

Supplementary Information

1. QCs and Characterization of the NOTA-mal-Nb

SEC

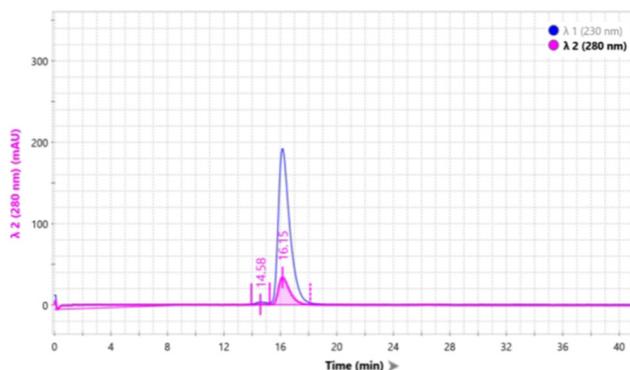


Figure S1. Size exclusion chromatography analysis (UV profile) of the purified NOTA-mal-(hPD-L1) Nb showing a 98% purity at 280 nm (pink line). Analysis at 230 nm (blue line) gives similar results.

SDS PAGE

2 μg and 10 μg of the conjugated Nb (NOTA-mal-hPD-L1) was analysed under non-reducing (2x LaemmLi Sample Buffer, Bio-Rad) and reducing conditions (LaemmLi Sample buffer supplemented with 5% dithiothreitol, DTT, Bio-Rad). A pre-stained ladder (PageRuler™ Prestained Protein Ladder, 10 to 180 kDa, ThermoFischer Scientific) and Bovin Serum Albumin (BSA, Bio-Rad Protein Assay Standard II) were used as reference on the SDS PAGE gel (Novex™ WedgeWell™ 16% Tris-Glycine Gel, ThermoFisher Scientific).

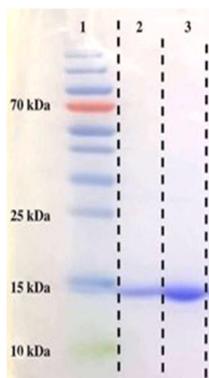


Figure S2. SDS PAGE result. Lane 1 = Pre-stained ladder; Lane 2 = 2 μg of purified NOTA-mal-(hPD-L1) Nb in non-reducing conditions; Lane 3 = 10 μg of NOTA-mal-(hPD-L1) Nb in non-reducing conditions.

