Biosensors

DOI: 10.1002/anie.201203684

A Selective Fluorescent Probe for Carbon Monoxide Imaging in Living Cells**

*Jing Wang, Jason Karpus, Boxuan Simen Zhao, Zheng Luo, Peng R. Chen, and Chuan He**

Carbon monoxide (CO) has long been primarily viewed as a toxic gas because of its strong affinity for hemoglobin.[1,2] However, the biological function of carbon monoxide and its roles as a cellular messenger in mammalian cells have recently attracted significant interest. Studies have shown that mammalian cells constantly generate carbon monoxide gas through the endogenous degradation of heme by a family of constitutive (HO-2) and inducible (HO-1) heme oxygenase enzymes.[3–6] Recent research has suggested that neural injury can be either ameliorated or exacerbated[7] by the HO–CO system. Overexpression or knockout of HO-1 in the mouse brain generates interesting phenotypes.[8] However, the underlying mechanisms and the exact roles that HO-1 and CO play in these systems are still unknown.

Currently, a major barrier to further elucidate the complex contributions of the HO–CO system to neural injury and other signaling events is the lack of methods for selectively tracking CO within biological systems. Current techniques for CO detection are limited, particularly under living conditions. These techniques include electrochemical sensors,[9] gas chromatography/reduction gas detectors (GC/RGD),[10–13] and quantum-cascade-laser technology, which allow detection of biogenic CO generation. However, there are currently no fluorescent probes capable of selective detection and monitoring of physiological levels of CO inside living cells.

We present here the design of a genetically encoded fluorescent probe that is capable of selectively imaging CO in living cells (Figure 1). We take advantage of the unique CO-binding selectivity of CooA, a dimeric CO-sensing heme protein from *Rhodospirillum rubrum*. CooA is a homodimer that contains one *b*-type heme per 25 kDa monomer and is capable of selectively binding to CO.[14,15] CooA consists of two major domains: a regulatory, effector-binding domain that contains the heme; and a DNA-binding domain.[16] The heme iron in the reduced CooA is six-coordinate with His77 and Pro2 serving as axial ligands.[17] The crystal structure and biochemical experiments show that CO displaces Pro2 and induces a conformation change that twists the long C helix between Phe132 and Asp134, thus allowing CooA to bind DNA and activate transcription of *coo* operon (Figure 1 A,B).[18,19] We decided to insert a fluorescent protein into this region with the intention of using the natural selectivity of CooA toward CO to construct an effective reporter (Figure 1 C).

Green fluorescent protein and its variants have long been used to develop genetically encoded intracellular reporters

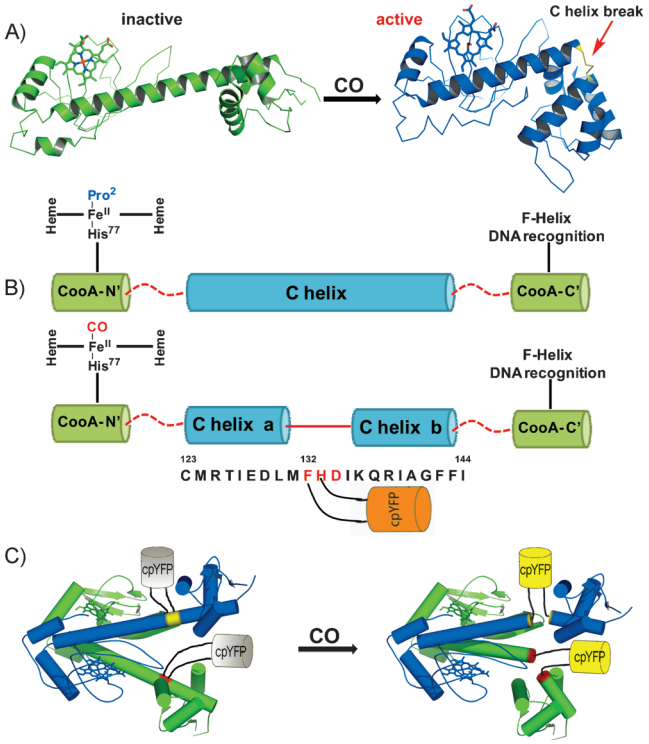

Figure 1. Design and construction of COSer. A) left: inactive monomer WT RrCooA (PDB No. 1FT9), and right: active monomer LLCooA (PDB No. 2HKX) after CO binding. Comparison of these two structures suggests that CO binding to CooA induces a conformational change. B) Upon CO binding, the long C helix of CooA is broken into two parts (C helix a and b) connected by residue 132 to 134. This flexible loop serves as the insertion point for our cpVenus. C) COSer contains a cpVenus inserted by two short linkers between residues 132 and 133 in each CooA monomer. The resulting structural change upon CO binding to CooA induces an increase in fluorescence of the probe.

