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FULL PAPER

Photosensitization of Molecular Oxygen on Graphene Oxide for Ultrasensitive Signal Amplification

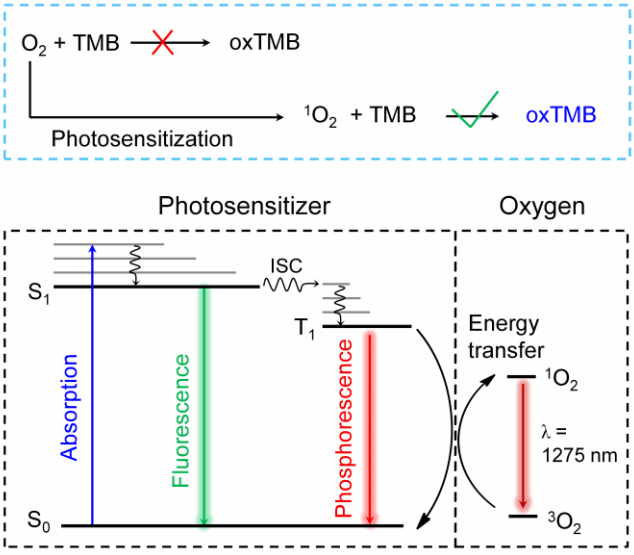
Xinfeng Zhang,[a] Li Deng,[a] Chengpeng Huang,[a] Jinyi Zhang,[b] Xiandeng Hou,[b] Peng Wu*[b] and Juewen Liu*[c]

Abstract: H_2O_2 and horseradish peroxidase (HRP) are commonly used together in bioassays. HRP is required to accelerate the reaction between a chromogenic substrate (e.g., 3,3',5,5'-tetramethylbenzidine, TMB) and H_2O_2 and thus amplifies the signal. We herein explore molecular oxygen for enzyme-free and H_2O_2-free oxidation, while still using the same colorimetric reaction. Restricted by spin selection rules, the ground-state triplet oxygen needs to be converted to the singlet state to oxide TMB. We used phloxine B (PB) as the photosensitizer for its excellent performance and safety. Under green light irradiation, each PB turnovers ~51 TMB molecules in 20 min, making PB a 'molecular enzyme mimic' for signal amplification. With its small size, multiple PB molecules were loaded on graphene oxide nanosheet to design a modified enzyme-linked immunosorbance (ELISA) assay (termed photosensitization immunosorbent assay, PISA), improving the 1: 1 enzyme/target ratio to n: 1. PISA was more sensitive for carcinoembryonic antigen than a commercial ELISA kit, and successfully measured the antigen in the serum of multiple cancer patients. This simple and green way of oxidation coupled with the small size of the photosensitizer and graphene oxide may enable many other applications in biosensor development, smart materials and energy harvest.

Introduction

In many bioassays, H_2O_2 and horseradish peroxidase (HRP) are used together for colorimetric signaling. One particularly important example is enzyme-linked immunosorbance assays (ELISA), a workhorse of current protein analysis.[1] By associating HRP with the target, detection signals are drastically amplified based on the catalytic turnover of HRP, allowing detection of proteins down to physiologically relevant levels.[2] However, typical ELISA requires conjugation of HRP with a secondary antibody. Efforts in eliminating HRP (or other enzymes)-conjugated antibodies have been attempted, for example, light scattering-based signaling through analyte-triggered aggregation of antibody-conjugated AuNPs.[3] However, most of them have not been adopted practically since they require the use of new assay instruments and often new chemicals that are unfamiliar to most end users. The cost of such infrastructure change is also very high.

The fundamental reason for the need of HRP is that HRP can drastically accelerate the chromogenic reaction between the substrates (e.g., 3,3',5,5'-tetramethylbenzidine, TMB) and H_2O_2 catalytically, and thus amplify the signal. We reason that both H_2O_2 and HRP can be eliminated altogether if another efficient oxidant can be found. Molecular oxygen (O_2) is the most readily-available oxidant, but direct oxidation of TMB by O_2 is kinetically inhibited by the spin state of O_2 due to spin-conservation restrictions: the ground-state O_2 is in the triplet form ($^3\Sigma$, Scheme 1), while TMB is in the singlet state. Their reaction is restricted by spin-conservation, since chemical reactions should occur in the same multiplicity according to the Wigners spin selection rule.[4]



Scheme 1. Replacing the conventional "H_2O_2 + HRP" by singlet oxygen produced from photosensitization for oxidation of TMB. O_2 cannot directly oxidize TMB. After photosensitization to generate singlet $^1O_2$, TMB can then be oxidized. The energy diagram of a photosensitizer and energy transfer to molecular oxygen is also shown.

