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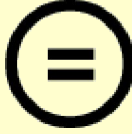
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Master's Thesis
석사 학위논문

Nature-Inspired Adhesive Catecholamine for Colorimetric Bioassays

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2020

Nature-Inspired Adhesive Catecholamine for Colorimetric Bioassays

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A thesis submitted to the faculty of DGIST in partial fulfillment of the requirements for the degree of Master of Science in the Department of Emerging Materials Science. The study was conducted in accordance with Code of Research Ethics[1]

05. 22. 2020

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Nature-Inspired Adhesive Catecholamine for Colorimetric Bioassays

Seunghwi Kim

Accepted in partial fulfillment of the requirements for the degree of Master of Science.

05. 22. 2020

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Abstract

Biomarkers are biological indicators of specific pathological conditions. By monitoring biomarkers, early disease diagnosis, and immediate treatment are possible. In conventional biomarker detection methods, complex protocols, additional equipment, and trained personnel are essential, limiting its application to a laboratory setting. Therefore, the colorimetric bioassay, which generates a visible signal in the presence of a target biomarker, has attracted attention. However, limitations of this assay type include low sensitivity and/or diffusion of the colorant resulting in the loss of spatial information. To address this limitation, we developed an enzyme-mediated adhesive colorant (EAC) platform. Unlike previous biomarker detection platforms that require additional equipment for the readout, in the EAC platform, pyrocatechol (PC) is oxidized/polymerized by horseradish peroxidase (HRP) to generate a colorimetric signal that can be detected with the naked eye. As a large number of PC are oxidized by a single HRP, signal amplification occurs. In addition, the chemical structure of the colorant is similar to polydopamine, a well-known universal surface adhesive, resulting in a unique adhesion property. That is, the generated adhesive colorant is attached in the vicinity of the HRP labeled target, allowing spatial analysis of the target. The broad use of the EAC platform was confirmed by applying the protocol to single-cell staining assays and paper-based bioassays.

Keywords: Biomarker, Catecholamine, Melanogenesis, Adhesive colorant, Colorimetric assay

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I. Introduction

Biomarkers are biological indicators that change qualitatively or quantitatively in response to specific pathological conditions or therapeutic interventions. (1, 2) The rapid detection of biomarkers has attracted attention as a possible early disease diagnostic tool, which would allow immediate treatment, with the potential for a positive effect on patient survival. (3, 4) Previous efforts to detect biomarkers include the use of immunohistochemistry, flow cytometry, polymerase chain reaction, etc. (5-9) Fluorescence, luminescence, and surface plasmon resonance, are common readouts obtained from these methods. Despite the high sensitivity and selectivity of these biotechnologies, they have multiple drawbacks, including time-consuming protocols and requirements of additional equipment and trained personnel, which limits their application to a laboratory environment.

The colorimetric bioassay is an emerging biomarker detection method due to its simplicity, selectivity, and visual signal, which can be detected by the naked eye without the requirement for additional equipment. (10-13) The use of gold nanoparticles as a colorimetric probe is a widely used method in lateral flow bioassays. (14, 15) By the antigen-antibody reaction, gold nanoparticles are bound to the antigen generating a clear and visible signal on the antigen-specific site. However, this assay has poor sensitivity due to the lack of signal amplification, limiting its application to highly concentrated biomarkers only. Another method that has been used in the conventional colorimetric bioassays is an enzyme-based system in which an enzyme is tagged onto a target antigen through the antigen-antibody reaction. This enzyme converts specific substrates into colorants. (16) In conventional enzyme-linked immunosorbent assay (ELISA) systems, the 3, 3’, 5, 5’-tetramethylbenzidine (TMB) is oxidized and converted into a colorant by horseradish peroxidase (HRP) bound to the antigen. (17) The signal is amplified, as a large number of TMB is catalyzed by a single HRP. (18, 19) The oxidized TMB, however, immediately diffuses into solution resulting in the loss of spatial information.

To address these limitations, we developed an enzyme-mediated adhesive colorant (EAC) platform inspired by melanogenesis, the process of natural pigment formation. In melanogenesis, enzyme-

mediated oxidation of catecholamines initiates polymerization of the catecholamines leading to the formation of colored macromolecules. In addition, the chemical structure of the generated colored macromolecule is similar to that of polydopamine, a well-known universal surface adhesive, resulting in the unique adhesive property. Using the EAC platform, catecholamine is oxidized by an enzyme bound to the target biomarker. This generates an adhesive colorant that immediately attaches to the enzyme-presenting site and forms a signal that can be detected by the naked eye. In addition, the EAC platform is expected to be applicable to a wide range of bioassays using various surfaces due to its unique adhesion property.

II. Experimental Section

Material

Pyrocatechol (PC), hydrogen peroxide (H_2O_2), laccase, tyrosinase, horseradish peroxidase (HRP), dopamine (DA), pyrogallol (PG), Diethylenetriamine (DETA), hydrogen chloride (HCl), bovine serum albumin (BSA), Tween 20, and formalin solution, neutral buffer were purchased from Sigma-Aldrich. 1X phosphate-buffered saline (1X PBS), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Corning. Nitrocellulose membrane and HyClone RPMI 1640 media with L-glutamine were purchased from GE Healthcare Life Science. α-fetoprotein (AFP) was purchased from PROSPEC. 96-well ELISA plate was purchased from BRANDplate®. Anti-α-fetoprotein antibody and anti-CD133 antibody were purchased from BioLegend. Goat anti-IgG (H+L) cross-absorbed secondary antibody conjugated with HRP was purchased from ThermoFisher. Anti-EGFR antibody and anti-EpCAM antibody were purchased from Abcam. Anti-MUC1 antibody was purchased from Fitzgerald Industries International. Dot-blot Apparatus was purchased from Bio-rad.

Catechol oxidation via representative oxidases

PC (0.5 μmol) was dissolved in 1X PBS (100 μL) containing H_2O_2 (0.5 μmol). 1X PBS (50 μL) containing representative oxidase, laccase, tyrosinase, or HRP (500 mU), was then added to the PC solution and incubated for 60 minutes. PC oxidation rate was monitored by measuring the absorbance from 230 to 900 nm using microplate reader (BioTek).

