Review

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# Through the looking glass – a new world of proteins enabled by chemical synthesis‡

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**'Chemical ligation' – *the regioselective and chemoselective covalent condensation of unprotected peptide segments* – has enabled the synthesis of polypeptide chains of more than 200 amino acids. An efficient total chemical synthesis of the insulin molecule has been devised on the basis of a key ester-linked intermediate that is chemically converted to fully active human insulin. Enzyme molecules of defined covalent structure and with full enzymatic activity have been prepared and characterized by high-resolution X-ray crystallography. A 'glycoprotein mimetic' of defined chemical structure and with a mass of 50,825 Da, has been prepared and shown to have full biological activity and improved pharmacokinetic properties. D-Protein molecules that are the mirror images of proteins found in the natural world have been prepared by total chemical synthesis. Racemic protein mixtures, consisting of the D-enantiomers and L-enantiomers of a protein molecule, form highly ordered centrosymmetric crystals with great ease; this has enabled the determination of the crystal structures of recalcitrant protein molecules. A protein with a novel linear-loop covalent topology of the peptide chain has been designed and synthesized and its structure determined by facile crystallization as the quasi-racemate with the D-form of the native protein molecule. We have developed an optimized total chemical synthesis of biologically active vascular endothelial growth factor-A; total synthesis of the mirror-image protein will be used to systematically develop D-protein antagonists of this important growth factor. The total chemical synthesis of proteins is now a practical reality and enables access to a new world of protein molecules.**


**Keywords:** chemical protein synthesis; chemical ligation; ester insulin; erythropoietin; glycoprotein mimetics; mirror-image proteins; racemic protein crystallography

## Background

> *"In the field of protein synthesis, it is my confident hope that tomorrow's deeds will catch up with today's titles, and that we shall truly be able to obtain enzymatically active proteins as synthetic substances: as materials composed of a single molecular species."*
> J. Rudinger [1]

The total chemical synthesis of proteins – particularly enzymes – was one of the grand challenges of 20th century organic chemistry. The great chemist Emil Fischer had, in early pursuit of that goal, articulated the 'peptide theory' of protein (and enzyme) structure and had gone on to pioneer the field of chemical peptide synthesis. In the three decades after the Second World War, organic chemists addressed with renewed enthusiasm this goal of total synthesis of protein molecules that have enzymatic activity. Novel methods of peptide synthesis were developed, including a wide range of protecting group and activation chemistries. Initially, these tools were applied to the chemical synthesis of biologically active peptides found in nature. Then, as the covalent and X-ray structures of enzyme protein molecules became available from the work of Anfinsen, Moore and Stein, Phillips, Harker, and others, these methods for the chemical synthesis of peptide chains were applied to the total chemical synthesis of enzymes. Classical solution methods of peptide synthesis culminated in the attempted total synthesis of 'consensus lysozyme' by Kenner and co-workers [2]. Also in the 1970s, Gutte and Merrifield reported the use of stepwise solid phase peptide synthesis to make RNase A with high enzymatic activity [3].

Nonetheless, despite the powerful methods that had been developed for the chemical synthesis of peptide chains, through the early 1990s reproducible total synthesis was limited to only the smallest proteins containing ~50 amino acids [4–7]. The main problems limiting the size of synthetically accessible peptide chains were the insolubility of protected peptide segments and racemization in solution synthesis, and the statistical accumulation of error peptide byproducts in stepwise solid phase synthesis.

In 2007, Durek reported the total chemical synthesis of human lysozyme (130 amino acid residues; eight Cys residues in four disulfides) and the determination of the high-resolution X-ray structure of the synthetic enzyme, which had full catalytic activity (Figure 1) [8]. Contemporaneously, Torbeev reported the total chemical synthesis of a 203 amino acid residue covalent dimer of the HIV-1 protease protein molecule, also characterized by high-resolution X-ray crystallography and with full enzymatic activity (Figure 2) [9]. Each of these total syntheses was the work of just one talented young scientist using manual synthetic methods. Clearly, a transformation of our ability to make proteins by chemical synthesis had taken place in the preceding ~15 years.

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**Biography**

**Stephen Kent** took his BSc in Chemistry and Biochemistry and his MSc (Thesis: 'Peptide sequences by mass spectrometry') in his native New Zealand. He completed a PhD in Organic Chemistry at the University of California, Berkeley (Thesis: 'Specific chemical modification and $^{13}$C-NMR properties of the lysine at the active site of alcohol dehydrogenase'). From 1974 to 1981, he worked with R. Bruce Merrifield at the Rockefeller University in New York. Subsequently, Kent held academic positions at the California Institute of Technology, Bond University, and The Scripps Research Institute before joining the University of Chicago where he is now Professor of Chemistry and Professor of Biochemistry and Molecular Biology.