The key to oxidize TMB by O_2 is to excite triplet O_2 to its singlet state ($^1O_2$, Scheme 1) or other reactive radicals (such as ·OH). However, direct excitation of triplet to singlet O_2 is also difficult, again restricted by the spin selection rule. Photosensitizers, dyes that possess a high intersystem crossing efficiency, can be used for overcoming this spin limitation through the photosensitization process.[5] Typically, absorbed photon

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energy is transferred from the excited triplet state of photosensitizing dyes to O_2, thus generating $^1O_2$ for oxidizing TMB (Scheme 1).[6]

We herein explored $^1O_2$ generated from photosensitization for replacing the combined use of H_2O_2 and HRP for oxidaataion of TMB oxidation in immunoassays. An enzyme-free colorimetric immunoassay format was developed. Instead of using HRP-labeled secondary antibody, we used a DNA aptamer for construction of the sensing complex. Association of photosensitizers with the ocomplex was achieved via the cartrigue of graphene oxide (GO), which is a thin atomic layer of oxidized graphene. GO is a highly useful analytical platform for adsorption of proteins, nucleic acids and small molecules.[7] Due to its small size, multiple photosensitizer molecules can be loaded on a GO nanosheet, improving the 1: 1 ratio of the HRP and target in conventional ELISA to n: 1, drastically increasing the sensitivity.

Results and Discussion

H_2O_2-free and enzyme-free oxidation of TMB

To test the hypothesis of direct TMB oxidation with molecular oxygen, a readily-available photosensitizer, phloxine B (PB), was employed here. As a derivative of fluorescein, PB possesses four bromine atoms of the xanthene ring and four chlorine atoms in the carboxyphenyl ring (Figure 1A). The presence of heavy bromine atoms favors intersystem crossing (ISC) to activate the triplet state, which further transfers energy to oxygen to generate $^1O_2$ (Scheme 1).[8] PB has already been explored as a photosensitizer for various applications.[9] In addition, water soluble PB has been approved as a color additive for drugs, foods and cosmetics.

As an easy-to-operate light source for photosensitization, a light emitting diode (LED, 3 V, 3 W) was used (Figure S1, S2).[10] Upon irradiation by the green LED in the presence of PB as a photosensitizer, TMB was successfully oxidized as indicated by the blue color (Figure 1B) as well as the characterized absorption spectra at ~655 nm (Figure 1C). According to the scheme of TMB oxidation,[11] the 655-nm absorption indicates the one-electron oxidation product (Figure 1D), and $^1O_2$ is converted to H_2O. PB (0.2 μM) alone has a very light red color due to its absorption peak at 540 nm (Figure 1B). Without the LED irradiation, PB alone failed to change the color of TMB. Without PB, LED irradiation alone also failed to induce TMB oxidation. Therefore, both PB and LED are required. These control experiments supported the mechanism proposed in Scheme 1. Besides, other common photosensitizer dyes, such as eosin Y and Rose Bengal, can also result in photosensitized oxidation of TMB (Figure S3), demonstrating the generality of this color development reaction.

Photosensitizers as a molecular enzyme mimic

The excellent sensitivity of ELISA comes from the fast catalytic turnover of HRP. Since each photosensitizer can be recycled multiple times between excitation and energy transfer, similar turnover effects might also be achieved to rival ELISA. In this regard, our photosensitizer might act as a molecular catalyst for photo-induced oxidation of TMB. For the example in Figure 1B, we only used 0.2 μM PB, while the added TMB was 200 mg/L

