Cell Encapsulation

DOI: 10.1002/anie.200902375

A Metal–Collagen Peptide Framework for Three-Dimensional Cell Culture**

*Marcos M. Pires, David E. Przybyla, and Jean Chmielewski**

The development of biocompatible three-dimensional scaffolds that enable cellular encapsulation and cell growth is of critical importance in the emerging fields of tissue engineering and regenerative medicine. Most currently available scaffolds that have shown promise are composed of either nonbioactive polymers or naturally derived biopolymers.[1] Covalently cross-linked polymers based on polyethylene oxide (PEO), poly(L-lactide) (PLLA), and poly(lactide-co-glycolic acid) (PLGA) have also received attention for use in tissue engineering as a result of their biocompatibility and biodegradability.[2] Peptide-based materials that mimic aspects of the three-dimensional matrix of cells have emerged as promising alternative designs, such as self-assembling peptide amphiphiles,[3] α helices,[4] β sheets,[5] and β-amino acid helices.[6]

We report herein on the design of a metal-triggered, self-assembling collagen peptide capable of incorporating multiple functionality into a hybrid three-dimensional scaffold for cell culture. First, we considered it essential that the core of our scaffold be composed of short, readily synthesized monomers that are capable of self-assembling upon the addition of an external stimulus. Second, the trigger should be compatible with living cells and should cause the rapid self-assembly of a three-dimensional network capable of cell entrapment and suitable for cell growth. Third, the assembly of the scaffold should be fully reversible under mild conditions to enable the dissolution of the matrix away from cells and tissues embedded within it. As many covalently cross-linked polymers do not have this property, their application is limited. Lastly, we envisioned that our scaffold should be composed of a collagen-like material to ensure that it had similar physical and biomechanical properties to those of natural collagen. We could thus generate a scaffold that more closely mimicked the extracellular matrix.

Currently, natural collagen scaffolds have many applications in three-dimensional cell culture and tissue engineering.[7] Self-assembling synthetic peptides have been explored as an alternative source of collagen material in an attempt to mimic and expand on the properties associated with collagen. A number of cleverly designed self-assembling collagen-mimetic peptides have been described.[8] To date, however, these collagen-based self-assembling systems have not been evaluated as three-dimensional scaffolds for cell encapsulation and cell culture. We now describe a metal-triggered collagen mimetic that assembles rapidly under physiological conditions into a fibrous three-dimensional scaffold.

The initial peptide design, NHbipy, consisted of repeating Pro-Hyp-Gly sequences (Figure 1) and three distinct metal-binding units: a nitrilotriacetic acid (NTA) unit at the N terminus of the peptide, a His_2 sequence at the C terminus, and a bipyridyl (bipy) moiety at a central position within the peptide. At the center of the peptide, a bipyridine moiety was incorporated in the side-chain amino group of a Pro-Lys-Gly tripeptide. This tripeptide was flanked by two sets of four repeating units of Pro-Hyp-Gly. Thus, our design combined terminal and central metal ligands capable of binding multiple metal ions into a collagen triple helix to generate a highly cross-linked three-dimensional scaffold.

The folding of NHbipy into a collagen triple helix in solution was deemed essential for the convergence of the metal ligands at the termini and the central core of the peptide. Therefore, circular dichroism (CD) studies were performed to probe the conformation of the modified collagen peptide. NHbipy was found to display a maximum absorption at 225 nm, a value that is indicative of a polyproline helix found in collagen model peptides (see the Supporting Information). Thermal denaturation studies demonstrated that NHbipy forms a stable triple helix at room temperature with a melting temperature of approximately 40 °C (see the Supporting Information). These findings support the premise that neither the NTA/His_2 nor the bipyridine modification prevents the peptide from adopting a triple-helical structure.

