pH-Tunable Oxidase-Like Activity of Cerium Oxide Nanoparticles Achieving Sensitive Fluorogenic Detection of Cancer Biomarkers at Neutral pH

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Supporting Information

ABSTRACT: The reliable and sensitive detection of cancerspecific biomarkers is important for the diagnosis and treatment of cancer. Hence, detection of these biomarkers has to be reliably and rapidly performed in diverse settings. A limitation of the conventional biomarker-screening method of enzyme-linked immunosorbent assay (ELISA) is the employment of labile components, such as hydrogen peroxide and horseradish peroxidase. Previously, we reported that nanoceria is able to oxidize various colorimetric dyes at acidic pH, such as 3,3′,5,5′-tetramethylbenzydine (TMB) and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (AzBTS), and an assay was designed for screening the folate receptor. Herein, we show that the ability of nanoceria to oxidize a substrate can be tuned by modulating the pH. Results showed that nanoceria can oxidize the nonfluorescent substrate ampliflu, either to the very stable fluorescent product resoruflun at pH 7.0 or to the nonfluorescent resazurin at pH 4.0. On the basis of these findings, we conjugated Protein G to immobilize antibodies on the surface of nanoceria, in order to detect the expression of prototypic cancer biomarkers at pH 7.0, such as the folate receptor and EpCAM. We found that within 3 h, nanoceria identified the expression of the folate receptor and EpCAM on lung carcinoma and breast adenocarcinoma cells, respectively. Traditional ELISA had a readout time of 15 h and a higher detection threshold, while requiring multiple washing steps. Considering these results and nanoceria’s ability to oxidize ampliflu to its stable fluorescent product at neutral pH, the use of antibody-carrying nanoceria in the lab and point-of-care molecular diagnostics is anticipated.

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Application of ampliflu, as its fluorescent readout quickly diminishes and has to be recorded within minutes upon initiating the HRP/H₂O₂ reaction.

Considering these limitations, there is a need to develop reaction conditions for the mild oxidation of ampliflu, yielding the intermediate fluorescent product (resorufin) without further oxidation to the nonfluorescent product (resazurin). Since nanoceria possess unique oxidase activity that can be tuned by changing the solution’s pH, behaving as a strong oxidant at acidic pH and weak oxidant at neutral pH, we hypothesized whether the oxidation of ampliflu to the fluorescent resorufin can be achieved by tuning the oxidase-like activity of nanoceria through the pH (Scheme 1b,c). We reasoned that at neutral pH, the weak oxidase-like activity of nanoceria could facilitate the partial oxidation of ampliflu to the fluorescent

Scheme 1. Schematic Showing the HRP/H₂O₂ and Nanoceria Mediated Oxidation of Ampliflu*
It has been previously reported that the use of high levels of hydrogen peroxide in ELISA results in inactivation of HRP and a decrease in the fluorescence of the oxidized substrate.\textsuperscript{14–16} In addition, pH also plays an important role in the oxidation of amplifu.\textsuperscript{15} Fluorescence imaging studies under UV illumination of 96-well plates containing amplifu (1.25 μg) with increasing concentrations of nanoceria (PNC) or HRP/H2O2 revealed that the HRP/H2O2 system quickly oxidized amplifu, developing a fluorescent product within minutes (Figure 2) that quickly decreased after 5 min, becoming practically undetectable. These results were expected, as it has been reported that HRP/H2O2 rapidly oxidizes amplifu to the fluorescent product resorufin at pH 7.0 but subsequently oxidizes resorufin to the nonflourescent resazurin.\textsuperscript{13} In contrast, when amplifu was oxidized with nanoceria at pH 7.0, fluorescent emission started to develop within an hour and continued increasing until reaching its maximum fluorescence within 24 h. The observed increase in fluorescence depended on the concentration of nanoceria; as the higher the amount of nanoceria, the higher fluorescence intensity was observed (Figure 2 and Figure S1 in the Supporting Information). This indicates that the mild oxidase activity of nanoceria at pH 7 was able to facilitate the oxidation of amplifu to the fluorescent intermediate resorufin without further oxidizing the substrate to the nonfluorescent product resazurin. Interestingly, nanoceria at pH 4.0 yielded no fluorescence, suggesting that nanoceria at pH 4.0 may primarily oxidize amplifu to the nonfluorescent resazurin (Figure 2). As expected, the HRP/H2O2 system at pH 4.0 was unable to generate a fluorescent signal due to the instability of horseradish peroxidase at this pH (Figure 2). Taking together, these results demonstrate that nanoceria is an ideal system for long ELISA readouts at pH 7.0, as opposed to the commonly used HRP/H2O2 system.

