Advantageous antibody microarray fabrication through DNA-directed immobilization: a step toward use of extracellular vesicles in diagnostics

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Abstract

Microarrays have been introduced to run multiple assays on a single platform. Since then, DNA and protein microarrays were developed to study both transcription and expression of genes. Protein microarray technology represents a powerful tool to get an insight into living systems. However, despite their enormous potential, the fabrication of protein arrays is affected by technological hurdles that limit their application. One of the major challenges is represented by the immobilization of proteins on solid surfaces. To overcome this limitation, DNA-directed immobilization (DDI) of proteins, an approach that exploits DNA-protein conjugates to transform a DNA microarrays into a protein array, has been developed. The adoption of DDI is limited as this approach requires the synthesis of DNA-protein conjugates. Herein, an optimized general protocol for DNA-protein ligation is provided and the conjugates are used to convert DNA arrays into antibody microarrays. Arrays obtained through DDI were used to capture and characterize extracellular vesicles (EVs), an emerging class of biomarkers. The proposed platform was tested against commercially available antibody microarrays, showing good performance combined with ease of fabrication.

Keywords

Microarray ï DNA-directed Immobilization ï Antibody ï DNA ï Extracellular Vesicles ï Diagnostics

1 Introduction

The concept of microarray was firstly introduced by Roger P. Ekins, who developed the so-called multi-analyte immunoassay in 1989 [1] and refers to the miniaturization of thousands of assays on a single substrate. During the following decade, DNA microarrays became widely adopted to simultaneously determine the expression levels of thousands of mRNA. However, gene expression profiling does not always reflect actual protein expression [2]. Since proteins are the key players in the majority of biological processes, there was a need for high-throughput technologies able to provide insights on protein functions and interactions. Protein microarrays were developed to fulfill this need [3].

In spite of their success, the potential of protein microarrays is still limited by a number of technological challenges. In fact, while DNA is a highly stable oligomer that possesses well defined physicochemical properties quite independent from its sequence, the term protein represents an heterogeneous class of biomolecules, generally more labile than DNA, all of them displaying different characteristics [4]. Moreover, proteins must retain their tertiary (or even quaternary) structure in order to maintain their biological activity. For this reason, the choice of an appropriate immobilization strategy that ensures protein availability and correct surface orientation is crucial. While nucleic acids are commercially available with a variety of chemical groups linked to their 5ô and 3ô ends that allow optimal surface orientation, proteins are more heterogeneous making it more difficult to devise immobilization protocols of general applicability.

Different immobilization approaches were developed (involving among others site-directed mutagenesis, heterobifunctional crosslinkers and affinity tags) but, in all the cases mentioned above, the protocol had to be optimized for each single protein [5].

In order to extend to proteins the technological robustness of DNA immobilization, an approach called DNA-directed immobilization (DDI) was developed in 1994 by Niemeyer et al [6]. DDI enables the translation of a DNA array into a protein array thanks to Watson-Crick base pairing between surface immobilized capture-DNAs and DNA-tagged antibodies [7].

Recent works have demonstrated that DNA linkers are excellent spacers to enhance the availability of antibody binding sites for bio-nanoparticle capture by decreasing the steric hindrance around the protein [8]. Due to these peculiar advantages, during the last two decades DNA-directed immobilization has been exploited in different fields, including proteomic studies, cell and microorganism arrays [7,9ï 11].

In the last few years, different approaches to obtain DNA-protein conjugates were developed, since these molecules offer the possibility to combine programmability of DNA with protein functionality [12]. This unique feature opens the door to applications such as immuno-PCR, proximity extension assays and DNA-PAINT [13]. However, there is a lack of simple and robust methods to conjugate proteins and DNA with approaches that can be easily practiced in laboratories that do not possess a strong chemical background.

In this work an optimized protocol for generating protein microarrays through DDI is presented. This simple and reproducible approach requires small amounts of inexpensive reactants and can be easily applied in every biological laboratory. We report here optimization of both, antibody conjugation and surface functionalization with DNA. The conjugation method is applicable to all proteins as it exploits a well-established reaction between amino group on lysine side chain and active ester. The microarray obtained by DDI was successfully used for capturing small extracellular vesicles (sEVs) on a single particle interferometric reflectance imaging sensor (SP-IRIS) [14]. Extracellular vesicles (EVs) are nanosized vesicles released from cells into bodily fluids which can mediate intercellular communication by transporting nucleic acids, proteins and lipids from one cell to another [15]. During the last decade, their great potential as biomarkers in diagnosis and prognosis of diseases has been demonstrated and many companies aim at exploiting EVs for targeted therapeutics [16]. EVs of endosomal origin, smaller than 200 nm in diameter [17] are involved in numerous biological processes, both physiological and pathological [18,19]. An innovative tool for their phenotyping is ExoViewÊ R100, an instrument commercialized by NanoView Biosciences (Boston, MA, USA) based on SP-IRIS technology that combines label free and fluorescence detection on a microarray platform [20].

DDI was compared with the classical immobilization approach used in ExoViewÊ R100 kit (which is based on an antibody microarray) in sEVs capturing from purified samples. Conventional and DDI microarrays provided excellent results, proving that DNA-directed immobilization of antibodies is a convenient approach for protein immobilization that allows to functionalize the same microarray platform with different proteins depending on end-user need, starting from a single stable array of oligonucleotides.

