Cross-Regulation of an Artificial Metalloenzyme

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Dedicated to Professor Roald Hoffmann on the occasion of his 80th birthday

Abstract: *Cross-regulation of complex biochemical reaction networks is an essential feature of living systems. In a biomimetic spirit, we report on our efforts to program the temporal activation of an artificial metalloenzyme via cross-regulation by a natural enzyme. In the presence of urea, urease slowly releases ammonia that reversibly inhibits an artificial transfer hydrogenase. Addition of an acid, which acts as fuel, allows to maintain the system out of equilibrium.*

*T*hanks to numerous attractive features, biocatalysis, relying on isolated enzymes, has gained increasing attention among the synthetic chemistry community.[1] To further improve on this asset, the focus is shifting towards enzyme cascades whereby an intermediate produced by a first enzyme acts as the substrate for a second enzyme etc. Such in-series assembled enzymes (Scheme 1a) allow to circumvent the time-consuming isolation and purification of intermediates. However, increasing the complexity of enzyme cascades often lead to suboptimal material flux as well as to inhibition.[2]

In living systems operating far from equilibrium, biochemical cascades have evolved to include signal transduction as well as metabolic and allosteric regulation safeguards that affect both the spatial control of intermediates and the temporal occurrence of events.[3]

Thanks to the progress in computational systems biology and genetic engineering, it has become possible to engineer in vivo synthetic gene networks to control some of the fundamental properties of living systems.[4] However, *understanding* at the molecular level the details of such complex, off-equilibrium (bio)chemical reaction networks remains challenging.[5–8]

In the past decade, artificial metalloenzymes (ArMs) have attracted increasing attention as alternative to both homogeneous catalysts and enzymes.[9] Artificial metalloenzymes result from the incorporation of an abiotic metal cofactor within a protein environment. In contrast to homogeneous catalysts, ArMs can be combined with natural enzymes to

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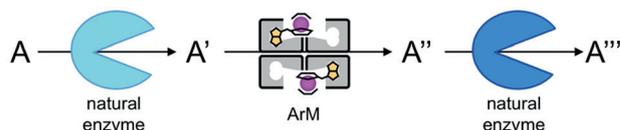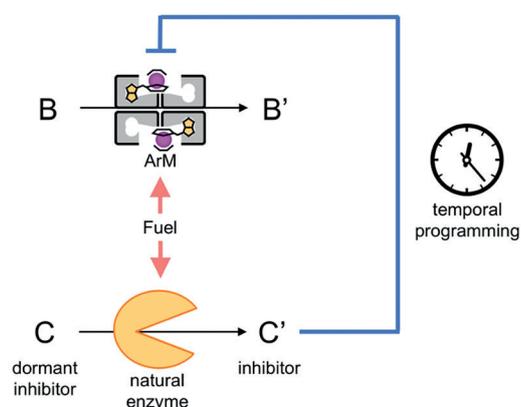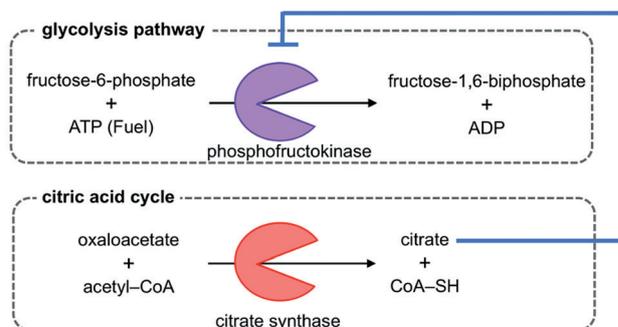
Scheme 1. Enzyme cascades, a) combined in series and b) in parallel. c) Phosphofructokinase, a key committed step of glycolysis, is inhibited by citrate (ArM is an artificial metalloenzyme).

afford enzyme cascades.[10] With the long-term goal of engineering artificial metabolic pathways,[11] we report on our efforts to introduce a programmable enzymatic temporal control of the activity of an artificial metalloenzyme.

