De Novo Design of Biomedical Polymers: Hybrids from Synthetic Macromolecules and Genetically Engineered Protein Domains

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SUMMARY: Genetic engineering technology provides a powerful tool for producing tailor-made protein polymers with pre-determined three-dimensional structures because exact control of the primary structure, composition and chain length can be achieved by manipulating the DNA sequence encoding the protein. Two approaches for the de novo design of bio-based systems using the coiled coil protein motif are discussed. One approach was to design hybrid hydrogels from synthetic macromolecules and coiled coil domains as crosslinks. This permits to impose the properties of the protein motif on the properties of the whole hydrogel. Temperature induced phase transition of the coiled coil domain resulted in a collapse of the hydrogel. The second approach was to construct a scaffold for epitope display based on genetically engineered peptides self-assembled on polystyrene surfaces. This construct may serve as a model for biorecognition studies with the aim to identify optimal ligand-receptor pairs. Such information is necessary for a rational design of targeted polymeric drug delivery systems.

Introduction

Traditional ways of biomaterials synthesis produced numerous materials with excellent properties. However, these synthetic pathways do not permit an exact control of chain length, sequence, and three-dimensional structure. For example, hydrogel biomaterials [1] have been studied for the last 45 years [2,3]. In recent years research has been concentrated on stimuli-sensitive hydrogels, i.e., materials whose degree of swelling is sensitive to small environmental changes, such as changes in pH [4,5], temperature [6-8], light [9], electric field [10], and biochemical stimuli [11]. The design of intelligent materials has to be based on reproducible synthesis resulting in a well-defined structure. Nonetheless, 45 years after the first synthesis of hydrogels [1] their detailed structure is far from being understood. The main problem is in the side reactions occurring during their synthesis. The most important ways to synthesize hydrogels are the crosslinking copolymerization [12], crosslinking of polymeric precursors [13], and polymer-polymer reactions [14]. Due to side reactions, networks

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prepared by these synthetic methods contain cycles, unreacted pendant groups, and entanglements [12-14].

Different synthetic methods result in different detailed structure of hydrogels [15]. The detailed structure of a hydrogel may influence its properties. For instance, biorecognition of ligands in hydrogels by enzymes is influenced by the structure of the ligand, the equilibrium degree of swelling, and the detailed structure of the network [16]. Degradation of hydrogels based on copolymers of N,N-dimethylacrylamide (which contain azoaromatic groups in the crosslinks) by azoreductase activity in the gastrointestinal tract depends on the method of synthesis [15].

It appears that new ways for the controlled synthesis of biomaterials are needed. The rapidly developing genetic engineering technology provides powerful tools for producing tailor-made biomaterials with predetermined three-dimensional structures, because exact control of the primary structure, composition and chain length of protein biomaterials can be achieved by manipulating the DNA sequence encoding the protein structure [17,18].

Genetically Engineered Biomaterials

Genetic engineering, or recombinant DNA technology, is being employed to produce protein-based biomaterials [19,20]. First, a structure of a target macromolecule (protein) is designed and translated into the genetic code. Then a gene encoding the target protein is synthesized chemically. For a longer or more complicated gene, it can be constructed either by step-wise ligation or polymerase chain reaction (PCR). Next the gene is ligated into a plasmid vector, a special circular DNA molecule, which is able to propagate with host cells. The production of the target protein by the host cells, most often bacteria, can be triggered or induced at any time. The expressed protein may accumulate as a soluble form in the cytoplasm, as an insoluble form in inclusion bodies, or may be secreted into the periplasm of the bacteria or into the culture media. One of the most convenient methods of purifying recombinant proteins is immobilized metal affinity chromatography (IMAC), which uses Ni(II) ions immobilized on agarose beads to preferentially bind target proteins containing terminal histidines.

There are distinct advantages of biosynthesis over chemical synthesis [17]. It is possible to obtain protein products with a very narrow and even uniform molecular weight distribution, whereas chemical synthesis inevitably results in a mixture of products with different chain lengths (Fig. 1). In biosynthesis, by defining the DNA sequence of the gene and by changing

the cell culture composition, exact control over the composition and stereochemistry of the target protein can be achieved. Through similar methods uncommon amino acids or amino acid analogs can also be incorporated into the protein chains [21,22]. By cutting and pasting gene segments through routine molecular biology procedures, different protein domains can be easily assembled, rearranged, and modified to generate new chimeras with novel or improved functions [23].

