DOI: 10.1002/anie.201100141

Bioconjugate Self-Assembly

Calcium Ions to Remotely Control the Reversible Switching of Secondary and Quaternary Structures in Bioconjugates**

*Romina I. Kühnle and Hans G. Börner**

Peptide–polymer conjugates have been in the focus of materials science for the last decade.[1–4] The versatility of synthetic polymers and the precision of peptides combine to give an interesting class of polymers. These biohybrid systems find applications as specific drug transporters, bioactive cell-growth scaffolds, morphogenetic systems for biomineralization, and components for nanoelectronics.[5–8]

Peptide segments in bioconjugates are able to guide self-assembly of various synthetic polymers in aqueous and organic media.[9–12] While gaining the possibility to program hierarchical superstructures, the regulation of the self-assembly processes is still rather limited.[13] Established triggers to manipulate peptide secondary structures are changes in pH value, temperature, or redox potential as well as enzymatic modification of peptides and metal-ion coordination to peptides.[14–17] Nevertheless, transfer of these principles to bioconjugates is not always straightforward.

Recently, approaches to advance the control of bioconjugate self-assembly were described. These involve 1) the incorporation of temporary structure defects ("switch segments") into the peptide of bioconjugates to inhibit β-strand formation. A minor change in pH value removed the defects and triggered self-assembly.[10] 2) Enzymatic dephosphorylation of phosphorylated peptide segments in a bioconjugate switched the system from non-aggregating to an active state.[18] Moreover, 3) protecting-group strategies were employed to activate self-assembly of bioconjugates, for example, by light or pH changes that trigger release of the protective groups.[19]

However, the established routes are irreversible and hence do not provide the possibility of a reversible switching process. In contrast, binding of metal ions is reversible and plays a critical role in protein function control by stabilizing certain structures or introducing conformational transitions.[20,21] Several model peptides have shown secondary-structure transitions upon metal-ion complexation.[22–24] Owing to their defined complexation behavior, transition metals such as Cu^{2+}, Zn^{2+}, Fe^{2+}, or Rh^{2+} were usually employed.[25–27]

Herein we report on a novel regulative mechanism for reversibly controlling the function of a peptide aggregator domain in peptide–polymer conjugates using calcium ions. While Ca^{2+} was previously used to cross-link preformed peptide fibrils,[28] the presented strategy involves control at the molecular peptide level. Activation and deactivation of the self-assembly function of bioconjugates is the result of a Ca^{2+}-controlled transition in peptide secondary structure, which triggers self-assembly or disassembly of fibrillar peptide–polymer nanostructures (Figure 1).

The bioconjugate **I** was synthesized by automated solid-phase-supported peptide synthesis, combining a poly(ethylene oxide) block (PEO, $M_n \approx 3200$) with an $(EL)_5nFG$ peptide segment. The peptide sequence consisted of five