Colorant generation of potential adhesive colorants

The phenolic precursor, DA, PC, or PG (0.5 μmol), was dissolved in 1X PBS (100 μL) containing H_2O_2 (0.5 μmol). 1X PBS (50 μL) containing HRP (500 mU) was then added to the phenolic precursor solution and incubated for 60 minutes.

To demonstrate the synergistic effect of amine and phenolic precursor, DETA (0.25 μmol) and H_2O_2

(0.5 μmol) was dissolved in 1X PBS (100 μL) and pH was adjusted to 7.4 with 1 M HCl right before the addition of PC (or PG, 0.5 μmol). 1X PBS (50 μL) containing HRP (500 mU) was then added to the PC (or PG) solution and incubated for 10 minutes. The colorant generation rate was monitored by measuring the absorbance from 230 to 900 nm using microplate reader.

Adhesion property of potential adhesive colorants

1 μL of HRP (1000, 300, 100, 30, 10, and 0 μU) dissolved in 1X PBS containing 0.1 wt% BSA was spotted onto the nitrocellulose membrane and dried in air for 5 minutes at room temperature. The HRP-immobilized membrane was then incubated in phenolic precursor solution, 1X PBS (3 mL) containing PC (or PG, 300 μmol) and H_2O_2 (60 μmol), for 10 minutes. To demonstrate the synergistic effect of amine and phenolic precursor, additional DETA (150 μmol) was added and pH was adjusted to 7.4 with 1 M HCl right before the addition of the PC (or PG). The adhesive colorant generated on the HRP-immobilized membrane was photographed and then quantified with ImageJ. For further analysis, we observed adhesive colorant generated on the HRP-immobilized membrane with the scanning electron microscope (SEM).

EAC platform for ELISA

100 μL of AFP (300, 100, 30, 10, 3, and 0 ng/mL) dissolved in 1X PBS was directly incubated in the commercialized 96-well ELISA plate for overnight at 4 °C and nonspecific binding site on the surface was blocked with 1X PBS containing 1 wt% BSA (1% BSA/PBS, 300 μL) for 1 hour. After washing of each well with 1X PBS containing 0.05 wt% Tween 20 (PBST) for 5 minutes three times, purified anti-α-fetoprotein antibody diluted in 1% BSA/PBS (1 μg/mL, 100 μL) was applied to the well for 2 hours. The unbounded antibodies were completely removed by three times of 5 minutes incubation with PBST. Goat anti-IgG (H+L) cross-absorbed secondary antibody conjugated with HRP diluted in 1% BSA/PBS (1 μg/mL, 100 μL) was subsequently added and incubated for 1 hour, followed by three times of 5 minutes incubation with PBST to wash excess antibodies. Finally, PC solution, 1X PBS (100 μL)

containing PC (25 mM), DETA (12.5 mM), and H_2O_2 (2.5 mM) at pH 7.4, was applied to the well and incubated for 30 minutes. The total amount of the resulting colorant was evaluated by measuring the absorbance at 360 nm. After brief washing of the well with PBST, surface attached adhesive colorant was quantified by measuring the absorbance at 360 nm. Data was normalized with the absorbance at 360 nm from the same bioassay conducted without primary antibody.

Single cell staining using adhesive colorant

Human colorectal cancer (CRC) cell lines, SW480 and SW620, were cultured in HyClone RPMI 1640 media with L-glutamine containing 10% FBS and 1% penicillin-streptomycin in the 24-well plate for 12 hours in the CO_2 incubator (37 °C, 5% CO_2). The cell was fixed by 30 minutes incubation with 4% paraformaldehyde at room temperature, and then immersed in 3% H_2O_2 to extinguish the endogenous peroxidase activity of the cell followed by brief washing with 1X PBS.

Nonspecific binding site on the surface of the cell was blocked with 1% BSA/PBS by 30 minutes incubation at room temperature and then cell was washed with 1X PBS three times. Anti-CD133 antibody, anti-EGFR antibody, anti-EpCAM antibody, or anti-MUC1 antibody diluted in 1% BSA/PBS (1 μg/mL, 500 μL) was incubated for 1 hour followed by washing with PBST three times. Goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody conjugated with HRP diluted in 1% BSA/PBS (1 μg/mL, 500 μL) was further incubated for 1 hour. Finally, after the removal of excess antibodies by washing with PBST three times, the PC solution, 1X PBS (500 μL) containing PC (25 mM), DETA (12.5 mM) and H_2O_2 (2.5 mM) at pH 7.4, was incubated for 30 minutes and then cell was washed with PBST briefly. The amount of target-specifically generated adhesive colorant on the cell membrane was quantified by measuring the absorbance at 360 nm. The data was normalized with the absorbance at 360 nm from the same bioassay conducted without primary antibody.

EAC platform for paper-based bioassays

100 µL of AFP (256, 128, 64, 32, 16, and 8 ng) was immobilized on the nitrocellulose membrane using Bio-Dot Apparatus followed by 1 hour incubation with 1% BSA/PBS to block nonspecific binding sites of the membrane. AFP-immobilized membrane was washed three times by 10 minutes incubation with PBST. Purified anti-α-fetoprotein antibody diluted in 1% BSA/PBS (1 µg/mL, 1 mL) was applied to the AFP-immobilized membrane. After 2 hours of incubation, unbounded antibodies were completely removed by incubating the membrane in PBST for 10 min three times. Goat anti-mouse IgG (H+L) cross-absorbed secondary antibody conjugated with HRP (1 µg/mL, 1 mL) was incubated with the membrane for another 1 hour and then washed with PBST by incubating membrane for 10 min three times. Finally, PC solution, 1X PBS (1 mL) containing PC (25 mM), DETA (12.5 mM), and H_2O_2 (2.5 mM) at pH 7.4, was employed to the AFP-immobilized membrane and incubated for 30 minutes. The resulting adhesive colorant on the AFP-immobilized surface was photographed and then quantified with ImageJ.

III. Results and Discussion

1. Introduction and advantage of enzyme-mediated adhesive colorant (EAC) platform

Figure 1 summarizes the overall process of the EAC platform. The target biomarker is labeled with an oxidase by an antigen-antibody specific reaction. Catecholamine, the precursor of the adhesive colorant, is oxidized by the enzymatic activity of oxidase. A single oxidase interacts with thousands of catecholamines leading to signal amplification, thus improved sensitivity of the colorimetric bioassay. Oxidized catecholamine undergoes polymerization, and by further non-covalent interactions, generates the adhesive colorant. The resulting adhesive colorant is immediately attached to the enzyme-presenting site, resulting in target biomarker visualization. The unique adhesion property of the adhesive colorant allows visualization of biomarkers on a wide range of surfaces without the assistance of additional equipment.

