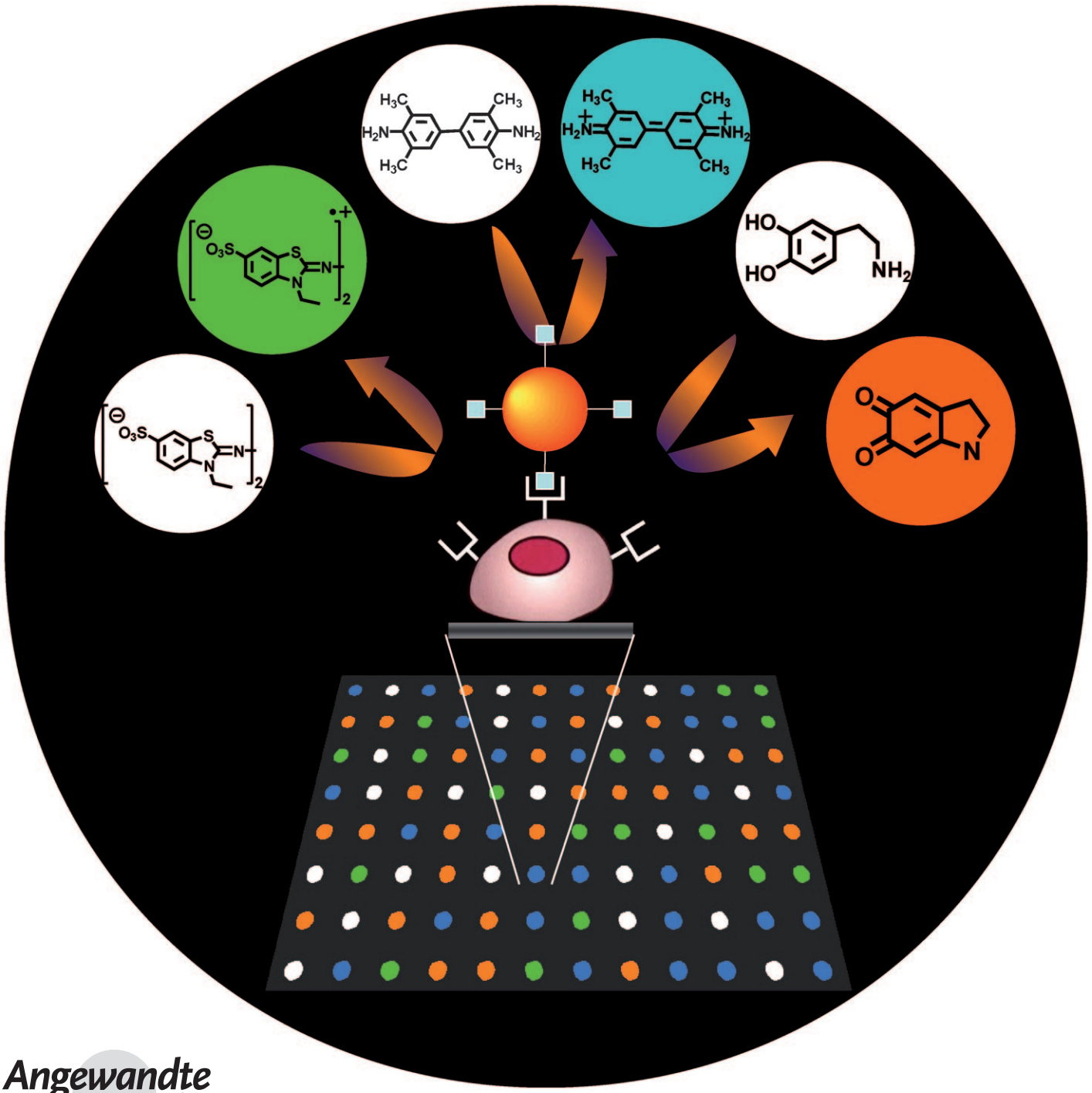
Nanoparticles

DOI: 10.1002/anie.200805279

Oxidase-Like Activity of Polymer-Coated Cerium Oxide Nanoparticles**

*Atul Asati, Santimukul Santra, Charalambos Kaittanis, Sudip Nath, and J. Manuel Perez**