2 Materials and Methods

2.1 Materials

Ammonium sulfate ($(NH_4)_2SO_4$), dibenzocyclooctyne-N-hydroxysuccinimidyl ester (DBCO-NHS ester), phosphate buffer saline tablets (PBS), Trizma base, 37% chloric acid (HCl), sodium phosphate (Na_3PO_4), sucrose monolaurate, sodium chloride (NaCl), ethanolamine, trehalose dehydrate and Amicon Ultra centrifugal filters (MWCO 100K) were purchased from Sigma Aldrich (St. Louis, MO, USA). All solvents were used as received. MCP-2 copolymer was purchased from Lucidant Polymers (Sunnyvale, CA, USA). Mouse anti-human CD9 IgG (clone MEM-61) and mouse anti-human CD63 IgG (clone MEM-259) were provided by Hansa BioMed Life Sciences Ltd (Tallinn, Estonia). Cy3-labeled rabbit anti-mouse IgG was purchased from Jackson ImmunoResearch (Baltimore, PA, USA). Oligonucleotides were synthesized by MWG-Biotech AG (Ebevsberg, Germany): see Supplementary Information for oligonucleotide sequences. These oligonucleotides were freeze-dried and resuspended in DI water at a final concentration of 100 µM before use. Untreated silicon chips with 100 nm thermal grown oxide (14 x 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. Spotting was performed using SciFLEXARRAYER S12 (Scienion, Berlin, Germany). Centrifugation was carried out using Eppendorf MiniSpin (Eppendorf, Hamburg, Germany). Fluorescence images were obtained using the ScanArray Lite confocal laser scanner and analyzed using ScanArray Express software (Perkin Elmer, MA, USA). Interferometric analysis of EVs was performed using ExoViewÊ R100 for image acquisition and NanoViewer 2.6.0 software for analysis (NanoView Biosciences Inc., MA, USA). Nanoparticle Tracking Analysis was performed with NanoSight NS300 using 3.2 Dev Build 3.2.16 software (Malvern Instruments Ltd, Malvern, United Kingdom).

2.2 General procedure for microarray chip preparation

Silicon chips were pre-treated with oxygen plasma for 10 min: the oxygen pressure was set to 1.2 bar with a power of 29.6 W. To coat the chips, MCP-2 copolymer was dissolved in DI water to a final concentration of 2% w/v and then diluted 1:1 with a solution of ammonium sulphate 1.6 M. The chips were immersed into each solution for 30 min, then rinsed with DI water, dried under a nitrogen stream, and finally cured at 80°C for 15 minutes.

A 100 µM solution of DNA was diluted in spotting buffer (150 mM sodium phosphate buffer, pH 8.5, containing 0.01% w/v sucrose monolaurate) at different concentrations,

while proteins were dissolved at 1 mg/mL concentration in PBS containing 50 mM trehalose. These solutions were printed onto coated silicon chips using a noncontact microarray spotter (sciFLEXARRAYER S12, Scienion, Berlin) equipped with an 80 µm nozzle.

Four hundred pL drops of spotting solution were deposited at room temperature and 65% humidity. Immediately after spotting, the chips were stored overnight in a sealed chamber filled at the bottom with sodium chloride saturated water (40 g/100 mL H_2O). After incubation, the chips were treated with a blocking solution of ethanolamine (50 mM in 0.1 M Tris/HCl buffer pH 9), at room temperature for 1 h. Then chips were rinsed with bidistilled water and dried.

2.3 Antibody conjugation using ssDNA

To a sodium azide free antibody solution (100 µL, 1 mg/mL) 2.46 µL of DBCO-NHS ester 4 mM (15 equivalents) were added and the mixture was allowed to react 30 min at room temperature. The reaction was quenched in 5 minutes at room temperature by adding 10 µL of 1M Tris/HCl pH 8.0. Unreacted DBCO-NHS ester was removed through centrifugation on Amicon Ultra 100 MWCO filters (3x5 min at 12.000 x g, adding PBS to reach 100 µL after each step). To perform DNA-conjugation, 6.67 µL (1 equivalent) of 100 µM azido-modified DNA-tag were added to reaction mixture and the so obtained solution incubated overnight at 37°C. Unreacted DNA-tag was removed through centrifugation on Amicon Ultra 100 MWCO filters (3x5 min at 12.000 x g, adding PBS to reach 100 µL after each step). Finally, 1 µL of 2% w/v sodium azide solution was added as preservative (final azide concentration 0.02%).

2.4 Immobilization of different DNA-antibody conjugates on silicon chips.

Nine silicon chips were coated and spotted with antiCD9, antiCD63, DNA-capture1 and DNA-capture2 (DNA probes were spotted at 3 different concentrations, i.e. 0.5, 10 and 25 µM) as described in Section 2.2. AntiCD9 and antiCD63 was conjugated with DNA-tag1 and DNA-tag2 respectively following the protocol described in Section 2.3. The chips were incubated, in triplicate, either with 5 µg/mL solutions of antiCD9-tag1 and antiCD63-tag2 or with PBS (as a negative control) for 1 h at room temperature. The chips were washed 10 min in PBS, rinsed with bidistilled water and dried under nitrogen stream and then incubated with a 10 µg/mL solution of Cy3-labeled rabbit anti-mouse IgG in PBS for 1 h at room temperature. The chips were washed 10 min in PBS, rinsed with bidistilled water and

dried under nitrogen stream. Fluorescence images were acquired using ScanArray Lite confocal laser scanner with 65% laser power and 55% PMT.

2.5 DNA-probe spotting optimization

Three silicon ExoViewÊ R100 oxide chips layered with 55 nm silicon oxide were coated using MCP-2 copolymer as described in section 2.2. DNA-capture alone or mixed in equimolar ratio with an oligonucleotide called DNA-stabilizer were diluted at different concentrations (5,10 and 25 μM for DNA-capture alone; 5+5, 10+10 and 25+25 μM for DNA-capture mixed with DNA-stabilizer) in spotting buffer (150 mM sodium phosphate buffer, pH 8.5, containing 0.01% sucrose monolaurate). The DNA solutions were printed on coated silicon chips as described in section 2.2. The chips were then scanned for label free particle detection using the ExoViewÊ R100 instrument and analyzed.