2. Catechol oxidation via representative oxidases

To commercialize the EAC platform, optimization of multiple factors is essential. In the EAC platform, the generation of colored macromolecules is initiated by enzyme-mediated catechol group oxidation.

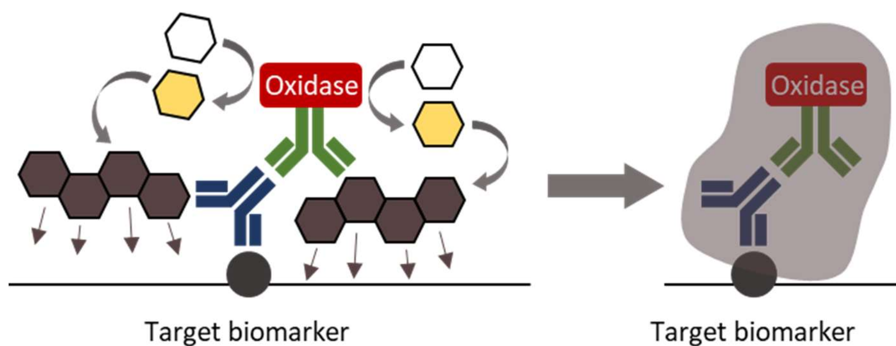

Figure 1. Schematic illustration of EAC platform.

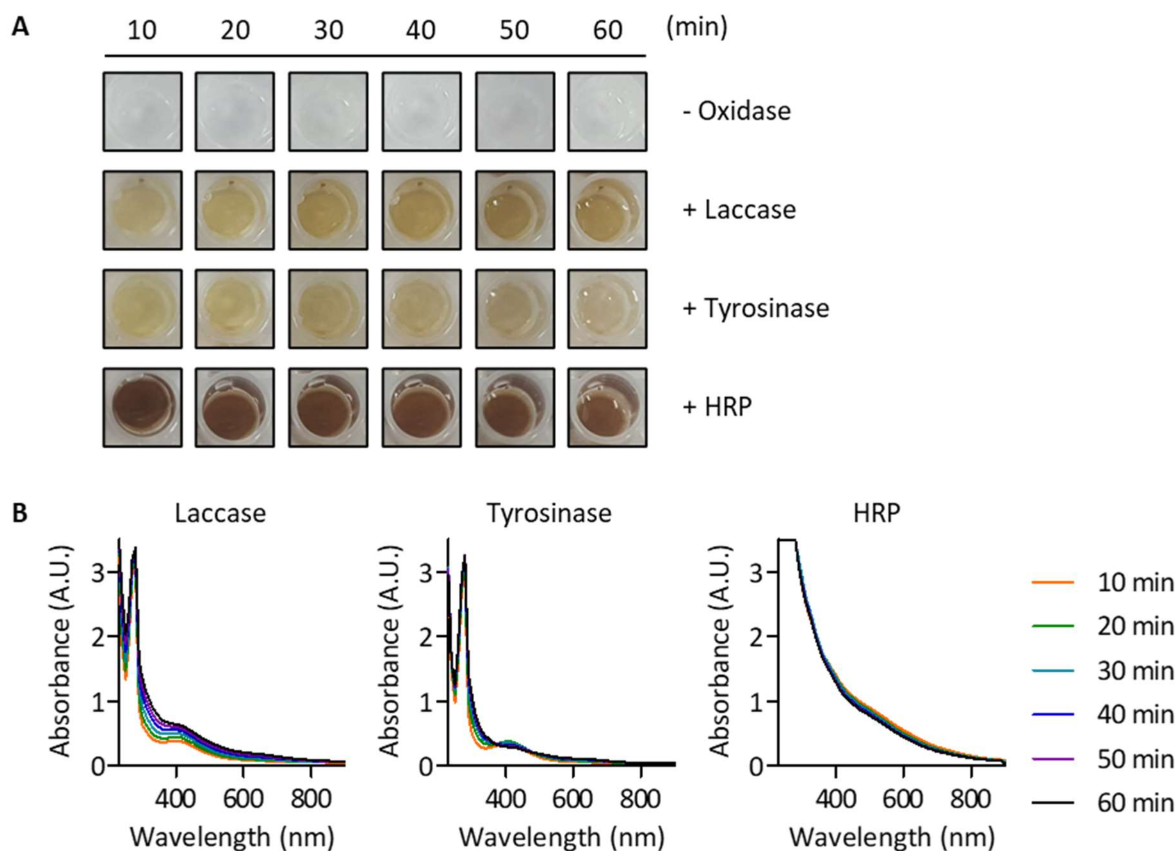

Figure 2. The efficiency of the representative oxidases. (A) Photograph of oxidized PC via representative oxidase, laccase, tyrosinase, and HRP. (B) The absorption spectrum in the UV-visible range (230-900 nm) of PC-based colorants generated by oxidase.

First, we evaluated the catechol oxidizing power of three representative oxidases, laccase, tyrosinase, and HRP. Laccase and tyrosinase are involved in natural melanogenesis, whereas HRP is commonly used in the conventional bioassays. Each oxidase was incubated for 60 minutes with 1X PBS containing PC and H_2O_2, required for enzyme activation. Oxidized PC underwent further polymerization, changing from and a colorless solution to dark brown. The efficiency of oxidases on PC oxidation can be verified by not only measuring the UV-visible absorbance but also by detecting signal with the naked eye (**Figure 2**). As shown in **Figure 2**, HRP significantly accelerated the generation of PC-based colorant compared to the other oxidases. Due to the remarkable oxidizing power of HRP, we chose HRP as the oxidase used in the EAC platform.

3. Colorant generation of potential adhesive colorants

Next, to determine the best adhesive colorant precursor, DA, PC, and PG, dissolved in 1X PBS containing H_2O_2, was incubated with HRP. DA, the smallest molecule containing both catechol group and amine group, slowly changed color to dark red over a period of 60 minutes. In contrast, PC and PG instantly polymerized and converted to colorant after the addition of HRP (**Figure 3A**). According to previous studies, under mild alkaline conditions, primary and secondary amines easily crosslink with PC through Michael Addition rather than Schiff's base reaction, forming oligomers/polymers. (20) Due to this synergistic effect of amine group with PC, the intensity of the polymerized PC dramatically increased in the presence of the amine group (**Figure 3B-C**).