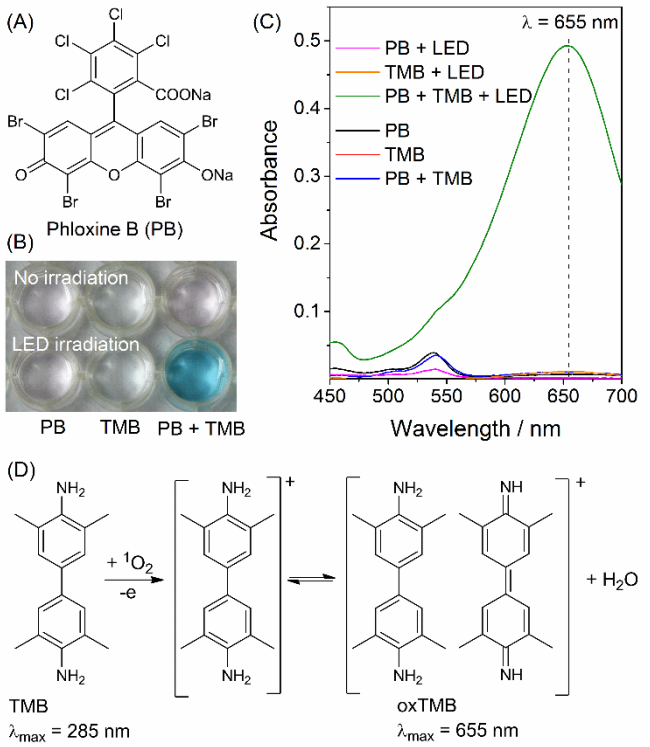

Figure 1. Confirmation of photosensitization-induced oxidation of TMB. (A) The structure of the photosensitizer phloxine B (PB). (B) A color picture of TMB oxidation. (C) The absorption spectra of TMB before and after photosensitized oxidation with PB. (D) A possible oxidation pathway of TMB by singlet oxygen. TMB concentration: 200 mg/L (833 μM); media pH: 4.5 glycine buffer (50 mM, containing 200 mM NaCl).

(833 μM). During the 20 min irradiation (comparable with a typical ELISA incubation time of 15 min), each PB converted ~51 TMB molecules (Figure S4, ε_{oxTMB} = 39 000 $M^{-1}\cdot cm^{-1}$), supporting PB acting as a "molecular enzyme mimic" for signal amplification. Control investigation showed that in the presence of 250 μM H_2O_2 (similar to the concentration of dissolved oxygen), the turnover number of HRP is ~3.8×10^5 in the same 20 min.

Compared to the "H_2O_2 + HRP" system, several advantages of using PB for TMB signaling are summarized here. First, PB can directly catalyze TMB oxidation with stable dissolved oxygen, while HRP needs less stable H_2O_2. Namely, the whole process is greener and more cost-effective. Dissolved oxygen, the source of singlet oxygen, is sufficient for TMB oxidation, since further bubbling of the reaction media with air or pure O_2 did not result in an appreciable signal increase (Figure S5). This implies the robustness of this reaction, insensitive to the concentration of oxygen over a wide range. This is attributed to that the reaction is limited by the energy transfer from PB (only 0.2 μM) to dissolved oxygen (> 200 μM). In addition, in such an open system, the consumed dissolved oxygen can be replenished from air during photosensitization.

Second, the size of PB is much smaller than that of HRP, making it easier for further signal amplification through assembling of multiple PB molecules (*vide infra*). Third,

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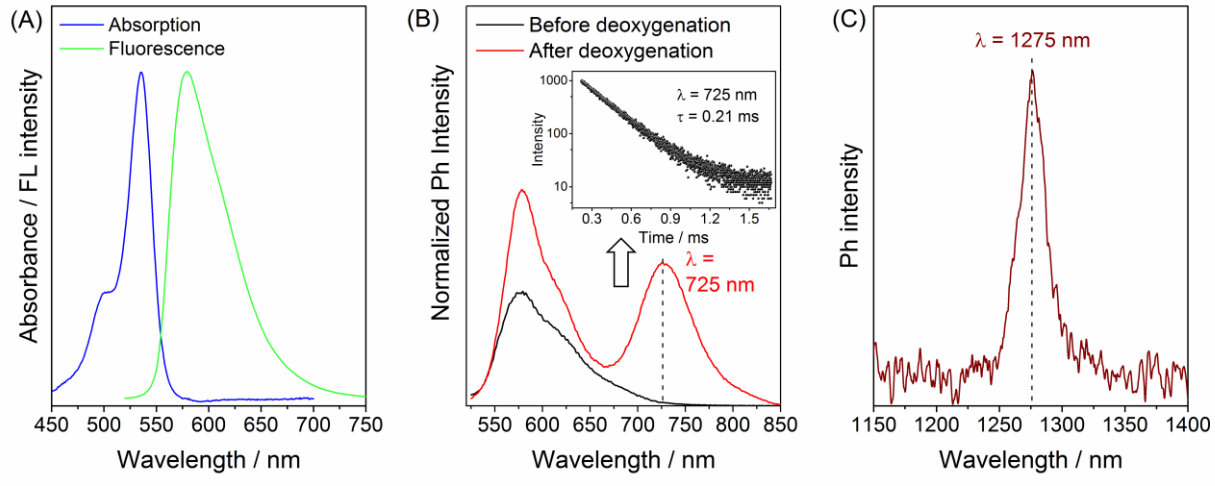


