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SUPPORTING INFORMATION

Development of an Actinium-225 Radioimmunoconjugate for Targeted Alpha Therapy against SARS-CoV-2

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Experimental Section

Caution: ^{225}Ac is a hazardous radionuclide that should be manipulated in specifically designated facilities in accordance with appropriate safety controls.

Chemicals and Materials

^{225}Ac trichloride was purchased from the National Isotope Development Center (Oak Ridge, TN). Metal solutions were prepared in standardized 0.1 N hydrogen chloride. S-2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (p-SCN-Bn-DOTA) was purchased from Macrocyclics (Plano, TX). Bradford reagent was obtained from Bio-Rad (Hercules, CA). Tris(2-carboxyethyl)phosphine -hydrochloride (TCEP), ammonium acetate, sodium acetate, sodium bicarbonate, sodium chloride, hydrogen chloride, ethylenediaminetetraacetic acid (EDTA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, N-hydroxysulfosuccinimide, Tween-20, poly-L-Lysine, bovine serum albumin (BSA), 30K Amicon Ultracentrifugal filter, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). HEPES buffer, MES buffer, Dulbecco's phosphate buffer saline (PBS), and Dulbecco's modified Eagle medium (DMEM) were purchased from Life Technologies (Carlsbad, CA). All solutions were prepared with Milli-Q water.

Antibody Characterization

S01 and nsIgG antibodies were obtained from ATUM (Newark, CA) as PBS solutions, and stored before use in aliquots at -80 °C. Antibody selection details had been previous reported elsewhere.[1] Epitope binning was performed via surface plasmon resonance (SPR) with a Carterra LSA platform equipped with a HC200M sensor chip (Carterra, Salt Lake City, UT). The assay was performed at 25 °C with HBSTE-BSA buffer, which contained 10 mM HEPES pH 7.4, 3 mM EDTA, 150 mM NaCl, 0.05 % Tween-20, and 0.5 mg/mL BSA. The SPR surface preparation was performed with 25 mM MES buffer (pH 5.5) supplemented with 0.05 % Tween-20. Before use, the chip was activated with a solution made of 130 mM 1-ethyl-3-(3-

dimethylaminopropyl) carbodiimide and 33 mM N-hydroxysulfosuccinimide in MES buffer. Antibody immobilization was carried out using 96PH at 15 µg/mL diluted in sodium acetate (10 mM, pH 4.3). The RBD2 antigen was injected for 4 min at 3.6 µg/mL (100 nM), followed by a 4 min injection of the analyte antibody at 30 µg/mL (200 nM). For each cycle, the surface was regenerated twice with 30 s pulses of elution buffer (Pierce IgG elution Buffer pH 2.8 with 1M NaCl). Data processing and analysis was carried out with the Epitope Tool (Carterra). For kinetic measurements, the RBD2 antigen was injected with 5 min association at six different concentrations, from at 500 nM down to 1.49 nM , and with 10 min dissociation.

Fluorescent microscopy was performed with HEK-293T cells displaying ACE2 (catalog number 631289, Takara Bio USA, San Jose, CA). The cells were grown in DMEM (supplemented with 10 % FBS). ACE2 receptor expression was activated by adding puromycin (1 µg/mL) into the cell media. Cells were seeded to 8-chambered borosilicate glass slides (Nunc LabTek, ThermoFisher Scientific, Waltham, MA) coated with poly-L-Lysine. Cells were fixed by treating them with 4 % paraformaldehyde in PBS for 15 min. The resulting cells were washed twice with PBS and blocked using 2 % BSA solution in PBS for at least 30 min. Regarding the binding assay, RBD2-sfGFP were pre-incubated with unlabeled antibodies for 1 h, and subsequently added to the chamber slides containing the ACE2 HEK-293T cells. Unbonded proteins were washed, and the binding was characterized with a Zeiss Axio Observer Z.1 (Carl Zeiss AG, Jena, Germany).

Antibody Conjugation and Characterization

200 µg of each antibody were buffer exchanged into sodium bicarbonate (pH = 9.5) using a 30k Amicon Ultracentrifugal filter. Adhering to the filter manufacturer's protocol, the antibodies were each filtered into the new buffer three times at 14,000 × g for 5 min, and recollected at 1,000 × g for 2 min. At pH of 9.5, a percentage of solvent exposed lysine side

chain amines along the antibody peptides begin to deprotonate, offering sites for conjugation to the bifunctional chelator, p-SCN-Bn-DOTA.

p-SCN-Bn-DOTA was first dissolved in DMSO to a concentration of 8 mg/mL, and then added in a 40:1 DOTA:antibody ratio to each S01 and nsIgG stocks. The reaction solutions were subsequently incubated at 37 °C for 60 min to allow for nucleophilic addition to occur between the isothiocyanate linker of p-SCN-Bn-DOTA and deprotonated amines of lysine side chains, forming stable thiourea linkages between antibody and chelator. The resulting antibody-DOTA solutions were buffer exchanged into ammonium acetate (pH = 5.5) to remove free p-SCN-Bn-DOTA molecules remaining in solution, and to prepare the conjugate solutions for radiolabeling.

