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An Enhanced Ascorbate Peroxidase 2/Antibody-Binding Domain Fusion Protein (APEX2-ABD) as a Recombinant Target-Specific Signal Amplifier

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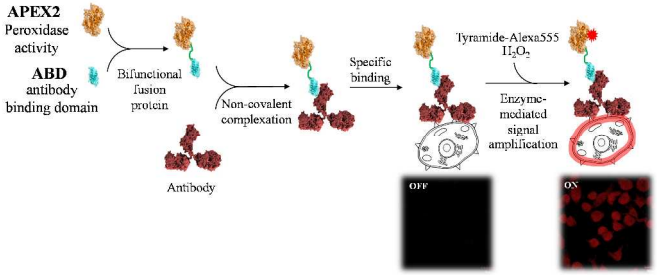
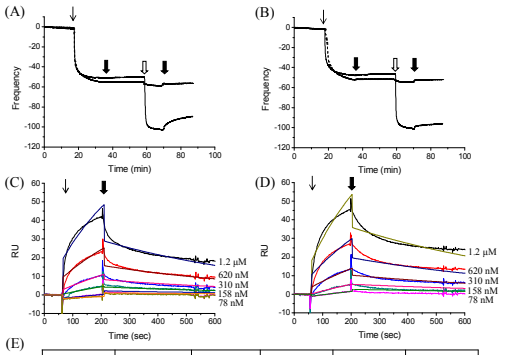
A recombinant target-specific signal amplifier was constructed by genetically fusing the enhanced ascorbate peroxidase 2 (APEX2) and an antibody-binding domain (ABD). The fusion protein APEX2-ABD possessed the peroxidase activity and the antibody-binding capability simultaneously and replaced the conventional HRP-conjugated secondary antibodies in a TSA assay for amplifying fluorescence signals.

The levels of individual proteins within a mammalian cell vary by many orders of magnitude (~10^1 -10^8 copies per cell). Many functionally important proteins frequently exist at very low levels and thus cannot be easily detected by simply using fluorescent-labeled primary and secondary antibodies or other routinely used affinity reagents. Therefore, in such cases it becomes necessary to use a signal amplification method, such as enzyme-mediated fluorescence probe labeling, to efficiently detect the low-abundant biomolecules in biological samples. In many signal amplification systems, the enzyme is linked to a target-specific affinity reagent such as an antibody either by direct conjugation or through a secondary complex, in order to obtain spatial information about the target biomolecules.[1] The enzyme repeatedly converts multiple copies of an inactive fluorogenic substrate into the active form to generate high-density labeling of a target protein in a space-controlled manner resulting in highly enhanced target-associated signal levels. Horseradish peroxidase (HRP) is the most commonly used enzyme for this purpose and it is conjugated to antibodies as a target-specific affinity reagent to confine its signal amplification in a limited area.[2]

In this study, we have constructed a recombinant peroxidase/antibody-binding domain fusion protein as a potential substitute for the conventional HRP-conjugated secondary antibodies in the enzyme-mediated signal amplification assay (Scheme 1). The enhanced ascorbate peroxidase 2 (APEX2)[3] was genetically fused with the antibody-binding domain of protein A, which has a high binding affinity and specificity for the Fc region of immunoglobulin G (IgG).[4] The broad binding capability of APEX2/antibody-binding domain fusion protein (APEX2-ABD) to various types of IgGs, derived from rabbit, rat, and mouse, was confirmed with quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) analyses. Further, the peroxidase activity of APEX2-ABD was verified using the Amplex Red hydrogen peroxide/peroxidase assay. Finally, the APEX2-ABD, instead of HRP-conjugated secondary antibody, was applied to a tyramide signal amplification (TSA) assay and a significant signal enhancement was observed in fluorescence cell imaging (Scheme 1).



Scheme 1. Construction of APEX2-ABD fusion protein and its applications for TSA assay as site-specific signal amplifier.



