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

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Supplementary Information

1 Material and Methods

1.1 Oligonucleotidic sequences

- *DNA-capture1:* 5'-NH_2-ACTTAGGACTCAGTACAGGATAGACTTGATATCGGTTGGA 3'
- *DNA-tag1:* 5'-Azide-AAAAATCCAACCGATATCAAGTCTA-3'
- *DNA-capture2:* 5'-NH_2-ATCCGACCTTGACATCTCTACCACTGCGACTAACTCTGTA-3'
- *DNA-tag2:* 5'-Azide-AAAAATACAGAGTTAGTCGCAGTGG-3'
- *DNA-capture3*: 5'-NH_2-ATATGTACCCACCGCATTCTCAGTCTGTTCATTCGTAGGC-3'
- *DNA-tag3*: 5'-Azide-AAAAAGCCTACGAATGAACAGACTG-3'
- *DNA-stabilizer3*: 5'-AGAATGCGGTGGGTACATAT-3'
- *DNA-capture4*: 5'-NH_2-AAAAAAAAAAAAAAAAAAAAACTCCAGTGCCAAGTACGAT -3'
- *DNA-tag4*: 5'-Azide-AAAAAATCGTACTTGGCACTGGAGT-3'
- *DNA-stabilizer4*: 5'-TTTTTTTTTTTTTTTTTTTT-3'
- *DNA-capture5*: 5'-NH_2-AAAAAAAAAAAAAAAAAAAATAATCTAATTCTGGTCGCGG-3'
- *DNA-tag5*: 5'-Azide-AAAAACCGCGACCAGAATTAGATT-3'

1.2 Optimization of DNA-antibody conjugation

1.2.1 Gel electrophoresis analysis. To 10 µL of a 1 mg/mL solution of antibody or antibody-DNA conjugate, 1 µL of 100 µM complementary DNA was added, and the resulting solution was diluted to 20 µL with sample buffer 2X (90 mM Tris, 90 mM boric acid, 2 mM EDTA, 1 mM sucrose, 0.08% xylene cyanol added with SYBR green 20X obtained diluting a 10.000X stock solution). The obtained samples were loaded on a 7% acrylamide-bisacrylamide gel. After electrophoresis, double strand DNA bands were revealed using a UV lamp while protein bands with Coomassie brilliant blue.

1.2.2 Strain-promoted azide-alkyne cycloaddition (SPAAC) reaction temperature optimization. The following protocol was repeated on three aliquots, each time changing SPAAC reaction temperature. To a polyclonal rabbit IgG 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 by adding 10 µL of 1M Tris/HCl, pH 8, and left 5 min at room temperature. Unreacted DBCO-NHS ester was removed through centrifugation on Amicon Ultra 10 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 of

azido-modified DNA-tag1 100 µM (1 equivalent) were added to the three aliquots and each of them was incubated overnight at different temperatures (4, 25 and 37°C). Unreacted DNA-tag1 was removed through centrifugation on Amicon Ultra 30 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 a preservative (final azide concentration 0.02%). An additional aliquot was treated as described above without performing the first purification step (for this aliquot SPAAC was carried out at 4°C).

1.2.3 Purification step optimization. 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 by adding 10 µL of 1M Tris/HCl pH 8 and left 5 min at room temperature. Unreacted DBCO-NHS ester was removed through centrifugation on Amicon Ultra 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 of azido-modified DNA-tag1 100 µM (1 equivalent) were added, and the mixture incubated overnight at 37°C. Unreacted DNA-tag was removed through centrifugation on Amicon Ultra 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 a preservative (final azide concentration 0.02%). The following protocol was repeated twice on aliquots “A” and “B”. For the aliquot “A”, a 10 MWCO centrifugal filter was used for the first purification, while a 30 MWCO filter was used for the second purification. For the aliquot “B” two filters with the same cutoff (100 MWCO) were used in both purification steps.

2 Discussion

We report here, step by step, the optimization work carried out to improve robustness and reproducibility of the synthesis. In particular we focused on two aspects that we hypothesize are most likely to have an impact on the overall yield of the synthesis: purification steps and temperature of SPAAC reaction between the DBCO-modified antibody and the azido-DNA.

We used a polyclonal rabbit IgG as a model system to study how the reaction was affected by experimental procedure. Firstly, we focused on the temperature of the SPAAC conjugation reaction. Three aliquots of polyclonal rabbit IgG were modified as described in

1.2.2. The obtained antibody-DNA conjugates, as well as the native antibody, were loaded on a 7% acrylamide-bisacrylamide gel together with their complementary DNA strand (DNA-capture1) and SYBR green for dsDNA UV detection. Another gel, prepared in the same way, was stained using Coomassie brilliant blue and the images are shown in Fig.S1.

