Development of a simple, sensitive and selective colorimetric aptasensor for the detection of cancer-derived exosomes

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Abstract

There is a growing need for cancerous exosome detection towards potential non-invasive cancer diagnosis. This study aims to develop a reliable colorimetric aptasensor for sensitive and specific detection of circulating cancer-derived exosomes. In this design, target exosomes were firstly captured by latex beads *via* aldimine condensation, followed by bio-recognition using a specific CD63 aptamer, which was conjugated to horseradish peroxidase (HRP) through biotin-streptavidin binding. Colorimetric detection was achieved in 10 min *via* enzymatic catalysis to produce dark colored polydopamine (PDA) from colorless substrate dopamine (DA) in especially prepared H_2O_2 reaction solution. The sensitivity was enhanced by *in situ* deposition of PDA around exosome particles to strengthen the developed colorimetric signal, which could be directly observed by naked eye. Signal quantification was carried out by absorbance measurement. The colour intensity correlates to the CD63 amount and the limit of detection can be as low as 7.7×10^3 particle/mL, improved by 3-5 orders of magnitude from conventional Dot-blot methods. The aptasensor showed specificity to HER2 and integrin αvβ6 positive, cell culture-derived, breast and pancreatic cancer-derived exosomes, respectively, when the correct aptamer sequence was used. Overall, a sensitive and selective colorimetric aptasensor was successfully developed for detecting cancer-derived exosomes facilitated by HRP-accelerated DA polymerization and *in situ* PDA deposition. This versatile aptasensor holds great potential for future development of point-of-care detection kits for cancer diagnosis in a clinical setting.

Keywords: exosome, aptamer, aptasensor, cancer diagnosis, integrin αvβ6, HER2

1. Introduction

Exosomes are extracellular, membrane- enclosed vesicles, of 30-150 nm in diameter and exhibit a biconcave "cup-shaped" morphology under electron microscopy (Théry et al., 2002). They are derived from most, if not all, eukaryotic cells through an endosomal pathway and circulate in body fluids (Raposo and Stoorvogel, 2013). Exosomes matter shed from tumorous tissues may exhibit clinical significance and can be identified as potential non-invasive cancer biomarkers as they reflect the genetics or proteins originating from parent tumours (Skotland et al., 2017). Proteomic analysis techniques have allowed for in depth study of the protein composition of exosomes and has shown common characteristic markers on the surface of exosomes. Some typical marker proteins include the heat shock proteins i.e. HSP90 and HSP70 and the tetraspanins such as CD9 and CD63. In the context of disease, early diagnosis generally renders the best chances of a cure and based on their reliability, biomarkers can provide an understanding of disease diagnosis, progression and response to treatment. For example, the human epidermal growth factor receptor 2 (HER2), a type 1 transmembrane glycoprotein, is overexpressed in several types of cancer, particularly, in breast cancer. Apart from potentially contributing to oncogenesis and promoting resistance to chemotherapy (Shah et al., 2018), (Kalluri and LeBleu, 2020), cancer cell derived HER2 exosomes are considered to be promising tumour biomarkers for clinical diagnosis (Kabe et al., 2018), (Arya et al., 2018). The ability to accurately quantify and identify tumour-derived exosomes may provide valuable insight into cancer diagnostics and have clinical efficacy on the prognosis of disease (Kalluri and LeBleu, 2020), (Xu et al., 2018), (Rayyan et al., 2018). Furthermore, exosomes may provide unique information regarding disease classification, as they contain a rich cargo of proteins, RNA, lipids and metabolites, reflecting the biological state of the originating cell type (Shao et al., 2018). They are also advantageous in that they are often very stable and can be stored for extended periods of time without degradation. Ultimately this reveals the potential clinical significance for the use of exosomes as biomarker tools.

Pancreatic cancer (PC) remains one of the deadliest malignancies with extremely low overall five-year survival probability. There is no official PC screening program around the world largely due to the absence of sensitive and specific markers, which has been one of the key confinements for early screening (Kamisawa et al., 2016). In clinical practice, carbohydrate antigen (CA) 19-9 is

the only one currently recommended for clinical use by the NCCN guidelines for PC (Paulista et al., 2017). Carcinoembryonic antigen (CEA) is also a commonly used blood-based PC biomarker. However, these markers are not tumour specific and is elevated in many hepatobiliary cancers (Winter et al., 2006). In this manner, the diagnostic paradigm based on the premise of markers such as CA19-9 experiences low diagnostic accuracy. Recent advances in the molecular pathology of PC have promoted the understanding of genetic properties. For example, KRAS mutation has been identified as recurrently mutated genes in PC (Kamerkar et al., 2017). Despite that, there is no dependable biomarker and the early recognition of PC is not adequate based on current biomarker cohort. Therefore, new biomarkers need to be identified urgently to bolster energizing zones of PC detection. Exosomes possess promising opportunities for biomarker discovery as their contents are largely derived from the originated tumours and more importantly, it allows non-invasive disease monitoring strategies mediated by exosomes in liquid biopsy.

Currently, existing conventional methods such as Western Blot, Nanoparticle Tracking Analysis (NTA) and flow cytometry have been adapted for exosome quantification and detection. However, limitations of these methods, such as the need for specialised equipment and complex software, mean they may not be appropriate in a clinical point-of-care diagnostic situation (Cheng et al., 2019). For example, although currently considered as one of the best methods available, NTA has a limited dynamic range and only provides an estimation of exosomes at high concentrations ($1 \times 10^7 – 1 \times 10^9$ particle/mL), thus needing volumes of greater concentrations to be diluted prior to testing (Liu et al., 2018). Additionally, NTA lacks specificity and flexibility to alter the settings on the software which means results will not always be exactly reproducible. Other detection methods also have a limited scope due to the insensitivity in smaller size ranges or a need for large volumes of sample. Moreover, during initial stages of cancer, this is rarely feasible as cancerous exosomes present in circulating fluid normally exist in small concentrations and to quantify these amounts absolute sensitivity is vital. Recently, there are many emerging biosensing methods reported for exosomes detection using aptamers in the modality of optical, electrochemical and electrical biosensors in the field of cancer and other diseases. Nanotechnology is playing a big role in enhancing their sensitivity, specificity and portability. Among the colorimetric biosensors for exosome detection, most used gold nanoparticles as colour reporter show limit of detection around

10^3-10^6 particle/μL. Our very recent systematic review provided a comprehensive overview on the latest developments and technical breakthroughs in this regard (Xu et al., 2020).