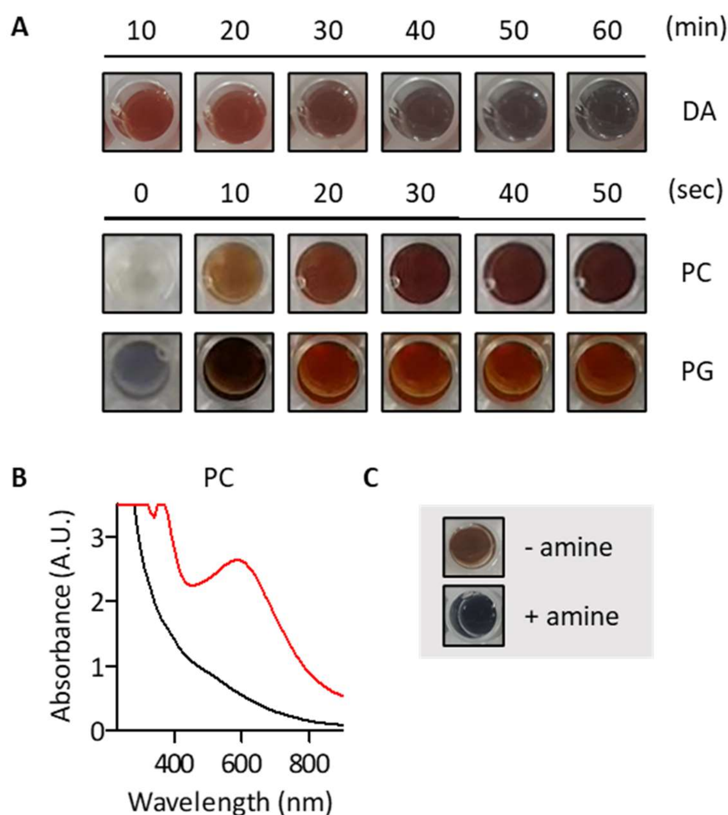

Figure 3. Colorant generation of adhesive colorant and the synergistic effect of amine group with PC and PG. (A) Photograph of oxidized DA, PC, and PG via HRP. (B) The absorption spectrum in the UV-visible range (230-900 nm) of the PC-based colorants generated by HRP in the absence of amine group (black line) and in the presence of amine group (red line). (C) Photograph of the PC-based colorant generated by HRP in the absence of amine group (top) and in the presence of amine group (bottom).

4. Limitations of conventional colorimetric bioassay using TMB

One advantage the EAC platform offers is the adhesion of colorant, which contributes to the preservation of spatial information. 3, 3', 5, 5'-Tetramethylbenzidine (TMB), the HRP substrate used in conventional enzyme-mediated colorimetric bioassays, is rapidly oxidized and converted to a colored molecule in the presence of HRP. However, the resulting colored molecule immediately diffuses into the solution, limiting its application only to ELISA-like in solution bioassays (**Figure 4**). On the other hand, in the EAC platform, the generated colorant is immediately attached to the target-presenting site due to its adhesion properties, preserving spatial information and expanding the application of colorimetric bioassays using various surfaces.

5. Adhesion property of potential adhesive colorants

To test the adhesion of potential adhesive colorant precursors, the HRP-immobilized nitrocellulose membrane was incubated for 10 minutes with DA, PC, or PG dissolved in 1X PBS containing H_2O_2. DA showed low sensitivity and slowly generated colorant responding only to highly concentrated HRP above 1000 μU. Compared to PC, PG is more hydrophilic due to an additional hydroxyl group. PG reacted with HRP and formed a number of colorants which are easily dispersed into the solution, due to the strong interaction between the three hydroxyl groups of PG and H2O. As a result, low adhesion and high background signal were observed.

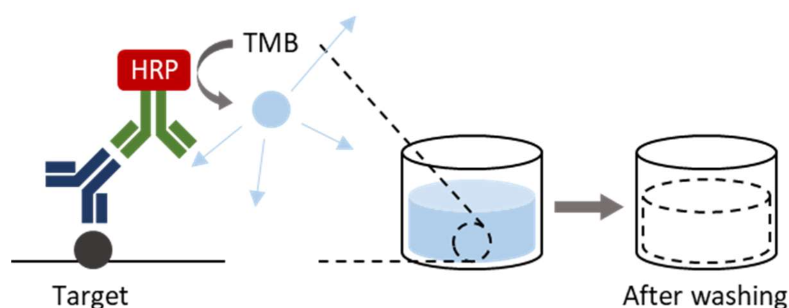

Figure 4. Schematic illustration of a conventional colorimetric bioassay, an ELISA, using TMB as the substrate for HRP.