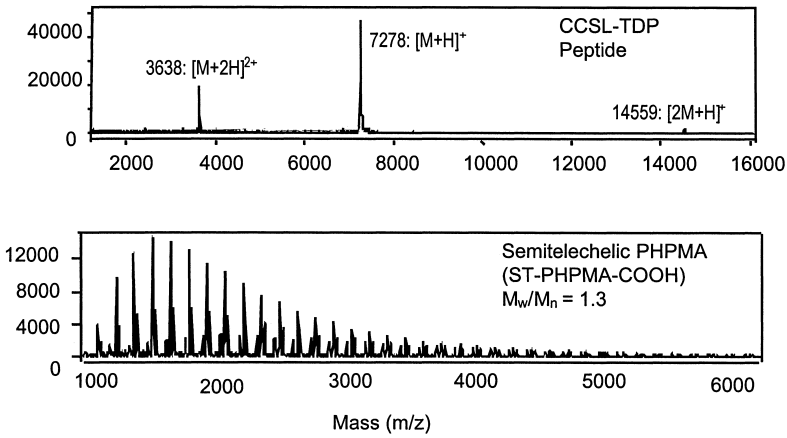


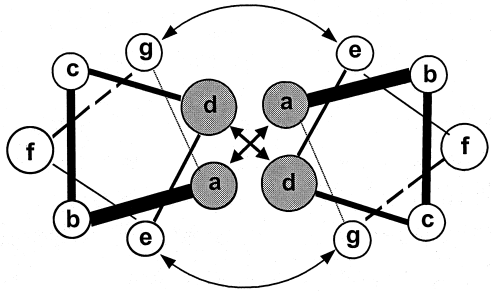
Fig. 1: Analysis of molecular weight distribution by matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS). a) Genetically produced coiled coil stem loop peptide (CCSL-TDP) [24]; b) semitelechelic poly[N-(2-hydroxypropyl)methacrylamide] prepared by radical polymerization in the presence of chain transfer agent (3-mercaptopropionic acid) [25].

Biological synthesis of protein-based biomaterials has already been used to create analogs of silk [26] and elastin [27], materials with more precise control of the three-dimensional structures [28], and molecules for biorecognition [29]. As the molecular basis of natural protein materials, such as that of the spider dragline silk [30], begins to unravel, the rational design of artificial protein materials will be greatly facilitated [31]. This field is now witnessing tremendous advances, and will probably play an integral role in the future of biomaterials development and research.

The Coiled Coils

One of the folding patterns of native proteins is called a coiled coil. It consists of two or more right-handed α-helices winding together and forming a slightly left-handed super-helix [31].

The primary structure of the coiled coil motif [32] is characterized by a heptad repeating sequence (Fig. 2) designated as "a, b, c, d, e, f, g", in which "a" and "d" are usually hydrophobic amino acid residues, while the others are polar. Two helices associate through a hydrophobic interface between "a" and "d" making "b", "c", and "f" face outward. More than two hundred native proteins such as muscle proteins, transcription factors, cytoskeletal proteins, cell and viral surface proteins, tumor suppressors, molecular motors, and many disease- and organ-specific auto-antigens have coiled coil domains which play important roles in the proper functioning of those proteins [33].



a-b-c-d-e-f-g-(a-b-c-d-e-f-g)$_n$-a-b-c-d-e-f-g

Fig. 2: A helical diagram of a parallel coiled coil homodimer. Residues "a" and "d" form the hydrophobic interface (thick arrows) which is the major stabilizing factor of the coiled coil structure. Electrostatic interactions between residues "e" and "g" (thin arrows) provide specificity for coiled coil association.

The distinctive feature of coiled coils is the specific spatial recognition, association, and dissociation of helices, making it an ideal model of protein biomaterials in which the higher structure may be predetermined by knowing the primary sequence. Consequently, various functional groups may be exactly positioned allowing specific intermolecular interactions to occur. Coiled coils based on native sequences or designed *de novo* have been used in the assembly of high avidity multivalent antibodies [34-36], the construction of conformationally defined combinatorial libraries [37,38], and the design of model proteins that mimic their natural counterparts [39,40] or the production novel proteins of biological and therapeutic value [41,42]. Here we report on the use of coiled-coil motifs in the design and synthesis of hybrid hydrogels and self-assembled epitope displays.