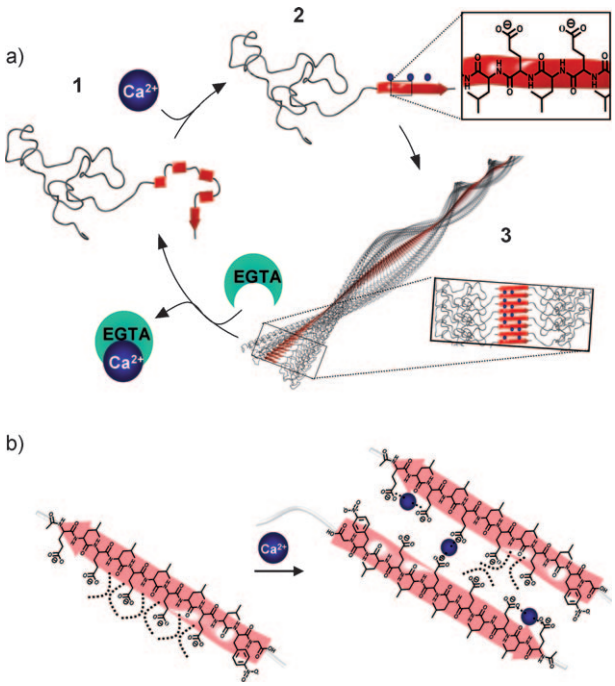


Figure 1. a) Idealized mechanism for the calcium-ion-regulated self-assembly of peptide–polymer conjugates (polymer depicted as a gray coil, peptide as red segments): 1) Deactivated bioconjugate without Ca^{2+}; the peptide adopts a random coil. 2) The addition of Ca^{2+} (blue spheres) stabilizes the peptide segment in a β strand that can self-assemble into twisted fibrils with β sheet core and polymer shell (3). Ca^{2+} can be removed using ethylene glycol tetraacetic acid (EGTA) competitive binders, which leads to disassembly of the fibrils; b) Intramolecular Coulomb repulsion destabilizes β-strand formation and is partially overcome by Ca^{2+} addition. (Chemical structures not drawn to scale.)

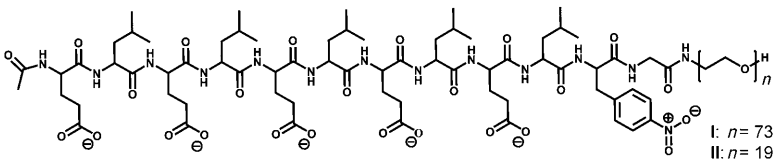
[*] R. I. Kühnle, Prof. Dr. H. G. Börner
Laboratory of Organic Synthesis of Functional Systems
Department of Chemistry, Humboldt Universität zu Berlin
Brook-Taylor-Strasse 2, 12489 Berlin (Germany)
Fax: (+49) 30-2093-7266
E-mail: h.boerner@hu-berlin.de

[**] The work was supported by the Center of Supramolecular Interactions Berlin (CSI Berlin), DFG (Emmy Noether BO1762/2) and the Max Planck Society. We thank Markus Antonietti for his support and K. Ostwald, J. Brandt, A. Heilig, and R. Pitschke for their contributions to the project.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201100141.

repeating diads of glutamic acid (E) and leucine (L). The alternating hydrophilic–hydrophobic pattern in the peptide sequence creates a high propensity to adopt a β-sheet secondary structure.[29] However, destabilization occurs because the carboxylic acid side chains of the glutamic acid residues are positioned in close proximity (Figure 1b). Above pH ≈ 5 the negative charges of the deprotonated COOH moieties shift the propensity for β-sheet formation towards statistical chain-segment conformation. At the C-terminal end, *para*-nitrophenylalanine (*n*F) was introduced as spectroscopic marker, and glycine residues provide a flexible spacer between the peptide and the PEO block. An inverse conjugation strategy on a PAP resin (PEO-attached polystyrene resin) using standard Fastmoc coupling was applied to synthesize the peptide–PEO conjugate. The fully deprotected bioconjugate was liberated from the support, and the chemical structure of **I** was confirmed by MALDI-TOF mass spectrometry and ^{1}H NMR and FTIR spectroscopy (see the Supporting Information).

The bioconjugate **I** was readily soluble in 0.17 mM tris(hydroxymethyl)aminomethane (TRIS)/HCl buffer (pH 8.9). Circular dichroism (CD) spectroscopy confirms that **I** adopts a statistical-coil conformation by showing the characteristic negative Cotton effect at $\lambda = 198$ nm (Figure 2). The CD spectrum of **I** without addition of Ca^{2+} remains stable for a period of at least five weeks (see the Supporting Information, Figure S5), thus suggesting a sufficient suppression of β-sheet formation. This conclusion was supported by atomic force microscopy (AFM) and transmission electron microscopy (TEM), which provided no evidence for the presence of anisotropic fibrillar nanostructures (see Figures S6 and S7).