Next, we set out to determine the ability of various metal ions to promote the self-assembly of NHbipy. Notably, all experiments were carried out in neutrally buffered solutions at room temperature. The addition of Ni^{II} to the NHbipy solution generated a turbid solution (Figure 1c). The assembly process is extremely rapid: the middle photograph in Figure 1c was taken approximately five seconds after the addition of $NiCl_2$. Similar results were observed for Co^{II}, Zn^{II}, and Co^{II}. To demonstrate that the metal ion mediated the assembly of the individual peptides, we performed chelation competition experiments with ethylenediaminetetraacetic acid (EDTA). When excess EDTA was added to the turbid solution containing Ni^{II}, the turbidity disappeared within minutes (Figure 1c).

The nature of metal-triggered NHbipy assembly was characterized by scanning electron microscopy (SEM). Sol-

[*] M. M. Pires,[+] D. E. Przybyla,[+] Prof. J. Chmielewski
Department of Chemistry, Purdue University
560 Oval Drive, West Lafayette, IN 47907 (USA)
Fax: (+1) 765-494-0239
E-mail: chml@purdue.edu

[+] These authors contributed equally.

[**] We are grateful to the NSF (0848325-CHE) for support of this research, to D. Sherman for assistance with SEM, and to E. Campanella for assistance with confocal microscopy.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200902375.

