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Aptamer-assisted Phage Display: Enhancing Checkpoint Inhibition with a Peptide and an Aptamer Targeting Distinct Sites on a Single PD-L1 Protein

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Reagents and materials

Rink amide resin, Fmoc-amino acids, (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate (COMU), N, N-Diisopropylethylamine (DIPEA) were purchased from chem-impex international. Piperidine was purchased from Bean Town Chemical (BTC). Triisopropyl silane (TIPS) was purchased from TCI (Tokyo Chemical Industry, USA). Acetonitrile (HPLC grade), Diethyl ether, Trifluoroacetic acid (TFA), Dichloromethane (DCM), and Dimethylformamide (DMF) were purchased from Sigma Aldrich. Ph. D-12 library was purchased from New England Biologics. Human PD-L1 protein was purchased from Sino biologics, USA.

Table S1. Phage bio-panning in the approach 1 (using the 10-min MJ5C incubation before washes).

Bio-panning Round	Amount of input Phage Library	Concentration of Aptamer used	Counter-selection time (with BSA)	Unamplified Phage (Output library)	Washing time (TBS-tween concentration)
1	6×10^{10}	-	-	4.3×10^{3}	10 min (0.1 %)
2	1×10^{11}	100 nM	30 min	2.15×10^{5}	10min (0.1 %)
3	3×10^{11}	100 nM	30 min	1.2×10^{6}	10 min (0.25 %)
4	3×10^{11}	200 nM	40 min	1.1×10^{6}	10 min (0.25 %)
5	3×10^{11}	200 nM	40 min	1.29×10^{5}	10 min (0.5 %)

Table S2. Phage bio-panning in the approach 2 (using three 1- min washes with MJ5C).

Bio-panning Round	Amount of input Phage Library	Concentration of Aptamer used	Counter-selection time	Unamplified Phage (Output library)	Washing time (TBS-tween concentration)
1	6×10^{10}	-	-	1.29×10^{4}	1 min x 3 (0.1%)
2	3×10^{11}	100 nM	30 min	4.3×10^{5}	1 min x 3 (0.1%)
3	3×10^{11}	100 nM	30 min	1.29×10^{6}	1 min x 3 (0.1%)
4	3×10^{11}	200 nM	40 min	3×10^{6}	1 min x 3 (0.25%)
5	3×10^{11}	200 nM	40 min	4.3×10^{6}	1 min x 3 (0.5%)

Phage display protocol

Bio-panning was performed using the commercially available Ph.D. -12 Phage Display Library (E8110S, New England Biolabs, MA) in competition with the anti-PD-L1 MJ5C aptamer. Initially, a Nunc Maxisorp high-binding 96-well plate (Ref# 44-2404-21) was coated with 1 µg of PD-L1 protein in a 100 µL of 0.1 M NaHCO3, pH 8.6 and incubated overnight at 4ºC. For one hour, the coated wells were blocked with 5 mg/ml of bovine serum albumin (BSA). After one hour, the plate was washed six times with 0.1% TBST. Next, the Phage library was added to the wells and incubated at room temperature for one hour, and the plate was washed 10 times to remove the unbound phages. The bound phages were eluted using 100µl of coating remaining solution to the well for 10 min. Then 100 µl of 0.2 M Glycine-HCl (pH 2.2) with 1 mg/ml BSA was added to the well with shaking for 10 min and mixed with the previous coating solution, followed by neutralization using 15 µL 1M Tris-HCl (pH 9.1).