[*] J. Wang, J. Karpus, Prof. Dr. C. He
Department of Chemistry, The University of Chicago
929 East 57th Street, Chicago, IL 60637 (USA)
E-mail: chuanhe@uchicago.edu
B. Zhao, Z. Luo, Prof. Dr. P. R. Chen, Prof. Dr. C. He
Beijing National Laboratory for Molecular Sciences (BNLMS), Department of Chemical Biology, College of Chemistry, and Synthetic and Functional Biomolecules Center, Peking University
Beijing 100871 (China)

[**] We thank Dr. C. Labno for assistance in all imaging experiments, Dr. S. W. Ragsdale for supplying CO-releasing molecules, and S. F. Reichard, MA for editing. This work was supported by the National Key Basic Research Foundation of China 2011CB809103, the US National Science Foundation International Collaboration in Chemistry between US Investigators and their Counterparts Abroad (CHE-0922998/NSFC 20921120404 to C.H.), and CHE-1213598 (C.H.).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201203684.

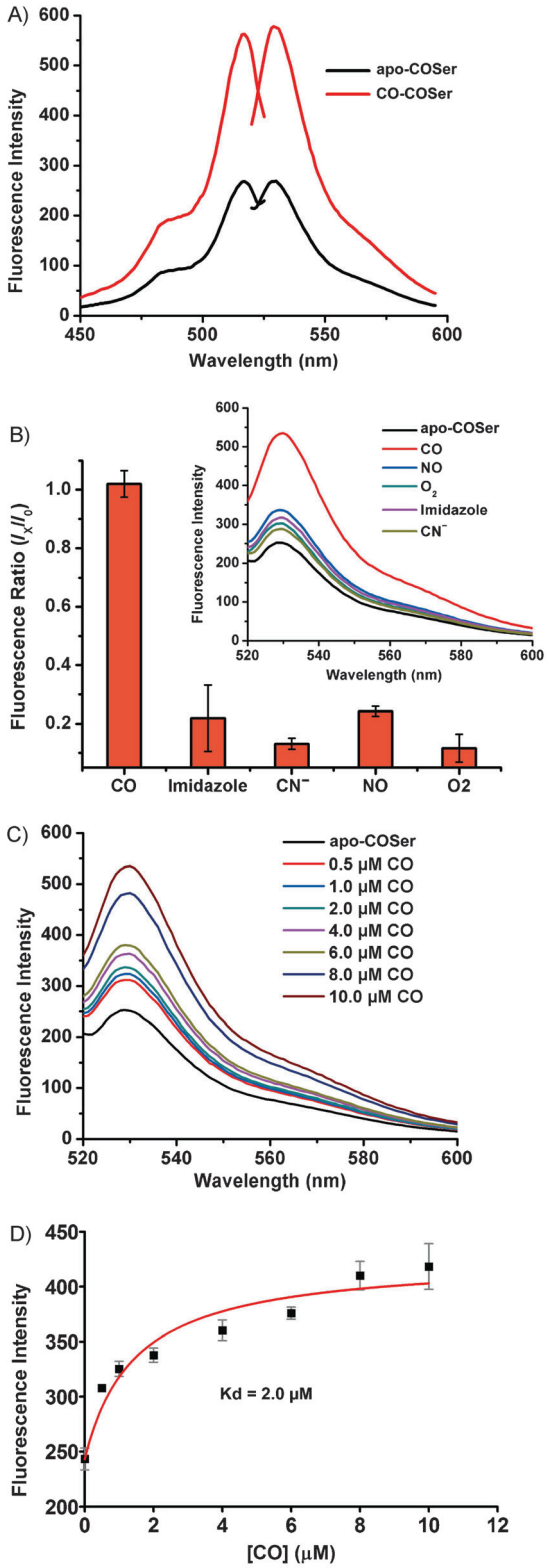

Figure 2. Characterization of COSer in vitro. A) Excitation and emission spectra for apo-COSer (black) and CO-COSer (red). B) Fluorescent response of COSer to various heme-binding ligands, including CO (10 μM), imidazole (100 μM), CN^- (100 μM), NO (20 μM), and O_2 (100 μM). C) COSer incubated with different concentrations of CO. D) Measurement of K_d value for COSer: fluorescence intensity at 528 nm was monitored as a function of CO concentration. Excitation: 516 nm, emission: 520–600 nm. All measurements were repeated three times.

for a variety of analytes.[20,21] A circularly permuted variant of yellow fluorescent proteins (cpYFP and cpVenus) has proven to be highly sensitive to the conformation of the protein. Previously, this method was successfully applied to probe reactive oxidative species (ROS) in living cells.[22,23] Through the use of fluorescence screening, a robust reporter system was created by introducing new N- and C-terminal residues with a flexible linker GGSGG.[24] This new version of cpVenus has been shown to be highly sensitive to conformational changes Recently, we have constructed a highly sensitive and selective biosensor for organic hydroperoxides (OHPs) by using this cpVenus construct with two short amino acid linkers, Ser-Ala-Gly and Gly-Thr-Gly.[23] Encouraged by this success, we sought to design a chimera construct by inserting cpVenus between residues Phe132 and His133 of CooA. This cpVenus-CooA fusion protein was constructed by PIPE cloning method (see Table 1 in the Supporting Information) and was named COSer (CO Sensor). After confirming the sequence of our newly constructed probe, we proceeded to characterize its sensitivity and selectivity.