Unique catalytic activities have been reported for nanoscale materials in recent years.[1] These size-dependent properties, which are often absent in the bulk materials, are the basis for the design of novel catalysts with multiple applications in energy storage, chemical synthesis, and biomedical applications.[2,3] Cerium oxide has been extensively used in catalytic converters for automobile exhaust systems, as an ultraviolet absorber, and as an electrolyte for fuel cells.[4–7] Most recently, it has been found that cerium oxide nanoparticles (nanoceria) possess antioxidant activity at physiological pH values, and the potential use of these materials in biomedical applications, such as protection against radiation damage, oxidative stress, and inflammation, has been reported.[8–12] The ability of these nanoparticles to act as an antioxidant resides on their ability to reversibly switch from Ce^{3+} to Ce^{4+}.[11] Furthermore, the synthesis of biocompatible dextran-coated nanoceria (DNC) and its enhanced stability in aqueous solution has been recently reported.[12]

Herein, we report that nanoceria has an intrinsic oxidase-like activity at acidic pH values, as it can quickly oxidize a series of organic substrates without any oxidizing agent (e.g. hydrogen peroxide). The observed activity is not only pH-dependent but is also dependent on the size of the cerium oxide nanoparticles as well as the thickness of the polymer coating. On the basis of these findings, we have designed an immunoassay in which folate-conjugated cerium oxide nanoparticles provide dual functionality by binding to folate-expressing cancer cells and facilitating detection by catalytic oxidation of sensitive colorimetric substrates (dyes). The unique pH-dependent oxidase-like activity of cerium oxide nanoparticles in aqueous media makes them a powerful tool for a wide range of potential applications in biotechnology and environmental chemistry.

For our first set of experiments, we investigated if a DNC preparation[12] could facilitate the oxidation of a series of organic dyes at low pH values. In these experiments, we selected 3,3′,5,5′-tetramethylbenzidine (TMB) and 2,2-azinobis(3-ethylbenzothizoline-6-sulfonic acid) (AzBTS), which upon oxidation develop either a blue (TMB) or green (AzBTS) color in aqueous solution.[13,14] These dyes are typically used as horseradish peroxidase (HRP) substrates in various bioassays, and most recently they have been used to demonstrate the peroxidase-like activity of iron oxide nanoparticles.[15] However, in these peroxidase-catalyzed reactions, hydrogen peroxide (H_2O_2) is required as the electron acceptor or oxidizing agent. In contrast, we have found that DNC catalyzes the fast oxidation (within minutes) of both TMB and AzBTS in the absence of hydrogen peroxide, as judged by the appearance of the characteristic color upon addition of the dyes to citrate-buffered solutions (pH 4.0) of the nanoparticles and by the corresponding UV/Vis spectrum (Figure 1a and Figure S1 in the Supporting Information).

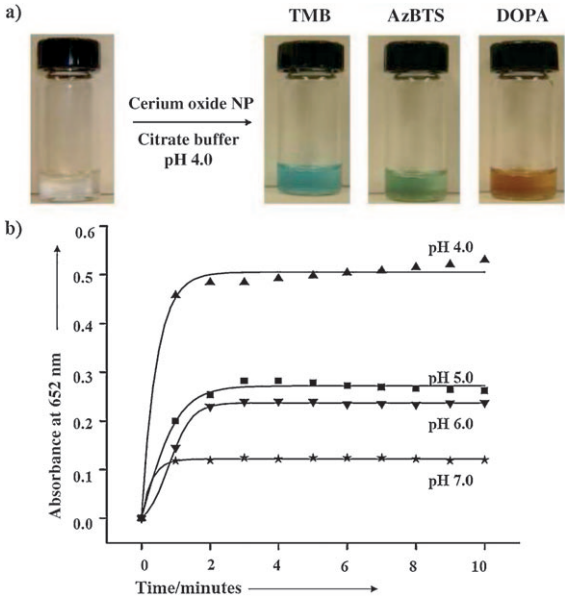


Figure 1. Formation of colored product owing to substrate oxidation is pH-dependent. a) Photographs show production of colored product upon addition of nanoceria to TMB, AzBTS, and DOPA at pH 4.0. b) Oxidation of TMB is pH-dependent, with the optimum activity at pH 4.0.