Hybrid Hydrogels

Hydrogels [1] as well as soft contact lenses [43] were designed by Wichterle and Lím. A detailed study of the relationship between the structure of hydrogels and their biocompatibility [44-48] resulted in their successful use in human medicine [49].

Hybrid hydrogels are usually referred to as hydrogel systems whose components are at least two distinct classes of molecules, for example, synthetic polymers and biological macromolecules, interconnected either covalently or noncovalently. They have been of particular interest, because it is possible to combine and superimpose the properties of the component molecules onto the hydrogels [50]. Water-soluble synthetic polymers have been crosslinked with molecules of biological origin, such as with oligopeptides [16,51,52], oligodeoxyribonucleotides [53], or with intact native proteins [54]. Very often there are several factors influencing the structure-property relationship of these systems making it difficult to engineer responsive hydrogels with predetermined properties [55].

We have designed new hybrid hydrogels assembled from water-soluble synthetic polymers and a well-defined protein folding motif, the coiled-coil. We found that these hydrogels underwent temperature-induced collapse due to the cooperative conformational transition of the coiled-coil protein domain. This system offers the possibility of using well-characterized water-soluble synthetic polymers, yet allows the hydrogel properties to be controlled and engineered with well-defined folding motifs of proteins [55].

Design of the hybrid hydrogel system [55]: Copolymers of N-(2-hydroxypropyl)methacrylamide (HPMA) [56] and a vinyl metal-chelating monomer N-(N',N'-dicarboxymethylaminopropyl)methacrylamide (DAMA), poly(HPMA-*co*-DAMA), were synthesized by radical copolymerization [55].

Two coiled-coils, CC1 and CC2, were studied. CC1 was a segment of the stalk region of *Drosophila* motor protein, kinesin. The cDNA of CC1 was extracted and amplified by Polymerase Chain Reaction (PCR) using a coding primer (5'-GGTCTAGAGTGGTCTGCGTTAACGAG-3') and a non-coding primer (5'-CCCCGGCGAGTCCAGCCTCGAGCC-3'). The PCR product was subcloned into the *NheI* and *AvaI* sites of a pET21a expression vector. CC2 contains a *de novo* designed coiled-coil sequence ($[VSSLESK]_6$), in which valine and leucine occupy the first and the fourth positions of the heptad repeating unit [57]. Specific charge patterns were also engineered in order to favor coiled-coil homodimerization. Complementary oligonucleotides encoding this sequence were designed using codons abundantly found in bacteria, chemically synthesized, selectively annealed and ligated to the *BamHI* and *EcoRI* sites of the pRSETB expression vector. Both DNA constructs were verified by direct sequencing. CC1 and CC2 with terminal histidine-tags were then expressed in *Escherichia coli* BL21(DE3)pLysS cells using isopropyl-β-thiogalactoside (IPTG) as an inducing agent. The proteins were purified from soluble cell lysate by Ni-NTA metal affinity chromatography. Their identity and purity were

verified by SDS-PAGE, amino acid analysis, and MALDI-TOF MS. Circular dichroism spectra of CC1 and CC2 indicated a highly α-helical coiled-coil conformation. Thermal melting study showed that CC1 unfolds cooperatively with a major mid-point temperature (T_m) of 35°C, whereas CC2 does not unfold below 90°C.

The two proteins were separately attached to poly(HPMA-*co*-DAMA) through a metal complex formed by the protein terminal histidine residues, Ni^{2+} ion, and iminodiacetate ligands from the HPMA copolymer backbone. A highly swollen hydrogel was formed upon coiled-coil multimerization (Fig. 3). Gels assembled using CC1 underwent a sharp volume transition upon heating with a mid-point around 39°C, which is in good agreement with the T_m of CC1 in solution. In contrast, gels containing CC2 did not show any appreciable change in volume [55].

These results seem to indicate that the properties of well-defined coiled-coil protein motifs can be imposed onto a hybrid hydrogel with synthetic polymeric backbones. It adds a new dimension to the field of "smart" hydrogel-based biomaterials providing the immense potential of tailoring material properties using genetically engineered proteins [50].