In this study, we aim to develop a sensitive method which would enable the detection of exosomes simply by exhibiting a colour change. The main premise of the concept involved capturing exosomes through the use of specific biotin-aptamers for exosomal marker (such as CD63) detection. A secondary horseradish peroxidase (HRP)-linked streptavidin is then added, which upon the addition of an HRP substrate produces a coloured signal. Taking advantage of the reported ability of HRP to catalyse dopamine (DA, colourless) to polydopamine (PDA, brown-black coloured) in the presence of H_2O_2, the reaction can be enhanced by approximately 300-fold over a short period of time (Li et al., 2017). PDA further reacts to the amine, sulfhydryl and phenol groups in exosomal surface proteins which could trigger *in situ* deposition of PDA around exosome particles. Therefore, the H_2O_2 accelerated catalysation of DA-PDA conversion can improve the detection in rate of reaction and the subsequently enabled site-specific PDA deposition in the vicinity of exosomes, which can significantly enhance the sensitivity by locally attracting and capturing a larger number of coloured reporting molecules, PDA. The developed aptamer-based colorimetric biosensor for exosome detection (ExoAptsSensor) was further employed for the detection of exosomes derived from breast and pancreatic cancer cells.

2. Materials and methods

2.1 Reagents and Materials

Exosomes were isolated from cell culture media. Please refer to supporting information for details. Anti CD63, HER2, integrin αvβ6 aptamers and a scrambled CD63 aptamer conjugated to Biotin on 3' end were acquired from Eurogentec. The sequences are as below. CD63 aptamer: 5'/CAC-CCC-ACC-TCG-CTC-CCG-TGA-CAC-TAA-TGC-TA/3'. HER2 aptamer: 5'/GGG-CCG-TCG-AAC-ACG-AGC-ATG-GTG-CGT-GGA-CCT-AGG-ATG-ACC-TGA-GTA-CTG-TCC/3'.
Integrin αvβ6 aptamer: 5'/CAC-ACA-TTC-CCG-TCC-TCG-ATA-AGT-CTA-GGC-TTA-GTG-CCA-CTT-ACT-TAA-TC/3'. Scrambled CD63 aptamer: 5'/ACT-CTT-CAC-CCG-CAC-ATT-CGC-ATC-GCC-CAA-GC/3'.

2.2 Development of ExoAptaSensor for the detection of exosomal common marker CD63

Briefly, 40 µL of exosome stock in PBS was incubated with 10 µL of latex beads (aldehyde/sulphate latex beads, 4% w/v, 4 µm, Sigma-Aldrich) for 15 min at room temperature (RT). Then 5 µl of 100 µM BSA was added to the exosome-beads mixture and incubated for 15 min at RT. Sterile PBS was added to top up to 1 mL for incubation for 60 min with mild agitation. After pellet the beads with centrifugation for 5 min at 580 g, the supernatant was removed and 1 mL of 100 mM glycine in PBS was added, followed by incubation for 30 min at RT. Similarly, the pellet was centrifuged for 5 min at 580 g. Biotinylated aptamer to CD63 (0.1 µM in 3% BSA/PBS, 200 µL) was added and incubated for 2 h at RT. Afterwards, the mixture was washed with 1 mL of PBS and incubated for 30 min with 100 µL of streptavidin-HRP conjugate prepared using VECTASTAIN® Elite® ABC HRP Kit (Vector Laboratories, UK) according to supplier's protocol. DA solution was freshly prepared by rapidly dissolving 10 mg of DA hydrochloride powder (Sigma-Aldrich, UK) in 2 mL of tris buffer (10 mM, pH 8.5), followed by quick addition of 40 µL of H_2O_2 (1 M). DA solution (100 µL) was added to the pellet after centrifugation for colour development for 10 min. The final colour of the samples can be directly observed by naked eye. To obtain the absorbance spectra, 100 µL of the sample was sampled to a clear-bottomed 96-well plate (Costar, USA) and scanned from 300 nm to 600 nm in a Microplate spectrophotometer (FLUOstar Omega, BMG LABTECH, UK). Absorbance values were recorded at 400 nm. The HER2 and integrin αvβ6 ExoAptaSensors were developed accordingly by replacing the aptamer against CD63 to HER2 and integrin αvβ6, respectively. The methodologies for conventional membrane-based assay (Dot-blot 1.0), enhanced membrane-based assay (Dot-blot 2.0) and bead-based exosome biosensor using antibody (ExoAbSensor) are described in supporting materials.

2.3 Assessment of the analytical performance of ExoAptaSensor

2.3.1 Sensitivity, limit of detection (LOD)

A variety of PANC-1 exosome solutions was made from stock (7.77×10^{12} particle/mL) by a ten-fold serial dilution and tested using the developed ExoAptaSensor using CD63 aptamer (CD63 ExoAptaSensor) as described. The absorbance signal was plotted against the log value of exosome concentration to establish the calibration curve. The LOD was determined by blank signal plus 3

standard deviations of the blank, which represents sample tested the same but without the addition of PANC-1 exosomes.

2.3.2 Specificity

The specificity of ExoAptaSensor was tested by targeting exosomes with specific marker CD63 against other samples including negative controls such as positively charged PEGylated liposome, neutral PEGylated liposome, neutral liposome, etc. Each type of sample was tested across range of concentrations between 10^4-10^{12} particle/mL. ExoAptaSensor developed by scrambled CD63 aptamer was also used for specificity test against a range of exosome concentrations in the range of 10^4-10^{12} particle/mL and the signal responses were recorded.

2.3.3 Accuracy

The CD63 expression was detected using the CD63 ExoAptaSensor for PANC-1, B16-F10, HEK-293 and P0403 exosomes at a concentration of 1×10^{11} particle/mL. In parallel, the CD63 on exosomes derived from these cell lines were also detected by bead-based flow cytometry assay according to our previous protocol (Faruqu et al., 2018). The results of CD63 expression obtained from flow cytometry assay were compared to that from ExoAptaSensor as a demonstration of the accuracy and reliability of the developed aptasensor.

2.4 Detection of cancer-derived exosomes using the ExoAptaSensor

The expression of HER2 was studied in $HER2^+$ breast cancer cell (HCC1954)-derived exosomes using a similar protocol described in section 2.2 by replacing the CD63 aptamer with HER2 aptamer. For specificity assessment, the developed HER2 ExoAptaSensor was used to detect other exosomes (as negative control) isolated from P0403, HEK-293, A549, HCT116 cell culture media, and FBS and healthy human plasma at a concentration of E10 exosome particle/mL. The calibration curve of HER2 ExoAptaSensor was established by measuring a serial concentration of $HER2^+$ exosomes derived from HCC1954. Similarly, the integrin αvβ6 expression on exosomes isolated from a PC cell line (P0403) was studied first and the specificity of integrin αvβ6 ExpAptaSensor was tested against other exosomes (as negative control) isolated from PANC-1, HEK-293, B16-F10, A549, HCT116 and G7 cell culture media, and FBS and healthy human

plasma in a concentration of E10 particle/mL. The calibration curve of integrin αvβ6 ExpAptaSensor was established by measuring a serial concentration of $\alpha v\beta 6^{+}$ exosomes derived from P0403.

