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ARTICLE TYPE

New method to amplify colorimetric signals of paper-based nanobiosensors for simple & sensitive pancreatic cancer biomarker detection

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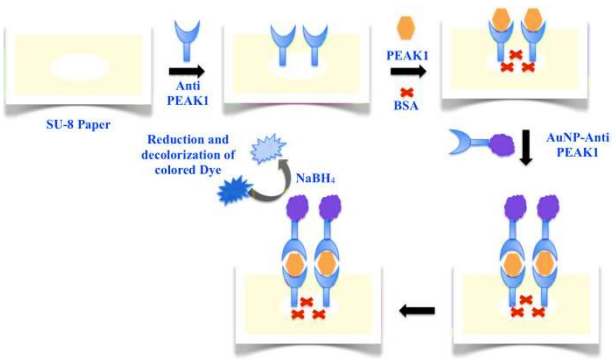
A low-cost, sensitive, and disposable paper-based immunosensor for instrument-free colorimetric detection of a pancreatic cancer biomarker PEAK1 was reported for the first time by capitalizing the catalytic properties of gold nanoparticle in colour dye degradation. This simple signal amplification method enhances the detection sensitivity by about 10 folds.

Early detection of a disease biomarker is crucial to the timely treatment of diseases.[1] There is an urgent need for rapid monitoring of disease biomarkers for cancer, a non-communicable disease that contributes a major reason for the increasing global mortality.[2, 3] According to World Health Organization, there are 8.8 million people who died worldwide due to cancer,[4] and the current data from the American Cancer Society forecast 1,688,780 new cases of cancer and 600,920 deaths in US.[5] 57% percent of new cancer cases arise in developing nations and the figure could reach 70% by 2050.[6, 7] Their cancer mortality now accounts for 70% of cancer deaths worldwide.[7-11]Among these cancer cases, pancreatic cancer caused an estimated 43,090 deaths in the US in 2017. Most of the pancreatic cancers occur in the exocrine pancreas, namelypancreatic ductal adenocarcinoma (PDAC). PDAC is the fourth-leading cause of cancer death in US, and the incidence of PDAC is increasing compared to other types of cancers. [5, 12-14] Since the pancreas is deep inside the body, regular screening may not identify the early progression of such a tumour. Additionally, the lack of symptoms at early stages makes it difficult to be identified. The appearance of the symptoms after PDAC spreads to other organs further decreases the 5-year survival rate to approximately 5%.[15] Therefore, a low-cost method for rapid and reliable early diagnosis of PDAC is in great need.

Recently, Keblar *et al.* discovered that a novel tyrosine kinase, PEAK1 (pseudopodium-enriched atypical kinase one, SGK269), could be used as a biomarker for PDAC.[13] Developing an immunosensor for PEAK1 that fulfills the conditions of a point-of-care tool with simple, sensitive, portable, rapid, low-cost and miniature features will be of great importance for the early clinical diagnosis of PDAC. However, traditional immunoassay methods with various sensing strategies such as radiation,[16] fluorescence,[17, 18] surface plasmon resonance (SPR),[19, 20] quartz crystal microbalance,[21] well-known enzyme-linked immunosorbent assays (ELISA),[22, 23] chemiluminescence [24] and elctrochemistry[25-28] require complex, expensive instruments and skilled operators. Furthermore, conventional methods including tissue immunohistochemistry and western blot for PEAK 1 measurement are invasive, cumbersome, costly, and they can only provide semi-quantitative results.[29, 30] Although electrochemical detection can provide quantitative data, it requires an expensive potentiostat, creating a challenge for point-of-care detection.[28] Considering these hindrances, a much simpler paper-based colorimetric assay method shows potential for low-cost point-of-care detection of the PDAC biomarker PEAK1, because of its simplicity, low cost, low volume of sample, and disposable nature. [23, 31, 32,33-40]

Gold nanoparticles (AuNPs) with 10-50 nm diameter are commonly used in various colorimetric assays, which exhibit red colour in a dispersed status and present a purple or blue colour at an aggregated state due to there interparticle distance and size-dependent localized surface plasmon resonance.[41,38] However, due to low sensitivity resulted from poor signal amplification, signal enhancement techniques such as enzyme cascade reactions or silver enhancement are often needed to enhance the sensitivity.[42, 43] Yet the use of enzyme and growth of palladium nanostructures on the surface of AuNPs makes the assay fairly complex. Taking advantage of the enzyme-like catalytic property of AuNPs,[44-46] herein, we report for the first time a new method to amplify the colorimetric signals based on catalytic activities of AuNPs towards azo dye degradation and have developed a novel disposable colorimetric immunosensor for the rapid POC detection of the PDAC biomarker PEAK1 on a paper-based microfluidic device.