Meanwhile, at pH 7.0, no significant oxidation of TMB or AzBTS was observed, even in the presence of hydrogen peroxide or upon overnight incubation, as judged by the absence of color development upon addition of nanoceria at this pH value (Figure S2 in the Supporting Information). Furthermore, pH-dependent studies of the DNC-catalyzed oxidation of TMB show that as the pH value of the buffered solution increases from pH 4.0 to 7.0, the ability of DNC to oxidize the dye decreases (Figure 1b). These results suggest that DNC behaves as an oxidation catalyst in a pH-dependent manner and performs optimally at acidic pH values.

To further verify the ability of nanoceria to behave as an oxidation nanocatalyst, we chose dopamine (DOPA), a catecholamine difficult to oxidize at low pH values.[16] Results showed that DNC facilitated the oxidation of DOPA in citrate buffer (pH 4.0) within minutes, producing the characteristic orange color corresponding to aminochrome, one of the major oxidation products of DOPA (Figure 1a). The formation of aminochrome by DNC was confirmed by UV/Vis studies, which show the appearance of the characteristic band at 475 nm (Figure S3 in the Supporting Information). However, in the absence of DNC, no apparent oxidation of DOPA occurs at pH 4.0, even after days of incubation. This result is in contrast to that for DOPA solutions in water or citrate buffer pH 7.0, in which DOPA slowly autoxidizes, developing the characteristic reddish-brown color after overnight incu-

[*] A. Asati, Dr. S. Santra, C. Kaittanis, Dr. S. Nath, Prof. J. M. Perez
Nanoscience Technology Center, Department of Chemistry and Burnett College of Biomedical Sciences
University of Central Florida
12424 Research Parkway, Suite 400, Orlando, FL 32826 (USA)
Fax: (+1) 407-882-2843
E-mail: jmperez@mail.ucf.edu
Homepage: http://www.nanoscience.ucf.edu/faculty/perez.php

[**] The authors acknowledge funding by the National Institutes of Health (CA101781) and an UCF-NSTC Start Up Fund, all to J.M.P.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200805279.

bation. Taken together, our results demonstrate that DNC is able to catalyze the oxidation of various organic molecules at acidic pH values.

It has been well established that the catalytic properties of nanomaterials often depend upon the size of the nanocrystal.[17] However, studies on the effect of the thickness of a polymer coating surrounding the nanoparticles are less common. This motivated us to study whether the nanoceria-catalyzed oxidation of these dyes is also dependent on nanoparticle size and on the thickness of the polymer coating. Our previously reported dextran-coated nanoceria (DNC) preparation was synthesized by an in situ procedure,[12] in which the dextran (10 kDa) is present in solution at the time of the initial formation of the cerium oxide nanocrystals. Under these conditions, the polymer influences both the nucleation and the growth of the initial nanocrystal, resulting in nanoparticles with a small nanocrystal core surrounded by a thin polymeric coating. In the case of DNC, we have obtained nanoparticles with a cerium oxide core of 4 nm surrounded by a thin coating of dextran for a total nanoparticle size (hydrodynamic diameter) of 14 nm. Meanwhile, a stepwise procedure in which the polymer is added at a specific time after initial formation of the nanocrystals can be adopted for the synthesis of nanoceria. This method has been reported for the synthesis of polymer-coated iron oxide nanoparticles and yielded nanoparticles with a thicker polymer coating than those from the in situ process.[18] Furthermore, slightly larger nanocrystal cores are also obtained using this method. Therefore, to study the effect of the polymeric coating thickness on the catalytic activity of nanoceria, we synthesized DNC nanoparticles using a stepwise method. In this method, the dextran polymer was added 60 seconds after initial formation of the nanocrystals, to yield a stepwise DNC (*sw*DNC) nanoparticle preparation with an average hydrodynamic diameter of 100 nm, approximately ten times bigger than the DNC nanoparticles obtained with the in situ method (*is*DNC). Moreover, another set of polymer-coated ceria nanoparticles was synthesized using poly(acrylic acid) (1.8 kDa). The use of a polymer with a smaller molecular weight in the synthesis of polymer-coated nanoceria is advantageous, because it allows the formation of nanoparticles with an even thinner coating than those obtained with dextran (10 kDa) using either the in situ or the stepwise method. Dynamic light scattering experiments show that for the in situ nanoceria preparations coated with poly(acrylic acid) (*is*PNC), the average hydrodynamic diameter of the nanoparticles was 5 nm; whereas for the stepwise preparation (*sw*PNC), a value of 12 nm was obtained (Figure S4 in the Supporting Information). As expected, smaller nanoparticles with a thinner polymer coating were obtained using the 1.8 kDa poly(acrylic acid) polymer. The average zeta potential value (ξ) for the DNC preparation was (-0.78 ± 0.4) mV, while for PNC $\xi = (-27.8 \pm 2.4)$ mV.