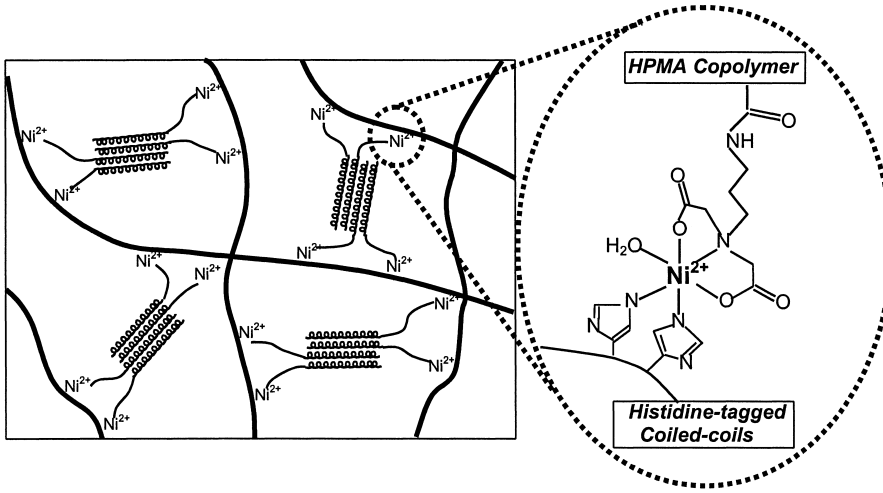


Fig. 3: Schematic structure of a hybrid hydrogel: water-soluble synthetic macromolecular chains (dark lines) are crosslinked through engineered coiled-coil protein domains. Chemical structure of the crosslink is shown in details.

Genetically Engineered Scaffolds for Display of Receptor Binding Epitopes

Multivalency: During the last decade we have designed, developed, and evaluated targetable HPMA copolymer - anticancer drug conjugates [reviewed in 58]. Various targeting moieties, such as carbohydrates [59], antibodies [60] or antibody fragments [61] have been used to achieve the biorecognizability of these conjugates. One of the main advantages is the possibility of attaching numerous biorecognizable moieties to one macromolecule. The biorecognition of HPMA copolymers containing side-chains terminated in N-acylated galactosamine by the asialoglycoprotein receptor was dependent on the amount of bound ligand [59]. A similar advantage of multivalent interactions (cooperative binding) was observed in the inhibition of virus-mediated agglutination of erythrocytes by polyacrylamides with pendant sialoside groups [62], lectin recognition of HPMA copolymers with pendant fucosylamine residues [63], and BCL_1 mouse lymphoma binding of short peptides fused via a semi-rigid hinge region with the coiled-coil assembly domains into a multivalent binding molecule [36].

Epitopes recognizable by immunocompetent cells: The Epstein-Barr virus (EBV) gp350/220 envelope glycoprotein mediates virus attachment to the EBV/C3dg receptor on human B lymphocytes [64] and specific binding to some receptors on human T cell lymphomas [65]. Two regions of amino acid similarity were found in the gp350 and C3d coding sequences and it was suggested that they may represent CD21 (CR2) binding sites of gp350/220. It was shown that multimeric forms of the N-terminal gp350/220 peptide, composed from nine amino acid residues (EDPGFFNVE), conjugated to albumin efficiently blocked recombinant gp350/220 and C3dg binding to B cells [64].

Recently, the biorecognition of HPMA copolymers containing the nonapeptide epitope were studied [66]. Attachment of the nonapeptide (NP) via a tetrapeptide (GFLG) side-chain to HPMA copolymers resulted in a tridecapeptide epitope (GFLGEDPGFFNVE) and in a modified biorecognition by B and T cells [66]. These conjugates interacted with Raji B-cells and CCRF-HSB-2 T-cells in a multipoint attachment mode, indicating that a cooperative effect occurred. A conjugate containing four tridecapeptide epitopes per HPMA copolymer chain possessed a binding activity (constant) that was one order of magnitude higher than the binding activity of a conjugate containing one epitope per macromolecule [66]. These data strongly suggested the possibility to manipulate the structure of epitopes with the aim to produce targetable drug delivery systems suitable for the treatment of lymphomas.

Genetically engineered scaffolds for epitope display: Detailed studies are needed to find the optimal epitope-receptor pair for the best biorecognition and targeting to immunocompetent cells. To this end, we established a model for biorecognition studies based on an epitope presentation scaffold – a coiled-coil stem loop (CCSL) peptide self-assembled on a solid substrate [24,37,67]. The CCSL peptide self-assembly represents a feasible model of exposing epitopes for biorecognition studies. A His-tagged CCSL peptide containing a tridecapeptide (TDP) epitope GFLGEDPGFFNVE (CCSL-TDP) was designed and synthesized genetically [24]. This peptide (Fig. 4) self-assembled in the presence of nickel on a polystyrene substrate covalently grafted with an N-(2-hydroxypropyl)methacrylamide copolymer containing nitrilotriacetic acid groups. The selective binding of Raji B cells to the CCSL-TDP coated surface but not to control surfaces indicated that cell attachment was mediated by the peptide, most probably by the loop domain [24,67]. It appears that the peptide self-assembly may represent a feasible model of displaying epitopes for biorecognition studies.