Scheme 1. Schematic depiction of the colorimetric detection of PEAK1 by paper-based nano-immunosensors, based on the AuNPs-catalyzed reduction of colour dye by $NaBH_4$.

Scheme 1 shows the main principle and procedures of the colorimetric detection of PEAK1 by the paper-based nano-immunosensor, based on the AuNPs-catalyzed reduction of a colour dye. In this work, the immunosensor for PEAK1 was fabricated using the photolithography technique on SU-8-treated chromatographic paper with detection zones (2 mm diameter),[33, 47] following each step in Scheme 1. First, paper detection zones were treated with 2 µL PEAK1 capture antibodies, Anti PEAK1 (20 µg/mL in Phosphate Buffered Saline, PBS), for 20 minutes and subsequently washed with PBS. Then, after the paper surface was blocked with a 0.5% BSA solution, 2 µL of different concentrations of PEAK1 were added and incubated for 15 minutes to facilitate the immuno recognition, followed by washing with PBS to remove the unbounded PEAK1. Next, the bioconjugate probes, AuNP-tagged Anti PEAK1 bioprobes, were added to the paper surface and incubated for another 20 minutes to establish the sandwich-structure immunocomplex. Finally, hydroxy naphthol blue (HNB) in the presence of $NaBH_4$ was added to amplify the signal for sensitive colorimetric detection, in which AuNPs catalyzed the degradation of azo dyes (i.e. HNB) in the presence of $NaBH_4$ and resulted in color changes. [45, 48-51]

Before the colorimetric signal amplification step, we first tested the AuNPs-based colorimetric detection of PEAK1 as a comparison (i.e. without signal amplification), as shown in Fig. 1. When adding different concentrations of the target PEAK1 to different detection zones (10^{-6}, 10^{-8}, and 0 g/mL PEAK1 for Fig. 1A(a)-(c), respectively), the immunocomplex showed different colours. The colour change was attributed to different amounts of AuNP bioprobes bound to different concentrations of PEAK1 during the immuno recognition. The absence of PEAK1 protein exhibited no colour change on the detection zones (Fig. 1A(c)), while the presence of 10^{-6} and 10^{-8} g/mL PEAK1 caused a purple colour (Fig. 1 A(a)) and a light pink colour (Fig. 1 A(b)), respectively, due to the SPR optical properties of AuNPs.[38] These colour changes demonstrated that AuNP-tagged Anti PEAK1 bioprobes could be used for the development of a colorimetric PEAK1 sensor. Nevertheless, the obscure color change in low concentrations of PEAK1 made it difficult for sensitive quantification of PEAK1 protein.

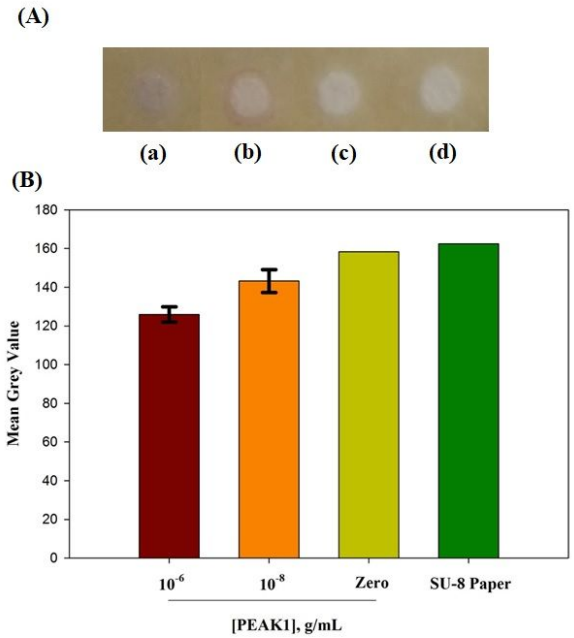


Fig. 1. Images (A) and gray values (B) of SU-8-treated paper detection zones (2 mm diameter) with (a) 10^{-6}, (b) 10^{-8}, (c) 0 g/mL PEAK1, and (d) a SU-8 paper detection zones before any immunoassay procedures.

To solve this impediment, we capitalized the catalytic properties of AuNPs to amplify colorimetric signals. To confirm the feasibility to use the developed immunoprobe for the degradation of an azo dye, and thus to develop a sensitive colorimetric immunosensor, we first studied the effects of the immunoprobe on an azo dye, hydroxy naphthol blue (HNB), in the presence of $NaBH_4$.

