Analyst

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Analyst*, 2017, **142**, 2322

Received 1st April 2017,
Accepted 13th May 2017

DOI: 10.1039/c7an00552k

rsc.li/analyst

Enzymatically activated reduction-caged SERS reporters for versatile bioassays†

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Here we report a facile strategy for activating reduction caged Raman reporters for surface-enhanced Raman scattering (SERS) with peroxidases. After selecting suitable caged reporters, versatile bioassays were developed. First, the bioassays for bioactive small molecules were developed. Then, the immunoassay was developed for C reactive protein (CRP), a biomarker for cardiovascular diseases.

Introduction

Activatable molecular reporters have recently attracted particular interest in bioanalytical, bioimaging and biomedical fields due to their extremely low background signals and smart response to various stimuli (such as pH, light, redox stress and enzymes).[1–14] For instance, hydrocyanines, the reduction caged forms of cyanines, were designed for *in vivo* imaging reactive oxygen species in living mice.[3] Photoactivatable fluorescent probes have recently been developed by reduction caging, which were subsequently employed for high-resolution imaging of microtubules.[5]

Despite the substantial progress, most of the currently developed activatable reporters are fluorescent ones. Only a few activatable molecular reporters have been developed for surface-enhanced Raman scattering (SERS), one of the most sensitive techniques for chemical and biomedical analysis.[2,15–28] SERS offers high sensitivity by confining Raman active reporters within the range of electromagnetic fields, which are usually originated from noble metal nanostructures (such as gold nanoparticles, AuNPs).[21,29–40] Therefore, the conversion of Raman inactive reporters into the active ones upon specific stimuli would provide novel activating strategies for SERS based bioassays. For example, a previous study by Ozaki *et al.* showed that Raman inactive *o*-phenylenediamine was converted into Raman active azoaniline with peroxidase and hydrogen peroxide. Based on this interesting activation mechanism, an ELISA for mouse IgG was developed by using peroxidase-conjugated antibodies.[15] Graham and co-workers reported several enzymatically cleavable caged SERS reporters, which were sophisticatedly designed and synthesized. In the presence of target enzymes (such as lipase and protease), the corresponding caged reporters would be activated and they produced SERS signals for measuring enzyme activities.[2,18,19]

Inspired by the early success, here we reported a facile enzymatic activation strategy for converting reduction caged Raman reporters to the active ones by peroxidase catalyzed oxidation with hydrogen peroxide. Such a strategy successfully avoided the sophisticated design and synthesis of the activatable SERS reporters. With the developed activation strategy, facile SERS assays were developed for two important bioactive small molecules, hydrogen peroxide and glucose. Moreover, the general application of the developed activation strategy was further illustrated by developing an immunoassay for the detection of cardiovascular diseases biomarker, C reactive protein (CRP).

Results and discussion

To demonstrate the feasibility of the proposed activation strategy, several commercially available molecules were tested. As shown in Fig. 1A, the reduction caged reporters were Raman inactive due to the absence of aromatic conjugation. However, when they were activated by oxidation with peroxidase (such as horseradish peroxidase (HRP)) and H_2O_2, the aromatic conjugation was restored. Therefore, the obtained Raman active reporters would give strong SERS signals. Leucomethylene blue (LMB), benzoyl LMB and leucomalachite green (LMG) were chosen as model Raman inactive reporters for testing. As

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† Electronic supplementary information (ESI) available: Experimental details and additional figures. See DOI: 10.1039/c7an00552k

