

Molecular Imprinting

International Edition: DOI: 10.1002/anie.201600205
German Edition: DOI: 10.1002/ange.201600205A β -Lactamase-Imprinted Responsive Hydrogel for the Treatment of Antibiotic-Resistant Bacteria

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Abstract: Antibiotics play important roles in infection treatment and prevention. However, the effectiveness of antibiotics is now threatened by the prevalence of drug-resistant bacteria. Furthermore, antibiotic abuse and residues in the environment cause serious health issues. In this study, a stimuli-responsive imprinted hydrogel was fabricated by using β -lactamase produced by bacteria for deactivating antibiotics as the template molecule. The imprinted hydrogel could initially trap β -lactamase excreted by drug-resistant bacteria, thus making bacteria sensitive to antibiotics. After the bactericidal treatment, the “imprinted sites” on the hydrogel could be reversibly abolished with a temperature stimulus, which resulted in the reactivation of β -lactamase to degrade antibiotic residues. We also present an example of the use of this antibacterial design to treat wound infection.

Despite significant improvements in biomedical technology, the global burden of infectious diseases remains high.^[1] Since the first introduction of penicillin, it was believed that antibiotics would put an end to bacterial infections.^[2] However, the effectiveness of antibiotics is threatened by the prevalence of antibiotic resistance.^[3] Nowadays, antibiotic-resistant pathogenic bacteria account for numerous treatment failures and mortality in the clinic. Another risk associated with antibiotics is their residues in the environment and animal products.^[4] These residues may cause health hazards and promote bacterial resistance. Therefore, the development of effective strategies to combat antibiotic resistance and residues is urgent for infection treatments.

The design of new antibiotics may address the urgent threat of drug resistance. However, the development of novel antibiotics is relatively slow,^[5] and the inevitable rise of resistance will ruin new antibiotics introduced in the near future. As an alternative, the suppression of bacterial resistance and restoration of the efficacy of conventional antibiotics is appealing. Among conventional antibiotics, β -lactam antibiotics are the most widely used and have a long history.^[6] However, bacteria have gradually developed resist-

ance to them.^[7] The expression of β -lactamase is the most pervasive resistance mechanism employed by bacteria.^[8] This enzyme can hydrolyze the β -lactam ring to deactivate these antibiotics.^[9] To inhibit β -lactamase, several molecules with the β -lactam core structure have been introduced.^[10] Unfortunately, such inhibitors usually upregulate the expression of β -lactamase. Some non- β -lactam inhibitors, including boronic acid derivatives and phosphonates, have also been designed.^[10] Although interesting, these small-molecule-based inhibitors often have poor selectivity,^[11] and none of them could resolve the problems of antibiotic resistance and antibiotic residues simultaneously.

Molecular imprinting has attracted wide interest for the fabrication of artificial receptors.^[12] During the imprinting process, the target template and functional monomers are first polymerized in place.^[13] After removal of the template, tailor-made recognition sites exist, which can rebind the target with high affinity and selectivity.^[14] As compared to antibodies, molecularly imprinted polymers are straightforward to prepare, cost-effective, and highly robust.^[14,15] These properties make them attractive for separations^[16] and sensing,^[17] as enzyme inhibitors^[18] and protein mimics,^[19] and for toxin clearance^[20] and cell/tissue imaging.^[21] More interestingly, intelligent imprinted polymers have been developed recently, in which the recognition sites can be switched on and off by triggers.^[22]

Inspired by these achievements, we have now developed a stimuli-responsive imprinted hydrogel to resolve the demanding challenges faced by antibiotic therapy (Figure 1). The intelligent imprinted hydrogel was fabricated by using β -lactamase as the template and *N*-isopropylacrylamide (NIPAAm) as the temperature-responsive monomer.^[23] It could recognize and trap the β -lactamase produced by bacteria, thus attenuating the bacterial resistance to antibiotics. After the killing of bacteria, the β -lactamase bound on the imprinted-polymer (IP) hydrogel could be reversibly activated by a temperature stimulus to degrade antibiotic residues. Accordingly, the imprinted hydrogel could act as an intelligent gate for β -lactamase to overcome antibiotic resistance and residues during different stages. Although imprinted polymers have exhibited some expanded bio-applications, this study is the first demonstration of imprinting β -lactamase for multifunctional antibacterial application.

