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Synthetic Erythropoiesis Protein

Bringing the Science of Proteins into the Realm of Organic Chemistry: Total Chemical Synthesis of SEP (Synthetic Erythropoiesis Protein)

*Stephen B. H. Kent**

chemical protein synthesis · EPO · glycoprotein mimetics · peptide-thioesters · protein design

Erythropoietin, commonly known as EPO, is a glycoprotein hormone that stimulates the production of red blood cells. Recombinant EPO has been described as "arguably the most successful drug spawned by the revolution in recombinant DNA technology". Recently, the EPO glycoprotein molecule has re-emerged as a major target of synthetic organic chemistry. In this article I will give an account of an important body of earlier work on the chemical synthesis of a designed EPO analogue that had full biological activity and improved pharmacokinetic properties. The design and synthesis of this "synthetic erythropoiesis protein" was ahead of its time, but has gained new relevance in recent months. Here I will document the story of one of the major accomplishments of synthetic chemistry in a more complete way than is possible in the primary literature, and put the work in its contemporaneous context.

1. EPO

1.1. Discovery, Isolation, and Molecular Structure

The existence of a biological factor that promoted the production of red blood cells was first suggested in 1906,[1] and confirmed by subsequent experiments. Erythropoietin itself was first isolated from human urine and prepared in pure form by Eugene Goldwasser and colleagues at the University of Chicago in 1977 as the culmination of a fifteen-year program.[2,3] Human urinary EPO was shown to be a glycoprotein that is made up of a 166-residue polypeptide chain containing four Cys residues that form two disulfide bonds (Figure 1).[4]

The structure of bacterially expressed nonglycosylated Met-Lys-[Asn24Lys,Asn38Lys,Asn83Lys]human EPO has been determined by NMR spectroscopy,[5] and by X-ray crystallography in complex with the extracellular domain of the EPO receptor protein (Figure 2).[6]

Human urinary EPO isolated from nature has an apparent molecular weight by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) of about 34000 and has a heterogeneous glycosylation pattern, notably with variable sialic acid content. Both natural human EPO and recombinant human EPO expressed in mammalian cells contain three N-linked complex glycans and one O-linked carbohydrate (Figure 3).[7]

1.2. Role of Glycosylation in EPO Biological Activity

It has been shown that deglycosylation of EPO isolated from natural sources does not affect its ability to bind to and stimulate receptor-expressing cells in vitro, but that deglycosylation abolishes biological activity in vivo.[8] Similar results were obtained for glycosylated and nonglycosylated forms of recombinant EPO.[9] Thus, glycosylation affects the rate at which EPO is cleared from the circulation in vivo but is not required for receptor binding and receptor activation.

1.3. Recombinant EPO as a Human Therapeutic

Human EPO was one of the first recombinant proteins developed as a human therapeutic,[10] initially for the treatment of anemia in renal dialysis patients who could thus avoid

[*] Prof. Dr. S. B. H. Kent
Department of Chemistry, University of Chicago
Chicago, IL 60637 (USA)
E-mail: skent@uchicago.edu
Homepage: http://chemistry.uchicago.edu/faculty/faculty/person/member/stephen-bh-kent.html

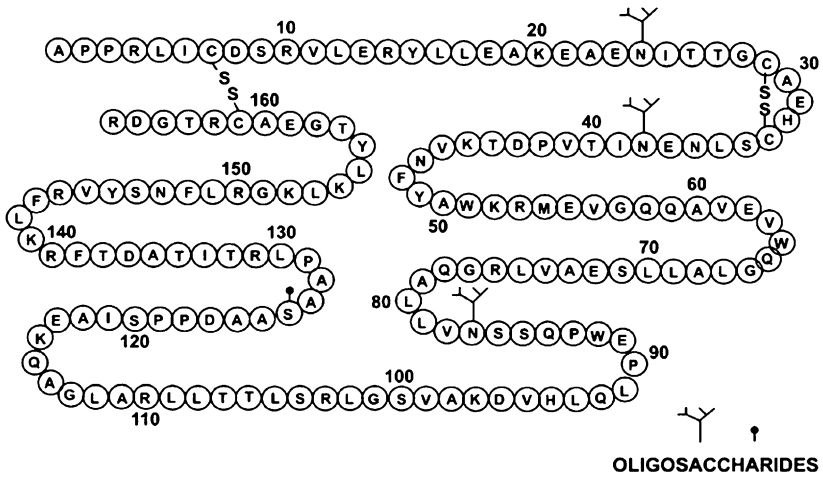

Figure 1. The chemical structure of EPO (taken from Ref. [51]).

