Volume 1 | Number 1 | Jan 2013 | Pages 1–100

ChemComm

Chemical Communications
www.rsc.org/chemcomm

ROYAL SOCIETY OF CHEMISTRY

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the **Information for Authors**.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard **Terms & Conditions** and the **Ethical guidelines** still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ChemComm

ARTICLE TYPE

Ultrafast colorimetric detection of nucleic acids based on the inhibition of the oxidase activity of cerium oxide nanoparticles

Moon Il Kim,[a,b] Ki Soo Park[a] and Hyun Gyu Park*[a]

A label-free colorimetric method to detect nucleic acids, which relies on target DNA induced shielding of the oxidase activity of CeO_2 NPs, is developed. With this novel strategy, target nucleic acids can be identified within a few minutes 10 and without the need for post-purification of PCR product.

Methods for detecting nucleic acids are in great demand in the areas of human genetics, clinical chemistry, cytology, and infectiology.[1] Common procedures for this purpose involve exponential amplification of target DNA sequences by using the 15 polymerase chain reaction (PCR)[2] followed by gel electrophoretic analysis.[3] However, the gel-based assays inevitably require tedious experimental procedures, specialized expertise, and long analysis times. An alternative to the use of conventional PCR coupled with gel electrophoresis, real-time PCR is now widely 20 used because it enables immediate detection of target DNA sequences during the amplification process.[4] This new method possesses many advantageous features including accurate quantification of target DNA, high sensitivity and specificity, and relatively short processing times.[5] Despite some of its distinctive 25 advantages, real-time PCR is limited by the need for special reagents (fluorescent probe or DNA-binding dye) and relatively expensive fluorescence instrumentation, which when combined critically limit applications in point-of-care testing (POCT) and facility limited environments, like those found in developing 30 countries where inferior healthcare facilities exist.[6] Thus, the development of simple and practical DNA screening methods that can be utilized in facility limited or POCT environments remains a significant goal.

Colorimetric strategies for DNA sensing are attractive because 35 they enable rapid visual detection without the need for sophisticated instrumentations. In this regard, colorimetric sensing methods employing noble metal nanoparticles (gold[7] and silver[8]) or conjugated polymers (polythiophenes[9] and polydiacetylenes[10]) have been investigated. Although these 40 systems rapidly and sensitively respond to target DNA, they possess several drawbacks including variabilities that depend on experimental environments (e.g., salt concentration, pH, etc) and that might lead to incorrect results. In addition, these types of sensing systems often require laborious pre- and/or post-45 modification procedures to conjugate the materials with probe oligonucleotides. To overcome these limitations, Li and Rothberg reported a method, which relies on single stranded DNA-induced inhibition of the salt-induced aggregation of gold nanoparticles; however, it still requires post-PCR processing of further

50 denaturation to prepare ssDNA from PCR products and annealing to induce hybridization of probe DNA with ssDNA from PCR products.[11]

Recently, the unique enzyme-like activities of certain nanoparticles have been utilized as the basis for new colorimetric 55 strategies for DNA detection. For example, the glucose oxidase activity of gold nanoparticles was employed in a novel system to detect DNA hybridization. The approach takes advantage of the remarkably different binding affinities of single (ss) and double stranded (ds) DNA to gold nanoparticles which leads to different 60 levels of inhibition of the oxidase activity of the nanoparticles.[12] We also devised a new colorimetric strategy to detect nucleic acids that utilizes target DNA induced inhibition of the peroxidase mimicking activity of magnetic nanoparticles.[13] These approaches have opened a new paradigm for the design of simple 65 and rapid methods to detect nucleic acids.