## Chemical Ligation

> *". . . the synthesis of slightly "generalized" enzymes, that is of simplified structures formally derived from the natural enzymes, . . . in such a way as to eliminate as many synthetic complications as possible while maintaining the structures which appear essential. If this should enable us to obtain a homogeneous substance with the appropriate enzymic properties, the loss of "naturalness" will have been worth it."*
> J. Rudinger [1]

The concept that enabled individual researchers to carry out the robust, reproducible total synthesis of enzyme protein molecules was 'chemical ligation', the chemoselective covalent condensation of unprotected peptides to give long polypeptide chain products [10]. The chemical ligation principle is shown in Figure 3. The key underlying concept is the formation of a non-native (analogue) structure at the site of covalent joining of the peptide segments; formation of this unnatural structure, not found in a protein's polypeptide chain, allowed the use of a pair of unique, mutually reactive functional groups – one on each of the peptide segments to be condensed – and thus did away with the need for protecting groups to direct the outcome of the condensation reaction. The result was a substantial simplification of the synthetic process and enabled the direct preparation of the long polypeptide chains found in protein molecules. Unprotected peptide segments are usually soluble in aqueous organic solvent mixtures and can be readily prepared by highly optimized SPPS, purified by reverse-phase HPLC, and characterized by LCMS. Chemoselective reaction with the formation of an unnatural structure at the ligation site also obviated racemization. Additionally, the full-length target polypeptide chain resulting from the chemical ligation of unprotected peptide segments is obtained in final form without the need for further chemical manipulations.

Thioester-forming chemical ligation, from the reaction of a peptide-thiocarboxylate and a bromoacetyl-peptide, was used in the initial demonstrations of chemical ligation for the total synthesis of proteins. Mirror-image forms of the HIV-1 protease enzyme molecule were prepared by thioester-forming chemical ligation and served to illustrate the reciprocal chiral specificity of mirror-image proteins acting on chiral substrates [11,12]. More complex protein constructs were also prepared using thioester-forming and oxime-forming ligation chemistries in combination [13]. Subsequently, thioester-forming chemical ligation was extended to the thioester-mediated formation of a native amide bond linking the two unprotected peptide segments ('native chemical ligation') [14].

## Ester Insulin

> *"In the 50 years since the determination of the insulin structure by Sanger, there have been sizable accomplishments in the chemical synthesis of insulin, but we still lack a high-yield synthesis of this molecule. This is an obstacle to the development of improved insulin analogs. . ."*
> J.P. Mayer, F. Zhang, and R.D. DiMarchi [15]

The total chemical synthesis of human insulin has been one of the principal synthetic objectives of organic chemistry. Reproducible total syntheses of human insulin and analogues were reported in the 1970s [4,5]. Nevertheless, because of the challenge of accurate recombination of the two-chain insulin molecule from individual synthetic A and B chains, an efficient total chemical synthesis of insulin has continued to elude the research community [15]. In the course of research aimed at a synthetic route to a chemical analogue of proinsulin, we noted that the side chain carboxyl of GluA4 was in contact with the side chain hydroxyl of ThrB30. We envisioned an ester bond linking those two side chains and set out to examine the feasibility of a total chemical synthesis of the insulin protein molecule via an 'ester insulin' intermediate (Figure 4). The ester-linked polypeptide precursor was prepared by a combination of SPPS and native chemical ligation; this depsipeptide chain folded efficiently at physiological pH in the presence of a redox couple to give 'ester insulin'. Multidimensional NMR and LCMS data were consistent with the formation of three native disulfide bonds in ester insulin. Simple saponification at 4°C gave fully active human insulin in greater than 90% yield [16].

Ester insulin is the key to the efficient total chemical synthesis of insulin analogues and will enable the application of the principles of medicinal chemistry to the further optimization of the properties of the insulin molecule for human therapeutic use [17].

## Erythropoietin

> *". . . access to homogeneous and structurally definable glycoproteins could be of significant value for the systematic study of the effects of protein glycosylation on biology-level performance. . . In particular, 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."*
> C. Kan and S.J. Danishefsky [18]

The glycoprotein erythropoietin (EPO) stimulates the production of erythrocytes and is widely used as a treatment for anemia; the recombinant EPO used as a human therapeutic is produced in genetically engineered mammalian cell cultures and consists of a number of different glycoforms with variable sialic acid content. A total synthesis of the EPO glycoprotein is a current objective for organic chemistry groups throughout the world in much the same way that insulin was in the 1970s. The goal is the production



  