The eluted Phage Library was amplified in 20 mL of ER2738 *E. coli* (OD600 at 0.02-0.04) with shaking vigorously at 37 ºC for 4 hours. The culture was then centrifuged at 12,000g for 10 min at 4 ºC, after which supernatant was transferred into a new tube with the addition of One-fourth volume of 20% polyethylene glycol (PEG) 2.5 M NaCl and incubated at 4ºC overnight. The PEG-precipitated phages were then centrifuged at 12,000g for 15 min at 4ºC. The phage pellet was resuspended in 500 µL of TBS, and that was the first round of the Phage library. This sub-library was subjected to phage tittering and used for the next round of screening. The Aptamer-assisted Phage bio-panning starts from the second round. In the second round, the crucial step of introducing competition for the phage library was carried out using the anti-PD-L1 aptamer, MJ5C, in two different ways. In the first approach, the plate was washed three times using TBST (50 Mm Tris-HCl (pH 7.5), 150 mM NaCl/0.1%/Tween 20), and then 100 µL of TBST containing 100 nM or 200 nM of MJ5C was added and incubated for 10 minutes. In the second approach, the plate was washed three times with the same concentrations of aptamer (1-minute incubation instead of the 10-minute incubation.). After that, both plates were then washed ten times with TBST, with an increase in the Tween concentration as the round proceeded, as mentioned in Table S1, to remove unbound phage. The bound phages were eluted using 100µl of coating remaining solution to the well for 10 min. Then 100 µl of 0.2 M Glycine-HCl (pH 2.2) with 1 mg/ml BSA was added to the well with shaking for 10 min and mixed with the previous coating solution, followed by neutralization using 15 µL 1M Tris-HCl (pH 9.1). Further rounds of screening were performed with the conditions as described in Table S1 and S2.

The Phage quantification from each round was done by performing phage tittering. In brief, 10 µL of serially diluted phage sub-libraries was mixed with 200 µL of mid-log phase of ER2738 cells, and then the mixture was added to 3mL of prewarmed top agar and covered on a prewarmed LB/IPTG/X-gal plate. After cooling the plate for 5 min, the plates were inverted and incubated at 37 ºC overnight. The phage plaques were counted, and the titers were calculated according to the plaque numbers and the corresponding dilution.

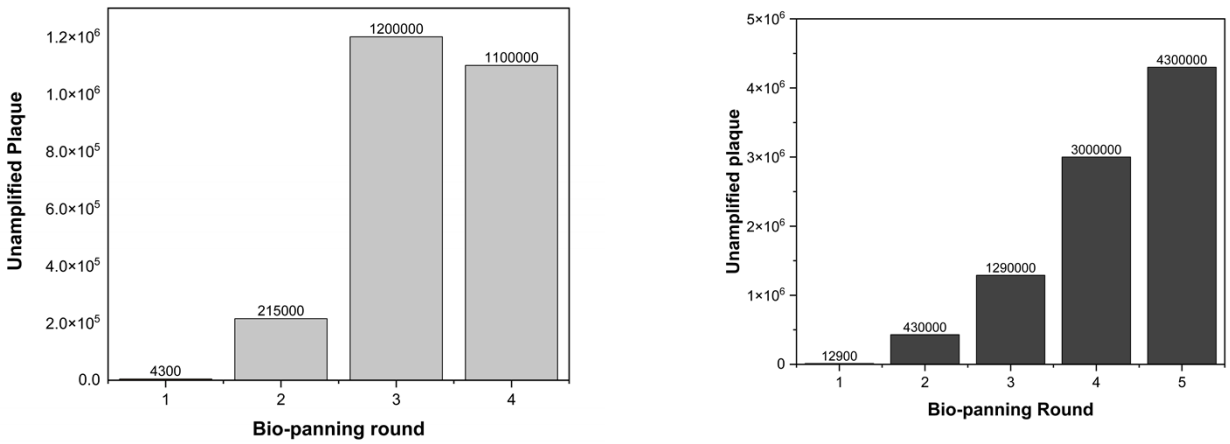


Fig S1. Bio-panning rounds for un-amplified phage numbers obtained from MJ5C incubation (left) and MJ5C washing (right)

Phage-based enzyme-linked immunosorbent assay.

We conducted a phage ELISA to identify phages that bind with PD-L1 protein in the presence of the MJ5C aptamer. To do this, we used a Nunc Maxisorp high-binding 96-well plate (Ref# 44-2404-21) and coated it with human PD-L1(0.5 µg) dissolved in 0.1 M NaHCO3 (pH 8.6), whereas the same amount of BSA protein was used as a control. The coated protein was incubated overnight at 4℃. After that, we blocked each well with 5 mg/ml BSA protein for an hour. After washing 6 times with 0.1% TBST, we added 5 x10^9 phages from each bio-panning cycle and again incubated them for an hour. We checked the phage binding in the presence of the MJ5C aptamer in two different ways. In the first way, we incubated the aptamer for 10 minutes with protein phage complexes. In another way, we washed each well three times with 100 nM of aptamer. Finally, we washed each well ten times with TBST to remove any unbound materials. The amount of phages bound to PD-L1 in the presence of MJ5C was determined by incubating them with HRP-conjugated anti-M13 monoclonal antibody (1:2000) for 20 minutes. We measured absorbance at 450 nm by reacting with the TMB substrate.