2.6 Separation and characterization of HEK EVs

2.6.1 HEK-derived sEVs separation by ultracentrifugation. Three days conditioned media from HEK-293 cells were harvested and centrifuged at 500 x g for 25 minutes. Supernatants were filtered with 0.22 mm filters (Merck Millipore) and centrifuged in a SorvallTM WX Ultracentrifuge (ThermoFisher Scientific, WX Ultra 100 #75000100) at 150.000 x g for 90 minutes at 4°C with a SureSpinTM 630 swinging bucket rotor (ThermoFisher Scientific) to pellet EVs. After supernatant was carefully removed, EV-containing pellet were resuspended in PBS and directly used for subsequent analysis.

2.6.2 HEK-derived sEVs characterization using Nanoparticle Tracking Analysis. The sample obtained as reported in paragraph 2.6.1 was analyzed using Nanosight NS300 (Malvern Panalytical, Malvern, UK). Videos were analyzed by the in-built NanoSight Software NTA 3.2 Dev Build 3.2.16. The Camera type, Camera level, and Detect Threshold were sCMOS, 14 and 4, respectively. The number of completed tracks in NTA measurements was 5 (a 60 seconds movie was registered for each measurement). Sample was diluted in PBS to a final volume of 1 mL. The ideal concentration was assessed by pre-testing the optimal particle per frame value (20-100 particles per frame).

2.7 EVs capture on antibodies immobilized by conventional and DDI approach

Nine SP-IRIS silicon chips were coated and spotted as described in section 2.2. Polyclonal rabbit IgG, mouse antiCD63 IgG and DNA-capture3 mixed with DNA-stabilizer3 (25 + 25

μM) were printed on silicon chips. After the chips were blocked, they were incubated with a 5 μg/mL solution of antiCD63-tag3 in PBS for 1 h at room temperature. The chips were washed 10 min in PBS, rinsed with bidistilled water, dried, mapped and analyzed using ExoViewÊ R100 to obtain prescan images. Then the chips, in triplicate, were incubated either with PBS or with EVs isolated from HEK cell culture media at two different concentrations ($1*10^8$ and $1*10^9$ particles/mL in PBS) for 2.5 h at room temperature. To compare conventional immobilization with DDI, three commercial microarray chips, part of the ExoView Tetraspanin Plasma kit, were also included in this experiment. In this case the chips were incubated with EVs isolated from HEK cell culture media ($1*10^9$ particles/mL in PBS) for 2.5 h at room temperature. After incubation, all chips were washed for 10 min in PBS, rinsed with bidistilled water, dried and incubated for 1 h at room temperature with fluorescently labeled antiCD81 solution, obtained by diluting 1:1000 in PBS the stock solution supplied with ExoView Tetraspanin Plasma kit (NanoView Biosciences, Boston, USA). The chips were then washed 10 min in PBS, rinsed with bidistilled water and dried. Silicon chips were analyzed using ExoViewÊ R100 to get postscan data.

3 Results and Discussion

EVs have demonstrated to be a potential source of biomarkers with an important role in diagnostics since their cargo and membrane composition mirror the cell from whom they originate. In particular, in liquid biopsy they allow to monitor cancerous phenotype evolution in patients by non-invasive blood draws, paving the way to precision-medicine [21]. Driven by the huge interest around the use of EVs in diagnostics, in the last few years, many methods to analyze them as well as their cargo were developed. A number of detection techniques, both label-based (e.g. nanoparticle tracking analysis, flow cytometry, digital PCR, microscopic imaging and DNA-PAINT) and label-free (Raman spectroscopy, atomic force microscope infrared spectroscopy, interferometric imaging), were developed [22].

Approaches to analyze EVs based on a protein microarray platform have also been introduced recently. Antibodies are printed onto a substrate, either a glass slide or a silicon chip, which enables the capturing of EVs through their surface or surface-associated proteins. Once captured on the surface, profiling of EVs can be performed by label-free or

fluorescence detection with labeled selected antibodies such as antiCD9, -CD63 and -CD81.

Recently, Nanoview Biosciences (Boston, MA, USA) has introduced ExoViewÊ R100, an instrument that provides EVs counting and phenotyping at single particle level by combining interferometric and fluorescent detection on an antibody microarray platform [20]. On a silicon/silicon oxide layered surface the light scattered by nanoparticles illuminated with LED light at a specific wavelength [23] produces images with areas of increased contrast that correlates with the diameter of the nanoparticle (the instrument is able to count individual nanovesicles ranging from 50 to 200 nm in diameter). The technique is called single particle interferometric reflectance imaging sensor (SP-IRIS) and allows to measure the number of particles captured by the different antibodies arrayed on the surface. This information is complemented by the integration on the same sensing platform of a fluorescence detection module. After capturing EVs, the chip is incubated with a mix of antibodies, each labeled with a different fluorophore. Colocalization of fluorescence and label free signals provides the protein expression profile of both surface and luminal proteins.

All systems based on microarray technology require optimization of spot morphology. This is particularly true for SP-IRIS that, being a high-magnification imaging technique, requires high quality spots since morphological features such as coffee rings, crystallization or other non-uniform structures prevents accurate detection of particles. However, the optimization of spotting conditions is time and material consuming, as many parameters must be varied to obtain homogeneous regions.