First, we tested the fluorescence properties of the protein probe in vitro. COSer was purified as previously described,[25] and the purity of COSer was estimated to be approximately 95% based on SDS-PAGE (Figure S1, Supporting Information). The protein was stored in a MOPS (25 mM)/NaCl (100 mM) buffer (pH 7.4; MOPS = 3-(*N*-morpholino)-propanesulfonic acid) and treated with 20 mM sodium dithionite (20 mM) to generate COSer [Fe^{II}] in the absence of O_2. The CO stock solution was prepared by purging CO gas into water (2.0 mL) at 1 atm. The concentration of dissolved CO was estimated to be 1 mM.[9,26] The protein sample was sealed in a cuvette after dilution to a concentration of 1 μM. All operations were performed under anaerobic conditions to prevent potential oxidation by air, although CooA is quite selective against dioxygen. As CO binds to the heme of CooA, the electronic and structural properties of the heme play a key role in the CO-sensing mechanism. We tested the absorption spectrum of COSer and found a heme occupancy of COSer of around 63% using the known extinction coefficient of 34.4 $mM^{-1}cm^{-1}$ (Figure S2, Supporting Information).[27] Prior studies also reported similar occupancy experimentally, which could be a result of our assay overestimating the amount of protein present, incomplete heme incorporation, or heme loss during the purification process.[27] With the exception of CO, no significant change in absorption of the electronic absorption spectrum was observed for a variety of small molecules tested (Figure S3, Supporting Information). Compared to the apo-COSer, the fluorescence emission (at 528 nm) of the purified COSer increased by factor two after incubation with CO (final concentration = 10 μM) for 10 minutes when excited at 516 nm (Figure 2A). We determined the selectivity of COSer by testing other small molecules, such as NO, O_2, CN^-, and imidazole, which are known to be good ligands for heme. A very good selectivity was observed for CO over these other biologically relevant ligands, including NO (20 μM) and O_2 (100 μM; both elicited only between 10% to 20% increase). Other relevant ligands, such as CN^- (100 μM) and imidazole (100 μM), showed limited fluorescence responses as well (Figure 2B, and Figure S4 in

the Supporting Information). Two other cellular species with known heme-binding abilities are H_2S and GSH, so we also tested the fluorescence response of our probe in excess of both of these analytes. Minimal response was observed with both Na_2S and GSH, which further demonstrates the specificity of the probe (Figures S5 and S6 in the Supporting Information). To further test the selectivity of our probe for CO, we performed competition assays with some of the more prevalent biological ligands. Incubation with GSH or CN^- gave only weak fluorescence response and subsequent addition of CO regenerated nearly the full response (Figures S7 and S8, Supporting Information). Taken together, these assays demonstrate that our probe has high selectivity that can be used for specific detection and imaging of CO in aqueous media at physiological pH. To obtain the CO-binding affinity of COSer, we treated the probe with a series of different concentrations of CO and monitored fluorescence response (Figure 2C). A binding curve was obtained and the K_d value was found to be 2.0 μM at pH 7.4 (Figure 2D). This K_d value agrees with the previously established affinity of CooA to CO.[28] The data also indicate that the probe can give noticeable responses (20–30%) at 1–2 μM levels of CO.