Cerium oxide nanoparticles (CeO_2 NPs) have been shown to promote rapid oxidation reactions of various organic substrates that generate colored products without the need for additional oxidizing agents (e.g., H_2O_2).[14] By employing CeO_2 NPs, color 70 generation takes place very rapidly within a few min, whereas other enzyme-like nanoparticles usually require several tens of min for color signal development.[14,15] In addition, because they are not beset by problems of enzyme instability, CeO_2 NPs have been used in place of horseradish peroxidase in more robust and 75 reliable immunoassay systems.[14,16] By taking advantage of the meritorious features of the oxidase activity of CeO_2 NPs, we have designed a new and convenient label-free colorimetric method to detect nucleic acids. The results of this investigation are described below.
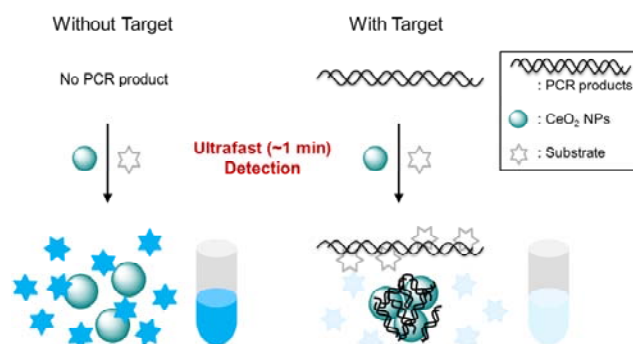
80 **Scheme 1** Schematic illustration of CeO_2 NPs-based, label-free, colorimetric detection of target DNA.

The overall strategy involved in devising the new DNA detection method is illustrated in Scheme 1. In the method, a sample containing the target DNA is first subjected to PCR amplification and then mixed with CeO_2 NPs (*ca.* 25 nm diameter), followed by addition of 3,3´,5,5´-tetramethylbenzidine (TMB), the color producing substrate. It is believed that the amplified target DNA will quickly adsorb on the surface of CeO_2 NPs owing to electrostatic interactions between the negatively charged phosphate backbone and the positively charged surface of the NPs.[14] As a consequence, direct contact of TMB with the NP surface, essential for promotion of the oxidation reaction, would be blocked. Moreover, negatively charged DNA also entraps the positively charged substrate TMB, which would also prevent free access of the substrate to the CeO_2 NPs. Finally, DNA present in the solution should also causes a certain level of aggregation of CeO_2 NPs, which would significantly decrease the availability of CeO_2 NPs surfaces and cause a further reduction of the oxidase activity.[14] These combined effects should cause a significant reduction of the oxidase activity of CeO_2 NPs and a corresponding reduction in the colorimetric signal produced by oxidation of TMB. In contrast, when amplified nucleic acids are not formed because target DNA is not present in the sample, CeO_2 NPs promoted oxidation of TMB should proceed in a normal manner to generate an intense blue colored product. Importantly, the differences in the intensities of colorimetric signals generated in these two cases should be distinguished easily even using the naked eye.
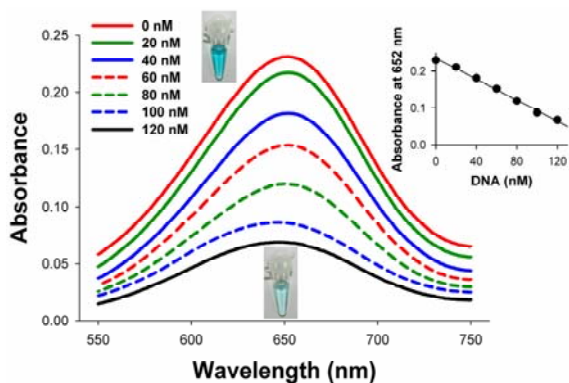

Fig. 1 UV-vis absorption spectra obtained from an assay system comprised of CeO_2 NPs and varying concentrations of model target DNA from C. trachomatis. The inset is a plot of the absorbance at 652 nm *vs.* the concentration of target DNA, and the images show a control sample without target DNA and a test sample containing target DNA at a concentration of 120 nM.