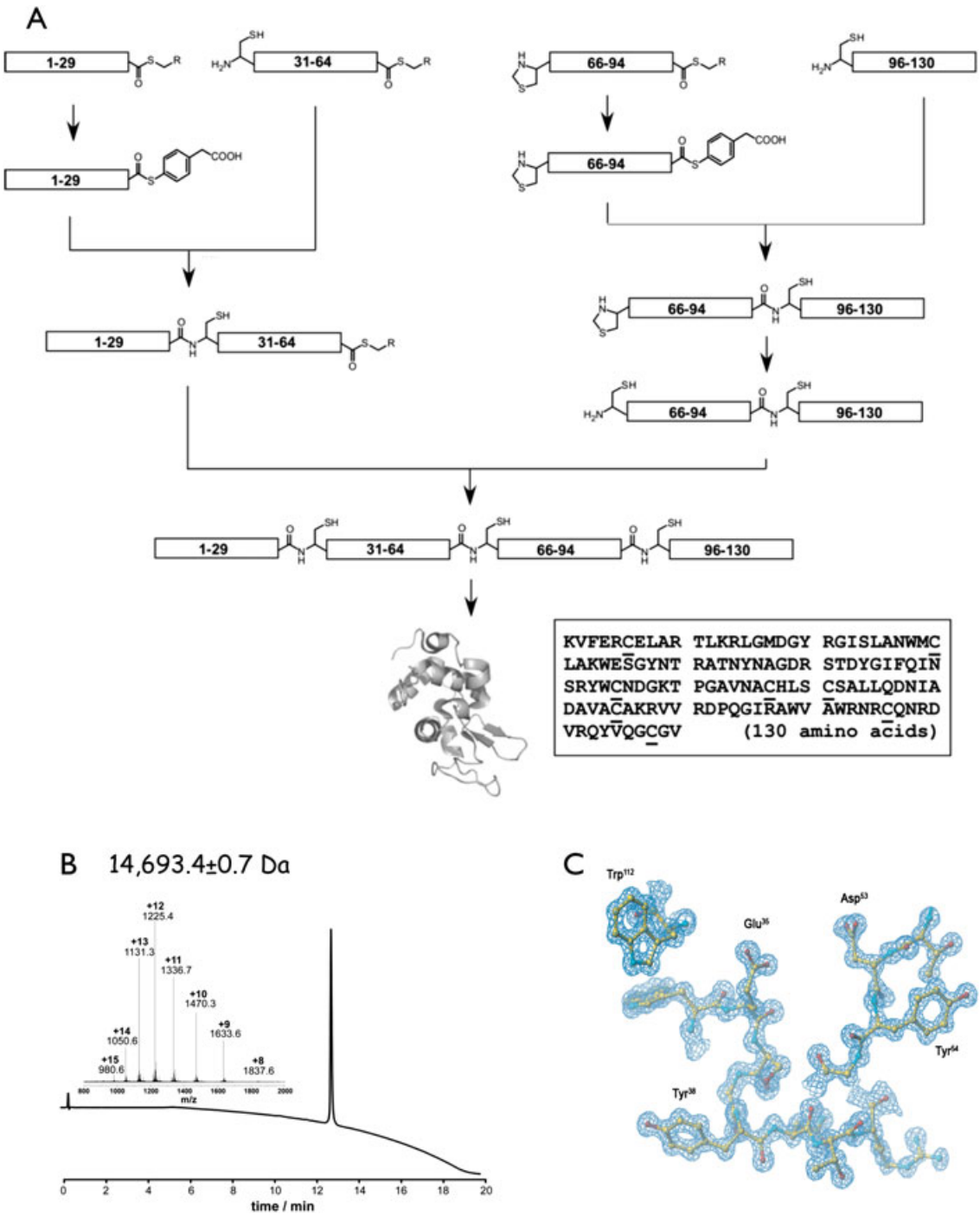


**Figure 1.** Total chemical synthesis of human lysozyme. (A) Scheme for the convergent chemical synthesis of the 130 amino acid polypeptide chain (sequence inset) and its folding to give human lysozyme containing four disulfide bonds; (B) LCMS characterization of the synthetic enzyme molecule; (C) a portion of the 2Fo-Fc electron density map with fitted structural model, used to determine the high-resolution (1.04 Å) X-ray structure of the synthetic enzyme [adapted from Ref. [8]].

of defined glycoforms in order to correlate the chemical structure of the EPO molecule with its biological activities [18].

In 2003, we reported the design and total chemical synthesis of 'synthetic erythropoiesis protein' (SEP), a glycoprotein mimetic of 50,825 Da with full biological activity and extended half-life *in vivo* (Figure 5) [19]. The target 166 amino acid polypeptide chain was assembled by the native chemical ligation of four synthetic peptides. Two of these peptide segments had identical covalently attached 'glycan mimetic' moieties; these branched oligo{ethyleneoxide-amide} molecules were made by conventional synthetic chemistry, were monodispersed, and had defined covalent structure. Each glycan mimetic was designed to increase the hydrodynamic volume of SEP, and each had four carboxylates to provide negative charges in the same way as a natural sialic acid. This synthetic *tour de force* involved more than 25 researchers and was carried out in a commercial research laboratory.

More recently, in our university laboratory, Suhuai Liu undertook the fully convergent synthesis of [Lys$^{24,38,83}$]EPO from five

  

