein; and (iii) modifying the genetic code of the recombinant protein for codon optimization. The first two of these involve a change in the primary structure of the protein whereas the third does not.

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Keywords: drug development; immunogenicity; drug safety; risk mitigation; quality by design.
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0165-6147/$ – see front matter.
Published by Elsevier Ltd. http://dx.doi.org/10.1016/j.tips.2013.08.005

There has been considerable progress in the engineering of therapeutic proteins during the past decade that has the potential for noteworthy improvements in product quality, therapeutic outcomes, and patient convenience. Consequently, engineered therapeutic proteins are rapidly becoming the norm rather than the exception as are more extensive alterations to the wild type sequence [7]. These changes present difficult regulatory decisions as to how much deviation from a wild type or parent molecule may be permitted in terms of protein engineering and the manipulation of the DNA sequence. Moreover, these decisions have to be made even as the technology and the underlying basic science are both evolving rapidly. In the past year alone the development of two bioengineered recombinant factor VIIa (rFVIIa) analogs, Vatreptacog alpha (http://www.novonordisk.com/include/asp/exe_news_attachment.asp?sAttachmentGUID=b7ff4a52-53ab-4a97-875c-156dedaf427a) and BAY86-6150 (http://www.investor.bayer.de/no_cache/en/news/investor-news/investor-news/show-NewsItem/1567) was discontinued in Phase III clinical trials due to safety issues. Similarly, peginesatide, a novel functional analog of erythropoietin, had to be recalled (http://www.fda.gov/Safety/Recalls/ucm340893.htm) less than a year after its March 2012 approval due to hypersensitivity reactions, including fatal anaphylaxis in some patients. These examples illustrate the risks associated with this class of drug products. In this review, our aim is to provide an overview of the emerging technologies that involve protein engineering and discuss these in the context of the underlying discoveries that are essential for science-based regulatory decisions. The review addresses these issues for all therapeutic proteins other than therapeutic antibodies, which constitute a separate class with distinctive properties.

Engineered proteins: therapeutics by design

Experience with first-generation recombinant therapeutic proteins suggests that most proteins do not make optimal drug products. Most therapeutic proteins are rapidly cleared from circulation and have a very short serum half-life and poor bioavailability [8]. They can elicit formation of antibodies with an inhibitory effect on drug availability and activity, and the production of some therapeutic proteins is very inefficient [9]. Finally, human proteins produced in heterologous expression systems such as *Escherichia coli* or yeast lack post-translational modifications which can sometimes lead to reduced activity or promote aggregation [6]. Protein engineering provides tools to address these issues. Moreover, as many of these technologies can be broadly applied to many different therapeutic proteins, they are developed as so-called platform technologies. Nonetheless, new platform technologies are not without drawbacks as will be discussed below.

Engineering proteins: the technologies

Fusion proteins

Protein fusion technologies exploit different properties of protein domains as 'modules' to generate new molecular entities with desirable properties. For example, the biological activity of one protein (or module) may be combined with the properties of another protein that extends the serum half-life. Most platform technologies that do this take advantage of mechanisms used by native proteins such as antibodies, serum albumin and transferrin, which persist in the serum for up to 25 days [10,11]. The Fc region of antibodies as well as human serum albumin are protected from degradation by pH-dependent recycling mediated by interaction with the neonatal Fc receptor, FcRn [12]. More recently, unstructured recombinant polypeptides called XTEN have been successfully used to generate fusion proteins with improved pharmacokinetic properties [13].

The proteins or polypeptides may be fused to the biologically active protein either by genetic engineering or by chemical crosslinking. The latter often involves the fusion of polymers other than proteins/polypeptides such as the covalent attachment of the polyethylene glycol (PEG) moiety (PEGylation) [14,15]. Moreover, platforms that rely on chemical crosslinking may also include genetic engineering of the therapeutic proteins to improve the efficiency of crosslinking or reduce the heterogeneity of the product. For instance, earlier attempts at chemical attachment of PEG moieties to proteins resulted in complex product mixtures which were difficult to characterize and monitor [16]. Subsequent advances addressed this problem by controlled PEGylation only at specific sites following site-directed mutagenesis [17]. Although this strategy reduces product heterogeneity, additional genetic modifications of the protein are required which can potentially trigger other unintended safety concerns (see below).

Table 1 lists fusion proteins (excluding monoclonal antibodies, antibody–drug conjugates, and fusion proteins with peptides and aptamers) in different stages of drug development. Table 1 has been classified on the basis of platform technologies used for bioengineering and then on the basis of the therapeutic area. As illustrated, engineered proteins have potential in many therapeutic areas. Also evident is the robust development of diverse technologies to modify properties of proteins to overcome difficulties in their clinical use or address different clinical needs.

As seen in Table 1, multiple technologies are in use to circumvent the same clinical predicament. Thus site-directed PEGylation, Fc fusion, Albumin fusion, XTEN fusion, etc., are all used to extend the biological half-life of therapeutic proteins. In some instances (such as the coagulation proteins Factor VIII and Factor IX), different manufacturers utilize separate platforms to address an identical unmet need in the same underlying product [18].

Protein engineering can also involve multiple alterations in therapeutic protein characteristics using more than one technology. A good example of such a product is rilonacept (Arcalyst®), which was approved by the FDA in 2008 for the treatment of cryopyrin-associated periodic syndromes. Rilonacept consists of the C terminus of the IL-1RAcP (interleukin-1 receptor accessory protein) ligand binding region fused to the N terminus of the IL-1RI (interleukin-1 receptor, type I) extracellular region which makes the protein a very potent (IC_{50} = 6.5 pM), high affinity (K_d = 1.5 pM) IL-1R antagonist. In addition, the protein is further engineered to include the Fc region of human IgG1, extending the half-life considerably [19] (Table 1).

Table 1. Bioengineered fusion proteins that have been approved or are under development[a]

Modified protein or drug	Generic name (brand name if applicable)	Effect of modification/general remarks
Fusion Fc proteins		
Immunosuppressive agents		
Extracellular domain TNFR/Fc-hIgG1	Etanercept (Enbrel®)	Half-life of 4.3 days.
Extracellular domain LFA3/Fc-hIgG1	Alefacept (Amevive®)	Half-life of 270 h. Administered once weekly.
Extracellular domain CTLA-4/Fc-hIgG	Abatacept (Orencia®)	Half-life of 13.1 days.
Extracellular domain CTLA-4/Fc-hIgG	Belatacept (Nulojix®)	Half-life of 8–10 days.
Extracellular domain IL-1R/Fc-hIgG	Rilonacept (Arcalyst®)	IL-1 trap. Half-life of 8.6 days.
Extracellular domain TACI/Fc-hIgG1	Atacicept	Administered once or twice weekly.
Antiangiogenic		
Extracellular domains VEGFR 1 + 2/Fc-hIgG	Aflibercept (Eylea®)	VEGF trap with a half-life of 1.7–7.4 days depending on dosage. Administered every 2 weeks.
Extracellular domains VEGFR 1 + 2/Fc-hIgG	KH902	NA
Angiopoietin 1 and 2 binding peptide/Fc-hIgG1	Trebananib (AMG386)	Half-life of 3.1–6.3 days depending on dosage. Administered weekly.
Thrombopoietin-stimulating factor		
Thrombopoietin binding peptide/Fc-hIgG1	Romiplostim (NPlate®)	Half-life of 3.5 days. Administered once weekly.
Coagulation factors		
Factor VIII–Fc-hIgG1	Factor VIII-Fc	Half-life of 15–16 h.
Factor IX/Fc-hIgG1	Factor IX-Fc	Half-life of ~50 h.
Enzyme replacement therapy		
TNSALP ectodomain and a terminal deca-aspartate motif/Fc-hIgG1	ENB-0040 (recombinant human TNSALP)	Half-life of 5 days.
Fusion albumin proteins		
Cytokines		
Interferon β/human albumin	Albuferon β	NA
Colony-stimulating factors (CSFs)		
G-CSF/human albumin	Neugranin	NA
Coagulation factors		
FVIIa/human albumin	rFVIIa-FP (CSL689)	Pharmacokinetic studies in rats demonstrated an extended half-life of 6- to 9-fold compared with wild type rFVIIa.
FIX/human albumin	rFIX-FP	Projected weekly to biweekly prophylaxis. FP extends half-life approximately 1.5–2-fold.
Hormones		
GLP-1 (7–36)/human albumin	Albiglutide	The product is fusion of two repeats of the human GLP-1 (7–36) with human albumin. The half-life of 4–7 days to allow once weekly injections.
Oncology		
Extracellular EphB4/human albumin	sEphB4-HSA	NA
Fusion XTEN proteins		
Coagulation factors		
FVIIa/XTEN	Patent	NA
FVIII/XTEN	Patent	NA
FIX/XTEN	Patent	NA
Hormones		
Glucagon/XTEN	Gcg-XTEN (AMX-808)	Projected daily to weekly dosing.
Exenatide/XTEN	E-XTEN (VRS-859)	Projected monthly dosing.
GLP2-2G/XTEN	AMX-256	Projected monthly dosing.
Ghrelin/XTEN	AMX-213	Projected monthly dosing.
C-peptide/XTEN	AMX-888	Projected monthly dosing.
hGH/XTEN	VRS-317	Projected monthly dosing.
Enzyme replacement therapy		
Alpha1antiTrypsin/XTEN	AMX-583	Projected daily to weekly dosing.
Fusion PolyXen proteins		
Cytokines		
Interferon α-2b/PolyXen (polysialylated interferon α-2b)	InferoXen®	NA
Colony-stimulating factors (CSFs)		
G-CSF/PolyXen (polysialylated G-CSF)	StimuXen™	NA
Erythropoiesis-stimulating agent (ESA)		
EPO/PolyXen (polysialylated EPO)	ErepoXen	Improved half-life.