We first synthesized the β -lactamase-imprinted responsive hydrogel (IP hydrogel). NIPAAm was chosen as the thermo-responsive monomer in combination with acrylamide as the hydrogen-bonding monomer. Furthermore, to mediate the polymerization in close proximity to the enzyme,^[18,24] a known small-molecule inhibitor of β -lactamase, 3-amino-

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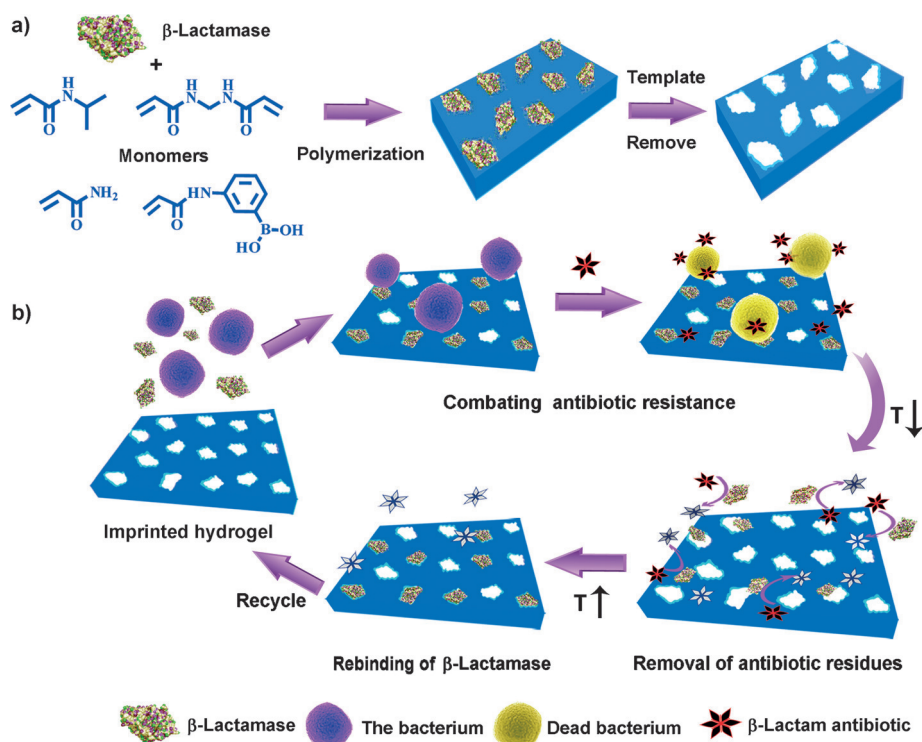


Figure 1. a) Fabrication of a temperature-responsive imprinted hydrogel with β -lactamase as the template. b) Bacteria could express β -lactamases to hydrolyze β -lactam antibiotics. The imprinted hydrogel bound β -lactamase and protected antibiotics from enzymatic degradation. After bactericidal treatment, the β -lactamase trapped in the hydrogel was released by a temperature stimulus and could then degrade antibiotic residues. The residual β -lactamase in solution could be rebound by the IP hydrogel to decrease their health risk.

phenylboronic acid (3-APBA),^[25] was used as the anchoring ligand. It was rendered polymerizable by coupling with acryloyl chloride. These functional monomers were first assembled with β -lactamase at 37°C and then polymerized between two glass plates. The β -lactamase templates and unreacted monomers were finally removed. A non-imprinted control hydrogel (NP hydrogel) was also prepared in an identical manner but without the addition of a template.