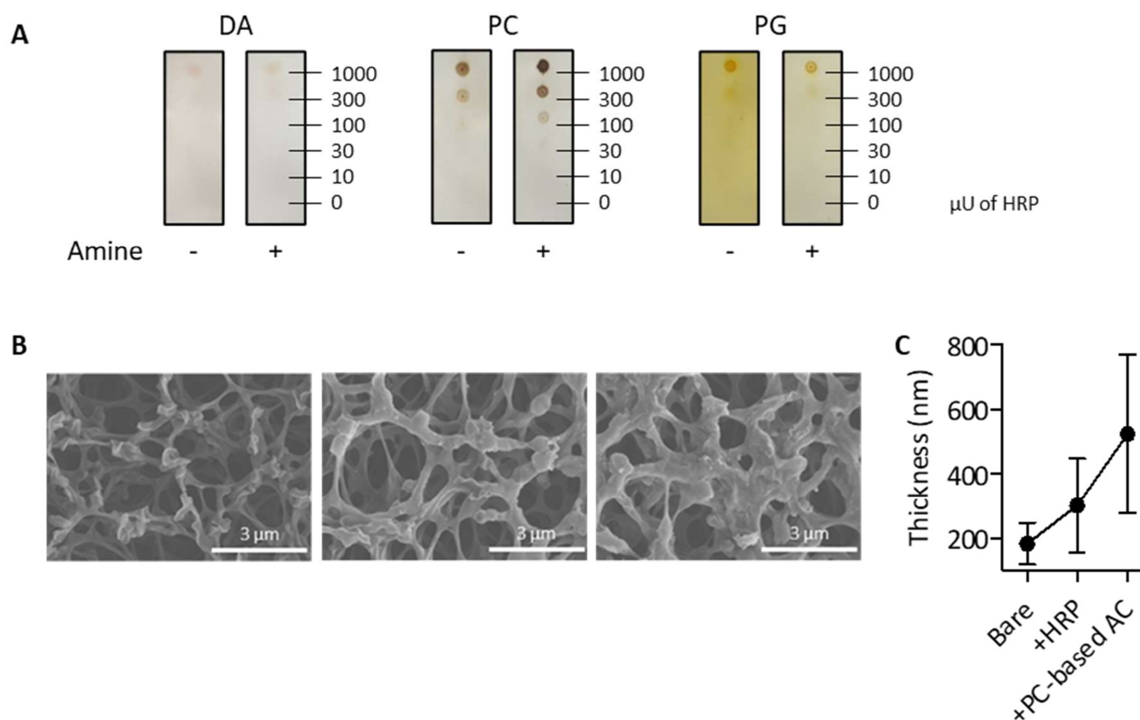

Figure 5. Adhesion property of potential adhesive colorant. (A) Visual observation of DA-based adhesive colorant (left), PC-based adhesive colorant (middle), and PG-based adhesive colorant (right), generated on the HRP-immobilized surface of the nitrocellulose membrane. (B) SEM image of bare nitrocellulose membrane (left), HRP-immobilized nitrocellulose membrane (middle), and PC-based adhesive colorant attached nitrocellulose membrane (right). (C) The thickness of the bare nitrocellulose structure (bare), HRP-immobilized nitrocellulose membrane (+HRP), and PC-based adhesive colorant attached nitrocellulose membrane (+PC-based AC) quantified with ImageJ. Data are mean ± SD (n=10).

Compared to DA and PG, PC showed higher sensitivity and adhesion strength. Moreover, the sensitivity and adhesion strength could be further increased by adding one equivalent of the amine groups (**Figure 5A**). After incubating the HRP-immobilized membrane with 1X PBS containing PC, DETA, and H_2O_2 for 10 minutes, membrane attached PC-based adhesive colorant could be observed using SEM. We observed that the thickness of the nitrocellulose structure dramatically increased after the incubation (**Figure 5B, C**).

6. EAC platform for ELISA

In order to optimize the concentration of PC and H_2O_2, PC solution (1X PBS containing PC, DETA, and H_2O_2) with various concentrations of PC and H_2O_2 was used as an HRP substrate in an ELISA, a conventional colorimetric bioassay. A model biomarker, alpha-fetoprotein (AFP), was immobilized on the surface of a commercialized 96-well ELISA plate and sequentially incubated with primary antibody and a secondary antibody conjugated with HRP. PC was oxidized and polymerized by HRP bound to AFP, forming adhesive colorant that immediately adhered to the target-specific surface. A visible signal was present, which can be detected with the naked eye. The signal remains even after vigorous washing (**Figure 6A**).

The intensity of the generated adhesive colorant did not increase with increasing concentration of PC. In a PC solution with PC above the critical concentration, PC was oxidized by HRP and simultaneously formed a large number of seeds, which interferes with polymerization, leading to reduced adhesion of the colorant (**Figure 6B**). In addition, HRP cannot be fully activated in the absence of sufficient H_2O_2. Interestingly, according to **Figure 6C**, a high concentration of H_2O_2 also prevents adhesive colorant formation. We assume that the radical generated by highly concentrated H_2O_2 attacks the oxidized PC, leading to the suppression of adhesive colorant generation. Using the optimized PC solution (1X PBS containing 25 mM PC, 12.5 mM DETA, and 2.5 mM H_2O_2), the adhesive colorant could be generated, and low-levels of AFP (30 ng/mL) could be detected (**Figure 6D**).

7. Single cell staining using adhesive colorant

The two colorectal cancer (CRC) cell lines, SW480, and SW620, which show distinctive protein expression levels, were chosen for testing using the EAC platform. (**Figure 7A**).