Next, we used these preparations of nanoceria to perform various kinetic studies and to assess the effect of the coating thickness and nanoparticle size on the catalytic activity of nanoceria. Results show that nanoceria's ability to oxidize TMB varies with nanoparticle size in the order *is*PNC (5 nm) > *sw*PNC (12 nm) > *is*DNC (14 nm) > *sw*DNC (100 nm). Interestingly, the nanoparticles with a thin poly(acrylic acid) coating (*is*PNC) have a higher catalytic activity than those with a thicker dextran coating (*sw*DNC; Figure 2).

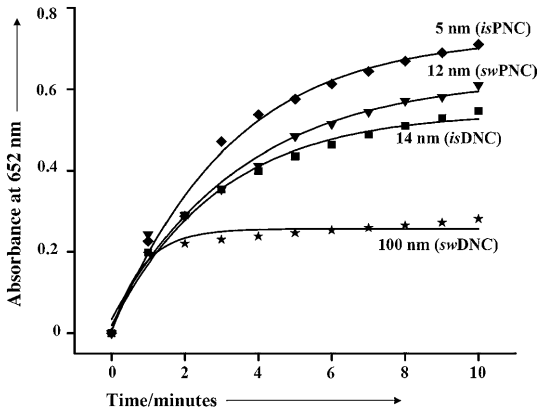


Figure 2. Nanoceria-promoted oxidation of TMB is size-dependent. At pH 4.0, smaller ceria nanoparticles show higher oxidase-like activity than larger nanoparticles.

This result might be attributed to the fact that nanoceria with a thin and permeable poly(acrylic acid) coating can facilitate the transfer of molecules to and from the nanoceria core surface faster than a thicker coating. Similar experiments were performed with AzBTS, which shows similar behavior to TMB (Figure S5 in the Supporting Information).

Next, we determined the steady-state kinetic parameters for the nanoceria-catalyzed oxidation of TMB. Typical Michaelis–Menten curves were obtained for both PNC and DNC (Figures S6 and S7 in the Supporting Information). Results show that as the hydrodynamic diameter of the nanoparticles increases, lower values for the Michaelis constant K_m and reaction rate V_{max} are obtained (Table 1). Similar results were observed with AzBTS. The fact that the nanoceria preparation with the smallest hydrodynamic diameter and thinnest coating (*is*PNC) displays the fastest kinetics (contrary to *sw*DNC) suggests that the thickness of the polymer coating plays a key role in the rate of oxidation of the substrate. Furthermore, kinetic studies of nanoceria (*is*PNC) at various pH values indicate faster kinetics at acidic pH values ($K_m = 3.8$, $V_{max} = 0.7$) and much slower kinetics at neutral pH values ($K_m = 1.3$, $V_{max} = 0.1$; Table 2), as expected. These results contrast those obtained using the enzyme HRP or iron oxide nanoparticles, for which slower kinetics are reported even in the presence of hydrogen peroxide.[15]

Table 1: Comparison of nanoceria's size-dependent kinetic parameters.[a]

Nanoceria	Size [nm]	K_m [mM]	V_{max} [μM s^{-1}]
*is*PNC	5	3.8	0.7
*sw*PNC	12	1.9	0.6
*is*DNC	14	1.8	0.5
*sw*DNC	100	0.8	0.3

[a] Data obtained at pH 4.0

Table 2: Nanoceria's oxidase-like kinetics are pH-dependent.[a]

pH	K_m [mM]	V_{max} [μM s^{-1}]
4.0	3.8	0.70
5.0	6.9	0.36
6.0	2.4	0.10
7.0	1.3	0.10

[a] Data obtained with *is*PNC.