The ability of the IP hydrogel to rebind β -lactamase was investigated by a batch binding approach at 37°C. As presented in Figure 2a, the adsorption capacity of the IP hydrogel increased as the initial β -lactamase concentration increased. In comparison with the NP hydrogel, the IP hydrogel clearly showed a higher affinity toward β -lactamase. The two types of hydrogels had the same monomer composition (see Figure S1 in the Supporting Information). Little difference in their macrostructure was observed by scanning electron microscopy (see Figure S2), thus excluding the possibility that differences in physical adsorption might result in the different binding capacity of the two hydrogels. To study binding selectivity, we used five non-template proteins as controls: cytochrome c (Cyt), bovine serum albumin (BSA), urease (Ure), lysozyme (Lys), and ovalbumin (OVA). Relatively low binding affinity of the IP hydrogel toward these control proteins was observed (Figure 2b).

After confirming the efficient recognition of β -lactamase by the IP hydrogel at 37°C, we studied the influence of

temperature on the rebinding process. The affinity between the IP hydrogel and β -lactamase decreased at 20°C (Figure 2c). The pNIPAm-based IP hydrogel exhibited temperature-responsive behavior. It became swollen at low temperatures (Figure 2d). During this process, the imprinted cavities in the hydrogel were no longer complementary to β -lactamase, thus weakening their interactions. We next studied the temperature-dependent release of β -lactamase. After being loaded with β -lactamase at 37°C, the IP hydrogel was immersed in a fresh solution at 20°C. The release of β -lactamase was clearly observed on the basis of the absorption change of the surrounding solution (see Figure S3). However, the release of β -lactamase at 37°C was much slower. The NP hydrogel showed serious leakage of β -lactamase at both 37 and 20°C (see Figure S4). These results proved that the temperature-responsive recognition sites on the IP hydrogel could control the binding and release of β -lactamase.

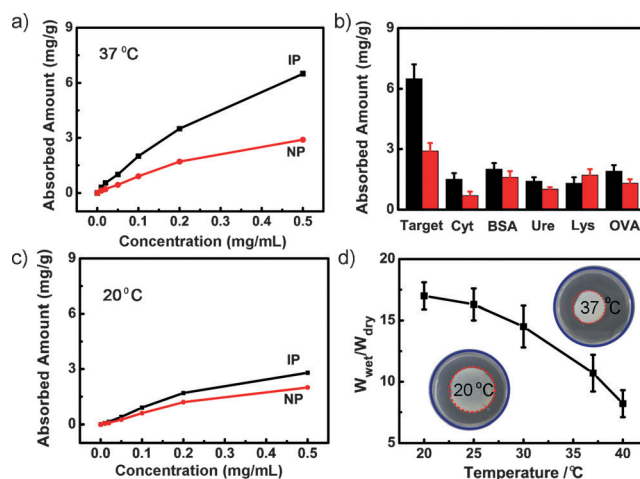


Figure 2. a) Binding isotherms of the IP (black line) and NP hydrogel (red line) toward β -lactamase at 37°C. b) Selective adsorption of β -lactamase by the IP (black) and NP hydrogel (red). c) Binding isotherms of the IP and NP hydrogel at 20°C toward β -lactamase. d) Weight change of the IP hydrogel at different temperatures. Inserts are photographs of the hydrogel at 20 and 37°C.

Next, the effect of the IP hydrogel on β -lactamase activity was investigated. Nitrocefin, a β -lactam antibiotic, was used to indicate the activity of β -lactamase with color readout. The solution of nitrocefin typically appeared yellow with an

absorption peak at 390 nm. After hydrolysis by lactamase, the nitrocefin solution gradually turned red with a new absorption band near 486 nm (see Figure S5). However, upon co-incubation with β -lactamase and the IP hydrogel, the nitrocefin solution was barely hydrolyzed at all and retained its original yellow color. The results confirmed that the IP hydrogel could inhibit β -lactamase activity, which was consistent with its binding capacity toward β -lactamase. Once bound to the IP hydrogel, the active site of the enzyme was presumed to be masked, thus decreasing its accessibility to the substrate. An increase in the amount of the IP hydrogel resulted in more efficient inhibition of β -lactamase (Figure 3a). At equivalent doses, the NP hydrogel clearly