To overcome this issue, we propose a different strategy to immobilize antibodies which can be implemented in any platform based on protein microarrays. In particular we demonstrate the usefulness of the proposed approach on the aforementioned SP-IRIS platform ExoViewÊ . The instrument normally uses a chip with subarrays of anti-tetraspanins (CD9, CD41a, CD63 and CD81) antibodies and isotype IgG negative controls.

DNA directed immobilization exploits arrays of DNA-capture probes to immobilize proteins conjugated with complementary DNA-tag sequences. Exploiting the complementarity between DNA strands, proteins can be site-selectively immobilized on a microarray substrate, turning a DNA microarray (which is more stable and easier to produce) into a protein array. This approach is not new [14] but in previous publication commercial kits of unknown composition were used for conjugating protein and DNA. In this work we disclose

a simple and robust strategy to enable DDI and demonstrate for the first time its use in EVs characterization. Both antibody conjugation and DNA microarray fabrication were optimized.

The reaction scheme for conjugation of an antibody to DNA is summarized in Fig.1, while the optimization of the conjugation step is fully described in Supplementary Information. In the first step the antibody was reacted with a bifunctional linker that presents an active ester (N-hydroxysuccinimde, NHS) at one end and a dibenzocyclooctine (DBCO) moiety on the opposite end. The NHS group reacts with Ůamine of lysine residues on the antibody surface, generating a DBCO-modified antibody. We targeted lysine residues for the first conjugation step because they are among the most abundant amino acids in proteins and are easily accessible being well exposed on protein surface. Both features are suitable for the development of a conjugation protocol applicable to different proteins. Moreover, lysine is one of the most commonly used amino acid for linking substrates to antibodies, and its modification has been exploited to synthesize clinically relevant antibody drug conjugates (i.e. Kadcyla® and Zevalin®) [24].

After removal of the unreacted bifunctional linker, a solution of DNA-tag, modified at 5ôend with an azide group, was added, in molar ratio, to the antibody and reacted overnight at 37°C. The strain-promoted azide-alkyne cycloaddition (SPAAC) biorthogonal reaction between azide and DBCO allows covalent conjugation of the DNA-tag with the antibody through formation of a stable triazole. Finally, the excess of unreacted DNA-tag was removed and sodium azide was added to a final concentration of 0.02% as a preservative.

The conjugation process allows modification of several antibodies in parallel with a reaction that requires small amounts of commercially available chemicals, short operating times and basic lab equipment (see Tab.S1 in Supplementary information).

Two different antibodies, directed against the EVs surface-markers CD9 and CD63, were reacted with two DNA sequences to form antiCD9-tag1 and antiCD63-tag2 conjugates. Then silicon/silicon oxide chips were coated with MCP-2 as described in Section 2.2. MCP-2 is 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 probes with retained capture efficiency and suppression of non-specific binding, that has extensively been used for biosensing [25,26]. NAS is the reactive unit inside the copolymer responsible for the immobilization of amino-modified oligonucleotides through an acylation reaction. The silicon chips were printed with unmodified antibodies and with DNA probes (DNA-

capture1 to immobilize antiCD9-tag1 and DNA-capture2 to bind antiCD63-tag1) using a noncontact microarray spotter. The DNA probes, dissolved in printing buffer at 3 different concentrations, 0.5, 10 and 25 μM were spotted on nine chips that were divided in three groups (three replicates each) and incubated with 5 μg/mL antiCD9-tag1, antiCD63-tag2, and PBS, as a negative control.

Surface immobilized antibodies were scanned for fluorescence detection after incubation with Cy3-labeled rabbit anti-mouse IgG. The results in Fig.2 clearly indicate each DNA-antibody conjugate binds specifically to its complementary surface immobilized DNA (Fig.2A-B). Incubation with PBS, the negative control, demonstrates the absence of nonspecific interactions between fluorescently-labeled secondary antibody and surface DNA (Fig.2C).

These results show that increasing the concentration of spotted oligonucleotide from 10 to 25 μM, leads only to a slight increment of captured antibody. At 25 μM, the amount of antibody captured by DDI is similar to that of the antibody covalently bound to the chip.

We then optimized spot morphology. Unfortunately 10 and 25 μM solutions of oligonucleotides do not provide spots suitable for detection on ExoViewÊ R100. In fact, they are inhomogeneous, with regions of different density and, most importantly, they are not flat (see Fig.3A). Due to spot roughness, the analysis software detects nanoparticles in the prescan step where only a small amount of background particles is detected when the spot quality is optimal. This artifact severely affects subsequent analysis.

In order to produce homogeneous spots, we investigated different options. First, three different concentrations of oligonucleotide DNA-capture3 (5, 10 and 25 μM) were spotted on silicon chips. The DNA-capture probe is a 40 mer oligonucleotide where 20 bases at 3ô end are complementary to the antibody tag sequence. The 5ôend bears the amino-linker and it is used to covalently bind the oligonucleotide to the surface. In an attempt to improve spot morphology, the 20 mer portion of the DNA-capture probe at the 5ôend was hybridized in solution, before spotting, with a complementary oligonucleotide called DNA-stabilizer that was added in equimolar ratio to the probe. The chips were blocked as described in Section 2.2 and then analyzed by label free interferometry. An increased concentration of DNA-capture led to lower particle counts. Moreover, adding the DNA-stabilizer to the DNA-capture caused a significant reduction of particle density in the prescan, (Fig.3B.). We hypothesize that, adding the stabilizer, which is complementary to the region of DNA close to the surface, produces a dsDNA region with lower propensity to

aggregate with nearby oligonucleotides. In addition, its enhanced rigidity in comparison with ssDNA leads to better orientation of the DNA strand.