On the basis of these findings, we hypothesized that nanoceria at pH 7.0 could provide better detection than the HRP/H2O2 system in cancer biomarker screening assays. Considering that at pH 7.0 nanoceria is able to oxidize amplifu to the stable fluorescent product resorufin, we reasoned that a more robust and stable fluorogenic detection system could be developed. Additionally, since no acidic conditions are required to achieve detection, the possibility of protein denaturation is reduced.

Furthermore, as nanoceria does not utilize hydrogen peroxide for a substrate’s oxidation due to the nanoparticles’ intrinsic oxidase-like activity, terminal nonfluorescent oxidation of amplifu is also minimal. Toward these objectives, we conjugated Protein G to the poly(acrylic acid)-coated nanoceria (PNC) (1.0 mM) suspended in buffers ranging from pH 1.0 to 8.0. Amplifu (1.25 μg) was then added, and the fluorescence emission of the suspension was recorded at different time intervals. Results showed that nanoceria was able to oxidize amplifu in a pH-dependent manner. Nanoceria quickly oxidized amplifu to a stable pink-colored fluorescent product within the pH range of 6.0 to 8.0 (Figure 1a,b). The fluorescent emission maximum of this product was at 585 nm, which corresponds to resorufin.\textsuperscript{13,14} This fluorescence emission intensity increased until reaching a plateau after 8 h. In contrast, when nanoceria oxidized amplifu in the pH range from 1.0 to 5.0, a weakly fluorescent product was obtained within an hour upon incubation of amplifu with the nanoparticles (Figure 1a,b). The fluorescent intensity of this oxidized product quickly decreased, eventually becoming nonfluorescent after 2 h. These results indicate that nanoceria in the pH range from 6.0 to 8.0 oxidized amplifu to the fluorescent resorufin with emission at 585 nm. In contrast, from pH 1.0 to 5.0 nanoceria transiently formed a fluorescent product that eventually was converted to nonfluorescent resazurin (Figure 1a).

Typically in a traditional immunoassay, a horseradish peroxidase (HRP)-labeled secondary antibody is utilized to assess the binding of a specific primary antibody to a particular target or surface receptor. This binding event is assessed by the ability of HRP/H2O2 to oxidize a substrate, such as amplifu. However, the
instability of HRP and H₂O₂, in addition to the H₂O₂-induced quenching of fluorogenic substrates, could result in erroneous results. Therefore, we reasoned that an antibody-Protein G-PNC conjugate would be more robust than current HRP-based assays, since neither HRP/H₂O₂ nor a secondary antibody would be needed for detection. First, we immobilized an antifolate-receptor antibody (Santa Cruz Biotech) on the Protein G-PNC conjugate. This antibody recognizes the folate receptor, which is overexpressed in many tumors. Experiments were performed using the lung cancer cell line A-549, which has been shown to overexpress this receptor. In control experiments, we used a breast carcinoma cell line (MCF-7) that does not express the folate receptor. Although both cell lines were grown in media having high amounts of folate (2.3 μM), subsequent treatments with the antifolate-receptor antibody-Protein G-PNC conjugate were carried out in 1× PBS having no folate. This ruled out the possibility of any false positive results or interference from the folate in the medium. In our initial experiments, both the lung carcinoma (A-549) and breast carcinoma (MCF-7) cells were plated at cell densities ranging from 0 to 6000 cells per well and incubated with the antifolate-receptor antibody-Protein G-PNC conjugate. The cells were then washed with 1× PBS and incubated with amplifu (25 μL of 0.5 mg/mL) for 3 h before fluorescence emission was recorded at 585 nm, using a microtiter plate reader. As expected, a target-concentration-dependent signal intensity was observed in the lung carcinoma cell line (Figure 3a), as opposed to the breast carcinoma cells that lacks the folate receptor (Figure 3b). The obtained fluorescent signal was stable even after 24 h, being able to detect as little as 1200 A-549 cells with the nanoceria conjugate.