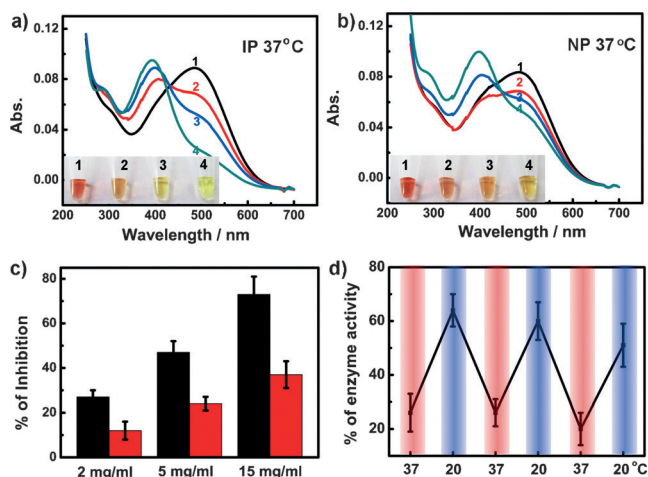


Figure 3. a, b) UV/Vis absorption of solutions of nitrocefin after treatment with different β -lactamase samples: a) β -lactamase was preincubated with (or without) the IP hydrogel at a concentration of 0 (sample 1), 2 (sample 2), 5 (sample 3), 15 mg mL⁻¹ (sample 4) at 37°C for 1.5 h; b) β -lactamase was preincubated with the equivalent dose of the NP hydrogel at 37°C. Inserts are the corresponding photographs of the nitrocefin solutions. c) Inhibition of β -lactamase activity by different amounts of the IP (black) or NP hydrogel (red). d) Inhibition and reactivation of β -lactamase by the IP hydrogel at 37 and 20°C.

exhibited a lower inhibition level (Figure 3b). Furthermore, when the hydrogels were synthesized without the anchoring monomer, they showed less effective β -lactamase binding and inhibition (see Figure S6). The activity of the enzyme was turned on when the IP hydrogel was cooled to 20°C (see Figure S7). β -Lactamase was released from the swollen IP hydrogel and hydrolyzed nitrocefin to give a red color. When the temperature was alternated between 37 and 20°C, the activity of β -lactamase in the presence of the IP hydrogel was inhibited, then restored, and then inhibited again (Figure 3d).

The potential of the IP hydrogel for antibacterial applications was then evaluated. Methicillin-resistant staphylococcus aureus (MRSA) bacteria were used as the model bacteria. Their viability was analyzed by a live/dead assay. The β -lactam antibiotic penicillin G, which is commonly used in the clinic, exhibited low inhibition of MRSA (Figure 4a). This result was consistent with previous studies and our nitrocefin tests (see Figure S8), which proved that MRSA could produce