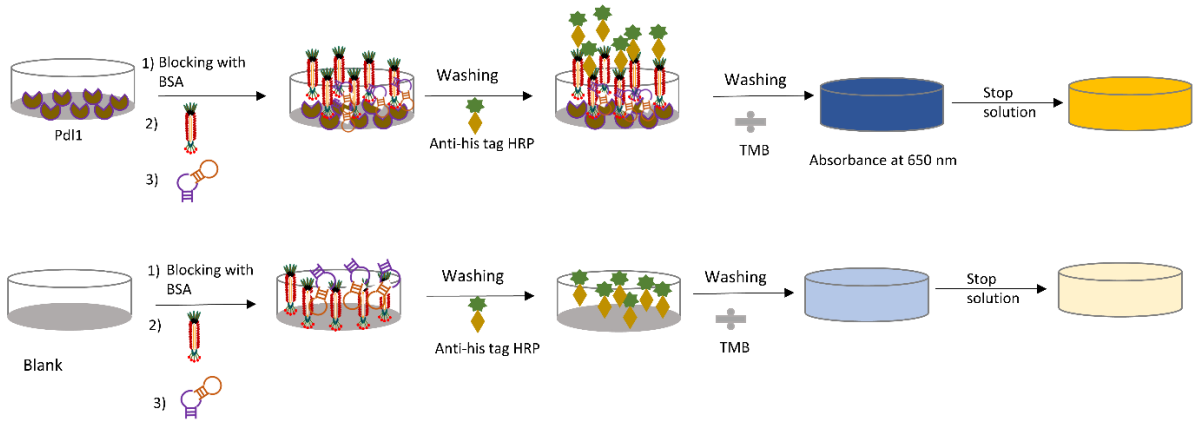


Fig S2. Schematic representation of phage-based ELISA in the presence of MJ5C.

