Supplementary Information for

# Automated fast-flow synthesis of the immune checkpoint receptors PD-1 and PD-L1

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# 1 General materials and methods

## 1.1 Reagents and solvents

All reagents were purchased and used as received unless otherwise specified. Fmoc-protected L-amino acids (Fmoc-Ala-OH x $H_2O$, Fmoc-Cys(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu), Fmoc-Val-OH, Fmoc-Trp(Boc)-OH, and Fmoc-Tyr(OtBu)-OH) were purchased from the Novabiochem line (Millipore-Sigma). L-Fmoc-His(Boc)-OH was purchased from Chem-Pep. L-Fmoc-Nle-OH was purchased from Chem-Impex. Fmoc-protected D-amino acids (Fmoc-Ala-OH x $H_2O$, Fmoc-Cys(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-His(Boc)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu), Fmoc-Val-OH, Fmoc-Trp(Boc)-OH, and Fmoc-Tyr(OtBu)-OH) were purchased from both the Novabiochem-line from Millipore-Sigma and Chem-Impex. Fmoc-D-Nle-OH was purchased from Chem-Impex. *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU, ≥97.0%), (7-azabenzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyAOP, ≥97.0%) were purchased from P3 Biosystems. *N,N*-Dimethylformamide (DMF) was purchased from EMD Millipore (Biosynthesis OmniSolv® grade, ≥99.9%) or VWR International (HiPerSolv CHROMANORM® for HPLC, ≥99.9%) and stored over AldraAmine trapping agents (for 1000 – 4000 mL DMF). Diisopropylethylamine (DIEA; 99.5%, biotech grade) was purchased from Sigma-Aldrich. Piperidine (ACS reagent, ≥99.0%), trifluoroacetic acid (TFA, HPLC grade, ≥99.0%), triisopropylsilane (TIPS, ≥98.0%), acetonitrile (HPLC grade), formic acid (FA, ≥95.0%), phenol (ACS reagent, ≥99.0%), diethyl ether ($Et_2O$, ≥99.7%, containing 1 ppm BHT as inhibitor), and 1,2-ethanedithiol (EDT, GC grade, ≥98.0%) were purchased from Sigma-Aldrich. H-Rink Amide (0.17-0.18 mmol/g loading) resin was purchased from PCAS Biomatrix. Water was deionized using a Milli-Q Reference water purification system (Millipore). Nylon 0.22 µm syringe filters were purchased from avantor VWR. 5 mL and 10 mL peptide synthesis reaction vessels were purchased from Torviq. Syringe tip caps were purchased from VWR. Guanidine HCl (GdnHCl, molecular biology grade, ≥99%), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, ≥99.5%), tris(hydroxymethyl) aminomethane (TRIS, ≥99.9%), tris(hydroxymethyl)aminomethane hydrochloride (TRIS HCl, ≥99.0%), monopotassium phosphate (KP*i*, ≥99.0%), dipotassium phosphate (≥ 99.0%), sodium chloride (NaCl, BioXtra, ≥99.5%), glycerol (≥99.5%), sodium hydroxide (≥98%), and hydrochloric acid (36.5-38%) were purchased from Sigma-Aldrich. A 0.5 M solution of tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Bond-Breaker™), was purchased from Thermo Fisher Scientific. 1,4-Dithio-DL-threitol (DTT, ≥99%) was purchased from Chem-Impex.

# 2 Peptide synthesis materials and methods

## 2.1 Automated Flow Peptide Synthesis (AFPS) protocol and set-up

All peptides were synthesized on an automated fast-flow system built in the Pentelute lab (one of either the “Amidator” or “Robosome” instruments). Capitalized letters refer to L-amino acids, lowercase letters refer to D-amino acids. Unless otherwise noted, the following settings were used for peptide synthesis:[1] flow rate = 40 mL/min, temperature = 90 °C (loop) and 85–90 °C (reactor). The 50 mL/min pump head pumps 400 µL of liquid per pump stroke; the 5 mL/min pump head pumps 40 µL of liquid per pump stroke. The standard synthetic cycle involves a first step of prewashing the resin at elevated temperatures for 60 s at 40 mL/min. During the coupling step, three HPLC pumps are used: a 50 mL/min pump head pumps the activating agent, a second 50 mL/min pump head pumps the amino acid and a 5 mL/min pump head pumps DIEA. The first two pumps are activated for 8 pumping strokes to prime the coupling agent and amino acid before the DIEA pump is activated. The three pumps are then actuated together for a period of 7 pumping strokes, after which the activating agent pump and amino acid pump are switched using a rotary valve to select DMF. The three pumps are actuated together for a final 8 strokes, after which the DIEA pump is shut off and the other two pumps continue to wash the resin for another 40 strokes. During the deprotection step, two HPLC pumps are used. Using a rotary valve, one HPLC pump selects deprotection stock solution and DMF. The pumps are activated for 13 pump strokes. Both solutions are mixed in a 1:1 ratio. Next, the rotary valves select DMF for both HPLC pumps, and the resin is washed for an additional 40 strokes. The cycle is repeated for all additional monomers.

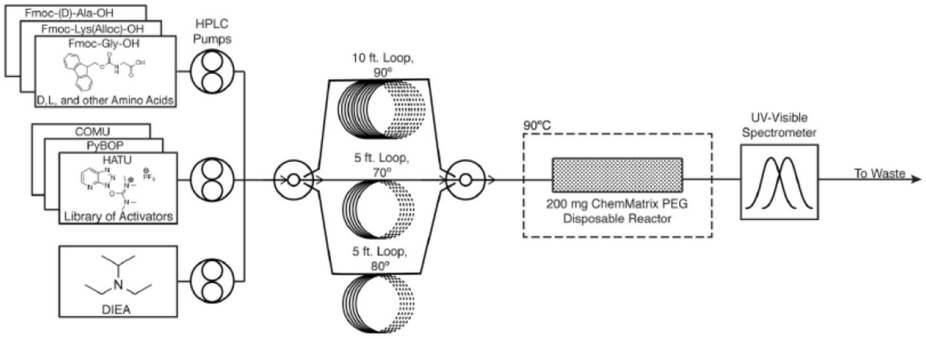



**Figure S1.** Schematic of the AFPS set-up used in this study.(1)

| Parameter | Conditions |
|---|---|
| Temperature | 85-90 °C in reactor, 60 °C in 5’ activation loop (for C and H), 90 °C in 10’ activation loop (for all other amino acids) |
| Flow rate | 40 mL/min |
| Coupling step | 0.40 M amino acids stock in amine-free DMF<br>0.38 M activator stocks in amine-free DMF<br>Coupling conditions:<br>HATU (13 pump strokes) for D, E, F, G, I, K, L, M, Nle, P, W, Y<br>HATU (26 pump strokes) for A, S<br>PyAOP (26 pump strokes) for C, H, N, Q, V, R, T |
| Deprotection step | 40% piperidine in amine-free DMF with 2% formic acid (13 pump strokes) |
| Washing steps | Amine-free DMF (40 pump strokes) |

**Table S1.** Optimized conditions used for the AFPS.[1]

## 2.2 Cleavage and work-up protocols

A fresh solution of a cleavage mixture (a ratio of 1 mL of cleavage mixture per μmol of peptidyl resin) was prepared according to one of the following recipes and used immediately.

