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Immobilization of biotinylated antibodies through streptavidin binding aptamer

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ABSTRACT

Liquid biopsy approaches are powerful strategies that potentially allow the diagnosis and prognosis of a number of diseases. The field is continuously and rapidly growing, encouraging the discovery of novel predictory biomarkers. Antibodies are usually exploited in sensors to validate biomarker candidates. Unfortunately, the immobilization of antibodies on the surface of sensors represents a challenging task. Immobilization strategies need to be optimized for each antibody, representing a huge obstacle to overcome in the discovery of new biomarkers. Herein we propose a novel strategy for the immobilization of antibodies, based on the use of a streptavidin-binding aptamer. Using this approach it is possible to immobilize antibodies on the surface of sensors with no need for optimization, with the only requirement for antibody to be biotinylated. The proposed strategy potentially paves the way towards a straightforward immobilization of antibodies on biosensors, making their use in biomarker validation more accessible.

1. Introduction

Starting from the introduction of the microarray concept by R. P. Ekins in 1989 [1], who first developed a multi-analyte immunoassay, the idea of miniaturizing thousands of assays on a single support has been widely applied. Firstly, DNA microarrays were developed to monitor the expression of multiple genes, then, to gather information regarding protein expression, functions, and interactions, protein microarrays came to light [2].

Unfortunately, the immobilization of multiple proteins on a sensor poses serious challenges. Proteins are a family of biomolecules with specific characteristics, as exemplified by the different behavior of soluble and transmembrane proteins. Each protein possesses a peculiar structure that needs to be maintained after immobilization on a solid surface to preserve its biological activity [3].

The ideal surface of a protein microarray substrate allows (i) preserving probe structure, (ii) optimizing its exposure towards the solution which contains the analyte, and possibly (iii) providing orientation (i.e., keeping the regions of the proteins responsible for the biological response away from the surface) [4].

These requirements also apply to immunoassays, which are biological assays where the probes printed on the sensor surface are antibodies [5]. Historically, antibodies are immobilized on the surface of microarrays by physical adsorption or chemical immobilization. While physical adsorption is the easiest way to immobilize proteins, it results in poor reproducibility, partly due to the detachment of antibodies from the surface. Chemical immobilization instead exploits the reactivity of amino acids side chains (e.g., amine groups of lysine or thiol group of cysteine), resulting in irreversible immobilization. Unfortunately, both these techniques may cause damage to antibodies by altering their structure due to multiple anchoring points on the surface [4].

A possible loophole has been found in exploiting bioorthogonal reactions to immobilize proteins. In this case, chemical groups that are not present in proteins can be introduced by means of chemical modification or by molecular engineering, thus producing recombinant proteins with specific chemical handles or tags. Despite the effectiveness of these approaches, they cannot be applied to all proteins. Moreover, different modification protocols should be optimized for each protein to obtain optimal immobilization [6].

In previous work, our group exploited the DNA-directed immobilization (DDI) of proteins to convert DNA microarrays (which are easier to produce and possess improved stability) into antibody microarrays [7].

Abbreviations: BSA, Bovine serum albumin; DDI, DNA-directed immobilization; EV, Extracellular vesicles; PBS, Phosphate buffer saline; PBS-M, PBS added with 2 mM $MgCl_2$.

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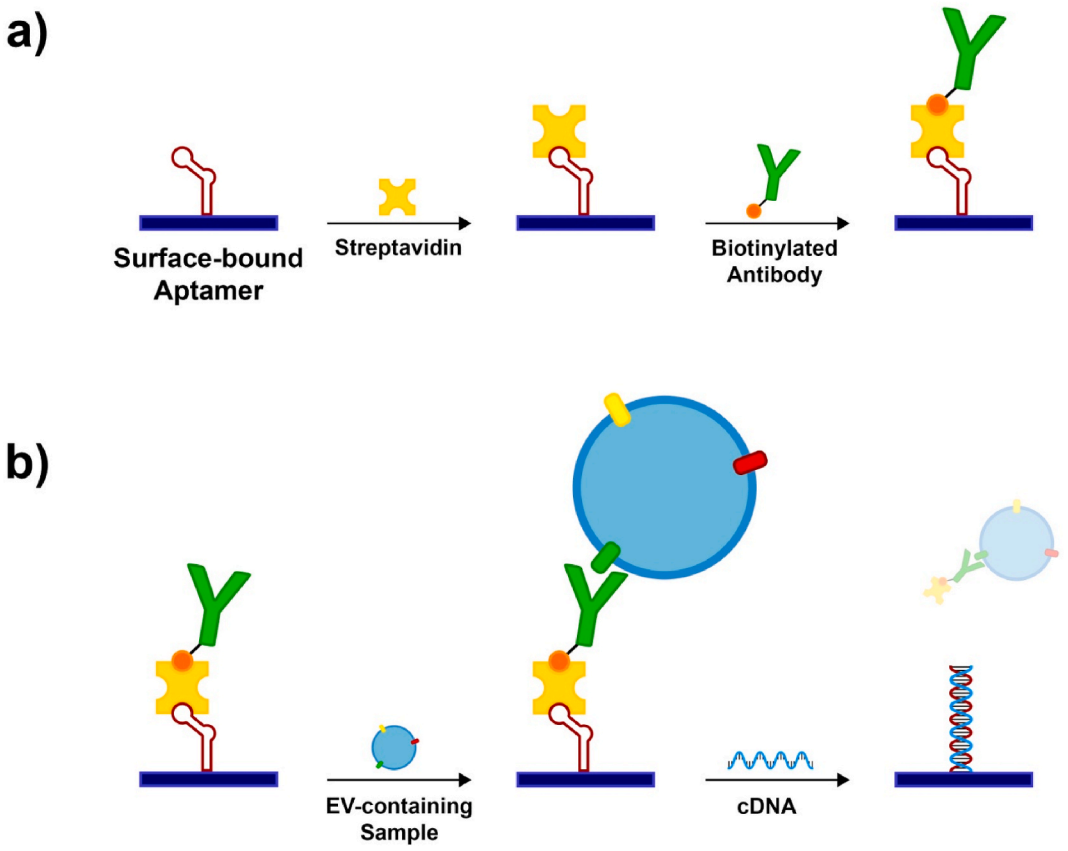


Fig. 1. Schematic representation of StrepApt5-mediated immobilization of antibodies on solid surfaces (a) and its use in EVs capture and release experiment using cDNA to promote detachment from the surface (b).