The validity and diagnostic capability of the new strategy was determined by employing the assay system comprised of CeO_2 NPs and model target nucleic acids from *Chlamydia trachomatis*, a common bacteria causing a sexually transmitted disease (STD).[17] A six-hundred base pair (bp) PCR product at 120 nM, a typical concentration of nucleic acids amplified by PCR,[10b] was produced using a clinical specimen from a patient infected with *C. trachomatis*. The PCR product along with a control not containing DNA were then subjected to the colorimetric assay procedure illustrated in Scheme 1. The results show that the control sample gives a clearly observed colorimetric response resulting from TMB oxidation by the CeO_2 NPs. On the other hand, a significantly reduced colorimetric signal is generated from the sample that contains target DNA. The extent of signal

reduction was determined to be *ca.* 70% based on a comparison of the absorbance intensities at 652 nm, the wavelength maximum of oxidized TMB. Moreover, plots of the intensities of colorimetric signals arising from analysis of diluted DNA samples *vs.* the concentration of target DNA display an excellent linearity (Fig. 1). These observations show that, as envisioned in creating the new strategy, target DNA significantly reduces the catalytic activity of CeO_2 NPs in a manner that is proportional to concentration (Fig. 1). Importantly, the blue color signals are generated within 1 min after mixing at RT, demonstrating that the CeO_2 NPs-based DNA detection method possesses an ultrafast capability.

40 bp dsDNA and PCR products with base pair lengths of 200, 600, and 900 bp, obtained from *C. trachomatis*, were analyzed using the new assay system in order to determine the effect of the DNA length on the reduction of oxidase activity of CeO_2 NPs (Fig. S1, ESI†). The results show that the 40 bp and 200 bp DNA reduced the oxidase activity of CeO_2 NPs only by *ca.* 25% and 50%, respectively, when compared with 600 bp DNA sample. This finding indicates that longer DNA causes more efficient reduction of the oxidase activity of CeO_2 NPs. However, no big difference is found to exist between the signal reduction caused by 600 bp and 900 bp DNA, showing that the new detection system is not sensitive to DNA base pair lengths over 600 bp. We also investigated the difference between ssDNA and dsDNA on the reduction of the oxidase activity of CeO_2 NPs (Fig. S2, see the ESI†).

In order to explore the factors involved in DNA induced suppression of the oxidase activity of CeO_2 NPs, we first evaluated adsorption of nucleic acids to the CeO_2 NPs surfaces. For this purpose, CeO_2 NPs were separated by using centrifugation for 30 s of a solution of CeO_2 NPs and target DNA that is preincubated for 1 min. The NP containing pellet was treated with Eva-green dye, which generates a fluorescence signal by intercalating into dsDNA.[13] Analysis by using confocal microscopy shows that the CeO_2 NPs display a fluorescence signal confirming that adsorption of DNA takes place on the surface of CeO_2 NPs (Fig. S3, ESI†). In contrast, a fluorescence signal was not observed from CeO_2 NPs that were not incubated with the target DNA.
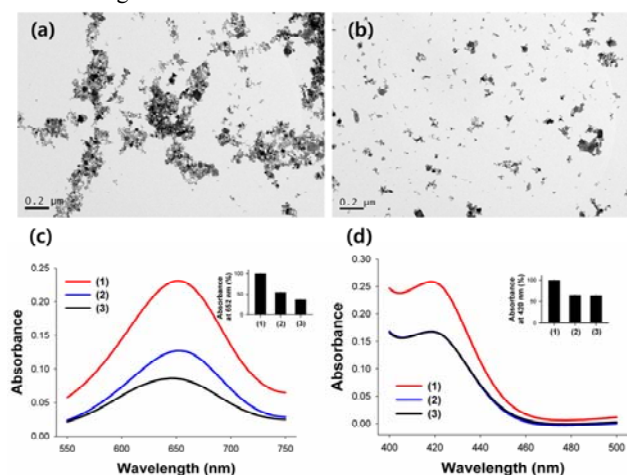

Fig. 2 TEM images of CeO_2 NPs in the presence (a) and absence (b) of target DNA, and reduction of oxidase activity of CeO_2 NPs induced by target DNA when TMB (c) or ABTS (d) is employed as a colorimetric substrate. UV-vis absorption spectra of a control sample without target DNA (1, red), a test sample containing target DNA after removal of DNA

remaining in the solution (2, blue), and a test sample containing target DNA before removal of DNA remaining in the solution (3, black). The inset contains bar graphs of the relative absorbance (%) when the absorbance from a control sample (red) was transformed to 100 *vs.* the absorbances from test samples containing target DNA.