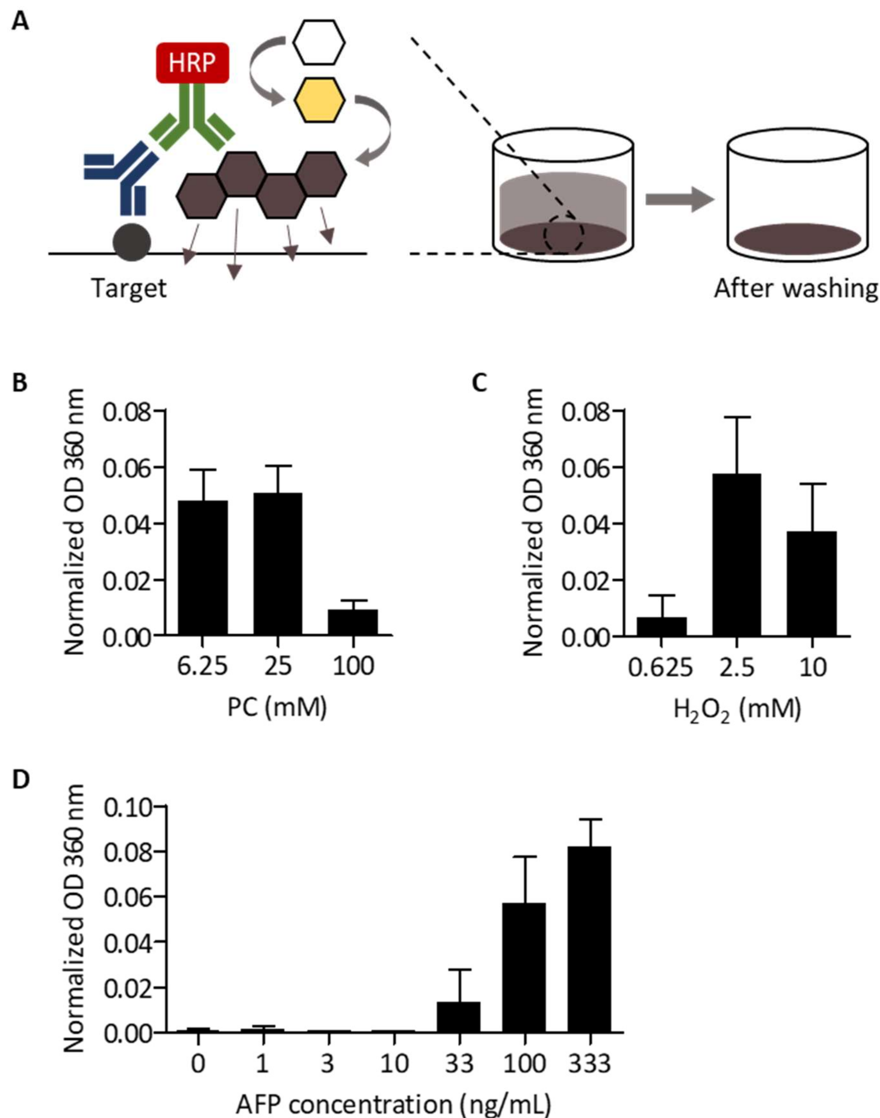

Figure 6. The EAC platform for ELISA. (A) A schematic illustration of an indirect ELISA detecting AFP using adhesive colorant. (B-C) Absorbance at 360 nm showing total colorant (black bar) and surface-attached adhesive colorant (red line) generated by detecting the HRP labeled to 10 ng AFP depending on (B) PC concentration and (C) H_2O_2 concentration of the PC solution. (D) Absorbance at 360 nm showing total colorant (black bar) and surface-attached adhesive colorant (red line) generated by detecting the HPR labeled to AFP of various concentrations. Data are mean ± SD (n=3).

Each of the fixed cell lines was incubated with a primary antibody specific to a transmembrane protein, such as CD133, EGFR, EpCAM, and MUC1. Subsequently, a secondary antibody conjugated with HRP was added to label the target transmembrane protein. After 30 minutes of incubation with the optimized PC solution, we successfully observed the generation of the adhesive colorant on the target

site using microscopy. Moreover, the intensity of the adhesive colorant correlated with the target protein expression level, enabling visualization of differences in protein expression (**Figure 7B**). Quantitative comparison was also possible by measuring the absorbance in the UV-visible range (**Figure 7C**).

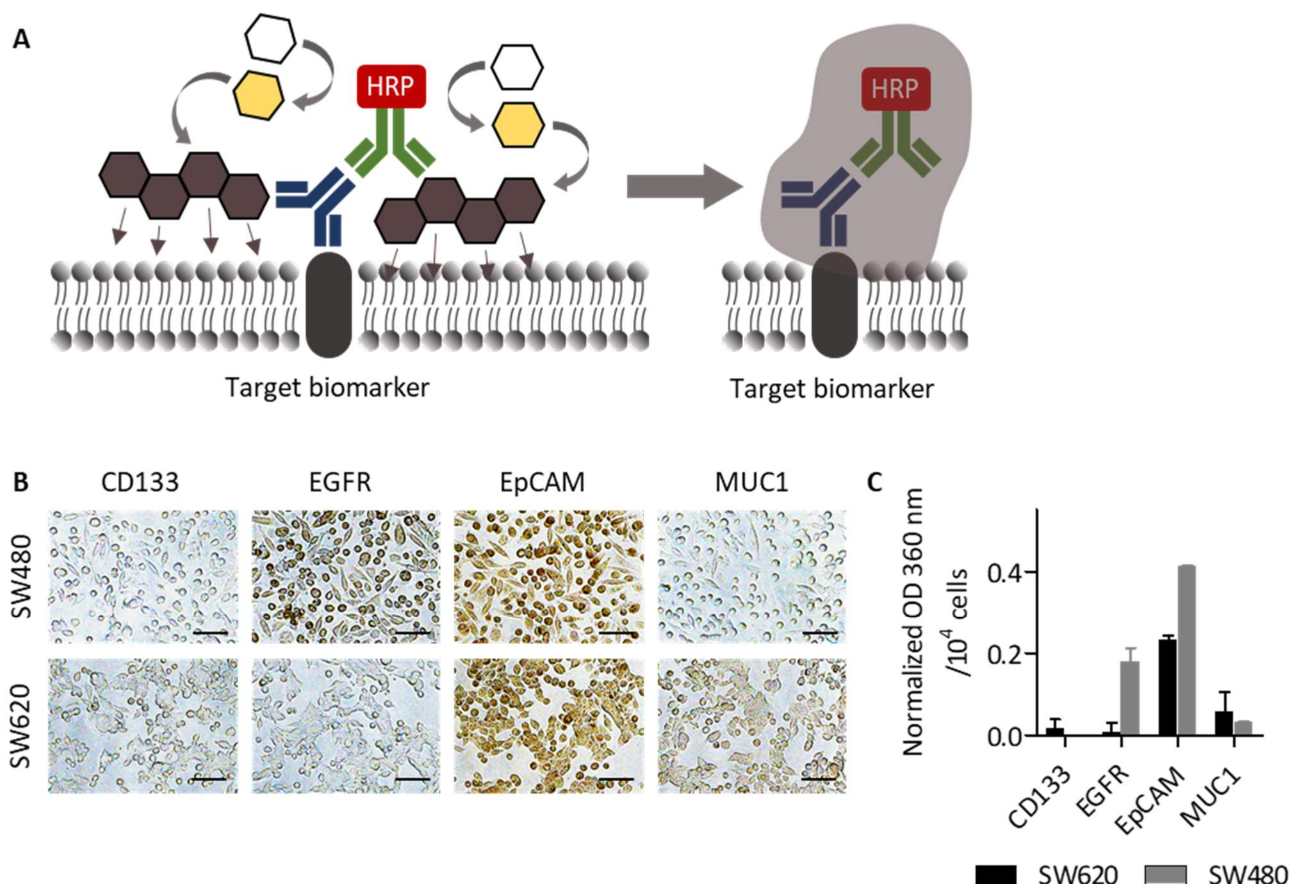

Figure 7. Single-cell staining using the EAC platform. (A) A schematic illustration showing the generation of adhesive colorant via HRP bound to the target transmembrane protein of the CRC cell. (B) Bright-field image of two different CRC cell lines, SW480 and SW620, stained with adhesive colorant. The adhesive colorant was generated by HRP bound to the target protein and attached to the target-specific site. The intensity of the resulting adhesive colorant correlated with the expression level of the target protein. Scale bars represent 30 µm. (C) Absorbance at 360 nm showing the intensity of the resulting adhesive colorant. Data are mean ± SD (n=3).