(E)

IgGs	k_{on}($M^{-1}s^{-1}$)	k_{off}(s^{-1})	K_A(M^{-1})	K_d(M)	Chi^2
Rabbit IgG	2.05×10^3	1.39×10^{-3}	1.47×10^6	6.79×10^{-7}	1.92
Mouse IgG	939	1.55×10^{-3}	6.06×10^5	1.65×10^{-6}	0.764
Rat IgG	882	1.17×10^{-3}	7.54×10^5	1.33×10^{-6}	1.42

Fig. 1 QCM resonance frequency change (-Δ*F*) profiles of APEX2-ABD (solid lines) and APEX2 (dashed lines) on the gold QCM sensor (thin arrows) and subsequent deposition of rabbit IgGs (A) and mouse IgGs (B) on the monolayer of APEX2-ABD (solid lines) and APEX2 (dashed lines). Buffer washings and IgG introduction are indicated as filled and open arrows, respectively. SPR analyses for APEX2-ABD binding to the rabbit IgGs (C) and mouse IgGs (D) for the immobilized gold SPR sensors. Introduction of buffer washings and APEX2-ABD are indicated as filled and thin arrows, respectively. (E) The values of dissociating constant (K_d), and association (k_{on}) and dissociation (k_{off}) rate constants for the indicated antibodies.

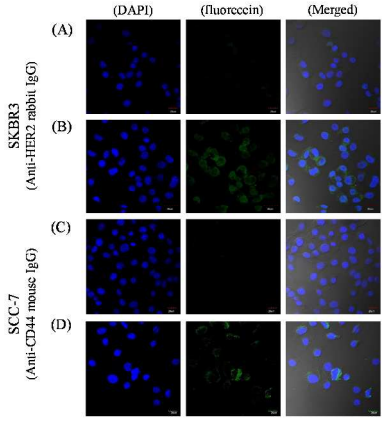


Fig. 2 Fluorescence microscopic images of SKBR3 cells (A, B) and SCC-7 cells (C, D) treated with fAPEX2-ABD (A, C), fAPEX2-ABD/Anti-Her2 rabbit IgGs (B), and fAPEX2-ABD/Anti-CD44 mouse IgGs (D). DAPI (left rows), fluorescein (middle rows), and merged (right rows) images are presented.

To construct a bifunctional fusion protein, which has both peroxidase activity and antibody binding capability, we prepared the gene encoding the APEX2 in pTRC99A expression vector and subsequently introduced a gene encoding an antibody-binding domain (ABD) of protein A at its C-terminal end. We used pTRC99A expression vector for APEX2 and APEX2 variant expressions, because heme incorporation into APEX2 is critical for its catalytic activity and slow expression of APEX2 driven by pTRC99A expression vector allows for efficient heme incorporation.[3c] A 27-amino acid long linker (KDPNSGGGLVARGSGGGCGGGTGGGS-GGG) was inserted between APEX2 and ABD to provide conformational flexibility, which would minimize the steric hindrance and ensure functional independency. In addition, we included one cysteine residue in the linker as a potential site for specific chemical conjugations, such as for a fluorescent probe.[5] The APEX2 and the fusion protein of APEX2 with the antibody-binding domain (APEX2-ABD) were overexpressed in *E. coli* as soluble cytosolic proteins. Since both APEX2 and APEX2-ABD had His tag at their N-termini, we purified these proteins individually using immobilized-metal affinity chromatography (IMAC) (Figure S1). Although both APEX2- and APEX2-ABD slowly released associated hemes, APEX2-ABD stayed intact at least a month at 4°C. The purified APEX2 and APEX2-ABD were separated as single bands on the SDS-PAGE with their corresponding molecular weights, i.e. 29425.0 Da and 38191.0 Da, respectively (Figure S2A, B). The absorption properties and secondary structures of APEX2 and APEX2-ABD were investigated with UV/Vis and circular dichroism (CD) spectroscopies. We observed strong absorption peaks at wavelengths 280 nm and 404 nm that represent the presence of protein and heme, respectively. We obtained a value of 2.2 as the ratio of 404 nm/280 nm for APEX2-ABD (Figure S2C). The CD spectra of APEX2 and APEX2-ABD had the characteristic shape of well-folded proteins. An ellipticity increase of APEX2-ABD at 195-205 nm is probably due to the high helical contents of the fused ABD (four helical bundles) (Figure S2D).