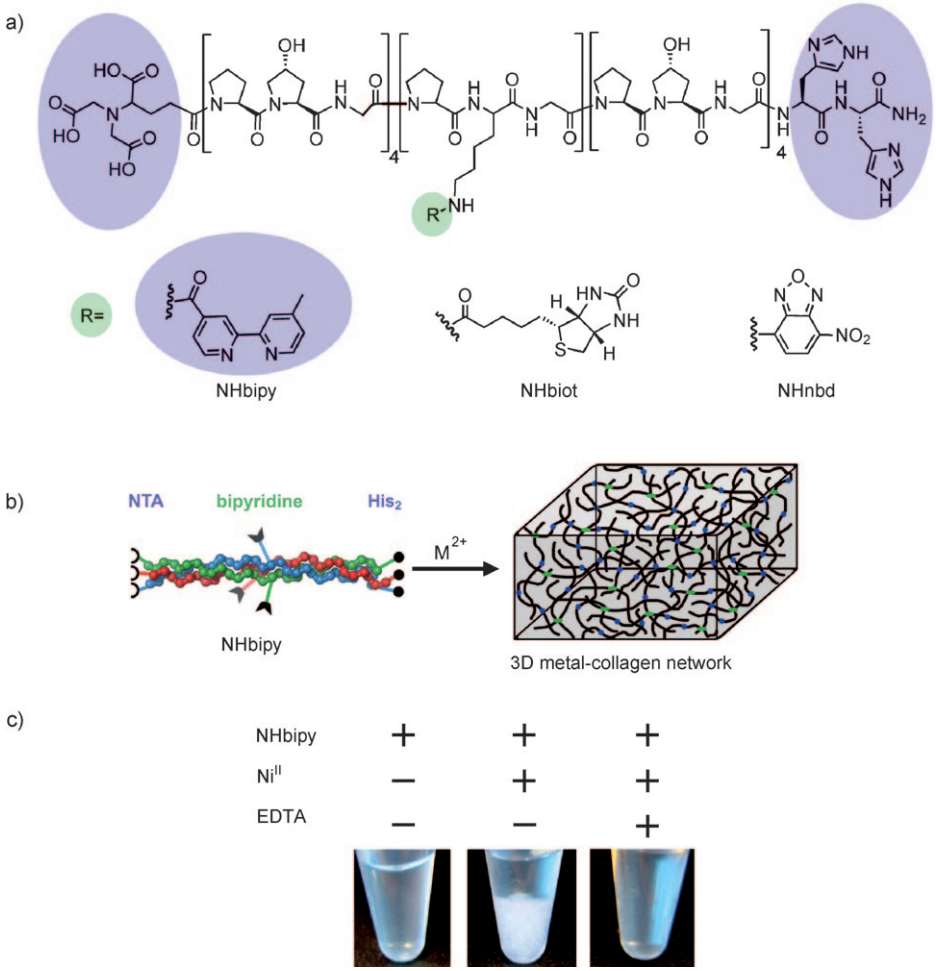

Figure 1. a) General structure of peptides NHbipy, NHbiot, and NHnbd (metal ligands are shaded in blue). b) Schematic representation of triple-helical NHbipy and its assembly into a cross-linked three-dimensional scaffold following the addition of metal ions. c) Photographs showing the visual change in a solution containing NHbipy (1 mM) upon the addition of $NiCl_2$ (2 mM). The addition of EDTA (1 mM) causes the disappearance of the turbidity.

utions containing NHbipy and various metal ions were imaged by SEM (Figure 2). Consistent with our design, the network was composed of highly cross-linked strands. Interestingly, some variation in the overall architecture was observed when the metal ion was varied. Specifically, Zn^{II} appeared to generate a less dense and more fibrous scaffold (Figure 2). By comparison, Co^{II}, Cu^{II}, and Ni^{II} all resulted in more cross-linking, with internal pores of approximately 5–20 μm in diameter. Although the use of different metal ions resulted in somewhat different morphologies of the network, the metal-to-peptide ratio appeared to have little or no effect on the type of cross-linked scaffold formed (see the Supporting Information).

The incorporation of two different metal ions in a single assembled species requires ligand systems with tunable affinities for different metal ions. As NHbipy contains both a bipyridine moiety and an NTA/histidine ligand system, it is conceivable that two distinct metal ions can be bound within the assembled network. We attempted to gain more control over the architecture of the scaffold by taking advantage of the dual-ligand assembly. We first used Ru^{II}, a metal ion expected to coordinate bipyridine but to leave the NTA and histidine ligands unbound. The Ru^{II} complex was generated by heating NHbipy (1 mM) with Ru^{III} (1 mM) at 90 °C for three hours. The peptide solution was then allowed to cool and refold into a triple helix. When this process (monitored by CD) was complete, Ni^{II}, Zn^{II}, Co^{II}, or Cu^{II} (1 mM) was added. These metals all have an affinity for the NTA and histidine ligands.

The resulting scaffolds were first examined by fluorescence microscopy (Figure 3a). A red fluorescent scaffold, indicative of a $(bipy)_3Ru^{II}$ complex, was observed.[9] Further characterization of the dual-metal scaffolds by energy-dispersive X-ray (EDX) spectroscopy showed that Ru^{II} was present along with the other metal ion (results not shown). The structure of these materials was characterized by SEM (Figure 3b) and compared to those obtained with single metals to determine whether the dual-metal scaffolds had unique architectures. The addition of Ru^{II} in combination with Cu^{II}, Zn^{II}, or Co^{II} was found to

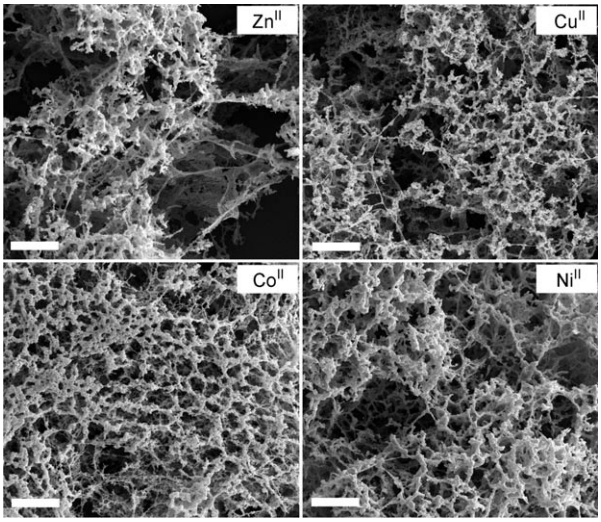

Figure 2. SEM images of the NHbipy peptide (1 mM) with specified metals (2 mM; scale bar: 5 μm).