The oxidase-like activity of ceria nanoparticles in slightly acidic aqueous solution makes them potentially useful as aqueous redox catalysts for the oxidation of water pollutants.[19] An immediate and equally important application of this technology is in the design of more robust and reliable TMB-based immunoassays using surface-modified nanoceria. In traditional ELISA, an HRP-labeled secondary antibody is utilized to assess the binding of a specific primary antibody to a particular target or surface receptor (Figure 3a). This binding event is assessed by the ability of HRP to oxidize a chromogenic substrate such as TMB in the presence of hydrogen peroxide. In traditional ELISA, the high rate of negative results is mainly attributed to 1) the instability of the antibodies that, when denatured, do not bind effectively to their target, 2) the instability of HRP that, when denatured, loses its peroxidase activity, and 3) the instability of hydrogen peroxide which, upon prolonged storage, decomposes and losses its ability to oxidize the substrate TMB in the presence of HRP. We hypothesized that a nanoceria-based detection approach would be more robust than current HRP-based assays, as no enzyme or hydrogen peroxide would be needed for detection (Figure 3b). The oxidase-like activity of the nanoceria, by itself, should facilitate the oxidation and corresponding color development. Using this assay, we can perform an immunoassay and identify the presence and concentration of a target faster and cheaper than using traditional ELISA.

For this purpose, nanoceria coated with poly(acrylic acid) (*is*PNC) was conjugated to folic acid using click chemistry (Scheme S1 and Figure S8 in the Supporting Information).[20–22] Folic acid is the ligand for the folate receptor, which is overexpressed in many tumors and cancer cell lines.[23,24] We hypothesized that a nanoceria conjugate with folic acid instead of an antifolate receptor antibody will make a more robust nanoprobe for our immunoassay. Experiments were performed using the lung cancer cell line (A-549), which overexpresses the folate receptor.[23,24] In control experiments, cardiac myocytes (H9c2) that do not overexpress the folate receptor were used.[25] In our first set of experiments, either A-549 or H9c2 cells (6000 cells) were incubated with an increasing amount of folate–cerium oxide nanoparticles in a 96-well plate for three hours and subsequently incubated with TMB (0.04 mM) for 30 minutes; product formation was monitored at 652 nm using a microtiter plate reader. As expected, folate–nanoceria-dependent binding was observed for the lung carcinoma cell line (A-549) compared to cardiac myocytes (H9c2), as judged by an increase in absorbance at 652 nm with increasing amount of folate–nanoceria (Figure 4). In another set of experiments, an increasing number of folate-positive lung carcinoma cells (1500 to 6000 cells) were treated with a constant amount of folate–ceria (5.0 μM). Results show an increase in the formation of TMB oxidation product (652 nm absorbance) with increasing number of A549 cells (Figure 5). This result was expected, as an increasing number of A549 cells translates into an increasing number of surface folate receptors available for binding to the folate–ceria nanoparticles. These results demonstrate the utility of cerium oxide nanoparticles as a detection tool, which arises from their dual functionality. Nanoceria-based assays outperform traditional sandwich ELISA, which requires hydrogen peroxide and an additional step to introduce an antibody carrying horseradish peroxidase (HRP antibody) to allow detection.