In order to program such an off-equilibrium control of the activity of an ArM, we reasoned that one could combine in parallel an enzyme that produces a reversible inhibitor of the ArM (Scheme 1b). Such cross-regulated biochemical networks play a key role in living systems (Scheme 1c). With

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only starting materials present at the onset of the reaction, both the enzyme and the ArM are active. As the product of both enzymatic reactions accumulate, the ArM is inactivated (Scheme 1b). Ideally, upon removal or consumption of the inhibitor, the activity of the ArM is restored. Building on our previous experience with artificial transfer hydrogenases (ATHase hereafter) based on the biotin-avidin technology,[9b] we set out to program the ATHase activity by combining it with an enzyme that produces an inhibitor.

In order to conveniently monitor the activity in real-time, we selected enrofloxacin **1** as an ATHase substrate.[12] The fluoroquinolone substrate **1** is a versatile antibiotic against both Gram-positive and Gram-negative bacteria. Upon reduction of the C=C double bond, the resulting β-ketoacid spontaneously decarboxylates to afford a yellow product **2** (Figure S1 in the Supporting Information). The carboxylate group of enrofloxacin **1** is essential for its antibacterial activity; its loss results in a significant increase of minimal inhibitory concentration.[13]

We selected an ATHase comprised of a biotinylated Cp*Ir(4,7-dihydroxy-1,10-phenanthroline) **3** (Cp* = 1,2,3,4,5-pentamethylcyclopentadienyl) and the streptavidin K121R variant (Sav K121R) (Scheme 2a). The ATHase [(Biot-Cp*)Ir(N^N)] **3**·Sav K121R was previously reported for its remarkable ATHase activity for the reduction of imines, using either NAD(P)H or formate as hydride source in the presence of a variety of enzymes, sharing a common intermediate (i.e. in-series enzyme cascades, Scheme 1a).[10c] A preliminary screen led to the identification of the following reaction conditions: ATHase (10 μM, 0.5 mol%) enrofloxacin **1** (2 mM), formate (2 M) and MOPS (0.1 M, pH 7.0). At 37°C, 96 turnovers (96 TON) were obtained after 8 hours.

Next, the pH-dependence of the [(Biot-Cp*)IrCl(N^N)] **3**·Sav K121R activity was evaluated by determining the initial rate of the reduction of enrofloxacin **1** by monitoring the appearance of the ketone **2** at 430 nm (Scheme 2b). A bell-shape pH-profile was observed, both for the free non-biotinylated cofactor [(Cp*)IrCl(N^N)] **4** as well as the ATHase [(Biot-Cp*)IrCl(N^N)] **3**·Sav K121R. As the ATHase [(Biot-Cp*)Ir(N^N)] **3**·Sav K121R is completely, but reversibly inhibited at pH > 8.0, we speculated that we may cross-regulate its activity by coupling it in parallel with an enzyme that produces a base as reaction product (Scheme 1b). To evaluate the stability of the ATHase upon cycling the pH from pH < 6.0 to pH > 8.5, the reaction mixture was repeatedly spiked with HCl and NaOH. Monitoring the absorbance at 430 nm unambiguously demonstrates the activity of the ArM towards enrofloxacin **1** in acidic medium. Addition of NaOH causes the ATHase to stall. Subsequent addition of HCl leads to a 30% erosion of ATHase activity for the second cycle. Gratifyingly, the activity for the second to fifth cycles remains constant (Scheme 2c and Figure S2).