We first evaluated the antibody-binding capability of APEX2-ABD. For this purpose, we used quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) that are used to measure the bimolecular interactions in real-time without any further labeling of the analytes.[6] QCM is sensitive to the deposition of molecules on the gold sensors used for this technique and hence, cumulative masses can be monitored in real-time. We previously demonstrated that many types of protein complexes could bind to the gold sensors without any chemical modifications.[6-7] Both APEX2 and APEX2-ABD were found to deposit efficiently on the gold sensors, like many other protein complexes (Figure 1A, B). The resonance frequencies (*F*) dramatically dropped upon the introduction of either APEX2 or APEX2-ABD (indicated by ↓ in Figure 1A, B) and formed a plateau-shaped graph under the continuous flow of the samples. This result suggests that binding of the protein complex to the gold sensors is strong and the surface of the gold sensors is completely saturated to form stable APEX2 or APEX2-ABD monolayers (Figure 1A, B). Subsequently, we introduced the solutions of rabbit (Figure 1A), mouse (Figure 1B), and rat (Figure S3A) IgGs, separately, over the APEX2 or APEX2-ABD monolayers formed on the QCM gold sensors. The resonance frequencies of the APEX2-ABD-monolayered QCM gold sensors drastically decreased (Figure 1A, B, solid lines), whereas those for the APEX2-monolayered QCM gold sensors did not show any significant change (Figure 1A, B, dashed lines). These results demonstrate that APEX2-ABD binds to the gold sensors in an orientation-controlled manner and thus, it can effectively capture various types of IgGs, including the mouse, rabbit, and rat IgGs.

Further, we performed SPR analysis to investigate the affinity of APEX2-ABD toward various antibodies quantitatively and compare its binding affinity for various IgGs. In contrast to QCM analysis, we first immobilized the various antibodies on the CM-5 SPR sensor and then subsequently introduced either APEX2 or APEX2-ABD during the experiment. The CM-5 sensor has a carboxylated-dextran surface coupled to a thin gold surface and hence, proteins can be covalently attached via amine coupling through the EDC/NHS reaction.[6a, 8] We immobilized three types of polyclonal antibodies that were derived from rabbit (Figure 1C), mouse (Figure 1D), and rat (Figure S3B) on the surface of the CM-5 sensor, individually. Further, 1 M ethanolamine solution was injected into the instrument to prevent any undesired conjugations between the residual reactive amine groups and the other samples. While the SPR response units (RU) did not change upon the introduction of APEX2 (↓, Figure S4A, B) regardless of the amount used, those for the APEX2-ABD gradually increased in a concentration-dependent manner (↓, Figure 1C, D). The IgGs derived from different species showed slightly

different dissociation constants. The rabbit IgGs showed the strongest binding with APEX2-ABD with a dissociation constant of 6.79×10^{-7} M. Moreover, the mouse and rat IgGs also showed a similar affinity toward APEX2-ABD (dissociation constants of 16.5×10^{-7} and 13.3×10^{-7} M, respectively, Figure 1E).

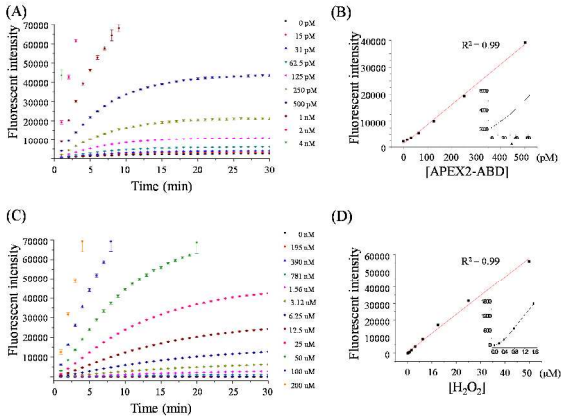


Fig. 3 Amplex® Red (AR) hydrogen peroxide/peroxidase assay with APEX2-ABD. Fluorescence intensities at 590 nm (excitation at 530 nm) were monitored for 30 min at various APEX2-ABD concentrations indicated in (A) and fluorescence intensity values at 15 min post-reaction are plotted against the different concentrations of APEX2-ABD in (B). The inset presents the fluorescence intensities at low concentrations of APEX2-ABD. These reactions were carried out with 25 μM of AR and H_2O_2. Fluorescence intensities at 590 nm (excitation at 530 nm) monitored for 30 min at various AR and H_2O_2 concentrations are indicated in (C) and fluorescence intensity values at 15 min post-reaction are plotted against the concentrations of H_2O_2 in (D). The inset shows the fluorescence intensities at low concentrations of H_2O_2. These reactions were carried out with 500 pM of APEX2-ABD and AR and H_2O_2 were maintained at 1:1 stoichiometry.