3. Results

3.1 The principle of the ExoAptaSensor for exosome detection

An aptasensor was developed for exosome detection (ExoAptaSensor). Figure 1A shows the schematic illustration of the proposed bead-based aptasensor with HRP accelerated DA polymerization and local PDA deposition for sensitive exosome detection. In brief, aldehyde latex microbeads are used to anchor exosomes by aldimine condensation. Surface markers of exosomes are captured by a CD63 specific aptamer, which is hypothesised to be more sensitive in detecting exosomes than the full IgG antibody. Streptavidin conjugated HRP is introduced (due to streptavidin's ability to strongly bind to biotin linker on aptamer with high affinity) followed by rapid addition of freshly prepared DA solution (colourless). HRP accelerated the colorimetric reaction leading to coloured product PDA after polymerisation. This product PDA is further deposited rapidly to the surface of target exosome in close proximity, due to outstanding reactivity of PDA to the amine, sulfhydryl and phenol groups of exosomal surface proteins (Figure 1B) (Lee et al., 2009), (Tokura et al., 2018), (Li et al., 2017). The generated colour in the substrate solution is quantifiable through absorbance (quantitatively) or visualisable by naked eye (semi-quantitatively or qualitatively) in a simple way. The absorbance signal recorded is correlated to the expression level of CD63 marker or specific disease biomarker on target exosomes. Therefore, this principle lays the foundation for the proposed ExoAptaSensor to be used for sensitive and specific detection of exosomal biomarkers in diseases diagnosis.

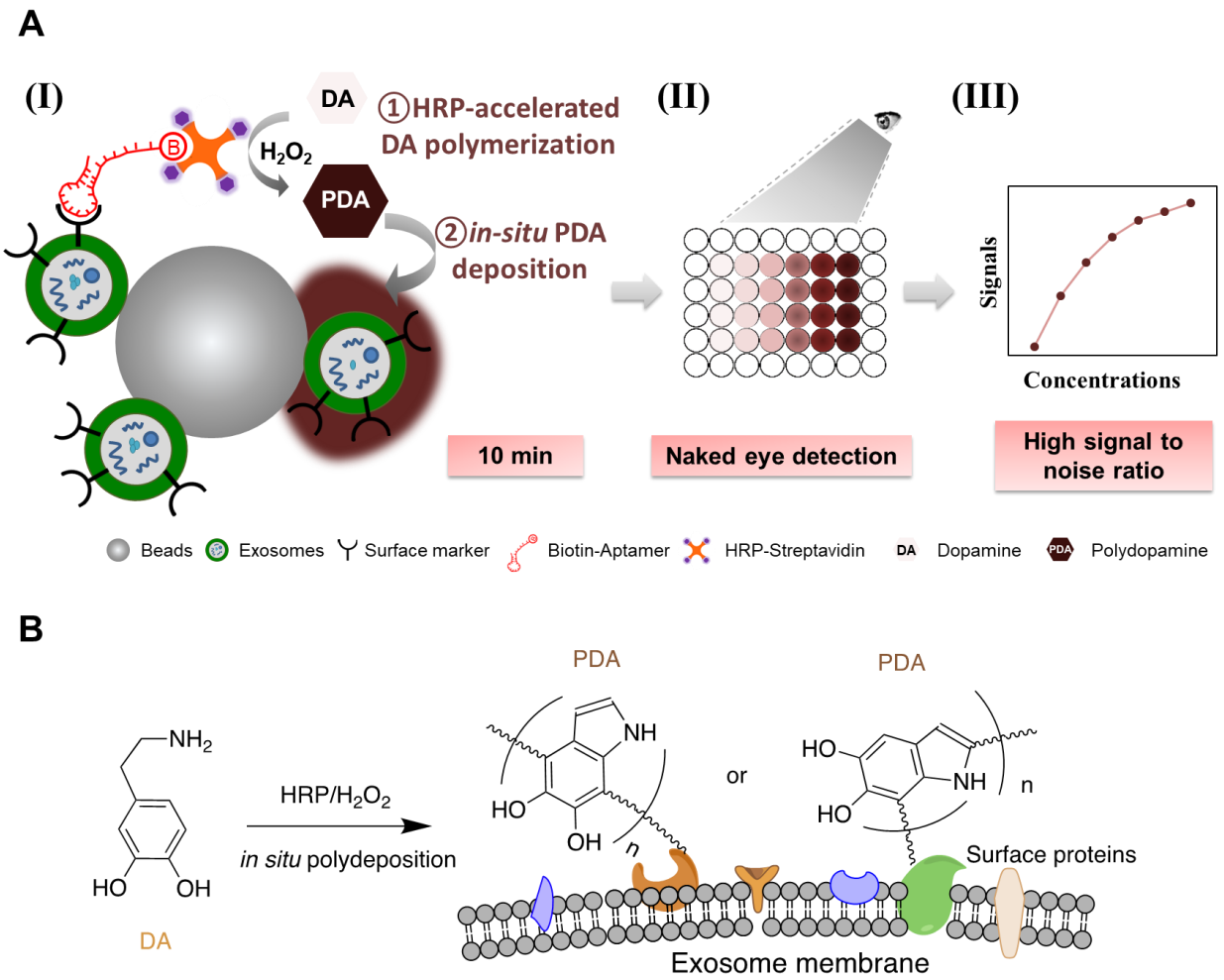

Figure 1. Schematic illustration of the proposed aptasensor with HRP accelerated dopamine polymerization and deposition for exosome detection (ExoAptaSensor). (A) (I) Exosomes anchored on sulphate/latex beads were captured by biotin conjugated aptamer specific to CD63, followed by incubation with streptavidin conjugated HRP for colorimetric reaction to convert colourless Dopamine (DA) into brown-black coloured Polydopamine (PDA) in 10 min in Tris buffer (pH 8.5). This oxidisation and polymerisation process are accelerated under HRP catalysis and hydrogen peroxide (H_2O_2) as the oxidant. (II) Colour developed by this aptasensor corelates to exosome concentrations and allows simple naked eye visualisation. (III) Absorbance signals of the product can also be quantified at 400 nm. (B) Scheme shows the poly-deposition process of PDA onto surface proteins of exosomes *in situ*.

Proof-of-concept study was first conducted. The HRP mediated colour reaction to catalyse DA to PDA with the presence of hydrogen peroxide (H_2O_2), the oxidant, was optimised by inclusion of different percentage strengths in DA solution and the colour change of products PDA deposited on nitrocellulose membrane monitored across a set time frame. Images of PDA deposition at the spot of HRP enzyme (Figure 2A) suggests that the colour intensity increases with time but seems not to further increase after a certain period of time for all H_2O_2 concentrations. This was

confirmed by quantified colour intensity curve against time that colour shows saturation at around 5 min for 1% and 2% H_2O_2 concentrations, around 10 min for 0.5%, and around 30 min for 0.2% (Figure 2B). The colour intensity of 0.5% peaked at 10 min after DA addition (Figure S1). The colour intensity increases first with increasing H_2O_2 concentration but decreases if the concentration of H_2O_2 is higher than 1%. This means H_2O_2 concentration affects the colour development. Figure 2A-B also suggested that 0.5% resulted in the highest PDA colour intensity, thus was established as an optimal H_2O_2 concentration.