Urease from *Canavalia ensiformis* (Jack bean, E.C. 3.5.1.5) was selected as alkali-generating enzyme to be integrated in parallel with the ATHase [(Biot-Cp*)Ir(N^N)] **3**·Sav K121R. Urea rapidly reacts with urease to afford carbon dioxide and two equivalents of ammonia. Addition of bromothymol blue allows to conveniently monitor the pH
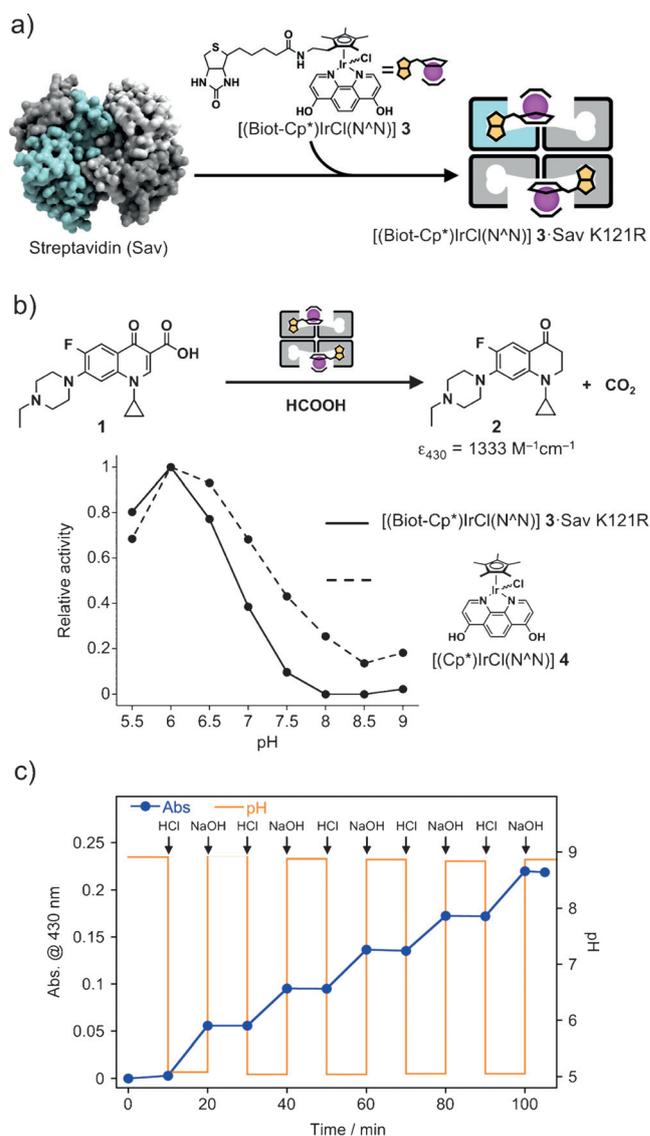
Scheme 2. a) Structure of the biotinylated iridium complex **3** and schematic representation of the corresponding artificial transfer hydrogenase (ATHase) tested for the reduction of enrofloxacin **1** using formate as hydride source. b) pH-dependence of enrofloxacin **1** reduction by ATHase [(Biot-Cp*)Ir(N^N)] **3**·Sav K121R (solid line) and by [(Biot-Cp*)Ir(N^N)] **4** (dashed line). c) Activity profile resulting from pH cycling upon addition of HCl and NaOH (see also Figure S2). Reaction conditions: 10 μM ATHase [(Biot-Cp*)Ir(N^N)] **3**·Sav K121R (or complex **4**), 1 mM enrofloxacin **1**, and 2 M HCOONa in 10 mM buffer at 37°C.

profile (i.e. reflecting the urease activity) by relying on the absorption ratio at 618 nm/501 nm (absorption maximum and isosbestic point, respectively) (Figure S3). In the presence of either [(Cp*)IrCl(N^N)] **4** or [(Biot-Cp*)IrCl(N^N)] **3**, no pH variation could be observed upon addition of urease and urea (Scheme S1). We conclude that either organometallic catalyst [(Cp*)IrCl(N^N)] **4** or bare cofactor [(Biot-Cp*)IrN^N] **3** inactivates the urease. In the presence of both the ATHase [(Biot-Cp*)IrN^N] **3**·Sav K121R and urease, the pH gradually increases upon addition of urea,

highlighting the compatibility of both ATHase and urease partners (Scheme S2).

Next, the activity of ATHase [(Biot-Cp*)Ir(N^N)] **3**·Sav K121R was evaluated in the presence of urease and urea. The initial pH was set to pH 9.0, where both urease and ATHase are inactive. Upon lowering the pH to 5.0 by addition of HCl$_{aq}$, both the ATHase and the urease are activated as reflected by the appearance of product **2** and the slow increase in pH. The ATHase remains active until pH 8 at which point, it is inhibited by the product of urease: ammonia

Scheme 3. a) Schematic representation of the pH-dependent activation of ATHase [(Biot-Cp*)Ir(N^N)] **3**·Sav K121R and urease. b) Time-dependent reduction of enrofloxacin **1** by the ATHase in the presence of urease and urea. The reaction was monitored at 430 nm (see Figure S1). c) (Re)activation of the ATHase (blue trace) and urease by addition of acid (orange trace). Experimental conditions; 25 μM ATHase [(Biot-Cp*)Ir(N^N)] **3**·Sav K121R, 1 mM enrofloxacin **1**, 0.1–0.4 mg mL^{-1} urease, 150–300 mM urea and 2 M HCOONa in 10 mM CHES (pH 9.0) at 37 °C. An aliquot of 5 M HCl (3.2 μL and 5 μL for 1st and 2nd addition, respectively to bring the pH down to 5.0) was added per 200 μL of the reaction mixture (see also Scheme S3).