Table 1 (*Continued*)

Modified protein or drug	Generic name (brand name if applicable)	Effect of modification/general remarks
Hormones		
Insulin/PolyXen (polysialylated insulin)	SuliXen®	Improved half-life.
Medusa hydrogel (poly L-glutamate backbone)		
Cytokines		
INF-XL		Improved half-life.
Hormones		
FT-105		Improved half-life.
***PEG*ylated proteins**		
Cytokines		
Interferon α-2a	*PEG*ylated interferon α-2a (Pegasys®)	The mean half-life is 160 h (range 84–353 h) compared with 5 h (range 3.7–8.5 h) for Roferon-A (recombinant interferon α-2a) that allows once weekly injections.
Interferon α-2b	*PEG*ylated interferon α-2b (Pegintron®, Sylatron®)	When compared with intron A (recombinant interferon α-2b) PegIntron (1 μg/kg) has approximately a 7-fold lower mean apparent clearance and a 5-fold greater mean half-life, permitting a reduced dosing frequency (generally injected once weekly).
Interferon β-1a	*PEG*ylated interferon β-1a	NA
Colony-stimulating factors (CSFs)		
Granulocyte colony-stimulating factor (G-CSF)	Pegfilgrastim (Neulasta®, Neulastim®)	Extends half-life to 15–80 h (injected once per chemotherapeutic cycle)
G-CSF	PEG-G-CSF/DA-3031	NA
G-CSF	PEG-G-CSF/MAXY-G34	NA
Erythropoiesis-stimulating agent (ESA)		
Erythropoietin (EPO)	Methoxy-PEG-epoetin beta (Mircera)	Allow for monthly administration. The half-life is 15–20 times longer than erythropoietin (134 h).
	Peginesatide (Omontys)	No amino acid sequence homology to erythropoietin. Extend half-life from once weekly injections to once monthly injections.
Coagulation factors		
FVIII	PEG-FVIII	Two versions are under development: full-length FVIII and B-domain-deleted FVIII.
FIX	PEG-FIX	NA
Thrombolytic therapy		
Staphylokinase	PEG-Sak	NA
Hormones		
Growth hormone mutant (B2036)	Pegvisomant (PEG-human growth hormone antagonist) (Somavert®)	*PEG*ylation decreases the antagonistic activity of B2036; however, the rate of clearance is greatly reduced compared with the un*PEG*ylated form with a mean half-life of approximately 6 days.
Growth hormone	PEG-hGH (ARX201)	Half-life of 4 days may potentially be suitable for weekly dosing, compared with current therapy that requires daily dosing.
Chemotherapy		
L-Asparaginase	Pegaspargase (Oncaspar®)	Sustained duration and prolonged effect compared with the native L-asparaginase. One dose of Oncaspar® can replace six to nine doses of D-asparaginase. *PEG*ylation enhances patient tolerability, providing a low rate of hypersensitivity reactions (2%). The plasma half-life is 3.2 ± 1.8 days and the compound is maintained in the body for 3 weeks.
Topoisomerase I-inhibitor	Etirinotecan pegol (NKTR-102)	Etirinotecan pegol achieved a 300-fold increase in tumor concentration as compared with a first-generation Topoisomerase I-inhibitor and a half-life greatly extending 50 days that ensures the drug remains in the circulation throughout the entire chemotherapy cycle.
Topoisomerase I-inhibitor	PEG-SN38 (10-hydroxy-7-ethyl-camptothecin) (EZN-2208)	The *PEG*ylated version allows parenteral delivery, increased solubility, higher exposure, more profound deoxyribonucleic acid (DNA) damage, inhibition of angiogenesis, and longer apparent half-life of SN38 as compared with Irinotecan (Camptosar).
Arginine deiminase (ADI)	ADI-PEG-20/PEG-arginine deiminase	NA
Arginase	PEG-arginase/BCT-100	NA
Glutaminase	PEG-glutaminase (PEG-PGA) plus 6-diazo-5-oxo-L-norleucine (DON)	NA
Human hyaluronidase (PH20)	PEGPH20	Dramatically increases the half-life of the compound in the blood and allows for intravenous administration.

Table 1 (*Continued*)

Modified protein or drug	Generic name (brand name if applicable)	Effect of modification/general remarks
Enzymes (for miscellaneous uses other than chemotherapy)		
Uricase	Pegloticase (KRYSTEXXA®)	In comparison to Rasburicase (recombinant urate oxidase), half-life is increased from approximately 8 h to 10–12 days, and immunogenicity of the foreign uricase protein is decreased. Allows injections every 2 to 4 weeks.
Adenosine deaminase (ADA)	PEG-bovine adenosine deaminase; pegademase bovine (ADAGEN®)	Extended half-life of 3 to more than 6 days.
Phenylalanine ammonia lyase (PAL)	rAvPAL-PEG	NA
Opioid antagonist		
Peripherally selective opioid antagonist	Naloxegol (NKTR-118)	Increased bioavailability and half-life as compared with Naloxol

[a]The list was compiled from publicly available sources. Information about products in development was obtained from research publications, reports of scientific meetings, and press notifications by individual companies. The list excludes monoclonal antibody based therapies and conjugates where the active moiety was a peptide or an aptamer. Abbreviations: NA, information not publicly available; TACI, transmembrane activator and calcium modulating ligand interactor; LFA, leukocyte function antigen; TNSALP, tissue-nonspecific isozyme of alkaline phosphatase; sEPHB4, soluble human Ephrin type-B receptor 4; VEGFR, vascular endothelial growth factor receptor.

The redundancy in the available platform technologies, the parallel development of multiple versions of the same therapeutic protein with improved clinical characteristics, and technical advancements in genetic engineering and protein purification all bode well for eventual success in transforming biologically important proteins into safe and effective drugs. Nonetheless, the rapid emergence of fundamentally very different technologies for protein modification can present both short- and long-term risks, which are difficult to predict or even to evaluate (see below).

Recombinant therapeutic proteins with a modified sequence

An expanding basic science knowledge base and the availability of relatively inexpensive technologies enable manufacturers to design and test multiple versions of the same protein product. Specific sequence changes inserted into the desired product are designed to address two main goals: one is to improve the production process for the manufacture of the protein and the other is to alter the characteristics of the protein to achieve specific clinical outcomes (Table 2).

Deletions, insertions, and point mutations in a wild type sequence are performed to increase the protein yield in a chosen host. For example, deletion of the B-domain of Factor VIII (Table 2), results in higher secretion of the protein into the media (and thus higher yield) as compared with the full-length form of Factor VIII [18].