Unfortunately, the DDI approach requires antibody modification with DNA tails. Despite the existence of easily applicable protocols and commercial kits, the requirement for the synthesis of DNA-protein conjugates still discourages many groups from using DDI. Additionally, not all antibodies available on the market are suitable for modification with DNA (e.g., antibodies requiring gelatin or BSA in their formulation).

In the present work, we describe a simple and robust approach for immobilizing antibodies on microarrays (see Fig. 1). It exploits the use of StrepApt5, a DNA aptamer that binds streptavidin [8]. The aptamer is immobilized on the solid surface and can be used to capture on the sensor streptavidin and biotinylated antibodies sequentially. The result is the immobilization of antibodies on the surface of a DNA microarray, with the only requirement for the antibody to be biotinylated (which represents the most common modification available on the market).

We exploited the aptamer-mediated immobilization of the antibody to generate a microarray surface able to bind extracellular vesicles (EVs) [9]. Capturing intact EVs is made difficult by the large size of these nanoparticles. However, the optimal exposure of antibodies on the surface, and a certain degree of flexibility of the probe has a remarkable impact in providing a stable immobilization. Antibodies immobilized via aptamer were demonstrated to fulfill these requirements. Additionally, the incubation with the sequence of DNA complementary to the aptamer allows the release of streptavidin (together with antibody and captured EV, see Fig. 1b) from the surface potentially enabling further analysis and/or the regeneration of the chip.

2. Material and methods

2.1. Materials

Ammonium sulfate ($(NH_4)_2SO_4$), phosphate buffer saline tablets (PBS), Trizma base, 37% chloric acid (HCl), sodium phosphate (Na_3PO_4), sucrose monolaurate, sodium chloride (NaCl), ethanolamine, trehalose dehydrate, magnesium chloride ($MgCl_2$), NHS-PEG_4-biotin, Amicon Ultra 100MWCO centrifugal filters, streptavidin and polyclonal rabbit IgG were purchased from Sigma Aldrich (St. Louis, MO, USA). Mouse anti-human CD9 IgG (clone MEM-61) and biotinylated mouse anti-human CD9 IgG (clone MEM-61) were a kind gift of Hansa BioMed Life Sciences Ltd (Tallinn, Estonia). Cy3-labeled Fab_2 fragment rabbit antimouse IgG and Cy3-labeled Fab fragment goat antihuman IgG were purchased from Jackson ImmunoResearch (Baltimore, PA, USA). Oligonucleotides were synthesized by MWG-Biotech AG (Ebevsberg, Germany). All oligonucleotide were modified in 5' position with a C6 amino-linker. Oligonucleotides were freeze-dried and resuspended in de-ionized water (DI water) at a final concentration of 100 μM before use. StrepApt5 underwent a folding procedure before immobilization on solid supports: aptamer solutions were heated at 95 °C for 8 min, transferred into ice at −20 °C for 10 min and finally left at room temperature for 15 min.

Untreated silicon chips with 100 nm thermal grown oxide (14 × 14 mm) were supplied by SVM, Silicon Valley Microelectronics Inc. (Santa Clara, CA, USA). NV10B silicon chips were supplied by NanoView Biosciences (Boston, MA, USA). Both chips were pretreated using a HARRICK Plasma Cleaner, PDC-002 (Ithaca, NY, USA), connected to an oxygen line. MCP-2 and MCP-6 copolmers were purchased from Lucidant Polymers Inc. (Sunnyvale, CA, USA). Spotting is performed using SciFLEXARRAYER S12 (Scienion, Berlin, Germany). Fluorescence

images were obtained using the ScanArray Lite confocal laser scanner and analyzed using ScanArray Express software (PerkinElmer, MA, USA). Interferometric and fluorescence analyses of EVs were performed exploiting SP-IRIS (Single Particle Interferometric Reflectance Imaging Sensor) technique using ExoView™ R100 for image acquisition and nanoViewer 2.6.0 software for analysis (NanoView Biosciences Inc., MA, USA). This instrument measures the number of single particles (ranging from 50 to 200 nm in diameter) captured on the chip surface as well as their size distribution.

2.1.1. Oligonucleotide sequences

- *StrepApt5: 5′-GGGAACGCACCGATCGCAGGTTTCCC-3′*
- *20A-StrepApt5:*

5′-AAAAAAAAAAAAAAAAAAAAGGGAACGCACCGATCG-CAGGTTTCCC-3′

- *cDNA: 5′-ACCTGCGATCGGTGCGTTCCC-3′*
- *COCU8: 5′-GCCCACCTATAAGGTAAAAGTGA-3′*
- *COCU11: 5′-TCACTTTTACCTTATAGGTGGGC-3′*

2.2. General preparation of microarray supports

Different silicon supports were used in various experiments. Silicon chips (14 × 14 mm) layered with 100 nm of thermal grown silicon dioxide were used in experiments requiring fluorescent detection, while NV10B silicon chips layered with 55 nm of thermal grown silicon dioxide were used for SP-IRIS experiments. Silicon supports were pretreated with oxygen plasma to clean and activate the surface. The oxygen pressure was set to 1.2 bar with a power of 29.6 W for 10 min. Then, chips were dipped into a 1% w/v solution of MCP-2 in 0,8 M aqueous ammonium sulfate. The slides were immersed into the coating solution for 30 min at room temperature, rinsed in bidistilled water, dried under a nitrogen stream, and then cured at 80 °C for 15 min.

Chips were spotted using a noncontact microarray spotter (sciFLEXARRAYER S12, Scienion, Berlin) equipped with an 80 μm nozzle. 400 pL of solution were spotted at room temperature and 65% humidity.