The concept of this ExoAptaSensor was proved by detecting CD63 on PANC-1 derived exosomes at concentration orders of 0 (blank), 10^3, 10^6, 10^8, and 10^{10} particles. The size, surface tetraspanins, and morphology of PANC-1 exosomes were characterised (Figure S2) for showing the properties of tested exosomes. Exosome samples with four different concentrations were detected using the proposed CD63 ExoAptaSensor. The developed colour in the sample solution was directly visualised by naked eye in 10 min, as shown in the digital images taken right after the colorimetric reaction (Figure 2C). Blank control resulted in minimal signal (almost transparent). This demonstrates the ability of this aptasensor for exosome detection in a simple and qualitative way. These tested samples were further scanned for their absorbance spectra at wavelengths from 300 to 600 nm. It was found that the peak value at around 400 nm increases with the increase in exosome concentrations (Figure 2D). This quantitative data reflects the fact that the proposed colorimetric aptasensor is capable of detecting exosomes by targeting exosomal surface markers (CD63 in this case). This data proved the concept of this colorimetric aptasensor and hence paved the way for the detection of cancer derived exosomes using the ExoAptaSenor.

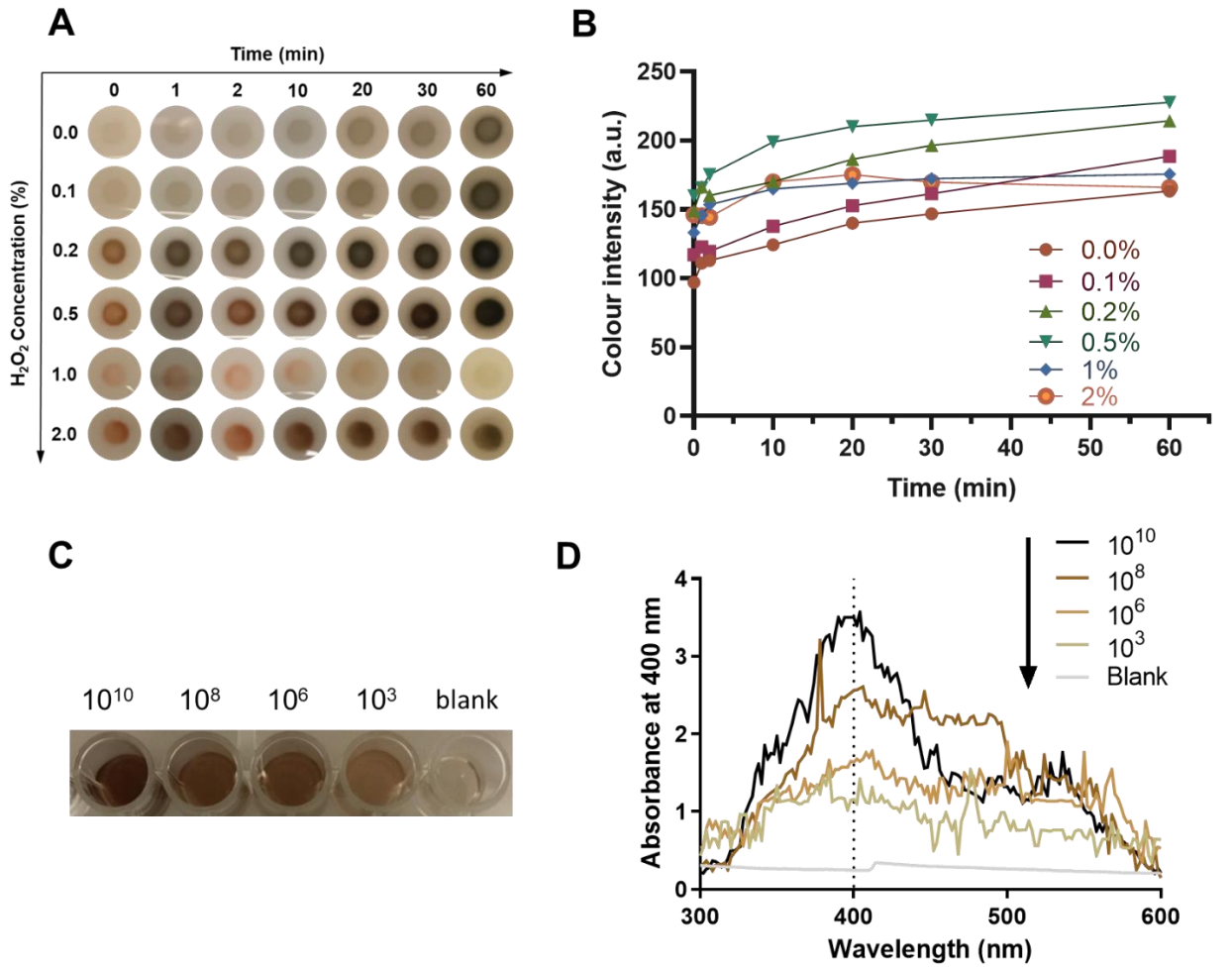

Figure 2. Proof-of-concept study of ExoAptaSensor. (A) Colour intensity of PDA deposition at target site of HRP where concentration of H_20_2 varied from 0% to 2%. (B) Colour intensity measured at intervals of 0, 1, 2, 10, 20, 30 and 60 min using ImageJ. (C) Digital images taken right after colour development of exosome samples in solution using the CD63 ExoAptaSensor. Colour intensity was dependent on exosome concentrations (10^{10}, 10^8, 10^6, 10^3, and 0 (blank) particles, respectively). (D) Absorbance spectra were obtained at wavelengths from 300 nm to 600 nm, with peak at 400 nm corelates with the changes in exosome concentrations. This supports the ability of the developed colorimetric aptasensor for exosome detection.

3.2 Evaluation of the sensitivity, specificity, and accuracy of the ExoAptaSensor

The performance of ExoAptaSensor including sensitivity, specificity and accuracy was assessed for the detection of exosomal surface marker CD63. A serial of concentrations of PANC-1 exosomes varying from 10^3-10^{11} (order of magnitude) particle/mL were tested. The colour intensity was calibrated at peak absorbance by a microplate spectrophotometer. The calibration curve (y=0.26x + 0.83, R^2=0.92) established by plotting the colour signal against the log value of exosome concentrations showed good liner relationships (Figure 3A). Colour intensity increased

with increasing exosome concentration, as shown in Figure 3B. The LOD of the developed ExoAptaSensor was calculated to be 7.77×10^3 particle/mL, which indicates the outstanding sensitivity of this ExoAptaSensor.