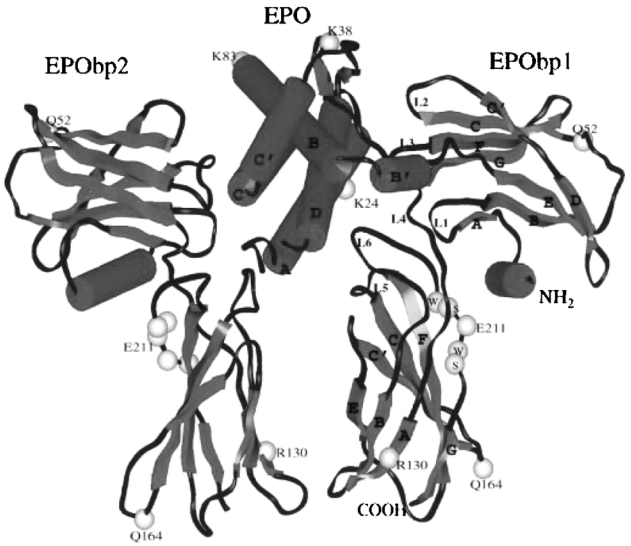

Figure 2. X-ray crystal structure of Met-Lys[Asn24 Lys,Asn38 Lys,Asn83 Lys]EPO bound to its receptor (taken from Ref. [6]).

repeated transfusions of whole blood. FDA-approved recombinant EPO is produced in mammalian CHO cells in tissue culture and has a 165-residue polypeptide chain with a sequence identical to that found in mature human EPO (but with the C-terminal Arg^{166} removed during expression).

Stephen Kent received BSc and MSc degrees in his native New Zealand, and a PhD (Organic Chemistry) from UC Berkeley. He then worked with Bruce Merrifield at the Rockefeller University from 1974–1981. Kent has held faculty positions at the California Institute of Technology and The Scripps Research Institute. From 1997 to 2001 he was Chief Scientist at Gryphon Sciences. Kent is currently Professor of Chemistry at the University of Chicago.

Recombinant EPO has heterogeneous glycosylation with a characteristic set of isoforms that differ from one another in sialic acid content and that have consequent distinct isoelectric points; in isoelectric focusing, the pattern of recombinant EPO isoforms is markedly different from that found in human EPO isolated from nature (Figure 4).[11]

This difference in the patterns of net charge for natural and recombinant EPO isoforms is the basis for tests that can identify the source of EPO found in blood samples taken from endurance athletes[11] The abuse of EPO by endurance athletes became widespread after its introduction as a human therapeutic, and EPO doping has been the subject of headlines in the popular press for more than a decade. Notwithstanding its abuse in sport, recombinant EPO as a human therapeutic is one of the success stories of the biotherapeutic industry, "*arguably the most successful drug spawned by the revolution in recombinant DNA technology*".[12]

1.4. EPO as a Target for Synthetic Organic Chemistry

In the late 1990s, at Gryphon Sciences (South San Francisco, California) a team of chemists, biochemists, and biologists set out to use total chemical synthesis to prepare a form of EPO of defined covalent structure, with full biological activity, and with improved therapeutic properties. Our design objectives included the unambiguous total synthesis of a modified form of the EPO polypeptide chain equipped with covalently attached monodisperse synthetic polymers designed to mimic the hydrodynamic and charge properties of the complex glycans found in natural EPO. It was anticipated that preparation of chemically synthesized "glycoprotein mimetic" forms of EPO in high purity and of defined covalent structure would enable the systematic variation of the structural features of the synthetic protein construct and lead to the development of an EPO analogue with improved drug properties.

Such an objective required the availability of effective chemistries for the total synthesis of protein molecules, an understanding of the molecular origins of the effects of

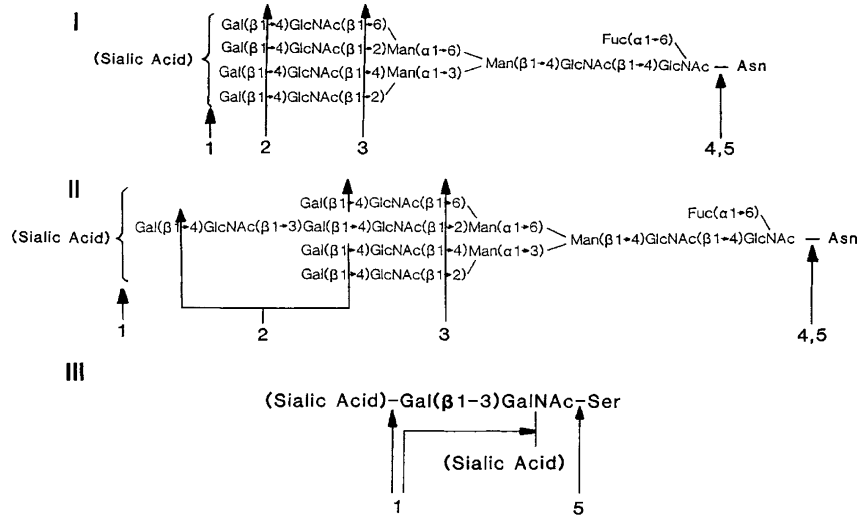

Figure 3. Structure of complex glycans found on recombinant EPO expressed in mammalian cells. The arrows show the sites of action of distinct glycosidases (taken from Ref. [7]).