To prepare spotting solutions, proteins were dissolved at 1 mg/mL in 50 mM trehalose in PBS, while amino-modified oligonucleotides were dissolved at different concentrations (5, 10, and 25 μM) in a solution of 150 mM sodium phosphate buffer containing 0.01% sucrose monolaurate at pH 8.5. After spotting, chips were stored overnight in a sealed chamber filled at the bottom with sodium chloride saturated water (40 g/100 mL H_2O). Finally, chips were treated with a blocking solution of ethanolamine (50 mM in 0.1 M Tris/HCl buffer pH 9 and 2 mM $MgCl_2$) at room temperature for 1 h, rinsed with bidistilled water, and dried.

2.3. System development using microarray supports

2.3.1. Optimization of aptamer concentration

3 silicon chips were prepared and spotted with StrepApt5 and 20A-StrepApt5 (both the aptamers were deposited on the surface at three different concentrations, namely 5, 10, and 25 μM) and 0.1 mg/mL Cy3-labeled Fab fragment goat antihuman IgG as a positive control as described in 2.2. Chips were incubated with 1 mg/mL streptavidin in 2 mM $MgCl_2$ in PBS (hereinafter referred to as PBS-M) for 1 h at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried under a nitrogen stream.

A second incubation was performed using 15 μg/mL biotinylated antiCD9 in PBS-M and was carried out for 30 min at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried under nitrogen stream. Then chips were incubated with 10 μg/mL Cy3-labeled Fab fragment rabbit anti-mouse IgG in PBS-M for 1 h at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$, and dried under nitrogen stream. Fluorescence images were acquired using ScanArray Lite confocal laser scanner with 65% laser power and 65% PMT.

2.3.2. Biotin-labeled antiCD9 capture and release

9 silicon chips were prepared and functionalized with 1 mg/mL streptavidin, 1 mg/mL antiCD9, 10 μM COCU8, 10 μM StrepApt5 and 0.1 mg/mL Cy3-labeled Fab fragment goat antihuman IgG as positive control as described in 2.2. Chips were incubated with 1 mg/mL streptavidin in PBS-M for 1 h at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried under nitrogen stream. Then chips were incubated with 15 μg/mL biotin-labeled antiCD9 in PBS-M for 30 min at room temperature, washed 10 min with PBS-M, rinsed with 2 mM $MgCl_2$ and finally dried under a nitrogen stream.

Then chips were divided into 3 groups. The first group was incubated with PBS-M for 30 min at room temperature, rinsed with 2 mM $MgCl_2$ and dried and nitrogen stream.

The second group was incubated with 1 μM Cy5-labeled cDNA in PBS-M for 30 min at room temperature, washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and finally dried under a nitrogen stream.

The third group was incubated with 1 μM Cy5-labeled COCU11 in PBS-M for 30 min at room temperature, washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and finally dried under a nitrogen stream.

Finally all chips were incubated with 10 μg/mL Cy3-labeled Fab fragment rabbit antimouse IgG in PBS-M for 1 h at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried under nitrogen stream.

Fluorescence images of all chips were acquired using ScanArray Lite confocal laser scanner with 65% laser power and 65% PMT for both red and green fluorescence.

2.4. Extracellular vesicles capture on microarray

2.4.1. Separation of EVs from HEK-293 cell culture medium by ultracentrifugation

HEK-293 cells were seeded on 150 mm dishes in DMEM culture medium supplemented with 10% EV-depleted FCS (obtained by recovering the supernatant after ultracentrifugation of the FCS at 150.000×*g* for 17 h), 2 mM L-Glutamine, 100. U/mL penicillin and 100 μg/mL streptomycin-sulfate. After 72 h incubation, the culture medium was collected and centrifuged (1500 rpm) for 25 min to remove cell debris. The obtained supernatant was filtered through 0.22 μm filter and then ultracentrifuged at 150.000×*g* for 2 h at 4 °C (Beckman Coulter). The EV containing pellet was resuspended in PBS.

2.4.2. Synthesis of biotinylated rabbit IgG

polyclonal rabbit IgG was dissolved into PBS at a final concentration of 1 mg/mL. To 100 μL of the so obtained solution, 3,15 μL of freshly prepared 8.5 mM NHS-PEG_4-biotin in PBS (40 equivalents) were added and the solution was allowed to react 30 min at room temperature. The reaction was quenched by adding 10 μL of 1 M Tris-HCl pH8. After 5 min at room temperature the unreacted biotin-linker was removed using Amicon Ultra 100MWCO centrifugal filters (4 × 5 min at 12,000×*g*, adding PBS to a final volume of 500 μL before each centrifugation). The final volume was set to 100 μL by adding PBS and the concentration (0.83 mg/mL) was assessed by Bradford protein assay.

2.4.3. Extracellular vesicles capture and release

15 silicon chips were functionalized with 1 mg/mL polyclonal rabbit IgG, 1 mg/mL antiCD9, 1 mg/mL streptavidin and 10 μM StrepApt5 as described in 2.2. Chips were incubated with 1 mg/mL streptavidin in PBS-M for 1 h at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried. 9 chips were then incubated with 15 μg/mL biotin-labeled antiCD9 in PBS-M for 30 min at room temperature, while the remaining chips were incubated with 15 μg/mL biotinylated polyclonal rabbit IgG in PBS-M (as negative control) under the same conditions. All chips were washed 10 min in PBS-M, rinsed

with 2 mM $MgCl_2$ and dried. Chips were scanned using ExoView™ R100.

Three chips, functionalized with biotin-labeled antiCD9 were incubated with PBS-M for 2.5 h at room temperature, while the remaing chips were divided into two groups and incubated with different concentrations of HEK-derived EVs in PBS-M ($5*10^{10}$ and $1*10^{9}$ particles/mL) for 2.5 h at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried.

Chips were finally scanned using ExoView™ R100.

2.4.4. Transfection and separation of CD9-RFP EVs from HEK-293 cell culture

Two millions cells were plated in a 10 cm^2 dish and incubated for 24 h. Transfection was performed by mixing 8 μg/dish of plasmid DNA with TransIT-X2transfection reagent (Mirus) in a 1:2 ratio in serum- and antibiotics-free medium. After 16 h the medium was replaced with fresh one. After transfection, EVs were separated by ultracentrifugation as described in Section 2.4.1.