8. EAC platform for paper-based bioassays

The EAC platform can be applied to colorimetric bioassays on various surfaces, such as paper-based assays. Conventional enzyme-mediated paper-based colorimetric assay accompanied colorant migration. Complex fabrication processes and additional apparatus is essential for previous paper-based colorimetric assay, in order to detect multiple samples simultaneously or to prevent diffusion of colorant. However, in the EAC platform, the adhesive colorant is expected to attach onto the HRP-presenting site of the nitrocellulose membrane, preserving spatial information.

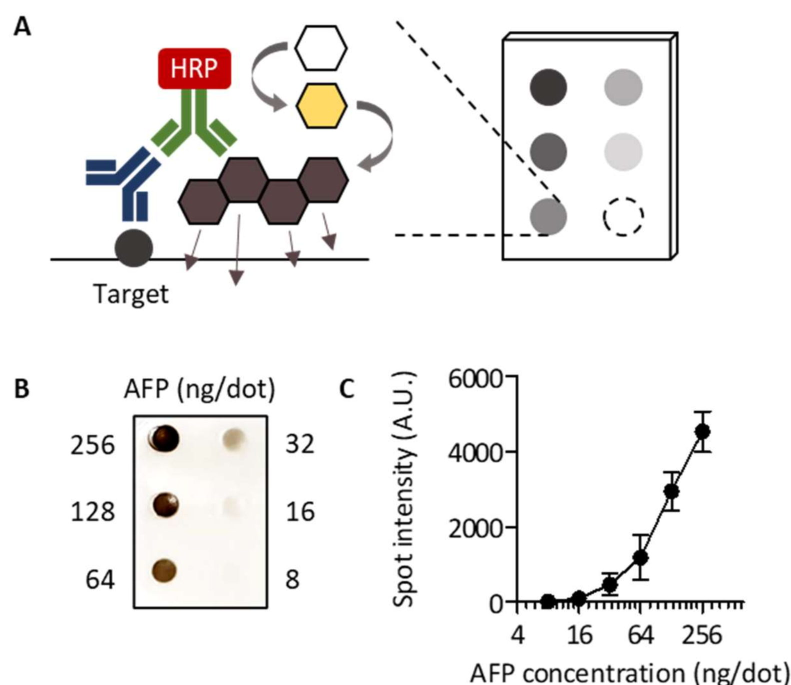

Figure 8. EAC platform applied to paper-based bioassay. (A) A schematic illustration of the dot-blot assay detecting AFP immobilized on the surface of the nitrocellulose membrane using adhesive colorant. (B) Visual observation of adhesive colorant. The colorant was generated by HRP tagged to immobilized AFP. The colorant attached to the target-specific surface to form a colorimetric signal. (C) The intensity of the resulting colorant quantified with ImageJ. Intensity correlates to the concentration of AFP immobilized on the surface. Data are mean ± SD (n=3).

Various concentration of AFP, a model biomarker, was immobilized on the surface of the nitrocellulose membrane by using apparatus. The AFP-immobilized membrane was incubated with primary antibody and subsequently, secondary antibody conjugated with HRP. Finally, the membrane was incubated in the optimized PC solution for 30 minutes. Consequently, the adhesive colorant formed and attached to the HRP labeled AFP-specific site (**Figure 8A**). The intensity of the adhesive colorant correlates with AFP concentration, thus allowing simultaneous visualization and approximate quantification of AFP (**Figure 8B**). The colorimetric signal could also be quantified using Image J (**Figure 8C**).

IV. Conclusion

In this study, we developed an enzyme-mediated colorimetric detection method, named the EAC platform. Using this method, thousands of PC molecules with one equivalent amine group were oxidized and subsequently polymerized to form adhesive colorants via a single HRP. This results in significant signal amplification. In addition, the adhesive colorant was immediately attached to the HRP-tagged surface, preserving spatial information. Using the EAC platform, we successfully visualized transmembrane biomarkers of CRC cells, including CD133, EGFR, EpCAM, and MUC1. The intensity of the adhesive colorant and level of biomarker expression correlated in the CRC cells. Furthermore, we confirmed that the EAC platform, which extends the boundaries of colorimetric bioassay, can be applied to 2D paper-based bioassays, such as dot-blot assay.

V. Reference

1. Golubnitschaja, O. and Flammer, J. "What are the biomarkers for glaucoma?" *Surv. Ophthalmol.*, 52(6), 2007, pp. S155-S161.
2. Wu, L. and Qu, X. G. "Cancer biomarker detection: recent achievements and challenges." *Chem. Soc. Rev.*, 44(10), 2015, pp. 2963-2997.
3. Li, M., Cushing, S. K., Zhang, J. M., Suri, S., Evans, R., Petros, W. P., Gibson, L. F., Ma, D. L., Liu, Y. X. and Wu, N. Q. "Three-Dimensional Hierarchical Plasmonic Nano-Architecture Enhanced Surface-Enhanced Raman Scattering Immunosensor for Cancer Biomarker Detection in Blood Plasma." *ACS Nano*, 7(6), 2013, pp. 4967-4976.
4. Stern, E., Vacic, A., Rajan, N. K., Criscione, J. M., Park, J., Ilic, B. R., Mooney, D. J., Reed, M. A. and Fahmy, T. M. "Label-free biomarker detection from whole blood." *Nat. Nanotechnol.*, 5(2), 2010, pp. 138-142.
5. Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., Henkelman, R. M., Cusimano, M. D. and Dirks, P. B. "Identification of human brain tumour initiating cells." *Nature*, 432(7015), 2004, pp. 396-401.
6. O'Hurley, G., Sjosted, E., Rahman, A., Li, B., Kampf, C., Ponten, F., Gallagher, W. M. and Lindskog, C. "Garbage in, garbage out: A critical evaluation of strategies used for validation of immunohistochemical biomarkers." *Mol. Oncol.*, 8(4), 2014, pp. 783-798.
7. Phillips, J. A., Xu, Y., Xia, Z., Fan, Z. H. and Tan, W. H. "Enrichment of Cancer Cells Using Aptamers Immobilized on a Microfluidic Channel." *Anal. Chem.*, 81(3), 2009, pp. 1033-1039.
8. Saeys, Y., Van Gassen, S. and Lambrecht, B. N. "Computational flow cytometry: helping to make sense of high-dimensional immunology data." *Nat. Rev. Immunol.*, 16(7), 2016, pp. 449-462.
9. Schamhart, D., Swinnen, J., Kurth, K. H., Westerhof, A., Kusters, R., Borchers, H. and Sternberg, C. "Numeric definition of the clinical performance of the nested reverse transcription-PCR for detection of hematogenous epithelial cells and correction for specific mRNA of non-target cell

origin as evaluated for prostate cancer cells." *Clin. Chem.*, 49(9), 2003, pp. 1458-1466.