Extensive data regarding protein structure, domains, and post-translation modifications have provided investigators with knowledge to change the protein sequence to alter pharmacokinetic properties as compared with the wild type protein or existing products. Insulin, one of the most studied proteins, is a classic example. Unlike most other areas of drug discovery, insulin analogs have been specifically designed using a systematic approach based on a detailed knowledge of biochemistry and the physiological actions of insulin. Analogs were developed to achieve a more physiological pattern of insulin replacement, thus promoting better glycemic control. Alterations in the amino acid sequence change both pharmacokinetics and pharmacodynamics when compared with subcutaneously injected regular insulin [20]. The rapid-acting insulin analogs (Table 2) have a fast onset and shorter duration of action because of changes to the B26–30 portion of insulin that inhibit formation of dimers and hexamers. The long-acting insulins were designed to achieve a protracted duration of action and a relatively smooth profile. Their use enables a tailored treatment to meet the specific needs of each patient.

Finally, site-directed mutagenesis can be used to leverage the cellular machinery to drive more extensive post-translational modifications. Glycosylation sequence motifs are introduced into a therapeutic protein [e.g., Aranesp (Darbepoetin alpha), an engineered erythropoietin] to exploit natural post-translational glycosylation. By targeting the number and location of these sites, the engineered protein has an increased negative charge and enhanced hydrodynamic size, which can reduce renal clearance, modulate receptor-mediated endocytosis, and mask proteolytic degradation sites [21].

Codon optimization

In 2004, a comparison of protein expression levels from wild type and codon-optimized genes, which included published reports up to that time, listed 41 examples [22]. There are now over 1500 published reports using optimized genes; PubMed searches show an exponential growth in the use of optimized genes since 2007 (Figure 1A). Although it is difficult to estimate how many optimized genes have entered the drug development pipeline, anecdotal evidence suggests widespread use of this technology [23]. In addition, codon optimized vectors are also being used during gene therapy [24].

There have been reports of spectacular increases, of up to 30-fold, in the expression of a specific recombinant protein following gene optimization [25]. However, the results are more modest when an algorithm is applied to a large set of proteins. Thus, for example, when 94 wild type and sequence optimized human genes were evaluated [26], although 70% of the optimized genes showed higher expression, the increase was in most cases less than 2-fold (Figure 1B). Moreover, wide variability in protein yields was observed, which is consistent with previous findings in smaller studies [22]. In recent years, a large number of

Table 2. Bioengineered proteins with modified sequences that have been approved or are under development[a]

Modified protein or drug	Generic name (brand name if applicable)	Purpose of modification
Cytokines		
Interleukin-2	Aldesleukin (Proleukin®)	Differs from native IL-2 in lack of glycosylation, the molecule has no N-terminal alanine, and a serine substituted for cysteine at amino acid position 125. Half-life is 85 min.
Interferon β-1b	Interferon β-1b (Betaseron, EXTAVIA®, Ziferon)	Substitutes serine for the cysteine residue found at position 17. It does not include the carbohydrate side chains found in the natural material. Mean half-life was 8 min to 4.3 h.
Interferon β-1b	Interferon β-1b (Betaferon®)	Contains serine instead of cysteine at position 17, lacks methionine at position 1, and lacks carbohydrate moieties. Levels remain above baseline throughout the 7-day (168-h) study period.
Colony stimulating factors (CSFs)		
G-CSF	Filgastrim (Neupogen)	Addition of an N-terminal methionine necessary for expression in *E. coli*. Because NEUPOGEN® is produced in *E. coli*, the product is non-glycosylated and thus differs from G-CSF isolated from a human cell.
Erythropoiesis stimulating agent (ESA)		
Erythropoietin (EPO)	Darbapoetin alpha (Aranesp)	Contains two additional N-linked oligosaccharide chains to extend half-life.
Coagulation factors		
rFVIII–B-domain-deleted	rFVII–B-domain-deleted (Refacto)	Multiple other drugs are under development; higher secretion of B-domain-deleted rFVIII versus rFVIII.
Hormones		
Basal insulins	Glargine (Lantus)	Glargine differs from human insulin in that the amino acid asparagine at position A21 is replaced by glycine and two arginines are added to the C terminus of the B-chain. The result is a relatively constant concentration/time profile over 24 h with no pronounced peak.
	Detemir (Levemir)	Detemir differs from human insulin in that the amino acid threonine in position B30 has been omitted, and a C14 fatty acid chain has been attached to the amino acid B29. Detemir has a relatively constant serum concentration over 24 h.
	Degludec (Tresiba)	Degludec differs from human insulin in that the amino acid threonine in position B30 has been omitted and a C16 fatty acid chain has been attached to the amino acid lysine B29 via a glutamic acid spacer. Thus, insulin degludec has an ultra-long half-life of approximately 40 h.
Short acting insulins	Lispro (Humalog®)	Lispro differs from human insulin in that the amino acid proline at position B28 is replaced by lysine and the lysine in position B29 is replaced by proline. Humalog is absorbed faster and has a shorter half-life as compared with regular insulin due to lack of hexamer formation.
	Aspart (Novorapid®, Novolog®)	Aspart differs from human insulin in that the amino acid proline at position B28 is replaced by aspartic acid. It has a faster absorption, onset of action, and shorter duration than regular human insulin.
	Glulysine (Apidra®)	Glulysine differs from human insulin in that the amino acid asparagine at position B3 is replaced by lysine and the lysine in position B29 is replaced by glutamic acid. It has a more rapid onset of action and shorter activity duration compared with regular human insulin.
	BIOD-123	Ultra-rapid acting recombinant human insulin formulation as compared to Humalog.
GLP-1 analogs	Liraglutide (Victoza)	Liraglutide is 97% homologous to native human GLP-1 and differs by substituting arginine for lysine at position 34 than attaching a C16 fatty acid (palmitic acid) with a glutamic acid spacer on a lysine residue at position 26. This allows a half-life of 13 h and once daily administration as compared with a half-life of less than 2 min of the human GLP-1.
Parathyroid hormone (PTH)	Teriparatide (Forteo)	Truncated PTH 1–34 amino acids instead of 1–84 amino acids identical to human PTH.
Leptin analog	N-Methionylleptin (Metreleptin)	NA

[a]The list was compiled from publicly available sources. Information about products in development was obtained from research publications, reports of scientific meetings, and press notifications by individual companies. The list excludes monoclonal antibody based therapies and conjugates where the active moiety was a peptide or an aptamer. Abbreviation: NA, information not publicly available.

publicly available algorithms for gene optimization have become available; Table 3 lists those for which active links are available. As these algorithms run the gamut from the straightforward use of natural codon biases to those that use multiple variables, a critical element of designing, evaluating, and improving gene optimization algorithms is to estimate the relative contribution of multiple variables either in the same algorithm or across algorithms.

Using some of the gene optimization algorithms depicted in Table 3, we calculated the RSCU score (see Figure 1 for calculation) of each codon of the wild type *F9* gene and optimized variants obtained using different algorithms (Figure 1C). The graph suggests that although many algorithms incorporate gene design variables in addition to codon usage, this parameter continues to be prominently reflected in the gene optimized product. The