2.4.5. Comparison between conventional immobilization and aptamer-mediated immobilization of antibodies

15 silicon chips were functionalized with 1 mg/mL antiCD9 and 10 μM StrepApt5 as described in 2.2. Chips were incubated with 1 mg/mL streptavidin in PBS-M for 1 h at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried. Chips were then incubated with 15 μg/mL biotin-labeled antiCD9 in PBS-M for 30 min at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried. Chips were scanned using ExoView™ R100.

Chips were incubated with different concentrations of CD9-RFP transfected EVs in PBS-M (namely $5*10^{10}$, $3*10^{9}$, $1*10^{9}$, $3*10^{8}$ and $1*10^{8}$, 3 chips were incubated with each concentration) for 2.5 h at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried. Chips were finally scanned using ExoView™ R100.

2.4.6. Fluorescent detection of captured EVs

3 silicon chips were functionalized with 1 mg/mL polyclonal rabbit IgG, 1 mg/mL antiCD9 and 10 μM StrepApt5 as described in 2.2. Chips were incubated with 1 mg/mL streptavidin in PBS-M for 1 h at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried. Chips were then incubated with 15 μg/mL biotin-labeled antiCD9 in PBS-M for 30 min at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried. Chips were scanned using ExoView™ R100.

Chips were incubated with HEK-derived EVs in PBS-M ($1*10^{10}$ particles/mL) for 2.5 h at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried.

For the fluorescent colocalization experiment, chips were incubated with Cy3-labeled antiCD63 and Cy5-labeled antiCD81 (both at 1 μg/mL in PBS-M) for 1 h at room temperature. Chips were washed 10 min in PBS-M, rinsed with 2 mM $MgCl_2$ and dried.

Chips were finally scanned using ExoView™ R100.

3. Results & discussion

Immunoassays on solid surfaces represent a standard tool to provide information on the presence and concentration of particular targets in a sample. Antibodies, especially IgGs, are commonly used as biological probes in immunoassays [5]. A crucial step in developing an immunoassay in a microarray format is choosing a suitable immobilization strategy to anchor many different antibodies on the sensor's surface.

Antibodies share the requirements for optimal immobilization of proteins. Thus, developing a strategy for anchoring antibodies on solid surfaces is not trivial, and immobilization protocols should be optimized for the single application. While it could be acceptable to optimize specific strategies of immobilization in the context of well-established biological assays with a limited number of probes, the need for optimization limits the validation of new biomarkers in research settings, where new antibodies are constantly being implemented in multiplex immunoassays.

Based on these considerations, the need for immobilization strategies of general applicability becomes evident.

Conversely to proteins, oligonucleotides can be easily immobilized with controlled density and spatial orientation. Nucleic acids share common characteristics that are almost independent from their sequence, and can be chemically synthesized and modified by the insertion of many different chemical groups both at 5′ and 3' end. As a result, developing biosensors functionalized with nucleic acids is easier, and the obtained substrates possess longer shelf lives.

In an attempt to combine the advantages of manufacturing DNA microarrays with the bulk of information given by protein microarrays, the DNA-directed immobilization (DDI) strategy has been developed [10]. DDI consists in fabricating a DNA microarray and synthesizing a protein-DNA conjugate. Proteins are immobilized on the sensors' surface through a DNA linker exploiting the interaction between the DNA tag and surface probe [11].

Our group successfully applied DDI to generate antibody microarrays for the multiplexed analysis of extracellular vesicles. The system demonstrated to give similar results when compared to commercial antibody microarrays, but without optimizing antibody immobilization [7]. Unfortunately, the need of synthesizing antibody-DNA conjugates, as well as low modification yields, discourages many groups from adopting the DDI strategy.

As an alternative, we herein disclose the aptamer-mediated immobilization of biotinylated antibodies. The proposed method (see Fig. 1) generates surfaces functionalized with StrepApt5, a DNA aptamer that binds streptavidin with high affinity ($K_d = 35$ nM), occupying two of the four biotin binding sites [8]. The aptamer-functionalized surface is then used to bind streptavidin, obtaining a surface coating that can immobilize biotinylated entities, in the specific case, antibodies.

Using this strategy, we developed an immunocapturing assay based on biotinylated antibodies, which are either commercially available or easily synthesizable using biotinylation kits, without needing to optimize antibody immobilization. Being StrepApt5 a DNA aptamer, the herein proposed approach requires (like DDI) the production of DNA microarrays that are inherently easier to fabricate and possess improved stability in comparison with protein arrays.

In addition, the use of StrepApt5 to mediate streptavidin immobilization on the surface provides a system that is stable during the target capture step (the aptamer stably retains its tertiary structure in 2 mM $MgCl_2$ in PBS). Moreover it enables a straightforward release of the target upon incubation with the strand of DNA complementary to the aptamer (cDNA), as shown in Fig. 1b, thus potentially allowing the regeneration of the sensors for multiple analysis.

To prove the effectiveness of this method, we demonstrated the immobilization of biotinylated antibodies in a reversible way using StrepApt5-anchored streptavidin in microarray tests.

Silicon chips were coated using MCP-2, a member of a family of copolymers widely used in biosensing [12–14]. MCP-2 is the commercial name for copoly (DMA-NAS-MAPS), a *ter*-copolymer of *N,N*-dymethilacrylamide (DMA) (97% in moles), *N*-acryloyloxysuccinimide (NAS) (2% in moles), and 3-(trimethoxysilyl)propyl methacrylate (MAPS) (1% in moles), which forms a 3-D layer for the immobilization of biomolecules with retained capture efficiency and suppression of non-specific binding [15].

