Supramolecular Enzyme Hybrids

DOI: 10.1002/anie.201308533

Constructing Hybrid Protein Zymogens through Protective Dendritic Assembly**

*David Y. W. Ng, Matthias Arzt, Yuzhou Wu, Seah Ling Kuan, Markus Lamla, and Tanja Weil**

Abstract: *The modulation of protein uptake and activity in response to physiological changes forms an integral part of smart protein therapeutics. We describe herein the self-assembly of a pH-responsive dendrimer shell onto the surface of active enzymes (trypsin, papain, DNase I) as a supramolecular protecting group to form a hybrid dendrimer–enzyme complex. The attachment is based on the interaction between boronic acid and salicyl hydroxamate, thus allowing the macromolecular assembly to respond to changes in pH between 5.0 and 7.4 in a highly reversible fashion. Catalytic activity is efficiently blocked in the presence of the dendrimer shell but is quantitatively restored upon shell degradation under acidic conditions. Unlike the native proteases, the hybrid constructs are shown to be efficiently taken up by A549 cells and colocalized in the acidic compartments. The programmed intracellular release of the proteases induced cytotoxicity, thereby uncovering a new avenue for precision biotherapeutics.*

The application of stimulus-responsive protective groups offering precise control of protein activity is an appealing approach in the preparation of protein-based drugs with potentially high therapeutic index.[1] In this context, nature introduces peptidic protecting groups to proteases (trypsin, pepsin, caspases) resulting in the formation of zymogens, which facilitate efficient intra/extracellular transportation and prevent unintended cellular degradation.[2] We were inspired by this transcendental efficacy and considered that the introduction of synthetic macromolecular protecting groups would provide a contemporary chemical perspective by masking the function of an enzyme through steric bulk prior to exposure to a designated stimulus.

Proteases play a central role in cancer progression and metastasis, regulating processes such as proliferation, apoptosis, differentiation, and evasion of the immune system.[3] The delivery of proteases is important in the elucidation of their function as well as the development of new protein therapeutics. As such, the design of a synthetic strategy incorporating a responsive "assemble and release" system mimicking the biosynthesis of zymogens represents an ideal model. Importantly, the innate proteolytic activity of proteases precludes them from most biological delivery systems (i.e. cell-penetrating peptides, fusion proteins).

The modification of active proteases to include stimulus-responsive moieties is challenged by protein denaturation and post-modification inactivation as well as by their chemoselectivity which often necessitates the use of mild bioorthogonal approaches.[4] Conventionally, site-specific modification through single-residue mutation has been developed to incorporate thermoresponsive steric handles near the catalytic site acting as a temperature-dependent switch.[5–7] This concept subsequently evolved into chemical strategies towards the development of protein–polymer conjugates assembled using in situ atom-transfer radical polymerization (ATRP).[8,9] However, the site-specific mutation of different classes of enzymes is often challenging owing to their highly diverse activity and stability, whereas metal-catalyzed reactions on proteins are plagued by the adsorption of metal contaminants. As a result, the application of these enzymatic constructs is still highly confined and underdeveloped.