To check the specificity of exosome detection, control experiments were performed using non CD63 expressing nanoparticles possessed the same size as exosomes and had similar lipid compositions (liposomes with varied surface charges). Low signals (close to background) were detected for all nanoparticles under all testing concentrations (Figure 3C, Figure S3). The aptasensor based on scrambled aptamer against CD63 (same nucleotide composition but different sequence) showed minimal signal (Figure 3D). Therefore, the minimal interference observed from similar lipid nanoparticles and scramble aptasensor suggests the great specificity of the developed aptasensor for exosomes.

To evaluate the accuracy of this assay as a detection method, results of CD63 expression performed on different exosome samples from the proposed ExoAptaSensor were compared to those of an established conventional antibody-based Bead-Exo flow cytometry assay (not DA-based), according to an established protocol by our group (Faruqu et al., 2018),(Faruqu et al., 2019). Three other exosome types of varying degrees of CD63 expression were measured, respectively (Figure 3E&F). Both techniques showed the same profile of CD63 expression, whereby PANC-1 exosomes gave the strongest reading, followed by P0403, B16-F10 and HEK-293 exosomes. The equivalent results gained from the in-house developed biosensing approach confirmed the accuracy of this assay by matching it to an established method. This consistency between the data sets also demonstrated the reliability of ExoAptaSensor in determining the expression level of surface markers on exosomes.

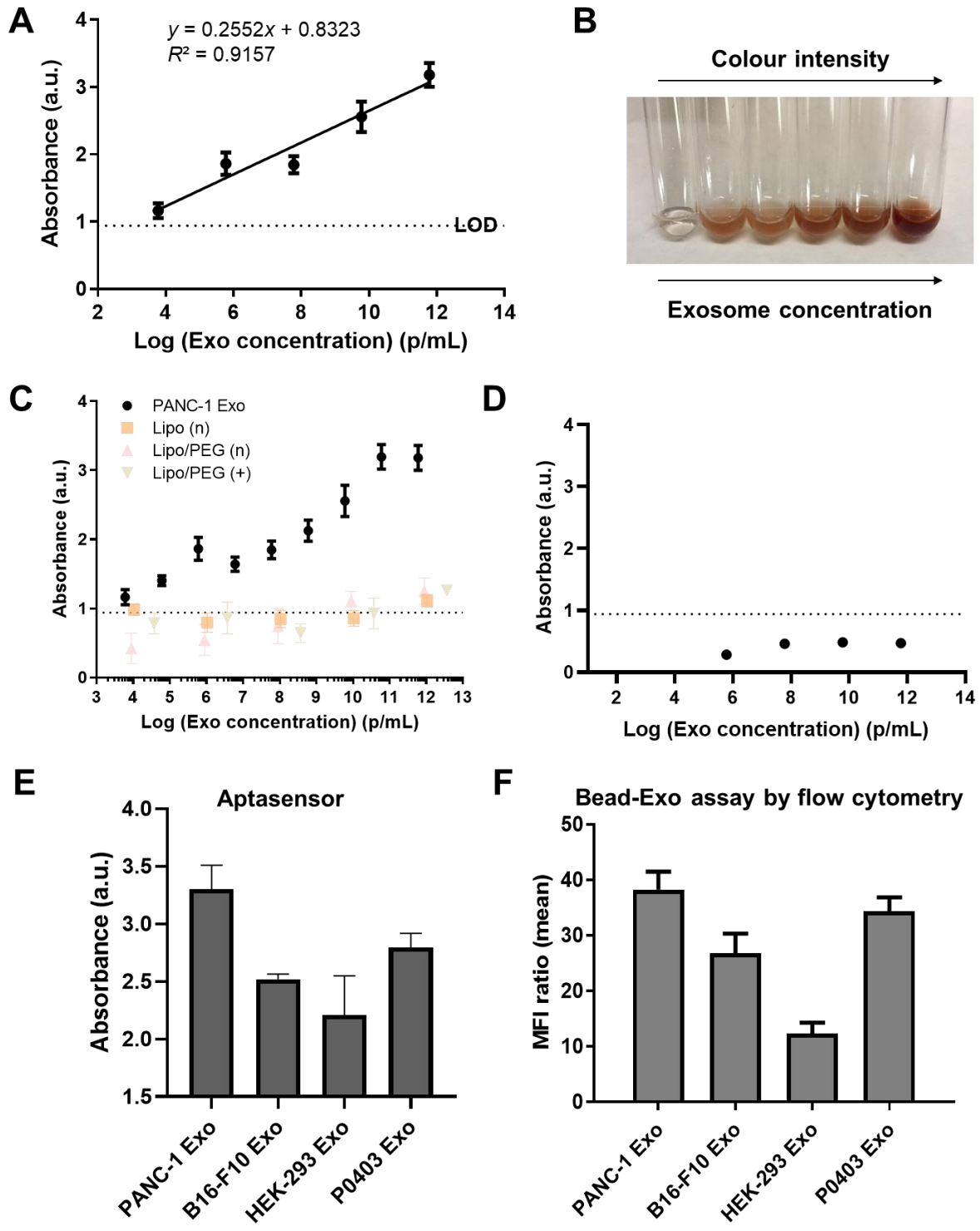

Figure 3. Performance assessment of ExoAptaSensor for the detection of exosomes. (A) Calibration curve of the aptasensor for PANC-1 exosomes. The limit of detection (LOD, dashed line) was established at 7.77×10^3 particle/mL. Values are expressed as mean ± SD (n=3). $y = 0.2552x + 0.8323$, $R^2 = 0.9157$. (B) This colour intensity increases with the exosome concentration for the samples corresponding to (A). The first sample in the left is reaction solution only as blank control. (C-D) Specificity assessment. (C) The CD63 ExoAptaSensor for sensing various targets including PANC-1 exosomes, exosome-like PEGylated

liposomes (+), PEGylated liposomes (neutral), and non-PEGylated liposomes (neutral). Values are expressed as mean ± SD (n = 3). (D) Detection results of ExoAptaSensor built on scrambled aptamer against CD63 for PANC-1 exosome at concentrations of 6E5, 6E7, 6E9, and 6E11 particle/mL. (E-F) Reliability/accuracy of CD63 ExoAptaSensor (E) for exosome analysis in comparison to established bead-flow cytometry assay (F) in terms of CD63 marker expression level on PANC-1, B16-F10 and HEK-293 exosomes at same concentration (1×10^{11} particle/mL), respectively. Values are expressed as mean ± SD (n=3).