Silicon chips were spotted with StrepApt5 and 20A-StrepApt5, as described in section 2.2. In particular, 20A-StrepApt5 is an oligonucleotide where the streptavidin-binding sequence is outdistanced from the surface by a 20-*mer* adenine tail on the 5' end. It was used to evaluate the effect of the distance between aptamer and the surface. Both the oligonucleotides were printed on chips at three different concentrations (5, 10, and 25 μM) and sequentially incubated with streptavidin, biotinylated antiCD9, and Cy3-labeled Fab_2 fragment rabbit antimouse IgG

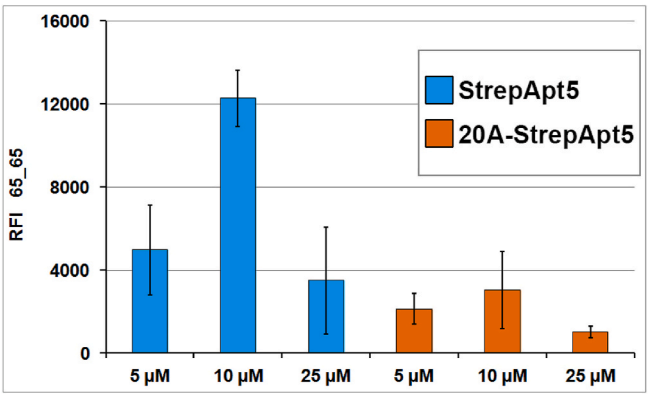


Fig. 2. Fluorescent detection of biotinylated antiCD9 immobilized on aptamer-bound streptavidin. Blue bars represent streptavidin immobilized on three different concentrations of StrepApt5. Orange bars represent streptavidin immobilized on three increasing concentrations of 20A-StrepApt5. Immobilized biotinylated antiCD9 was revealed upon incubation with Cy3 labeled secondary antibody. The results are the mean of three independent experiments, error bars represent standard deviation.

to enable fluorescent detection of the antibody. Results of Fig. 2 clearly show the same trend for both aptamers, where the highest degree of immobilization is reached when the DNA is spotted at a concentration of 10 μM; using lower or higher concentrations the fluorescent signal decreases.

These results suggest that there is a critical immobilization density for the aptamer (reached with a particular spotting concentration), at which the immobilization of streptavidin is optimal. At this critical density, the aptamer retains an appropriate folding, and lower concentrations predictably produce lower signals. Instead, at higher densities, interactions between complementary regions in adjacent strands occur, hindering the correct aptamer folding and thus limiting streptavidin immobilization. The results also show that the presence of the spacer in 20A-StrepApt5 reduces streptavidin immobilization levels, probably because the spacer promotes interaction between contiguous strands. Based on this experiment, 10 μM StrepApt5 was chosen as the best spotting condition to be used in the following experiments.

A simple experiment was designed to demonstrate the reversibility of antibody-streptavidin immobilization on aptamer and its specificity. Silicon chips were coated using MCP-2 and functionalized with streptavidin, antiCD9, StrepApt5, and COCU8 (a ssDNA sequence that does not bind StrepApt5, used as the negative control). The chips were sequentially incubated with streptavidin, biotinylated antiCD9, and Cy3-labeled Fab_2 rabbit anti-mouse IgG. Then, chips were divided in 3 groups: the first was analyzed without performing additional incubations, the second was incubated with Cy5-labeled cDNA (which is complementary to StrepApt5) while the third was incubated with Cy5-labeled COCU11 (the strand of DNA complementary to COCU8, an unrelated sequence used as a negative control). The experimental scheme is summarized in Fig. 3. With this experimental protocol, a green fluorescence signal appears on antiCD9 either spotted on the surface or immobilized though streptavidin, and incubated with Cy3 labeled anti-antibody (Fig. 3B). Addition of a Cy5 labeled complementary antibody turns the signal from green to red as a result of the annealing of the strand complementary to the aptamer sequence (Fig. 3C). The results of Fig. 3 show that using this approach, biotinylated antiCD9 can be immobilized on aptamer-bound streptavidin with high density (around half of the antibody immobilized on streptavidin directly printed on the surface) and effectively released through incubation with a cDNA complementary to the aptamer. Moreover, the bottom panel (Fig. 3D) demonstrates that the release is sequence-selective. In fact, when the chip is incubated with COCU11, the biotinylated antibody is not released from StrepApt5.

We, therefore, demonstrated that using streptavidin immobilized on StrepApt5 it is possible to generate a platform for selective capture of biotinylated antibodies. Additionally, we provided the proof of concept that biotinylated antibodies can be released from the surface upon incubation with cDNA.

In order to better understand the behavior of the herein developed system and prove the efficient binding between the sensing elements, we functionalized silica microbeads with StrepApt5 as already described in our previous work [16] and characterized the rate of functionalization at each single step (see Supplementary data).

Aptamer-directed antibodies were then tested in the immunocapturing of extracellular vesicles (exploiting the recognition of CD9, a transmembrane protein commonly found on the surface of EVs). EVs are biological nanoparticles surrounded by a lipid bilayer that are gaining interest as biomarkers in liquid biopsy approaches [17–22]. Since their dimension sensibly outranges the size of an antibody, the antibody must be immobilized on the surface in a way that guarantees a certain degree of flexibility to increase its capture efficiency.