We further investigated whether the APEX2-ABD/IgG complex could specifically recognize and selectively bind *in vitro* to their targets guided by the bound antibodies with using fluorescence cell imaging.[6a] We prepared two different cell lines, SKBR-3 and SCC-7, as well as the IgGs corresponding to these target cell lines. The anti-HER2 IgGs (rabbit) and anti-CD44 IgGs (mouse or rat) were used for SKBR-3 (Figure 2B) and SCC-7 cell lines (Figure 2D and S5), respectively. The APEX2-ABD was labeled using fluorescein-5-maleimide (fAPEX2-ABD)[5] and was mixed with the antibodies to generate the fAPEX2-ABD/IgG complex. The antibodies were not labeled with any fluorescent dye in order to keep them intact and active. The two individual cell lines were treated with the complex of fAPEX2-ABD/IgGs (including the corresponding IgGs). In the case of cells treated with fAPEX2-ABD alone, we did not observe any green fluorescence (Figure 2A, C, and S5), clear green fluorescent cell images were observed for the samples treated with fAPEX2-ABD/IgG complexes (Figure 2B, D, and S5). This result indicates that the ABD portion of APEX2-ABD binds to the Fc region of the IgGs regardless of their origins. This allows for an effective exposure of the variable region of the bound antibodies leading to the IgG-guided target specific binding of the fAPEX2-ABD/IgG complexes.

To measure the catalytic activity of APEX2-ABD, we used *N*-acetyl-3,7-dihydroxyphenoxazine, which is the Amplex® Red reagent (AR), as a reporter molecule. The peroxidase enzyme is known to oxidize the non-fluorescent AR to fluorescent resorufin in the presence of hydrogen peroxide (H_2O_2).[9] HRP has most widely been used for the Amplex® Red hydrogen peroxide/peroxidase assays to detect the trace amounts of H_2O_2.[9-10] The conversion of AR to resorufin in the presence of H_2O_2, resulting in an increased fluorescent intensity, could be a good check for the peroxidase activity of APEX2-ABD. For this purpose, we first varied the concentrations of APEX2-ABD with excess amounts of AR and H_2O_2 (25 μM each) and monitored the fluorescence intensity changes at a wavelength of 590 nm (Figure 3A). The fluorescence intensities at 590 nm were observed to rapidly increase and reach a

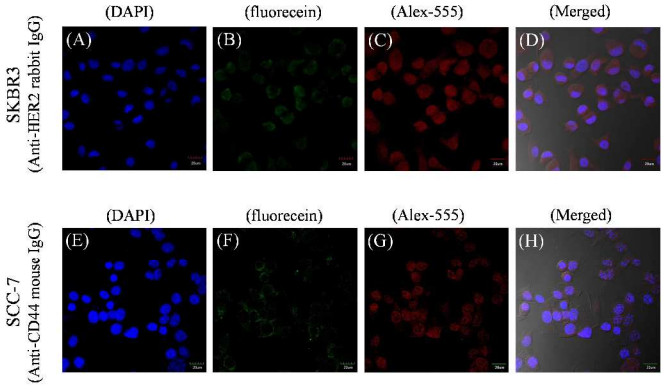


Fig. 4 Fluorescent microscopic images of SKBR3 cells (A-D) and SCC-7 cells (E-H) treated with fAPEX2-ABD/Anti-Her2 rabbit IgGs (A-D) and fAPEX2-ABD/Anti-CD44 mouse IgGs (E-H), respectively, before TSA treatment (B, F) and after TSA treatment (C, G) are shown. DAPI (A, E), fluorescein (B, F), Alexa-555 (C, G) and merged (D, H) images are presented.

plateau at approximately 15 min post-reaction for a concentration of 500 pM for APEX2-ABD. These signals were saturated at the concentrations of APEX2-ABD higher than 500 pM. We plotted the fluorescence intensities at 15 min post-reaction at each of the concentrations of APEX2-ABD and they were found to increase linearly (Figure 3B). The fluorescence intensities also showed a linear increase in AR and H_2O_2 concentration-dependent manner and the APEX2-ABD complex was able to detect as low as 1 μM of H_2O_2 (Figure 3C and D).[9]

We have confirmed that APEX2-ABD has both peroxidase activity and antibody-binding capability in an orientation-controlled manner. In order to utilize these two functions together, we applied the APEX2-ABD complex in a tyramide signal amplification (TSA) assay as a substitute for the HRP-conjugated secondary antibodies. TSA assay is an antibody-based high content imaging assay that has been widely used for providing greatly enhanced sensitivity for cell and tissue applications.[2] It is an enzyme-mediated detection method that utilizes the catalytic activity of peroxidase to generate high-density labeling for a target protein or nucleic acid sequence *in situ*. In this assay, a dye-conjugated tyramide derivative is employed as a target-specific labeling reagent.[2] The deposition of activated dye-conjugated tyramide derivatives on the target site results in localized enhancement of the fluorescence signal. The HRP-conjugated secondary antibodies are a key player in the TSA assay by acting as a site-specific signal amplifier. They selectively bind to the primary antibodies, which in turn bind to the specific target sites and locally activate the fluorescent dye-conjugated tyramide derivatives. This results in the localized labeling of adjacent proteins, thus significantly enhancing the fluorescence signals.