3.3 Comparison of ExoAptaSensor with ExoAbSensor, Dot-blot 2.0, and Dot-blot 1.0

The sensitivity of ExoAptaSensor (CD63) was compared to conventional membrane-based dot blot assay (Dot-blot 1.0), enhanced membrane-based dot blot assay by HRP accelerated deposition of PDA (Dot-blot 2.0), and bead-based Exo biosensor using antibody as bio-recognition element (ExoAbSensor), for the detection of CD63 on exosomes (Figure 4). Dot-blot 1.0: exosomes on a nitrocellulose membrane is recognised by primary antibody (mouse anti human CD63 IgG), which is then captured by HRP conjugated secondary antibody (goat anti mouse IgG-HRP). Substrate from the Enhanced chemiluminescence (ECL) kit is added to allow visualisation of dot colour under gel imaging system. Dot-blot 2.0: Substrate is replaced with DA dissolved in Tris buffer on the basis of Dot-blot 1.0, to allow DA-PDA oxidisation and polymerisation accelerated under HRP+H_2O_2 catalysis. ExoAbSensor basically has the same design as the ExoAptaSensor but the only difference is to use anti-CD63 as the bio-recognition element, which is conjugated to HRP for colorimetric reaction in the solution. Dot-blot assays are carried out in solid basis—membrane, and ExoAbSensor and ExoAptSensor are carried out in aqueous solution environment. A serial dilution of PANC-1 exosome samples were tested using the four methods in parallel. It was concluded that the final LOD for exosome detection using the DA-PDA/anti-CD63 based Dot-blot 2.0 assay was 7.77×10^8 particle/mL with optimal H_2O_2 concentration of 0.5% and 10-min reaction time. This was a 2-log increase in the sensitivity in comparison to the commercial ECL substrate-, anti CD63-, membrane- based Dot-blot 1.0 assay. This result also suggested that the optimisation of the colorimetric substrate resulted in a more rapid and cheaper assay (Dot-blot 2.0) by utilising less reagent and consequently giving a more economical and efficient process. Moreover, the as-prepared ExoAbSensor could further increase the sensitivity to another two orders of magnitude to be ~10^6 particle/mL by conducting the exosomal CD63 detection in "solution format" rather than membrane based "solid substrate" as Dot-blot 2.0 demonstrated. In comparison, the LOD of ExoAptaSensor was calculated to be 7.77×10^3 particle/mL, significantly improving the sensitivity

further by 1000 times. The data indicated that the CD63 aptamer is more sensitive than CD63 antibody in this application and the current format of ExoAptaSensor was superior to ExoAbSensor, Dot-blots 2.0 and 1.0.

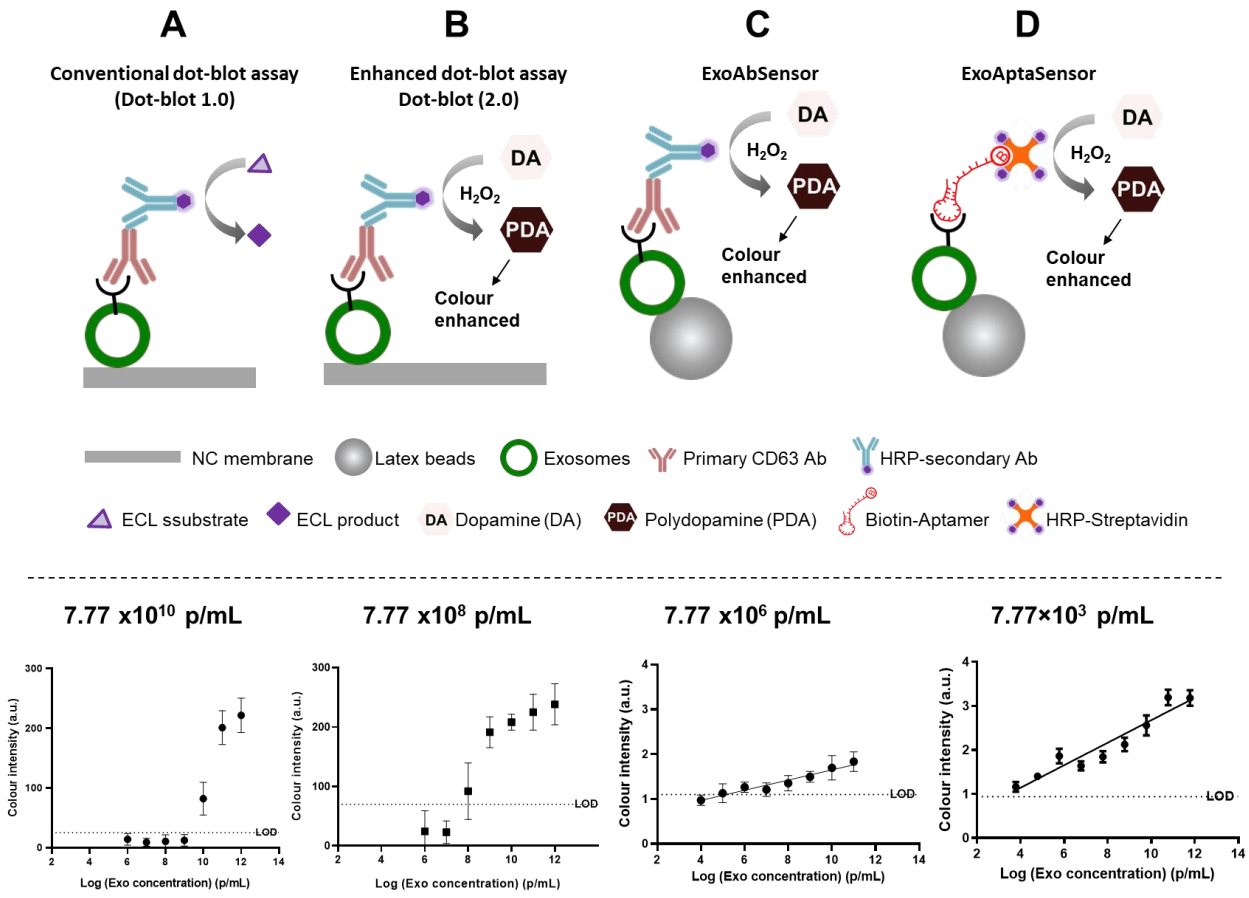

Figure 4. Comparison of ExoAptaSensor to the antibody-based exosome biosensor (ExoAbSensor) and two types of membrane-based dot-blot assays (Dot blot 2.0 and Dot blot 1.0). (A) Conventional dot-blot assay (Dot-blot 1.0) for PANC-1 exosome detection. PANC-1 exosomes (10 μL) sampled on a nitrocellulose membrane was recognised by primary mouse anti human CD63. HRP conjugated secondary antibody (goat anti mouse IgG) was then added. Substrate from the Enhanced chemiluminescence (ECL) kit was added to allow visualisation under gel imaging system. (B) Enhanced dot-blot by HRP accelerated Dopamine (DA) polymerization and deposition (Dot-blot 2.0) for PANC-1 exosome detection. Substrate is replaced with DA dissolved in Tris buffer (pH 8). DA (colourless) is slowly oxidised in oxygen and polymerises to form Polydopamine (PDA, brown-black). This process is accelerated under HRP catalysis and hydrogen peroxide (H_2O_2) as the oxidant. (C) ExoAbSensor in similar design as ExoAptaSensor but only difference is to use antibody against CD63 as the bio-recognition element. (D) The scheme of ExoAptaSensor.