In subsequent studies, we immobilized an anti-EpCAM antibody (Santa Cruz Biotech) on the Protein G-PNC conjugate and screened MCF-7 cells for the expression of this protein. EpCAM (37–40 kDa) is overexpressed on the plasma membrane of most human epithelial cancers, including the MCF-7 breast cancer cell line, which makes this biomarker a promising tumor-associated antigen for detection and therapy. Results showed an increasing trend in fluorescence intensity as the number of MCF-7 cells increased (Figure 4a). Moreover, the signal from each well was stable even after 24 h. As a negative control, we used human embryonic kidney cells (HEK293), as these cells lack the EpCAM receptor. As expected, a nominal signal was observed with these cells (Figure 4b). In addition, saturation experiments adding excess anti-EpCAM antibody to the MCF-7 cells, before addition of the EpCAM antibody nanoceria conjugate, abrogated the signal (data not shown). These results clearly indicate that the anti-EpCAM antibody conjugated to nanoceria (PNC) via Protein G was able to
selectively assess the presence of the EpCAM receptor in MCF-7 cancer cells. In our previously published report, we used folate-conjugated nanoceria to monitor the expression of folate receptor utilizing TMB as the chromogenic substrate. However, TMB, being a colorimetric substrate, may not provide good quantification and sensitive detection as opposed to a fluorogenic substrate like ampliflu. In addition, the low pH required for detection using nanoceria and TMB could limit that assay, particularly when proteins including antibodies and other labile biomacromolecules (HRP) are used.

Finally, we compared our results with those obtained with the colorimetric TMB detection of EpCAM in MCF-7 cells at pH 7.0 and 4.0 using the EpCAM antibody-Protein G-PNC conjugate. Results show that when detection was performed with TMB (0.04 mM) at pH 7.0, quantification of the MCF-7 cells was not possible, as low absorbance at 652 nm was obtained (Figure 5a).

However, in experiments performed at pH 4.0, TMB identified EpCAM in a higher number of cells (Figure 5b), in contrast with ampliflu at pH 7.0 (Figure 4) that had a lower detection limit. Overall, these results indicate that the nanoceria-mediated oxidation of a fluorogenic substrate at neutral pH provides a more sensitive immunoassay with an improved detection limit for cellular ELISA applications. The fluorogenic substrate ampliflu works efficiently at pH 7.0, eliminating any extreme conditions, such as the acidic pH and oxidizing agents (H₂O₂).

In summary, we demonstrated that the oxidase-like activity of nanoceria can be tuned by changing the pH of the solution, facilitating the mild oxidation of a substrate to yield a product with enhanced fluorescent properties. Specifically, we showed that ampliflu can be oxidized at pH 7 by nanoceria to the fluorescent product resorufin, without further oxidizing the substrate to the nonfluorescent product resazurin. This unique capability of nanoceria is due to its mild oxidizing activity at neutral pH that prevents further oxidation of the substrate to resazurin in the absence of hydrogen peroxide. The selective nanoceria-mediated oxidation of ampliflu can be used to develop sensitive cell-based ELISA, offering various advantages over the HRP/H₂O₂ ELISA. The complexities associated with the HRP/H₂O₂ system, such as instability, quenching/bleaching of the fluorogenic substrate and other labile components in immunoassays, can be potentially avoided when a nanoceria-antibody conjugate system is employed. In addition, nanoceria-mediated oxidation of a fluorogenic substrate is independent of hydrogen peroxide, introduces stability in the assay, increases sensitivity, and is an ideal candidate for long readouts, overall rendering it a more reliable setup for target detection. Finally, when compared to gold nanoparticle based ELISA methods that use quartz crystal microbalances (QCM) or surface plasmon resonances (SPR)
detectors that require complex instrumentation,26 the fluorescent signal generated by the nanoceria based ELISA method can be easy to read visually under UV-illumination or by a fluorimeter. Furthermore, when compared to iron oxide based ELISA methods that take advantage of the intrinsic peroxidase activity of these nanoparticles in the presence of hydrogen peroxide,25 our method has the unique advantage that no hydrogen peroxide is needed to oxidize the fluorogenic substrate, facilitating detection. Taken together, the use of antibody-immobilized nanoceria based ELISA is expected in the clinic and field as a robust nanoprobe for efficient and sensitive cellular assays.

**EXPERIMENTAL SECTION**

Synthesis of PAA-Coated Nanoceria (PNC). Polyacrylic acid-coated nanoceria was synthesized as described by Asati et al.12

Nanoceria Mediated Oxidation of Ampliflu at Various pH (1–8). Polyacrylic acid-coated nanoceria (PNC) (1.0 mM) was suspended in PBS buffer at different pH ranging from pH 1.0 to pH 8.0. Then ampliflu (1.25 μg) was added, and the fluorescence emission spectrum of the suspension was recorded at different time point intervals (UV–visible and fluorescence emission).