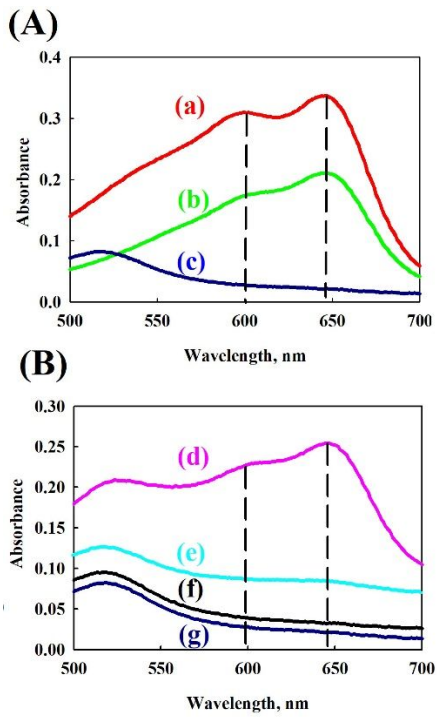
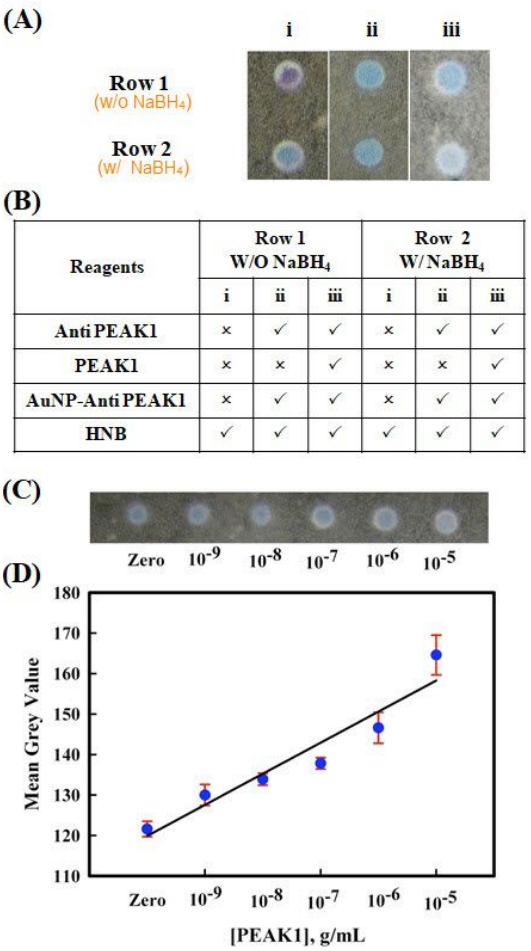


Fig.2. (**A**) UV-vis absorption spectra for (a) HNB, (b) HNB with $NaBH_4$, and (c) HNB + $NaBH_4$ + AuNP-AntiPEAK1 after 30 minutes. **(B)** UV-vis absorption spectra (d-g) at 0, 5, 30, and 40 minutes after the addition of AuNP-AntiPEAK1 to the mixture of HNB and $NaBH_4$, respectively.

UV-vis absorption spectra studies were carried out with a Beckman Coulter DU 730 UV spectrometer to clarify the importance of the immunoprobe on the degradation of HNB. As shown in Fig.2, HNB exhibited well-defined absorption peaks centred at 600 and 645 nm (Curve (a) in Fig. 2A). After the addition of 0.1 M $NaBH_4$ (Curve (b) in Fig. 2A), there was no typical change in the absorption spectra noticed (i.e. the same absorption peaks centred at 600 and 645 nm), which indicates that the reduction of HNB did not occur (note the absorbance in the y-axis was offset for easy comparison). Interestingly, even after keeping the mixture for a long period of time, no changes were observed. The obtained results are similar to a previous report by Li *et. al*,[45] in which the addition of $NaBH_4$ failed to promote the degradation of an azo dye (mythyl orange). In contrast, in the presence of AuNP-Anti PEAK1,the absorption peaks of HNB disappeared gradually as the reaction proceeded in Fig. 2A(c). We further studied the spectra of the mixture of HNB + $NaBH_4$ at different times after adding AuNP-AntiPEAK1. It can be found from Figure 2B that the complete degradation of HNB (disappearance of HNB absorption peak) took place after 5 minutes (Fig .2B(e)). This clearly indicated HNB was degraded in the presence of AuNPs-tagged immunoprobes, and AuNP-Anti PEAK1 indeed exhibited good catalytic properties. Thus, AuNPs-catalyzed degradation of HNB can be used as a colorimetric indicator for PEAK1 detection.