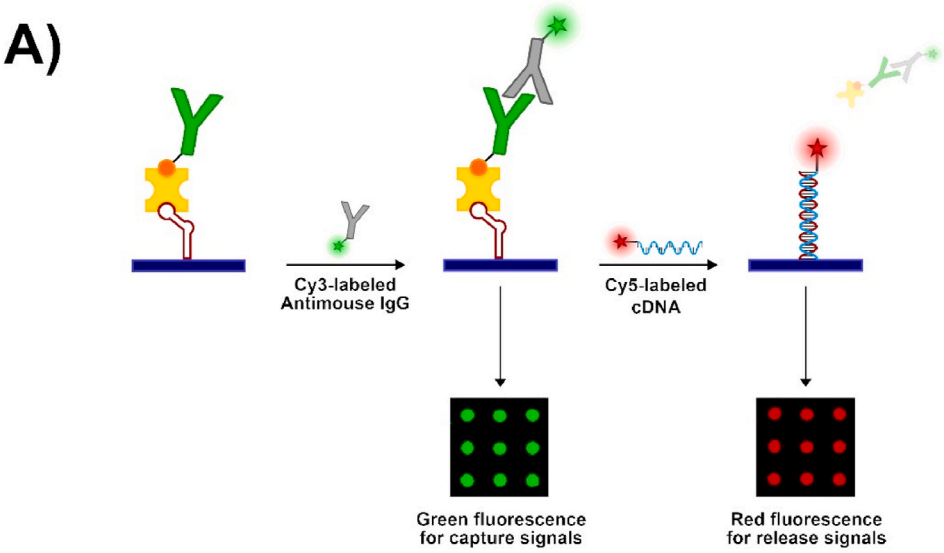
To evaluate the ability of antibodies immobilized via StrepApt5 to capture EVs, we developed a test in which the EVs were detected with Exoview™ R100, a commercial detector for microarray developed by Nanoview Biosciences (Boston, MA, USA). This Instrument digitally detects and sizes EVs by exploiting label free interferometry [23]. Silicon chips were coated using MCP-2 and functionalized with rabbit IgG, antiCD9, streptavidin, and StrepApt5, as described in Section 2.2. The chips were incubated with 1 mg/mL streptavidin and then with a biotinylated antibody (either antiCD9 or rabbit IgG). Using this experimental design, both streptavidin and aptamer spots on the microarray surface captured biotinylated antibodies.

Chips were incubated with EVs purified from HEK-293 cell culture supernatant by ultracentrifugation, diluted with PBS-M to a final concentration of $5*10^{10}$ particles/mL, and finally analyzed using label-free interferometry. As a negative control, chips functionalized with biotinylated antiCD9 were incubated with PBS-M under the same conditions and analyzed. Results (see Fig. 4A) show that EVs are efficiently captured on StrepApt5 spots only when the chips are functionalized with antiCD9. On the contrary, only low background signals are obtained when the aptamer's spot is incubated with rabbit IgG or with PBS-M. This experiment confirms the capture selectivity.

Since the number of EVs captured on antiCD9, either conventionally immobilized or aptamer-directed on the surface, is relatively high (suggesting a saturation of the signal), the same experiment was repeated incubating chips with a lower concentration of EVs (i.e. $1*10^9$ particles/mL) and the results are shown in Fig. 4B. The aptamer-directed antiCD9 shows improved affinity for EVs compared to antiCD9 conventionally immobilized on the surface or captured on the sensor only via streptavidin.

In order to shed light into this different behavior of the antibody in capturing EVs, we performed an additional experiment, considering a broader range of EV concentrations. Silicon chips were functionalized with antiCD9 and StrepApt5 as described in Section 2.2. Chips were sequentially incubated with streptavidin and biotinylated antiCD9 to perform aptamer-mediated immobilization of the antibody. Chips were then incubated with different concentrations of CD9-RFP transfected EVs (chosen in order that presence of CD9 was not a limiting factor in immunocapturing) and analyzed using Exoview™ R100.

Results, shown in Fig. 5, clearly show a different behavior of the same antibody. In fact, antiCD9 immobilized via aptamer has demonstrated to perform better in capturing EVs at lower concentrations if compared with the same antibody directly printed on the surface of chip. Increasing the number of vesicles in the sample, the difference in captured EVs using the two immobilization strategies progressively reduces, while at the highest concentration (namely $5*10^{10}$ particles/mL) the situation is reversed, since conventionally immobilized antiCD9 binds a slightly higher number of vesicles in comparison with antibody immobilized via StrepApt5. A reasonable explanation is that at high