That nucleic acids absorb CeO_2 NPs surfaces was also demonstrated by using energy dispersive X-ray spectroscopy analysis (Fig. S4, ESI†). This technique shows that elements associated with both the CeO_2 NPs (Ce and O) and DNA (N and P) are present in the NP pellet obtained after removal of the solution containing DNA. In addition, analysis of TEM images probing NP surfaces (Fig. 2a and 2b) and the particle size distributions by measuring dynamic light scattering (DLS) (Fig. S5a, ESI†), show that CeO_2 NPs preincubated with nucleic acids, aggregate to a greater extent than those that are not treated with nucleic acids. The presence of nucleic acids on the aggregated CeO_2 NPs was also confirmed by the negative value of zeta potential (Fig. S5b, ESI†). This finding indicates that negatively charged nucleic acids induce aggregation of positively charged CeO_2 NPs. The combined results demonstrate that nucleic acids adsorb to surfaces and induce aggregation of CeO_2 NPs, which contributes to the reduction of catalytic activity of the NPs.

The amount of nucleic acids adsorbed on the CeO_2 NPs was estimated by measuring the quantity of nucleic acids remaining in solution after centrifugal removal of the DNA absorbed CeO_2 NPs. As shown in Table S1, greater than 50% of the applied nucleic acids bind on the surface of the CeO_2 NPs. To assess the contribution made by unbound nucleic acids remaining in solution to inhibition of catalytic activity of the CeO_2 NPs, colorimetric assays to assess oxidase activity were performed before and after removal of the nucleic acids from solutions of DNA and the NPs and then compared to those arising from a control not containing DNA. The results (Fig. 2c) show that a significant reduction in the colorimetric signal occurs even after removal of the nucleic acids in the solution. However, the degree of color intensity reduction is *ca.* 70% of that of a solution containing nucleic acids in the supernatant. This observation demonstrates that nucleic acids in solution and those absorbed on NP surfaces both contribute to the reduction of oxidase activity of CeO_2 NPs.

We propose that electrostatic interactions between positively charged TMB and the negatively charged DNA molecules present in the solution hinders substrate transfer to the CeO_2 NPs and causes a reduction in the efficiency of the colorimetric signal producing oxidation reaction. To gain evidence to support the validity of this assumption, the negatively charged substrate, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), was used instead of TMB in the assay system.[18] The results show that PCR products arising from target DNA cause a reduction in the color intensity generated by oxidation of ABTS. In this case however, the extent of reduction is significantly lower than that arising when positively charged substrate TMB is employed (Fig. 2d). Importantly, no significant differences were observed in the degree of colorimetric signal reduction before and after removal of the unbound nucleic acids remaining in the assay solution (Fig. 2d; 2,3). Importantly, this finding shows that nucleic acids in the solution do not contribute to reduction of the colorimetric signal producing oxidation reaction of ABTS. Thus, it appears that nucleic acids in the solution do not interact with and cause aggregation of negatively charged ABTS whereas, as we proposed, their interaction with the positively charged TMB

inhibits oxidation by CeO_2 NPs.