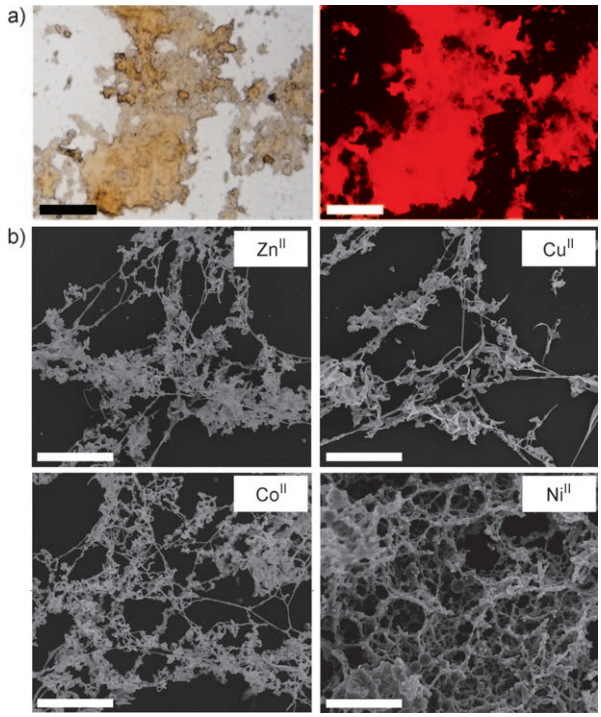

Figure 3. Incorporation of two distinct metal ions into the NHbipy scaffold. a) Bright field (left) and fluorescence (right) microscopy images of NHbipy (1 mM) with the dual-metal combination Ru^{II}/Ni^{II} (1 mM each; scale bar: 50 μm). b) SEM images of NHbipy (1 mM) with the dual-metal combinations Ru^{II}/Zn^{II}, Ru^{II}/Cu^{II}, Ru^{II}/Ni^{II}, and Ru^{II}/Co^{II} (1 mM in each metal; scale bar: 4 μm).

drastically affect the scaffold shape: the dual-metal materials were found to consist of long rope-like strands with significantly less cross-linking than that observed for the corresponding single-metal materials. The Ru^{II}–Ni^{II} scaffold more closely resembled that obtained with Ni^{II} alone, but with a somewhat more open morphology.

Following the discovery that NHbipy assembles rapidly into a fibrous mesh, we sought a general method that would enable the display of biofunctional moieties within the three-dimensional framework of the scaffold. Although NHbipy mimics an important component of the extracellular matrix (ECM), namely a three-dimensional collagen matrix, the ECM is notoriously heterogeneous and highly complex in composition. Consequently, for an NHbipy-based scaffold to be useful in the field of regenerative medicine, it should be modular to enable the integration and display of different components. To demonstrate this concept, we synthesized a peptide with a biotin handle in place of the bipyridine moiety (NHbiot, Figure 1). Since NHbiot retains the NTA/histidine "sticky" ends, we expected it to be incorporated reliably into the growing biopolymer of NHbipy; thus, the scaffold would be decorated with biotin. NHbiot was coincubated in varying concentrations with NHbipy and metal ions to generate scaffolds with the potential of displaying biotin moieties with increasing density. These scaffolds were treated with fluorescein-labeled streptavidin, and fluorescence microscopy images indicated that NHbiot was incorporated in the matrix (Figure 4). These results demonstrate that it is possible