1) **Reagent K**: trifluoroacetic acid (TFA) (82.5% v/v), water (5% v/v), phenol (5% v/v, melted in 60 °C water bath prior to use), thioanisole (5% v/v), and 1,2-ethanedithiol (EDT) (2.5% v/v).
2) **Met-reducing cleavage cocktail**: trifluoroacetic acid (TFA) (77.5% v/v), thioanisole ($5\%_{v/v}$), trimethylsilyl chloride (TMSCl) (5% v/v), dimethyl sulfide ($Me_2S$) (5% v/v), triisopropyl silane (TIPS) (5% v/v), 1,2-ethanedithiol (EDT) (2.5% v/v), and triphenylphosphine ($PPh_3$) ($5\%_w$). Adapted from[2].

The cleavage mixture was added to the peptidyl resin in a fritted syringe capped with a luer-tip cap. The syringe was placed on a nutating mixer at room temperature (r.t.) for 3.5 h. At the end of the incubation time, the cleavage supernatant in the reaction syringe was expelled into a 50 mL Falcon tube, and the resin was retained by the internal frit of the reaction syringe. An additional aliquot of TFA (2 mL) was used to wash the resin. Chilled diethyl ether was added to the cleavage supernatant to precipitate the peptide. The Falcon tube was centrifuged at for 10 min at 4000 rcf while maintaining the temperature at 4 °C. The supernatant was discarded and the solid pellet was briefly dried under a $N_2$ stream. The dried peptide was dissolved in 5 mL of a 50:50 mixture of $H_2O$/ACN+0.1% TFA. Vortexing and sonication was used to aid solubilization of the peptide. The mixture was purged with a $N_2$ flow and incubated at 40 °C for 30 min after which time the mixture was flash-frozen with liquid nitrogen, and lyohpilized for at least 16 h.

## 2.3 Preparative High-Performance Liquid Chromatography (Prep HPLC)

Preparative HPLC purification was performed on an Agilent mass-directed purification system (1260 Infinity LC and 6130 Single Quad MS) equipped with a Timberline Instruments TL105 HPLC column heater. The crude lyophilized peptide powder was weighed in a 50 mL Falcon tube and dissolved in a denaturing buffer consisting of 6 M GdnHCl, 100 mM TRIS HCl at pH 7. The mixture was sequentially vortexed and sonicated until no visible precipitate remained. Depending on the solubility of the peptide sequence, additional denaturing buffer was required. The mixture was passed through a 0.22 μm nylon syringe filter. We performed an analytical HPLC run (1) to generate a focused gradient for the preparative HPLC (2). The gradient for the preparative HPLC was determined based on the %B at which the desired protein eluted in the analytical run.

### 1) Analytical HPLC

Column: Agilent Zorbax 300SB-C3 PrepHT, 21.1 x 100 mm, 5 μm
Flow Rate: 20 mL/min
Solvents: A: $H_2O$+0.1% TFA, B: ACN+0.1% TFA
Column Temperature: 50 °C
Gradient: 0-10 min isocratic 5% B, 10-45 min linear to 65% B, 40-45 min linear to 95% B
Loading: ca. 2 mg crude protein

### 2) Focused gradient preparative HPLC

To generate the final preparative HPLC method we centered the focused gradient around the %B at which the desired protein sequence elutes as determined from the analytical run. We used a 0.25% B/min gradient.

Column: Agilent Zorbax 300SB-C3 PrepHT, 21.1 x 100 mm, 5 μm
Flow Rate: 20 mL/min
Solvents: A: $H_2O$+0.1% TFA, B: ACN+0.1% TFA

Column Temperature: 50 °C
Gradient: see Section 3 for gradient used for each protein
Loading: ca. 20-30 mg crude protein

### 2.4 Liquid Chromatography-Mass Spectrometry (LC-MS)

Analysis was performed on an Agilent 1290 Infinity HPLC coupled to an Agilent 6550 Q-TOF with Dual Jet Stream ESI ionization and iFunnel. MS was run in positive ionization mode, extended dynamic range (2 GHz), and low mass range (*m/z* in range 100 to 1700).

**Method 1 – 1-91% B in 10 min (Aeris C4)**

Column: Phenomenex Aeris Widepore C4 200 Å, 150 x 2.1 mm, 3.6 µm
Flow Rate: 0.4 mL/min
Solvents: A: $H_2O$+0.1% FA, B: ACN+0.1% FA
Column Temperature: r.t.
Gradient: 0-2 min isocratic 1% B, 2-8 min linear to 91% B, 8-10 min linear to 95% B (MS data acquisition from 2 to 8 min)

**Method 2 – 1-91% B in 10 min (Zorbax C3)**

Column: Zorbax 300-SB C3, 2.1 x 100 mm, 5 µm
Flow Rate: 0.4 mL/min
Solvents: A: $H_2O$+0.1% FA, B: ACN+0.1% FA
Column Temperature: r.t.
Gradient: 0-2 min isocratic 1% B, 2-8 min linear to 91% B, 8-10 min linear to 95% B (MS data acquisition from 2 to 8 min)

### 2.5 Analytical High-Performance Liquid Chromatography (HPLC)

Analytical HPLC was carried out on an Agilent 1290 series system with UV detection at 214 nm.

**Method 1 – 5-65% B in 30 min (Zorbax C3)**

Column: Zorbax 300-SB C3, 2.1 x 100 mm, 5 µm
Flow Rate: 0.4 mL/min
Solvents: A: $H_2O$+0.1% TFA, B: ACN+0.08% TFA
Column Temperature: r.t.
Gradient: 0-5 min isocratic 5% B, 5-35 min linear to 65% B, 35-40 min linear to 95% B

**Method 2 – 5-65% B in 60 min (Zorbax C3)**

Column: Zorbax 300-SB C3, 2.1 x 100 mm, 5 µm
Flow Rate: 0.4 mL/min
Solvents: A: $H_2O$+0.1% TFA, B: ACN+0.08% TFA
Column Temperature: r.t.
Gradient: 0-5 min isocratic 5% B, 5-65 min linear to 65% B, 65-70 min linear to 95% B

**Method 3 – 1-91% B in 15 min (BEH C4)**

Column: ACQUITY UPLC Protein BEH C4 300 Å, 2.1 x 50 mm, 1.7 µm
Flow Rate: 0.4 mL/min
Solvents: A: $H_2O$+0.1% TFA, B: ACN+0.08% TFA
Column Temperature: r.t.
Gradient: 0-1 min isocratic 1% B, 1-16 min linear to 91% B, 16-17 min isocratic 91% B

**Method 4 – 1-91% B in 15 min (Zorbax C18)**

Column: Zorbax 300-SB C18 RRHD, 2.1 x 50 mm, 1.8 µm
Flow Rate: 0.5 mL/min

Solvents: A: $H_2O$+0.1% TFA, B: ACN+0.08% TFA
Column Temperature: r.t.
Gradient: 0-1 min isocratic 1% B, 1-16 min linear to 91% B, 16-17 min isocratic 91% B

# 3 Synthesis of peptides

## 3.1 Considerations for installing biotin-$PEG_{12}$ handle

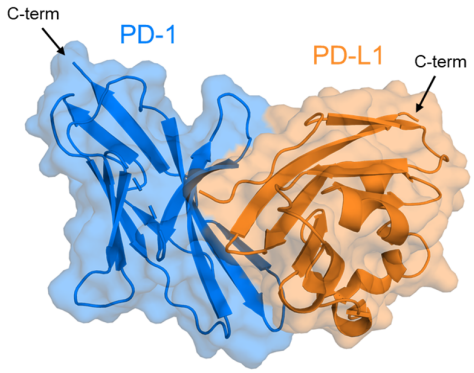
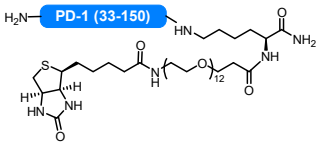



**Figure S2.** The C-termini of PD-1 and PD-L1 were chosen to install the biotin-$PEG_{12}$ handle to minimize interactions with the PD-1/PD-L1 binding interface (pdb 4ZQK).