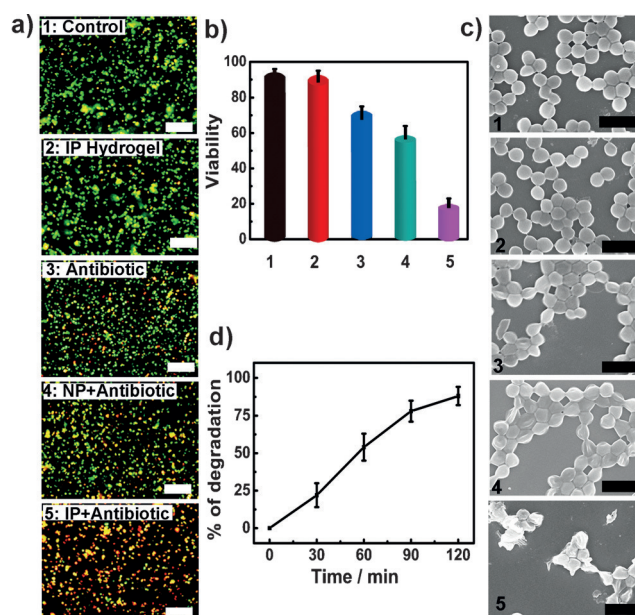


Figure 4. a) Live-dead fluorescence imaging of bacteria samples. Living cells showed a green signal, and dead cells showed a red signal. Scale bars: 20 μ m. b) Viability analysis of bacteria samples. c) SEM images of bacteria samples. Scale bars: 2 μ m. The bacteria samples 1–5 were prepared by incubating bacteria with phosphate-buffered saline (PBS), the IP hydrogel, penicillin G, the NP hydrogel + penicillin G, and the IP hydrogel + penicillin G, respectively. d) Degradation of antibiotic residues after the β -lactamase-loaded IP hydrogel was cooled to 20°C.

β -lactamase to destroy the antibiotic. However, in the presence of the IP hydrogel, the efficacy of the antibiotic was greatly enhanced, and the bacterial viability decreased by nearly 80 % (Figure 4b). As a control, the IP hydrogel alone without an antibiotic exhibited little toxicity. This result implied that the IP hydrogel did not directly act as an antibacterial agent but rather as an adjuvant to enhance the efficacy of the antibiotic. It could trap and inhibit β -lactamase excreted by bacteria, thus making bacteria sensitive to conventional antibiotics. Notably, although the free APBA monomers showed some inhibitory effect, the inhibition capability of the IP hydrogel was mainly derived from the imprinting effect, not direct inhibition by APBA (see Figure S9). As compared with the IP hydrogel, the NP hydrogel with the same monomer composition caused some but a significantly lower decrease in the bacterial resistance. Besides viability analysis, the growth of bacteria was evaluated with a disk diffusion assay (see Figure S10). Only in the presence of the IP hydrogel could the antibiotics dramatically inhibit the bacterial growth. Morphology changes of the bacteria were investigated by SEM (Figure 4c). In the untreated and IP-hydrogel-treated samples, bacteria typically had a round shape with a smooth cell wall. After antibiotic treatment, only a small amount of cells were deformed. However, most of the cells were seriously deformed and some were even lysed upon co-incubation with the IP hydrogel and the antibiotic. These results showed that the IP hydrogel could improve the antibacterial efficiency of β -lactam antibiotics. Control protein-imprinted hydrogels were synthesized

to verify the effect of β -lactamase imprinting on the antibacterial process. The sizes of control template proteins were larger than, or smaller than, or similar to that of β -lactamase. However, these control hydrogel-based systems showed lower antibacterial efficiency than that of the β -lactamase-imprinted hydrogel (see Figure S11; for the batch-to-batch performance of the IP hydrogel, see Figure S12).

In addition to suppressing antibiotic resistance, the IP hydrogel-based system could be further utilized to degrade antibiotic residues (Figure 1). After the killing of bacteria, the antibacterial system containing the IP hydrogel and the antibiotic was cooled to 20 °C. The residual antibiotic in solution was monitored by a microbiological method. About 80 % of the antibiotic was gradually degraded within 2 h (Figure 4d). At low temperature, the β -lactamase bound by the IP hydrogel was released into solution and could hydrolyze the residual antibiotic molecules. Although the direct addition of β -lactamase has been reported for the elimination of antibiotic residues, residual β -lactamase is also a health risk.^[26] In our system, after the degradation of the antibiotic, the β -lactamase in solution could be conveniently rebound into the IP hydrogel at 37 °C, which avoided the side effect of β -lactamase. Thus, the IP hydrogel provided an efficient and safe method to eliminate antibiotics. It could be used repeatedly for further cycles after washing.