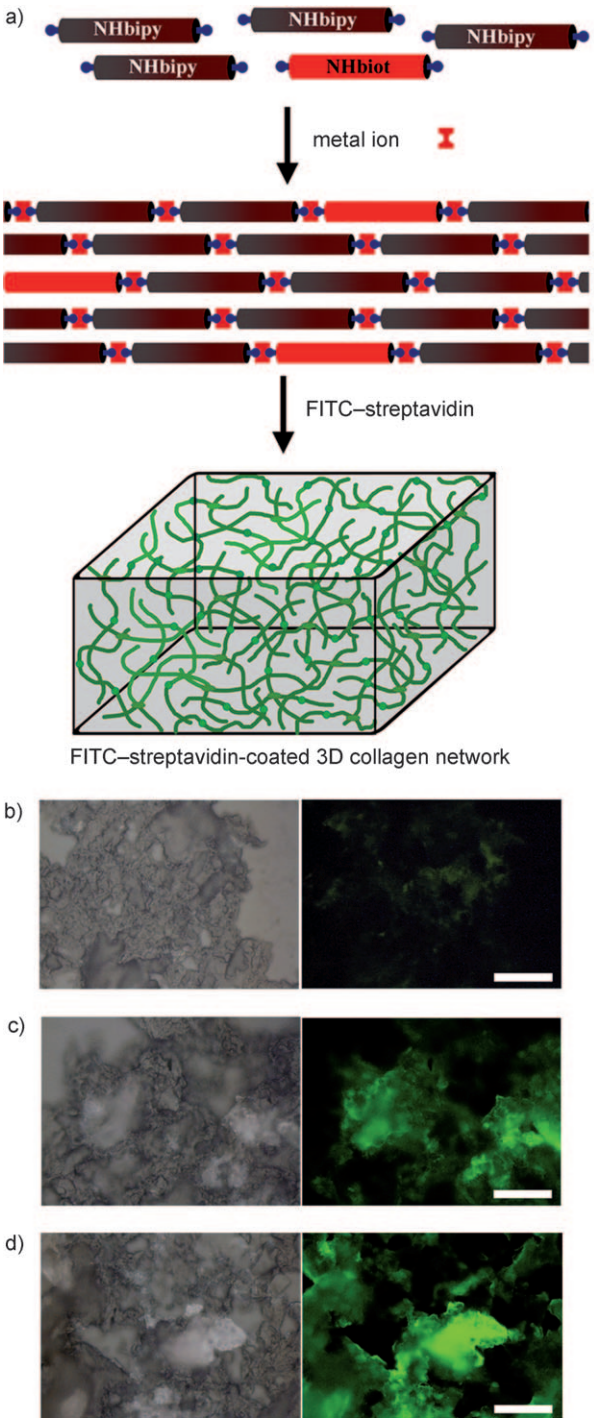

Figure 4. a) Overall strategy for the incorporation of biofunctionality within the three-dimensional matrix composed of NHbipy, NHbiot, and Ni^{II}. b–d) Bright field (left) and fluorescence (right) microscopy images of the network with increasing amounts of NHbiot: b) 0%, c) 16%, d) 33% (scale bar: 200 μm). Fluorescence results from the binding of FITC–streptavidin. FITC = fluorescein isothiocyanate.

to incorporate different functionalities into the three-dimensional matrix, and that these functional groups are surface-exposed to enable interaction with their respective binding partners. This particular example demonstrates that one could

potentially construct designer collagen scaffolds that display numerous biofunctional moieties with no additional steps in the assembly procedure.

Finally, we set out to determine whether the NHbipy-based matrices could be used to encapsulate cells, and whether they would enable normal cellular growth. Our previous results for the use of NHbiot in combination with NHbipy demonstrated that it is possible to incorporate other collagen peptides with central modifications and NTA/histidine ligands at the ends. We extended this strategy to a new peptide, NHnbd (Figure 1), which contains a nitrobenzoxadiazole (NBD) fluorophore anchored to the central lysine residue. Labeling of the assembled matrix with NHnbd could enable convenient tracking of the scaffold through the use of standard fluorescence microscopy techniques. Upon the addition of metal ions to a solution containing NHbipy (1 mM) with a small fraction of NHnbd (20 μM), a highly fluorescent collagen network was formed. This result further confirmed the possibility of incorporating different collagen sequences into the scaffold (see the Supporting Information).