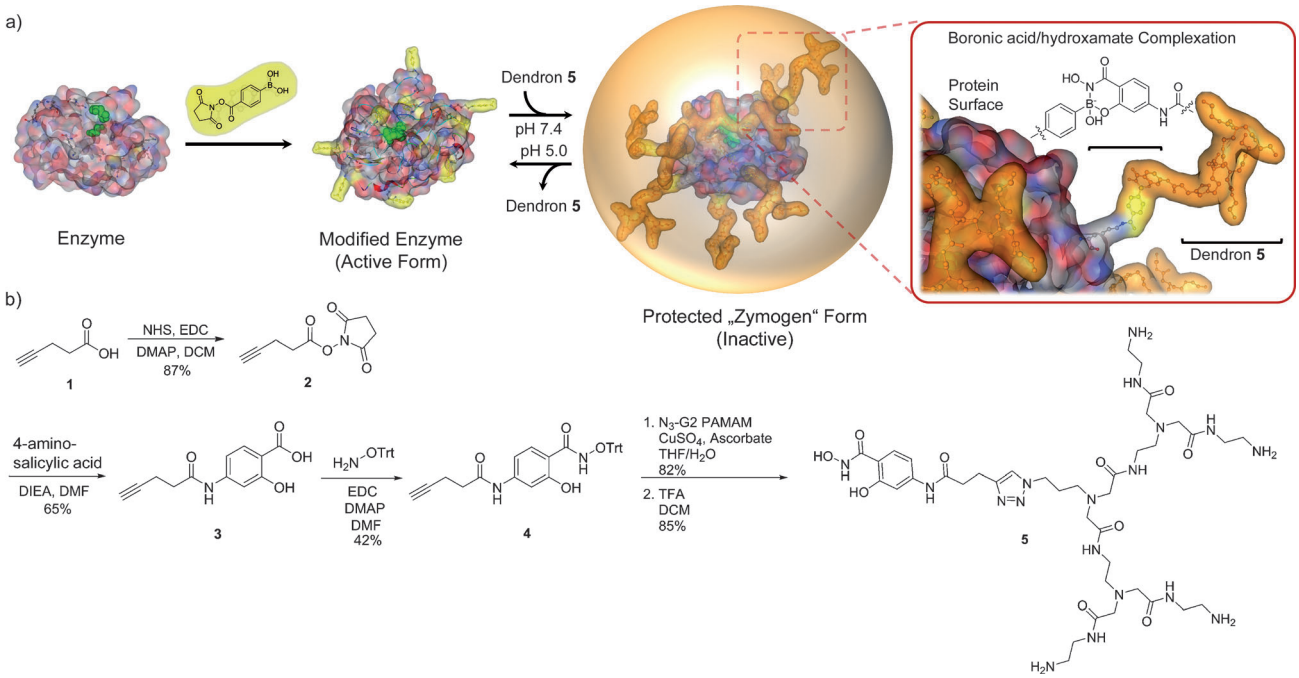
In this context, macromolecular protecting groups involving metal-free reactions under physiological conditions for the protection/deprotection of enzyme catalytic sites offer a distinct advantage over existing systems. Inspired by recent results on engineered aptamers[10] as macromolecular protecting groups, we report herein the use of a stimulus-responsive dendritic core–shell system that self-assembles into a supramolecular dendrimer–enzyme complex in a facile and bioorthogonal manner. Small defined dendrimer segments (dendrons) provide a high volume to molecular weight ratio due to their branched structure and thus serve as an ideal candidate as a sterically demanding, bulky protecting group.[11] In addition, the dendrons confer additional pharmacologically attractive properties (increased uptake, potency)[12] depending on their surface functionalities.[13] These dendrons are self-assembled onto the enzyme utilizing an acid-labile boronic acid/salicyl hydroxamate based ligation method, resulting in a pH-responsive dendrimer–enzyme hybrid which functions as a synthetic zymogen (Scheme 1 a). We have chosen catalytically unique enzymes (trypsin, papain, DNase I), each representing an enzyme class with high therapeutic significance, to demonstrate the broad applicability of this strategy.[3]

The synthesis of the dendrimer protective group is based on the bifunctional second-generation poly(amido)amine (PAMAM) dendron bearing a single azido group as well as four primary amino groups (N_3-G2 PAMAM, Scheme 1b)

[*] D. Y. W. Ng, M. Arzt, Y. Wu, Dr. S. L. Kuan, M. Lamla, Prof. Dr. T. Weil
Institute of Organic Chemistry III, Ulm University
Albert-Einstein-Allee 11, 89081 Ulm (Germany)
E-mail: tanja.weil@uni-ulm.de
D. Y. W. Ng, Prof. Dr. T. Weil
Max Planck Institute for Polymer Research
Ackermannweg 10, 55128 Mainz (Germany)

[**] This work was supported by the Deutsche Forschungsgemeinschaft (DFG) within the SFB 625, the BMBF project Biotechnologie 2020+, and the ERC Synergy Grant 319130-BioQ. S.L.K. is grateful to the Alexander von Humboldt Foundation for providing a research fellowship.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201308533.