To demonstrate the aforementioned proof-of-concept for the POC detection of PEAK1, we dropped coated HNB onto paper detection zones and dried it at room temperature. As appeared in Fig.3A-B, HNB produced a purple colour, and in the presence of $NaBH_4$ it turned into a sky blue colour, which was probably attributed to the change of pH value since $NaBH_4$ is a base. When there was zero g/mL PEAK1, the light blue colour remained the same (Fig. 3A), whether there was $NaBH_4$ or not. However, it is interesting to note the colour changed from light blue to colourless, when 10^{-6} g/mL PEAK1 was added onto complete immunosensor detection zones constructed by following the immunoassay steps depicted in Scheme 1, in the presence of HNB and $NaBH_4$. The colour change was mainly due to the catalytic degradation of the colour of HNB in the presence of $NaBH_4$ by AuNPs from the immunocomplex that was confirmed by XPS (Fig. S1). On the contrary, as for zero g/mL PEAK1, there was no AuNP-Anti PEAK1 for the immuno recognition and no AuNPs to bleach the dye, resulting in no colour change. This indicates the feasibility of using AuNPs-catalyzed degradation of HNB for the colorimetric detection of biomolecules.



Reagents	Row 1 W/O $NaBH_4$			Row 2 W/ $NaBH_4$		
	i	ii	iii	i	ii	iii
Anti PEAK1	×	✓	✓	×	✓	✓
PEAK1	×	×	✓	×	×	✓
AuNP-Anti PEAK1	×	✓	✓	×	✓	✓
HNB	✓	✓	✓	✓	✓	✓

Fig.3. (A-B) Images for the reactions between (i) HNB, (ii) HNB + Zero g/mL PEAK1 sandwich immunocomplex, and (iii) HNB + 10^{-6} g/mL PEAK1 sandwich immunocomplex in the absence (top row) or in the presence (bottom row) of $NaBH_4$ on paper detection zones. (C) Images and (D) the calibration curve of the paper-based immunosenor for the colorimetric detection of different concentrations of PEAK1.

After the feasibility test, different concentrations of PEAK1 ranging from zero to 10^{-6} g/mL PEAK1 were tested using the paper-based nano-immunosensor catalyzed by AuNPs for colorimetric detection. The colour changes from blue (zero g/mL PEAK1) to colorless (10^{-6} g/mL PEAK1) at different concentrations of PEAK1 (Fig. 3C) could be recognized by the naked eye. Nevertheless, to obtain quantitative data, we measured the grey values of digitally photographed detection zones with different concentrations of PEAK1 using ImageJ software.[52-54] It can be clearly observed from Fig. 3D that the grey intensity values increased gradually as the concentrations of PEAK1 varied from low (10^{-9} g/mL) to high (10^{-5} g/mL). The calibration plot exhibited a linear range between 10^{-5} to 10^{-9} g/mL PEAK1, with a squared correlation coefficient of 0.975. As mentioned earlier, the degradation of HNB was directly proportional to the amount of AuNP-Anti PEAK1 bound to different concentrations of PEAK1 during the formation of the sandwich immunoassay on the paper-based device. With increased concentration of PEAK1, the AuNPs presented on the detection zones increased accordingly, which

facilitated the decolourization of HNB. The limit of detection (LOD) was calculated to be 1.0 ng/ mL PEAK1 by considering three times the standard deviation above the blank. This LOD was about 10-fold lower than that of AuNPs-based colorimetric detection without signal amplification, indicating higher detection sensitivity from our new AuNPs-catalyzed colorimetric immunoassay.

In conclusion, we have developed a novel, cost-effective, and simple paper-based colorimetric immunoassay method for the sensitive detection of a pancreatic cancer biomarker, PEAK1, by utilizing the catalytic activity of AuNPs in decolourization of a coloured azo dye without employing an enzyme or Ag and Au enhancement technique for signal amplification. This AuNPs-catalyzed colorimetric immunoassay on a paper-based device can detect 1.0 ng/mL level of PEAK1 in an hour, which enhances the detection sensitivity by 10 folds than an AuNPs-based colorimetric immunoassay without signal amplification (LOD of ~10.0 ng/mL PEAK1). The method is also simpler and faster than conventional ELISA techniques that require long time (e.g. about 30 hours[55]) and sophisticated instruments, and thus it could be used for the early diagnosis of PDAC at the point of care. Along with (I) faster assays from paper-based devices, some other major benefits of paper-based devices in this work include: (II) It is a low-cost platform; and (III) The white colour of paper makes the device usually have low background and high contrast in colour changes, thus making it highly suitable for colorimetric detection. In addition, the proposed detection technique can be used for the development of other biosensing methods for point-of-care diagnosis from different biological specimens. To address the issue of interference from blood, the incorporation of blood filtration membranes or plasma separation membrane onto a paper-based device will endow to circumvent colour interference in colorimetric assays.[55-56]

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Notes and references

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† Electronic Supplementary Information (ESI) available: [materials and chemicals list and prepration of AuNPs bioconjugate]. See DOI: 10.1039/b000000x/

‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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Catalytic properties of gold nanoparticle in colour dye degradation is utilized to amplify colorimetric detection signal of a low-cost paper-based immunosensor for instrument-free detection of a pancreatic cancer biomarker PEAK1.