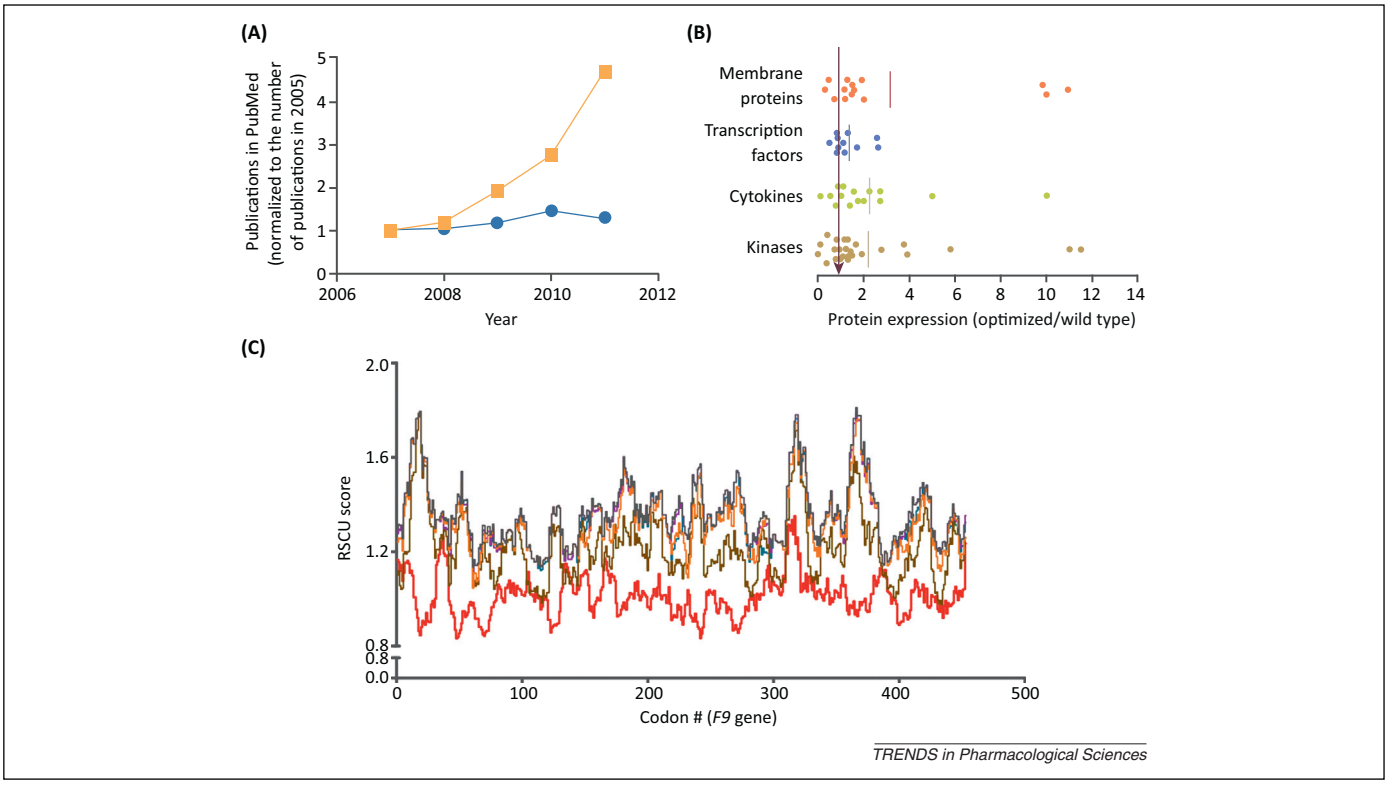

Figure 1. **(A)** Increase in total number of publications in PubMed (blue circles) compared with the increase in entries of unique publications that involve the use of codon optimized genes for protein expression (orange squares). Owing to the difference in the size of the datasets, both sets were normalized to 1 for 2007 to allow a comparison. Data presented cover the 5 years from 2007 to 2011. **(B)** Change in protein expression of different genes as a result of codon optimization. Proteins from different protein classes depicted on the *y*-axis were codon optimized using the same gene optimization algorithm. Each filled circle is the change in protein expression of a unique protein in that class [26]. The purple arrow represents no change in expression (ratio of optimized/wild type expression = 1). **(C)** The human *F9* gene was codon optimized using different gene optimization algorithms (see Table 3). The graph depicts the RSCU scores for each codon of the wild type (red line) and different codon optimized *F9* genes. The RSCU measures the preference of a gene or genome for a particular codon over synonymous alternatives. RSCU values were calculated for codons within the human genome, based on the observed imbalanced codon usage using the table available from the Kazusa Codon Usage Database (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=9606). For a codon which appears $N(c)$ times and is one of S synonymous representations for an amino acid which appears $N(a)$ times, $RSCU = S\,N(c)/N(a)$.

widespread use of codon usage, however, is not supported by experimental observations, which show poor correlation between the number of rare codons in a gene and protein expression [27]. Similarly, a systematic study [28] generated approximately 40 variants each of two genes where the codon adaptation index (CAI) ranged from <0.3 to >0.9. There was no correlation between the CAI and the expression of the two genes ($R^2 = 0.0013$ and 0.0029, respectively). Unfortunately, most reports in the literature use gene optimization as a tool, and hypothesis driven studies to validate these tools and/or side-by-side comparisons of algorithms with respect to experimental outcomes are rarely performed. Therefore, it is difficult to provide an unequivocal evaluation of the algorithms or the individual design parameters. However, a recent report that compared a wild type enzyme with two mutant forms, one composed entirely of frequent codons and the other composed entirely of rare codons, illustrates a significant potential pitfall. In this study, synonymous variants of a key enzyme coding gene in *Methylobacterium extorquens* were generated. Mutant gene expression, enzyme production, enzyme activity, and fitness were all significantly lower than wild type. What is particularly worrying (from the observation in Figure 1C that most gene optimizing algorithms appear to introduce frequent codons across the length of the gene) is that encoding the gene using only rare codons decreased fitness by 40%, whereas an allele coded entirely by frequent codons decreased fitness by more than 90% [29].

In addition to the effectiveness of this strategy in increasing the protein yield, there is now a critical mass of literature [30], which indicates that codon usage also regulates the speed of translation, correct protein folding, and the coordinated expression of functionally related proteins. Thus, although codon optimization does not alter the amino acid sequence of the resulting protein, this technology can also potentially result in safety issues (see below and Box 1 for a more detailed discussion).

Engineering therapeutic proteins: the pitfalls

Emerging combinatorial engineering technologies offer very efficient ways to identify protein variants with improved biological properties [8]. These technologies, however, rely much less on a knowledge base of structural, computational, or biochemical information about the ligand–receptor system, making it that much more difficult to identify potential risks for candidate drugs. Some of the concerns of this advanced technology are altered protein–protein interactions, aggregations, and immunogenicity.

For example, studies have demonstrated that bioengineered insulin analogs also exhibit an altered affinity to the insulin-like growth factor 1 receptor (IGF1R). Although

Table 3. Publicly available algorithms for gene optimization

Gene composer	http://www.genecomposer.net/
Gene designer	http://www.dna20.com/genedesigner2/
JCat	http://www.jcat.de/Start.jsp
Optimizer	http://genomes.urv.es/OPTIMIZER/
Visual gene developer	http://www.visualgenedeveloper.net/
Gene optimizer	http://secure.eurogentec.com/product/research-custom-genes.html
Codon optimizer	http://www.innovationsinmedicine.org/software/Tm-normalized-oligo-design/codopt/
Reverse Translate	http://bioinformatics.org/sms2/rev_trans.html
UpGene	http://www.vectorcore.pitt.edu/upgene/upgene.html
Genome compiler	http://www.genomecompiler.com/
TinkerCell	http://www.tinkercell.com/

there is insufficient clinical data at this juncture, the altered affinity for the IGF1R is a cause for concern [31]. This is because *in vitro* data and animal studies suggest that the altered affinities for IGF1R can make the insulin analogs more mitogenic. Given the life-long use of the insulin analogs and the large populations involved, this controversy surrounding a bioengineered product has direct public health ramifications. Regulatory agencies also recognize that protein engineering may affect subpopulations differently. Thus, the package insert for insulin glargine (Lantus) cautions against use in pregnant women and in children under the age of 6 years, because of lack of clinical data in these groups (http://products.sanofi.us/lantus/lantus.html).

Post-marketing clinical studies and recalls also illustrate the potential risks associated with engineered protein therapeutics. Two influential clinical studies reported early this year compared peginesatide (Omontys), a functional analog of erythropoiesis stimulating agents (ESAs), to first- and second-generation ESAs [32,33]. Peginesatide is a PEGylated synthetic peptide which mimics the structure of erythropoietin and promotes red cell development. Peginesatide was non-inferior to standard ESAs in increasing and maintaining target hemoglobin levels. Moreover, both groups had comparable cardiovascular composite safety end-points in patients receiving hemodialysis. However, in patients not receiving hemodialysis there was an increased incidence of sudden death, unstable angina and arrhythmia, and the rate of acute kidney failure and back pain was twice as high in patients receiving peginesatide. In February 2013 and unrelated to these studies, all lots of peginesatide were recalled from the market due to serious hypersensitivity reactions in some patients (http://www.fda.gov/Safety/Recalls/ucm340893.htm).