Using UV three bands of dsDNA were detected corresponding to: 1) the unreacted DNA-tag1 (the band with higher mobility) and 2) the antibody-DNA conjugate. The native antibody cannot be seen using UV detection because it is not detectable using SYBR green staining. As it can be easily seen, the intensity of the band corresponding to antibody-DNA conjugate increases by raising the conjugation temperature (lane 1 to lane 3). This result is also confirmed by Coomassie brilliant blue staining. For this reason we chose 37°C as the best condition to run SPAAC reaction. In the lower region of the gel, two different bands were detected with UV. We hypothesize that the band with slightly higher mobility corresponds to unreacted DNA-capture1 itself, while the band with lower mobility corresponds to DNA-capture that has reacted with the remaining DBCO-NHS ester that is still present in the mixture despite the first purification.

A second aliquot of antibody was conjugated following the same protocol (SPAAC reaction at 4°C) except that the first purification step was not performed. Using this procedure only a small amount of antibody is effectively conjugated to DNA, while the majority of it is modified only with DBCO (see upper band in Fig.S1, lane2). This is due to the presence of DBCO-NHS ester in excess that quenches azido groups of oligonucleotides during the conjugation step.

The low reaction yield together with the presence of DNA-capture1 in lanes 3, 4 and 5 indicates that purification steps are essential and need improvement. Therefore we optimized the cutoff of centrifugal filters. We used a 100 kDa cutoff for both the purification steps and then we compared the purity of the samples by gel electrophoresis (Fig.S2). The two bands of non-conjugated DNA, although still present, are less intense and the quality of the tagged antibody compatible with its use in immunoassay.

Step #	Process	Time
1	Antibody modification with DBCO + purification	1.5 h *
2	Azido-DNA conjugation	Overnight
3	Final purification	0.5 h

Table S1*. Steps for antibody-DNA-conjugate synthesis and time needed to perform each step. (*) time required if buffer exchange procedure is needed.*