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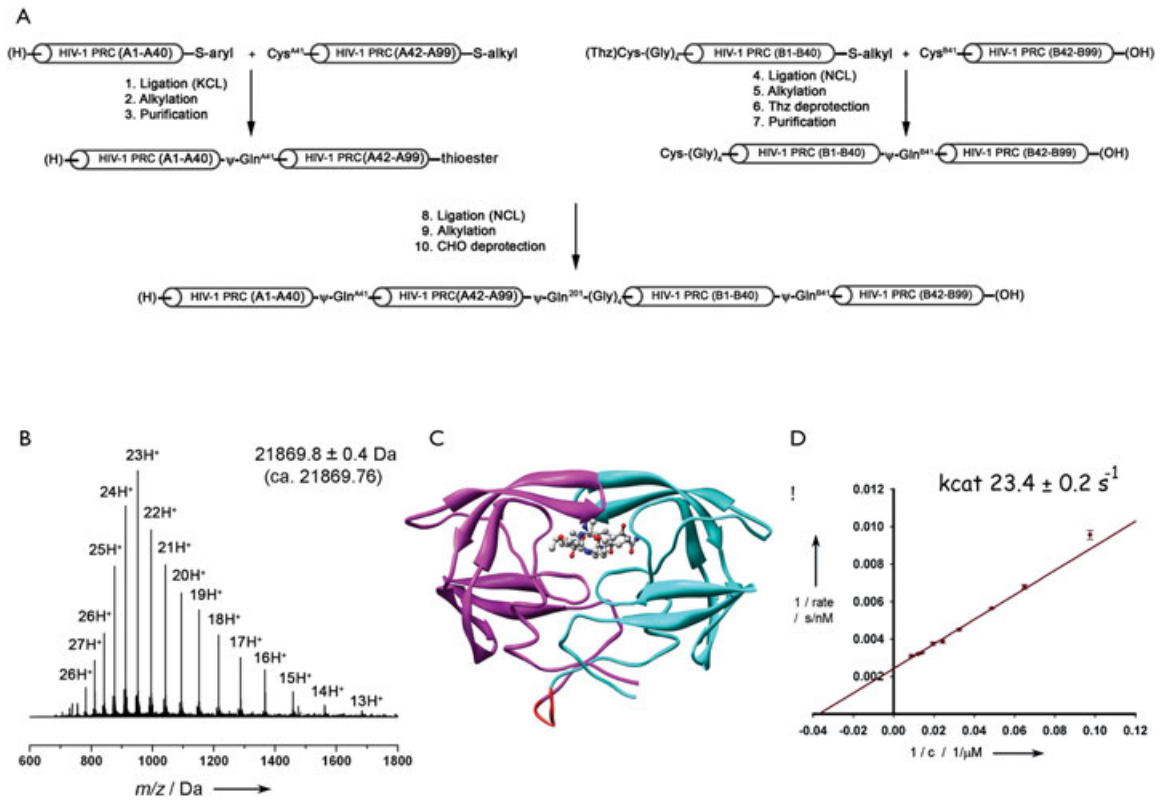


**Figure 2.** Total chemical synthesis of a covalent dimer of HIV-1 protease. (A) Scheme for the convergent synthesis of the 203 amino residue polypeptide chain from four synthetic peptide segments; (B) Fourier transform ion cyclotron resonance electrospray mass spectrometric characterization of the synthetic enzyme molecule; (C) X-ray structure of the synthetic enzyme; (D) catalytic activity of the synthetic enzyme (adapted from Ref. [9]).

synthetic peptide segments by convergent chemical ligation (Figure 6) [20]. By contrast with the synthesis of synthetic erythropoiesis protein a decade earlier, synthesis of [Lys$^{24,38,83}$]EPO was accomplished by a single individual using manual methods. A key feature of the approach was the use of kinetically controlled ligation [21] in a convergent synthetic scheme; another key aspect of the synthesis was selective desulfurization of Cys residues in the presence of protected Cys(Acm) residues, enabling native chemical ligation at Xaa-Ala sites [22,23]. Final removal of the Acm groups and folding with concomitant formation of two native disulfides gave synthetic [Lys$^{24,38,83}$]EPO that had full biological activity in a factor-dependent cell line proliferation assay. In collaboration with Danny Lee in the Margaret Brimble Lab at the University of Auckland, we are extending this work to the use of triazole-forming 'click' chemistry for the total chemical synthesis of a neoglycan form of EPO. This work is based on our recent report of the compatibility of native chemical ligation and triazole-forming click chemistry in the preparation of synthetic neoglycopeptides [24].

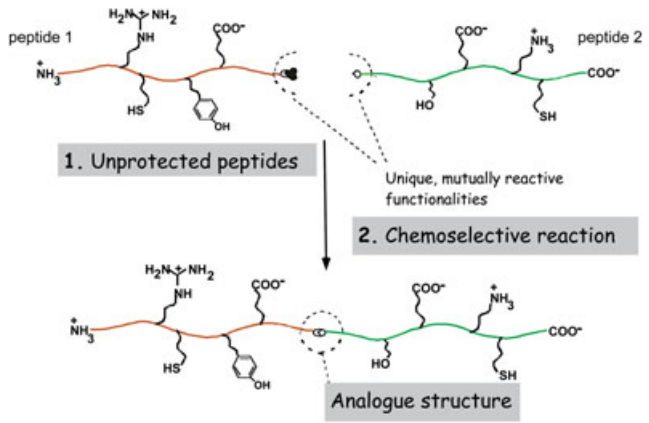


**Figure 3.** The chemical ligation principle [10].

## Racemic Protein Crystallography

> "*. . . more importantly, since we expect* P$\bar{1}$ *to be significantly more probable than other symmetries, we predict that racemic protein mixtures will crystallize more readily than samples consisting only of the biological enantiomer.*"
> S.W. Wukovitz and T.O. Yeates [25]

In 2008, we reported the use of racemic protein crystallography to determine the X-ray structure of the snow flea antifreeze protein (sfAFP). This novel protein was found to have an unprecedented structure made up entirely of polyproline type II helices connected by reverse turns [26]. In the course of that work, we observed that a racemic mixture made up of D-sfAFP and L-sfAFP crystallized much more readily than the L-sfAFP protein alone. This facile crystallization was also observed for a quasi-racemic mixture of D-sfAFP with a L-[$SeCH_2$]sfAFP.[1] In a prescient theoretical paper describing the frequency of occurrence of the 'biological' space groups in protein crystallography, Wukovitz and Yeates had predicted the more facile crystallization of racemic

[1]The protein molecule L-[$SeCH_2$]sfAFP contains a 'pseudo-Gln' residue made by alkylating selenocysteine with bromoacetamide, where the D-protein contains D-Gln, to produce a heavy atom analogue to solve the phase problem in X-ray analysis [26].