Concentration-Dependent Oxidation of Ampliflu via Nanoceria and HRP/H2O2. Various concentrations of nanoceria (1.0, 4.0, 10, and 20 μM) and HRP (0.25, 0.1, 0.5, and 0.75 μM) were used, while keeping the hydrogen peroxide concentration (20.0 μL of 0.03%) constant. The amount of ampliflu used for the experiment was 1.25 μg (stock 0.5 mg/mL solution in DMSO/PBS). The reaction was carried out in a 96-well plate, and pictures were taken at different time points.

Fluorescence Intensity Measurement of Concentration Dependent Oxidation of Ampliflu Using Nanoceria. In a 96-well plate, 1.25 μg of ampliflu (stock 0.5 mg/mL solution in DMSO/PBS) was added into different wells and treated with various concentration of nanoceria (1.0, 4.0, 10, and 20 μM), and the intensity was measured at 3 h using a TECAN’s infinite M200 PRO fluorescence plate reader with an excitation filter at 525/25 nm and a 590/25 nm emission filter.

Conjugation of Protein G to Poly(acrylic acid)-Coated Nanoceria (PNC). Carboxidimide Chemistry. To a 1.0 mL suspension of PAA-coated nanoceria (50.0 mg Ce/mL) in MES buffer (1.0 mL, pH = 6.0), a solution of EDC (40.0 mg) and NHS (26.0 mg) in MES buffer (0.5 mL) was added and incubated for 3 min. To the resulting reaction mixture, Protein G (3.0 mg) in 0.25 mL PBS (0.25 mL, pH = 7.4) was added dropwise, incubated for 2 h at room temperature, and then incubated at 4 °C for 3 h. The resulting solution was then dialyzed against DI water to remove unbound Protein G and other reagents, using a cellulose membrane [molecular weight cutoff (MWCO) 50 000]. The concentration of Protein G conjugated on the nanoparticle was determined through the BCA assay. The results indicate that the protein concentration of the Protein-G-PNC was 0.33 μg/mL. The final preparation was stored at 4 °C until further use.

Immobilization of Antibody on Protein-G-Conjugated Nanoceria. To immobilize antibodies on nanoceria, 7.0 μL of the antibody (antifolate and anti-EpCAM, SantaCruz Biotech) was added to 1.0 mL of solution of Protein-G-PNC and allowed to incubate overnight at 4 °C. The final concentration of antibody on nanoceria was 1.4 μg/mL.

Cellular ELISA Using Ampliflu As a Substrate. Lung cancer (A-549) and breast carcinoma (MCF-7) were obtained from ATCC. Lung cancer cells were grown in Kaighn’s modification of Ham’s F12 medium (F12K) supplemented with 5% fetal bovine serum, 1-glutamine, streptomycin, amphotericin B, and sodium bicarbonate. All cell lines were maintained at 37 °C, 5% CO2 in a humidified incubator. Cells were plated in a 96-well plate at densities of 0 to 6000 cells per well. After 24 h growth, they were treated with 25.0 μL of antibody-Protein G-PNC conjugate. For the lung carcinoma cell line, antifolate-receptor antibody-Protein G-PNC conjugate was used, and for the breast carcinoma cells and human embryonic kidney 293 cells (HEK293), the EpCAM antibody-Protein G-PNC conjugate was utilized. Nanoparticle conjugates were allowed to incubate with the cells overnight. Afterward, the cells were washed with 1× PBS and then incubated with 50.0 μL of 0.5 mg/mL ampliflu in PBS for 3 h to allow development of the fluorescent product. The product of the reaction was quantified fluorimetrically, using a TECAN’s infinite M200 PRO fluorescence plate reader with an excitation filter at 525/25 nm and 590/25 nm emission filter.

**Cellular ELISA Using Saturation with Free Anti-EpCAM Antibody.** MCF-7 cells were preincubated with free anti-EpCAM antibody (0.05 μg/mL) for 3 h. The rest of the procedure is same as above.

**Cellular ELISA Using TMB As Substrate.** TMB was purchased from Sigma. Similar experiments were performed as described in previous section with the breast carcinoma cell line. After incubation with nanoceria conjugate and washing, 100 μL of (0.04 mM) TMB was added and incubated for 3 h. The product of the reaction was quantified spectrophotometrically at 652 nm using a Synergy HT plate reader (BIOTEK).

**ASSOCIATED CONTENT**

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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