## 3.2 Programmed cell death protein 1 – PD-1

### Sequence

**Uniprot ID Q15116, residues 33-150, mutations M70Nle C93S M108Nle (119mer)**
NPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRXSPSNQTDKLAAFPEDRSQPGQDSRFRVTQL
PNGRDFHXSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTERRAEK(PEG$_{12}$-Biotin)
X=L-Nle

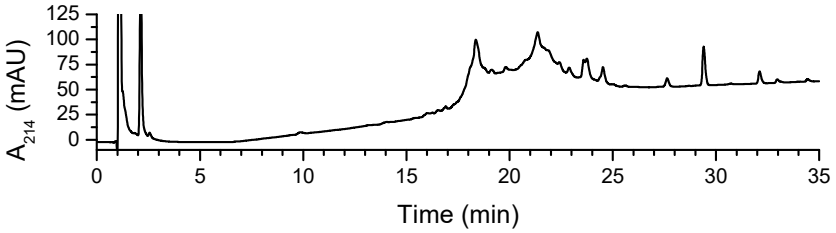



Resin: 131 mg of H-Rink amide ChemMatrix LL (0.17 mmol/g), yielding C-terminal amide
Synthesis time: 6 h
Cleavage protocol: Reagent K (3 h, r.t.), 72 mg crude protein

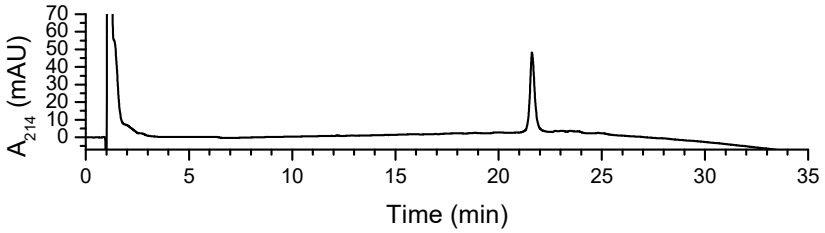
### Preparative HPLC purification

Column: Agilent Zorbax 300SB-C3, 9.4 x 250 mm, 5 µm
Flow Rate: 4 mL/min
Solvents: A: $H_2O$+0.1% TFA, B: ACN+0.1% TFA
Column Temperature: 60 °C
Gradient: 0-10 min isocratic 5% B, 10-25 min linear to 30% B, 25-85 min linear to 50% B, 85-90 min linear to 95% B.
Yield of purified protein: 3.2 mg (1%), MW 14129 g/mol, $MW_{w/TFA}$ 16068 g/mol

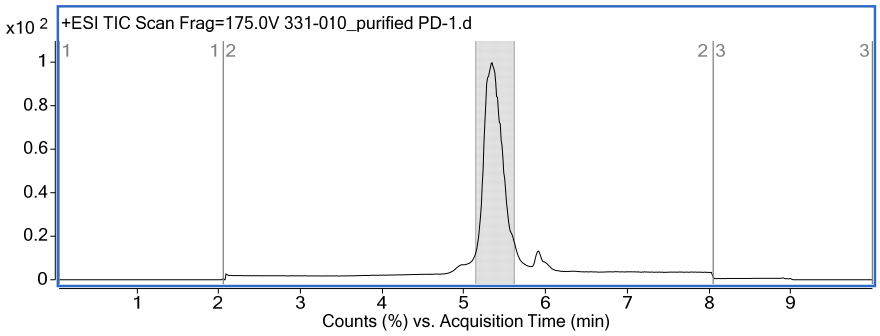
**Analytical HPLC trace of crude protein (Method 1)**



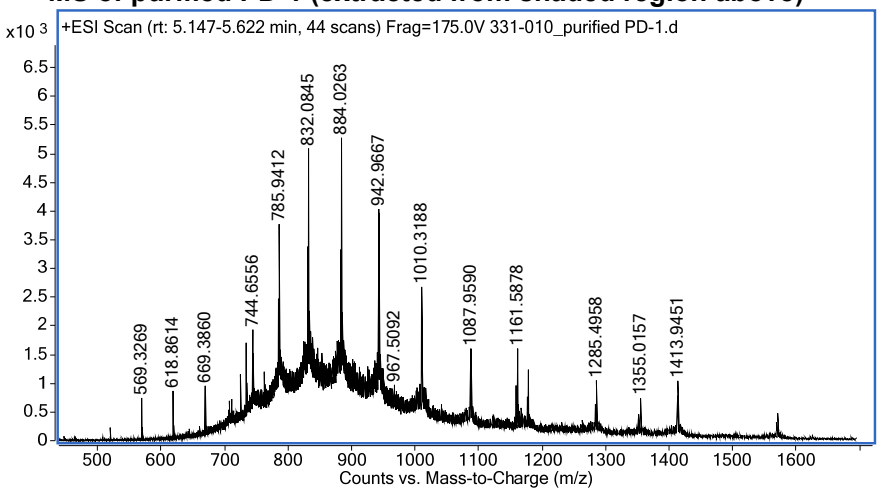

**Analytical HPLC trace of purified PD-1 (Method 1)**



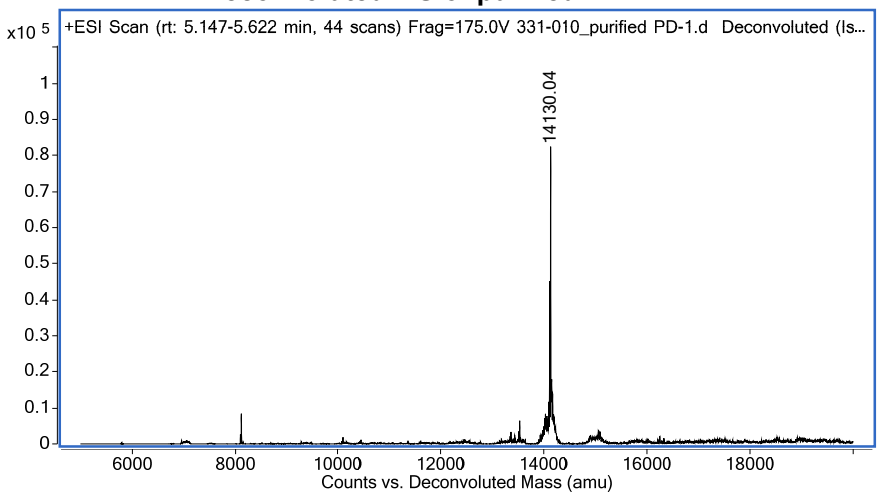
**LC-MS total ion count chromatogram (TICC) of purified PD-1 (Method 1)**