Scheme 1. a) Synthesis and supramolecular dendritic assembly for the construction of hybrid zymogens. Catalytic residues are highlighted in green. b) Synthesis of the salicyl hydroxamate core dendron.

which has been described previously.[14] The azido core group of the dendron was converted into a salicyl hydroxamate moiety by a copper-catalyzed azide–alkyne cycloaddition. Salicyl hydroxamates have been previously reported to "click" with aryl boronic acids to form a stable boronate complex.[15] The kinetics and orthogonality of this fast complexation have been studied and utilized to perform efficient peptide ligation.[16] To construct the dendron core group, 4-pentynoic acid was activated by *N*-hydroxysuccinimide and coupled with 4-aminosalicyclic acid to afford **3**. Condensation of the ethynyl salicyclic acid **3** with trityloxyamine in excess afforded **4** in moderate yield. The resulting protected ethynyl hydroxamic acid underwent copper-catalyzed cycloaddition with the Boc-protected azido G2 PAMAM dendron with high yield. Subsequent deprotection using trifluoroacetic acid in the presence of triisopropylsilane as a radical scavenger afforded the final product **5** as a trifluoroacetate salt.

The native enzymes trypsin, papain, and DNase I were functionalized by condensation of their surface lysine residues with an excess of preactivated 4-carboxyphenylboronic acid (Scheme 1 a). As the catalytic domains of the proteases primarily do not involve lysine residues, the surface lysines become an attractive target for modification.[17–19] The modified proteins were purified using ultrafiltration and characterized by MALDI-TOF mass spectrometry (Figure S1 in the Supporting Information). Trypsin, papain, and DNase I were functionalized with an average of ten, ten, and four boronic acid residues, respectively, due to their different numbers of solvent-accessible lysines. The degree of functionalization for trypsin and papain is 90%, whereas only 50% of the surface lysines were modified for DNase I. These modified proteins were subsequently assayed for their catalytic activity, which was found to be $>80\%$ of that of their native counterparts (Figure S2).

The construction of the dendritic assembly with dendron **5** was monitored using a fluorescence assay based on Alizarin Red S. Alizarin Red S complexes with each aryl boronic acid on its catechol moiety quantitatively to produce a fluorescence complex ($\lambda_{ex}=495$ nm, $\lambda_{em}=600$ nm).[20] As observed, the fluorescence quenching upon the addition of dendron **5** corresponds to the displacement of Alizarin Red S from the protein surface (Figure 1 a). The affinity constants of boronic acid/catechol and boronic acid/salicyl hydroxamate at pH 7.4 were reported to be $800\,\text{M}^{-1}$ and $17\,800\,\text{M}^{-1}$, respectively,[16,21] allowing the quantification of the number of boronic acid groups and dendrons bound on each enzyme molecule through a fluorescence-based titration. For each protein, the endpoint of the titration with dendron **5** (10 equiv for trypsin and papain, 4 equiv for DNase I) corresponds to the results obtained earlier by MALDI-TOF analysis.

To evaluate the protection efficiency of the dendritic assembly, the proteolytic activity of the boronic acid modified proteases (trypsin and papain) were tested and quantified using N_α-benzoyl-L-arginine 4-nitroanilide hydrochloride as the substrate. The dendrons were first conjugated onto both proteins by incubating the two components at pH 7.4 prior to the analysis. Incidentally, the activity of the hybrid construct was reduced to $<10\%$, suggesting that the catalytic site of the enzymes was sterically blocked by the attached dendrons (Figure 1 b,c). Upon reduction to pH 5.0, the dendrons dissociated and were removed by ultrafiltration and the activity of the enzymes was measured again at pH 7.4. The recovery of activity upon the dissociation of dendrons was calculated to be $>90\%$, supporting the reversibility of the boronic acid/salicyl hydroxamate binding. To further substan-