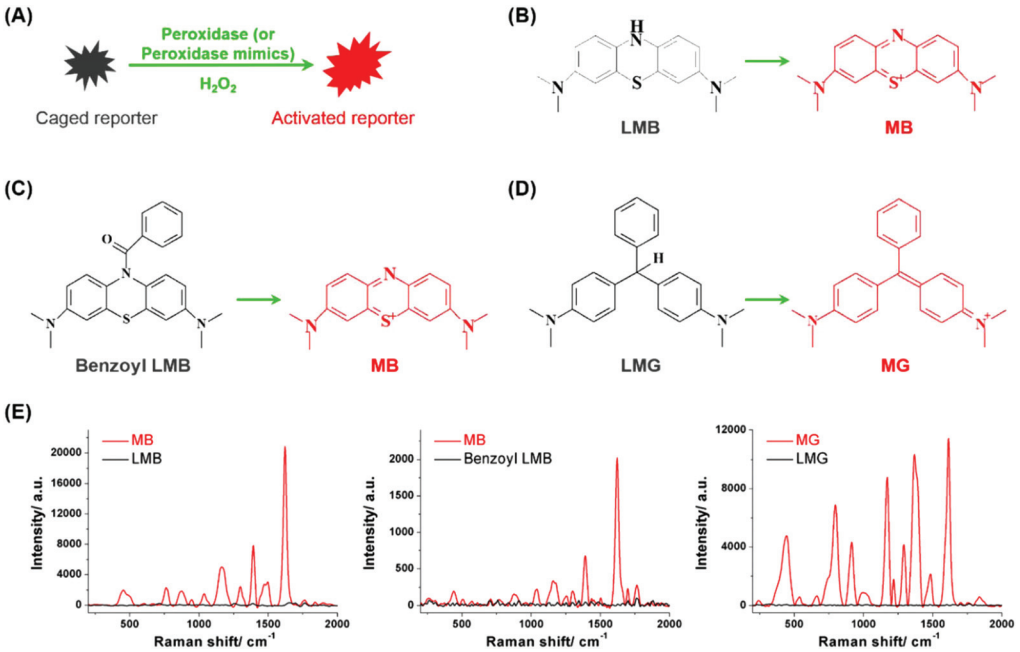

Fig. 1 (A) Schematic illustration of activating reduction caged Raman reporters with peroxidase (or peroxidase mimics) and H_2O_2 to produce Raman active reporters for SERS measurements. (B)–(D) Conversion of reduction caged Raman reporters to the corresponding Raman active reporters. (E) Typical SERS spectra of reduction caged Raman reporters and the corresponding Raman active reporters after activation.

shown in Fig. 1E, none of them produced detectable SERS signals. However, when they were activated, all the Raman active products (*i.e.*, MB and MG) produced significant SERS signals. While the hydrophobicity and solubility of benzoyl LMB and LMG are similar (hydrophobicity: $\log P_{\text{benzoyl LMB}} = 3.277 \pm 0.641$ and $\log P_{\text{LMG}} = 6.407 \pm 0.407$; solubility: benzoyl LMB = 5.5×10^{-3} g L^{-1} and LMG = 2.6×10^{-4} g L^{-1} at 25 °C), the activation efficiency of benzoyl LMB was lower than those of LMB and LMG, which was probably due to its weaker interaction with peroxidases. For both of LMB and LMG, they could be efficiently activated. However, LMB was not very stable and could be auto-oxidized in air. Therefore, LMG was chosen for further studies. Interestingly, we found that hemoglobin with peroxidase activity could also be used to activate the reduction caged reporters, demonstrating the generality of the proposed activation strategy with peroxidases and peroxidase-like enzymes (Fig. S1†). As shown in Fig. S2,† the activation of reduction caged Raman reporters by peroxidases was also verified by UV-visible absorption spectra.

After establishing the enzymatic strategy for activating reduction caged Raman reporters for SERS measurements, the bioassays for H_2O_2 and glucose were developed. To show the generality of the proposed activation strategy, hemoglobin instead of HRP was used for the two bioassays. As shown in Fig. 2A and B, the presence of H_2O_2 oxidized LMG into MG, the latter would then give strong SERS signals when it was mixed with citrate-protected AuNPs. The SERS signals displayed a good response to different concentrations of H_2O_2 from 0 to 5 mM (Fig. 2C). A linear response curve from 20 μM to 500 μM was obtained (Fig. S3†).