Finally, the new assay method was applied to the direct analysis of PCR products arising from unpurified genomic DNA. As can be seen by viewing the graph in Fig. 3, an obvious reduction of colorimetric signal takes place even when unpurified PCR products (600 bp) at 100 nM are assayed using the CeO_2 NPs system. Although the extent of signal reduction (*ca.* 50%) is lower than when purified PCR product is used (*ca.* 70%), the reduced color intensity associated with unpurified PCR products is still easily distinguished from a negative control using the naked eye.
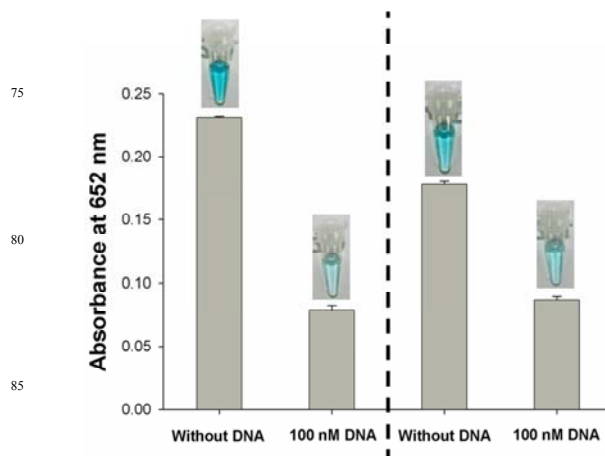

Fig. 3 The extent of absorbance reduction obtained from the CeO_2 NPs-based assay system with (left graph) or without prior purification steps (right graph). The images show a control sample without target DNA and a test sample containing target DNA at a concentration of 100 nM.

In conclusion, a new, simple and ultrafast colorimetric method for the detection of nucleic acids has been developed. The assay relies on target DNA induced inhibition of the oxidase activity of CeO_2 NPs. By using the new technique, DNA samples can be analyzed by naked-eye detection within a few minutes without the need for post-purification of PCR product, chemical modifications of either the CeO_2 NPs or DNA, and tedious salt treatment steps, all of which are generally required in gold nanoparticle-based DNA detection systems. The clinical utility of the simple CeO_2 NPs-based colorimetric detection strategy was successfully demonstrated by its use in diagnosing *C. trachomatis* using a human urine sample. As a consequence of its many advantageous features, the new assay procedure should find wide use in POCT applications. Furthermore, the strategy employed to design the new DNA sensor has the potential for being used to create assay systems to detect single nucleotide polymorphisms coupled with allele-specific PCR and biomolecules that are rationally designed to inhibit the oxidase activity of CeO_2 NPs.

Notes and references

ᵃ Department of Chemical and Biomolecular Engineering (BK 21+ program), KAIST, Daejeon 305-701, Republic of Korea. E-mail: hgpark@kaist.ac.kr; Tel.: +82-42-350-3932; Fax: +82-42-350-3910
ᵇ Department of BioNano Technology, Gachon University, Gyeonggi-do
5 *461-701, Republic of Korea*