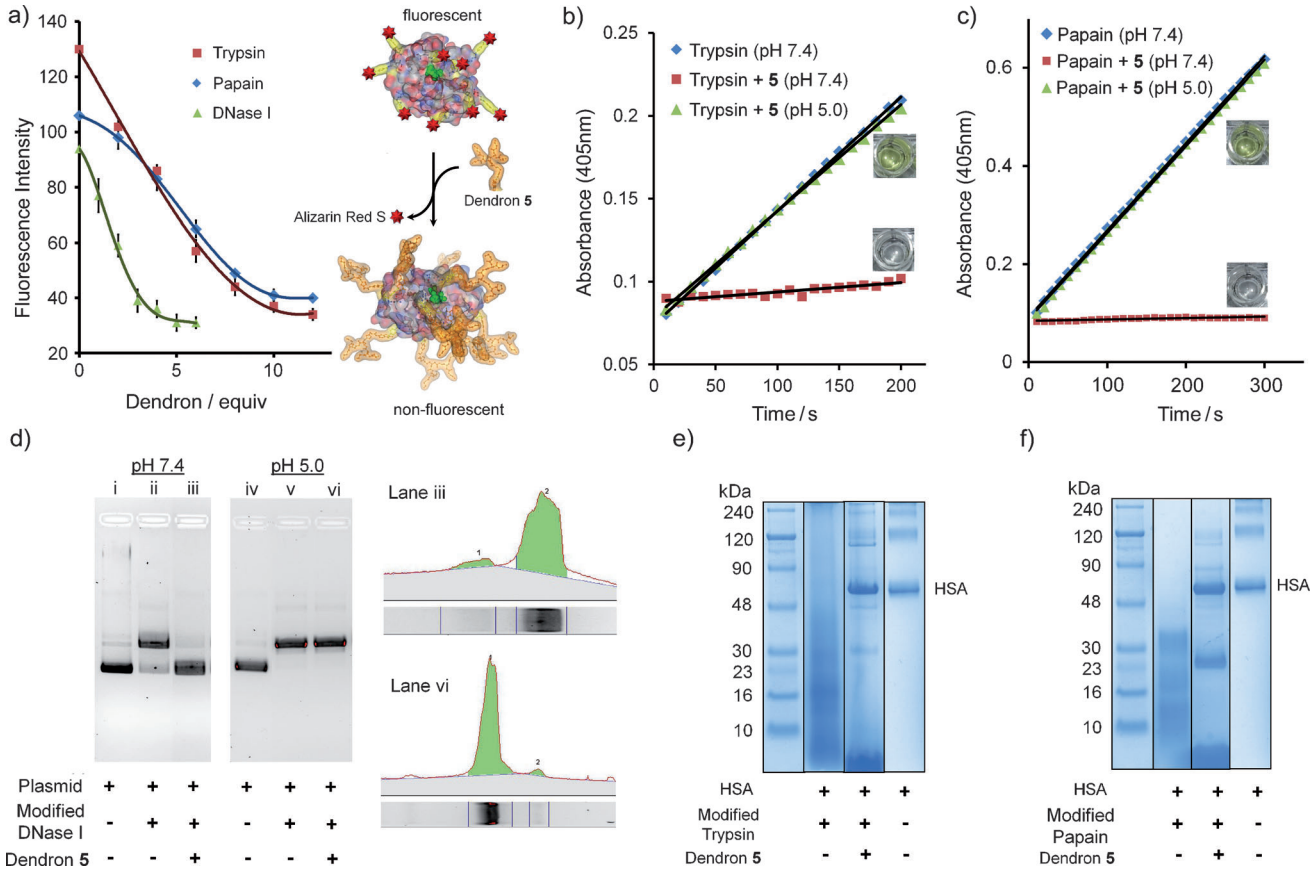


Figure 1. a) Fluorescence titration curves displaying the stoichiometric substitution of the fluorogenic Alizarin Red S/boronic acid complex on each protein by dendron **5**. b,c) Colorimetric enzyme kinetics for modified trypsin and papain based on N_{α}-benzoyl-L-arginine 4-nitroanilide hydrochloride as a molecular substrate. d) Agarose gel electrophoretic analysis, using SYBR safe stain, of the activity of modified DNase I on pUC19 DNA plasmid. Fluorescence intensity of bands in lanes iii and vi are shown in detail. e,f) SDS-PAGE analysis of the activity of modified trypsin and papain on human serum albumin as a protein substrate.