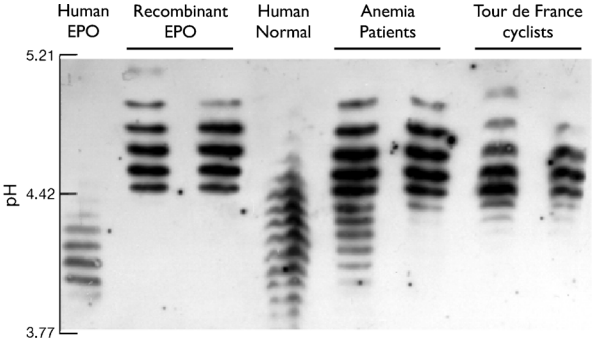

Figure 4. Isoelectric focusing of natural and recombinant forms of EPO (taken from Ref. [11]).

glycosylation on the biological properties of EPO, and the development of novel monodisperse "glycan mimetic" polymers to emulate those effects, all employed to give a chemical EPO analogue as a single molecular species with carefully tailored properties.

2. Chemical Protein Synthesis

2.1. History

More than 100 years ago Emil Fischer declared the preparation of protein molecules to be an important objective of synthetic chemistry. In his remarks upon receiving the second Nobel Prize in Chemistry, Fischer said: "*Nevertheless, the chemical enigma of Life will not be solved until organic chemistry has mastered another, even more difficult subject, the proteins, in the same way as it has mastered the carbohydrates.*"[13] Franz Hofmeister and Fischer independently proposed the peptide theory of protein covalent structure, namely that proteins consisted of linear polymers of α-amino acids joined by amide ("peptide") bonds. In the following five decades, Fischer and his scientific descendants and chemists throughout the world developed a variety of sophisticated methods for the total synthesis of peptides by chemical reactions carried out in organic solvents.[14]

It was not until the determination of the complete covalent structure of the 51-residue insulin molecule by Fred Sanger in the late 1950s,[15] that the amino acid sequence information necessary for the total synthesis of a protein became available: "*One significant result of this work is that it demonstrates that insulin, and probably other proteins, are homogeneous substances with unique structures and are not merely statistically random polymers of amino acids. This brings the science of proteins into the realms of classical organic chemistry and opens up the way to similar studies of the many other proteins that exist in nature and hence to a better understanding of the chemistry of life.*"[16]

By the 1970s ever more powerful solution synthetic methods had led to the preparation of crystalline insulins with full biological activities.[17] In parallel with these developments, the solid-phase peptide synthesis (SPPS) method was introduced by Bruce Merrifield.[18] SPPS is the archetype for polymer-supported organic synthesis; the SPPS method made synthetic peptide chemistry widely accessible to researchers and had a profound impact on the number of peptides prepared by chemical means.

However, inherent features of both the solution- and solid-phase synthetic approaches still prevented the practical, reproducible total synthesis of larger protein molecules of more typical size.[19] For SPPS, peptide coproducts that arise from incomplete reaction or from competing side reactions are resin-bound; thus byproducts accumulate throughout a synthesis and give rise to complex product mixtures. For solution synthesis, fully protected peptide segments frequently had poor solubility in organic solvents, leading to slow reactions and poor condensation yields. Activation of protected peptide segments led to racemization at the C-terminal residue under basic reaction conditions. In addition, for both the solution and SPPS methods there are distinct inherent features that hinder effective purification of intermediate

products and their analytical characterization.[19] Consequently, chemical synthesis of proteins containing polypeptide chains of more than roughly 50 amino acids remained a daunting and highly questionable undertaking.

The state of synthetic protein chemistry seven decades after Fischer was eloquently summarized by George Kenner in his 1977 Royal Society Bakerian Lecture describing the ultimately unsuccessful effort to synthesize the enzyme lysozyme by synthetic organic chemistry solution methods: "*… the vitality of organic synthesis is far from exhausted by the efforts of the previous decades. Nevertheless such investigations, intriguing though they be, do not tackle the fundamental problem alluded to earlier in this lecture, namely the simulation in the laboratory of Nature's marvellous intramolecular coupling of carboxyl and amino groups on the template of protein biosynthesis… What is required is a general method for coupling sections of a polypeptide chain by virtue of an intramolecular condensation.*" [20]

2.2. Chemical Ligation

In the early 1990s, just such a method as Kenner had envisaged was developed, based on the principle of "chemical ligation":[21] the chemoselective covalent condensation of unprotected peptides, enabled by formation of an unnatural analogue structure at the ligation site (Figure 5A).