**MS of purified PD-1 (extracted from shaded region above)**




**Deconvoluted MS of purified PD-1**

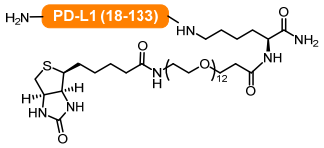



Calculated $[M+H]^+$ m/z 14130.3

Observed $[M+H]^+$ m/z 14130.0

### 3.3 Programmed cell death protein ligand 1 – PD-L1

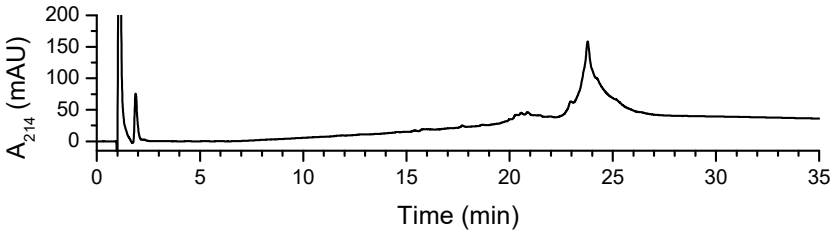
**Sequence**
**Uniprot ID Q9NZQ7, residues 18-133, mutations M36Nle M59Nle M115Nle (117mer)**
AFTVTVPKDLYVVEYGSNXTIECKFPVEKQLDLAALIVYWEXEDKNIIQFVHGEEDLKVQHSSYRQRA
RLLKDQLSLGNAALQITDVKLQDAGVYRCXISYGGADYKRITVKVNAPK(PEG$_{12}$-Biotin)
X=L-Nle



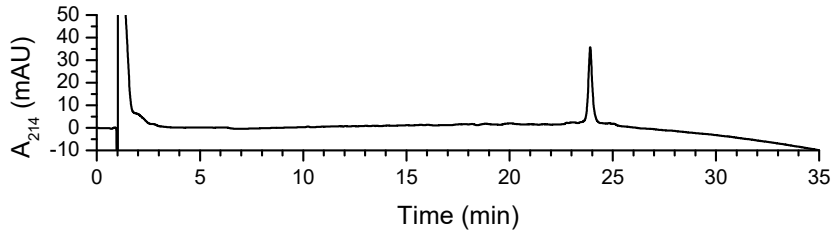

Resin: 133 mg of H-Rink amide ChemMatrix LL (0.17 mmol/g), yielding C-terminal amide
Synthesis time: 6 h
Cleavage protocol: Reagent K (3 h, r.t.), 134 mg crude protein

**Preparative HPLC purification**
Column: Agilent Zorbax 300SB-C3 PrepHT, 21.1 x 100 mm, 5 µm
Flow Rate: 20 mL/min
Solvents: A: $H_2O$+0.1% TFA, B: ACN+0.1% TFA
Column Temperature: 50 °C
Gradient: 0-10 min isocratic 5% B, 10-25 min linear to 35% B, 25-85 min linear to 50% B, 85-90 min linear to 95% B.
Yield of purified protein: 9.2 mg (3%), MW 14114 g/mol, $MW_{w/TFA}$ 16052 g/mol

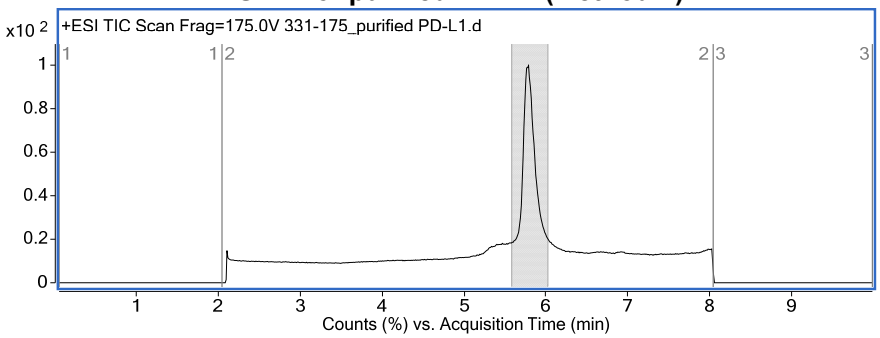
**Analytical HPLC trace of crude protein (Method 1)**



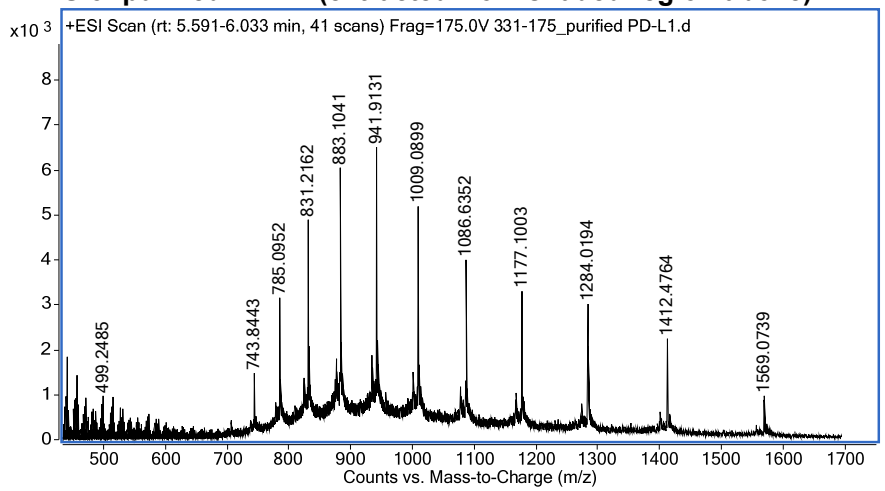

**Analytical HPLC trace of purified PD-L1 (Method 1)**



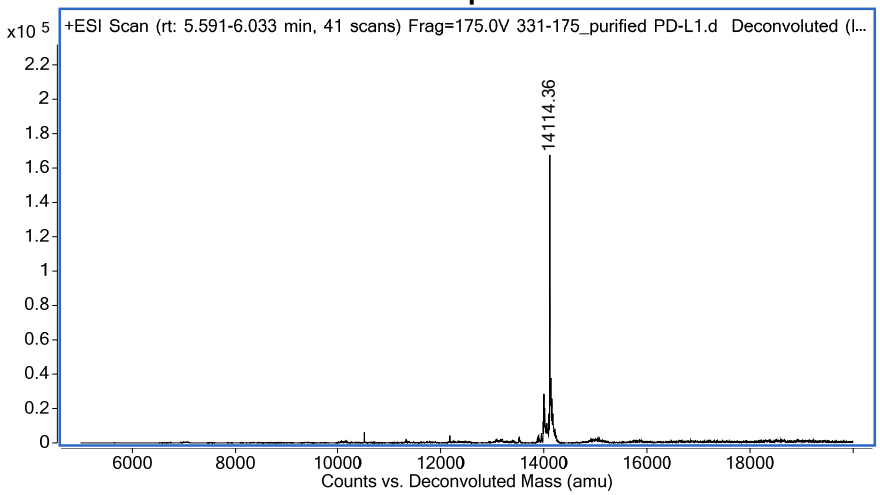
## LC-MS TIC of purified PD-L1 (Method 1)