To confirm the utility of our probe for use in imaging CO fluctuations inside living cells, we transfected HeLa cells with a COSer-containing expression vector (Figure S9, Supporting Information). Cells were prepared for imaging experiments and a solution that contained CO was then added to the cells. While some background fluorescence was observed even in the absence of supplemental CO (Figure 3A), the signal intensity increased after incubation with CO (5 μM; Figure 3B). Higher CO concentrations (i.e., 10 μM) generated an even more significant response (Figure 3C and D). To ensure that our probe is selective for CO inside cells, a variety of other biologically relevant small molecules were tested. Incubation with NO (40 μM), O_2 (100 μM), Na_2S (100 μM), or GSH (100 μM) failed to generate a significant response (Figure 4A–D, and Figure S10A–D in the Supporting Information). Differential interference contrast (DIC) transmission measurements after CO, NO, O_2, H_2S, and GSH addition

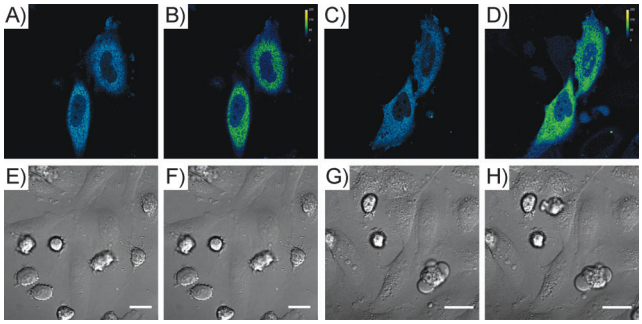

Figure 3. COSer response to different concentrations of CO in living HeLa cells. A–D) HeLa cells expressing COSer without added CO (A and C; control experiments), and with added CO (B: 5 μM; and D: 10 μM). E–H) DIC images of live HeLa cells before and after treatment with CO. Compared with control experiments, HeLa cells showed an increase in fluorescence of 24% after incubation with 5 μM CO, and 35% after incubation with 10 μM CO. Scale bars: 20 μm.

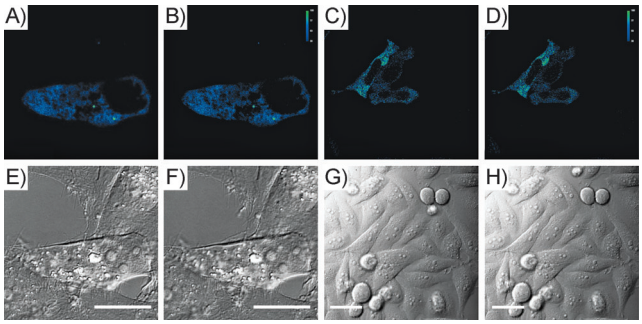

Figure 4. COSer response to a variety of biologically relevant gases in living HeLa cells. A–D) HeLa cells expressing COSer without additional analyte (A and C; control experiments), and with the addition of B) NO (40 μM), and D) O_2 (100 μM). All images were taken 20 min after addition of analyte. E–H) DIC images of live HeLa cells before and after the treatment of NO and O_2. Compared with control experiments, HeLa cells showed no significant increase in fluorescence after incubation with NO or O_2. Scale bars: 20 μm.

(Figures 3E–H and 4E–H, and Figure S10E–H in the Supporting Information) confirmed that the cells were viable throughout the imaging experiments. However, the levels that are needed to saturate the probe are higher than those used in vitro. While the response is still satisfactory, it appears as if the CO is having difficulty entering the cell, or that upon CO uptake it may bind to other metallocenters before reaching the probe. We also tested the performance of the probe in HEK293FT cells, which displayed a similar fluorescent response (Figure S11, Supporting Information).