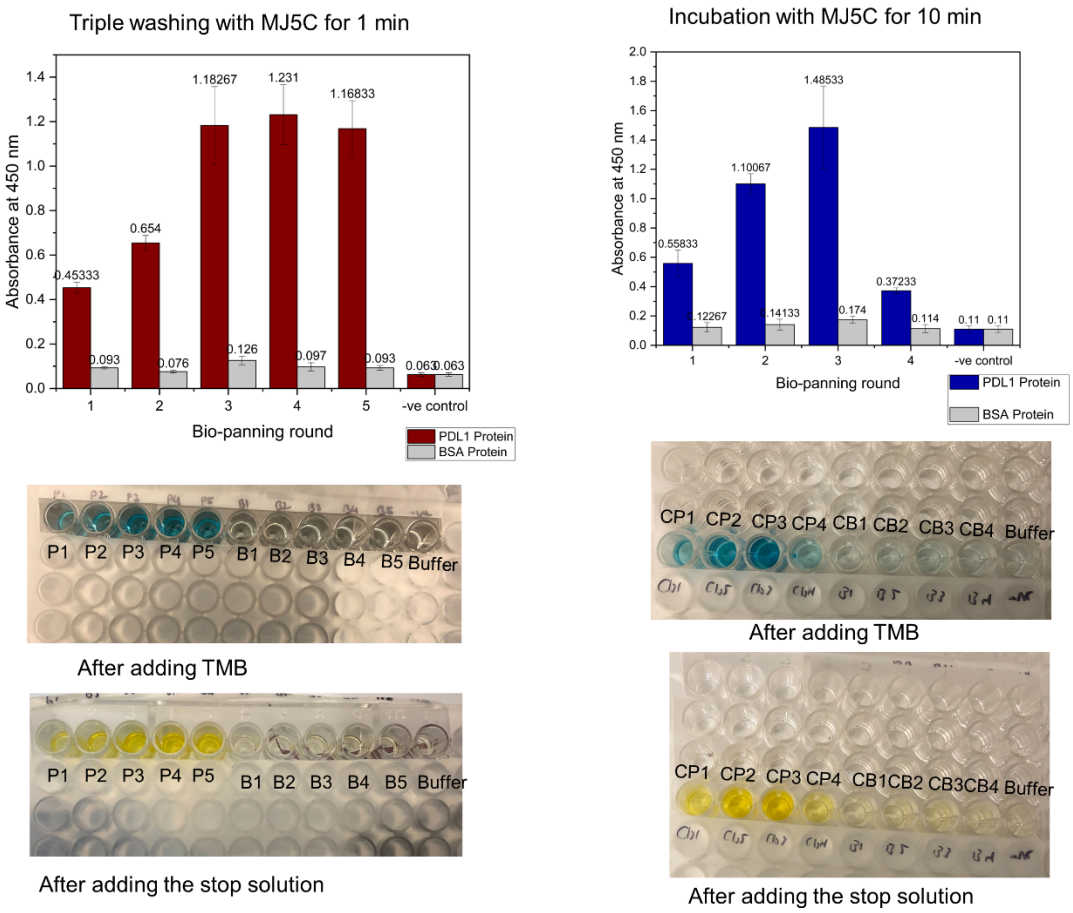


Fig S3. Phage-based competitive ELISA with the MJ5C aptamer for all rounds of bio-panning for APD. Phages from bio-panning rounds 1 to 5 for PD-L1 protein with 3 times washing using the MJ5C aptamer are represented by P1, P2, P3, P4, and P5, respectively. B1, B2, B3, B4, and B5 represent phages from bio-panning rounds 1 to 5 for the control BSA protein. On the other hand, CP1, CP2, CP3, and CP4 represent phages from bio-panning rounds 1 to 4 for PD-L1 protein with 10 minutes of incubation using the MJ5C aptamer, while CB1, CB2, CB3 and CB4 represent phages from bio-panning rounds 1 to 3 for the control BSA protein.

Table S3. Peptide sequences obtained from two approaches of Aptamer-assisted Phage Display.

Approach 1 (10 min incubation) competitive bio-panning

Sr. no	Peptide Sequence	%repetition
1	VTFQSSNEVLSN	25% (5/20)
2	AMPQGAALVTKS	25% (5/20)
3	QAFISLEDGTTG	20% (4/20)

Approach 2 (3-time washing) competitive bio-panning.

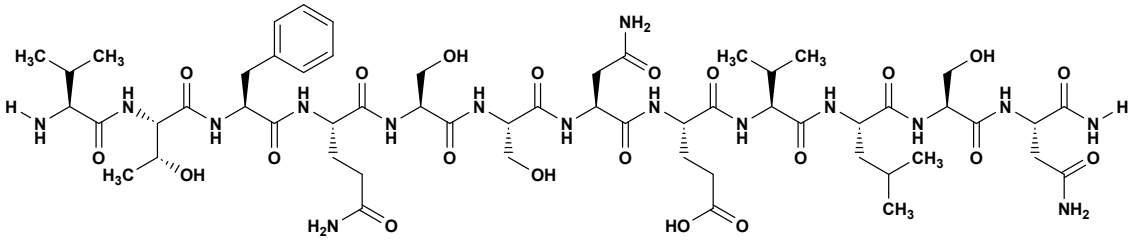
Sr. no	Peptide Sequence	%repetition
1	VTFQSSNEVLSN	70% (14/20)
2	GDASLRGPFLHG	20 % (4/20)
3	QMGFMTSPKHSV	10% (2/20)

Peptide Synthesis, HPLC Purification, and Mass Characterization

Solid Phase Peptide Synthesis technique was used for peptide synthesis. The rink amide resin was used as the solid support. The 50 µmole (100 mg) of resins were soaked in dry DMF for 1 h for swelling and then deprotection was done for removal of Fmoc using 20% piperidine in dry DMF (10 min x 2). For coupling amino acids, the complex of amino acid: DIEA: COMU (150 µmol: 300 µmol: 150 µmol) was used for each coupling in the peptide sequence. Capping was introduced after each coupling. Washing was done by DMF x2, DCM X 1, DMF X 2, DCM X 1, and DMF X 2. After the last coupling, the resin was washed by DMF X 2, DCM 1, DMF X 2, DCM X 1, and MeOH x 6, and was dried under the vacuum in hood. For cleavage, 0.1-0.5 mL of cleavage mixture was added in a ratio of 18:1:1 TFA: H2O: TIPS and incubated for 3 h. For peptide precipitation, pre-cold ether (10 mL) was used, and the solution was centrifuged for 10 min in the hood. The pellet was dried and purified by RP-HPLC.