Even small changes in the amino acid sequence can have a considerable effect on the safety profile of a medication. A recombinant human coagulation Factor VIIa (NovoSeven) was approved by the FDA in 1999 and has had an excellent safety profile. Subsequently, two fast acting analogs of Factor VIIa, Vatreptacog alpha (Novo Nordisk) and BAY86-6150 (Bayer), were developed which differed from the parent molecule at only three and four amino acid positions, respectively. The development of both products was discontinued earlier this year due to the development of inhibitory antidrug antibodies that could potentially interact with the endogenous FVIIa in patients (http://www.novonordisk.com/include/asp/exe_news_attachment.asp?sAttachmentGUID=b7ff4a52-53ab-4a97-875c-156dedaf427a; http://www.investor.bayer.de/no_cache/en/news/investor-news/investor-news/showNewsItem/1567).

The examples described above involve alterations in the amino acid sequence of the therapeutic protein. However, recent studies show that even synonymous changes in the gene (as in codon optimizing strategies) can result in conformational changes in proteins with significant physiological effects [30]. There are many ways the sequence of a gene can influence protein expression (Box 1) and each of these parameters can have multiple interactions with the cellular machinery. Thus, a synonymous nucleotide change may be made for the purpose of altering local mRNA structure, but the change may also have an effect on global mRNA structure, the kinetics of translation, etc. Finally, evaluating individual variables becomes even more complicated because most gene optimization strategies involve several simultaneous substitutions. It has therefore been advocated [9] that gene optimization be treated as a multidimensional optimization problem. There is only one report of multivariate optimization used for gene optimization, but the approach appears to be promising [28]. In this study, 81 individual genes encoding two proteins were synthesized and expression levels estimated. The results contradict the concept that mimicking the codon bias of the host alone will ensure high protein expression. However, a good correlation ($R^2 = 0.77$) was obtained for the measured and predicted expression based on a model using partial least squares regression.

Although the systematic analysis of gene design parameters shows promise for the improvement of gene optimization, these strategies focus on protein yield. Such an endpoint would perhaps be adequate for the production of industrial enzymes. However, this is an insufficient criterion for successful drug development and licensure because a critical mass of scientific literature has challenged the dogma (for reviews see [30,34]) that all the information necessary for a protein to fold correctly is embedded in the amino acid sequence [35]. One response to these findings could be a lack of support for all codon optimization in the development, licensure, and regulation of recombinant therapeutic proteins. Alternatively, codon optimized therapeutic proteins could be evaluated on a case-by-case basis similar to any other bioengineered product that includes changes at the amino acid level.

Characterizing engineered proteins

The preceding sections suggest that although innovative protein engineering technologies can add considerable value to therapeutic proteins, these strategies are not free from accompanying risk and it is likely that efficacy and pharmacokinetics of the modified protein will also be altered. Thus, the scientific issues surrounding the licensure of a bioengineered product are similar to those of a novel unmodified therapeutic protein. In many of these instances, such as for example, immunogenicity, regulatory agencies

encourage a risk-based approach and drug manufacturers formulate and implement risk management plans [36].

Risk assessment for therapeutic proteins has two important components (Figure 2). The first is to determine what changes (if any) the proposed alterations are likely to have on the characteristics of the protein. The second is to determine whether the alterations in the protein characteristics have clinical consequences. The risk due to the proposed change is then a product of the severity of the clinical consequence and the probability that it will occur. The reliability of such an assessment depends on the underlying knowledge base for the particular protein being developed as a therapeutic and the platform technology used to modify it. Equally important are analytical tools that can measure outcomes at different stages of the product life cycle.

To illustrate this paradigm, we consider six relatively common and potentially adverse consequences of bioengineering a protein therapeutic: changes in the tertiary structure, an increase in microheterogeneity, increased immunogenicity, changes in PK/PD (pharmacokinetics/pharmacodynamics), decreased bioactivity, and aggregation (Figure 3A). Similarly, bioengineered products can broadly be divided into those that involve the insertion of one or several mutations or deletions in the protein, those that are genetically or chemically linked to another

Box 1. Codon usage affects both efficiency and fidelity of protein translation

Codon usage and tRNA abundance control both the amount of protein (efficiency of translation) as well as the quality (fidelity of translation) of protein synthesized. Thus, tRNA content and codon usage bias generally correlate (Figure IA) for highly expressed genes. The tRNA codon usage bias can be exploited to increase protein yield and is an important component of codon optimization strategies (Figure IC). Although the roles of codon usage biases within and across genomes are still poorly understood, a critical mass of evidence suggests that codon usage regulates translation fidelity. The genomes of higher organisms have evolved to contain not only higher tRNA copy numbers but also exhibit a greater range of copy numbers (Figure IB). In addition, enzymes, such as t-RNA-dependent adenosine deaminase (ADAT), can modify base 34 of the anticodon and increase the decoding capacity of an individual codon (Figure IC). The role of synonymous mutations in determining mRNA structure and stability is an important emerging area of study [49,50]. A landmark study of 154 genes of green fluorescent protein with random changes at synonymous sites showed that although more stable mRNA structures can result in higher protein expression, synonymous mutations that selectively make the local structure in the region of the start codon more stable [51] result in lower expression levels (Figure ID). This is probably because the less stable local structure can facilitate translation initiation. Similarly, it is becoming increasingly evident that codon bias [34] affects the rate of translation [52] and that mRNA mediated control of the kinetics of protein translation may be necessary to balance translation efficiency with fidelity [53–56]. New approaches, novel technologies, and genomic data permit the elucidation of genome wide trends to successfully model plausible scenarios for control of protein folding mediated by synonymous codons, that is, 'rules' that have wide applicability. Genome wide analyses show that codon usage enforces translation fidelity and that specific mechanisms seem to regulate protein folding mediated by synonymous codons. For example, a consistent feature of mRNA transcripts is that a cluster of rare codons is overrepresented in the first 30–50 codons [57] (Figure IE). These so-called ramps are ubiquitous and slow translation elongation, thus spacing the ribosomes on the mRNA which in turn prevents ribosome congestion, stalling and misfolded or truncated proteins [58]. The slow ramp in the early part of the gene is often followed by rapid translation. One pattern that accomplishes this is the use of the same codon when the same amino acid recurs in a sequence [59] as the reuse of a tRNA confers an advantage [60,61] (Figure IF).