To demonstrate that our collagen–metal framework was capable of cellular encapsulation, we added metal ions to a solution containing NHbipy/NHnbd and HeLa cells (stained with Hoechst 33342 nuclear dye) in Dulbeco modified Eagle medium with 10% serum. Fluorescence microscopy imaging confirmed that the fluorescently labeled NHbipy scaffold formed, and that it was capable of efficiently encapsulating cells with various metal ions (see the Supporting Information). With the Ni^{II} matrix, HeLa cells (blue nuclei) were found to be fully surrounded by the fibrous collagen-based network (green) and remained associated within the assembled matrix (Figure 5a). This result was confirmed by cryo-SEM imaging of the Ni^{II} matrix containing cells (Figure 5b). To confirm the viability of cells within the Ni^{II} matrix, we stained live HeLa cells with the acetomethoxy derivative of calcein (calcein AM) and performed MTS assays on the encapsulated cells (Figure 5c; see also the Supporting Information; MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium). The cells within the Ni^{II} scaffold showed similar viability to that of cells cultured on normal tissue-culture plates. Overall, we found that HeLa cells continued to proliferate and remain viable when encapsulated within the NHbipy scaffold even after several days of culture (monitored for 5 days). Furthermore, calcein AM staining showed that cells released from the Ni^{II} matrix by treatment with EDTA were also viable (see the Supporting Information).

In conclusion, we have designed a collagen-based peptide that self-assembles into a three-dimensional scaffold through the bidirectional coordination of metal ions. Assembly was triggered by a variety of metal ions, and it was possible to generate multiple alternative scaffold structures. The speed with which the NHbipy peptide assembles in the presence of metal ions under physiological conditions, and the subsequent disassembly of the scaffold upon treatment with a mild chelating agent, make precise temporal control of the construction of the scaffold possible with this system. This modular system was also extended to the incorporation of other collagen peptides on the basis of the NTA/histidine strategy to enable fluorescence tracking of the polymer and interaction with streptavidin. Most importantly, we demonstrated that this collagen-peptide scaffold encapsulates and retains human endothelial cells with no observable cytotoxic effects. Investigations are underway to probe the versatility of our scaffold for applications in tissue engineering and regenerative medicine.

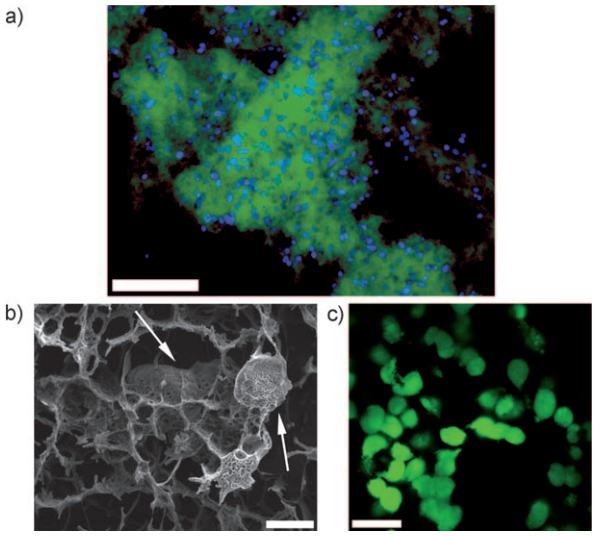

Figure 5. Visualization of cell encapsulation within NHbipy and NHbipy/NHnbd matrices. a) Fluorescence microscopy image of HeLa cells (blue nuclei) encapsulated within the NHbipy/NHnbd–Ni^{II} matrix (scale bar: 200 μm). b) Cryo-SEM image of HeLa cells encapsulated within the NHbipy/NHnbd–Ni^{II} matrix (scale bar: 10 μm). Representative sectioned cells imbedded in the matrix are indicated by arrows. c) Confocal microscopy image of encapsulated HeLa cells stained with calcein AM in the NHbipy–Ni^{II} matrix (scale bar: 20 μm).