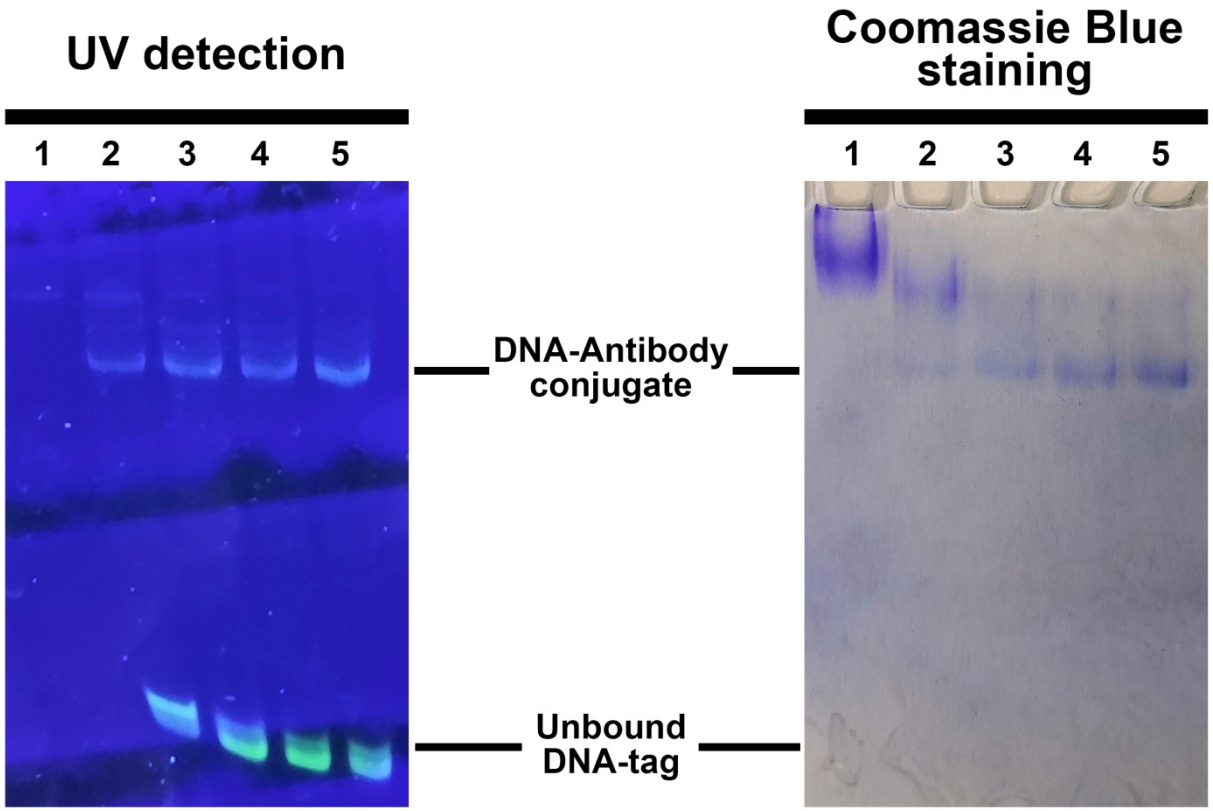

***Figure S1**. Gel Electrophoresis of tagged-antibodies obtained with different conjugation protocols. Lane1: Native IgG. Lane2: product conjugated with DNA at 4°C without purification after the first step. The upper band corresponds to the DBCO-modified antibody. Lane3: SPAAC reaction performed at 4°C. Lane4: SPAAC reaction performed at 25°C. Lane5: SPAAC reaction performed at 37°C.*

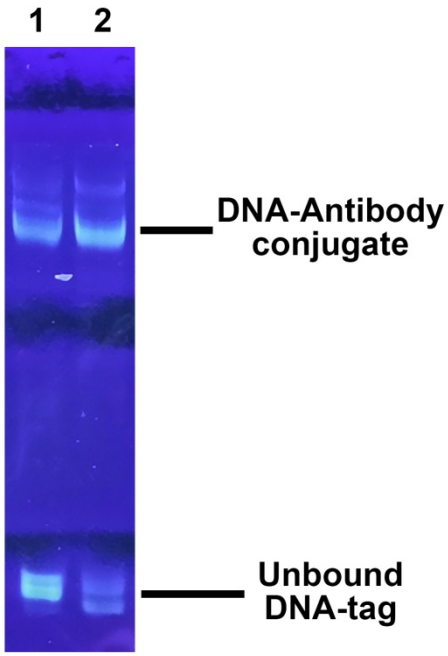

***Figure S2**.Gel Electrophoresis of DNA-antibody conjugates obtained using two different purification protocols: aliquot "A" (lane1) and "B" (lane2).*

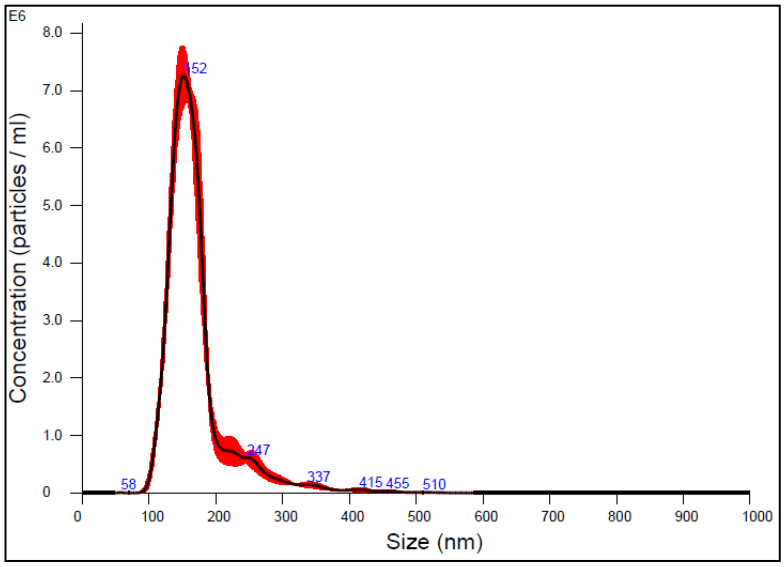

***Figure S3**. Nanoparticle Tracking Analysis of extracellular vesicles purified from HEK-293 cell line via ultracentrifugation.*