The origin of the chemical ligation concept has been recounted.[22] Initially we used thioester-forming ligation chemistry to demonstrate the utility of the approach (Figure 5B). Shortly thereafter, we developed "native chemical ligation" (Figure 5C), the thioester-mediated, amide-forming covalent condensation of unprotected peptide segments at Xaa-Cys ligation sites.[23] Native chemical ligation was developed as a conceptual extension of thioester-forming chemical ligation, and in accord with the chemical ligation concept a non-native thioester moiety is initially formed at the ligation site. The success of native chemical ligation is based on a correct understanding of the reacion mechanism. Key is the use of a suitable thioaryl catalyst to ensure that the initial thioester-forming step is both rapid and reversible, and that consequently the reaction is regioselective—only the thioester formed at an N-terminal Cys residue can undergo irreversible intramolecular rearrangement to give the final amide-linked ligation product.[24,25] Native chemical ligation is carried out in aqueous solution at neutral pH; chaotropes such as 6 M guanidinium hydrochloride enable the general use of millimolar peptide concentrations with consequent useful reaction rates and near-quantitative ligation yields. To this day, native chemical ligation remains the most effective ligation chemistry.

The chemical ligation principle and the native chemical ligation reaction have had a transformative impact on our ability to make proteins by total chemical synthesis.[26] Chemoselective reaction of unprotected peptide segments is practical and robust. This simple concept at one fell swoop overcame all the issues that bedevilled the classic maximal protection/organic solution approach previously taken by synthetic organic chemists for total protein synthesis: unprotected peptides are almost invariably soluble in aqueous 6 M guanidinium hydrochloride at millimolar concentrations, and native chemical ligation reactions proceed in a matter of hours to give near-quantitative yields of ligation products in the condensation of even large polypeptide segments. Because of the neutral reaction conditions used, racemization is not observed.

Furthermore, the use of high-purity well-characterized unprotected synthetic peptide segments overcame the limi-

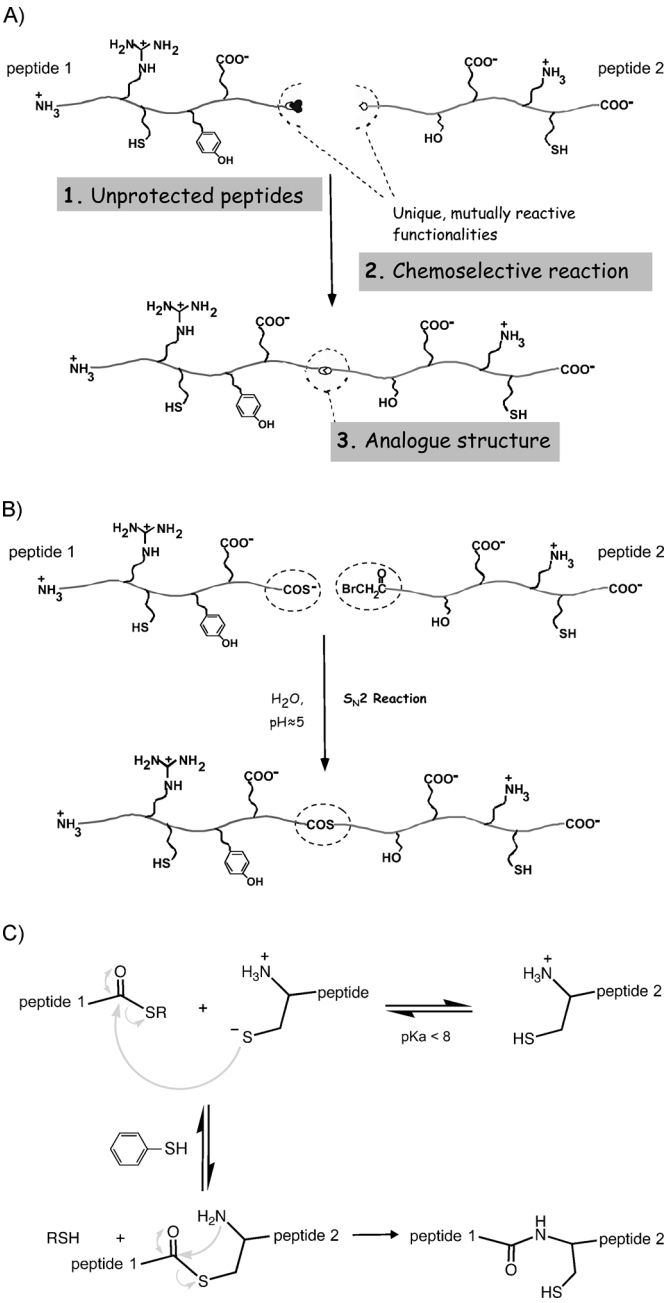

Figure 5. Chemical ligation of unprotected peptide segments. A) Chemoselective covalent condensation enabled by formation of an unnatural analogue structure at the ligation site;[21] B) Thioester-forming chemical ligation;[21] C) Native chemical ligation: the initial ligation product contains an unnatural thioester that rearranges by intramolecular nucleophilic attack to give a native peptide (amide) bond at the ligation site.[23]