  

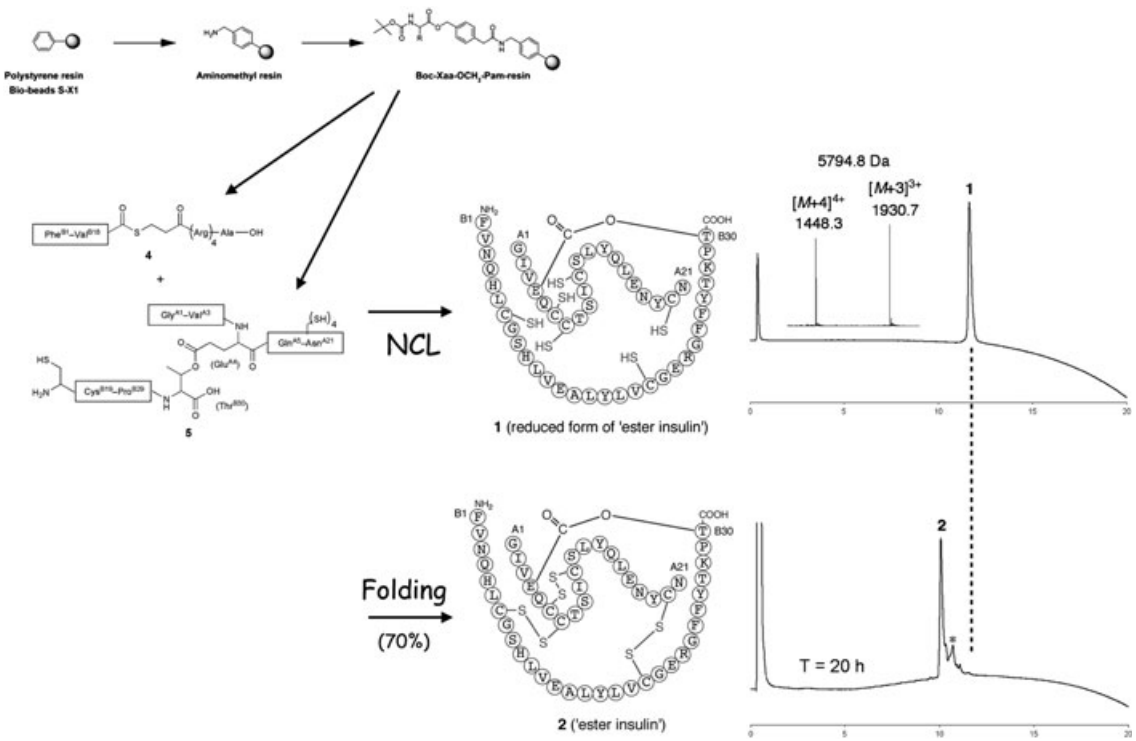


**Figure 4.** Ester insulin (adapted from Ref. [16]).

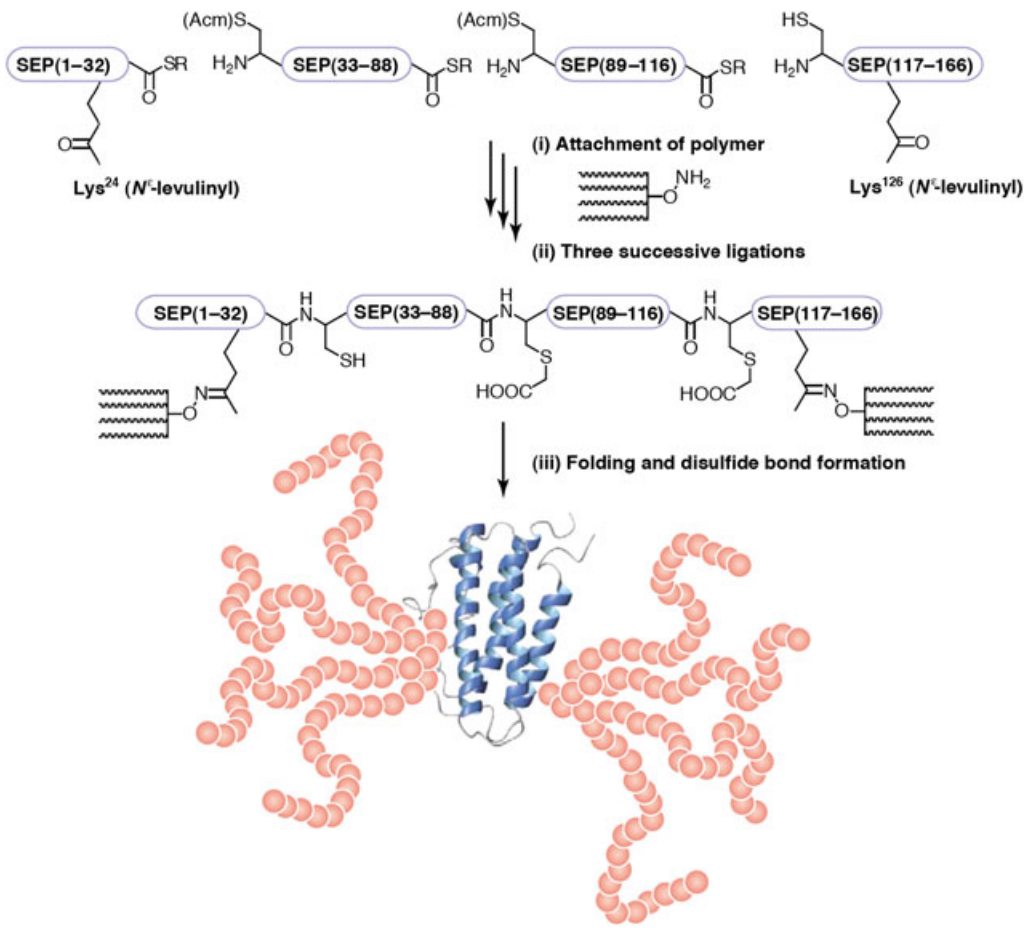


**Figure 5.** Synthetic erythropoiesis protein (reprinted from [32], with permission from Elsevier).



  