Finally, we present an example of the use of this antibacterial design to treat wound infection.^[27] As heavily hydrated materials, hydrogels can protect wounds in a suitably moist healing environment. First, the biocompatibility of our IP hydrogel was studied. More than 95 % of cells cultured on the IP-hydrogel surface were alive after 24 h (see Figure S13). We then tested the IP hydrogel in serum to confirm its feasibility for use in biological samples (see Figure S14). For infection treatment, the backs of mice were slashed and injected with MRSA to build the infected-wound model. The wounds of the mice were treated with PBS buffer, with the IP hydrogel alone, with penicillin G alone, with the NP hydrogel + penicillin G, or with the IP hydrogel + penicillin G. During the whole therapeutic process, the wounds treated with the IP hydrogel + antibiotics did not show erythema, and they formed scabs after therapy. The wounds in the other four groups had different levels of erythema (Figure 5a). To assess the bactericidal effect, we excised the wound tissues to quantify the number of bacteria on them (Figure 5b,c). From the grown colonies, we could see that the combination of the IP hydrogel and the antibiotic resulted in the most effective wound antibacterial therapy. The control protein-imprinted hydrogels were also tested for infection treatment. As compared with the β -lactamase-imprinted hydrogel, these control hydrogels showed an unsatisfactory therapeutic effect (see Figure S15). In this study, the imprinted polymers were prepared in the form of hydrogel layers for treating wound infection. They could also be prepared readily in other forms, such as nanoparticles. Nanostructured imprinted materials have a high surface-to-volume ratio, and the binding capacity and kinetics would be further improved.

In summary, we have prepared a β -lactamase-imprinted responsive hydrogel for multifunctional antibacterial applications. The IP hydrogel could trap β -lactamase excreted by

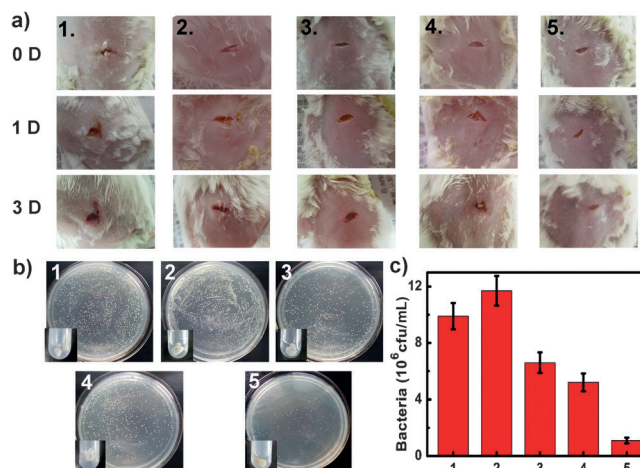


Figure 5. a) Wounds of mice after 1 and 3 days of therapy. The mice samples 1–5 were treated with PBS buffer, the IP hydrogel, penicillin G, the NP hydrogel + penicillin G, and the IP hydrogel + penicillin G, respectively. b) The bacteria separated from wound tissue were cultured on agar plates. Inserts are the wound tissue. c) Number of bacteria in the wound tissue of each sample.

drug-resistant bacteria, thus making the bacteria sensitive to antibiotics. It could thus act as an adjuvant to enhance the efficacy of conventional antibiotics against drug-resistant bacteria. After the killing of bacteria, β -lactamase bound on the IP hydrogel could be reactivated by a temperature stimulus for the degradation of antibiotic residues. With thermoresponsive “ β -lactamase-recognition sites”, the IP hydrogel might resolve two challenging issues in antibiotic therapy: the developed drug resistance and antibiotic residues. We hope our study will guide the use of smart imprinted polymers for multifunctional biomedical applications.

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- [1] K. E. Jones, N. G. Patel, M. A. Levy, A. Storeygard, D. Balk, J. L. Gittleman, P. Daszak, *Nature* **2008**, *451*, 990–993.
- [2] M. A. Kohanski, D. J. Dwyer, J. J. Collins, *Nat. Rev. Microbiol.* **2010**, *8*, 423.
- [3] S. B. Levy, B. Marshall, *Nat. Med.* **2004**, *10*, S122–S129.
- [4] D. I. Andersson, D. Hughes, *Nat. Rev. Microbiol.* **2014**, *12*, 465–478.
- [5] P. Fernandes, *Nat. Biotechnol.* **2006**, *24*, 1497–1503.
- [6] C. N. Wivagg, R. P. Bhattacharyya, D. T. Hung, *J. Antibiot.* **2014**, *67*, 645.
- [7] T. L. Harris, R. J. Worthington, C. Melander, *Angew. Chem. Int. Ed.* **2012**, *51*, 11254–11257; *Angew. Chem.* **2012**, *124*, 11416–11419.

