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Communication

Cholesterol determination using protein-templated fluorescent gold nanocluster probes

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We describe the development of a fluorescent biosensor platform for soluble cholesterol based on bovine serum albumin-stabilized gold nanocluster probes co-dissolved with cholesterol oxidase (ChOx) in a surfactant emulsion system. Selective enzymatic oxidation of cholesterol to cholest-4-en-3-one by ChOx produces stoichiometric amounts of H_2O_2 by-product, generating a quenching response signaling the presence of cholesterol at clinically relevant levels (LOD ~12 μM).

According to guidelines provided by the National Cholesterol Education Program (NCEP), although cholesterol is an essential lipid for human health (an essential structural component of cell membranes, for example), elevated individual plasma cholesterol levels exceeding 6.2 mM (240 mg/dL) are associated with poor cardiovascular conditions, posing a substantial risk for increased incidence of atherosclerosis and hypertension, contributing factors underlying coronary heart disease, myocardial infarction, and stroke.[1] For obvious reasons, intense research is being pursued for diagnostics of cholesterol levels as well as other potential markers of overall health and cardiovascular risk. Particularly sought are methods which offer operational simplicity and can be implemented in resource limited settings. Currently, options for determining cholesterol levels include amperometric biosensors,[2, 3] high-performance liquid chromatography (HPLC) and other separation-based methods,[4, 5] ultracentrifugation,[6] and various enzymatic methods.[7, 8] Among these approaches, enzyme-based sensors are generally best suited to match the rigorous demands for sensitivity simultaneously coupled with high selectivity (see entries in Table S1 of the Electronic Supplementary Information, ESI†).

Metallic nanoclusters (NCs), composed of ligand-protected, atomically-precise clusters of a few to tens of metal atoms, constitute a state of matter intermediate between molecules and crystalline solids.[9, 10] The size of metallic NCs, typically smaller than 2 nm, results in uniquely tunable optical and catalytic properties.[11-14] Amongst the metal NCs, gold NCs have received considerable attention in biosensing and imaging applications due to their biocompatibility and bright fluorescence (Fig. 1).[15] Recently, Ying and co-workers reported the facile, single-pot preparation of protein-templated gold NCs using bovine serum albumin (BSA) as a mineralizing agent.[16] In recent years, BSA and several additional proteins (*e.g.*, pepsin, lysozyme, horseradish peroxidase)[17] have been used to generate fluorescent noble metal NCs of various kinds. Within the realm of optical sensing, fluorescent metal NCs have been used to detect hydrogen peroxide (H_2O_2),[17, 18] heavy metal ions (*e.g.*, Hg^{2+}, Zn^{2+}, Cd^{2+}, Cr^{3+}, Cu^{2+}),[19-22] amino acids,[23] dopamine,[24, 25] and ascorbic acid,[26] amongst other examples. A number of groups have also demonstrated quenching of the fluorescence from such nanoclusters as a result of enzymatic proteolysis by trypsin or other proteases.[14, 27, 28] Of particular relevance to the current study, the Dong group recently employed biomolecule-stabilized AuNCs for glucose detection based on the quenching brought on by enzymatically-generated H_2O_2.[29]

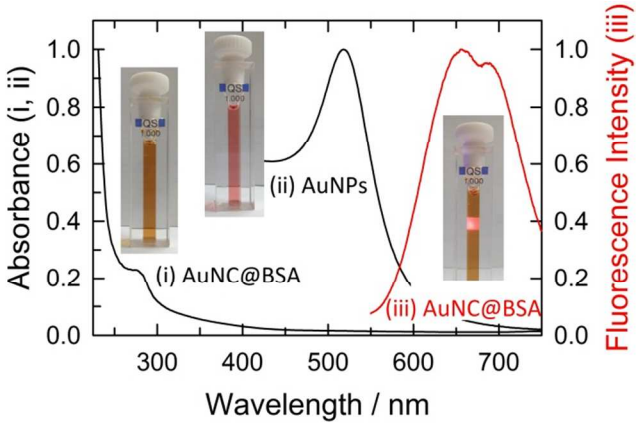

Fig. 1 Optical features of (i) absorption and (iii) emission under 405-nm laser pointer excitation for AuNCs@BSA. For comparison, the wine-red appearance of a typical dispersion of citrate-stabilized gold colloids (~20 nm AuNPs) with a localized surface plasmon resonance band near 518 nm is shown alongside the corresponding extinction spectrum in (ii).