10. Song, Y. J., Wei, W. L. and Qu, X. G. "Colorimetric Biosensing Using Smart Materials." *Adv. Mater.*, 23(37), 2011, pp. 4215-4236.
11. Wang, X. D., Chen, H. X., Zhou, T. Y., Lin, Z. J., Zeng, J. B., Xie, Z. X., Chen, X., Wong, K. Y., Chen, G. N. and Wang, X. R. "Optical colorimetric sensor strip for direct readout glucose measurement." *Biosens. Bioelectron.*, 24(12), 2009, pp. 3702-3705.
12. Zhang, Y., Hu, J. and Zhang, C. Y. "Sensitive Detection of Transcription Factors by Isothermal Exponential Amplification-Based Colorimetric Assay." *Anal. Chem.*, 84(21), 2012, pp. 9544-9549.
13. Gao, Z. Q., Deng, K. C., Wang, X. D., Miro, M. and Tang, D. P. "High-Resolution Colorimetric Assay for Rapid Visual Readout of Phosphatase Activity Based on Gold/Silver Core/Shell Nanorod." *ACS Appl. Mater. Interfaces*, 6(20), 2014, pp. 18243-18250.
14. Hwang, S. G., Ha, K., Guk, K., Lee, D. K., Eom, G., Song, S., Kang, T., Park, H., Jung, J. and Lim, E. K. "Rapid and simple detection of Tamiflu-resistant influenza virus: Development of oseltamivir derivative-based lateral flow biosensor for point-of-care (POC) diagnostics." *Sci. Rep.*, 8, 2018, pp. 12999
15. Zhong, Y. H., Chen, Y. J., Yao, L., Zhao, D. P., Zheng, L., Liu, G. D., Ye, Y. W. and Chen, W. "Gold nanoparticles based lateral flow immunoassay with largely amplified sensitivity for rapid melamine screening." *Microchim. Acta*, 183(6), 2016, pp. 1989-1994.
16. Ye, H. H., Yang, K. K., Tao, J., Liu, Y. J., Zhang, Q., Habibi, S., Nie, Z. H. and Xia, X. H. "An Enzyme-Free Signal Amplification Technique for Ultrasensitive Colorimetric Assay of Disease Biomarkers." *ACS Nano*, 11(2), 2017, pp. 2052-2059.
17. Fu, G. L., Sanjay, S. T., Zhou, W., Brekken, R. A., Kirken, R. A. and Li, X. J. "Exploration of Nanoparticle-Mediated Photothermal Effect of TMB-H2O2 Colorimetric System and Its Application in a Visual Quantitative Photothermal Immunoassay." *Anal. Chem.*, 90(9), 2018, pp. 5930-5937.
18. Lequin, R. M. "Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA)."

Clin. Chem., 51(12), 2005, pp. 2415-2418.

19. Fanjul-Bolado, P., Gonzalez-Garia, M. B. and Costa-Garcia, A. "Amperometric detection in TMB/HRP-based assays." *Anal. Bioanal. Chem.*, 382(2), 2005, pp. 297-302.
20. Qiu, W., Wu, G. and Xu, Z. “Robust Coatings via Catechol-Amine Codeposition: Mechanism, Kinetics, and Application.” *ACS Appl. Mater. Interfaces*, 10(6), 2018, pp. 5902-5908.

요약문

바이오마커는 병리학적 상태의 변화를 알려주는 지표이며 바이오마커를 검출함으로써 질병의 초기 진단이 가능하다. 기존 바이오마커 검출 방법은 복잡한 프로토콜을 거쳐 전문가에 의해 진행되었으며 결과 판독에 추가 장비가 필요했기 때문에 일반 가정에서 사용될 수 없었다. 따라서 표적 바이오마커의 유무에 따라 눈에 보이는 신호를 형성해 결과 판독에 추가 장비가 필요 없는 비색 분석법이 최근 주목을 받고 있다. 하지만 기존에 사용되던 비색 분석법의 낮은 감도 또는 분자 프로브의 즉각적인 확산으로 인한 공간적 표적 분석의 한계에 의해 그 활용성이 떨어졌다. 따라서 우리는 효소에 의해 증폭된 신호가 표적이 존재하는 공간에 접착함으로써 기존 비색 분석법의 문제점을 해결할 수 있는 enzyme-mediated adhesive colorant 플랫폼 (EAC 플랫폼)을 디자인했다. 이전의 바이오마커 검출 플랫폼이 결과값을 얻기 위해 추가 기기가 필요한 것과 다르게 EAC 플랫폼에서는 피로카테콜 용액이 바이오마커에 표지 된 산화효소에 의해 산화/고분자화 되어 맨눈으로 검출가능한 비색 신호를 형성한다. 또한 생성된 비색 신호의 화학구조는 만능 접착제인 폴리도파민의 화학구조와 매우 유사하여 접착력을 가진다. 즉, 형성됨과 동시에 산화효소가 존재하는 부근에 접착되기 때문에 신호의 고정화를 통한 바이오마커의 공간적 분석을 가능하게 한다. 더 나아가 단일 세포 염색 분석과 paper-based bioassay인 Dot-blot assay 등에 해당 플랫폼을 적용시킴으로써 플랫폼의 폭 넓은 활용가능성을 입증했다.

핵심어: 바이오마커, 카테콜아민, 멜라닌 생성반응, 접착성 착색제, 발색 기반 분자검출법