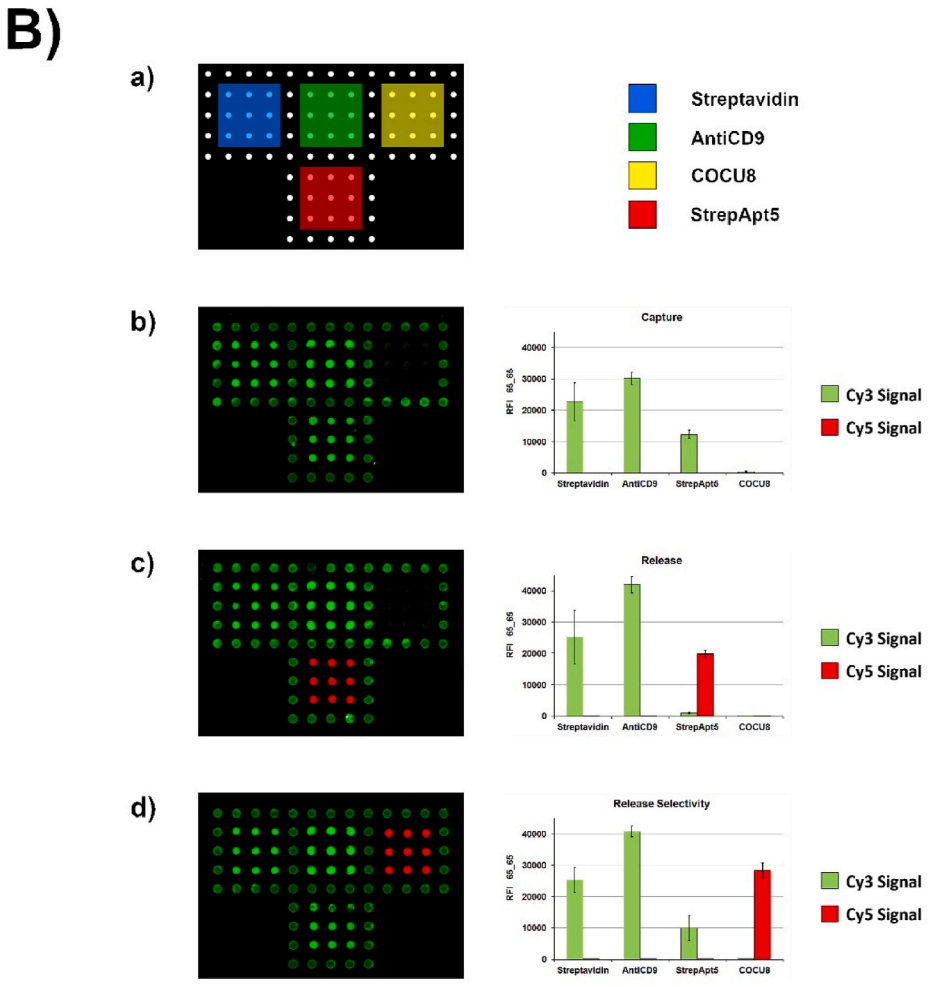


Fig. 3. (A) Schematic representation of experimental outline. Biotin-labeled antiCD9 immobilized on aptamer-bound streptavidin is revealed upon incubation with Cy3 labeled secondary antibody, producing green fluorescence signal. Cy5-labeled cDNA was used to release antibody-streptavidin complex from the aptamer, causing the switch from green to red fluorescence. (B) Overview of results. a) Spotting scheme. b) Capture experiment, chips not incubated with cDNA. c) Release experiment, chips incubated with cDNA to release the antibody. d) Chips incubated with COCU11 to prove selectivity of release step.

concentrations, where the number of EVs is not a limiting factor, the higher amount of antibody is immobilized the more vesicles are captured on the surface, while lowering the concentration of analytes the way of antibody immobilization becomes an important parameter to provide effective immunocapturing of EVs.

As expected, the choice of the immobilization strategy is crucial for the capture of bulky targets such as EVs. In particular, this study confirms that an optimal distance from the surface combined with an increased degree of flexibility of the probe, is crucial for efficient EV capturing as already suggested by our previous work on DDI

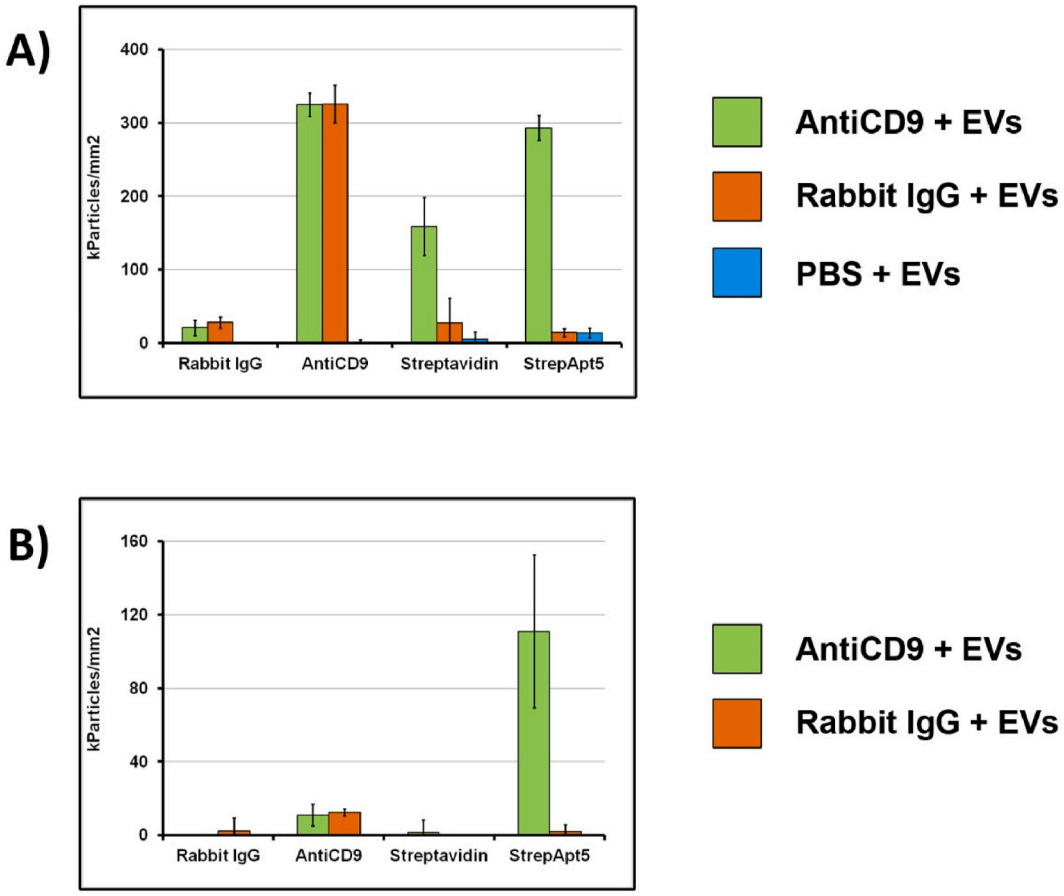


Fig. 4. Number of EVs detected by label free interferometry on microarray chips. Streptavidin and StrepApt5 spots are functionalized either with antiCD9 or rabbit IgG as described in Section 2.4.3. a) Results for chips incubated with PBS-M or $5*10^{10}$ particles/mL EVs. b) Results for chips incubated with $1*10^{9}$ particles/mL EVs.