(Scheme 3b). Addition of HCl$_{aq}$, restores 86% of the initial ATHase's activity, highlighting the reversible inhibition of the ATHase (Scheme 3c). As can be appreciated, the activity of the ATHase (as revealed by the increase in absorbance at 430 nm) correlates with the pH variation (monitored by the ratio of absorbance at 618 nm/501 nm in the presence of bromothymol blue as pH indicator) (Scheme 3c, Figure S4 and Scheme S4). In the presence of urease, the ATHase stalls below pH 7.0, instead of pH > 8.0 for the isolated ATHase (Scheme 2b). We speculate that this arises from the formation of ammonia from urea which competes with formate as a ligand for the Ir-center thus leading to an earlier onset of (reversible) inhibition. Upon fine-tuning the concentrations of urea and the urease, the activity window of the ATHase can be time-programmed (Scheme 3b).

Enzymatic logic gates producing a pH change as output have been reported. In such systems, urease and esterase are used to produce base and acid, respectively.[14] Building upon this, we hypothesized that one could use a dormant activator to substitute HCl, and thus program the progressive onset of

Scheme 4. a) Schematic representation of time-dependent reduction of enrofloxacin **1** by ATHase [(Biot-Cp*)Ir(N^N)] **3**·Sav K121R programmed by esterase activation and urease inhibition. b) Time dependent reduction of enrofloxacin **1** in the presence of ATHase, urease and esterase (solid line), ATHase and esterase (dashed line) and ATHase and urease (dotted line). Experimental conditions: 25 μM ATHase [(Biot-Cp*)Ir(N^N)] **3**·Sav K121R, 1 mM enrofloxacin **2**, 0.2 mg mL^{-1} urease, 150 mM Urea, 0.5 mg mL^{-1} esterase, 80 mM ethyl butyrate and 2 M HCOONa in 10 mM CHES (pH$_{initial}$ = 9.0) at 37 °C.

ATHase activity. We evaluated the performance of the ATHase [(Biot-Cp*)Ir(N^N)] **3**·Sav K121R in the presence of urease, that produces the alkali inhibitor and the esterase from porcine liver (E.C. 3.1.1.1) and its substrate ethyl butyrate, the dormant activator (Scheme 4). Upon addition of ethylbutyrate to a solution containing urea (the dormant deactivator), enrofloxacin **1**, the ATHase, urease and the esterase, a slow onset of ATHase activity was observed as well as a slow inhibition resulting from the production of ammonia. In the absence of esterase, no product **2** could be detected.

In summary, we have demonstrated the temporal control of an ATHase upon combining it in-parallel with an enzyme that slowly releases an inhibitor as a response to an external stimulus. The reaction network is maintained off-equilibrium by addition of acid which acts as an activator to fuel the reaction. Upon stalling, neutralization of the inhibitor by addition of a fast activator allows to restore enzymatic activity. Accordingly, the operational time-window of the ATHase can be programmed at will. This self-regulating, off-equilibrium feature is orchestrated by a feedback mechanism reminiscent of complex cellular networks (Scheme 1c). We anticipate that the concept of temporal catalyst activation will contribute to engineer complex, bottom-up (bio)chemical reaction networks that combine both natural and artificial enzymes. In particular, the prospect of exploiting a pH switch, may allow to specifically turn on the ATHase activity in cancer cells where the pH is lower than in healthy cells.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: artificial metalloenzymes · biocatalysis · cross-regulation · hydrogenation · urease

[1] a) A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, *Nature* **2001**, *409*, 258–268; b) A. J. J. Straathof, S. Panke, A. Schmid, *Curr. Opin. Biotechnol.* **2002**, *13*, 548–556; c) R. N. Patel, *ACS Catal.* **2011**, *1*, 1056–1074; d) J. H. Schrittwieser, J. Sattler, V. Resch, F. G. Mutti, W. Kroutil, *Curr. Opin. Chem. Biol.* **2011**, *15*, 249–256; e) U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185–194; f) M. T. Reetz, *J. Am. Chem. Soc.*

2013, *135*, 12480–12496; g) J. A. Rollin, T. K. Tam, Y. H. P. Zhang, *Green Chem.* **2013**, *15*, 1708–1719; h) N. J. Turner, M. D. Truppo, *Curr. Opin. Chem. Biol.* **2013**, *17*, 212–214.