2. ESI-Q-ToF

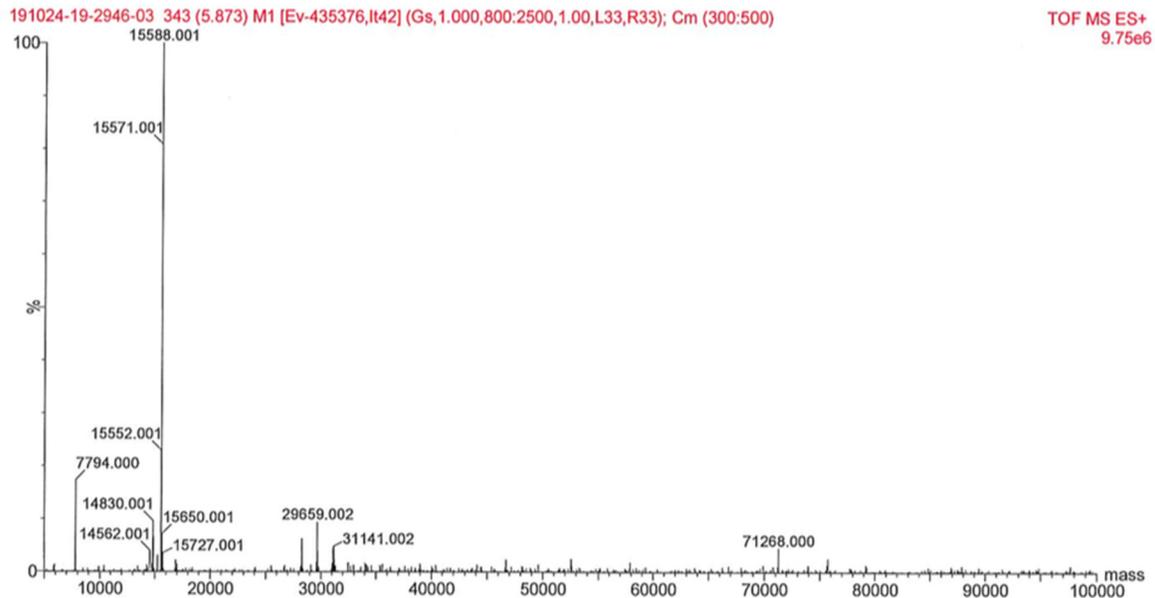


Figure S3. Mass determination analysis of the NOTA-mal-Nb showing the mass peak at 15588 Da (calculated = 15588 Da) and the deamidated compound at 15571 Da. Starting Nb (15165 Da) or Nb-dimer (30330 Da) are not visible

3. Surface Plasmon Resonance

Measurements were performed on a Biacore T200 device (GE Healthcare) at 25°C and using Hepes-buffered saline (HBS; 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Tween 20) as running buffer. The recombinant protein was dissolved to 10 µg/mL in 10 mM NaOAc pH 5.0 for immobilization on a CM5 sensor chip using linkage chemistry with 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide (EDC) and *N*-hydroxy-succinimide (NHS). Unreacted EDC-NHS linkers were blocked with 1 M ethanolamine-HCl.

The modified Nbs were tested for affinity on immobilized human PD-L1 protein in SPR. To this end, 9 different Nb dilutions were allowed to bind to the target protein for 120 sec and dissociation was monitored for 160 sec. The equilibrium dissociation constant K_D was calculated by fitting the obtained sensor-grams to theoretical curves, assuming 1-to-1 binding geometries, using Biacore Evaluation software.

4. Thermostability of the Nb (melting temperature)

The melting temperature of the starting Nb and NOTA-modified Nb was determined using the Protein Melting program of a RealTime PCR machine. Samples were prepared by mixing 12.5 µg of Nb with 7.5 µL of Cypro Orange dye (Thermo fisher, 300 x dilution) in PBS to a 25 µl final volume. Blank samples contained NH₄OAc. Samples were prepared in triplicates.