Moreover, glucose detection has attracted particular attention due to its important roles in diabetes and brain energy metabolism. When glucose oxidase (GOx) was coupled with hemoglobin and an activatable SERS reporter, a sensitive and selective bioassay for glucose detection was developed (Fig. 2D). As shown in Fig. 2D, glucose was catalytically oxidized by GOx to produce H_2O_2. The newly formed H_2O_2 subsequently oxidized LMG into MG. MG then produced SERS signals for measurements when it was mixed with AuNPs. As shown in Fig. S4A,† the presence of glucose indeed produced strong SERS signals. The SERS signals displayed a good response to different concentrations of glucose (Fig. 2E). A linear response curve from 0 to 200 μM was obtained (Fig. S4B†). Moreover, due to the high specificity of GOx, the bioassay exhibited excellent selectivity toward glucose detection against other saccharides (such as fructose, lactose, and maltose) (Fig. S4C†). The results clearly demonstrated the good sensitivity and selectivity of the developed SERS bioassay for glucose detection.

We further showed that the currently proposed activation strategy was also applicable to the immunoassay. To this end, CRP, a biomarker for cardiovascular diseases, was chosen as the target of interest for the immunoassay. A sandwich assay format was used, where CRP was captured with capture antibodies immobilized onto agarose beads. Then HRP-conjugated detection antibodies were applied to form the sandwich assay (Fig. 3A). The conjugated HRP would activate reduction caged LMG with H_2O_2 and generate SERS active MG for measurements. As shown in Fig. 3B, the SERS signals of MG exhibited a good response to different concentrations of CRP. A linear

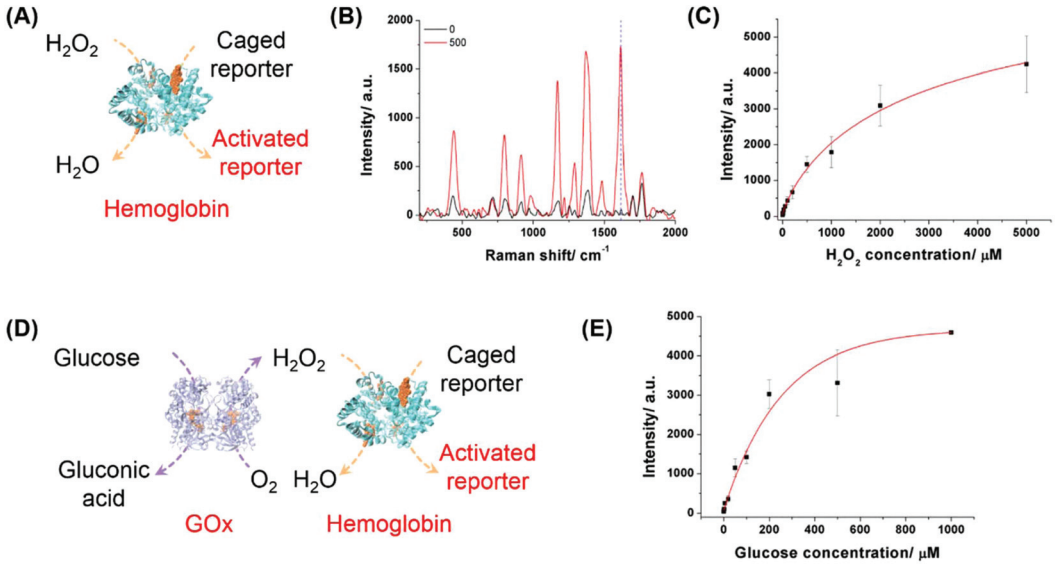

Fig. 2 (A) Schematic illustration of H_2O_2 detection with reduction caged Raman reporter and hemoglobin. (B) SERS spectra in the absence and presence of 500 μM H_2O_2. (C) Plots of Raman intensity of MG at 1615 cm^{-1} *versus* H_2O_2 concentrations. (D) Schematic illustration of glucose detection with reduction caged Raman reporter, glucose oxidase (GOx) and hemoglobin. (E) Plots of Raman intensity of MG at 1615 cm^{-1} *versus* glucose concentrations. Error bars indicate standard deviations of three independent measurements.