Peptide Sequence: VTFQSSNEVLSN, calculated molecular weight-: 1322.39 g/mol.

observed molecular weight-: 1322.05 g/mol.



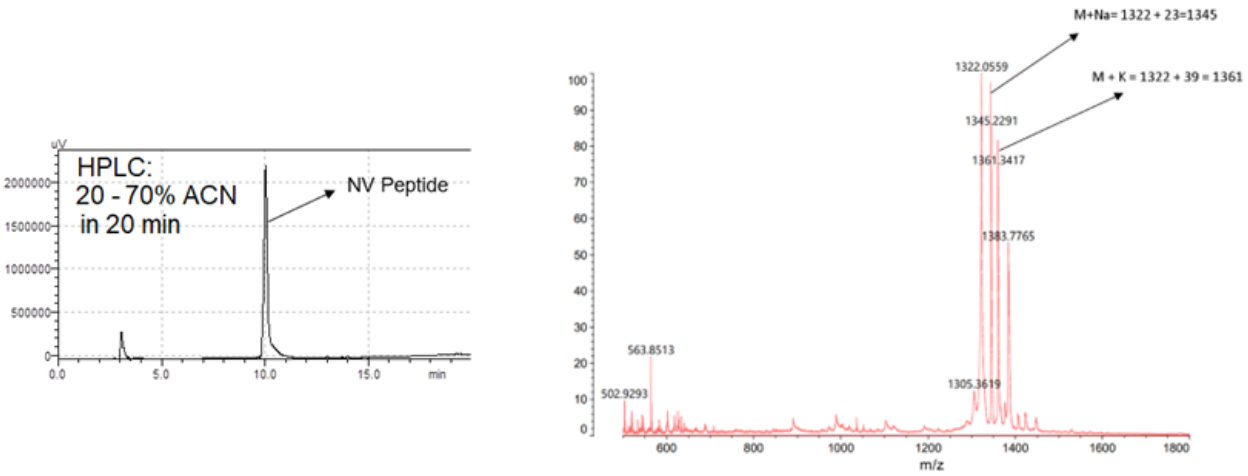
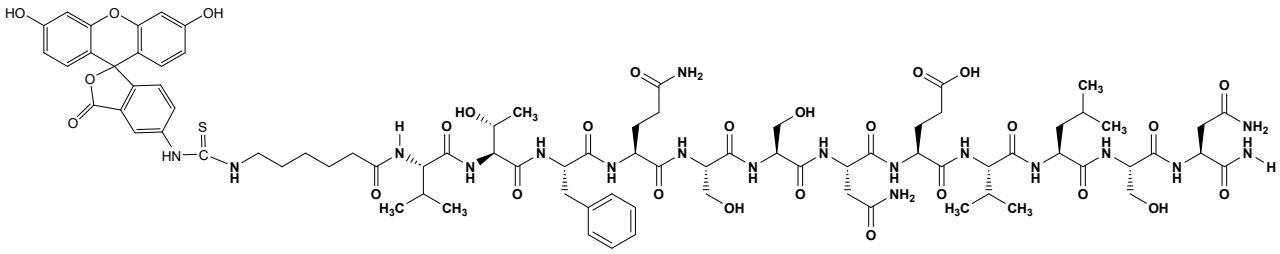


Fig S4. RP-HPLC analysis and MALDI-TOF-MS spectrum for the NV peptide.

FITC- labeled Peptide.

Peptide Sequence: FITC- Ahx- VTFQSSNEVLSN, calculated Molecular weight-: 1826.0 g/mol. observed molecular weight-: 1826.0 g/mol.