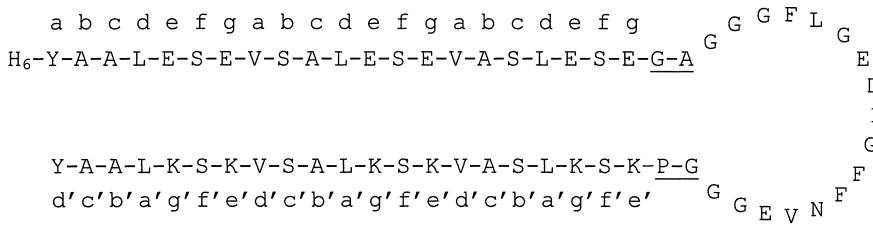


Fig. 4: The amino acid sequence of the CCSL-TDP peptide containing the TDP epitope in the loop region (one letter abbreviations were used for amino acid residues). For MALDI-TOF MS spectra see Fig. 1A. Adapted from [24].

Design of an antiparallel coiled-coil stem loop protein: The double-stranded cDNA, encoding the antiparallel CCSL-TDP peptide sequence, was chemically synthesized as six single-stranded oligonucleotides between 64 to 75 nucleotides long. The complementary strands were annealed to form three duplexes in which the one in the middle was phosphorylated. The three duplexes were ligated to the *NdeI* and *EcoRI* sites of the expression vector pRSETB. The correct construct was verified by restriction enzyme digestion and direct sequencing [24]. The two ends of the sequence of the complementary DNA encoding the coiled coil stem loop (CCSL) peptide matched the restriction enzyme sites of *NdeI* and *EcoRI*, respectively so that the DNA segment could be readily inserted into a plasmid vector digested with these two enzymes. Several other unique restriction enzyme sites were incorporated for easy detection

of the successful insertion. The *KasI* and *ApaI* sites corresponding to the hinges of the peptide were specially designed for later modification of the loop [24].

E. coli BL21(DE3) cells were transformed with the recombinant vector and the target peptide expression was induced by IPTG. The target peptide was purified by metal affinity chromatography, analyzed by SDS-PAGE for purity and MALDI-TOF MS for the correct molecular mass (Fig. 1A). The secondary structure of the peptide in solution was characterized by circular dichroism (CD).

Modification of a solid substrate with a copolymer containing Ni $^{2+}$-chelating groups: Polystyrene was chosen as a substrate. Attachment of the polymer containing Ni^{2+}-chelating groups to the substrate surface by physical and chemical methods has been studied. Based on preliminary data the covalent attachment of the surface modifying copolymer was chosen [24]. A monomer containing a photoreactive benzophenone (BP) group, 4-methacrylamido benzophenone (MABP), and a comonomer containing a nitrilotriacetic acid (NTA) group, 2-methacrylamidobutyl nitrilotriacetic acid (MABNTA), were synthesized [24]. Copolymerization of HPMA, MABNTA, and MABP gave a copolymer containing two kinds of functional groups: NTA and BP. NTA is a tetradentate ligand that forms stable bonds with hexacoordinate Ni^{2+}.

The copolymer was attached to the surface by UV-initiated photografting. Specifically, a copolymer solution in PBS was added to a Falcon 1008 polystyrene Petri dish and then exposed to 366nm UV irradiation for 1 h. The dish was washed with water, ethanol and again with water to remove unbound copolymer. The grafting of the copolymer was visualized by a significant increase in the water wettability of the surface (the static water contact angle changed from 83 deg before grafting to 50 deg after grafting). The dish was then treated with NaOH solution followed by $NiSO_4$ solution to form a surface containing complexed Ni. N1s and Ni2p3 peaks in the ESCA spectrum indicated that the copolymer was bound to the surface and the surface contained complexed Ni [24].