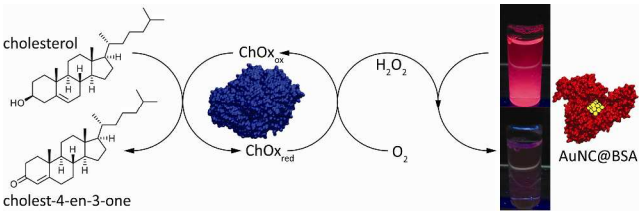

Fig. 2 Schematic representation of the sequence of events involved in cholesterol biosensing employing AuNCs@BSA as fluorescent reporters.

In this work, we investigate BSA-protected gold nanoclusters (hereafter, abbreviated as AuNCs@BSA) as quenchable fluorescent targets for enzyme-mediated cholesterol sensing. AuNCs@BSA were prepared by minor modification of the

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methods reported previously by Ying and co-workers (see ESI for details).[16] Our biosensing approach, schematically summarized in Fig. 2, entails the oxidation of cholesterol to cholest-4-en-3-one catalyzed by cholesterol oxidase (ChOx) to generate stoichiometric quantities of H_2O_2 as by-product. In turn, H_2O_2 degrades the Au–S bonds at the surface of the AuNC which are essential to nanocluster integrity. In particular, the thiol group of Cys residues plays a pivotal role in AuNC@BSA stabilization.[29, 30] In this regard, it is likely that H_2O_2 acts as an oxidant to generate disulfides or sulfonates which desorb from the AuNC surface, destabilizing its structure. Overall, these changes manifest in a quenching of the observed AuNC@BSA photoluminescence in a manner proportional to the original cholesterol concentration.

In spite of the tidy logic of the approach outlined by Fig. 2, however, whilst being readily soluble in organic solvents (*e.g.*, ethanol, chloroform, acetone), cholesterol is notoriously insoluble in water (a solubility of only 0.095 mg L^{-1} (246 pM) at 30 °C), the obvious solvent of choice for most enzymatic assays. This is an imposing technical hurdle not faced in previous oxidase-mediated sensors utilizing AuNCs@BSA probes, such as the glucose oxidase example used in the detection of glucose,[29] wherein a fully aqueous system could be employed.

Seeking a resolution to our solubility issues, we initially considered a number of surfactant-containing aqueous solvent systems. One system we considered consisting of 1.0 vol% Brij® 93 ($C_{18}H_{35}(OCH_2CH_2)_{\sim 2}OH$) plus 10 vol% isopropanol. Although this afforded improved cholesterol solubility, the deleterious impact on enzyme activity did not meet our requirements. Noting that neat surfactant would surely deactive most proteins, we ultimately selected an aqueous solution of Triton™ X-100 (TX-100, polyethylene glycol *p*-(1,1,3,3-tetramethylbutyl)-phenyl ether). Explicitly, 0.24 M TX-100 (*aq*) was found adequate for preparing cholesterol stock solutions near 13 mM.[31]

After combining a small volume of AuNCs@BSA with 0.24 M TX-100, however, an opaque, milky solution resulted, making reliable optical measurements difficult. We determined that the volumetric ratio of phosphate buffered saline (PBS) to TX-100 must be at least 1:1 in order to achieve an optically-transparent medium agreeable for spectroscopy. PBS was included to aid in protein stabilization, but it should be stressed that even dilute TX-100 exerts substantial influence over the fluorescence characteristics of AuNCs@BSA. This is illustrated in Fig. S1. As can be seen, after addition of AuNCs@BSA to a PBS/TX-100 mixture, the fluorescence signal shows a slow decay over the course of an hour or so before equilibrating to the new medium. This decay can be tentatively ascribed to the attachment of the hydrophobic aromatic domain of the TX-100 surfactant to hydrophobic regions of BSA, partially unfolding the protein.[32] As a consequence, for consistency, we aged all AuNCs@BSA samples in PBS/TX-100 media for 3 h prior to injection of ChOx.

Based on monitoring the fluorescent signal change of AuNCs@BSA pre-equilibrated in cholesterol-containing PBS/TX-100 following injection of ChOx, the ChOx activity showed a striking dependence on TX-100 concentration. As displayed in Fig. S2, when the solvent contained less TX-100 (3.7 *vs.* 18.5 vol% 0.24 M TX-100), not only was the initial fluorescence intensity much higher, but the fluorescence signal also decayed more rapidly, indicating a higher ChOx activity (*i.e.*, faster H_2O_2 formation kinetics).