[2] a) M. Bujara, M. Schumperli, R. Pellaux, M. Heinemann, S. Panke, *Nat. Chem. Biol.* **2011**, *7*, 271–277; b) X. Chen, C. Zhang, R. Zou, K. Zhou, G. Stephanopoulos, H. P. Too, *PLoS One* **2013**, *8*, e79650; c) C. Hold, S. Billerbeck, S. Panke, *Nat. Commun.* **2016**, *7*, 12971.

[3] a) D. E. Koshland, Jr., A. Goldbeter, J. B. Stock, *Science* **1982**, *217*, 220–225; b) D. Bray, *Nature* **1995**, *376*, 307–312; c) B. N. Kholodenko, *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 165–176; d) J. E. Ferrell, Jr., T. Y. Tsai, Q. Yang, *Cell* **2011**, *144*, 874–885.

[4] a) B. Novák, J. J. Tyson, *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 981–991; b) J. Kim, E. Winfree, *Mol. Syst. Biol.* **2011**, *7*, 465; c) J. Hasty, D. McMillen, J. J. Collins, *Nature* **2002**, *420*, 224–230; d) R. Kojima, D. Aubel, M. Fussenegger, *Curr. Opin. Chem. Biol.* **2015**, *28*, 29–38; e) M. Xie, V. Haellman, M. Fussenegger, *Curr. Opin. Biotechnol.* **2016**, *40*, 139–148.

[5] a) A. Zhabotinsky, *Biofizika* **1964**, *9*, 306–311; b) W. Jahnke, A. T. Winfree, *J. Chem. Educ.* **1991**, *68*, 320–324.

[6] a) G. M. Whitesides, R. F. Ismagilov, *Science* **1999**, *284*, 89–92; b) J. M. Lehn, *Angew. Chem. Int. Ed.* **2015**, *54*, 3276–3289; *Angew. Chem.* **2015**, *127*, 3326–3340.

[7] a) L. Heinen, A. Walther, *Soft Matter* **2015**, *11*, 7857–7866; b) B. A. Grzybowski, W. T. S. Huck, *Nat. Nanotechnol.* **2016**, *11*, 585–592.

[8] a) S. N. Semenov, A. S. Y. Wong, R. M. van der Made, S. G. J. Postma, J. Groen, H. W. H. van Roekel, T. F. A. de Greef, W. T. S. Huck, *Nat. Chem.* **2015**, *7*, 160–165; b) A. S. Y. Wong, S. G. J. Postma, I. N. Vialshin, S. N. Semenov, W. T. S. Huck, *J. Am. Chem. Soc.* **2015**, *137*, 12415–12420; c) S. N. Semenov, L. J. Kraft, A. Ainla, M. Zhao, M. Baghbanzadeh, V. E. Campbell, K. Kang, J. M. Fox, G. M. Whitesides, *Nature* **2016**, *537*, 656–660; d) J. J. Armao, J. M. Lehn, *J. Am. Chem. Soc.* **2016**, *138*, 16809–16814; e) B. Yurke, A. J. Turberfield, A. P. Mills, Jr., F. C. Simmel, J. L. Neumann, *Nature* **2000**, *406*, 605–608; f) J. Boekhoven, A. M. Brizard, K. N. Kowlgi, G. J. M. Koper, R. Eelkema, J. H. van Esch, *Angew. Chem. Int. Ed.* **2010**, *49*, 4825–4828; *Angew. Chem.* **2010**, *122*, 4935–4938; g) J. Boekhoven, W. E. Hendriksen, G. J. M. Koper, R. Eelkema, J. H. van Esch, *Science* **2015**, *349*, 1075–1079; h) J. Boekhoven, J. M. Poolman, C. Maity, F. Li, L. van der Mee, C. B. Minkenberg, E. Mendes, J. H. van Esch, R. Eelkema, *Nat. Chem.* **2013**, *5*, 433–437; i) S. Ogi, T. Fukui, M. L. Jue, M. Takeuchi, K. Sugiyasu, *Angew. Chem. Int. Ed.* **2014**, *53*, 14363–14367; *Angew. Chem.* **2014**, *126*, 14591–14595; j) T. Heuser, A. K. Steppert, C. M. Lopez, B. Zhu, A. Walther, *Nano Lett.* **2015**, *15*, 2213–2219; k) T. Heuser, E. Weyandt, A. Walther, *Angew. Chem. Int. Ed.* **2015**, *54*, 13258–13262; *Angew. Chem.* **2015**, *127*, 13456–13460; l) E. Jee, T. Bansagi, A. F. Taylor, J. A. Pojman, *Angew. Chem. Int. Ed.* **2016**, *55*, 2127–2131; *Angew. Chem.* **2016**, *128*, 2167–2171; m) S. G. J. Postma, I. N. Vialshin, C. Y. Gerritsen, M. Bao, W. T. S. Huck, *Angew. Chem. Int. Ed.* **2017**, *56*, 1794–1798; *Angew. Chem.* **2017**, *129*, 1820–1824.