In order to more accurately generate CO inside cells, we employed CO-releasing molecules (CO-RMs) that have been tested in a number of in vivo assays.[29–34] These compounds elicited a significant response, thus demonstrating that the delivery of CO is attainable by storing the gas in a stable chemical form (carbonyl/transition-metal complex). This alternate method of delivery is important, as it could carry and supply CO to tissues in a more controllable fashion than can be achieved with CO gas. These complexes provided an alternative method to deliver CO to cells and could have possibly mitigated the difficulty we had in getting CO into the cells by using simple CO solutions. HeLa cells were prepared for imaging and then incubated with varying amounts of CO-RM2. The signal intensity significantly increased (≈58%) after incubation with 5 μM CO-RM2, and even showed a noticeable response (≈34%) with only 1 μM of CO-RM2 (Figure 5A–D). A similar fluorescent response was observed when incubating with 10 μM CO-RM2 (Figure S12, Supporting Information), thus suggesting that the fluorescent response had reached its maximum and the probe had been saturated. This is an improvement over the response we obtained when we used the saturated CO solution, thus indicating that the lower response we observed previously is likely a result of a problem with CO uptake/delivery. By adding the CO-releasing compound rather than using the CO-saturated solution, we ensured the CO would be released inside the cells and more closely mimic real biological conditions. To ensure that the response was a result of the release of CO, we also performed a vehicle control experi-

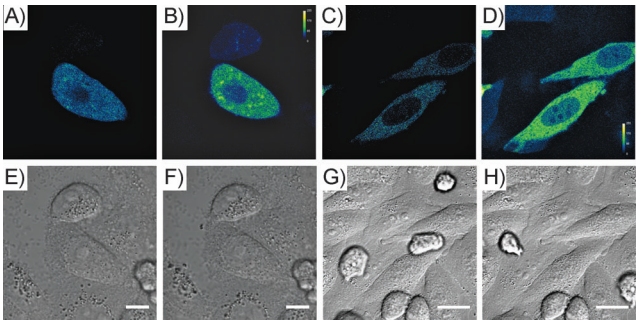

Figure 5. COSer response to incubation with CO-releasing compound (CO-RM2) in living HeLa cells. HeLa cells prior to (A, C) and after (B, D) addition of CO-RM2 (B: 1 μM for 20 min; D: 5 μM for 10 min). E–H) DIC images of live HeLa cells before and after treatment with CO-RM2. Compared with control experiments, HeLa cells showed an increase in fluorescence of 34% after incubation with 1 μM CO-RM2, and 58% after incubation with 5 μM CO-RM2. Scale bars: 10 μm (E and F), 20 μm (G and H).

ment. Dimethylsulfoxide (DMSO) proved unable to elicit a response on its own (Figure S13 in the Supporting Information). We also tested the fluorescence response of COSer to CO-RM2 in vitro and found the results consistent with our previous data using a saturated CO solution. The fluorescence increased by a factor of almost two after incubation with 8–10 μM CO-RM2 (Figure S14, Supporting Information). Finally, we performed real-time imaging of HeLa cells with the addition of 5 μM CO-RM2, which demonstrated a fast response for in situ monitoring of CO levels in living cells (Figure S15, Supporting Information). In contrast, only a minimal fluorescence increase was observed in HeLa cells following the treatment with 100 μM O_2 (Figure S16, Supporting Information). The aforementioned results suggest that our COSer probe is both sensitive and selective in living cells, and also demonstrate its utility for future studies of CO signaling.

In conclusion, we have successfully developed a sensitive and selective fluorescence probe named COSer for CO imaging in living cells. Among known gasotransmitters, probes for NO[35–38] and H_2S[39–43] have been reported. This new probe fulfills the urgent need for monitoring CO production and signaling in biological systems. Future work will focus on tracking endogenous CO production, in particular by heme oxygenases, as well as determining potential signaling events in mammalian cells associated with CO.

Experimental Section

Expression and purification of COSer: The gene encoding the full-length COSer was cloned into pET28a vector (Novagen). The protein carrying a C-terminal 6×His tag was expressed in *E. coli* strain BL21 (DE3). The overnight bacterial culture were diluted 1:100 fold into fresh LB and grown for additional 3 h to reach OD600 = 0.6, at which point IPTG (1.0 mM) was added to start the induction at 16°C for 16 h. Lysis of bacteria was obtained by sonication and the expressed protein was purified by a HisTrap™ HP column (GE) operated with FPLC™ system (GE) according to standard protocol.