## MS of purified PD-L1 (extracted from shaded region above)




## Deconvoluted MS of purified PD-L1




Calculated $[M+H]^+$ m/z 14114.8

Observed $[M+H]^+$ m/z 14114.4

# 4 Refolding, purification and characterization of synthetic proteins

## 4.1 Methods

### 4.1.1 Determination of protein concentration $A_{280}$

All measurements were recorded on a Tecan Spark plate reader using a NanoQuant plate. For each sample or buffer blank to be measured, 2 µL was added to each read position. Solution containing the protein of interest was added to 3 spots as 2 µL aliquots, and the absorbance at 280 nm recorded. The molar extinction coefficient of each protein sequence was estimated based on the sequence of the protein via Expasy Swiss Institute of Bioinformatics (web.expasy.org/protparam/). Protein concentration was calculated using Beer's law.

### 4.1.2 Analytical Size Exclusion Chromatography (SEC)

Analytical SEC was carried out on an Agilent 1290 Infinity series system with UV detection at 214 nm and 280 nm.

Column: Superdex 75 Increase 5/150 GL
Flow Rate: 0.25 mL/min
Buffer: 50 mM HEPES, 150 mM NaCl, pH 7.5 or 20 mM TRIS, 200 mM NaCl, pH 7.5
Column Temperature: r.t.

All buffers were filtered through a 0.22 µm PES bottle top filter (Corning). The SEC column was equilibrated in the running buffer for at least 2 column volumes before analysis. The sample was applied to the SEC column and eluted over 15 min (3.75 mL). A gel filtration standard (BioRad) containing Thyroglobulin (bovine) 670 kDa, γ-globulin (bovine) 158 kDa, ovalbumin (chicken) 44 kDa, myoglobin (horse) 17 kDa, and vitamin B12 1350 Da was analyzed prior to protein analysis in the same running buffer.

### 4.1.3 Preparative size exclusion chromatography (SEC)

Preparative SEC was carried out on an Agilent 1290 Infinity series system with UV detection at 214 nm and 280 nm.

Column: Superdex 75 Increase 10/300 GL
Flow Rate: 0.7 or 0.8 mL/min
Buffer: 10 mM TRIS, 150 mM NaCl, pH 8.0 or 10 mM TRIS, 20 mM NaCl, pH 8.0
Column Temperature: r.t.

All buffers were filtered through a 0.22 µm PES bottle top filter (Corning). The SEC column was equilibrated in the running buffer for at least 2 column volumes before analysis. The sample was applied to the SEC column and eluted over 30 min at 0.8 mL/min (24 mL) or 40 min at 0.7 mL/min (28 mL). A gel filtration standard (BioRad) containing Thyroglobulin (bovine) 670 kDa, γ-globulin (bovine) 158 kDa, ovalbumin (chicken) 44 kDa, myoglobin (horse) 17 kDa, and vitamin B12 1350 Da was analyzed prior to protein analysis in the same running buffer.

## 4.2 Protein refolding

### 4.2.1 PD-1 refolding

Purified PD-1 (920 µg) was dissolved in a denaturing buffer (6 M GdnHCl, 100 mM TRIS, pH 8.0) at 10 mg/mL in a plastic tube. An aliquot of a tris(2-carboxyethyl)phosphine hydrochloride (TCEP) solution (345 mM) was added to the denatured protein (5 equiv.) and the solution incubated for 5 min at r.t.. LC-MS was used to verify the protein was converted to the reduced form. The protein solution was diluted with denaturing buffer (6 M GdnHCl, 100 mM TRIS·HCl, pH 8.0) at 0.94 mg/mL and cooled to 4 °C. The protein solution was then rapidly diluted to 0.2 mg/mL protein concentration using a refolding buffer yielding final composition of 1.7 M GdnHCl, 100 mM TRIS, 1 mM Cys, 1 mM cystine, pH 8.0 and incubated at 4 °C for 16 h in a

plastic tube. Next, the protein was dialyzed at 4 °C for 16 h against 100 mM TRIS, pH 8.0 using a 3.5 kDa MWCO dialysis cassette (Slide-A-Lyzer, Thermo Scientific). The protein solution was concentrated using a 10 kDa MWCO spin concentrator (Amicon Ultra, Millipore Sigma). During the concentration step we observed precipitate formation. Purification was performed using preparative SEC (see Section 4.1.3). Isolated fractions were analysed by LC-MS and the ones containing PD-1 were pooled (40 µg, 4% refolding yield). Protein solutions were concentrated and flash frozen prior to storage at -80 °C.