In addition to stabilization of the AuNC@BSA signal, not surprisingly, the aqueous content also proved essential to maintenance of ChOx activity. Ultimately, we decided upon an aqueous detergent medium containing as little TX-100 as possible (with the remainder PBS) in order to afford adequate cholesterol solubility without completely extinguishing ChOx activity. Thus, the final composition of our assay solvent mixture was mostly (96.3 vol%) PBS with just 3.7 vol% 0.24 M TX-100. This composite solvent proved satisfactory for retention of ChOx activity without incurring obvious unfolding or unpredictable spectroscopic changes in AuNCs@BSA, whilst allowing for cholesterol solubility at levels suitable for clinical analysis.

In order to establish proof of concept for our biosensing strategy outlined in Fig. 2, we conducted an experiment comprising a positive sample group alongside two negative controls each lacking an essential component of the enzymatic/sensory sequence. The positive sample group represented by a) in Fig. 3 contained all components necessary for the tandem ChOx/AuNCs@BSA enzyme–reporter system to respond to the presence of cholesterol. Profile a) gives a typical temporal response of the cholesterol-containing AuNCs@BSA assay when triggered by ChOx injection. As can be seen, there is an immediate but rather slow decay in fluorescence signal, reaching a maximum degree of quenching of ~55% after 5 h. In control b) which lacked cholesterol, being otherwise identical to the sample group, no such quenching was observed. Shown alongside is the result of control experiment c) containing cholesterol but no ChOx. Clearly, these control experiments do not result in quenching, confirming the presumed role of H_2O_2 in the sensory response, as depicted in the scheme outlined in Fig. 2.

Although the quenching mediated by the H_2O_2 by-product produced via reaction between cholesterol and ChOx was qualitatively as expected, the overall rate of fluorescence loss is much slower than we anticipated. We interpret our results on the basis of the presence of millimolar TX-100 leading to deactivation of ChOx, a result consistent with literature observations.[33] This underscores the importance of solvent composition in enzymatic assays and suggests some avenues forward (*vide infra*). In order to confirm that the slow kinetics of H_2O_2 formation were responsible for the sluggish sensory response, we carried out control experiments in which H_2O_2 was directly injected into AuNC@BSA dissolved in purely aqueous PBS as well as in the PBS/TX-100 emulsion system. The outcome of this experiment, revealed in Fig. S3, is that the quenching is similarly rapid for the two different solvent systems; *i.e.*, ~70% quenching was observed within 5 min. This experiment clearly confirms that stunted enzymatic production of peroxide controls the rate of quenching and not some change in peroxide diffusion rate or an altered quenching mechanism resulting from the presence of the emulsion system.

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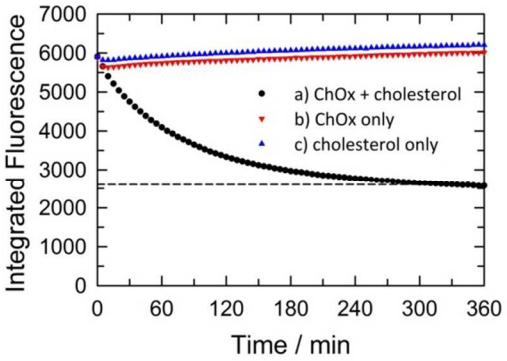

Fig. 3 Integrated fluorescence intensity (630–640 nm) of AuNCs@BSA as a function of time following cholesterol (or dummy) injection. Sample a) contains both cholesterol and ChOx. Control b) contains ChOx but no cholesterol and c) was challenged with cholesterol but no ChOx enzyme was present in the assay mixture. Excitation wavelength is 350 nm.

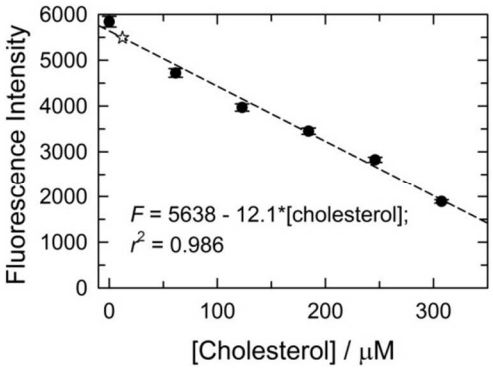

Fig. 4 Calibration curve constructed from the integrated fluorescence intensity of AuNCs@BSA in ChOx-containing aqueous emulsion following a 6 h incubation in the presence of different cholesterol concentrations. The star indicates the LOD based on three times the standard devation (3σ) of the signal for the cholesterol-free sample. (Conditions as per Table S3, ESI)