- [8] a) R. Liu, R. Liew, J. Zhou, B. Xing, *Angew. Chem. Int. Ed.* **2007**, *46*, 8799; *Angew. Chem.* **2007**, *119*, 8955; b) J. Zhang, Y. P. Chen, K. P. Miller, M. S. Ganewatta, M. Bam, Y. Yan, M. Nagarkatti, A. W. Decho, C. Tang, *J. Am. Chem. Soc.* **2014**, *136*, 4873.
- [9] Z. Yang, P.-L. Ho, G. Liang, K. H. Chow, Q. Wang, Y. Cao, Z. Guo, B. Xu, *J. Am. Chem. Soc.* **2007**, *129*, 266–267.
- [10] S. M. Drawz, R. A. Bonomo, *Clin. Microbiol. Rev.* **2010**, *23*, 160–201.
- [11] M. Philipp, M. L. Bender, *Proc. Natl. Acad. Sci. USA* **1971**, *68*, 478–480.
- [12] L. Chen, S. Xu, J. Li, *Chem. Soc. Rev.* **2011**, *40*, 2922–2942.
- [13] a) G. Wulff, J. Liu, *Acc. Chem. Res.* **2012**, *45*, 239–247; b) J. Li, C. E. Kendig, E. E. Nesterov, *J. Am. Chem. Soc.* **2007**, *129*, 15911–15918; c) E. V. Piletska, G. Stavroulakis, L. D. Larcombe, M. J. Whitcombe, A. Sharma, S. Primrose, G. K. Robinson, S. A. Piletsky, *Biomacromolecules* **2011**, *12*, 1067–1071; d) C. Zheng, X.-L. Zhang, W. Liu, B. Liu, H.-H. Yang, Z.-A. Lin, G.-N. Chen, *Adv. Mater.* **2013**, *25*, 5922–5927.
- [14] M. J. Whitcombe, I. Chianella, L. Larcombe, S. A. Piletsky, J. Noble, R. Porter, A. Horgan, *Chem. Soc. Rev.* **2011**, *40*, 1547–1571.
- [15] a) K. Haupt, K. Mosbach, *Chem. Rev.* **2000**, *100*, 2495–2504; b) J. Svenson, I. A. Nicholls, *Anal. Chim. Acta* **2001**, *435*, 19–24.
- [16] a) Y. Ma, G. Pan, Y. Zhang, X. Guo, H. Zhang, *Angew. Chem. Int. Ed.* **2013**, *52*, 1511–1514; *Angew. Chem.* **2013**, *125*, 1551–1554; b) J. Orozco, A. Cortés, G. Cheng, S. Sattayasamitsathit, W. Gao, X. Feng, Y. Shen, J. Wang, *J. Am. Chem. Soc.* **2013**, *135*, 5336–5339.
- [17] a) W. Bai, D. A. Spivak, *Angew. Chem. Int. Ed.* **2014**, *53*, 2095–2098; *Angew. Chem.* **2014**, *126*, 2127–2130; b) W. Zhang, W. Liu, P. Li, H. Xiao, H. Wang, B. Tang, *Angew. Chem. Int. Ed.* **2014**, *53*, 12489–12493; *Angew. Chem.* **2014**, *126*, 12697–12701; c) Y.-J. Zhao, X.-W. Zhao, J. Hu, J. Li, W.-Y. Xu, Z.-Z. Gu, *Angew. Chem. Int. Ed.* **2009**, *48*, 7350–7352; *Angew. Chem.* **2009**, *121*, 7486–7488; d) D. Cai, L. Ren, H. Zhao, C. Xu, L. Zhang, Y. Yu, H. Wang, Y. Lan, M. F. Roberts, J. H. Chuang, M. J. Naughton, Z. Ren, T. C. Chiles, *Nat. Nanotechnol.* **2010**, *5*, 597–601; e) X.-A. Ton, B. Tse Sum Bui, M. Resmini, P. Bonomi, I. Dika, O. Soppera, K. Haupt, *Angew. Chem. Int. Ed.* **2013**, *52*, 8317–8321; *Angew. Chem.* **2013**, *125*, 8475–8479.