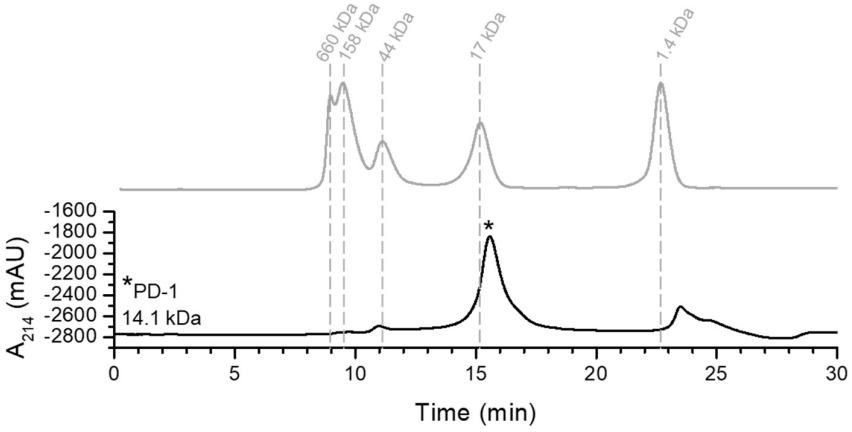



**Figure S3.** SEC purification trace of refolded PD-1.

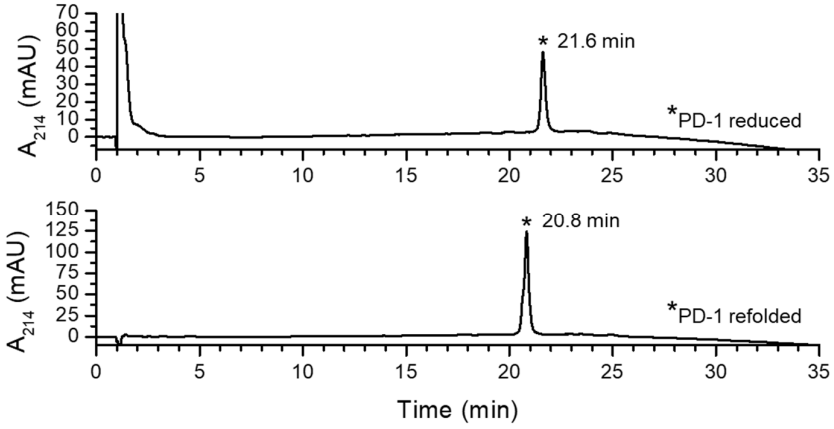



**Figure S4.** Analytical HPLC trace of refolded PD-1 (Method 1).

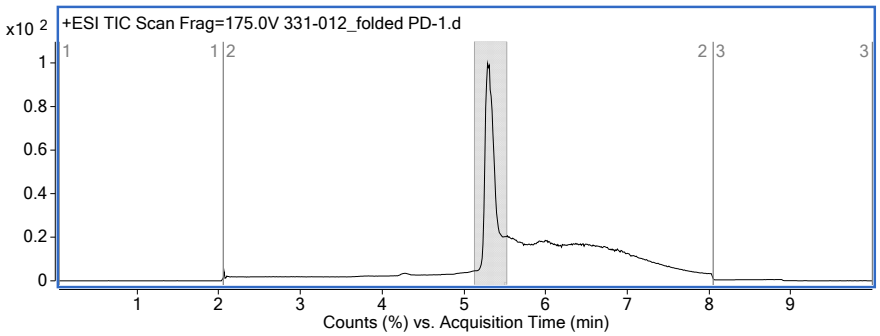



**Figure S5.** LC-MS TICC of refolded PD-1 (Method 1).

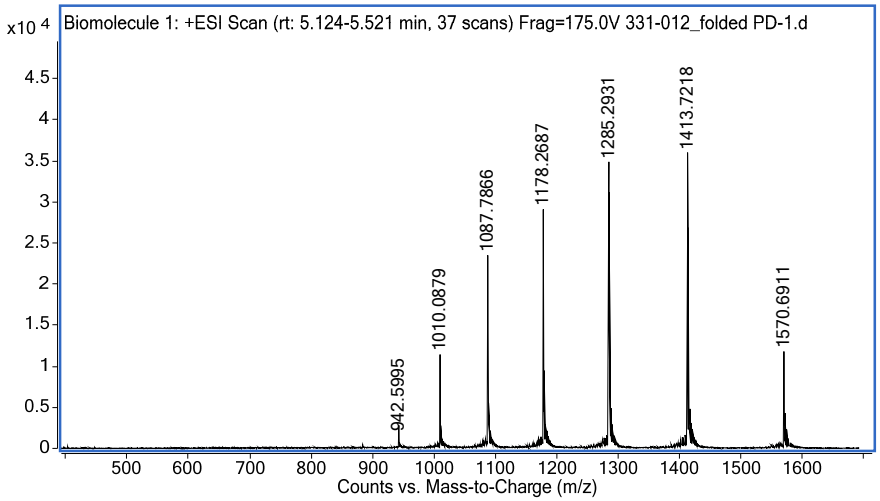



**Figure S6.** MS of refolded PD-1.

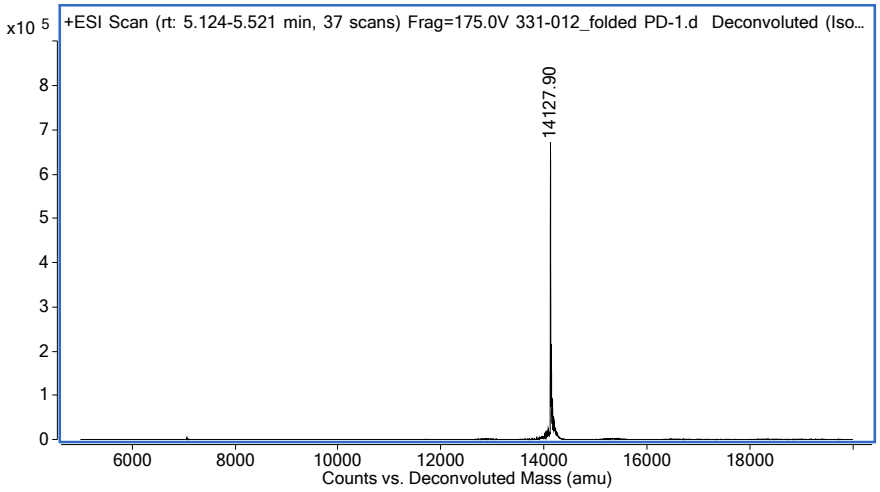



**Figure S7.** Deconvoluted MS of refolded PD-1. Calculated $[M+H]^+$ m/z 14128.3. Observed $[M+H]^+$ m/z 14127.9

### 4.2.2 PD-L1 refolding

Purified PD-L1 (890 µg) was dissolved in a denaturing buffer (6 M GdnHCl, 50 mM TRIS, pH 8.0) at 0.9 mg/mL in a low binding plastic tube. An aliquot of a tris(2-carboxyethyl)phosphine hydrochloride (TCEP) solution (50 mM) was added to the denatured protein (5 equiv.) and the solution incubated for 5 min at r.t.. LC-MS was used to verify the protein was converted to the reduced form. The protein solution was diluted with denaturing buffer to 0.09 mg/mL and cooled to 4 °C. First, the protein was dialyzed at 4 °C for 16 h against 50 mM TRIS, 100 mM NaCl, 0.25 mM GSH, 0.25 mM GSSG, 10%$_v$ glycerol, pH 8.0 using a 10 kDa MWCO dialysis cassette (Slide-A-Lyzer, Thermo Scientific). Second, the protein was dialyzed at 4 °C for an additional 16 h against 50 mM TRIS, 100 mM NaCl, 10%$_v$ glycerol, pH 8.0 using the same dialysis cassette. The protein solution was concentrated using a 10 kDa MWCO spin concentrator (Amicon Ultra, Millipore Sigma). Purification was performed using preparative SEC (see Section 4.1.3). Isolated fractions were analysed by LC-MS and the ones containing PD-L1 were pooled (15 µg, 2% refolding yield). Protein solutions were concentrated and flash frozen prior to storage at -80 °C.

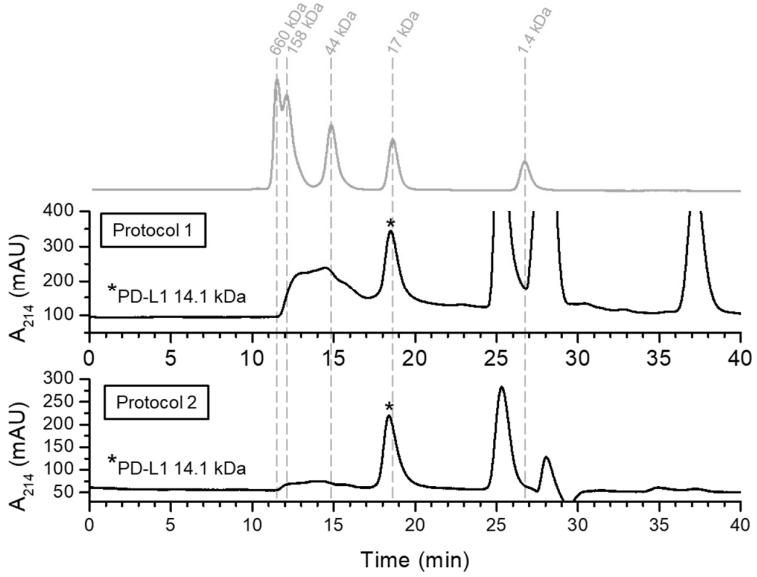



**Figure S8.** SEC purification trace of refolded PD-L1. Protocol 1: 1st step dilution in refolding buffer (100 mM TRIS, 1 M L-Arg, 100 mM NaCl, 2 mM EDTA, 0.25 mM GSH, 0.25 mM GSSG, 10%$_v$ glycerol, pH 8.0, 4 °C), 2nd step dialysis (10 mM TRIS, pH 8.0, 4 °C), SEC purification as described above. Protocol 2: as described above.