Figure 2. Confirmation of the photosensitized generation of singlet oxygen ($^1O_2$) from of PB. (A) Absorption and fluorescence emission spectra of PB. (B) Room-temperature phosphorescence spectra (delayed by 50 μs) of PB before and after deoxygenation. Inset: the corresponding phosphorescence decay after deoxygenation. (C) A near-infrared phosphorescence emission spectrum of singlet oxygen from PB in D_2O. PB concentration was 40 μM and the excitation was at 510 nm.

termination of the TMB oxidation in the "H_2O_2 + HRP" system is often achieved via addition of H_2SO_4, while the PB system can be easily turned off by removing the LED light. Finally, the noncovalent association of PB avoids the labeling process thus reducing the cost.

Direct catalytic oxidation of TMB by molecular oxygen is analogous to the oxidase activity. Currently, few oxidase enzymes were used in ELISA assays. Compared with some reported oxidase mimics (e.g., CeO_2 nanoparticles[12]), photosensitizers are advantageous in its reproducibility, structural tunability,[13] and much smaller size.

Photosensitization-mediated oxidation

After confirming the signalling value of PB, we then studied the mechanism of singlet oxygen production by photosensitization. The absorption and fluorescence of PB are in the range of 450-560 nm and 550-700 nm, respectively (Figure 2A). After deoxygenation with N_2, a new emission band emerged at 670-770 nm (Figure 2B), which was assigned as the phosphorescence of PB based on its longer emission wavelength and a very long lifetime (~0.21 ms, inset of Figure 2B). Moreover, by exciting PB in D_2O, the characteristic near-infrared phosphorescence of singlet oxygen at 1275 nm was observed (Figure 2C), which is consistent with the generation of singlet oxygen via energy transfer (Scheme 1).[6] According to the results of Redmond and

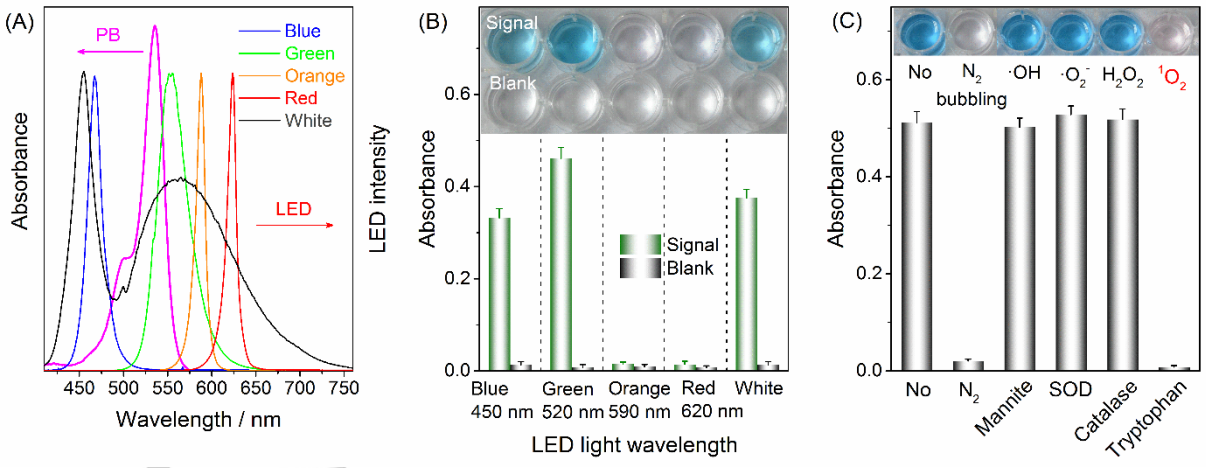


Figure 3. Confirmation of direct oxidation of TMB by photosensitized generation of singlet oxygen. (A) Absorption spectra of TMB before and after photosensitized oxidation with PB. (B) Spectral overlap between the absorption spectrum of PB and lighting wavelength of various LEDs. (C) TMB oxidation efficiency assisted by blue, cyan, green, orange, red and white LEDs. (D) Identification of singlet oxygen for TMB oxidation via specific ROS scavengers. PB concentration: 200 nM; TMB concentration: 200 mg/L; LED irradiation time: 20 min; pH: 4.5.

Gamlin,[14] the singlet oxygen yield (Φ_Δ) of PB was about 0.50 relative to the Rose Bengal standard.