3.4 Analysis of cancer-derived exosomes using ExoAptaSensor

HER2 has been reported to be expressed on breast cancer derived exosomes. For the demonstration of the ability in detecting cancer biomarkers, the HER2 expression level on cancerous exosomes was determined by HER2 ExoAptaSensor, in which HER2 aptamer was used as bio-recognition element instead of CD63 aptamer for this biosensor development. First, HER2 expression was verified on breast cancer cell (HCC1954) derived exosomes using flow cytometry (Figure 5A). The HER2 ExoAptaSensor was found to be specific against HER2 expressing HCC1954 exosomes and the exosomes derived from PC cell line (P0403), epithelial cell line (HEK-293), lung cancer cell line (A549), colorectal cancer cell line (HCT116), fetal bovine serum (FBS), and healthy human plasma caused minimal inference (Figure 5B). This data agrees with reported HER2 expression level in P0403 (no), HEK-293 (minimal), A549 (low), HCT116 (low) cell line, and FBS (no) (LaBonte et al., 2011), (Hosseinzadeh Colagar et al., 2013), (Bunn et al., 2001), (LaBonte et al., 2011), (Barok et al., 2018). The calibration curve for HER2 detection was established by testing a range of $HER2^+$ exosomes (y=0.2094x + 0.5857, R^2=0.97948) (Figure 5C). The LOD was found to be at in the order of 10^{4-5} particle/mL. The calibration curve could be used to predict the number of $HER2^+$ exosomes in a sample for cancer detection.

Since the current format of the developed ExpAptaSensor can detect breast-cancer derived exosomes based on known/validated exosomal biomarker--HER2, this biosensor was furthered used to detect PC by analysing PC-derived exosomes. As is known, there is a lack of valid exosome biomarker for PC diagnosis. Herein, the exosomal integrin αvβ6 is hypothesized to be a new potential diagnostic target for PC. First, the integrin αvβ6 expression was confirmed on PC cell (P0403) derived exosomes by flow cytometry (Figure 5D). The integrin αvβ6 ExoAptaSensor was fabricated by using the specific aptamer against integrin αvβ6. The specificity was confirmed by the fact that this biosensor only responds significantly to $\alpha v\beta 6^+$ P0403-derived exosomes, rather than a range of exosomes derived from PANC-1, HEK-293, B16-F10, A549, HCT116, G7, FBS, and healthy human plasma (Figure 5E), which are expected to be negative (Bi et al., 2019), (Fedele et al., 2015). Figure 5F shows the calibration curve for exosomal αvβ6 detection (y=0.2027x + 0.4338, R^2=0.9592) and the LOD was around the order of 10^{4-5} particle/mL. This assay validated the integrin αvβ6 aptamer and this aptasensor method can be employed in future for the detection of integrin $\alpha v\beta 6^+$ exosomes from cancer patients with PC. The data laid foundation for the clinical

HER2 or integrin αvβ6 testing in patients derived cancerous exosomes, with a possibility to be extended to detection other exosomal biomarkers.

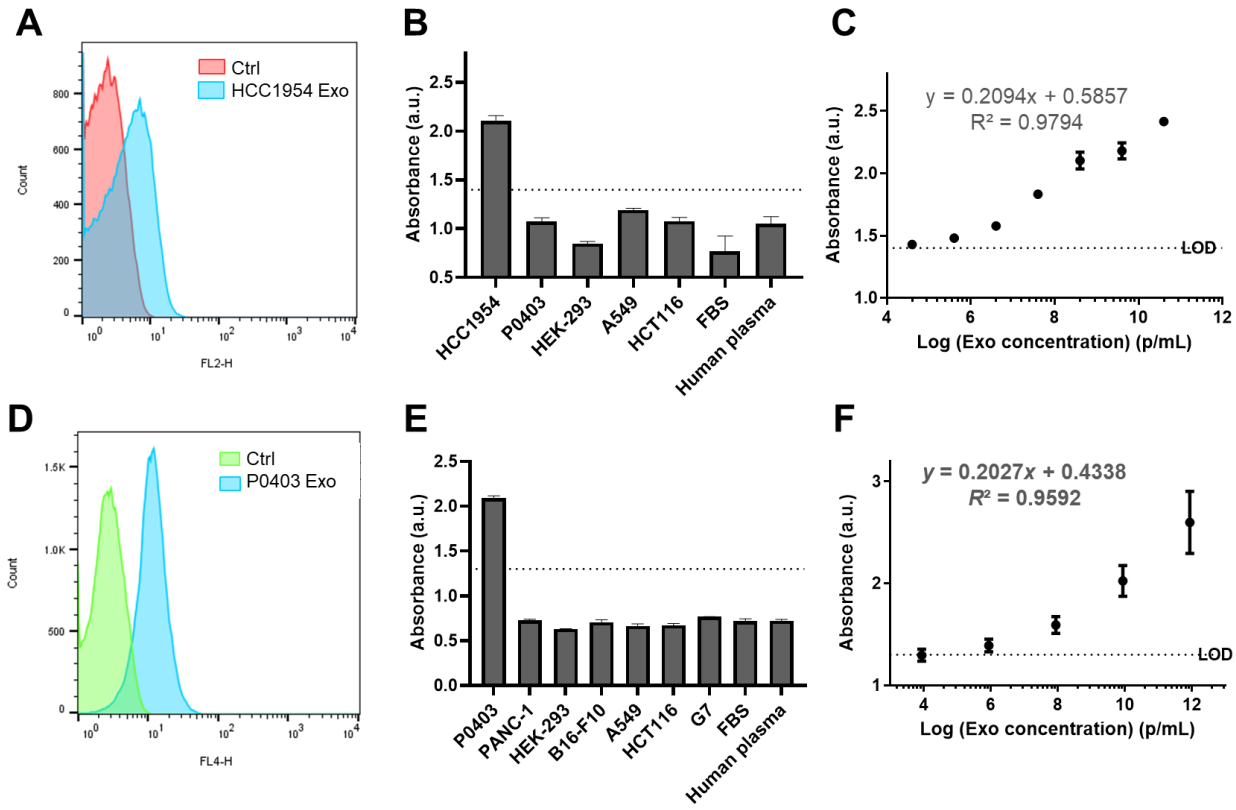

Figure 5. Exosomal biomarker analysis by the ExoAptaSensor. (A-C) Colorimetric HER2 ExoAptaSensor for the detection of $HER2^+$ exosomes. Aptamer against HER2 was used for bio-recognition. (A) HER2 expression was confirmed on breast cancer cell (HCC1954) derived exosomes by flow cytometry. (B) Specificity test of HER2 ExoAptaSensor for exosomes isolated from HCC1954, P0403, HEK-293, A549, HCT116 cell culture media, and FBS and healthy human plasma. Each sample contained E10 exosome particle/mL. (C) Calibration results for HCC1954 exosome detection. (D-F) Colorimetric integrin αvβ6 ExoAptaSensor for the detection of $αvβ6^+$ exosomes. Aptamer against integrin αvβ6 was used for bio-recognition. (D) αvβ6 expression was confirmed on PC cell (P0403) derived exosomes by flow cytometry. (E) Specificity test of αvβ6 ExoAptaSensor for exosomes isolated from P0403, PANC-1, HEK-293, B16-F10, A549, HCT116 and G7 cell culture media, and FBS and healthy human plasma. Each sample contained E10 particle/mL. (F) Calibration results for P0403 exosome detection.