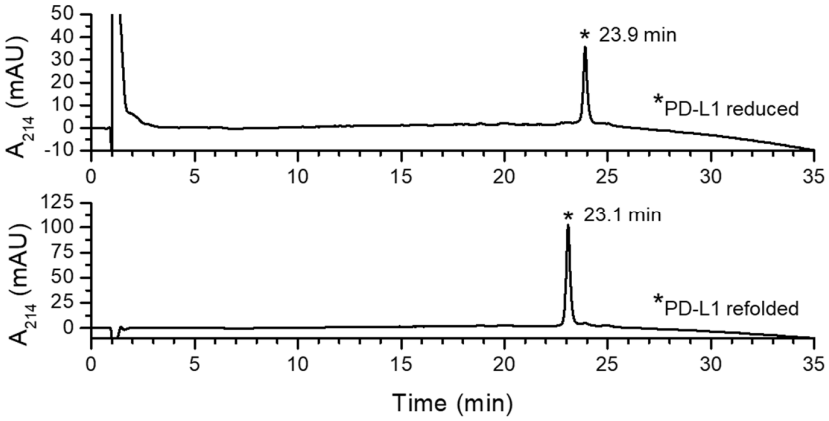



**Figure S9.** Analytical HPLC trace of refolded PD-L1 (Method 1).

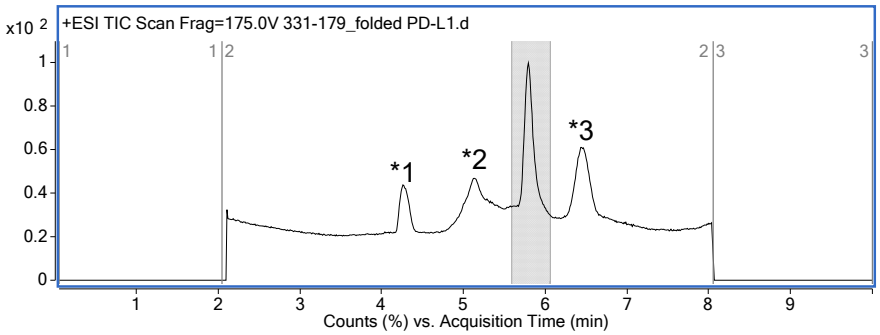



**Figure S10.** LC-MS TICC of refolded PD-L1 (Method 1). *1 and *2 non-protein impurities, *3 PEG contamination (see Figure S13).

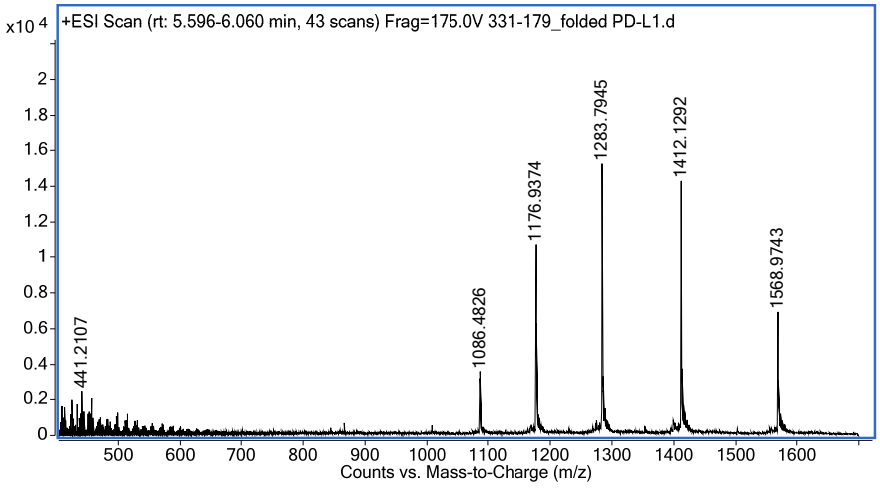



**Figure S11.** MS of refolded PD-L1.

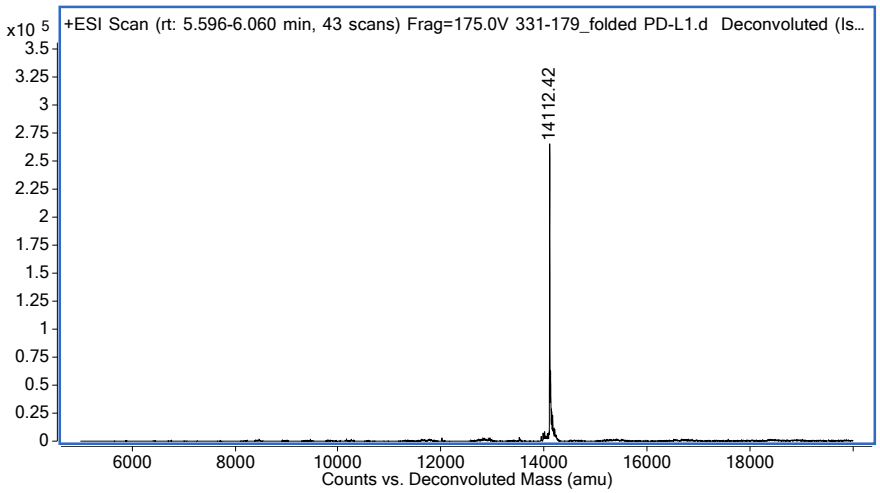



**Figure S12.** Deconvoluted MS of refolded PD-L1. Calculated $[M+H]^+$ m/z 14112.8. Observed $[M+H]^+$ m/z 14112.4.

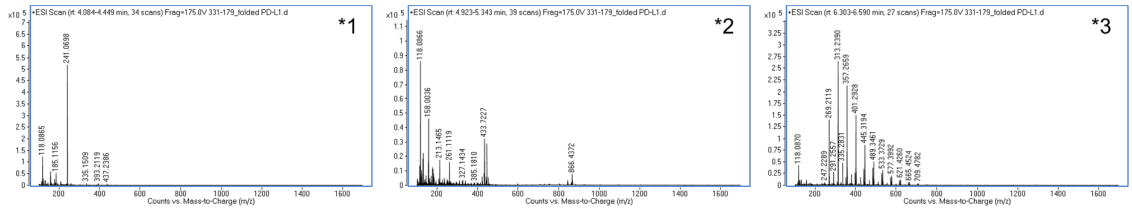



**Figure S13.** MS of impurities in Figure S10.

### 4.3 Biolayer Interferometry (BLI) assay

Biolayer interferometry was performed using a Gator Plus Next Generation Biolayer Interferometry instrument. All assays were performed at 30 °C and stirred at 1000 rpm. Streptavidin-coated probes (Gator Bio cat. #160029) were soaked in pM buffer (Gator Bio cat. #120044) for at least 10 min prior to analysis. Recombinant human PD-L1 was purchased from Abcam (25 kDa). Recombinant human PD-1 (His Tag, calculated molecular weight 17.4 kDa, molecular weight calculated approximately as 41.1 kDa due to glycosylation) was purchased from Sino Biological Inc. Atezolizumab (anti-PD-L1, molecular weight 145 kDa) was purchased from Selleckchem. Assays were performed in 384 well-plates (80 µL per well). The assay was performed in 5 steps:

1. Baseline: probes immersed in wells with pM buffer (30 s)
2. Loading: probes immersed in wells with a solution of synthetic biotinylated protein in pM buffer to allow for immobilization (120 s)
3. Baseline: probes immersed in wells with pM buffer (30 s)
4. Association: probes immersed in wells with a dilution series of recombinant protein in pM buffer to obtain association curve
5. Dissociation: probes immersed in wells with pM buffer to obtain dissociation curve

Specific times used for loading, association, and dissociation are listed for each individual assay below. Synthetic protein-only and recombinant protein-only were used for background subtraction. Apparent dissociation constants ($K_D$) were calculated using a 1:1 binding model with the global fit $R_{max}$ unlinked algorithm or with the steady state model as implemented in the Gator Bio analysis software. The experimental sensorgrams were acquired with the GatorPlus software and replotted with OriginPro.

### 4.3.1 Synthetic PD-1 binding to recombinant PD-L1

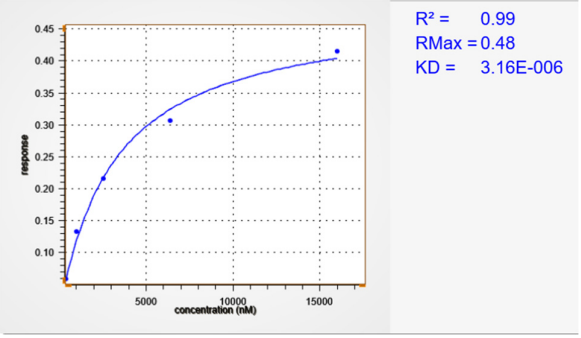



**Figure S14.** Steady-state analysis plot of BLI curves. The measured dissociation constant ($K_D$) is 3.16 μM. Association (180 s). Dissociation (240 s). Synthetic PD-1 concentration for loading 100 nM. Recombinant PD-L1 concentrations 16 μM, 6.4 μM, 2.6 μM, 1.0 μM, 400 nM.

### 4.3.2 Synthetic PD-L1 binding to recombinant PD-1

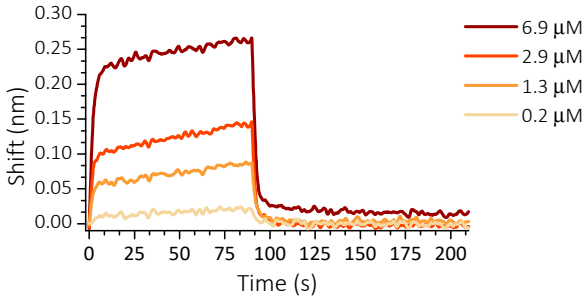



**Figure S15.** BLI assay of synthetic PD-L1 with recombinant PD-1.

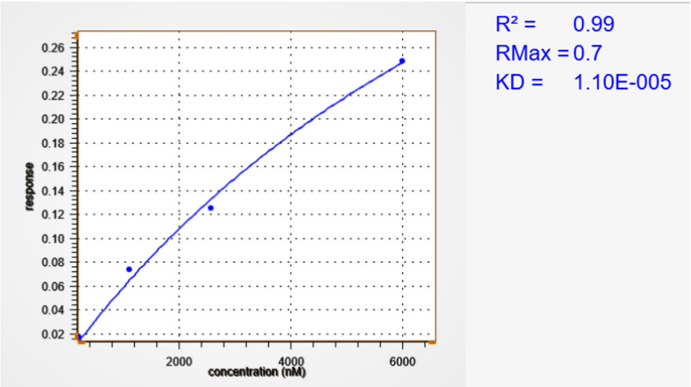



**Figure S16.** Steady-state analysis plot of BLI curves. The measured dissociation constant ($K_D$) is 11.0 μM. Association (180 s). Dissociation (240 s). Synthetic PD-L1 concentration for loading 100 nM. Recombinant PD-1 concentrations 16 μM, 6.86 μM, 2.94 μM, and 540 nM.

### 4.3.3 Synthetic PD-L1 binding to recombinant Atezolizumab

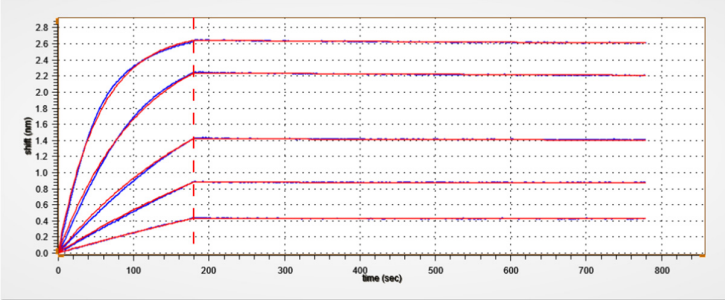



**Figure S17.** Association and dissociation curves (*blue*) and calculated fitted curves (*red*). Measured kinetic constants are $k_{on}$ = 3.62·10$^{5}$ ± 6.92·10$^{2}$ M$^{-1}$s$^{-1}$ and $k_{off}$ = 2.09·10$^{-5}$ ± 7.61·10$^{-7}$ s$^{-1}$. The estimated dissociation constant is $K_D$ = 59 pM. The slow off-rate does not allow accurate determination of the $k_{off}$ using dissociation curve fitting analysis (global fit, 1:1 model). Association (180 s). Dissociation (600 s). Synthetic PD-L1 concentration for loading 100 nM. Recombinant Atezolizumab concentrations 50 nM, 25 nM, 12.5 nM, 6.25 nM, and 3.13 nM.

# 5 References

(1) Hartrampf, N.; Saebi, A.; Poskus, M.; Gates, Z. P.; Callahan, A. J.; Cowfer, A. E.; Hanna, S.; Antilla, S.; Schissel, C. K.; Quartararo, A. J.; Ye, X.; Mijalis, A. J.; Simon, M. D.; Loas, A.; Liu, S.; Jessen, C.; Nielsen, T. E.; Pentelute, B. L. Synthesis of Proteins by Automated Flow Chemistry. *Science* **2020**, *368* (6494), 980–987. https://doi.org/10.1126/science.abb2491.

(2) Nandhini, K. P.; Alhassan, M.; Veale, C. G. L.; Albericio, F.; de la Torre, B. G. Methionine-Containing Peptides: Avoiding Secondary Reactions in the Final Global Deprotection. *ACS Omega* **2023**, *8* (17), 15631–15637. https://doi.org/10.1021/acsomega.3c01058.