Conjugation efficiency was assessed by determining the number of chelators bound per antibody unit, and retention of antibody native macrostructure integrity throughout conjugation. To evaluate antibody conjugate macrostructure, polyacrylamide gel electrophoresis (PAGE) was performed on each antibody before and after conjugation to p-SCN-Bn-DOTA. Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry with an AB SCIEX TF4800 system (AB Sciex, Framingham, MA) was performed on each antibody and antibody conjugate. The median mass shift between the pre- and post-conjugation antibodies was used to determine the median number of chelators stably conjugated per antibody.

Radioimmunoconjugate Radiolabeling and Characterization

2 mCi of ^{225}Ac was dissolved into 0.1 M HCl, resulting in a molar concentration of 15.3 µM. ^{225}Ac was then injected into S01-DOTA, and nsIgG-DOTA in a 1:200 ^{225}Ac:DOTA ratio based upon the median number of chelators stably conjugated per antibody previously determined by MALDI-TOF mass spectrometry. The resulting solutions were each incubated at 45 °C for 90 min, before being buffer exchanged into 1X PBS using 30K Amicon

Ultracentrifuge filters in order to remove any free ^{225}Ac ions remaining in solution. Five rounds of filtration (14,000 × *g* for 5 min each) were completed for the samples during the buffer exchange, with recollection of the samples (1,000 × *g* for 2 min), which included the accurate measurement of the volume of solution recovered from the filters. This volume measurement would be used to calculate the activity concentration (μCi/μL) of the radioimmunoconjugates and radiochemical yield.

Characterization of both radioimmunoconjugates was performed in three steps. A Bradford assay was used to determine the concentration and yield of protein (antibody) remaining in each treatment after the final buffer exchange. Activities of the injection solutions were determined by gamma spectroscopy via a P-Type High Purity Germanium gamma spectrometer using the 218.00 keV line of ^{221}Fr. Before counting, samples were allowed to sit at rest for more than 1 hour prior to measurements to allow ^{221}Fr to reach secular equilibrium with ^{225}Ac, allowing the ^{221}Fr activity to be equivalent to ^{225}Ac. Radio thin layer chromatography was performed with a BioScan System 200 (10 mm, high efficiency collimator from Eckert & Ziegler, Wilmington, MA) and silica gel/glass plates to determine the percentage of total ^{225}Ac radioactivity present in solution that is stably bound to antibody-conjugated chelator. The mobile phase was made of deionized water (pH shifted to 10.5 with KOH), and 50 mM EDTA to move the free metal.

Cell Viability Studies

The *in vitro* studies were performed on freshly prepared radioimmunoconjugates, which were serial-diluted in PBS (10 mM, pH 7.4) to achieve the desired activity. The experiments were performed with commercial HEK-293T cells that display the spike protein on their surface (catalog number P30908, Innoprot, Bilbao, Spain). The immunofluorescence assay results demonstrating the presence of SARS-CoV-2 spike protein in the cell surface are publicly available at Innoprot's technical data sheet. The cells were seeded at 5,000 cells/well

in 96-well plates (Corning Incorporated, Corning, NY). The cells were grown in DMEM medium supplemented with 10 % FBS, and kept at 37 °C in 5 % CO_2 / 95 % air atmosphere. At 12 h post-seeding, the medium was replaced by fresh one, and the cells were treated with additional media containing the various radioimmunoconjugates (the serial dilutions were performed in PBS, and later in cell medium), for a total volume of 150 µL per well. After treatments, the cells were further incubated for 4 days. Cell viability was assessed using CellTiter-Glo assay (Promega, Madison, WI) following the manufacturer's protocol. Luminescence was recorded with a SpectraMax Plus 96-well plate reader (Molecular Devices, Sunnyvale, CA), and the results were normalized to the no-treatment control for each plate. Each experiment was repeated independently three times, and results were expressed as means ± one standard deviation.

Table S1. Amino acid sequence of the complementarity-determining regions of S01. These regions were included into a standard IgG1 scaffold to obtain the full S01 antibody.