Photosensitization efficiency is directly related to the light absorption of the sensitizer to reach the excited state (Scheme 1). As shown in Figure 3A, the absorption spectrum of PB overlaps the best with that of the green LED. Note the intensity of the white LED is lower than that of the green LED in the green spectral range. Also, green LED yields a lower background signal. Indeed, TMB oxidation had the highest efficiency with the green light (Figure 3B), and the yield increased almost linearly with the illumination time (Figure S4).

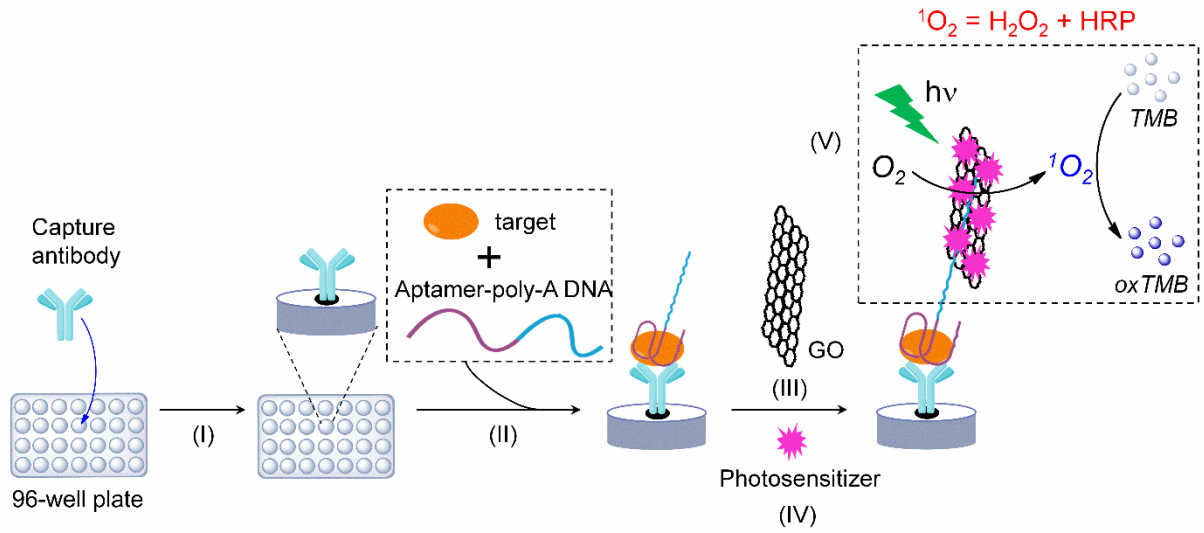
The involvement of singlet oxygen in TMB oxidation was further confirmed through deoxygenation by N_2 and adding various scavengers (Figure 3C). Without O_2, no TMB photo-oxidation was observed, indicating that reactive oxygen species (ROS) from dissolved O_2 (type-I and type-II photosensitization[6]) is responsible for the oxidation. ROS-specific scavengers, namely mannite for ·OH, tryptophan for $^1O_2$, superoxide dismutase (SOD) for $\cdot O_2^-$, and catalase for H_2O_2,[15] were then explored. Only tryptophan effectively inhibited the reaction, indicating that singlet oxygen was indeed responsible for TMB oxidation.

Ultrasensitive photosensitization immunosorbent assay (PISA) on a graphene platform

Based on the above results, photosensitizers can play a similar role as that in the "H_2O_2 + HRP" system for TMB color production. Given its analytical advantages, we proposed a new colorimetric immunoassay scheme without H_2O_2 or HRP, namely photosensitization immunosorbent assay (PISA). As shown in Scheme 2, the protocol is very much like that of the conventional ELISA. Importantly, the spectrometer familiar to ELISA users can still be used. The target protein is first bound by the immobilized capture antibody. Instead of using HRP-labeled secondary antibody, we herein used a DNA aptamer appended toa poly-A DNA.[16] The poly-A region was intended for binding to a graphene oxide (GO) sheet.[17] With an aromatic structure, PB can also be adsorbed by GO (Figure S6). The adsorption of both DNA and dyes by GO has been reported previously.[18] Here we also confirmed the adsorption of poly-A DNA (blue part of the DNA in Scheme 2) and PB by GO via batch experiments (Figure S7). To facilitate the association of photosensitizer with the antibody-target-aptamer sandwich complex, GO was explored for sequential adsorption of the poly-A containing DNA (step III) and the photosensitizer (step IV). After washing away the unbounded GO and PB, the green LED was turned on for color development from TMB oxidation and the signal at 655 nm was measured for quantification.