tations, caused by the statistical accumulation of resin-bound byproducts, on the size of synthetically accessible peptides. Now, whatever size peptides can be made by SPPS, use of chemical ligation enables the doubling and doubling again of the maximum-size polypeptide chain that can be prepared by total synthesis. With the advent of chemical ligation, fully functional protein constructs of more than 20 kDa immediately became accessible by total synthesis.[27,28]

2.3. Synthesis of Peptide-Thioesters

Efficient preparation of the peptide-thioesters used as building blocks in native chemical ligation is key to successful chemical protein synthesis. Commonly used SPPS methods are not suitable for the preparation of peptides containing a preformed thioester, because the thioester moiety is very susceptible to attack by amine nucleophiles. Both the piperidine used to remove the N^{α}-Fmoc protecting group at each step of Fmoc chemistry SPPS and the neutralized N-terminal amino groups of the resin-bound peptide chains at each step of standard Boc chemistry SPPS will react with any thioester present (Fmoc = fluorenylmethoxycarbonyl, Boc = *tert*-butoxycarbonyl). A practical way to circumvent the susceptibility of thioester-containing peptides during SPPS is "in situ neutralization" Boc chemistry:[29] at each stage of the synthesis, the peptide-resin containing a preformed thioester is neutralized in the presence of a high concentration of the incoming activated Boc-amino acid, thus avoiding exposure of the thioester moiety to amine nucleophiles. This simple and effective SPPS chemistry was originally developed in order to minimize peptide chain aggregation during synthesis, and is a straightforward way of making peptide-thioesters for use in chemical protein synthesis.[30]

2.4. Purification and Analytical Control

Peptide-thioesters are completely stable to the conditions normally used for handling synthetic peptides (typically 0.1 % trifluoroacetic acid (TFA) in water/acetonitrile solvents). The crude deprotected peptides are readily purified by reverse-phase HPLC and the purification is monitored by analytical reverse-phase HPLC mass spectrometry (LCMS). Use of unprotected peptide segments has enabled routine analytical control of chemical ligation reactions by high-resolution methods such as LCMS, and has enabled the straightforward purification of synthetic intermediates and final products by preparative HPLC. Finally, because the synthetic protein products are obtained directly in unprotected form, they are readily characterized by LCMS for purity and correct mass; such precise mass measurement serves as a useful confirmation that a protein molecule of the correct structure has in fact been obtained.

3. Synthetic Erythropoiesis Protein (SEP)

By the late 1990s a set of practical protein synthesis chemistries had been developed.[31] This enabled us to undertake the serious challenges involved in the redesign of a glycoprotein drug molecule with the goal of improving its therapeutic properties. Our objective was a "designer glycoprotein mimetic", that is, a glycoprotein mimetic of defined chemical structure with full EPO biological activity and enhanced pharmacokinetic (PK) properties, prepared by total chemical synthesis. Aspects considered in the design of the SEP molecule included modifications of the target polypeptide structure in order to facilitate its synthesis while retaining biological activity, and the replacement of the complex N-linked glycans found in both natural and recombinant EPO by polymer moieties intended to provide similar hydrodynamic and charge properties. The ultimate goal was to use total chemical synthesis to systematically improve the therapeutic properties of EPO.

3.1. Design

The covalent structure of the target SEP protein molecule is shown in Figure 6.[32] The 166-residue polypeptide chain in SEP included the C-terminal Arg^{166} residue found in human EPO isolated from natural sources but not found in recombinant human EPO. Amino acid substitutions were made at