On the basis of spot morphology (shape and homogeneity) and lower number of background particle detected during the prescan, we chose to use 25 μM DNA-capture prehybridized with DNA-stabilizer at the same concentration as the best condition for printing DNA on microarray chips.

We tested DDI for EV-capturing with optimized spotting conditions using DNA tagged antiCD63 antibody. CD63 is a tetraspanin found on the membrane of many extracellular vesicles. An azide-free mouse antiCD63 IgG, a gift from Hansa BioMed (Tallin, Estonia), was conjugated with DNA-tag3 as described in Section 2.3. A series of SP-IRIS chips where coated and spotted with polyclonal rabbit IgG (as negative control), antiCD63 immobilized through conventional NHS-amine coupling, and antiCD63 immobilized via DDI following the procedure described in Section 2.2. The chips were statically incubated with 20 μL of a 5 μg/mL solution of antiCD63-tag3 in PBS for 1 h at room temperature (see Section 2.4). To test the system, we used sEVs derived from HEK-293 cell line, obtained through ultracentrifugation whose quality was confirmed by Nanoparticle Tracking Analysis (NTA, data shown in Fig.S3). The chips were divided into three groups and prescan images were acquired with ExoViewÊ R100. The three groups were separately incubated with purified EVs at two concentrations, $1*10^9$ and $1*10^8$ particles/mL, and with PBS as the negative control. The chips were then incubated a fluorescently labeled antiCD81 antibody, that is part of ExoView Kit and postscan images were taken. The results are shown in Fig.4. As expected, no vesicles were detected on rabbit IgG neither in label free nor in fluorescence mode. DNA directed antiCD63 showed good capture ability in both detection modes with small standard deviations, confirming the suitability of DDI for EV-capturing. Surprisingly, when the same antibody was directly spotted on the chip, the fluorescence signal was lower and almost no particles were detected label free. It is possible that the conditions used for the immobilization were not optimal to preserve the antibody activity, confirming how spotting optimization is crucial in protein microarrays.

In Fig.5A we focus on the results provided by DDI antiCD63 spots. As it can be seen, there is a good correspondence between label free and fluorescence signals in the negative control as well with the two EV concentrations tested. Moreover, label free and fluorescence counts scale linearly with the concentration of EVs analyzed (Fig.5B-C).

Combining ExoViewÊ R100 label and label-free counts we assessed the number of particles that were simultaneously detected by the two modes. This is an important

indication of the quality of the analysis. In fact, particles that presents only label free signals may be extracellular vesicles but also protein aggregates and other entities that are erroneously recognized as particles. Likewise, fluorescent only signals may come from EVs smaller than 50 nm but also from non-specific interactions between the fluorescent antibody and the surface.

We found that only 20% of particles (in the range of 50-200 nm in diameter) were simultaneously detected by label free interferometry and fluorescence (Fig.S4) but it has to be noted that this percentage may depend on the low co-expression on the same vesicle of CD63 and CD81. We also measured the size distribution of colocalized particles captured using the higher EV concentration tested ($1*10^9$ particles/mL) and it was compatible with that of an EV population (Fig.S5).

Finally we compared the results on our custom array with those provided by the ExoView Tetraspanin Plasma kit. We repeated the on-chip incubation with the higher EV concentration ($1*10^9$ particles/mL) using the same protocol used in the experiment of figure 5. Label free particle counts and fluorescence signals are shown in Fig.6. In general, signals detected with ExoView kit showed a higher standard deviation, compared to those provided by DDI chips except for antiCD9 spots.

No signals were detected on the negative control spots (mouse IgG) and on antiCD41a (a marker for platelet-derived vesicles, which are not supposed to be found in EVs isolated from cell culture media), while a high number of particles was detected on the other anti-tetraspanins antibodies (CD9, CD63 and CD81).

We measured label free, fluorescence and colocalized counts on antiCD63 spots (data shown in Fig.S6A). Comparing results obtained with different immobilization approaches, it was demonstrated that, while colocalized and fluorescence signals are quite similar in the two cases, the label free signal in the ExoView chip is almost double than that obtained with custom array. The percentage of colocalized particles on ExoView chip is 8.4% (lower than the 20.8% registered using DDI, see Fig.S6B). Since the two types of chips were incubated with exactly the same sample, the observed difference might indicate a greater specificity of DNA-directed antiCD63.

Finally we considered the size distribution of the fraction of particles detected simultaneously as label and label-free particles on ExoView and on our custom chips. On both types of substrates, the number of particles captured by the isotype control (mouse IgG) is negligible while antitetraspanins antibodies capture particle of EVs-compatible size (see Fig.S7A and B).

4 Conclusions

We confirmed DNA-directed immobilization of proteins as a viable method for transforming DNA microarrays into antibody microarrays. We optimized the conditions to synthesize DNA-antibody conjugates and to spot DNA on silicon chips, obtaining an easy to handle fabrication of protein microarrays. DDI approach was successfully exploited to capture EVs from diluted samples and compared with conventional antibody microarrays, demonstrating an excellent performance while maintaining ease of fabrication.

5 Acknowledgements

This work was supported by European Union Horizon 2020 under Grant 766466: INDEX, Integrated Nanoparticle isolation and Detection system for complete on-chip analysis of Exosomes.

The authors are grateful to R. Vago (Urological Research Institute, Division of Experimental Oncology, IRCCS San Raffaele Scientific Institute, Milan, Italy) for providing HEK-derived EV samples.

6 Appendix A. Supplementary information

7 Conflicts of interest

The authors declare no conflict of interest.