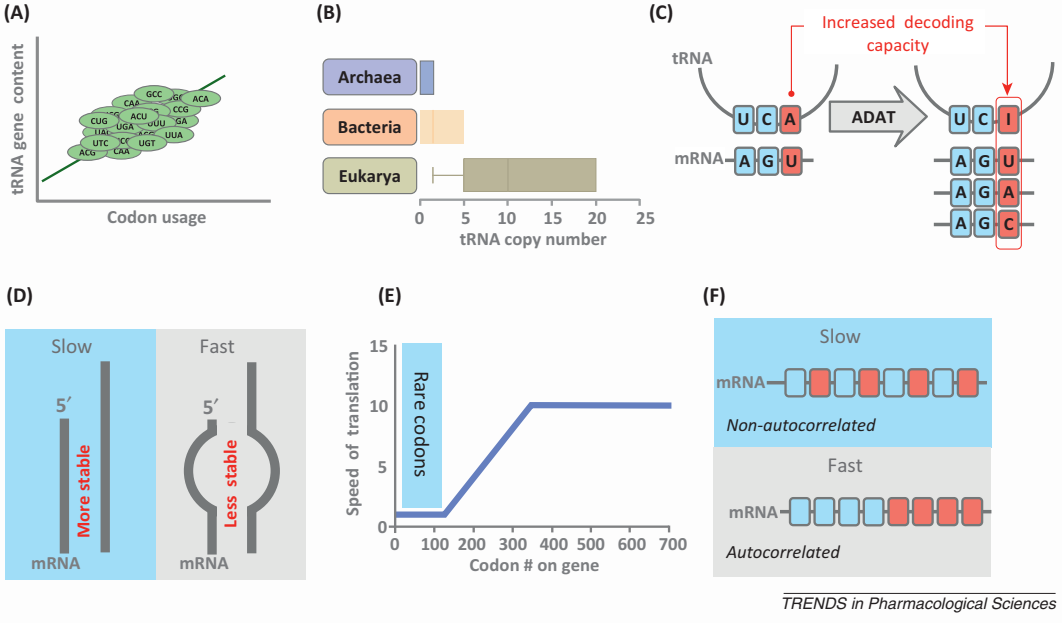

Figure I. **(A)** Codon usage bias and tRNA content generally correlate for highly expressed genes. **(B)** The genomes of higher organisms contain higher tRNA copy numbers and also exhibit a greater range of copy numbers. **(C)** Enzymes, such as t-RNA dependent adenosine deaminase (ADAT) can modify base 3 of the anticodon and increase the decoding capacity of an individual codon. **(D)** Synonymous mutations that selectively make the local structure in the region of the start codon more stable result in lower expression levels. **(E)** A consistent feature of mRNA transcripts is that a cluster of rare codons is overrepresented in the first 30–50 codons and constitute so-called ramps which slow translation elongation. **(F)** The occurrence of the same codon when the same amino acid recurs in a sequence permits reuse of the tRNA, which can increase the speed of translation.

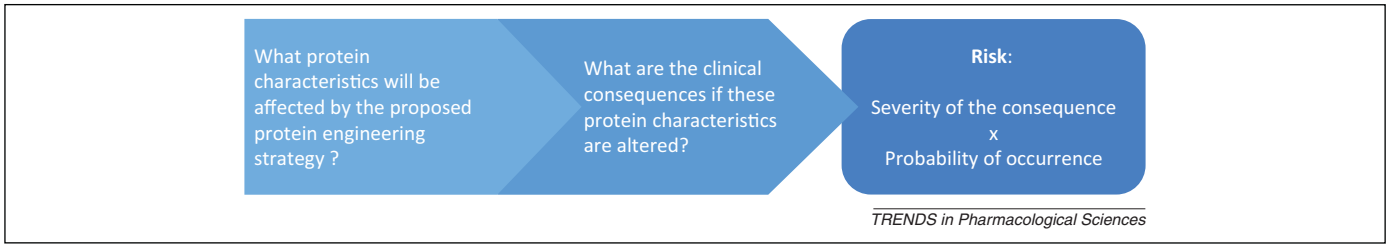

Figure 2. Schematic showing a risk assessment strategy for engineered therapeutic proteins.

protein or polymer and those whose genetic code is altered to achieve codon optimization. On the basis of the nature of the alteration, it is possible to assign a plausible risk for affecting different characteristics of that particular protein therapeutic. As a case in point, an engineered protein with a single point mutation is unlikely to result in a major conformational change, whereas there is moderate to high probability that a fusion protein will have a different conformation from the parent molecule. Likewise, there is a strong likelihood that a molecule with extensive codon optimization will exhibit increased microheterogeneity due to the demands on cellular machinery but the protein will not show increased T cell mediated immunogenicity because the primary amino acid sequence is unaltered.

In Figure 3A, we posit the likelihood of affecting each of the six different protein characteristics for three different protein engineering platforms. The ability to assess the risk associated with a particular modification for an individual protein therapeutic will depend on many factors including manufacturing experience with the unmodified protein, other products using the same platform technology that have been used in the clinic, and the published scientific literature. Although risk assessment provides guidance as to which product characteristics are more likely to be affected by a particular change, it does not replace product characterization. In Figure 3B, we illustrate the current state of the art with respect to assessing the product characteristics shown in Figure 3A using analytical tools, preclinical animal studies, and clinical studies.

Charts such as those illustrated in Figures 2 and 3 can be useful in risk assessment, product characterization, and risk mitigation strategies. It is advantageous to detect alterations in protein characteristics that could potentially affect the safety or efficacy of the product early in product development, but this is not always possible. For example, although there is a moderate to high likelihood of a conformational change for all protein engineering platforms, a significant gap remains in monitoring the tertiary and quaternary structures of proteins during drug development and manufacture [37]. Furthermore, the availability of robust and reliable tools to measure a protein characteristic is of limited utility unless a clinical correlation can be established. There is, for instance, a choice of sensitive and reliable *in vitro* and *ex vivo* assays and animal models for determining the immunogenicity of therapeutic proteins, but these correlate very poorly with clinical outcomes (http://www.fda.gov/downloads/Drugs/. . ./Guidances/UCM192750.pdf).

Just as there are many ways the sequence of a gene can influence protein expression and conformation, there are numerous ways in which an alteration in the protein conformation can affect the safety and efficacy of the product. Therefore, the choice of the protein quality attributes measured as well as the framework of the risk–benefit analyses become important. A reduction in bioactivity of a modified therapeutic protein has been deemed acceptable in some cases if offset by an accompanying increase in serum half-life [38,39]. However, the use of bioactivity alone as a defining marker may be too simplistic as it does not take into account additional risks associated with the modification. For example, a truncated and PEGylated form of human megakaryocyte growth and development factor (MGDF-PEG) increased platelet counts in patients with idiopathic thrombocytopenic purpura (ITP) in an initial clinical study. Nonetheless, the clinical development had to be halted because MGDF-PEG elicited an antibody response in some patients that neutralized MGDF-PEG and the endogenous thrombopoietin (TPO) leading to severe and persistent thrombocytopenia in normal individuals [40]. Similarly, PEG is a non-biodegradable polymer and can induce renal tubular vacuolation in animals, although with unaltered clinical pathology [41].

Assessing engineered proteins using design of experiments principles

Therapeutic proteins are inherently complex molecular entities that exhibit considerable microheterogeneity. The manufacture of a therapeutic protein is far more complicated than that of a small molecule drug, for example, a typical small molecule drug has approximately 100 critical process steps, whereas a protein therapeutic has more than 5000 [42]. Although even subtle alterations in the sequence of a gene can influence protein expression and conformation and thereby the safety and efficacy of the product, a conformational change in a protein does not necessarily affect the safety and/or efficacy of a protein. Thus, even if one has the tools to measure all these changes in product attributes, the challenge is still to separate those that correlate to clinical outcomes and those that do not. The problem is analogous to determining the critical quality attributes during process engineering. In a univariate approach, experiments are performed to determine the relationship between a factor and the desired response. However, in the real world, there may be several factors affecting a certain response or a combination of responses. Therefore, a structured experimental design for multivariate analysis is helpful. Design of experiment (DoE) strategies have been used with varying degrees of success in the implementation of quality-by-design (QbD). Central to a successful QbD program is an understanding of the relationship between the process and the critical