Overall, these results demonstrate that, individually, the presence of cholesterol or ChOx is insufficient to bring about fluorescence quenching of AuNCs@BSA. Thus, although the kinetics are not as rapid as we had projected, it appears that that concept indeed offers promise for the selective optical determination of cholesterol based on the relative magnitude of quenching achieved after incubation with cholesterol for a few hours. It is noteworthy that, when challenged with glucose, no quenching was observed, the profile appearing similar to the results for controls b) and c) within Fig. 3 (data not shown). We note that, following quenching by H_2O_2, AuNC@BSA spectra display slightly blue-shifted emission peaked at 635 nm instead of 643 nm (Fig. S4). We note that a similar shift occurs when AuNCs@BSA are directly quenched by the addition of H_2O_2, suggesting that this phenomenon is related at least in part to changes in the local BSA fold surrounding the AuNC induced by H_2O_2 generation.

We next tested the relationship between the steady-state fluorescence signal arising from AuNCs@BSA and the concentration of cholesterol used in the assay. We employed the integrated fluorescence intensity from 630 to 640 nm instead of the intensity at a fixed wavelength in order to achieve improved reliability (signal-to-noise, S/N) in the construction of the calibration curve. As shown in Fig. 4, a reasonably linear calibration plot for cholesterol was achieved over the concentration range 0–300 μM, with a correlation coefficient of 0.986 and a limit of detection (LOD) of 12 μM defined by three times the standard deviation (3σ) of the probe signal in the absence of cholesterol. Given that individual plasma cholesterol levels fall in the mM regime, this sensitivity is more than ample for performing assays on diluted biological specimens.

To summarize, we have shown proof of concept for using AuNCs@BSA as a fluorimetric substrate for the enzyme-based detection of cholesterol, a vital analyte that is essentially insoluble in water. The use of a specific oxidase enzyme provided target selectivity, however, the surfactant-based medium used to achieve cholesterol solubility compromised the ChOx activity, hindering the reaction rate. Although the ChOx enzyme kinetics were indeed found to be sluggish—a fact we can clearly attribute to the detergent content of the solvent system employed, a circumstance necessitated by the poor aqueous solubility of cholesterol—these results do bode well for other challenging (water-insoluble) analytes, and of course *any* oxidase substrate amenable to fully-aqueous systems. Finally, we note that we have recently designed a series of task-specific ionic liquids (TSILs) comprising glycol-substituted cations paired to acetate and shown that these TSILs promote carbohydrate dissolution (up to 10 wt% cellulose and 80 wt% D-glucose) while concurrently maintaining the transesterification activity of lipase B from *Candida antarctica*.[34] In the light of our present results, an optimization of the solvent system based on bio-compatible TSILs which simultaneously foster cholesterol dissolution with better retention of native enzyme activity might also be feasible for improving AuNCs@BSA/ChOx tandem based cholesterol sensing, an intriguing prospect we are keen to explore in future work.

References and notes

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† Electronic Supplementary Information (ESI) available: Supplemental figures and experimental details. See DOI: 10.1039/b000000x/

1. M. Nauck, W. März and H. Wieland, *Clin. Chem.*, 2000, **46**, 436-437.
2. Y. Tong, H. Li, H. Guan, J. Zhao, S. Majeed, S. Anjum, F. Liang and G. Xu, *Biosens. Bioelectron.*, 2013, **47**, 553-558
3. S. Cao, L. Zhang, Y. Chai and R. Yuan, *Talanta*, 2013, in press.
4. W. Chen, P. Li, S. Wang, J. Dong and J. Z. Li, *Clin. Chem.*, 1993, **39**, 1602-1607.
5. A. S. Csallany, S. Kindom, P. Addis and J.-H. Lee, *Lipids*, 1989, **24**, 645-651.
6. A. G. Olsson, *Acta Med. Scand.*, 1975, **581**, 1.
7. J. Prasad, A. Joshi, R. D. Jayant and R. Srivastava, *Biotechnol. Bioeng.*, 2011, **108**, 2011-2021