Formation of AP-CCSL self-assembled monolayers and interaction of displayed epitopes with lymphocytes: The self-assembly was designed to produce a defined surface that exposes an epitope, which would be recognized by (corresponding) receptor positive cells [24]. This defined biorecognizable surface consists of a self-assembled layer of AP-CCSL-TDP epitope, which is anchored to a metal-chelating synthetic polymer coated substrate through Ni^{2+} complexation (Fig. 5).

The Raji cells selectively bound to the surface containing self-assembled CCSL-TDP peptide but not to control surfaces [24]. This indicates that there are no specific interactions between

the cells and the polystyrene substrate, the copolymer, or a coiled coil peptide that does not contain the epitope. Therefore, the cell attachment was mediated by the CCSL-TDP peptide, most probably by the TDP epitope in the loop domain. Not surprisingly, when the cell density was increased, more cells were available to interact with the exposed surface ligands, thus more cells attached to the self-assembled CCSL-TDP surface [24].

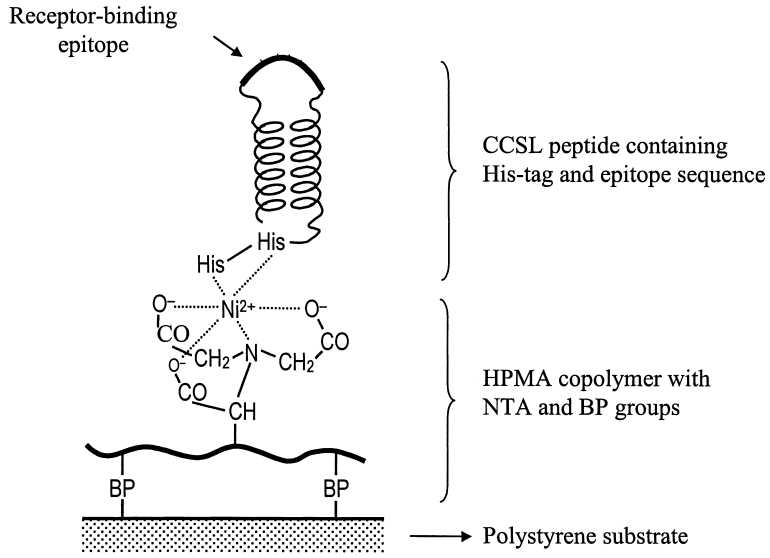


Fig. 5: Schematic representation of the CCSL peptide self-assembly on polystyrene substrate. Modified from ref. 24.

To test the role of the TDP region and the specificity of cell interaction with the CCSL-TDP peptide, a cell attachment study was also performed on different surfaces with adsorbed CCSL-TDP peptide. In the absence of nickel, the CCSL-TDP peptide probably interacts with the copolymer surfaces mainly through nonspecific interactions rather than Ni-His chelation involving the His-tag region. Since the peptide was not well oriented to expose the loop domain on the surface, fewer cells attached. The absence of cells on the polystyrene-CCSL surface may indicate that the nonspecific interaction between the peptide and polystyrene results in the TDP region being buried rather than being exposed on the surface. This is not unreasonable considering that the loop region is slightly more hydrophobic than the stem regions where the hydrophobic residues tend to be located in the interface of the α-helices.
In summary, the current data [24,67,68] seem to suggest that the CCSL peptide self-assembly on a solid substrate may represent a feasible model for displaying epitopes for biorecognition studies.

Conclusions

The design and synthesis of genetically engineered polymeric biomaterials is a reflection of biological approaches to the solution of materials science and engineering problems. The possibility to design new materials with exactly predetermined structures and properties will lead this field beyond copying structures developed by Mother Nature. New structures, not found in nature, will emerge as smart biomaterials and drug delivery systems. The design of a right-handed four-stranded coiled coil motif with repeating sequences of eleven amino acid residues is just one example of the potential of this approach. The predicted structure of the motif was correct to 0.2 Å [69].