To increase the self-assembly tendency of the peptide–PEO conjugate, different amounts of calcium chloride were added to solutions of **I**. Calcium ions were chosen owing to their biocompatibility. Moreover, Ca^{2+} interaction with carboxylates is known to be strong, as binding is driven by both enthalpy and entropy.[30] The addition of Ca^{2+} may screen the negative charges of the carboxylates and thus enable the peptide segment to adopt a β strand, which triggers self-assembly of the bioconjugate. The structural transition from random coil to β sheet could be monitored by CD spectroscopy (Figure 2; see also Figure S8). About five minutes after addition of five molar equivalents Ca^{2+} per COOH group of the bioconjugate, the characteristic Cotton effects for a β-sheet structure were evident ($\lambda = 195$ (+) and 216 nm (−)). The intensity of the signals increased with increasing calcium ion concentration and reached a maximum at 8 equiv Ca^{2+} per COOH (see Figure S4).

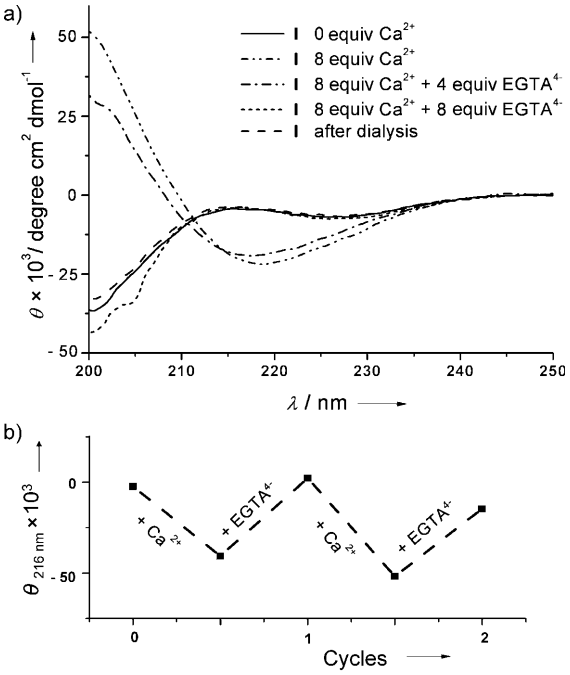


Figure 2. a) CD spectra of **I** before and after addition of Ca^{2+} and subsequently of EGTA. b) Cycling diagram of sequential secondary structure transitions of **I**, prepared by monitoring the CD signal change of the characteristic negative Cotton effect for a β-sheet structure at 216 nm, depending on repetitive Ca^{2+} and $EGTA^{4-}$ addition. (After the last EGTA addition, the structural transition occurred within three days to reach about 70% conversion.)

TEM and AFM analysis of the solutions of **I** prior to and after addition of 8 equiv Ca^{2+} per COOH were consistent with the CD data. While no anisotropic structures were found in the absence of Ca^{2+}, the addition of Ca^{2+} resulted in fibrils several hundreds of nanometers in length (Figure 3). Interestingly, TEM revealed a helical fine structure of the fibrils, and a trend to form stiff helical dimers was evident (inset Figure 3).

To prove the reversibility of both secondary and quaternary structure, the sodium salt of EGTA was added to the solution with the bioconjugate fibrils. EGTA is a competitive

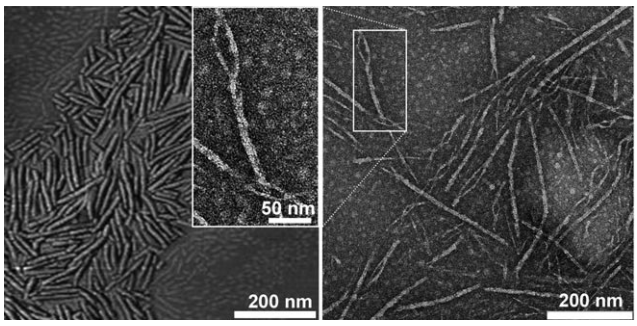


Figure 3. Twisted fibrils of conjugate **I** after addition of eight equivalents of calcium ions. Left: AFM phase image ($z = 150$ mV) on mica substrate; right TEM image (negatively stained).