To demonstrate the proposed assay, carcinoembryonic antigen (CEA) was chosen as the model target. CEA is a broadband cancer biomarker associated with colorectal cancer, liver cancer and pancreatic cancer.[19] To provide the immuno-sandwich complex, an antibody and an aptamer for CEA[20] were used.

A series of experimental conditions, including the length of the poly-A region, the concentration of PB, and the buffer conditions, were optimized for the immunosensing (Figure S8-S10). Under the optimal condition, the color (absorbance) of TMB solutions was gradually deepened with increasing concentration of CEA (Figure 4A). The resulting color change allowed for visual detection of CEA down to as low as 0.05 ng/mL. Plotting the absorbance of oxTMB (@655 nm) with the concentration of CEA yields a linear range of 0.02-5.0 ng/mL. The limit of detection (LOD, 3σ) was evaluated using a spectrophotometer to be 0.009 ng/mL, which is comparable to other amplified or labled immunoassays (Table S2). It is worth noting that the reference



Scheme 2. Scheme of the H_2O_2-free, non-enzymatic colorimetric immunoassay based on photosensitization: (I) capture antibody is attached onto the surface of 96-well plate; (II) if the target is present, capture antibody and the aptamer both bind to the target and a sandwich complex is formed. In the absence of target biomarker, aptamer is removed by subsequent washing; (III) and (IV) GO is added for adsorption of poly-A DNA; (V) photosensitization-induced color development from TMB oxidation.

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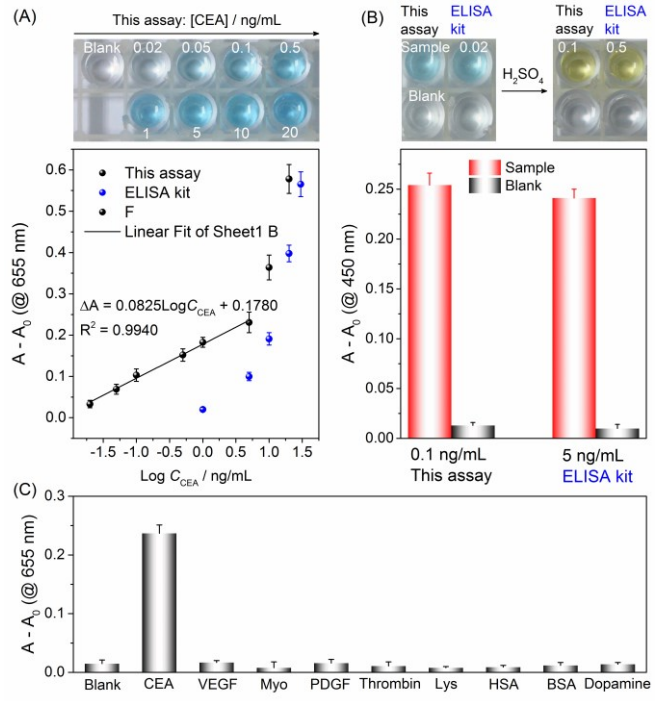


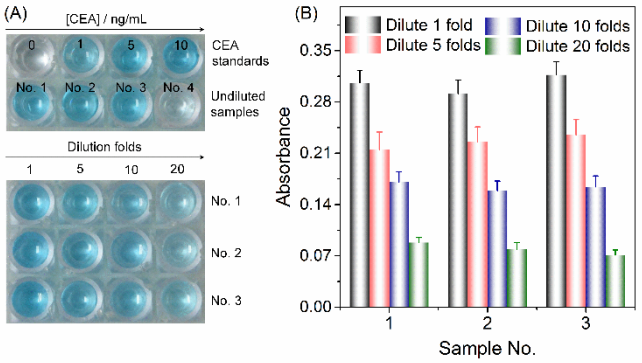
Figure 4. Analytical performance of the photosensitization-based colorimetric immunoassay: (A) color change and the absorbance of oxTMB in the presence of increasing amounts of CEA by the proposed immunoassay; (B) sensitivity comparison of the proposed assay with commercial ELISA kit; and (C) selectivity of the proposed assay for CEA. VEGF: vascular endothelial growth factor; Myo: myoglobin; PDGF: platelet-derived growth factor; Lys: lysozyme; HSA: human serum albumin; BSA: bovine serum albumin. The concentrations for CEA and other competing proteins were 5 ng/mL and 500 ng/mL, respectively.

values in serum of healthy people are normally lower than 3.0 ng/mL. Hence, our assay is sensitive enough for the real clinical diagnosis. When tested against other common proteins, the proposed assay showed excellent selectivity for CEA (Figure 4C), retaining the excellent performance of conventional ELISA.