Received: May 4, 2009
Revised: July 8, 2009
Published online: September 15, 2009

Keywords: cell encapsulation · collagen peptides · metal–organic frameworks · self-assembly · three-dimensional scaffolds

[1] a) C. Fischbach, H. J. Kong, S. X. Hsiong, M. B. Evangelista, W. Yuen, D. J. Mooney, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 399–404; b) L. G. Griffith, M. A. Swartz, *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 211–224; c) E. B. Lavik, H. Klassen, K. Warfvinge, R. Langer, M. J. Young, *Biomaterials* **2005**, *26*, 3187–3196.

[2] a) R. Langer, J. P. Vacanti, *Science* **1993**, *260*, 920–926; b) J. M. Anderson, M. S. Shive, *Adv. Drug Delivery Rev.* **1997**, *28*, 5–24; c) R. Langer, D. A. Tirrell, *Nature* **2004**, *428*, 487–492; d) H. J. Kong, D. J. Mooney, *Nat. Rev. Drug Discovery* **2007**, *6*, 455–463.

[3] J. D. Hartgerink, E. Beniash, S. I. Stupp, *Science* **2001**, *294*, 1684–1688.

[4] a) M. G. Ryadnov, D. N. Woolfson, *Nat. Mater.* **2003**, *2*, 329–332; b) M. Zhou, D. Bentley, I. Ghosh, *J. Am. Chem. Soc.* **2004**, *126*, 734–735; c) K. L. Lazar, H. Miller-Auer, G. S. Getz, J. P. Orgel, S. C. Meredith, *Biochemistry* **2005**, *44*, 12681–12689; d) Y. Zimenkov, S. N. Dublin, R. Ni, R. S. Tu, V. Breedveld, R. P. Apkarian, V. P. Conticello, *J. Am. Chem. Soc.* **2006**, *128*, 6770–

6771; e) H. Dong, S. E. Paramonov, J. D. Hartgerink, *J. Am. Chem. Soc.* **2008**, *130*, 13691 – 13695.
[5] a) S. G. Zhang, T. Holmes, C. Lockshin, A. Rich, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 3334 – 3338; b) L. Haines-Butterick, K. Rajagopal, M. Branco, D. Salick, R. Rughani, M. Pilarz, M. S. Lamm, D. J. Pochan, J. P. Schneider, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 7791 – 7796; c) K. Murasato, K. Matsuura, N. Kimizuka, *Biomacromolecules* **2008**, *9*, 913 – 918.
[6] W. C. Pomerantz, V. M. Yuwono, C. L. Pizzey, J. D. Hartgerink, N. L. Abbott, S. H. Gellman, *Angew. Chem.* **2008**, *120*, 1261 – 1264; *Angew. Chem. Int. Ed.* **2008**, *47*, 1241 – 1244.
[7] A. Alavi, D. G. Stupack, *Methods Enzymol.* **2007**, *426*, 85 – 101.
[8] a) S. E. Paramonov, V. Gauba, J. D. Hartgerink, *Macromolecules* **2005**, *38*, 7555 – 7561; b) F. W. Kotch, R. T. Raines, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 3028 – 3033; c) S. Rele, Y. H. Song, R. P. Apkarian, Z. Qu, V. P. Conticello, E. L. Chaikof, *J. Am. Chem. Soc.* **2007**, *129*, 14780 – 14787; d) M. A. Cejas, W. A. Kinney, C. Chen, J. G. Vinter, H. R. Almond, K. M. Balss, C. A. Maryanoff, U. Schmidt, M. Breslav, A. Mahan, E. Lacy, B. E. Maryanoff, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 8513 – 8518; e) D. E. Przybyla, J. Chmielewski, *J. Am. Chem. Soc.* **2008**, *130*, 12610 – 12611; f) M. M. Pires, J. Chmielewski, *J. Am. Chem. Soc.* **2009**, *131*, 2706 – 2712.
[9] A. Juris, V. Balzani, F. Barigelletti, S. Campagna, P. Belser, A. Vonzelewsky, *Coord. Chem. Rev.* **1988**, *84*, 85 – 277.