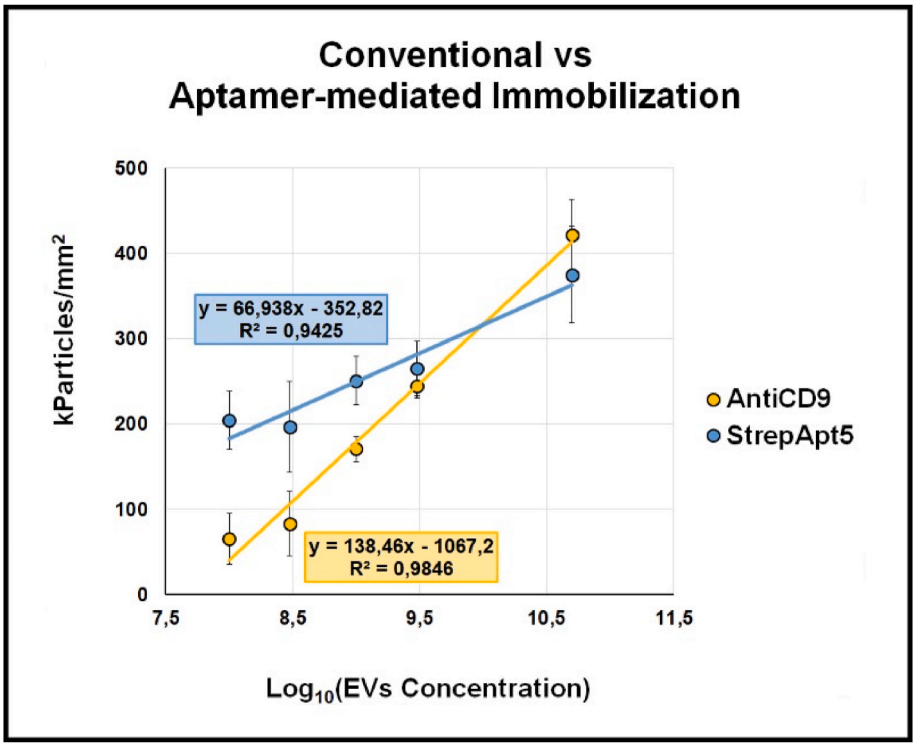


Fig. 5. Number of EVs captured on antiCD9 immobilized through conventional spotting (yellow dots) or aptamer-mediated immobilization (blue dots). On the X axis, EVs concentration is intended as particles/mL.

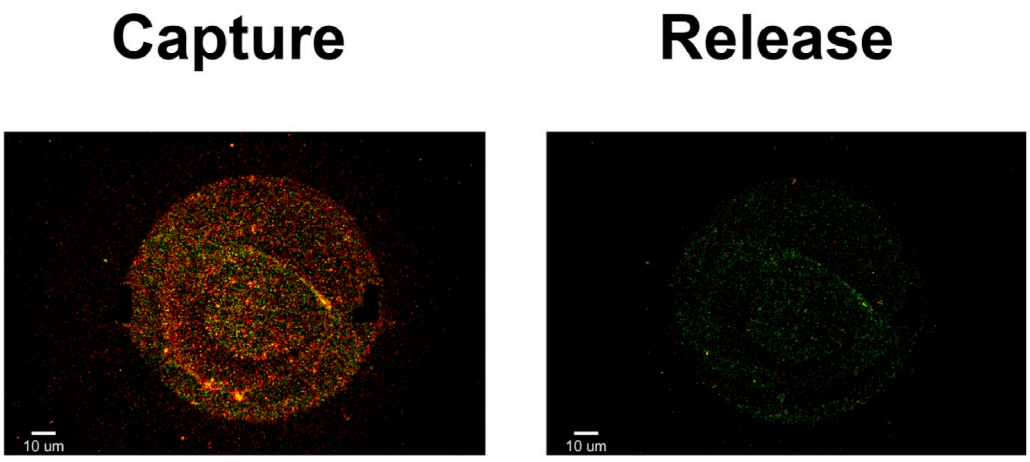


Fig. 6. Green and red fluorescence signals on StrepApt5 spots after EV-capture step and after cDNA-mediated release.

immobilization [7,24]. This peculiar feature makes this approach appealing for systems aimed at capturing large analytes such as EVs but potentially also viruses as well as lipid and synthetic nanoparticles, while it is supposed to have a lower impact on smaller targets (e.g. small molecules, proteins and nucleic acids). In fact, in the latter case the interaction with the antibody is relatively easier and the overall binding is influenced mostly by the amount of antibody immobilized on the surface (that is higher through conventional spotting as previously demonstrated, see Fig. 4B panel b).

Finally, to demonstrate that EVs captured on the surface could be released upon incubation with cDNA, the silicon chips were incubated with a mix of fluorescently-labeled antibodies (namely Cy3-labeled antiCD63 and Cy5-labeled antiCD81). Fig. 6, left panel, shows a high resolution fluorescence image of a single spot of EVs immobilized on antiCD9 antibody immobilized on StrepApt5 and labeled with the mixture of fluorescently labeled antibodies. The red and green fluorescence signals detected on the spot, further confirm that the nanoparticles captured on the aptamer-directed antiCD9 are actually EVs. The chip was then incubated with cDNA and, as shown in Fig. 6, right panel, the fluorescence signal dramatically decreased, proving how EVs captured on the surface using the aptamer-mediated approach can be effectively released from the spot by the competition of the DNA sequence complementary to the aptamer.

4. Conclusions

We developed an innovative strategy to immobilize biotinylated antibodies on the surface of sensors. The proposed strategy is based on the immobilization of StretpApt5, a DNA aptamer that binds streptavidin, on the surface of a solid support. In this way, it is possible to generate a surface where streptavidin is captured in a reversible way. On one hand, streptavidin can be exploited to immobilize biotinylated entities like we did using antibodies. On the other side, by incubating the surface with the sequence of DNA complementary to the aptamer, it is possible to remove streptavidin (together with captured antibody). Despite more experiments are needed to elucidate the effectiveness and actual applicability of this approach for sensors surface's regeneration and further analysis of EVs, it clearly shows how aptamer folding is necessary to bind streptavidin and indirectly immobilize biotinylated antibodies.

We demonstrated that this approach works finely on microarray supports, and we applied it to capture extracellular vesicles. As a result, antibody immobilized via aptamer shows an improved efficiency in capturing EVs (especially at lower concentrations) when compared to the same antibody not only directly immobilized on the surface of the microarray chip, but also immobilized on covalently bound streptavidin via a biotin linker.

In summary, we developed an immobilization strategy for biotinylated antibodies that improves the exposure of the protein and thus its efficiency. Additionally, spotting optimization for antibodies is no longer required, making the generation of antibody-based sensors easier.

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Credit author statement

Dario Brambilla – Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft, Visualization. Laura Sola – Methodology, Writing – Review&Editing. Francesco Damin – Methodology, Writing – Review&Editing. Alessandro Mussida – Writing – Review&Editing. Marcella Chiari – Conceptualization, Writing – Review&Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2023.124847.

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