5. Stability Studies

In Vitro Stability Studies of [⁶⁸Ga]Ga-NOTA-mal-(hPD-L1) Nb

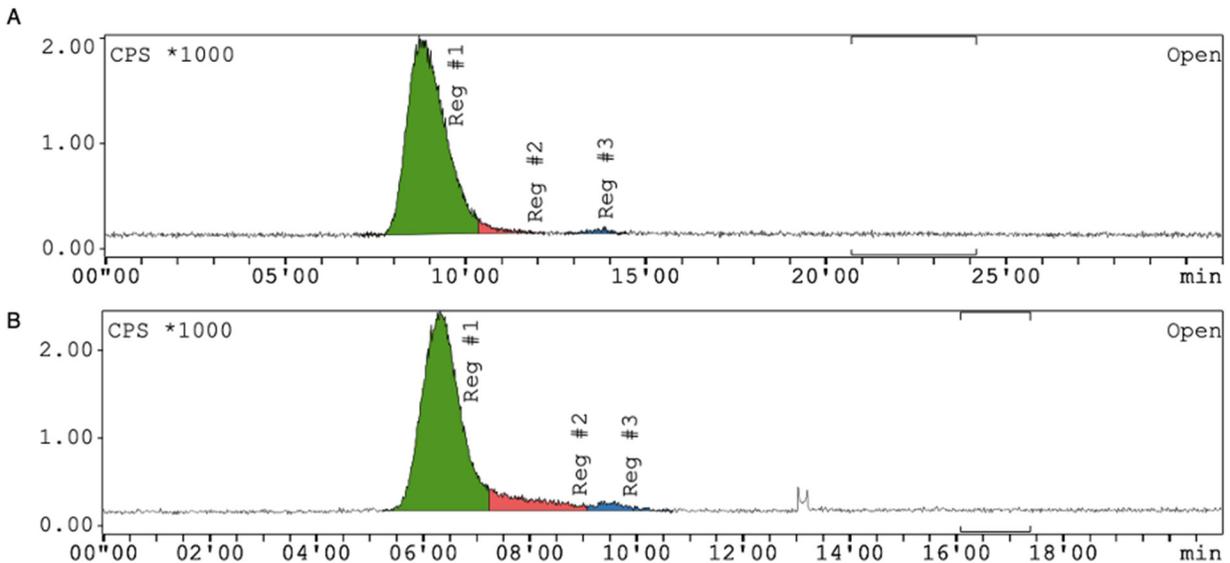


Figure S4. *In vitro* stability study of [⁶⁸Ga]Ga-NOTA-mal-(hPD-L1) Nb. (A) Radio-SEC showing > 95% radiochemical purity of [⁶⁸Ga]Ga-NOTA-mal-(hPD-L1) Nb after 180 min in injection buffer at room temperature. (B) Radio-SEC showing > 85% radiochemical purity of [⁶⁸Ga]Ga-NOTA-mal-(hPD-L1) Nb after 180 min in human serum at 37°C.

⁶⁸Ga-labeling and Stability of NOTA-mal-(hPD-L1) Nb after Two Months Storage

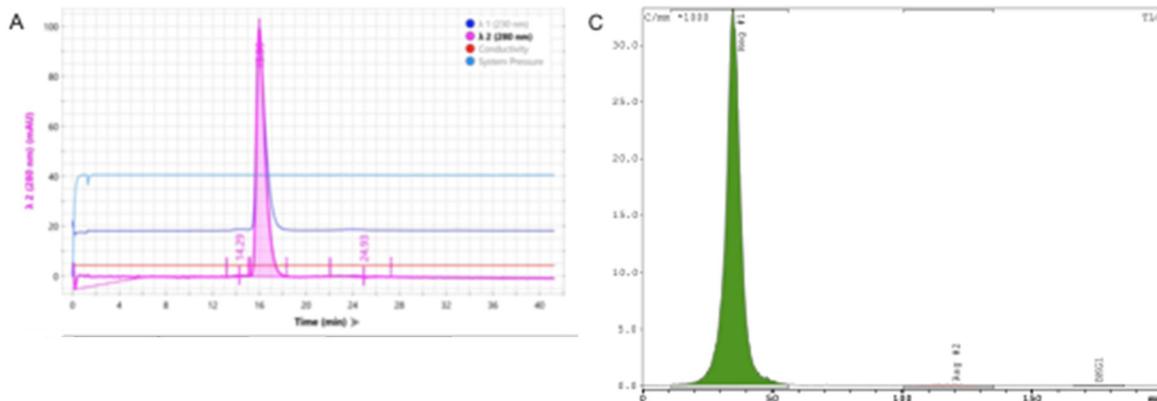


Figure S5. Stability of NOTA-mal-(hPD-L1) Nb after two months storage at -30°C in 0.1 M NH₄OAc. (A) Size exclusion chromatography analysis of NOTA-mal-(hPD-L1) Nb at 280 nm showing > 98% purity. Rt(Nb) = 15.99 min, Rt(dimer) = 14.29 min, Rt(degradation) = 21–28 min. (B) Radio-iTLC of [⁶⁸Ga]Ga-NOTA-mal-(hPD-L1) Nb (after purification) after 180 min in injection buffer showing a RCP > 99%. Rf(⁶⁸Ga-Nb) = 0, Rf(free ⁶⁸Ga) = 1.