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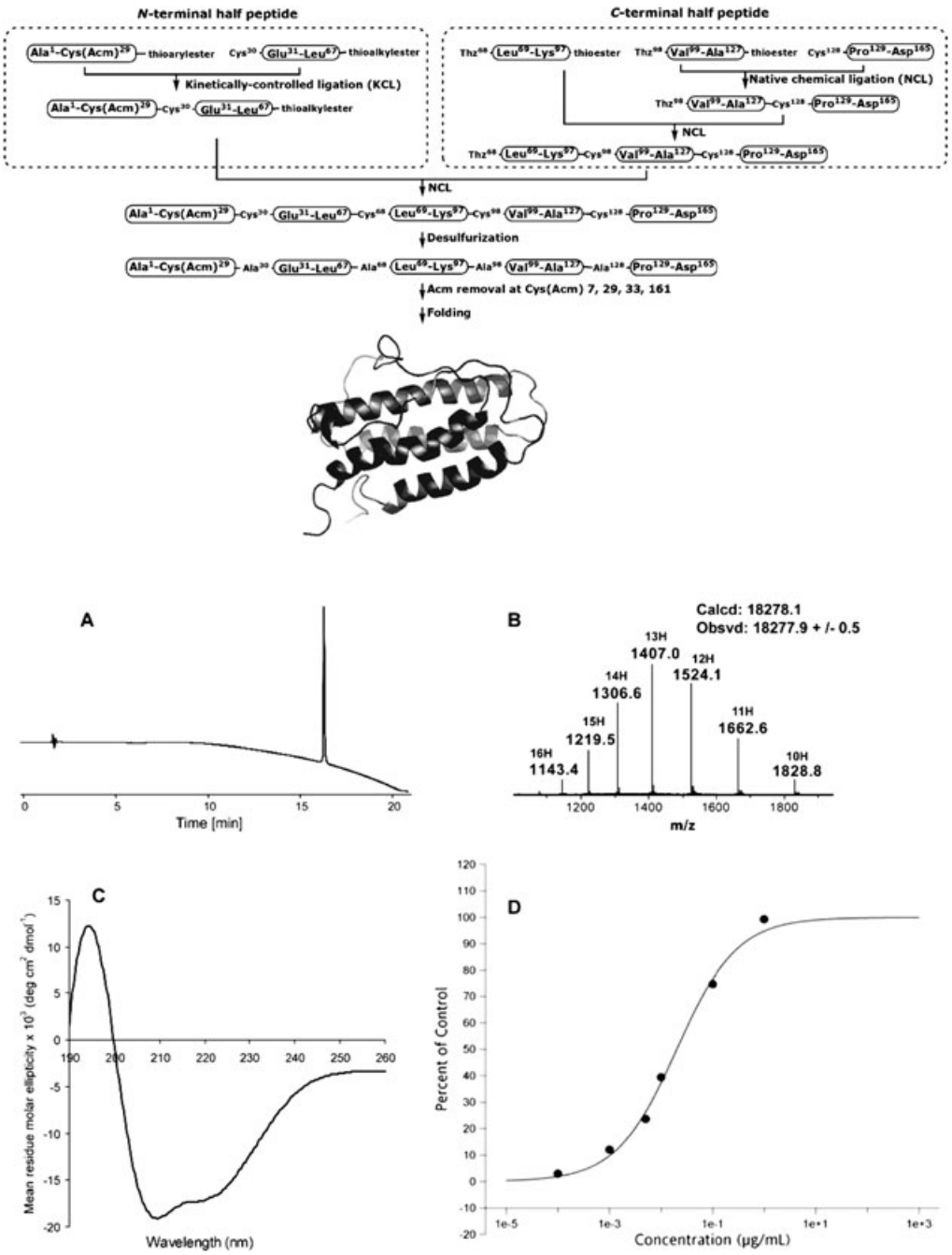


**Figure 6.** Total chemical synthesis of [Lys$^{24,38,83}$]EPO (adapted from Ref. [20]).

protein mixtures in centrosymmetric space groups [25]. In multiple instances, encompassing more than a dozen proteins, we have verified this prediction.