We employed same cell line/antibody pairs that are used for cell imaging (Figure 2). The two cell lines i.e., SKBR-3 and SCC-7, were treated with the corresponding fAPEX2-ABD/IgG complexes (i.e. SKBR-3 with fAPEX2-ABD/anti-HER2 rabbit IgG and SCC-7 with fAPEX2-ABD/anti-CD44 mouse IgG). Further, these treated cell lines were incubated with a mixture of Alexa-555-conjugated tyramide and 0.0015% H_2O_2 for 10 min. The fluorescence microscopic images (Figure 4 and S6) showed that the fAPEX2-ABD/anti-HER2 rabbit IgG and fAPEX2-ABD/anti-CD44 mouse IgG were bound to their appropriate target cells, i.e. SKBR-3 and

SCC-7, respectively (Figure 4B and F). However, very strong red fluorescence signals were observed in the case of both the cell lines, SKBR-3 and SCC-7, upon treatment with Alexa-555-conjugated tyramide and H_2O_2. This was probably observed due to multiple labeling of the target cells by the red fluorescent dyes mediated by the peroxidase enzyme, APEX2 (Figure 4C and G). This data suggests that APEX2-ABD/IgG complexes recognize their target cells and efficiently bind to them as guided by the complexed antibodies and subsequently activates the Alexa-555-conjugated tyramide to label the adjacent surface proteins of the target cells in the presence of H_2O_2 amplifying the red fluorescence signals.

Antibodies have been widely used as a ligand for the selective detection of biomarkers *in vitro* and/or *in vivo*. This is because they have extremely high binding affinities and specificities toward their target molecules and they virtually offer an almost unlimited range of specific targeting moieties.[11] To avoid a direct chemical modification of the targeting (or primary) antibodies or to enhance their detection capabilities, secondary antibodies conjugated with enzymes, such as HRP, are frequently used. However, there is a species variation in the Fc region of the targeting antibodies and each secondary antibody recognizes it in a species-limited manner and selectively binds to it. Since APEX2-ABD can bind to the Fc regions of various types of IgGs, it can be applied to a variety of targeting antibodies, without altering the targeting capability of the primary antibodies. Furthermore, APEX2-ABD possesses both peroxidase activity and IgG-binding capability simultaneously and hence, chemical conjugations or modifications are not necessary. Although HRP is a powerful and most commonly used peroxidase in immunoassays, it is extremely difficult to manipulate and express it in the bacterial system due to presence of complex glycosylations and disulfide bonds.[12] In contrast, APEX2-ABD can be easily and inexpensively overexpressed in large quantities in *E. coli* systems, as we have demonstrated in this study. According to our knowledge, this study is the first report about successfully producing a bifunctional fusion protein with APEX2 in a bacterial expression system and applying this fusion protein for immunoassays. Although there is a large scope for further improvements, the APEX2-ABD fusion protein reported in this study could offer new opportunities for developing diverse types of APEX2-associated target-specific signal amplifiers and spatial-resolved membrane proteome mapping tool-kits.

In this study, we genetically combined APEX2 and the antibody-binding domain to generate a bifunctional APEX2-ABD fusion protein. We successfully overexpressed it in *E. coli* and purified it in large quantities using the simple principle of affinity chromatography. APEX2-ABD exhibited both the peroxidase activity and capability of binding to a broad variety of IgGs derived from rabbit, mouse, and rat, in an orientation-controlled manner. Further, APEX2-ABD spontaneously formed non-covalent complexes with antibodies via simple protein-protein interactions and these complexes selectively bound to their target cells. In the presence of Alexa-555-conjugated tyramide and H_2O_2, APEX2-ABD remained bound to the target site and activated Alexa-555-conjugated tyramide, resulting in labeling of the adjacent surface proteins of the target cells and thus, significantly amplifying the red fluorescence signals. Thus, in conclusion APEX2-ABD was successfully used as a target-specific signal amplifier in combination with primary targeting antibodies for fluorescence cell imaging.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details and characterization data of APEX2-ABD are available. See DOI: 10.1039/c000000x/

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