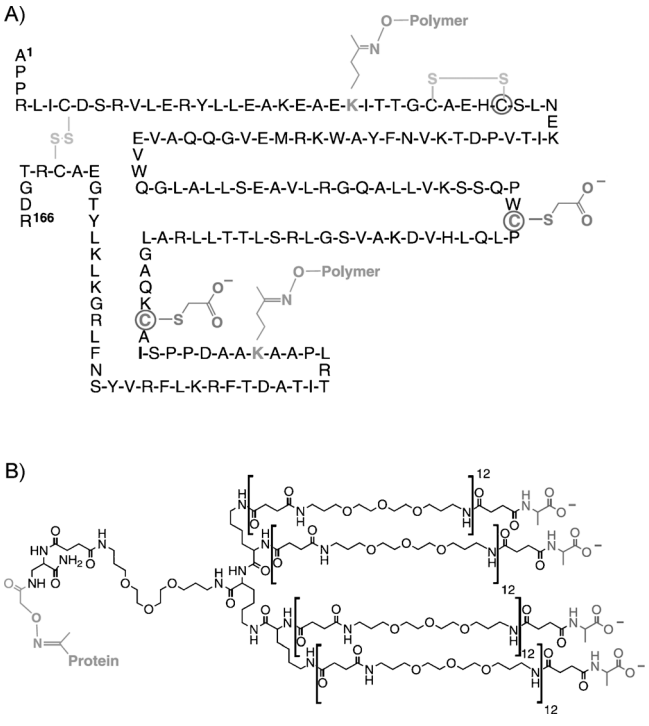

Figure 6. Synthetic erythropoiesis protein (SEP). A) Covalent structure of SEP; B) Structure of the "glycan mimetic" moiety, equipped with charge control units carrying a negative charge. This branched polymer was monodisperse with a molecular formula of $C_{737}H_{1354}N_{112}O_{267}$ and had a molecular weight of 16057.2 (average isotope composition) (taken from Ref. [32]).

positions 24, 89, 117, and 126 in order to provide suitable ligation sites for attachment of branched polymer chains and for condensation of the unprotected peptide segments (see Section 3.2.).

The N-linked complex glycans found in natural and recombinant human EPOs were replaced by a "glycan mimetic" moiety, a monodisperse branched polymer that was systematically designed to emulate the larger hydrodynamic volume and other properties of the natural glycoprotein. Larger hydrodynamic volume retards renal clearance by glomerular filtration. Carboxylate-containing charge control moieties were built into the branched polymer in order to assume the role of the sialic acids by providing the negative charges known to also influence (retard) clearance by renal filtration.[8,9] Chemical synthesis of the novel branched polymer construct was based on Keith Rose's precision oligomer synthesis using 4,7,10-trioxa-1,13-tridecanediamine/succinic anhydride (TTD-SUCC) chemistry,[33] and involved a combination of polymer-supported organic synthesis with synthesis in solution. The resulting monodisperse branched polymer contained a single aminooxy functionality for attachment to the ketone functionality of a levulinic acidmodified Lys residue by an oxime-forming chemoselective reaction.[34] An Ala residue was attached by its N-terminus to each of the TTD-SUCC oligomer branches to provide negative charges to mimic the effect of the sialic acid carboxylates in the human EPO glycoprotein.

3.2. Synthesis

The synthetic scheme used for the preparation of SEP is shown in Figure 7. The polypeptide chain was assembled from four synthetic peptide segments by consecutive native chemical ligations, starting from the C-terminal segment. Ligation-site Cys residues 117 and 89 were alkylated to give unnatural "pseudo-Glu" residues in place of the natural Glu residues found at those positions; natural Cys residue 161 was protected as the orthogonal Cys(picolyl) derivative during the alkylation. The final ligation was at natural Cys residue 33, with unprotected side chains at natural Cys residues 7 and 29. The C-terminal and N-terminal peptide segments each contained a copy of the branched polymer attached by an oxime bond to the side chain of a Lys residue modified with levulinic acid. These polymer attachment points were at residues 24 and 126 in the polypeptide chain, natural glycosylation sites in human EPO. Attachment of the polymer at these positions was not expected to interfere with binding to the EPO receptor as judged from the co-crystal structure of EPO bound to its receptor external domains.[6]

3.3. Analytical Characterization

The full-length 166 amino acid polymer-modified SEP polypeptide chain was purified by reverse-phase HPLC, and then folded with concomitant formation of the two native