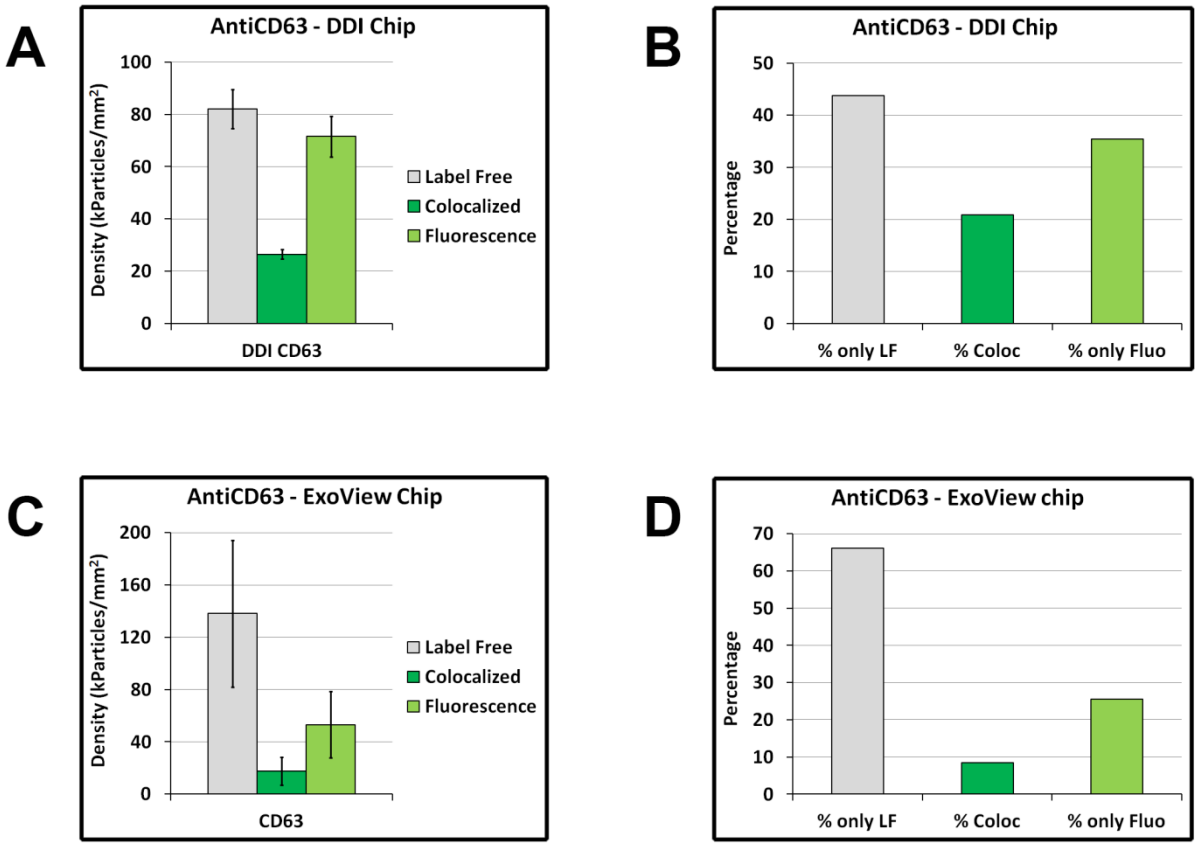

***Figure S4**. Label free and fluorescence detection of EVs captured on microarray chip using either DNA-directed antiCD63 (A and B) and covalently immobilized antiCD63 (C and D). (A;C) Dark green data represents colocalized particles, which are particles detected by label free and fluorescence detection modes*

simultaneously. (B;D) Percentage of label free only, fluorescent only and colocalized signals obtained incubating the chips with 5*10^9 EVs/mL.*

(*) For percentage measurement the density of colocalized particles has been subtracted from total label free and fluorescence densities and measured as follows:

- **Total label free particle density (L)**
- **Total fluorescence particle density (F)**
- **Colocalized particle density (C)**
- **Total particle density (T) = (L - C) + (F - C) + C**
- **Label free percentage = [(L – C) / T] * 100**
- **Fluorescence percentage = [(F – C) / T] * 100**
- **Colocalized percentage = (C / T) * 100**

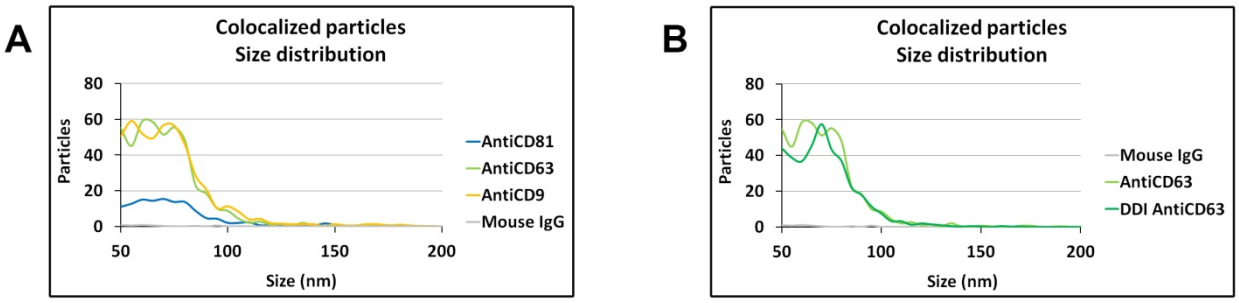

***Figure S5**. (A) Size distribution of colocalized purified EVs captured on ExoView chip by antitetraspanins antibodies and isotype control. (B) Size distribution of colocalized EVs captured by antiCD63 printed on microarray (light green line) or DNA-directed (dark green line) on custom array.*