- [18] A. Cutivet, C. Schembri, J. Kovensky, K. Haupt, *J. Am. Chem. Soc.* **2009**, *131*, 14699–14702.
- [19] a) A. Poma, A. Guerreiro, M. J. Whitcombe, E. V. Piletska, A. P. F. Turner, S. A. Piletsky, *Adv. Funct. Mater.* **2013**, *23*, 2821; b) Y. Zhang, C. Deng, S. Liu, J. Wu, Z. Chen, C. Li, W. Lu, *Angew. Chem. Int. Ed.* **2015**, *54*, 5157; *Angew. Chem.* **2015**, *127*, 5246.
- [20] Y. Hoshino, H. Koide, T. Urakami, H. Kanazawa, T. Kodama, N. Oku, K. J. Shea, *J. Am. Chem. Soc.* **2010**, *132*, 6644–6645.
- [21] a) Y. Liu, S. Fang, J. Zhai, M. Zhao, *Nanoscale* **2015**, *7*, 7162–7167; b) D. Yin, S. Wang, Y. He, J. Liu, M. Zhou, J. Ouyang, B. Liu, H.-Y. Chen, Z. Liu, *Chem. Commun.* **2015**, *51*, 17696–17699; c) S. Shinde, Z. El-Schich, A. Malakpour, W. Wan, N. Dizeyi, R. Mohammadi, K. Rurack, A. Gjörlöf Wingren, B. Sellergren, *J. Am. Chem. Soc.* **2015**, *137*, 13908–13912; d) S. Kunath, M. Panagiotopoulou, J. Maximilien, N. Marchyk, J. Sängler, K. Haupt, *Adv. Healthcare Mater.* **2015**, *4*, 1322–1326.
- [22] a) L. Li, Y. Lu, Z. Bie, H.-Y. Chen, Z. Liu, *Angew. Chem. Int. Ed.* **2013**, *52*, 7451–7454; *Angew. Chem.* **2013**, *125*, 7599–7602; b) G. Pan, Q. Guo, Y. Ma, H. Yang, B. Li, *Angew. Chem. Int. Ed.* **2013**, *52*, 6907–6911; *Angew. Chem.* **2013**, *125*, 7045–7049; c) Y. Hoshino, R. C. Ohashi, Y. Miura, *Adv. Mater.* **2014**, *26*, 3718–3723.
- [23] H. Liu, X. Liu, J. Meng, P. Zhang, G. Yang, B. Su, K. Sun, L. Chen, D. Han, S. Wang, L. Jiang, *Adv. Mater.* **2013**, *25*, 922–927.
- [24] A. A. Vaidya, B. S. Lele, M. G. Kulkarni, R. A. Mashelkar, *J. Appl. Polym. Sci.* **2001**, *81*, 1075–1083.
- [25] a) S. J. Cartwright, S. G. Waley, *Biochem. J.* **1984**, *221*, 505–512; b) G. S. Weston, J. Blazquez, F. Baquero, B. K. Shoichet, *J. Med. Chem.* **1998**, *41*, 4577–4586.
- [26] Z. Xu, H. Wang, S. Huang, Y. Wei, S. Yao, Y. Guo, *Anal. Chem.* **2010**, *82*, 2113–2118.
- [27] H. Sun, N. Gao, K. Dong, J. Ren, X. Qu, *ACS Nano* **2014**, *8*, 6202–6210.

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