Ca^{2+} binder, and CD spectroscopy confirmed a transition in peptide secondary structure (β sheet→coil) upon EGTA addition (Figure 2). This indirect evidence for fibril disassembly was confirmed by TEM and AFM, neither of which provided evidence for the presence of extended fibrillar structures (see Figures S6 and S7). The system could be successfully brought back to the starting point by using dialysis to quantitatively remove the Ca^{2+}–EGTA complexes from the bioconjugate solution. After dialysis, CD spectroscopy showed a bioconjugate with a peptide segment that adopted a statistical chain-segment conformation (Figure 2). Interestingly, the switch is fully reversible, and multiple cycles can be repeated even without dialyzing off the regulators (Ca^{2+}–EGTA). Figure 2b shows that two on/off cycles can be performed. The last transition from β sheet to random coil took three days to reach about 70% conversion. This effect might be due to the system being overloaded with salt, partially shielding the charges of the glutamic acid side chains (see Figure S9).

Taking the observations into account, a mechanism for calcium-ion-regulated reversible self-assembly of **I** can be proposed (Figure 1). The negative net charge of the peptide segment of the bioconjugate prevents self-assembly. Owing to inter- and intramolecular Coulomb repulsion, the bioconjugate adopts a random-coil structure. After Ca^{2+} ion addition, the negative charges are partially screened. Thus the peptide can form a β strand, which proceeds to form fibrillar β-sheet structures. Similar to assemblies of congener bioconjugates, the facial amphiphilicity of the peptide–polymer β sheet leads to interfibrillar association, thus generating more complex structures. It is noteworthy that extended β-sheet assemblies are usually low-energy states, and their formation is frequently not reversible. However, the design of the peptide sequence apparently provides enough driving force to promote disassembly upon removal of the Ca^{2+} regulator.

The postulated mechanism is in good agreement with the observed fibril dimensions. For conjugate **I** nanofibers with a rather uniform height of about (6.1 ± 1.0) nm could be confirmed by AFM. TEM showed very persistent fibers with widths around (10.0 ± 1.4) nm. These fibers consist of two entwined fibrils with widths of about (4.5 ± 1.1) nm and thus similar dimensions as observed previously with comparable PEO–peptide nanotapes (Figure 3 and Figures S6 and S7).[31]

To elucidate the impact of the length of the PEO block on the Ca^{2+}-regulated self-assembly process, a second conjugate **II** was synthesized. This conjugate comprised an identical peptide sequence but a shorter PEO block with $M_{n,PEO} \approx 800$ (see the Supporting Information). The conjugate **II** was readily soluble at pH 8.9. CD spectroscopy revealed a peptide segment in an unstructured random coil, which was stable for at least six months. In contrast to the 8 equiv Ca^{2+} per COOH necessary to effectively switch the secondary structure of **I**, the bioconjugate **II** required as little as 0.5 equiv Ca^{2+} per COOH to rapidly trigger the structural transition (coil→β sheet, see Figures S4 and S5). A better stabilization of the peptide segment could be expected from the longer PEO block, which prevents self-assembly. As **II** exhibits a shorter PEO block than **I**, the random-coil conformation is less stabilized. Thus, **II** requires a less intense Ca^{2+} trigger (see Figure S4).