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References

1. O. Wichterle, D. Lím, *Nature* **185**, 117 (1960)
2. Y. Osada, J. Gong, *Prog. Polym. Sci.* **18**, 187 (1993)
3. N.A. Peppas, *Curr. Opin. Colloid Interface Sci.* **2**, 531 (1997)
4. J. Kopeček, J. Vacík, D. Lím, *J. Polym. Sci. A-1*, **9**, 2801 (1971)
5. P.F. Kiser, G. Wilson, D. Needham, *Nature* **394**, 459 (1998)
6. R. Yoshida, K. Uchida, Y. Kaneko, K. Sakai, A. Kikuchi, Y. Sakurai, T. Okano, *Nature* **374**, 240 (1995)
7. G. Chen, A.S. Hoffman, *Nature* **373**, 49 (1995)
8. Y.H. Bae, T. Okano, S.W. Kim, *Makromol. Chem., Rapid Commun.* **9**, 195 (1988)
9. A. Suzuki, T. Tanaka, *Nature* **346**, 345 (1990)
10. I.C. Kwon, Y.H. Bae, S.W. Kim, *Nature* **354**, 291 (1991)
11. E. Kokufuta, Y.Q. Zhang, T. Tanaka, *Nature* **351**, 302 (1991)
12. H. Brøndsted, J. Kopeček, *Biomaterials* **12**, 584 (1991).
13. P.-Y. Yeh, P. Kopečková, J. Kopeček, *J. Polym. Sci., Part A: Polym. Chem.* **32**, 1627 (1994)
14. H. Ghandehari, P. Kopečková, P.-Y. Yeh, J. Kopeček, *Macromol. Chem. Phys.* **197**, 965 (1996)
15. P.-Y. Yeh, P. Kopečková, J. Kopeček, *Macromol. Chem. Phys.* **196**, 2183 (1995)
16. V. Šubr, R. Duncan, J. Kopeček, *J. Biomat. Sci. Polym. Ed.* **1**, 261 (1990)
17. J. Cappello, *Trends Biotechnol.* **8**, 309 (1990)
18. K.P. McGrath, M.J. Fournier, T.L. Mason, D.A. Tirrell, *J. Am. Chem. Soc.* **114**, 727 (1992)
19. F.A. Ferrari, J. Capello, in: *Protein-Based Materials*, K. McGrath, D. Kaplan (Eds.), Birkhäuser, Boston 1997, p. 37
20. J.G. Tirrell, M.J. Founier, T.L. Mason, D.A. Tirrell, in: *Protein-Based Materials*, K. McGrath, D. Kaplan (Eds.), Birkhäuser, Boston 1997, p. 61
21. C.J. Noren, S.J. Anthony-Cahill, M.C. Griffith, P.G. Schultz, *Science* **244**, 182 (1989)
22. T.J. Deming, M.J. Fournier, T.L. Mason, D.A. Tirrell, *Macromolecules* **29**, 1442 (1996)
23. S.C. Stinson, *Chem. Eng. News,* July 16, 1990, p. 26
24. A. Tang, C. Wang, R.J. Stewart, J. Kopeček, *Bioconjugate Chem.* **11**, 363 (2000)
25. Z.-R. Lu, P. Kopečková, Z. Wu, J. Kopeček, *Bioconjugate Chem.* **9**, 793 (1998)
26. J. Cappello, J. Crissman, M. Dorman, M. Mikolajczak, G. Textor, M. Marquet, F. Ferrari, *Biotechnol. Prog.* **6**, 198 (1990)