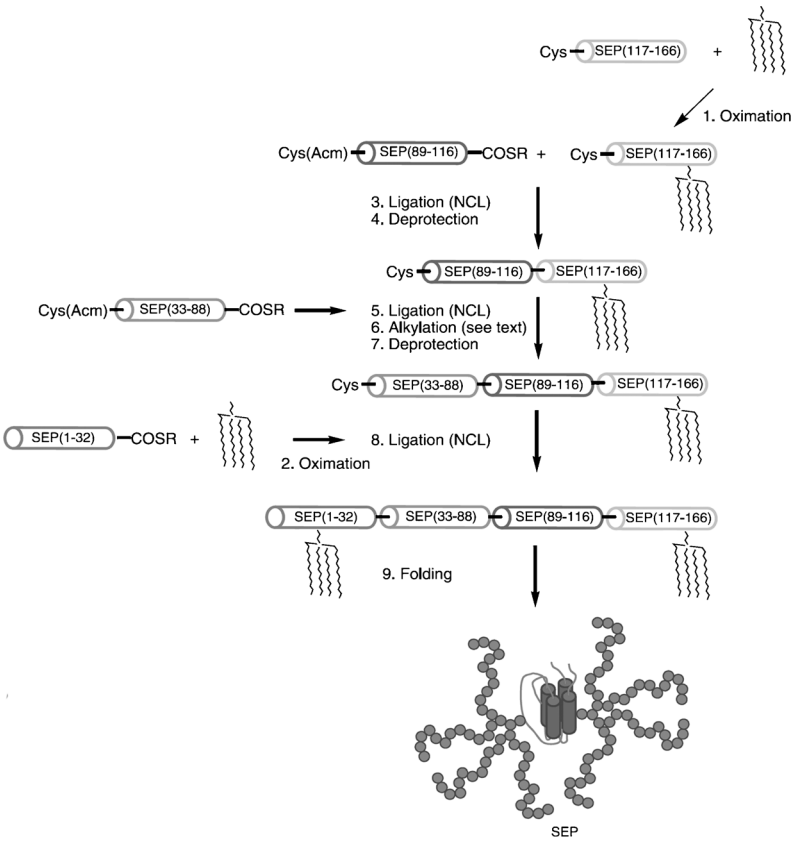

Figure 7. Strategy for the total chemical synthesis of synthetic erythropoiesis protein (SEP) (taken from Ref. [32]).

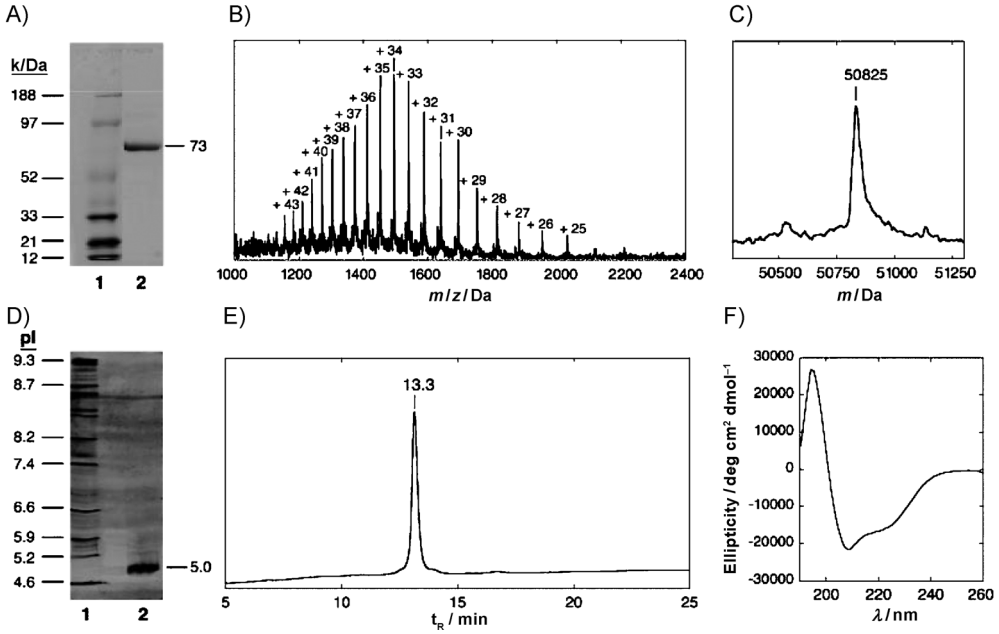

Figure 8. Analytical characterization of SEP. A) SDS-PAGE, apparent molecular weight 73 000; B) Electrospray mass spectrum showing multiple charge states; C) Reconstructed mass spectrum giving a measured molecular mass of (50 825 ± 10) Da; D) IEF of SEP gave a single band with pI = 5.0; E) Analytical HPLC of SEP; F) CD spectrum of SEP (taken from Ref. [32]).

disulfide bonds to give the SEP protein molecule.[32] The total synthesis of SEP was repeated a number of times, and amounts in excess of 100 milligrams of high-purity protein were produced. Chemically synthesized SEP met the highest standards for analytical characterization available at that time: the polymer-modified synthetic protein ran as a single band in SDS-PAGE; in isoelectric focusing (IEF), SEP formed a single band with pI = 5.0; SEP was of high purity by LCMS, and had a mass of (50 825 ± 10) Da; and SEP was a folded highly helical protein as evaluated by CD spectroscopy (Figure 8).

3.4. Biological Activity

Finally, the biological properties of SEP were evaluated. SEP was fully active in a factor-dependent cell line proliferation assay in vitro, and had full in vivo biological activity in mouse hematocrit assays (Figure 9). Importantly, as intended in the original design of this glycoprotein mimetic molecule, SEP had improved PK properties: SEP was cleared from the circulation at less than half the rate of first-generation recombinant EPO (Figure 9).[32]

3.5. A Designed Glycoprotein Mimetic

A glycoprotein mimetic of novel structure and a mass of 50 825 Da was prepared in homogenous form by total chemical synthesis. Synthetic erythropoiesis protein had full biological activities, and had improved pharmacokinetic properties compared with first-generation recombinant human EPO.