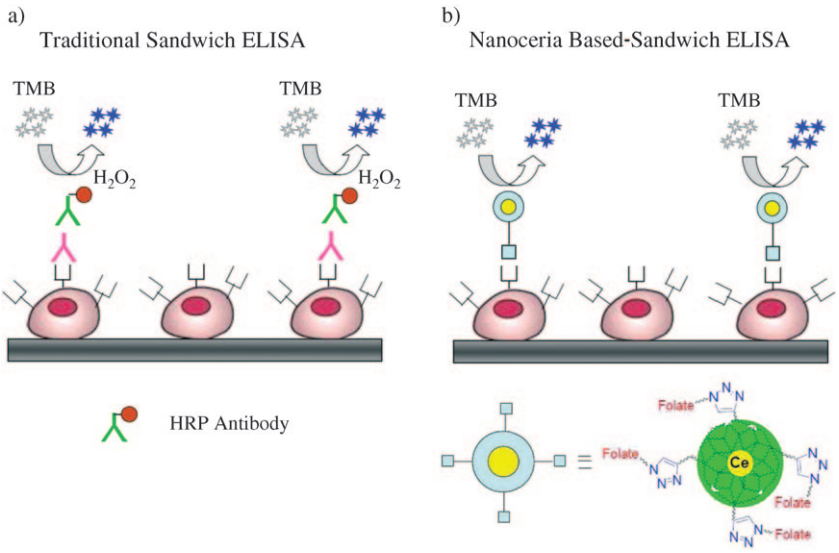


Figure 3. Comparison of traditional ELISA (a) and nanoceria-based ELISA (b). In traditional ELISA, an HRP antibody is utilized as secondary antibody that, upon hydrogen peroxide treatment, facilitates the oxidation of TMB, resulting in color development. In nanoceria-based ELISA, the oxidase-like activity of nanoceria facilitates the direct oxidation of TMB without the need of HRP or hydrogen peroxide.

In conclusion, we report that ceria nanoparticles possess unique oxidase-like activity, as they can facilitate the fast oxidation of organic dyes and small molecules in slightly acidic conditions without the need of hydrogen peroxide. When compared to other systems that require peroxides or proteins (such as oxidases and peroxidases), our polymer-coated nanoceria is a more robust and water-soluble redox nanocatalyst, as it is not susceptible to denaturation or decomposition. Furthermore, conjugation with targeting ligands makes nanoceria an effective nanocatalyst and detection tool in immunoassays. Taken together, these results demonstrate that this unique aqueous oxidase-like activity of nanoceria can be used in a wide range of new potential applications in biotech-

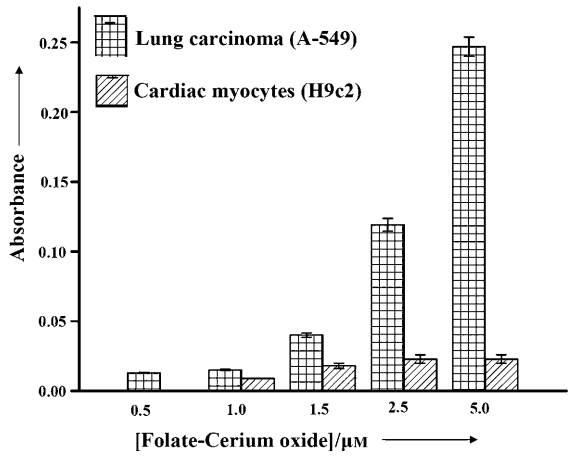


Figure 4. Nanoceria-mediated ELISA detection of folate receptor expressing cells. Folate–nanoceria associating with A-549 cells, which overexpress the folate receptor, effectively oxidizes TMB.

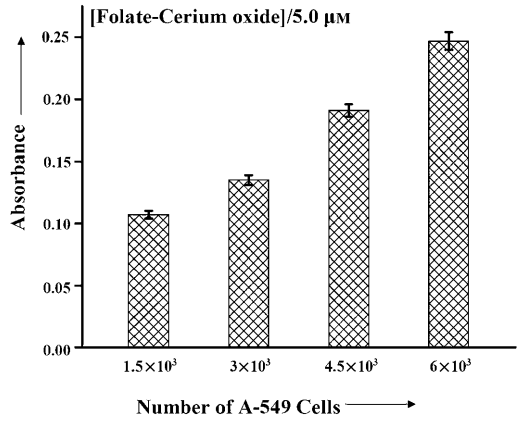


Figure 5. Nanoceria-based immunoassay is sensitive to the number of folate-positive cells (folate-receptor-expressing cells A-549).

nology, environmental chemistry, and medicine.