1 (a) C. Wagener, *J. Mol. Med.* 1997, **75**, 728; (b) D. M. Morens, G. K.
10 Folkers, A. S. Fauci, *Nature* 2004, **430**, 242.
2 R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, H. A. Erlich, *Science* 1988, **239**, 487.
3 (a) O. Smithies, *Genetics* 1995, **139**, 1; (b) D. H. Van Winkle, A. Beheshti, R. L. Rill, *Electrophoresis* 2002, **23**, 15.
15 4 (a) R. Higuchi, C. Fockler, G. Dollinger, R. Watson, *Nat. Biotechnol.* 1993, **11**, 1026; (b) S. Tyagi, F. R. Kramer, *Nat. Biotechnol.* 1996, **14**, 303; (c) L. G. Lee, C. R. Connell, W. Bloch, *Nucleic Acids Res.* 1993, **21**, 3761.
5 M. J. Espy, J. R. Uhl, L. M. Sloan, S. P. Buckwalter, M. F. Jones, E. A.
20 Vetter, J. D. C. Yao, N. L. Wengenack, J. E. Rosenblatt, F. R. Cockerill, T. F. Smith, *Clin. Microbiol. Rev.* 2006, **19**, 165.
6 D. Mabey, R. W. Peeling, A. Ustianowski, M. D. Perkins, *Nat. Rev. Microbiol.* 2004, **2**, 231.
7 (a) C. A. Mirkin, R. L. Letsinger, R. C. Mucic, J. J. Storhoff, *Nature*
25 1996, **382**, 607; (b) K. Sato, K. Hosokawa, M. Maeda, *J. Am. Chem. Soc.* 2003, **125**, 8102; (c) H. X. Li, L. Rothberg, *P. Natl. Acad. Sci. USA* 2004, **101**, 14036; (d) J. J. Storhoff, A. D. Lucas, V. Garimella, Y. P. Bao, U. R. Muller, *Nat. Biotechnol.* 2004, **22**, 883; (e) K. Sato, K. Hosokawa, M. Maeda, *Nucleic Acids Res.* 2005, **33**, 5; (f) Y. L. Jung, C.
30 Jung, H. Parab, T. Li, H. G. Park, *Biosens. Bioelectron.* 2010, **25**, 1941.
8 (a) J. S. Lee, A. K. R. Lytton-Jean, S. J. Hurst, C. A. Mirkin, *Nano Lett.* 2007, **7**, 2112; (b) D. G. Thompson, A. Enright, K. Faulds, W. E. Smith, D. Graham, *Anal. Chem.* 2008, **80**, 2805; (c) R. Kanjanawarut, X. D. Su, *Anal. Chem.* 2009, **81**, 6122.
35 9 (a) H. A. Ho, M. Boissinot, M. G. Bergeron, G. Corbeil, K. Dore, D. Boudreau, M. Leclerc, *Angew. Chem. Int. Ed.* 2002, **41**, 1548; (b) H. A. Ho, A. Najari, M. Leclerc, *Accounts Chem. Res.* 2008, **41**, 168.
10 (a) D. J. Ahn, E. H. Chae, G. S. Lee, H. Y. Shim, T. E. Chang, K. D. Ahn, J. M. Kim, *J. Am. Chem. Soc.* 2003, **125**, 8976; (b) Y. K. Jung, T.
40 W. Kim, J. Kim, J. M. Kim, H. G. Park, *Adv. Funct. Mater.* 2008, **18**, 701.
11 H. Li, L. J. Rothberg, *J. Am. Chem. Soc.* 2004, **126**, 10958.
12 X. X. Zheng, Q. Liu, C. Jing, Y. Li, D. Li, W. J. Luo, Y. Q. Wen, Y. He, Q. Huang, Y. T. Long, C. H. Fan, *Angew. Chem. Int. Ed.* 2011, **50**,
45 11994.
13 K. S. Park, M. I. Kim, D. Y. Cho, H. G. Park, *Small* 2011, **7**, 1521.
14 A. Asati, S. Santra, C. Kaittanis, S. Nath, J. M. Perez, *Angew. Chem. Int. Ed.* 2009, **48**, 2308.
15 H. Wei, E. Wang, *Chem. Soc. Rev.* 2013, **42**, 6060.
50 16 A. Asati, C. Kaittanis, S. Santra, J. M. Perez, *Anal. Chem.* 2011, **83**, 2547.
17 (a) J. A. Brinkman, M. Z. Rahmani, W. E. Jones, A. K. Chaturvedi, M. E. Hagensee, *J. Clin. Virol.* 2004, **29**, 230; (b) L. Hafner, K. Beagley, P. Timms, *Mucosal Immunol.* 2008, **1**, 116; (c) B. Y. Won, D. W. Lee, S.
55 C. Shin, D. Y. Cho, S. S. Lee, H. C. Yoon, H. G. Park, *Biosens. Bioelectron.* 2008, **24**, 665; (d) W. Y. Chung, Y. L. Jung, K. S. Park, C. Jung, S. C. Shin, S. J. Hwang, D. Y. Cho, S. H. Cha, S. K. Lim, H. G. Park, *Biosens. Bioelectron.* 2011, **26**, 4314.
18 F. Q. Yu, Y. Z. Huang, A. J. Cole, V. C. Yang, *Biomaterials* 2009, **30**,
60 4716.

ChemComm Accepted Manuscript