Compared to a conventional ELISA kit (Figure 4A and Figure S11), our PISA exhibited a much lower LOD (0.009 ng/mL for the proposed assay versus 0.43 ng/mL for ELISA kit). Note that with 20 min incubation, we could not obtain any measurable color below 1 ng/mL CEA for the ELISA kit. Beyond this concentration, it has a slightly higher slope (~2-fold) than that of our PISA assay. As can be seen from Figure 4B, the color (after H_2SO_4 termination, a typical step in conventional ELISA) and TMB absorbance (450 nm) of 0.1 ng/mL CEA by this assay are comparable to those of 5 ng/mL CEA by the conventional ELISA. Here, due to the small size of photosensitizers, multiple photosensitizer molecules can be loaded on each GO sheet, leading to n: 1 ratio of the photosensitizer and the target for further signal amplification. We measured the turnover number of HRP with 250 μM H_2O_2 to be 3.8×10^5 TMB in 20 min. The slope of the ELISA kit is twice of that the PISA assay. Since each PB can turnover 51 TMB molecules in 20 min, we estimated that $n = 3.8 \times 10^5 / (51 \times 2) = 3725$. Therefore, even though individual PB is not as efficient as HRP, the larger number of PB associated with each target can overcompensate its individual rate. This is similar to the loading of multiple HRP enzymes on AuNPs.[21] Also, using GO as a cartrigue allows on-line noncovalent association of the photosenstizier with the aptamer, thus avoding the labeling process.

Detecting cancer biomarker in patient serum samples

To test the application of our assay in real samples, we collected serum samples from three cancer patients from a local hospital and another one from a healthy volunteer. We then used our assay to measure the CEA cancer marker. A few standard spiked samples were prepared, and visual observation indicated that all the patient samples were blue, while the healthy sample was colorless (Figure 5A, top panels). This indicates that simple visual detection of cancer marker is possible.



(C) Analytical results of CEA in serum samples (ng/mL)

No	Clinical test	Un-diluted	1-fold	5-fold	10-fold	20-fold
1	7.99	> 5	7.08 ± 0.28	8.35 ± 0.37	7.56 ± 0.15	8.11 ± 0.14
2	7.29	> 5	6.87 + 0.22	7.04 + 0.23	7.41 + 0.16	7.39 + 0.11
3	8.49	> 5	7.74 ± 0.20	8.78 ± 0.23	8.32 ± 0.17	8.57 = 0.12
4		0.37 ± 0.08[a]				

Sample No. 1, 2 and 3 are from cancer patients, and 4 is from a healthy volunteer.
a: the recovery for spiking 1 ng/mL of CEA is 95%.

Figure 5. Analytical results of human serum samples for CEA by the proposed photosensitization-induced colorimetric immunoassay using visual observation (A) and spectrometer detection (B). (C) The CEA concentration measured by our assay at various dilution factors and a comparison with the standard clinical test. The assay conditions were the same as those in Figure 4.

We then made a serial dilution of the patient samples up to 20-fold (Figure 5A, bottom panels), and a color gradient was observed as expected. The absorbance of these samples were measured (Figure 5B) and compared with a standard curve to calculate the CEA concentrations. The analytical results for CEA in these samples agreed well with those by clinical tests or featured satisfactory spike-recovery at each dilution level (Figure 5C). As can be seen from Figure 5C, the CEA levels in patient serum samples are all higher than the threshold of healthy people (3 ng/mL), while that for a healthy volunteer (0.37 ng/mL) is lower than the threshold value. Therefore, our assay was highly sensitive (e.g., allowing 20-fold dilution) and accurate (within 10% difference from the standard value). Such results support the

potential of exploring photosensitization-based immunoassay for clinical diagnosis.

Test of a second cancer marker

All the above work used CEA as the target molecule. To test the generality of our PISA, vascular endothelial growth factor (VEGF) was further assayed. VEGF is a signal protein produced by cells to stimulate vasculogenesis and angiogenesis,[22] and its over-expression is also associated with cancer development. We also obtained a VEGF concentration dependent color signal (Figure S12), and the color change allowed visual detection of VEGF down to 0.1 nM, which is 10~200-fold more sensitive than previous assays.[23]

Conclusions

In summary, photosensitizer PB was explored for activation of molecular oxygen to directly oxidize TMB, eliminating unstable and costly enzymes and H_2O_2 required for traditional immunoassays. Namely, singlet oxygen plays the same role as that of the "H_2O_2 + HRP" system. A simple PISA was developed, allowing visual detection of CEA as low as 50 pg/mL, and it also works in serum from real patients and even allowed 20-fold sample dilution. Our method keeps the same color producing agent and instruments familiar with most researchers and users of ELISA, making it more easily accepted by end-users. The same signaling method could also be extended to other analytical platforms and this will be a topic of future research.