tiate this observation, the dendronized proteases were tested at pH 7.4 against human serum albumin (HSA) as a protein substrate followed by SDS-PAGE analysis. The addition of dendron **5** to both boronic acid modified proteases significantly blocked the proteolytic activity, with observable native albumin (67 kDa) clearly seen (Figure 1 e,f). Correspondingly, we found further support for the reversibility of the protective dendritic shell when the dendrons were removed at pH 5.0 prior to the proteolytic digestion (Figure S3). In a similar context, the activity of modified DNase I was tested against a 2000 base pair plasmid (pUC19) in which the degradation of the supercoiled plasmid into its linear form was effectively blocked in the presence of dendron **5** at pH 7.4 and subsequently recovered at pH 5.0 (Figure 1 d).

The bioorthogonality and possible aggregation behavior of the dendritic assembly was investigated using fluorescence polarization. The interaction between the boronic acid modified protease with dendron **5** demonstrated an expected increase in size and displayed consistency in serum-fortified DMEM (Figure S4). The absence of aggregation behavior of these dendronized proteins[22] and the specificity of the boronic acid/salicyl hydroxamate interaction agree with previous reports.[16]

In addition to conferring pH-dependent "assemble and release" properties to the modified enzymes, these positively charged dendrons facilitate cellular uptake through electrostatic interactions with the negatively charged cell membrane. The uptake proceeds by means of clathrin-dependent endocytosis and intracellular release is driven by the proton sponge effect as described previously.[14,23] Consequently, these modified proteases showed efficient dendron-mediated uptake which was observed by confocal microscopy (Figure 2). As the dissociation of the dendritic shell directly relied on low intracellular pH, localization of these constructs in acidic compartments was necessary for the release of the active protease. Colocalization studies using LysoTracker, which is fluorescent in acidic organelles, directly showed that the dendronized proteins were indeed located within these cellular compartments. In contrast, the nondendronized proteins displayed minimal cellular uptake.

Following cellular entry facilitated by the dendritic shell, these proteases could become active when the attached dendrons dissociate, directly causing rampant proteolytic degradation and cell death. Hence, upon addition of these dendronized proteases into A549 cells, the impact on cell viability and their potency was immediately observed after 3 h of incubation at 37 °C and 5% CO_2. As controls, the