AFM images show fibers with rather uniform heights around (5.3 ± 0.6) nm. TEM revealed twisted fibers with widths of about (9.4 ± 1.4) nm. These stiff objects are composed of fibrils with (6.1 ± 1.4) nm widths. Despite the more sensitive switching, the disassembly of fibers composed of **II** was feasible by the competitive complexation of Ca^{2+} with EGTA. The transition in secondary structure was confirmed by CD (Figure 4a). AFM and TEM measurements

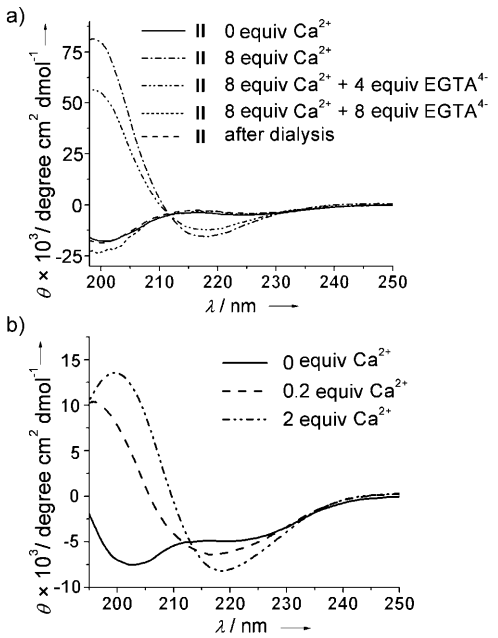


Figure 4. CD spectra showing conjugate **II** before and after addition of calcium and subsequently of EGTA (a) and non-PEGylated peptide before and after calcium addition (b).

supported the CD data, showing disassembly of fibrillar structures (see Figure S6, S7). As for conjugate **I**, conjugate **II** could also be brought back to its starting point by removing the Ca^{2+}–EGTA complexes from the bioconjugate solution by dialysis. As depicted in Figure 4a, the conjugate **II** adopts a random-coil conformation after dialysis (i.e., after an entire cycle).

Compared to the conjugates **I** and **II**, the peptide without PEO seems to have an intrinsic tendency to adopt the β-sheet structure. The CD spectrum of the denatured peptide prior to Ca^{2+} addition shows that the peptide exists partially with β-sheet structure, even if the random-coil structure dominates (Figure 4b). Furthermore, after aging, a 1 wt% solution of the peptide without calcium provides a clear, self-supporting hydrogel after several days (see Figure S10). The structural transition toward the β sheet can be accelerated by Ca^{2+} addition, as indicated by CD (Figure 4b). Consistent with the pioneering work of Mutter et al. on peptide PEGylation, the PEO stabilizes secondary peptide structures and slows down structural transitions (in the case of **I** and **II** the statistical segment conformation).[32] No secondary structure transition can be observed for weeks to months (see Fig-

ure S5). This finding suggests that the design of peptides with switchable structures requires amino acid sequences that possess a delicate balance between the two secondary structures.

In conclusion, it was demonstrated that introduction of negative charges into peptide aggregator domains of peptide–PEO conjugates effectively prevented their self-assembly. Calcium ion addition was shown to be a powerful tool to stimulate the formation of hierarchical nanostructures. The self-assembly process was fully reversible upon calcium ion removal by the addition of EGTA as competitive Ca^{2+} binder. This work might contribute to the understanding of the implication of calcium in amyloid formation and inspire new pathways to reversibly control micro- and nanostructure formation in hybrid materials.

Received: January 7, 2011
Published online: April 8, 2011

Keywords: bioconjugates · nanofibers · peptides · secondary structure · self-assembly