Recently, we designed an analogue of the model protein crambin in which an ion pair between the side chain guanidinium of Arg10 and the α-carboxylate of Asn46 was replaced by a covalent (amide) bond [27]. This protein was designed to contain a novel linear-loop polypeptide chain covalent topology not yet observed in nature. We set out to chemically synthesize the linear-loop polypeptide chain, to fold it to form a protein molecule, and to determine the X-ray structure of this novel protein analogue. In order to effect the synthesis, we developed the 'kinetically controlled ligation' method for the condensation of a peptide-thioarylester with a Cys-peptide-thioalkylester to give a product peptide-thioalkyl-ester

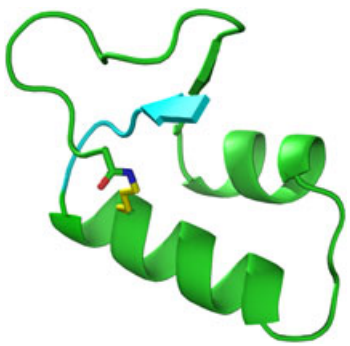

**Figure 7.** X-ray structure of a novel topological analogue of crambin (adapted from Ref. [27]).



  

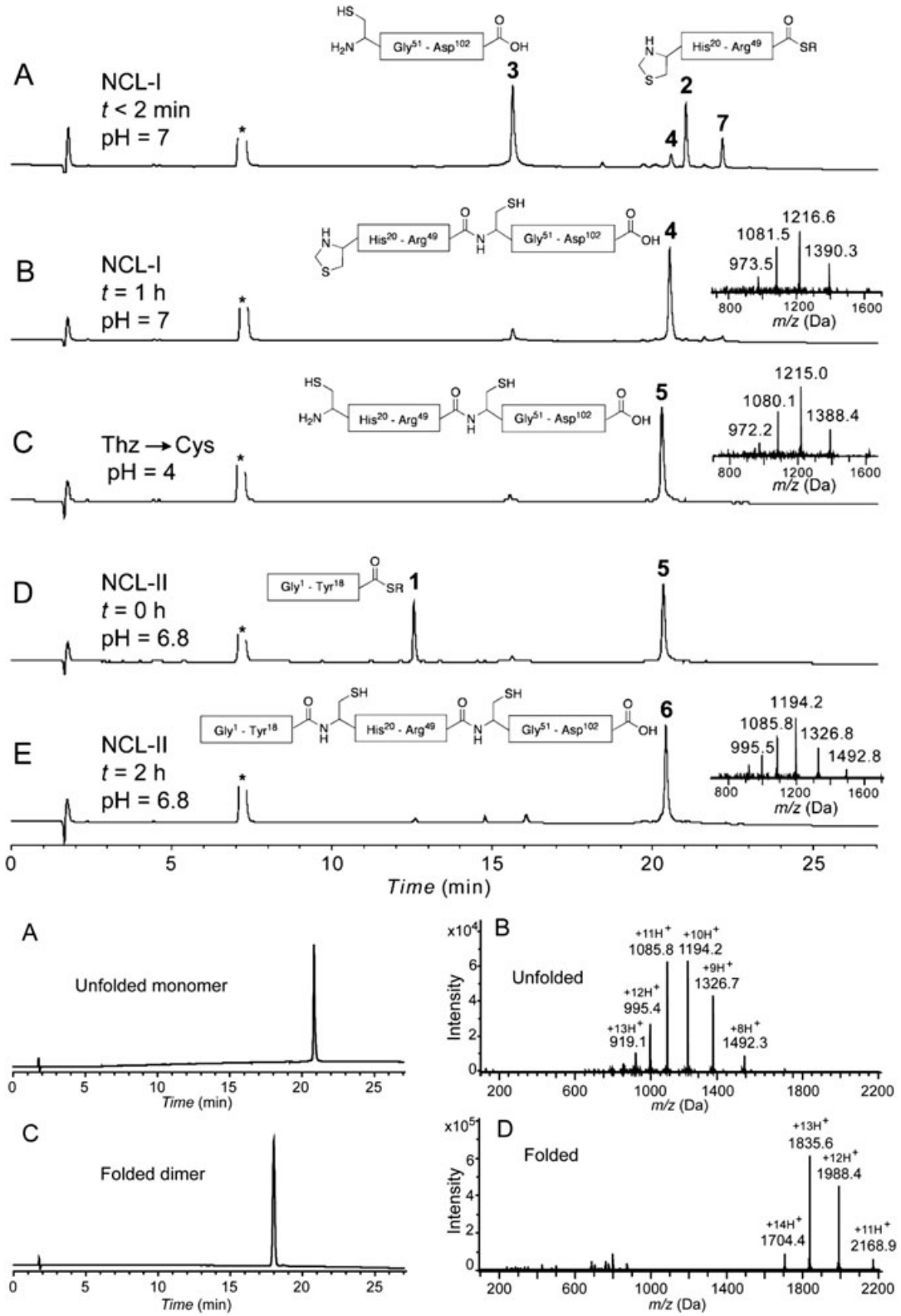


**Figure 8.** Total chemical synthesis of VEGF-A. The observed mass of the unfolded monomer was 11,932.2 ± 0.7 Da; the observed mass of the folded synthetic VEGF-A protein molecule was 23,848.7 ± 1.2 Da (adapted from Ref. [29]).

  

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[21]; then, we applied kinetically controlled ligation to the synthesis of the key branched thioester intermediate, followed by intramolecular native chemical ligation and folding with concomitant formation of three disulfide bonds as shown by LCMS [27].