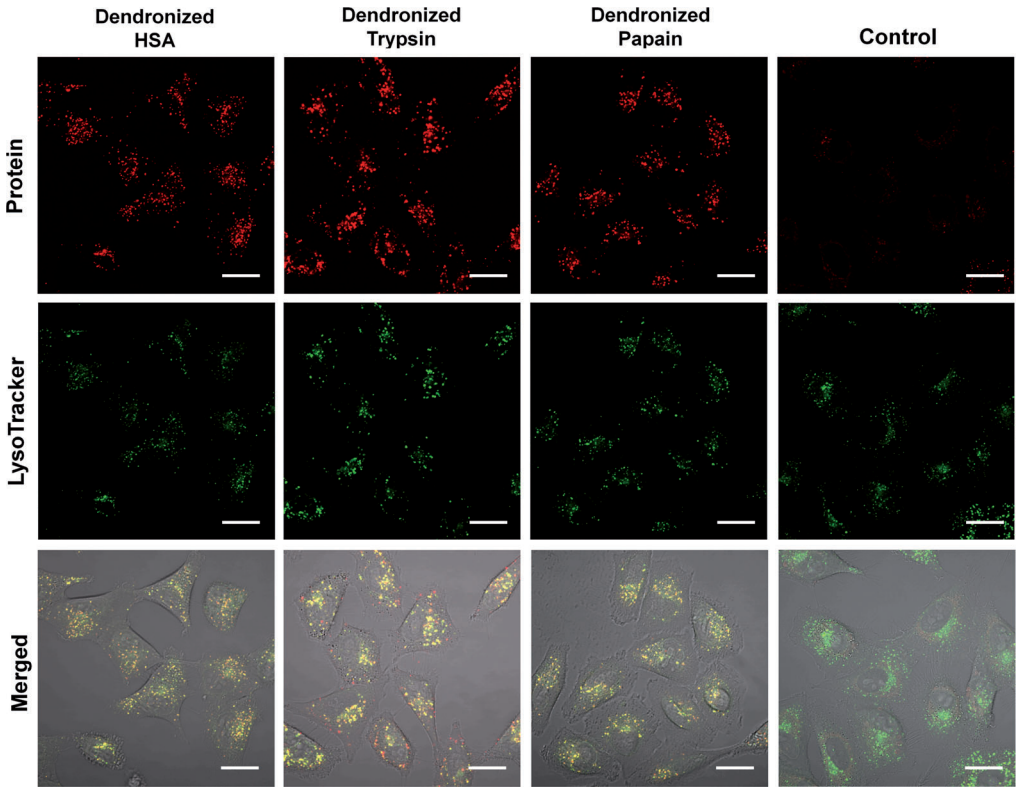


Figure 2. Confocal laser scanning microscopy using A549 cell lines. Colocalization of dendronized proteins (using Dylight 647-conjugated proteins) performed with LysoTracker. The control observation is representative of nondendronized HSA, trypsin, and papain. Scale bar: 20 μm.

respective native proteases, covalently dendronized G2-HSA and dendron **5** were used for comparison (Figure 3). The noncytotoxic behavior of both covalently and noncovalently dendronized HSA at much higher concentrations implied that the observed toxicity of the dendronized proteases cannot be attributed to the dendrons themselves or the cationic dendron–protein hybrid structure. These observations suggested that the dendritic shell not only plays an important role in the intracellular transportation but also as a benign steric moiety.

In conclusion, we have presented the synthesis of a sterically demanding dendritic supramolecular protecting group, which is responsive to changes between pH 7.4 and pH 5.0, by utilizing a bioorthogonal ligation method based on boronic acid/salicyl hydroxamate complexation. The dendritic protecting group shields the large binding sites of macromolecules in a highly reversible fashion. In addition, the formation of a dendritic PAMAM shell imparts the capability for efficient membrane translocation and localization into the acidic intracellular compartments. These attributes are of high significance since the assembly and disassembly of the dendritic shell occurs at physiological conditions, conferring stability at near-neutral pH and release inside acidic cellular lysosomes. The resulting active proteases in the cell severely decreased the cell viability. In perspective, this integrated bioorthogonal dendritic assembly provides a contemporary dimension in creating highly efficient hybrid zymogens as smart protein therapeutics.

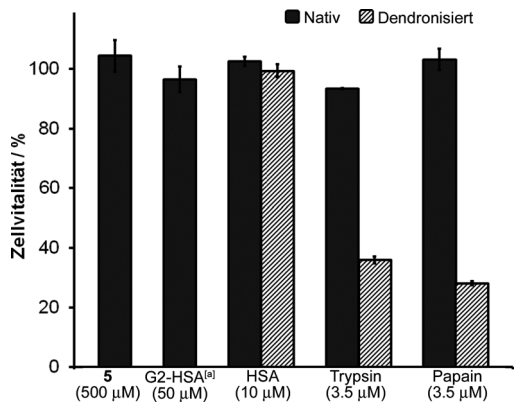


Figure 3. Comparison of the cytotoxicities of native proteases and their dendronized counterparts. Cell viability was referenced against blank controls. Data expressed as mean ± SEM, $n = 3$. [a] G2-HSA was constructed by covalently conjugating 32 G2 PAMAM dendrons onto human serum albumin as previously reported.[22]