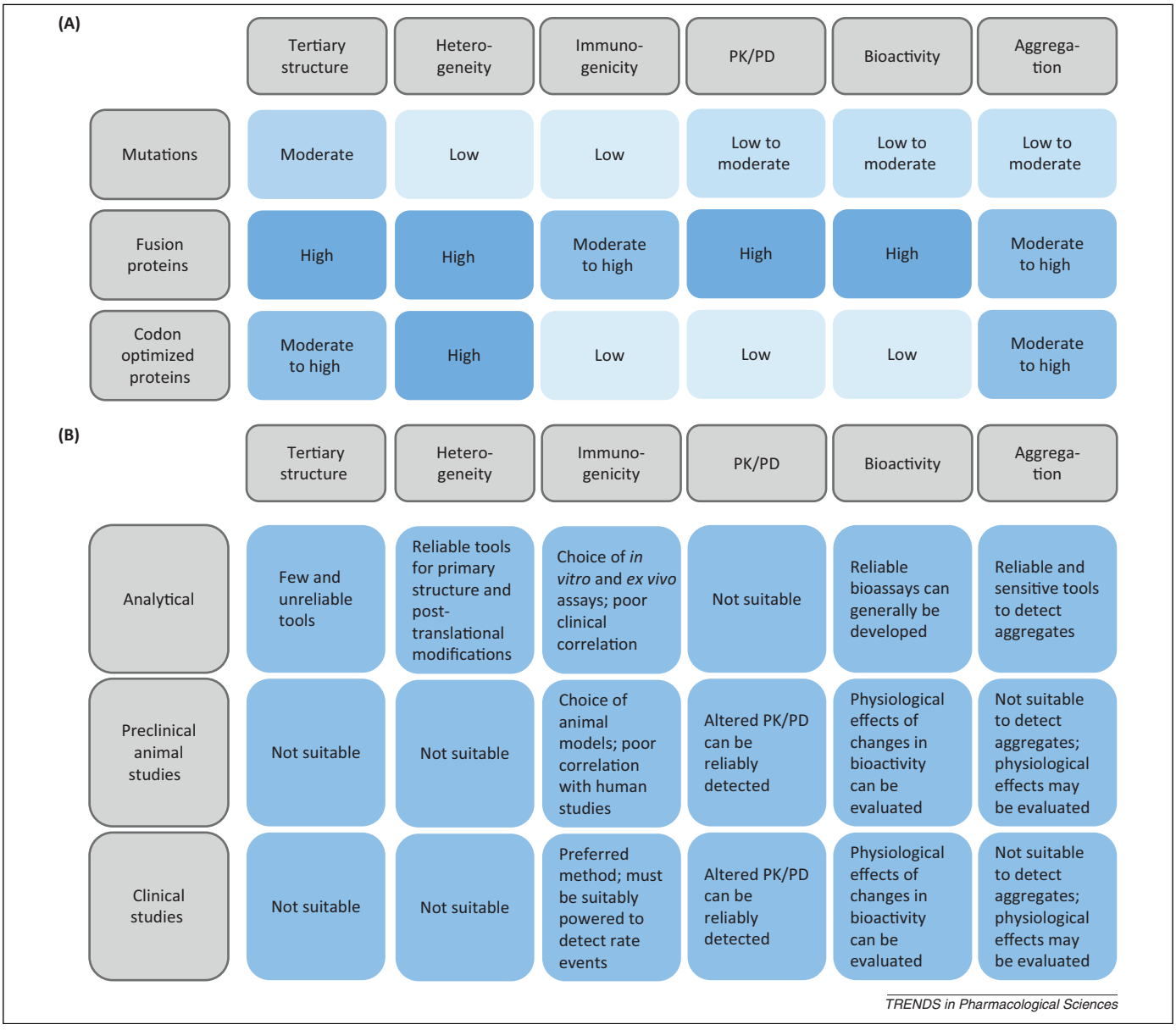

Figure 3. **(A)** Risk assessment of three principal protein engineering strategies, namely mutations, fusion proteins, and codon optimization, with respect to six attributes of therapeutic proteins that can affect safety and/or efficacy. The assessment would be applicable to therapeutic proteins other than therapeutic antibodies which are synthesized in mammalian cells. **(B)** Chart illustrating the current state of the art with respect to monitoring the product attributes described in (A) using analytical, preclinical, and clinical strategies.

quality attributes (CQAs) of a product, as well as the association between CQAs and the clinical properties of a product [43].

The application of DoE principles for assessing engineered biological systems *per se* (as opposed to the process engineering) is in its infancy with few examples [43,44]. Nonetheless, the underlying mathematics of multivariate optimization has a long history, is well established, and has been used successfully to find solutions to wide ranging problems that include Sir James Lind's classic study to find a cure for scurvy [45], predicting the movie preferences of Netflix® customers (http://www.stat.osu.edu/~dmsl/GrandPrize2009_BPC_BellKor.pdf), QSAR small molecule drug design [46], and QbD [43]. These multivariate optimization tools provide a framework for experimentation in which several variables and variable combinations can be simultaneously tested for sequence–function relationships. The success of this approach for any individual therapeutic protein will depend on the identification of relevant variables, the technology available to quantify these variables, and, most importantly, determination of the relative clinical effects of the individual variables. The pace of implementation of DoE approaches may initially be slow due to the complexity of these products and limited clinical experience. This is comparable to the experience with introducing DoE principles in the context of manufacturing processes for biotechnology based products, which regulatory agencies strongly encourage (see [42]), where there is now considerable progress. This experience has also resulted in development of risk analysis models, screening and optimization designs, for use in instances where one has limited information, and these concepts are

Box 2. Protein engineering strategies and adverse effects

In a draft guidance for immunogenicity testing of therapeutic proteins (http://www.fda.gov/downloads/Drugs/. . ./Guidances/UCM192750.pdf), the FDA recommends that for fusion proteins, multiple assays should be developed to measure immune responses to both domains of the fusion molecule as well as to the neoantigen formed at the junction of the components. These so-called neoantigens are synthetic peptide sequences that serve as junctions, linkers, etc., during protein engineering. Such sequences do not exist in nature and are thus potentially immunogenic 'foreign' antigens. Neoantigens are generated not only during the design of fusion proteins but also when a protein is truncated or otherwise modified. For example, the human *F8* gene encodes a Factor VIII protein of 2351 amino acid residues with a domain structure of signal peptide A1-A2-B-A3-C1-C2. Experimental and clinical evidence suggest that the 908 amino acid B domain is not required for function [62]. BDD forms of FVIII have been deemed advantageous for development as drug products because of higher expression levels and greater product homogeneity [63]. Not all BDD molecules are identical; at least two constructs of the BDD–FVIII have been reported [62,64] and these have slightly different linker regions. Of these, one is a marketed drug FVIII product whereas the other is in development. The heat maps depicted in Figure I illustrate computational estimations of the binding of peptides from the two neoantigens generated in the two BDD–FVIII molecules to common MHC-Class II alleles using the predictive tool NetMHCIIpan version 2.0 [65]. Note that the color bar shows only the high affinity range ($K_d \leq 50$ nM). The peptide–MHC-II binding is a necessary condition for eliciting anti-FVIII antibodies and the binding affinity can be a marker for immunogenicity [47,48]. The two heat maps show that the neoantigens from product A bind the protein products of more MHC-II alleles than product B. More important is the observation that neoantigens from both products A and B bind only to a few MHC-II proteins. This suggests that the potential risk of these products is not the same for all patients but is dependent on the MHC-II repertoire of the individual patient. The lower left panel shows the prevalence of the MHC-II alleles shown in the heat maps in the US population (the alleles that form high affinity peptide–MHC-II complexes with products A and B are shown in blue and tan, respectively). The lower right panel shows immunogenicity scores for all overlapping 15-mer peptides comprising the neoantigens in products A and B. The central (position 8) amino acid for each peptide is depicted above the graph. The immunogenicity score is the (normalized) sum of prevalence percentages over DRB1* alleles that bind a given peptide with high affinity (≤50 nM). This plot suggests that neoantigens from product A are more likely to bind to MHC-II alleles than neoantigens from product B. However, it is important to clarify that although peptide–MHC-II binding is a necessary condition for eliciting an immune reaction, it is not a sufficient condition. It is also noteworthy that in small clinical trials (which are common for rare diseases such as hemophilia A) sufficient patients with individual MHC-II alleles to statistically test the hypothesis that the neoantigen elicits the immune response may not always be recruited. This is one reason that a decade after a BDD–Factor VIII therapeutic protein was introduced the relative immunogenicity of full-length and BDD–Factor VIII therapeutic proteins remains controversial [66].