[1] H. G. Börner, *Prog. Polym. Sci.* **2009**, *34*, 811.
[2] H. D. Maynard, S. Y. Okada, R. H. Grubbs, *Macromolecules* **2000**, *33*, 6239.
[3] J. C. M. van Hest, D. A. Tirrell, *Chem. Commun.* **2001**, 1897.
[4] P. De, M. Li, S. R. Gondi, B. S. Sumerlin, *J. Am. Chem. Soc.* **2008**, *130*, 11288.
[5] H. G. Börner, *Macromol. Rapid Commun.* **2011**, *32*, 115.
[6] A. K. H. Hirsch, F. Diederich, M. Antonietti, H. G. Börner, *Soft Matter* **2010**, *6*, 88.
[7] S. Kessel, A. Thomas, H. G. Börner, *Angew. Chem.* **2007**, *119*, 9181; *Angew. Chem. Int. Ed.* **2007**, *46*, 9023.
[8] E. Jahnke, I. Lieberwirth, N. Severin, J. P. Rabe, H. Frauenrath, *Angew. Chem.* **2006**, *118*, 5510; *Angew. Chem. Int. Ed.* **2006**, *45*, 5383.
[9] T. S. Burkoth, T. L. S. Benzinger, D. N. M. Jones, K. Hallenga, S. C. Meredith, D. G. Lynn, *J. Am. Chem. Soc.* **1998**, *120*, 7655.
[10] J. Hentschel, E. Krause, H. G. Börner, *J. Am. Chem. Soc.* **2006**, *128*, 7722.
[11] J. Hentschel, H. G. Börner, *J. Am. Chem. Soc.* **2006**, *128*, 14142.
[12] L. Ayres, K. Koch, P. Adams, J. C. M. van Hest, *Macromolecules* **2005**, *38*, 1699.
[13] H. G. Börner, H. Kühnle, J. Hentschel, *J. Polym. Sci. Part A* **2010**, *48*, 1.
[14] A. Top, K. L. Kiick, C. J. Roberts, *Biomacromolecules* **2008**, *9*, 1595.
[15] C. J. Bowerman, B. L. Nilsson, *J. Am. Chem. Soc.* **2010**, *132*, 9526.
[16] R. J. Williams, R. J. Mart, R. V. Ulijn, *Biopolymers* **2010**, *94*, 107.
[17] K. Pagel, T. Vagt, B. Koksch, *Org. Biomol. Chem.* **2005**, *3*, 3843.
[18] H. Kühnle, H. G. Börner, *Angew. Chem.* **2009**, *121*, 6552; *Angew. Chem. Int. Ed.* **2009**, *48*, 6431.
[19] T. B. Yu, J. Z. Bai, Z. B. Guan, *Angew. Chem.* **2009**, *121*, 1117; *Angew. Chem. Int. Ed.* **2009**, *48*, 1097.
[20] Z. Grabarek, *J. Mol. Biol.* **2006**, *359*, 509.
[21] L. Belkacemi, I. Bedard, L. Simoneau, J. Lafond, *Cell Calcium* **2005**, *37*, 1.
[22] S. N. Dublin, V. P. Conticello, *J. Am. Chem. Soc.* **2008**, *130*, 49.
[23] J. J. Dong, J. E. Shokes, R. A. Scott, D. G. Lynn, *J. Am. Chem. Soc.* **2006**, *128*, 3540.
[24] Q. Y. Dai, C. Xiao, M. X. Dong, Z. G. Liu, Z. Y. Sheng, F. J. Castellino, M. Prorok, *Peptides* **2009**, *30*, 866.
[25] K. Pagel, T. Seri, H. von Berlepsch, J. Griebel, R. Kirmse, C. Böttcher, B. Koksch, *ChemBioChem* **2008**, *9*, 531.
[26] D. E. Przybyla, J. Chmielewski, *J. Am. Chem. Soc.* **2010**, *132*, 7866.
[27] A. N. Zaykov, B. V. Popp, Z. T. Ball, *Chem. Eur. J.* **2010**, *16*, 6651.
[28] M. A. Greenfield, J. R. Hoffman, M. O. de La Cruz, S. I. Stupp, *Langmuir* **2010**, *26*, 3641.
[29] W. X. Wang, M. H. Hecht, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 2760.
[30] C. G. Sinn, R. Dimova, M. Antonietti, *Macromolecules* **2004**, *37*, 3444.
[31] D. Eckhardt, M. Groenewolt, E. Krause, H. G. Börner, *Chem. Commun.* **2005**, 2814.
[32] V. N. Rajasekharan Pillai, M. Mutter, *Acc. Chem. Res.* **1981**, *14*, 122.