[9] a) M. E. Wilson, G. M. Whitesides, *J. Am. Chem. Soc.* **1978**, *100*, 306–307; b) T. Heinisch, T. R. Ward, *Acc. Chem. Res.* **2016**, *49*, 1711–1721; c) F. Yu, V. M. Cangelosi, M. L. Zastrow, M. Tegoni, J. S. Plegaria, A. G. Tebo, C. S. Mocny, L. Ruckthong, H. Qayyum, V. L. Pecoraro, *Chem. Rev.* **2014**, *114*, 3495–3578; d) T. Ueno, S. Abe, N. Yokoi, Y. Watanabe, *Coord. Chem. Rev.* **2007**, *251*, 2717–2731; e) T. Hayashi, Y. Sano, A. Onoda, *Isr. J. Chem.* **2015**, *55*, 76–84; f) J. C. Lewis, *Curr. Opin. Chem. Biol.* **2015**, *25*, 27–35; g) P. J. Deuss, R. den Heeten, W. Laan, P. C. Kamer, *Chem. Eur. J.* **2011**, *17*, 4680–4698; h) O. Pàmies, M. Diéguez, J. E. Bäckvall, *Adv. Synth. Catal.* **2015**, *357*, 1567–1586; i) J. Bos, G. Roelfes, *Curr. Opin. Chem. Biol.* **2014**, *19*, 135–143.

[10] a) V. Köhler, Y. M. Wilson, M. Dürrenberger, D. Ghislieri, E. Churakova, T. Quinto, L. Knörr, D. Häussinger, F. Hollmann, N. J. Turner, T. R. Ward, *Nat. Chem.* **2013**, *5*, 93–99; b) Y. Okamoto, V. Köhler, C. E. Paul, F. Hollmann, T. R. Ward, *ACS Catal.* **2016**, *6*, 3553–3557; c) Y. Okamoto, V. Köhler, T. R. Ward, *J. Am. Chem. Soc.* **2016**, *138*, 5781–5784.

[11] a) M. Jeschek, R. Reuter, T. Heinisch, C. Trindler, J. Klehr, S. Panke, T. R. Ward, *Nature* **2016**, *537*, 661–665; b) H. M. Key, P. Dydio, D. S. Clark, J. F. Hartwig, *Nature* **2016**, *534*, 534–537.

[12] T. Heinisch, K. Langowska, P. Tanner, J. L. Reymond, W. Meier, C. Palivan, T. R. Ward, *ChemCatChem* **2013**, *5*, 720–723.

[13] a) I. Turel, *Coord. Chem. Rev.* **2002**, *232*, 27–47; b) S. T. Nguyen, X. Ding, M. M. Butler, T. F. Tashjian, N. P. Peet, T. L. Bowlin, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5961–5963.

[14] a) E. Katz, V. Privman, *Chem. Soc. Rev.* **2010**, *39*, 1835–1857; b) M. Motornov, J. Zhou, M. Pita, V. Gopishetty, I. Tokarev, E. Katz, S. Minko, *Nano Lett.* **2008**, *8*, 2993–2997; c) I. Tokarev, V. Gopishetty, J. Zhou, M. Pita, M. Motornov, E. Katz, S. Minko, *ACS Appl. Mater. Interfaces* **2009**, *1*, 532–536.