4. Discussion

In this study, a new concept was proposed for exosome detection. We investigated the potential of a simple and cost-effective colorimetric biosensor for sensitive and specific detection of exosomes facilitated by the novel approach of HRP-accelerated DA polymerisation and *in situ*/local formation and deposition of PDA at the target site.

The innovation of this work is to achieve a highly sensitive, specific and reliable detection of cancer-derived exosomes using a simple colorimetric readout. The advantages of this aptasensor include the relatively simple and fast detection while achieving outstanding sensitivity in comparison to many other current approaches.

The detection limit of 7.77×10^3 particle/mL (~8 particle/μL), enhanced *via* HRP accelerated DA poly-deposition around exosomal surface, which makes the ExoAptaSensor one of the most sensitive biosensors for exosome detection reported in the literature (Théry et al., 2018), (Cheng et al., 2019), (Chia et al., 2017), (Xu et al., 2020) and other conventional methods such as the enzyme-linked immunosorbent assay (ELISA) and flow cytometry (Shao et al., 2018). Based on literature search, most current colorimetric biosensors for exosome detection needs gold nanoparticles (AuNPs) for colour reporting and the LODs of those are around 10^3-10^6 particle/μL (Xu et al., 2020). Hence in comparison, the aptasensor in this study is very competitive in terms of sensitivity and cost. Additionally, ExoAptaSensor offers simplicity advantages and substitution of complex instrument such as polymerase chain reaction (PCR) (Ramirez et al., 2018). Compared to electrochemical luminescence (ECL) only visible under large equipment such as Gel Doc systems, the ExoAptaSensor can be used in a solution format so the colour change can be easily quantified by measuring absorbance. Since only small volume in the microliter range is required to run the assay, the aptasensor is suitable to be adapted in a finger-prick based kit for future cancer screening. Many factors could contribute to the tremendous enhancement in sensitivity. Firstly, HRP accelerated DA polymerization and PDA driven self-deposition locally around the target exosomes enhanced two orders of magnetite in the sensitivity compared to ECL kits as regular HRP substrate (Dot-blot 2.0 vs Dot-blot 1.0), due to the speedy colorimetric catalysis and signal *in situ* amplification. Secondly, microbeads as the anchoring base for immobilising exosome particles advantage over nitrocellulose membrane, resulting in the improvement of sensitivity with 2 more orders of magnetite (ExoAbSensor vs Dot-blot 2.0). The liquid-based detection format allows more adequate interaction between exosomal surface antigens and enzyme conjugated bio-capture/recognition element (antibody) than membrane based solid detection format, resulting in increased capacity for the antibody-enzyme complexes as reporting system. Thirdly, the use of antibodies has been shunned in favour of aptamers which exhibit superior levels of specificity and

sensitivity to their target. In fact, aptamers have been reported to possess an intrinsic advantage regarding their sensitive recognition, excellent specificity and high affinity (Kim et al., 2014), (Tombelli et al., 2005), (Xu et al., 2015). Our data from the use of aptamers agrees with this. Therefore, these key steps made a big difference for the development of a robust biosensor for sensitive exosome detection especially when combined with the DA-PDA amplification approach (Scheme in Figure 1A).

The coloured product PDA produced by the enzymatic reaction may exist in two forms, one is anchored on exosome surface and the other is "free floating" in the solution. In this work, the two forms were not separated, as both resulted from the aptamer conjugated HRP catalysation and should be taken into account, to accurately quantify the expression level of the biomarker of interest. A termination step is essential to include especially when colour continues to change over the measurement time window. In this study, the absorbance plateaued after 10 min when optimal conditions were used. A termination step was therefore not employed in this assay to cut down on the number of steps and minimise interference from additional reagents. Although the final readout step requires only 10 min, the whole assay needs ~ 4h from sampling to final readout. This is still much quicker than Western blotting and flow cytometry for example. With all advantages in mind, it is envisaged that the aptasensor could successfully detect common exosomal marker CD63, as well as disease markers HER2 and integrin αvβ6. With all the above-mentioned advantages, the aptasensor can also be used in biomarker cancer discovery and early cancer detection.

We further explored the potential to cultivate this biosensor on a selective basis. Though the beads (aldehyde) covalently bind to exosomes (NH_2), it offers limited selectivity towards exosomes. The specificity of this ExoAptaSensor heavily rely on the specificity of aptamer. Hence, before implementing the measurement of exosomal biomarkers, the selectivity of the aptamer used was evaluated. Fortunately, the aptamers to CD63, HER2, and integrin αvβ6 involved in this study all shows excellent specificity to the targets, respectively. This definitely lays a solid foundation for the reliable and sensitive detection of the interesting markers on exosomes.

The role of integrins in cancer growth and metastasis spread has been reported in many publications (Hoshino A, Costa-Silva B, Shen TL, 2015), (Hamidi et al., 2016), (Bandyopadhyay

and Raghavan, 2009). Among the integrin family, integrin αvβ6 has been evident to express on exosomes. For example, the integrin αvβ6 is transferred intercellularly *via* exosomes for prostate cancer (Fedele et al., 2015). However, to the best of our knowledge, there is no report showing exosomal integrin αvβ6 to be diagnostic cancer biomarkers. This is the first study to hypothesise that integrin αvβ6 could be a potential exosomal biomarker for PC detection. Due to limit of clinical samples, the measurement of αvβ6 on exosomes in biofluids (e.g. blood, urine, saliva) from PC patients has not yet been conducted, but such studies are needed to validate the feasibility of the developed ExoAptaSensor for clinical use. The early detection of PC is extremely challenging at the moment, due to the obstacles both in the identification of novel PC makers and in the absence of robust analytical tools. Parallel progress in both is required to push the field of PC early detection closer to realisation. The proposed approach is the first to propose a new exosomal marker that could be feasible for PC diagnosis, the impact of which, if successful in clinical trial, would be remarkable.

5. Conclusions

In summary, a highly sensitive and selective colorimetric aptasensor was developed for the detection of cancer-derived exosomes based on HRP-accelerated, fast and localised polydopamine *in situ* deposition. The limit of detection is as low as 7.77×10^3 particle/mL for CD63-based exosomes detection. This aptasensor was used for accurate colorimetric quantification of HER2 and integrin αvβ6-expression exosomes derived from cell cultures. Future studies will focus on the validation of clinical patients' samples and adapting it into a point-of-care device for cancer early detection and diagnosis.

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Competing Interests

The authors have declared that no competing interest exists.

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