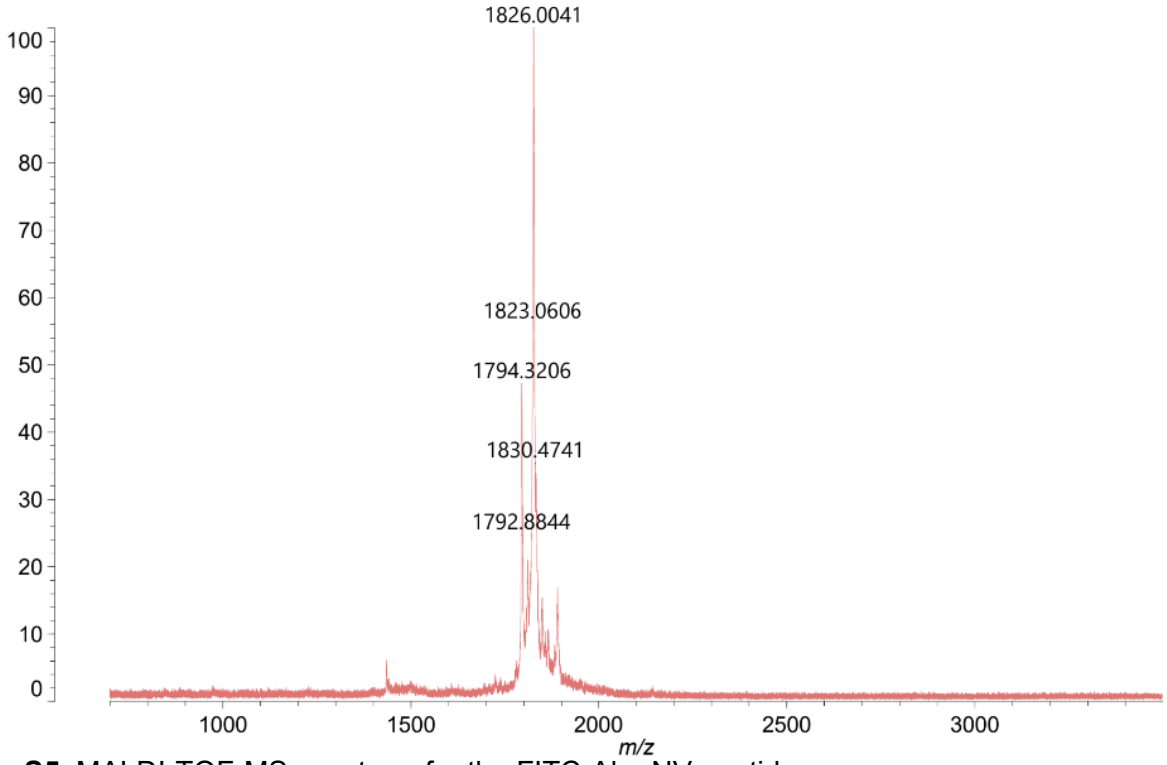


Fig S5. MALDI-TOF-MS spectrum for the FITC-Ahx-NV peptide.

Flow cytometry analysis

Three microliters of Ni-NTA magnetic beads (G Bioscience Lot # 211106) diluted 100 times from the original tube were used for three samples. For each sample, the protein used was 20 picomole. The mixture of protein and bead was incubated for 2 h at room temperature. The mixture was washed twice with PBST (0.1% Tween) and then the required amount of FITC-labeled aptamer and unlabeled peptide were added. The mixture was incubated for another 2 h. The complex was washed twice with 200 µL of PBST (0.1%) and then resuspended with 90 µL of PBS for flow cytometry (Catalog # 0500-4005; Guava easyCyte 5HT), counting approximately 5000 events. Each experiment was run for three trials to calculate the mean fluorescence intensity (X_C).