Live-cell fluorescence imaging: Hela cells were grown in DMEM media with 10% FBS and penicillin/streptomycin (Invitrogen). 24 h after plating, the cells were transfected with pcDNA-COSer using Lipofectamine™ LTX transfection reagent (Invitrogen). 24 h after transfection, cells were treated with CO-saturated solutions of different concentrations, CO-releasing compounds, or other small molecules (40 μM NO and 100 μM O_2). The acquisition of image data and synchronization of the illumination were performed on a fixed cell DSU spinning confocal microscope (Leica).

Received: May 12, 2012
Revised: July 26, 2012
Published online: August 28, 2012

Keywords: biosensors · carbon monoxide · fluorescent probes · heme proteins · living cells

[1] C. G. Douglas, J. S. Haldane, J. B. Haldane, *J. Physiol.* **1912**, *44*, 275.
[2] J. B. Haldane, *Biochem. J.* **1927**, *21*, 1068.
[3] M. D. Maines, *Annu. Rev. Pharmacol. Toxicol.* **1997**, *37*, 517.
[4] R. Tenhunen, H. S. Marver, R. Schmid, *J. Biol. Chem.* **1969**, *244*, 6388.
[5] L. Yi, P. M. Jenkins, L. I. Leichert, U. Jakob, J. R. Martens, S. W. Ragsdale, *J. Biol. Chem.* **2009**, *284*, 20556.
[6] J. D. Gardner, L. Yi, S. W. Ragsdale, T. C. Brunold, *J. Biol. Inorg. Chem.* **2010**, *15*, 1117.
[7] J. Wang, S. Dore, *Brain* **2007**, *130*, 1643.
[8] A. S. Ahmad, H. Zhuang, S. Dore, *Neuroscience* **2006**, *141*, 1703.
[9] Y. Lee, J. Kim, *Anal. Chem.* **2007**, *79*, 7669.
[10] A. A. Kosterev, F. K. Tittel, W. Durante, M. Allen, R. Kohler, C. Gmachl, F. Capasso, D. L. Sivco, A. Y. Cho, *Appl. Phys. B* **2002**, *74*, 95.
[11] H. J. Vreman, R. J. Wong, T. Kadotani, D. K. Stevenson, *Anal. Biochem.* **2005**, *341*, 280.
[12] R. A. Dercho, K. Nakatsu, R. J. Wong, D. K. Stevenson, H. J. Vreman, *J. Pharmacol. Toxicol. Methods* **2006**, *54*, 288.
[13] Y. Morimoto, W. Durante, D. G. Lancaster, J. Klattenhoff, F. K. Tittel, *Am. J. Physiol. Heart. Circ. Physiol.* **2001**, *280*, H483.
[14] G. P. Roberts, R. L. Kerby, H. Youn, M. Conrad, *J. Inorg. Biochem.* **2005**, *99*, 280.
[15] S. Aono, *Acc. Chem. Res.* **2003**, *36*, 825.
[16] R. L. Kerby, H. Youn, M. V. Thorsteinsson, G. P. Roberts, *J. Mol. Biol.* **2003**, *325*, 809.
[17] S. Inagaki, C. Masuda, T. Akaishi, H. Nakajima, S. Yoshioka, T. Ohta, B. Pal, T. Kitagawa, S. Aono, *J. Biol. Chem.* **2005**, *280*, 3269.
[18] W. N. Lanzilotta, D. J. Schuller, M. V. Thorsteinsson, R. L. Kerby, G. P. Roberts, T. L. Poulos, *Nat. Struct. Biol.* **2000**, *7*, 876.
[19] M. Borjigin, H. Li, N. D. Lanz, R. L. Kerby, G. P. Roberts, T. L. Poulos, *Acta Crystallogr. Sect. D* **2007**, *63*, 282.
[20] O. Griesbeck, *Curr. Opin. Neurobiol.* **2004**, *14*, 636.
[21] J. Zhang, R. E. Campbell, A. Y. Ting, R. Y. Tsien, *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906.
[22] V. V. Belousov, A. F. Fradkov, K. A. Lukyanov, D. B. Staroverov, K. S. Shakhbazov, A. V. Terskikh, S. Lukyanov, *Nat. Methods* **2006**, *3*, 281.
[23] B. Simen Zhao, Y. Liang, Y. Song, C. Zheng, Z. Hao, P. R. Chen, *J. Am. Chem. Soc.* **2010**, *132*, 17065.
[24] J. J. Chiang, I. Li, K. Truong, *Biotechnol. Lett.* **2006**, *28*, 471.
[25] D. Shelver, M. V. Thorsteinsson, R. L. Kerby, S. Y. Chung, G. P. Roberts, M. F. Reynolds, R. B. Parks, J. N. Burstyn, *Biochemistry* **1999**, *38*, 2669.
[26] *Handbook of Chemistry and Physics* (Ed.: D. R. Lide), CRC, Boca Raton, FL, **1990–1991**.
[27] D. Shelver, R. L. Kerby, Y. He, G. P. Roberts, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 11216.