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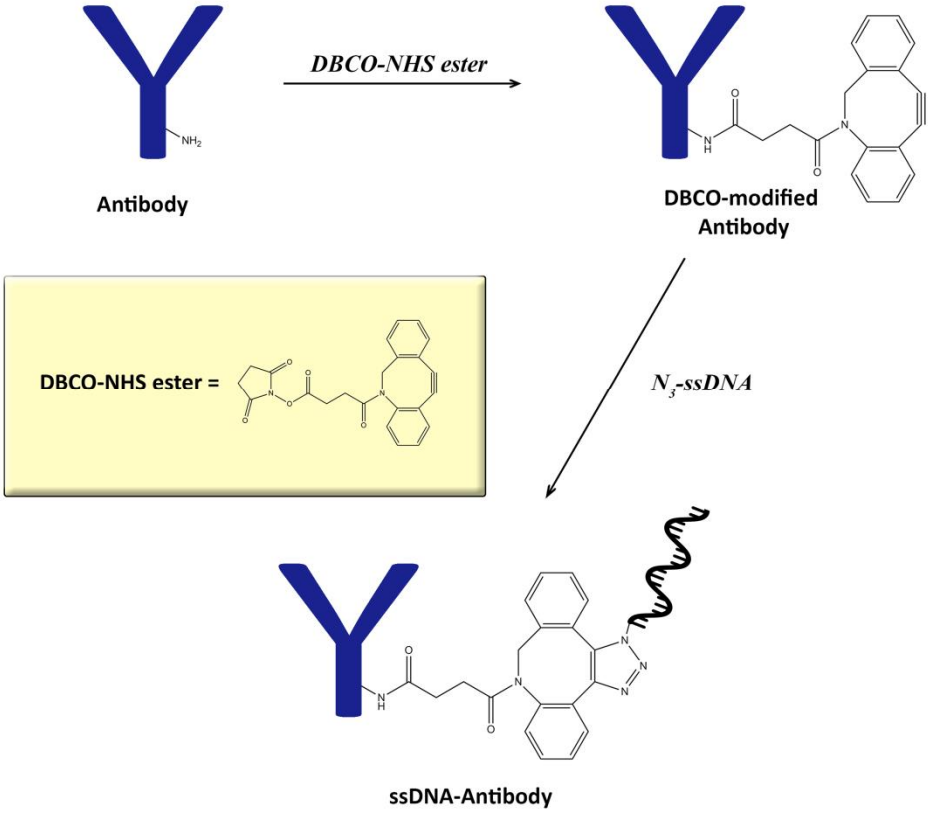
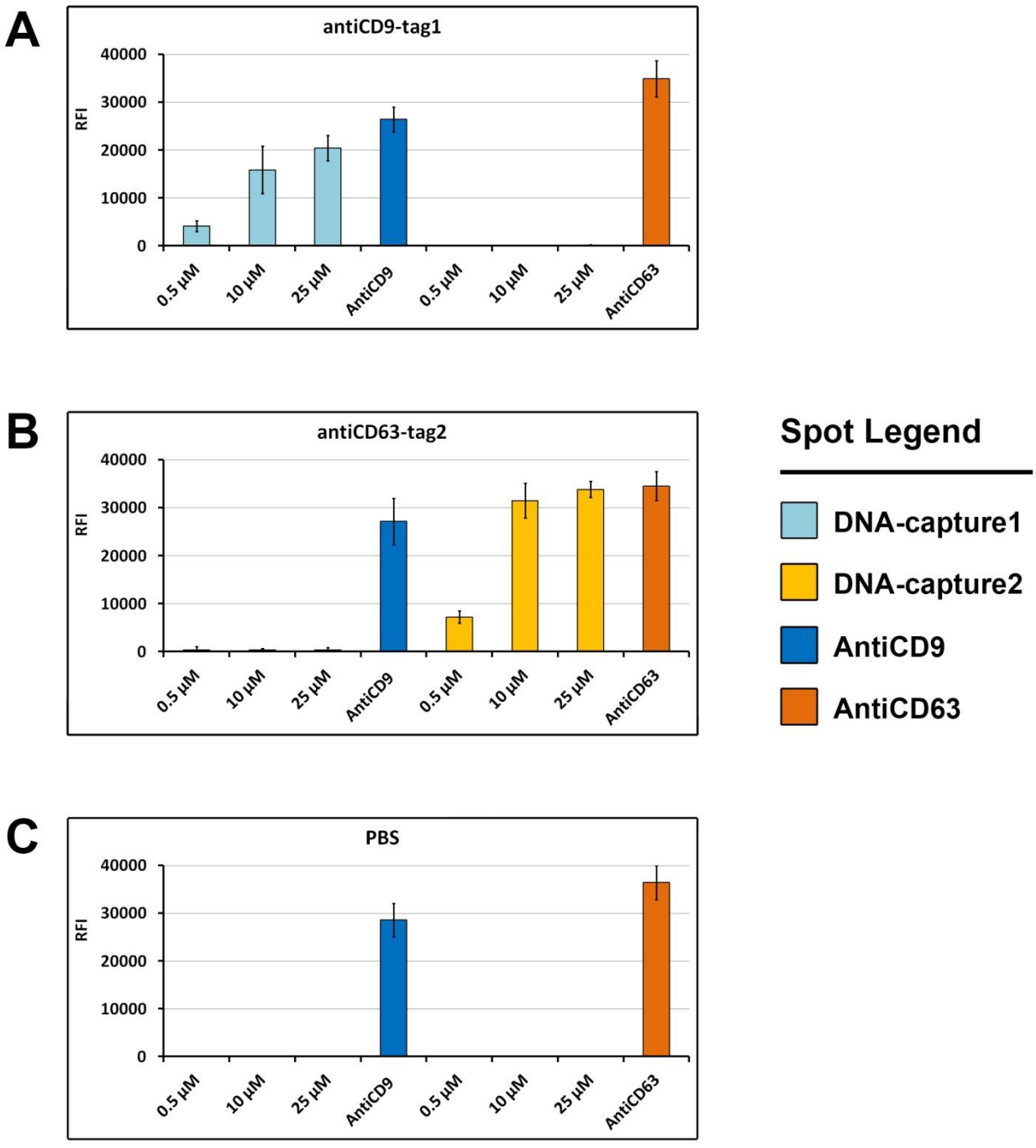