Received: September 30, 2013

Keywords: dendrimers · enzymes · supramolecular chemistry

[1] P. Plainkum, S. M. Fuchs, S. Wiyakrutta, R. T. Raines, *Nat. Struct. Mol. Biol.* **2003**, *10*, 115.
[2] A. V. Karginov, F. Ding, P. Kota, N. V. Dokholyan, K. M. Hahn, *Nat. Biotechnol.* **2010**, *28*, 743.
[3] C. López-Otín, L. M. Matrisian, *Nat. Rev. Cancer* **2007**, *7*, 800.
[4] E. M. Sletten, C. R. Bertozzi, *Angew. Chem.* **2009**, *121*, 7108; *Angew. Chem. Int. Ed.* **2009**, *48*, 6974.
[5] Z. Ding, C. J. Long, Y. Hayashi, E. V. Bulmus, A. S. Hoffman, P. S. Stayton, *Bioconjugate Chem.* **1999**, *10*, 395.
[6] P. De, M. Li, S. R. Gondi, B. S. Sumerlin, *J. Am. Chem. Soc.* **2008**, *130*, 11288.
[7] P. S. Stayton, T. Shimoboji, C. Long, A. Chilkoti, G. Ghen, J. M. Harris, A. S. Hoffman, *Nature* **1995**, *378*, 472.
[8] W. Gao, W. Liu, T. Christensen, M. R. Zalutsky, A. Chilkoti, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 16432.

[9] M. Yan, J. Du, Z. Gu, M. Liang, Y. Hu, W. Zhang, S. Priceman, L. Wu, Z. H. Zhou, Z. Liu, T. Segura, Y. Tang, Y. Lu, *Nat. Nanotechnol.* **2010**, *5*, 48.
[10] A. A. Bastian, A. Marcozzi, A. Herrmann, *Nat. Chem.* **2012**, *4*, 789.
[11] D. Astruc, E. Boisselier, C. Ornelas, *Chem. Rev.* **2010**, *110*, 1857.
[12] L. Albertazzi, B. Storti, L. Marchetti, F. Beltram, *J. Am. Chem. Soc.* **2010**, *132*, 18158.
[13] S. K. Hamilton, E. Harth, *ACS Nano* **2009**, *3*, 402.
[14] D. Y. W. Ng, J. Fahrer, Y. Wu, K. Eisele, S. L. Kuan, H. Barth, T. Weil, *Adv. Healthcare. Mater.* **2013**, DOI: 10.1002/adhm.201200419.
[15] M. L. Stolowitz, C. Ahlem, K. A. Hughes, R. J. Kaiser, E. A. Kesicki, G. Li, K. P. Lund, S. M. Torkelson, J. P. Wiley, *Bioconjugate Chem.* **2001**, *12*, 229.
[16] S. B. Y. Shin, R. D. Almeida, G. Gerona-Navarro, C. Bracken, S. R. Jaffrey, *Chem. Biol.* **2010**, *17*, 1171.
[17] C. Q. Pan, J. S. Uumer, A. Herzka, R. A. Lazarus, *Protein Sci.* **1998**, *7*, 628.
[18] P. Carter, J. A. Wells, *Nature* **1988**, *332*, 564.
[19] H. A. Chapman, R. J. Riese, G.-P. Shi, *Annu. Rev. Physiol.* **1997**, *59*, 63.
[20] G. Springsteen, B. Wang, *Chem. Commun.* **2001**, 1608.
[21] G. Springsteen, B. Wang, *Tetrahedron* **2002**, *58*, 5291.
[22] S. L. Kuan, B. Stöckle, J. Reichenwallner, D. Y. W. Ng, Y. Wu, M. Doroshenko, K. Koynov, D. Hinderberger, K. Müllen, T. Weil, *Biomacromolecules* **2013**, *14*, 367.
[23] L. Albertazzi, M. Serresi, A. Albanese, F. Beltram, *Mol. Pharm.* **2010**, *7*, 680.