[28] M. Puranik, S. B. Nielsen, H. Youn, A. N. Hvitved, J. L. Bourassa, M. A. Case, C. Tengroth, G. Balakrishnan, M. V. Thorsteinsson, J. T. Groves, G. L. McLendon, G. P. Roberts, J. S. Olson, T. G. Spiro, *J. Biol. Chem.* **2004**, *279*, 21096.
[29] L. S. Nobre, J. D. Seixas, C. C. Romao, L. M. Saraiva, *Antimicrob. Agents Chemother.* **2007**, *51*, 4303.
[30] P. Sawle, J. Hammad, I. J. Fairlamb, B. Moulton, C. T. O'Brien, J. M. Lynam, A. K. Duhme-Klair, R. Foresti, R. Motterlini, *J. Pharmacol. Exp. Ther.* **2006**, *318*, 403.
[31] R. Motterlini, B. E. Mann, T. R. Johnson, J. E. Clark, R. Foresti, C. J. Green, *Curr. Pharm. Des.* **2003**, *9*, 2525.
[32] J. E. Clark, P. Naughton, S. Shurey, C. J. Green, T. R. Johnson, B. E. Mann, R. Foresti, R. Motterlini, *Circ. Res.* **2003**, *93*, 2e.
[33] L. S. Nobre, F. Al-Shahrour, J. Dopazo, L. M. Saraiva, *Microbiology* **2009**, *155*, 813.
[34] H. Song, H. Zhao, Y. Qu, Q. Sun, F. Zhang, Z. Du, W. Liang, Y. Qi, P. Yang, *J. Periodontal Res.* **2011**, *46*, 48.
[35] Y. Katayama, S. Takahashi, M. Maeda, *Anal. Chim. Acta* **1998**, *365*, 159.
[36] N. Soh, Y. Katayama, M. Maeda, *Analyst* **2001**, *126*, 564.
[37] M. H. Lim, D. Xu, S. Lippard, *Nat. Chem. Biol.* **2006**, *2*, 375.
[38] M. H. Lim, S. Lippard, *Acc. Chem. Res.* **2007**, *40*, 41.
[39] C. Liu, J. Pan, S. Li, Y. Zhao, L. Y. Wu, C. E. Berkman, A. R. Whorton, M. Xian, *Angew. Chem.* **2011**, *123*, 10511; *Angew. Chem. Int. Ed.* **2011**, *50*, 10327.
[40] A. R. Lippert, E. J. New, C. J. Chang, *J. Am. Chem. Soc.* **2011**, *133*, 10078.
[41] Y. Qian, J. Karpus, O. Kabil, S. Y. Zhang, H. L. Zhu, R. Banerjee, J. Zhao, C. He, *Nat. Commun.* **2011**, *2*, 495.
[42] C. S. Lim, G. Masanta, H. J. Kim, J. H. Han, H. M. Kim, B. R. Cho, *J. Am. Chem. Soc.* **2011**, *133*, 11132.
[43] H. Peng, Y. Cheng, C. Dai, A. L. King, B. L. Predmore, D. J. Lefer, B. Wang, *Angew. Chem.* **2011**, *123*, 9846; *Angew. Chem. Int. Ed.* **2011**, *50*, 9672.