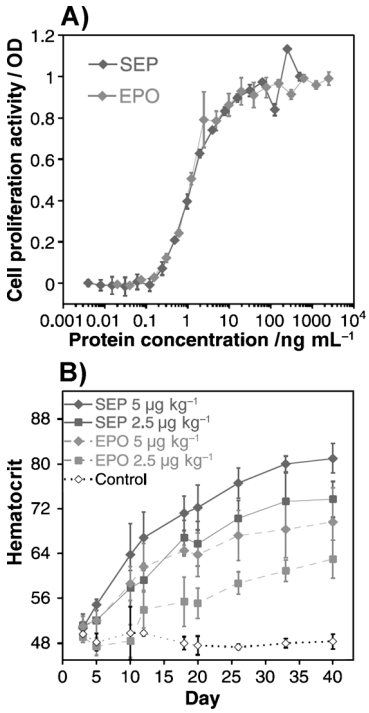

Figure 9. Biological activity of SEP in vitro and in vivo. A) Factor-dependent cell line proliferation in vitro; B) Hematocrit in mice in response to SEP (dark gray) or recombinant EPO (light gray) (taken from Ref. [32]).

4. Sequelae

The design and total chemical synthesis of SEP recounted above demonstrated that synthetic chemistry could be employed to systematically improve the properties of a pro-

tein biotherapeutic by the use of designed chemical moieties not found in nature. There have been a number of developments since that work was done.

4.1. New Synthetic Chemistry

Our ability to use synthetic organic chemistry for the preparation of protein molecules has been greatly enhanced by recent developments in chemical protein synthesis. These include: one-pot synthesis by sequential native chemical ligations, used in combination with masking of N-terminal Cys residues as the thiazolidine (Thz);[35,36] development of an improved thioaryl catalyst for native chemical ligation;[25] and fully convergent synthesis enabled by kinetically controlled ligation.[33–39]

The current focus of a number of organic chemistry research groups on the preparation of EPO protein molecules with native carbohydrate structure is leading to numerous important contributions to improved methods for the chemical synthesis of proteins. These include the extension of Yan and Dawson's native chemical ligation and subsequent desulfurization for ligation at Xaa-Ala sites,[40] to a wide range of Xaa-Yaa sites;[41–44] the introduction of a soluble catalyst for selective desulfurizations;[45] the use of complex/remodeled glycans isolated from natural sources in the synthesis of glycoproteins;[46] and the use of expressed protein ligation for the semisynthesis of defined EPO glycoforms.[47]

4.2. Structural Characterization of Synthetic Proteins

In addition to new synthetic methods, there have been important advances in the characterization of synthetic protein products. Because of the complex macromolecular nature of proteins, it is essential that synthetic proteins be subjected to rigorous analytical characterizations. These include: homogeneity by LCMS and by a charge-based method such as isoelectric focusing (IEF); covalent structure by precise mass measurement across the entire product peak in LCMS or by direct infusion of the purified synthetic protein; formation of a single, unique folded protein structure by multidimensional NMR spectroscopy;[38] and elucidation of the synthetic protein structure by X-ray crystallography including racemic protein crystallography.[38,39,48] Only after meticulous characterization has established the purity and chemical structure of a synthetic protein molecule should its biological activity be evaluated.

4.3. Future Prospects

As eloquently summarized in Danishefsky's recent Minireview,[49] research over the past decade has brought glycopeptide synthetic methods to the verge of a total synthesis of EPO with complex native glycans. This work will enable the fruitful application of synthetic organic chemistry to the preparation of EPO glycoforms of defined structure and evaluation of their biological properties: "… *de novo chemical synthesis might provide a powerful path to designed glycoproteins as it offers, in principle, precise structural control while providing opportunities for systematic variation of both the structures of the glycodomains and their location within the peptide sequence.*" [50]

The pathway to designed glycoproteins, blazed by the design and total chemical synthesis of SEP, will then be available for the systematic tuning of the properties of EPO and other glycoproteins.

5. Summary and Perspectives

The total chemical synthesis of SEP—a designed glycoprotein mimetic with improved properties—was a milestone in synthetic organic chemistry applied to proteins, and vividly illustrates the power of applying the principles of medicinal chemistry to improving the properties of protein therapeutics.

On a broader note, application of synthetic organic protein chemistry in conjunction with advanced physical methods will greatly facilitate the elucidation of the molecular basis of protein function. In the future it will prove possible to design and build enzyme molecules from scratch. Using synthetic chemistry and employing the full range of available chemical functionalities, these de novo designed enzymes will act as powerful and selective catalysts for any arbitrary chemical transformation. Then, the science of proteins will truly be a part of the realm of organic chemistry.

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