6. Tumor Targeting

Biodistribution Profile

Table S1. Biodistribution and tumor uptake 1 h 20 post-injection of the site-specifically [⁶⁸Ga]Ga-NOTA-mal-(hPD-L1) Nb in hPD-L1^{POS} and hPD-L1^{NEG} tumor bearing athymic nude mice (N = 12 and N = 6 / group, respectively), mean of %IA/g for each organ or tissue, with the standard deviation (SD).

| | hPD-L1 ^{POS} | | hPD-L1 ^{NEG} | |
|-----------------------------------|-----------------------|------|-----------------------|------|
| | %IA/g | SD | %IA/g | SD |
| Blood | 0.38 | 0.13 | 0.42 | 0.03 |
| Heart | 0.17 | 0.12 | 0.15 | 0.01 |
| Lungs | 0.34 | 0.13 | 0.44 | 0.13 |
| Liver | 0.97 | 0.45 | 0.87 | 0.26 |
| Spleen | 0.54 | 0.35 | 0.36 | 0.11 |
| Pancreas | 0.10 | 0.03 | 0.12 | 0.01 |
| Kidneys | 27.90 | 5.07 | 23.90 | 7.63 |
| Stomach (without content) | 0.11 | 0.04 | 0.13 | 0.04 |
| Small intestine (without content) | 0.24 | 0.42 | 0.13 | 0.03 |
| Large intestine (without content) | 0.12 | 0.03 | 0.14 | 0.02 |
| White fat from pelvis | 0.17 | 0.29 | 0.10 | 0.03 |
| Muscle | 0.08 | 0.04 | 0.11 | 0.04 |
| Bone | 0.14 | 0.06 | 0.18 | 0.03 |
| Lymph nodes | 0.19 | 0.06 | 0.28 | 0.10 |
| Brown fat | 0.13 | 0.04 | 0.13 | 0.03 |
| Tumour | 1.86 | 0.67 | 0.42 | 0.03 |

Flow Cytometry

hPD-L1 expression on the cells from the dissected tumors was assessed. The dissected tumors stored in PBS (max. 12 h) were cut, placed in 5 mL RPMI medium and treated using a gentleMACS™ dissociator. 150 μ L of Collagenase from *Clostridium histolyticum* (Sigma Aldrich, 10.000 U/mL in PBS) and 150 μ L of Dispase (Sigma Aldrich, 32 mg/mL in water) were added to the mixture and incubated at 37°C for 40 min. 2 μ L of DNase (1 mg/mL in PBS) was added to the mixture and treated 2 times on the gentleMACS™ dissociator. After filtration and centrifugation, red blood cell lysis buffer was added. The mixture was centrifuged, and the pellet was incubated with 100 μ L of anti-mouse CD16/32 Antibody (clone 93, BioLegends, 1/200 dilution in PBS/BSA) for 10 min at RT. The pellets were incubated 30 min at 4°C with either 20 μ L of isotype control solution (PE-CF594 Mouse IgG1, k Isotype Control, Clone X40 RUO, BD Horizon, 1.6/100 μ L of PBS/BSA) or 20 μ L of staining solution (PE-CF594 Mouse Anti-Human CD274, Clone MIH1 RUO, BD Horizon, 1.6/100 μ L of PBS/BSA). Samples were resuspended in PBS/BSA for FC reading (BD FACSCelesta™, BD Biosciences). % of cells expressing hPD-L1 is measured as the difference between the % of positive cells from the stained sample and the % of positive cells from the isotype control sample.

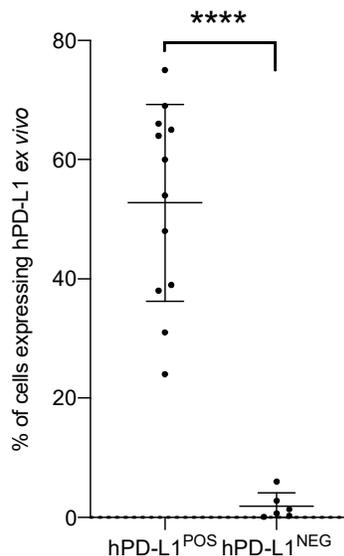


Figure S6. *Ex vivo* assessment of hPD-L1 expression by flow cytometry. hPD-L1 expression in the hPD-L1^{POS} tumors after dissection of the animals, expressed in % of cells expressing hPD-L1, as compared with the hPD-L1^{NEG} tumors (****; $p < 0.0001$).