Our challenge was to determine the folded structure of this novel protein molecule. Attempts to crystallize the protein analogue on its own were not successful. The topological analogue ('topologue') protein molecule was readily crystallized from a quasi-racemic mixture with D-crambin, and the structure was solved by molecular replacement using inverted coordinates for L-crambin. The high-resolution X-ray data showed that the topological analogue had a unique folded structure in which the N-terminal of the polypeptide chain penetrated the macrolactam ring and is pinned in that position by two disulfide bonds (Figure 7) [27]. Facile crystallization of the quasi-racemic protein mixture was key to determination of the structure of this novel topological protein analogue.

## Mirror-Image Protein Therapeutics

> "*A method is described that uses a biologically encoded library for the identification of D-peptide ligands, which should be resistant to proteolytic degradation. In this approach, a protein is synthesized in the D-amino acid configuration and used to select peptides from a phage display library expressing random L-amino acid peptides. For reasons of symmetry, the mirror images of these phage-displayed peptides interact with the target protein of the natural handedness.*"
> Peter S. Kim and associates [28]

In 1996, Peter Kim and colleagues at the Whitehead Institute at the Massachusetts Institute of Technology published a novel 'mirror image phage display' technique for the systematic development of D-peptide ligands for a natural protein target molecule [28]. This ingenious method uses the mirror-image D-protein form of target protein molecule to screen phage-displayed peptide libraries and select high-affinity peptide ligands. The selected peptide ligands are chemically synthesized in mirror–image D-peptide form; because of the reciprocal nature of chiral interactions at the molecular level, these D-peptides will have the same specificity and affinity for the natural L-protein form of the target molecule. The limiting feature of this approach to the development of D-peptide ligands is the ability to synthesize the natural target as a D-protein molecule.

In collaboration with the Sachdev Sidhu Lab at the University of Toronto, we have set out to extend Kim's approach to screen phage-displayed protein libraries against the mirror-image D-protein form of vascular endothelial growth factor (D-VEGF-A). First, we set out to establish efficient chemical synthesis of L-VEGF-A (Figure 8) [29]. The VEGF-A protein molecule is a covalent homodimer: each 102 amino acid residue monomer polypeptide chain has three intrachain disulfides, and there are two disulfide bonds connecting the monomers in the VEGF-A molecule. The target 102 residue polypeptide chain was made from three synthetic peptide segments by sequential 'one pot' native chemical ligation reactions. A single purification step gave high purity product with the correct mass. Folding was carried out at pH 8.4 in aqueous 0.15 M guanidine·HCl in the presence of a redox couple and, over a period of several days, gave a near quantitative yield of folded VEGF-A with the correct mass by direct infusion electrospray mass spectrometry [29]. Synthetic L-VEGF-A was fully active in a human umbilical vein endothelial cell proliferation assay; the chemically synthesized protein was crystallized, and its X-ray structure was determined to 1.8 Å resolution and was identical, within experimental error, to the reported structure of recombinant VEGF-A [29].

The mirror-image protein D-VEGF-A has been prepared using a similar synthetic strategy and screened against phage-displayed libraries of variants of a small protein scaffold. These results will be reported elsewhere [30].

## Conclusion

> *"The chemical ligation approach... breaks the conceptual shackles imposed by the peptide bond, frees us from the linear paradigm of the genetic code, and opens the world of proteins to the entire repertoire of chemistry."*
> S. Kent and associates [31]

Total protein synthesis based on modern chemical ligation methods enables the reproducible preparation of protein molecules of a size that less than 20 years ago was considered to be inaccessible to chemistry. Key to this ability is the covalent chemical condensation of unprotected peptide segments by means of an *unnatural* link between the two reacting segments. In the native chemical ligation refinement of this approach, the initial thioester-linked intermediate rearranges to give a native amide bond at the ligation site. Enzymes can be prepared in crystalline form and with full catalytic activity. An efficient route to the total chemical synthesis of human insulin and analogues has been devised on the basis of the use of an ester-linked synthetic intermediate that folds in quantitative yield and that can be simply converted in high yield to fully active human insulin. Thus, two of the grand challenges of synthetic protein chemistry have been successfully realized.

Modern chemical ligation methods have also enabled the design and total chemical synthesis of 'synthetic erythropoiesis protein', a glycoprotein mimetic of defined chemical structure with a molecular weight of 50,825 g/mol that has full biological activity and an extended half-life *in vivo*.

Most significantly, chemical protein synthesis enables the routine preparation of 'mirror-image' D-protein molecules. Mirror-image enzyme molecules display reciprocal chiral specificity – a D-enzyme will act only on the mirror image of the natural substrate. The highly ordered crystals necessary for determination of high-resolution X-ray structures form with great ease from racemic protein mixtures. This facile crystallization of racemic protein mixtures overcomes the principal obstacle to determination of protein structures by X-ray diffraction and has proven useful for the determination of the structure of novel proteins and novel protein analogues. Mirror-image protein molecules can also be used to develop D-peptide (and D-protein) ligands specific for the natural form of a target protein molecule. Such D-peptide molecules are invisible to biochemical metabolism and may have advantages as human therapeutics.

In the future, we can anticipate the elucidation of important biological questions enabled by chemical protein synthesis and the broader application of mirror-image proteins to the development of novel human therapeutics.

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