View Article Online
DOI: 10.1039/C3AN01548C

8. K.-E. Kim, T. G. Kim and Y.-M. Sung, *J. Nanopart. Res.*, 2012, **14**, 1-9.
9. G. Schmid, *Chem. Rev.*, 1992, **92**, 1709-1727.
10. L. Shang, S. Dong and G. U. Nienhaus, *Nano Today*, 2011, **6**, 401-418.
11. M. D. Hughes, Y.-J. Xu, P. Jenkins, P. McMorn, P. Landon, D. I. Enache, A. F. Carley, G. A. Attard, G. J. Hutchings and F. King, *Nature*, 2005, **437**, 1132-1135.
12. A. A. Herzing, C. J. Kiely, A. F. Carley, P. Landon and G. J. Hutchings, *Science*, 2008, **321**, 1331-1335.
13. H. Kawasaki, K. Hamaguchi, I. Osaka and R. Arakawa, *Adv. Funct. Mater.*, 2011, **21**, 3508-3515.
14. J. Zheng, P. R. Nicovich and R. M. Dickson, *Ann. Rev. Phys. Chem.*, 2007, **58**, 409.
15. C. Zhou, M. Long, Y. Qin, X. Sun and J. Zheng, *Angew. Chem. Int. Ed.*, 2011, **123**, 3226-3230.
16. J. Xie, Y. Zheng and J. Y. Ying, *J. Am. Chem. Soc.*, 2009, **131**, 888-889.
17. F. Wen, Y. Dong, L. Feng, S. Wang, S. Zhang and X. Zhang, *Anal. Chem.*, 2011, **83**, 1193-1196.
18. Y. Xia, W. Li, M. Wang, Z. Nie, C. Deng and S. Yao, *Talanta*, 2013, **107**, 55-60.
19. S. Liu, F. Lu and J.-J. Zhu, *Chem. Commun.*, 2011, **47**, 2661-2663.
20. L. Yan, Y. Cai, B. Zheng, H. Yuan, Y. Guo, D. Xiao and M. M. F. Choi, *J. Mater. Chem.*, 2012, **22**, 1000-1005.
21. H. Liu, X. Zhang, X. Wu, L. Jiang, C. Burda and J.-J. Zhu, *Chem. Commun.*, 2011, **47**, 4237-4239.
22. I. M. Raimundo and R. Narayanaswamy, *Sens. Actuators, B*, 2003, **90**, 189-197.
23. M. Wang, Q. Mei, K. Zhang and Z. Zhang, *Analyst*, 2012, **137**, 1618-1623.
24. Y. Tao, Y. Lin, J. Ren and X. Qu, *Biosens. Bioelectron.*, 2013, **42**, 41-46
25. P. Biji and A. Patnaik, *Analyst*, 2012, **137**, 4795-4801.
26. X. Wang, P. Wu, X. Hou and Y. Lv, *Analyst*, 2013, **138**, 229-233.
27. X. Wang, Y. Wang, H. Rao and Z. Shan, *J. Braz. Chem. Soc.*, 2012, **23**, 2011-2015.
28. Y. Wang, Y. Wang, F. Zhou, P. Kim and Y. Xia, *Small*, 2012, **8**, 3769-3773.
29. L. Jin, L. Shang, S. Guo, Y. Fang, D. Wen, L. Wang, J. Yin and S. Dong, *Biosens. Bioelectron.*, 2011, **26**, 1965-1969.
30. C. Vericat, M. Vela, G. Benitez, P. Carro and R. Salvarezza, *Chem. Soc. Rev.*, 2010, **39**, 1805-1834.
31. M. K. Ram, P. Bertoncello, H. Ding, S. Paddeu and C. Nicolini, *Biosens. Bioelectron.*, 2001, **16**, 849-856.
32. S. K. Patra and M. K. Pal, *Eur. J. Biochem.*, 1997, **246**, 658-664.
33. L. Pollegioni, G. Gadda, D. Ambrosius, S. Ghisla and M. S. Pilone, *Biotechnol. Appl. Biochem.*, 1999, **30**, 27-33.
34. H. Zhao, G. A. Baker, Z. Song, O. Olubajo, T. Crittle and D. Peters, *Green Chem.*, 2008, **10**, 696-705.

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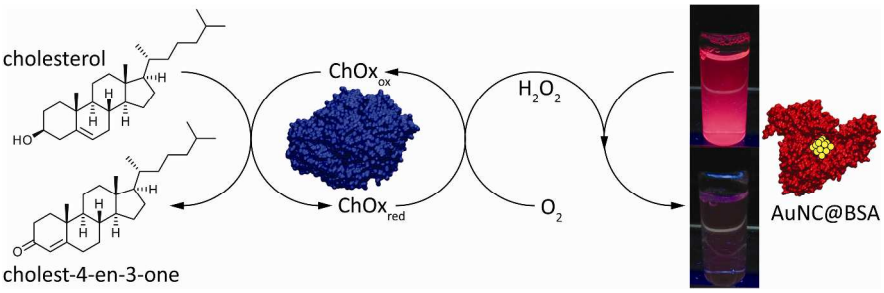
DOI: 10.1039/C3AN01548C



Protein-stabilized gold nanoclusters plus cholesterol oxidase in an aqueous emulsion comprise a biosensory tandem toward selective fluorimetric cholesterol determination.