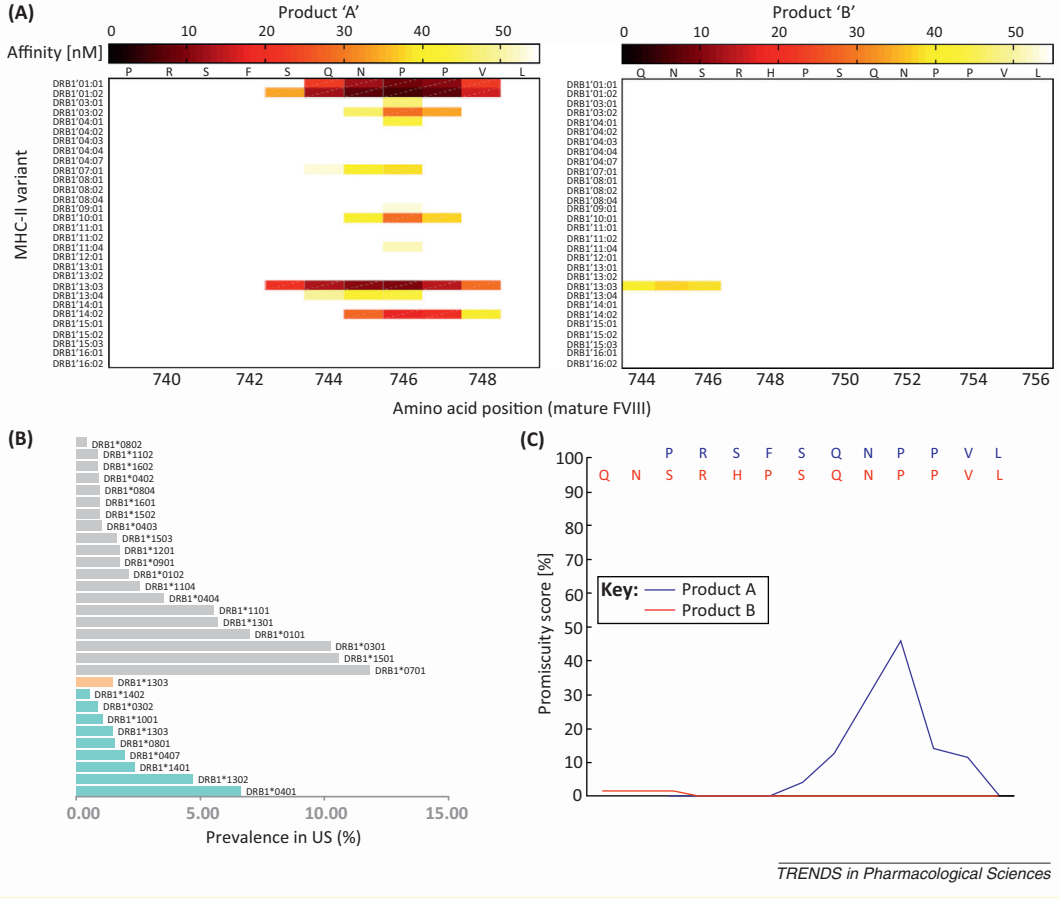

Figure I. **(A)** Heat maps depicting the predicted peptide–MHC-II binding affinities. The peptides represent neoepitopes spanning the synthetic junction (*x*-axis) of two β-domain-deleted (BDD) products (product 'A' and product 'B'). We examined a 32-residue segment of this BDD–rFVIII protein that included 16 amino acids on each side of the synthetic junction. Percentile ranks were determined for the binding of 18 overlapping 15-mer peptides covering the synthetic BDD junction to each of 30 MHC-II molecules using the consensus method (http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html). A lower percentile rank indicates higher binding affinity. **(B)** The graph depicts the prevalence, in the US population, of MHC-II variants that bind to neoepitopes from product A (cyan bars) and product B (tan bar). **(C)** Promiscuity scores for overlapping neoepitopes from product A (blue) and product B (red). The score represents the fraction of DRB1 MHC-II molecules that each peptide binds to (see [67] for details).

clarified in the International Conference on Harmonization (ICH) Q8 and ICH Q9 documents. Thus, the application of these strategies to the design of the molecular entity *per se* (as opposed to the manufacturing process) will also become easier with the rapid technological progress in the development of robust analytical tests, an expanding base of scientific and clinical data for specific products, and platform technologies. Furthermore, the knowledgebase will also expand as more clinical experience is gained with engineered therapeutic proteins and with the reports of post-marketing monitoring of these products.

Pharmacogenetic considerations in engineering therapeutic proteins

When engineering a therapeutic protein involves a change in the primary sequence it is possible that the modified protein will affect different individuals or populations differently. Of particular concern are rare adverse events which may be difficult to detect even in clinical trials. The most obvious consequence of introducing novel sequences (which do not exist in nature) into a protein is immunogenicity. The presentation of 'foreign' sequences by Class II Major histocompatibility complex (MHC-II) proteins is a necessary condition for initiating an immune response against therapeutic proteins. MHC-II proteins are among the most polymorphic in the human genome and, consequently, artificial sequences introduced into engineered proteins would bind to the MHC-II repertoire of individual patients with varying affinities. Several reports in the literature suggest that the stability of a peptide–MHC-II complex may be a marker for the subsequent immunological reaction [47,48]. In Box 2, we exemplify these concepts using a β-domain deleted (BDD)–FVIII therapeutic protein which is obtained by engineering the wild type protein and involves the introduction of a novel junction. It should be emphasized that the relative immunogenicity of full-length and β-domain-deleted–FVIII products remains controversial, and the increased risk of β-domain-deleted products to any class of patients has not been demonstrated. Nonetheless, computational tools and *in vitro* and *ex vivo* assays provide information that can be useful while assessing the potential risks associated with an engineered protein therapeutic. This in turn can be used in designing and analyzing clinical trials.

Concluding remarks

The drug development pipeline shows that engineered therapeutic proteins are rapidly emerging as a critical component of the overall healthcare industry and have the potential to improve and expand therapeutic options in many disease areas. However, the primary function of regulatory agencies is to ensure that patient safety is not compromised. Given the complexity of therapeutic proteins and the rapid adoption of sophisticated new technological platforms, the regulation of these products is challenging. There are, for example, significant lacunae in analytical techniques to comprehensively characterize therapeutic proteins.

Moreover, given the complex interactions of multiple variables, the development of more sensitive and accurate analytical tools alone may not be sufficient to make clinical predications and thus it may be crucial to understand the mechanism of action of therapeutic proteins [37]. A case in point is the €34.9 million ABIRISK (Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK) project (http://www.abirisk.eu/) funded by the Innovative Medicines Initiative (IMI), a public–private partnership between the EU and European Federation of Pharmaceutical Industries and Associations. One of the major goals of this concerted effort is to assemble diverse databases into a single immunogenicity databank to generate analytical and computational tools that can accurately predict clinical manifestations of immunogenicity. Large initiatives such as these, accumulating clinical data, and experience with specific therapeutic proteins and advances in detailed understanding of disease mechanisms and adverse events are all prerequisite to the application of mathematical tools such as DoE for a more formal evaluation of engineered therapeutic proteins. In the short to medium term however, these products will need to be evaluated on a case-by-case basis to identify risks and measure changes in physicochemical characteristics associated with the risks. It will also not be possible to determine up front how much deviation from the parent molecule may be permitted in terms of protein engineering and manipulation of the DNA sequence. In fact, the scientific literature discussed in this perspective suggests that all genetic changes must be considered potentially risky.

Given our current limited ability to correlate changes in protein structure and function from analytical tests to clinical outcomes, clinical data will continue to play a critical role in evaluating engineered therapeutic proteins. Even clinical trials may not always provide definitive answers, particularly for products used in the long-term management of chronic diseases and those used to treat rare diseases. In the absence of a critical mass of scientific and clinical knowledge with respect to the platform technologies used to bioengineer protein therapeutics it may be prudent for drug manufacturers to conduct Phase IV post-marketing surveillance (PMS) studies. Registries maintained by the drug manufacturers, patient advocacy groups, or agencies such as the Centers for Disease Control are another option for the surveillance of spontaneously reported adverse events. Similarly, it may be desirable to biobank patient samples during Phase III studies as new scientific knowledge, assays, etc., often emerge through investigator-initiated research after a drug is commercially available.

Although the development and regulation of engineered therapeutic proteins will continue to be a challenge, these molecular entities represent a critical component of the overall healthcare industry. The rapid progress of many different scientific disciplines holds promise for more predictable criteria for the licensure of these products and, eventually, less burdensome regulatory requirements.

Acknowledgments

We thank Dr Jay Epstein and Dr Basil Golding for helpful discussions. We thank Mr Ofer Kimchi, Mr Andrew Wu, and Ms Yini Qi for editorial assistance. This work was supported by funds from the Laboratory of Hemostasis, Center for Biologics Evaluation and Research (C.K-S. and Z.E.S.) and the Food and Drug Administration's Modernization of Science program (Z.E.S.).

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