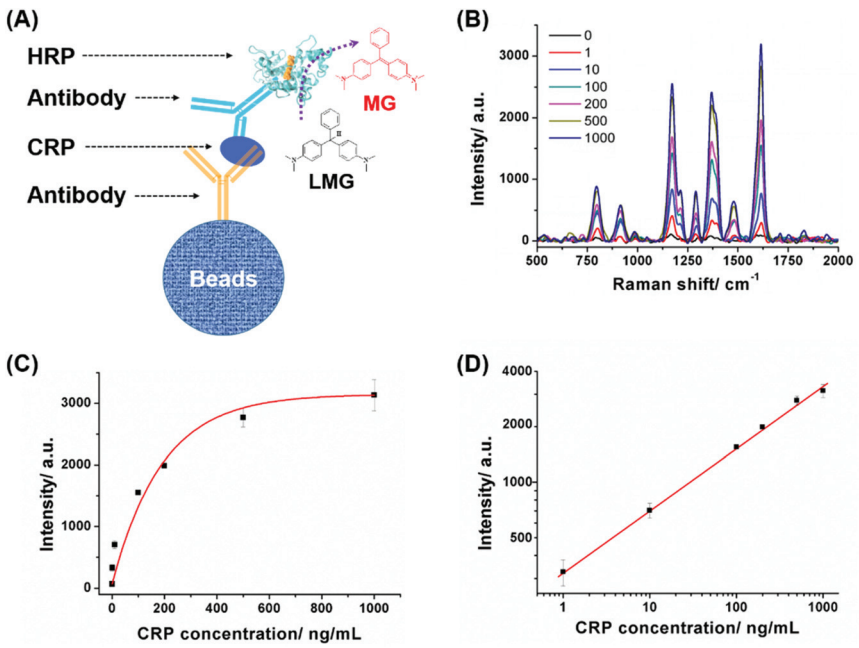

Fig. 3 (A) Schematic illustration of a sandwich immunoassay for CRP detection with reduction caged Raman reporters. (B) SERS response to different concentrations of CRP. (C) Plots of Raman intensity of MG at 1615 cm^{-1} *versus* CRP concentrations. (D) Logarithmic scale plots of Raman intensity of MG at 1615 cm^{-1} *versus* CRP concentrations. Error bars indicate standard deviations of three independent measurements.

curve for CRP from 1 to 1000 ng mL^{-1} on a logarithmic scale was obtained (Fig. 3C and D). Moreover, as high as 10 mg mL^{-1} BSA did not show significant interference towards CRP detection (Fig. S5†). These results demonstrated that the current bioassay had good sensitivity and selectivity towards CRP detection.

Conclusions

In summary, we have demonstrated an interesting enzymatic strategy to activate reduction caged reporters for SERS bioassays. Several reduction caged reporters (such as LMB, benzoyl LMB, and LMG) were oxidatively converted into

Raman active ones (*i.e.*, MB and MG) with H_2O_2 and peroxidases. By further exploring such enzymatic activation mechanisms, various facile bioassays have been developed. H_2O_2 and glucose detection was achieved by using hemoglobin and LMG. It was then showed that when a peroxidase conjugated antibody was used, an immunoassay for important biomarkers (such as CRP) could be developed. The current study not only provided a reliable new approach to activating caged reporters but also developed versatile bioassays for biologically important targets. Considering the vast number of nanomaterials with peroxidase mimicking activities, it is expected that the reduction caged reporters can be activated by these enzyme mimics for wide applications in future.[41–44]

Acknowledgements

This work was supported by the National Natural Science Foundation of China (21405081), the 973 Program (2015CB659400), the PAPD program, the Shuangchuang Program of Jiangsu Province, the Six Talents Summit Program of Jiangsu Province, the Open Funds of the State Key Laboratory of Analytical Chemistry for Life Science (SKLACLS1704), and the Thousand Talents Program for Young Researchers. We thank Professor Shuming Nie for insightful discussions and comments.

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