Received: October 28, 2008
Published online: January 7, 2009

Keywords: cerium · immunoassays · nanotechnology · oxidases · oxidation

[1] D. Astruc, F. Lu, J. R. Aranzaes, *Angew. Chem.* **2005**, *117*, 8062; *Angew. Chem. Int. Ed.* **2005**, *44*, 7852.
[2] F. Raimondi, G. G. Scherer, R. Kotz, A. Wokaun, *Angew. Chem.* **2005**, *117*, 2228; *Angew. Chem. Int. Ed.* **2005**, *44*, 2190.
[3] J. H. Choi, F. T. Nguyen, P. W. Barone, D. A. Heller, A. E. Moll, D. Patel, S. A. Boppart, M. S. Strano, *Nano Lett.* **2007**, *7*, 861.
[4] M. Chen, P. Z. Zhang, X. M. Zheng, *Catal. Today* **2004**, *93–95*, 671.
[5] T. Masui, M. Yamamoto, T. Sakata, H. Mori, G. Adachi, *J. Mater. Chem.* **2000**, *10*, 353.
[6] C. Laberty-Robert, J. W. Long, E. M. Lucas, K. A. Pettigrew, R. M. Stroud, M. S. Doescher, D. R. Rolison, *Chem. Mater.* **2006**, *18*, 50.
[7] C. Laberty-Robert, J. W. Long, K. A. Pettigrew, R. M. Stroud, D. R. Rolison, *Adv. Mater.* **2007**, *19*, 1734.
[8] R. W. Tarnuzzer, J. Colon, S. Patil, S. Seal, *Nano Lett.* **2005**, *5*, 2573.
[9] J. P. Chen, S. Patil, S. Seal, J. F. McGinnis, *Nat. Nanotechnol.* **2006**, *1*, 142.
[10] J. Niu, A. Azfer, L. M. Rogers, X. Wang, P. E. Kolattukudy, *Cardiovasc. Res.* **2007**, *73*, 549.
[11] M. Das, S. Patil, N. Bhargava, J. F. Kang, L. M. Riedel, S. Seal, J. J. Hickman, *Biomaterials* **2007**, *28*, 1918.
[12] J. M. Perez, A. Asati, S. Nath, C. Kaittanis, *Small* **2008**, *4*, 552.
[13] P. D. Josephy, R. P. Mason, T. Eling, *Cancer Res.* **1982**, *42*, 2567.
[14] C. Henriquez, C. Aliaga, E. Lissi, *Int. J. Chem. Kinet.* **2002**, *34*, 659.
[15] L. Z. Gao, J. Zhuang, L. Nie, J. B. Zhang, Y. Zhang, N. Gu, T. H. Wang, J. Feng, D. L. Yang, S. Perrett, X. Yan, *Nat. Nanotechnol.* **2007**, *2*, 577.
[16] M. Bisaglia, S. Mammi, L. Bubacco, *J. Biol. Chem.* **2007**, *282*, 15597.
[17] M. Shokouhimehr, Y. Piao, J. Kim, Y. Jang, T. Hyeon, *Angew. Chem.* **2007**, *119*, 7169; *Angew. Chem. Int. Ed.* **2007**, *46*, 7039.
[18] H. Lee, E. Lee, D. K. Kim, N. K. Jang, Y. Y. Jeong, S. Jon, *J. Am. Chem. Soc.* **2006**, *128*, 7383.
[19] B. Meunier, *Science* **2002**, *296*, 270.
[20] M. G. Finn, H. C. Kolb, V. V. Fokin, K. B. Sharpless, *Prog. Chem.* **2008**, *20*, 1.
[21] H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem.* **2001**, *113*, 2056; *Angew. Chem. Int. Ed.* **2001**, *40*, 2004.
[22] M. A. White, J. A. Johnson, J. T. Koberstein, N. J. Turro, *J. Am. Chem. Soc.* **2006**, *128*, 11356.
[23] H. Yuan, J. Miao, Y. Z. Du, J. You, F. Q. Hu, S. Zeng, *Int. J. Pharm.* **2008**, *348*, 137.
[24] M. E. Nelson, N. A. Loktionova, A. E. Pegg, R. C. Moschel, *J. Med. Chem.* **2004**, *47*, 3887.
[25] N. Parker, M. J. Turk, E. Westrick, J. D. Lewis, P. S. Low, C. P. Leamon, *Anal. Biochem.* **2005**, *338*, 284.