EIVMTQSPSSLSASVGDSVTITCQASQDISNYLNWYQQKPGKAPKLLIYDASNLETGVPSRFSGGGSGTDFTLTITSLQPEDIATYYCQQYDNFPPTFGPGTKVDIKSG GSTITSYNVYYTKLSSSDTQVQLVESGGGLVKPGGSLRLSCAASGFTVGSNYMSWVRQAPGKGLEWVSVIYSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRA EDTAVYYCARGSSGAWYFDLWGRGTLVTVSS

Light region (red), heavy region (green), and linker between regions (grey).

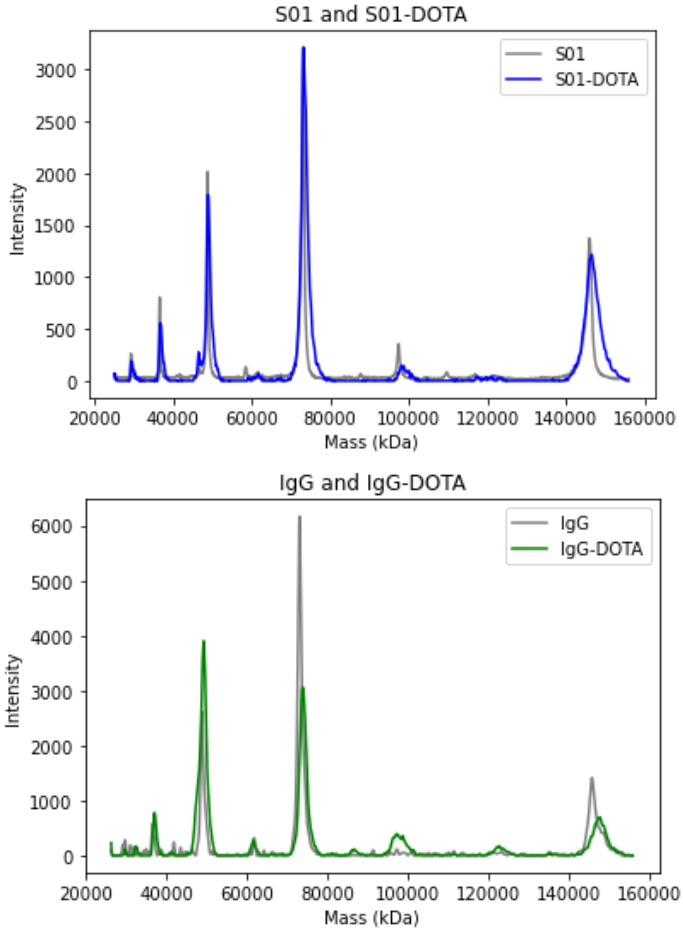

Figure S1. Full mass spectra of antibodies and DOTA-antibodies. Each plot is an overlay of the antibody pre- and post-conjugation with p-SCN-Bn-DOTA.

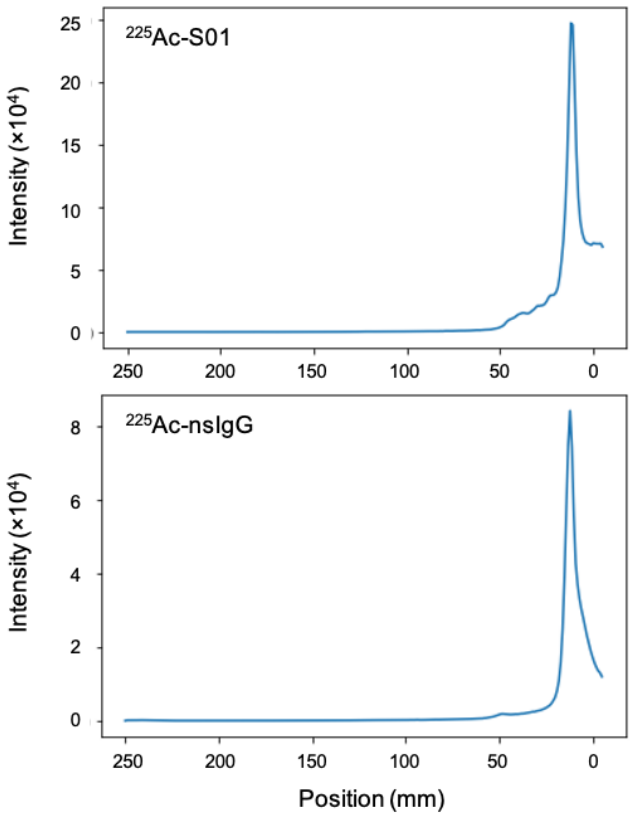

Figure S2. Radio thin layer chromatography of radioimmunoconjugates.

References

1. A. M. Lillo, G. S. Waldo, N. Velappan and H. T. B. Nguyen, *US Department of Energy* 2021, DOI: 10.2172/1760553.