27. D.T. McPherson, C. Morrow, D.S. Minehan, J. Wu, E. Hunter, D. Urry, *Biotechnol. Prog.* **8**, 347 (1992)
28. M.T. Krejchi, E.D.T. Atkins, A.J. Waddon, M.J. Fournier, T.L. Mason, D.A. Tirrell, *Science* **265**, 1427 (1994)
29. K. McGrath, D. Kaplan, *Mater. Res. Soc. Symp. Proc.,* **292**, 83 (1993)
30. A.H. Simmons, C.A. Michal, L.W. Jelinski, *Science,* **271**, 84 (1996)
31. D.A. Tirrell, *Science* **271**, 39 (1996)
32. A. Lupas, *Trends Biochem. Sci.* **21**, 375 (1996)
33. J.G. Adamson, N.E. Zhou, R.S. Hodges, *Curr.Biol.* **4**, 428 (1993)
34. P. Pack, A. Plückthun, *Biochemistry* **31**, 1579 (1992)
35. P. Pack, K. Müller, R. Zahn, A. Plückthun, *J. Mol. Biol.* **246**, 28 (1995)
36. A.V. Terskikh, J.-M. Le Doussal, R. Crameri, I. Fisch, J.-P. Mach, A.V. Kajava, *Proc. Natl. Acad. Sci. USA* **94**, 1663 (1997)
37. R. Miceli, D. Myszka, J. Mao, G. Sathe, I. Chaiken, *Drug Des. Discov.* **13**, 95 (1996)
38. N. Suzuki, I. Fujii, *Pept. Chem.* **34**, 437 (1996)
39. S.F. Betz, P.A. Liebman, W.F. DeGrado, *Biochemistry* **36**, 2450 (1997)
40. S. Lee, T. Kiyota, T. Kunitake, E. Matsumoto, S. Yamashita, K. Anzai, G. Sugihara, *Biochemistry* **36**, 3782 (1997)
41. D.H. Lee, J.R. Granja, J.A. Martinez, K. Severin, M.R. Ghadiri, *Nature* **382**, 525 (1996)
42. M.J.F. Waterman, J.L.F. Waterman, T.D. Halazonetics, *Cancer Res.* **56**, 158 (1996)
43. US 3,496,254 (1964), Czechoslovak Academy of Sciences, inv.: O. Wichterle.
44. L. Šprincl, J. Vacík, J. Kopeček, D. Lím, *J. Biomed. Mater. Res.* **5**, 197 (1971)
45. L. Šprincl, J. Kopeček, D. Lím, *J. Biomed. Mater. Res.* **5**, 447 (1971)
46. J. Kopeček, L. Šprincl, H. Bažilová, J. Vacík, *J. Biomed. Mater. Res.* **7**, 111 (1973)
47. L. Šprincl, J. Kopeček, D. Lím, *Calc. Tiss. Res.* **13**, 63 (1973)
48. J. Kopeček, L. Šprincl, Polymers in Medicine (Wroclaw) **4**, 109 (1974)
49. Z. Voldřich, Z. Tománek, J. Vacík, J. Kopeček, *J. Biomed. Mater. Res.* **9**, 675 (1975)
50. C. Wang, R.J. Stewart, J. Kopeček, in: *Polymers for Medical Applications in the Twenty First Century*, R.M. Ottenbrite, S.W. Kim (Eds.), Technomic, Lancaster, 2000, in press
51. K. Ulbrich, J. Strohalm, J. Kopeček, *Biomaterials* **3**, 150 (1982)
52. L. Chen, J. Kopeček, R.J. Stewart, *Bioconjugate Chem.* **11**, 734 (2000)
53. S. Nagahara, T. Matsuda, *Polymer Gels Networks* **4,** 111 (1996).
54. A.A. Obaidat, K. Park, *Pharmaceutical Res.* **13,** 989 (1996).
55. C. Wang, R.J. Stewart, J. Kopeček, *Nature* **397**, 417 (1999)
56. J. Kopeček, H. Bažilová, *Eur. Polym. J.* **9**, 7 (1973)
57. T.J. Graddis, D.G. Myszka, I.M. Chaiken, *Biochemistry* **32**, 12664 (1993)
58. J. Kopeček, P. Kopečková, T. Minko, Z.-R. Lu, *Eur. J. Pharm. Biopharm.* **50**, 61 (2000)
59. R. Duncan, L.C.W. Seymour, L. Scarlett, J.B. Lloyd, P. Rejmanová, J. Kopeček, *Biochim. Biophys. Acta* **880**, 62 (1986)
60. V. Omelyanenko, C. Gentry, P. Kopečková, J. Kopeček, *Int. J. Cancer* **75**, 600 (1998)
61. Z.-R. Lu, P. Kopečková, J. Kopeček, *Nature Biotechnol.* **17**, 1101 (1999)
62. M. Mammen, S.-K. Choi, G.M. Whitesides, *J. Am. Chem. Soc.*. **118**, 3789 (1996)
63. R.C. Rathi, P. Kopečková, J. Kopeček, *Macromol. Chem. Phys.* **198**, 1165 (1997)
64. G.R. Nemerow, R.A. Houghten, M.D. Moore, N.R. Cooper, *Cell* **56**, 369 (1989)
65. D. Watry, J.A. Hedrick, S. Siervo, G. Rhodes, J.J. Lamberti, J.D. Lambris, C.D. Tsoukas, *J. Exp. Med.* **173**, 971 (1991)
66. V. Omelyanenko, P. Kopečková, R.K. Prakash, C.D. Ebert, J. Kopeček, *Pharmaceutical Res.* **16**, 1010 (1999)
67. A. Tang, C. Wang, R.J. Stewart, J. Kopeček, *J. Control. Release,* (2001) in press
68. A. Tang et al., in preparation.
69. P.B. Harbury, J.J. Plecs, B. Tidor, T. Alber, P.S. Kim, *Science* **282**, 1462 (1998)