Experimental Section

Chemicals. 3,3',5,5'-tetramethylbenzidine (TMB) was from Aladdin (Shanghai, China). Phloxine B (PB), sodium hydroxide, hydrochloric acid, dimethyl sulfoxide (DMSO) and phosphate (KH_2PO_4) were purchased from Kelong Reagent Co. (Chengdu, China). CEA antibody, CEA ELISA kits (monoclonal primary anti-human CEA antibody from mouse and polyclonal secondary anti-human CEA antibody from rabbit) and all the DNA sequences were purchased from Shanghai Sangon Biotechnology Co., Ltd., and the DNA sequences are listed in Table S1. CEA standard solution was purchased from Abcam Co., (Shanghai, China). Ultrapure water with conductivity of 18.2 MΩ·cm was used thorough in this work.

Instruments. UV-vis absorption spectra were recorded on a UV-5200PC spectrophotometer (Shanghai Yuanxi Instrument, Shanghai, China). Fluorescence and phosphorescence (delay time of 50 μs) of PB were measured with a Fluoromax-4P spectrofluorometer (Horiba JobinYvon). Phosphorescence lifetime and the singlet oxygen near infrared emission were measured with a Fluorolog-3 spectrofluorometer (Horiba JobinYvon). For collection of the phosphorescence lifetime, an S-460 SpectraLED (460 nm, Horiba Scientific) and a picosecond photon detection module (PPD-850, Horiba Scientific) were explored as the excitation source and the detector, respectively. LEDs (3 V, 3 W, irradiance of 7.5 mW/cm^2) were obtained from Yichuang Technology Co., Zhuhai, China. The photographs were taken with a Nikon D300S digital camera equipped with a Nikon AF-S VR 105 mm f/2.8G IF-ED Macro Lens without any filters.

Immunoassays. The capture antibody (2 mg/mL, Sangon Biotech Co. Ltd., Shanghai, China) was diluted 3-fold in carbonate buffer solution immediately before use. The steps for the immunoassay was similar to those of ELISA and detailed information was given as follows. For immobilization of the capture antibody, a 96-well microtiter plate was coated with capture antibody solution (0.66 mg/mL, 200 μL) and incubated overnight at 4 °C. Each well was carefully washed four times with ultrapure water and two times with PBS buffer (pH 7.2, 20 mM), and blocked with 250 μL of 20% BSA incubation at room temperature for 2 h. After washing (the same as above), 100 μL of CEA (or serum samples) was added to each well, incubated at room temperature for 2 h, and washed again. To each well, 100 μL of CEA aptamer was added (eventual concentration of 500 nM), incubated at room temperature for 2 h, and washed again. To each well, 200 μL of GO (0.5 mg/mL) was added, incubated at room temperature for 1 h, and washed again. To each well, 50 μL of PB (200 μM) was added and incubated for 0.5 h. Afterwards, the residue PB was removed away carefully with a pipette. For washing, 4 times with ultrapure water and two times with glycine buffer (50 mM, pH 4.5, containing 200 mM NaCl and 20% ethanol). To each well, glycine buffer (50 mM, pH 4.5, containing 200 mM NaCl and 20% ethanol) containing TMB (200 mg/L) was added and subjected to green LED irradiation for 20 min. The 96-well microtiter plate was taken photo with a camera and then the absorbance was measured with the UV-vis spectrometer.

The procedures for the conventional ELISA with the commercial CEA ELISA kit were similar to those above, except that the aptamer was replaced with secondary antibody conjugated with HRP. After formation of the sandwich immunocomplex, H_2O_2 and TMB were added for signaling.

Serum sample preparation. Human serum samples were collected from a local hospital (containing EDTA). Three samples were from cancer patients and one from a healthy volunteer. The patients and the volunteer were informed about the study and gave their consent. Before analysis, the serum samples were diluted properly.

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Keywords: photosensitization • TMB oxidation • immunoassay • colorimetric • graphene oxide

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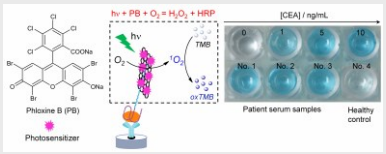

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