DBCO-NHS ester
Antibody
DBCO-modified Antibody
DBCO-NHS ester =
N_3-ssDNA
ssDNA-Antibody

Figure 1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30000
20000
10000
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0.5 µM
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10 µM
25 µM
AntiCD63
B
antiCD63-tag2
RFI
40000
30000
20000
10000
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10 µM
25 µM
AntiCD9
0.5 µM
10 µM
25 µM
AntiCD63
Spot Legend
DNA-capture1
DNA-capture2
AntiCD9
AntiCD63
C
PBS
RFI
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10000
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25 µM
AntiCD63

Figure 2

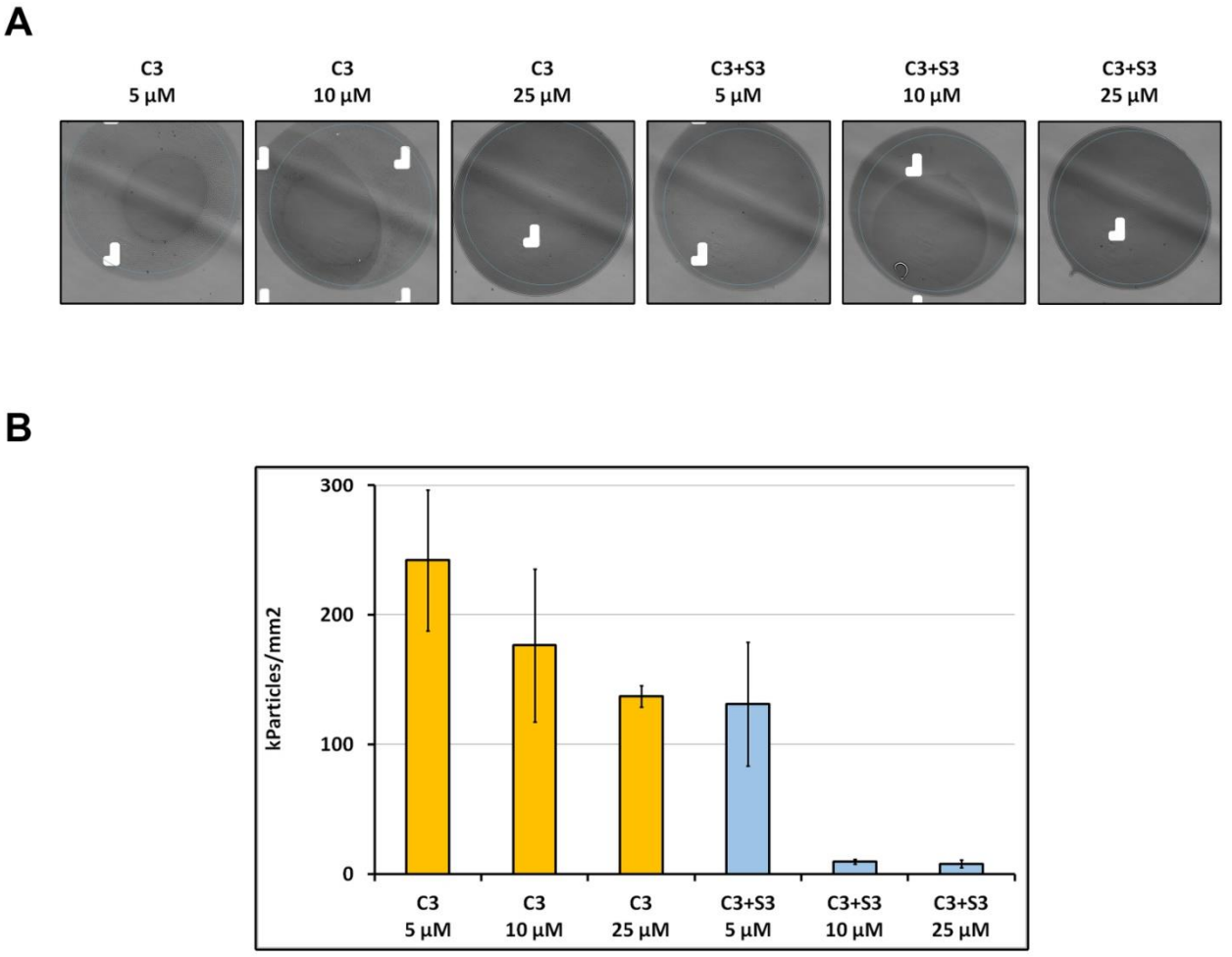

Figure 3

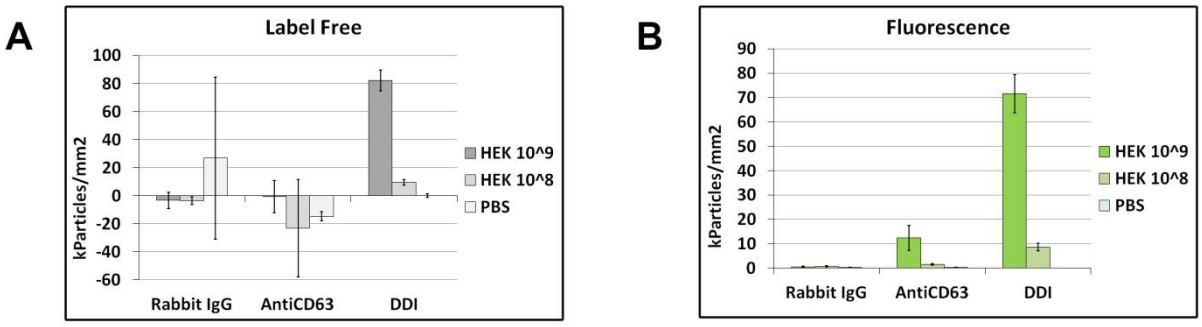

Figure 4

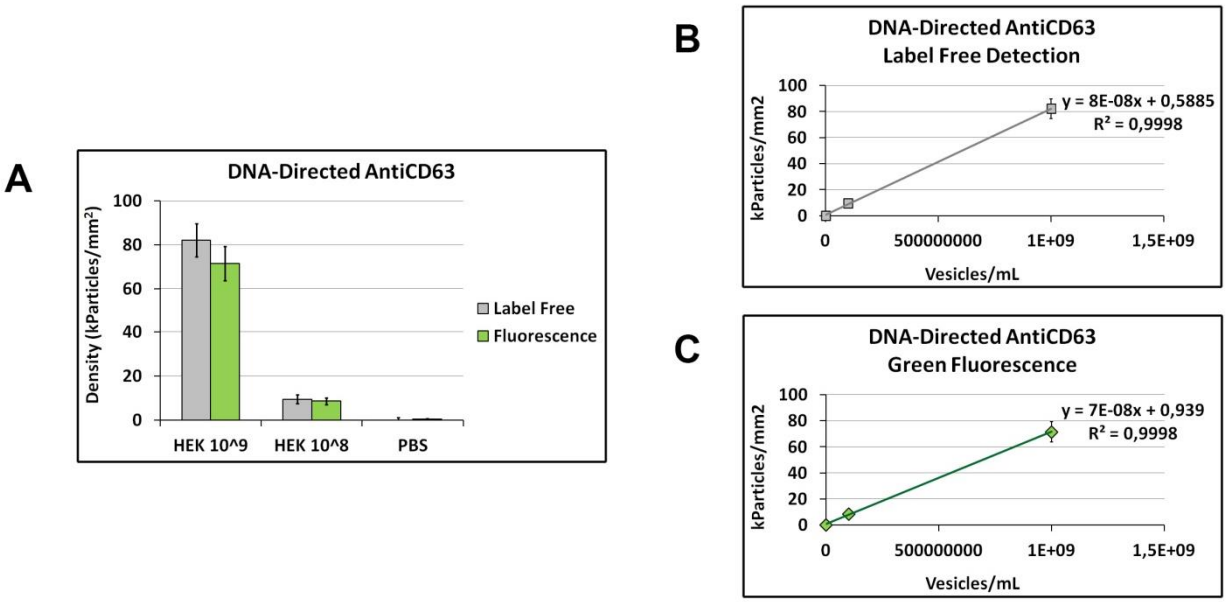

Figure 5

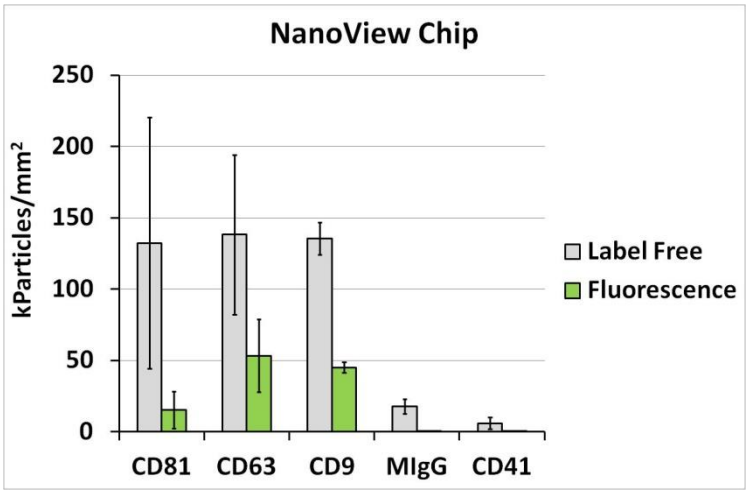

Figure 6

***Figure 1**. Synthetic route for DNA-antibody conjugate preparation.*

***Figure 2**. Antibody capture on DNA-capture strands through DDI. Fluorescence data for (A) chips incubated with antiCD9-tag1, (B) chips incubated with antiCD63-tag2 and (C) chips incubated with PBS.*

***Figure 3**. (A) Label free images of DNA spotted under different conditions. (B) Density of particles detected by ExoView® R100 during prescan step using different spotting conditions. Both DNA-capture3 alone (C3) and DNA-capture3 prehybridized with DNA-stabilizer3 (C3+S3) were printed on chip at three different concentrations.*

***Figure 4**. Density of extracellular vesicles immobilized on polyclonal rabbit IgG and antiCD63. The antibodies were immobilized both through amine coupling and DNA-directed immobilization (antiCD63 and DDI labels, respectively). Label free (A) and fluorescence (B) signals detected by ExoView R100 are shown .*

***Figure 5**. Label free and fluorescence detection of single extracellular vesicles captured on microarray chip using DNA-directed antiCD63 (A). Linear regressions of the same data are shown for label free (B) and fluorescence (C) detection.*

***Figure 6**. Particles captured from HEK-derived EVs on antibody array measured using label free interferometry (gray bars) and fluorescence (green bars).*