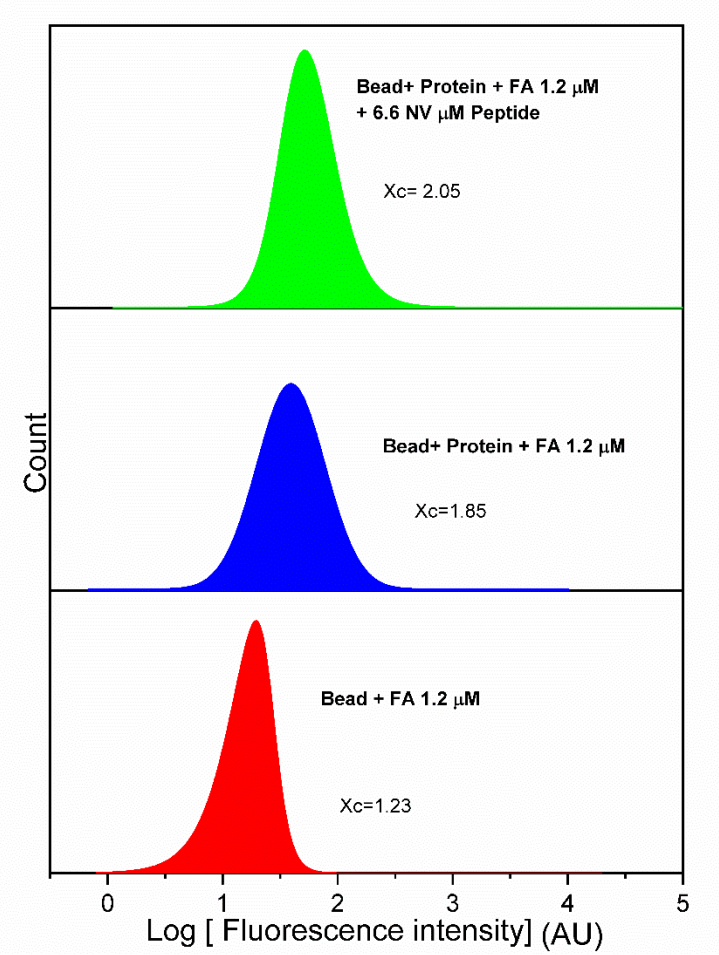


Fig S6. Flowcytometry analysis for the competition assay for FITC labelled MJ5C aptamer and the NV peptide (No FITC).

Fluorescence microscope-based binding assay

A volume of 2 µL of Hispur Ni-NTA resin, was washed twice with 500 µL PBST (0.1% tween). The resin was then diluted in a 50 µL PBS buffer, and 20 pmole of the protein was added. This mixture was incubated at room temperature for 1 h. The Ni-NTA resin/protein complex was washed twice with PBST (0.1% tween) to remove any free protein molecules. The complex was then resuspended in 50 µL of PBS buffer. Subsequently, 10 µM of FITC-labeled peptide was used to bind with the protein/bead complex for 2 h. The protein/bead/FITC-peptide complex was washed twice with PBST (0.1% tween) and resuspended with 50 µL PBS buffer. Finally, 20 µL of the mixture was used for the fluorescence measurement. The fluorescence images were collected using both the green fluorescence and the transmitted light channels by the digital inverted fluorescence microscope (Invitrogen EVOS FL).

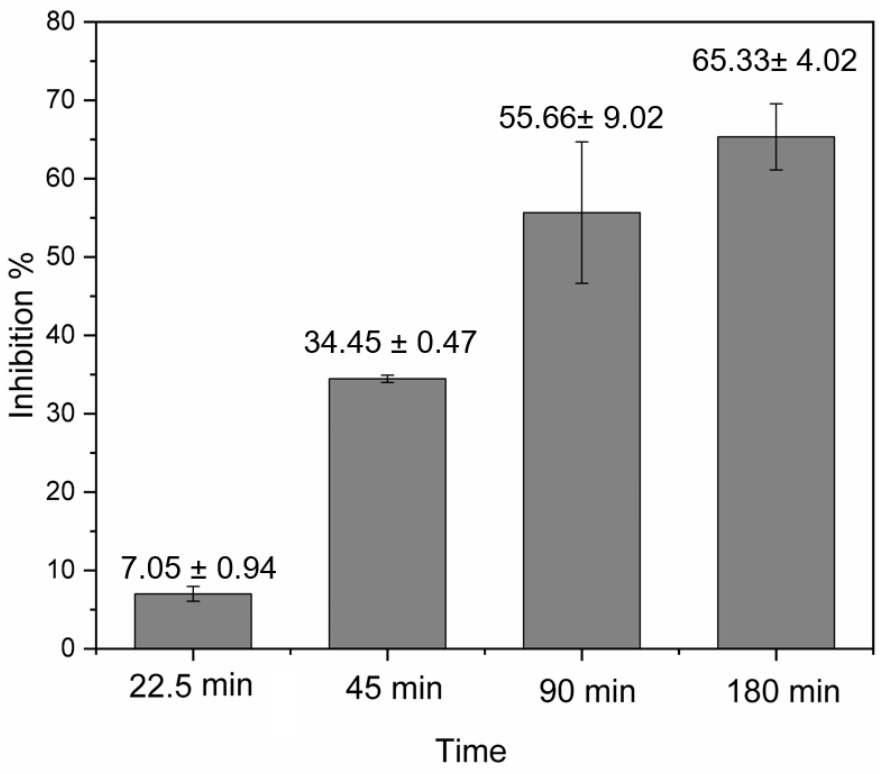


Fig S7. Inhibition efficacies (%) of NV Pep + MJ5C Apt against PD-1/ PD-L1 at different incubation time.