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# High-Throughput, Quantitative Analysis of Peptide-Exchanged MHCI Complexes by Native Mass Spectrometry

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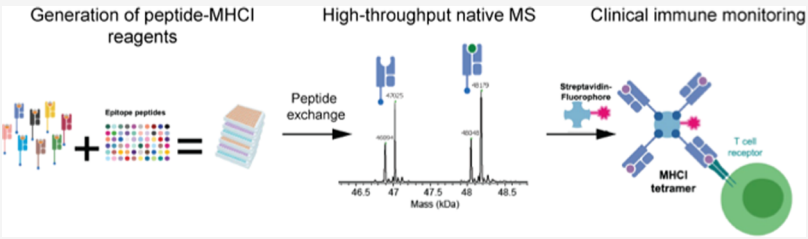


**ABSTRACT:** Immune monitoring in cancer immunotherapy involves screening CD8+ T-cell responses against neoantigens, the tumor-specific peptides presented by Major histocompatibility complex Class I (MHCI) on the cell surface. High-throughput immune monitoring requires methods to produce and characterize small quantities of thousands of MHCI−peptide complexes that may be tested for a patient's T-cell response. MHCI synthesis has been achieved using a photocleavable peptide that is exchanged by the neoantigen; however, assays that measure peptide exchange currently disassemble the complex prior to analysis—precluding direct molecular characterization. Here, we use native mass spectrometry (MS) to profile intact recombinant MHCI complexes and directly measure peptide exchange. Coupled with size-exclusion chromatography or capillary-zone electrophoresis, the assay identified all tested human leukocyte antigen (HLA)/peptide combinations in the nanomole to picomole range with minimal run time, reconciling the synthetic and analytical requirements of MHCI−peptide screening with the downstream T-cell assays. We further show that the assay can be "multiplexed" by measuring exchange of multiple peptides simultaneously and also enables calculation of $Vc_{50}$, a measure of gas-phase stability. Additionally, MHCI complexes were fragmented by top-down sequencing, demonstrating that the intact complex, peptide sequence, and their binding affinity can be determined in a single analysis. This screening tool for MHCI−neoantigen complexes represents a step toward the application of state-of-the-art MS technology in translational settings. Not only is this assay already informing on the viability of immunotherapy in practice, the platform also holds promise to inspire novel MS readouts for increasingly complex biomolecules used in the diagnosis and treatment of disease.

## INTRODUCTION

Current efforts in cancer vaccine development involve harnessing the patient's immune system to identify and clear tumor cells.[2−4] One strategy consists of engineering a patient's T cells to target the antigenic peptides containing the "non-self" mutations identified in a tumor through genomic sequencing.[3] Nonetheless, this modality of personalized cancer immunotherapy (CIT) requires knowledge of what a patient's T cells will recognize as immunogenic. For this reason, "immune monitoring" involves screening a patient's T-cell responses against neoantigens, which are the peptides processed and presented on Major histocompatibility complex Class I (MHCI) upon production by tumor-specific mutations.[1]

MHCI are cell-surface, noncovalent, heteromeric complexes made up of the human leukocyte antigen (HLA) and the $\beta$-2-microglobulin (B2M, 11 kDa).[5,6] Humans have six different HLA alleles; however, the HLA allele is highly polymorphic: nearly 20,000 HLA class I alleles have been identified.[7] These proteins are expressed in virtually all somatic cells, recognizing and signaling the physiological state of a cell to the effector cells of the immune system (*e.g.*, T cells). They do this by presenting neoantigen peptides on the cell surface.[8] Peptides are generated by proteasomal degradation of cytosolic proteins and then transported to the endoplasmic reticulum and bound to MHCI molecules after recognition based on peptide length and sequence.[4] After transport to the cell surface, the peptide−MHCI complex helps determine histocompatibility. In healthy cells, presented peptides are autologous to those tolerated by CD8+ T cells. However, when a cell begins to express mutant sequences, for example, in a cancer tumor, the "non-self"

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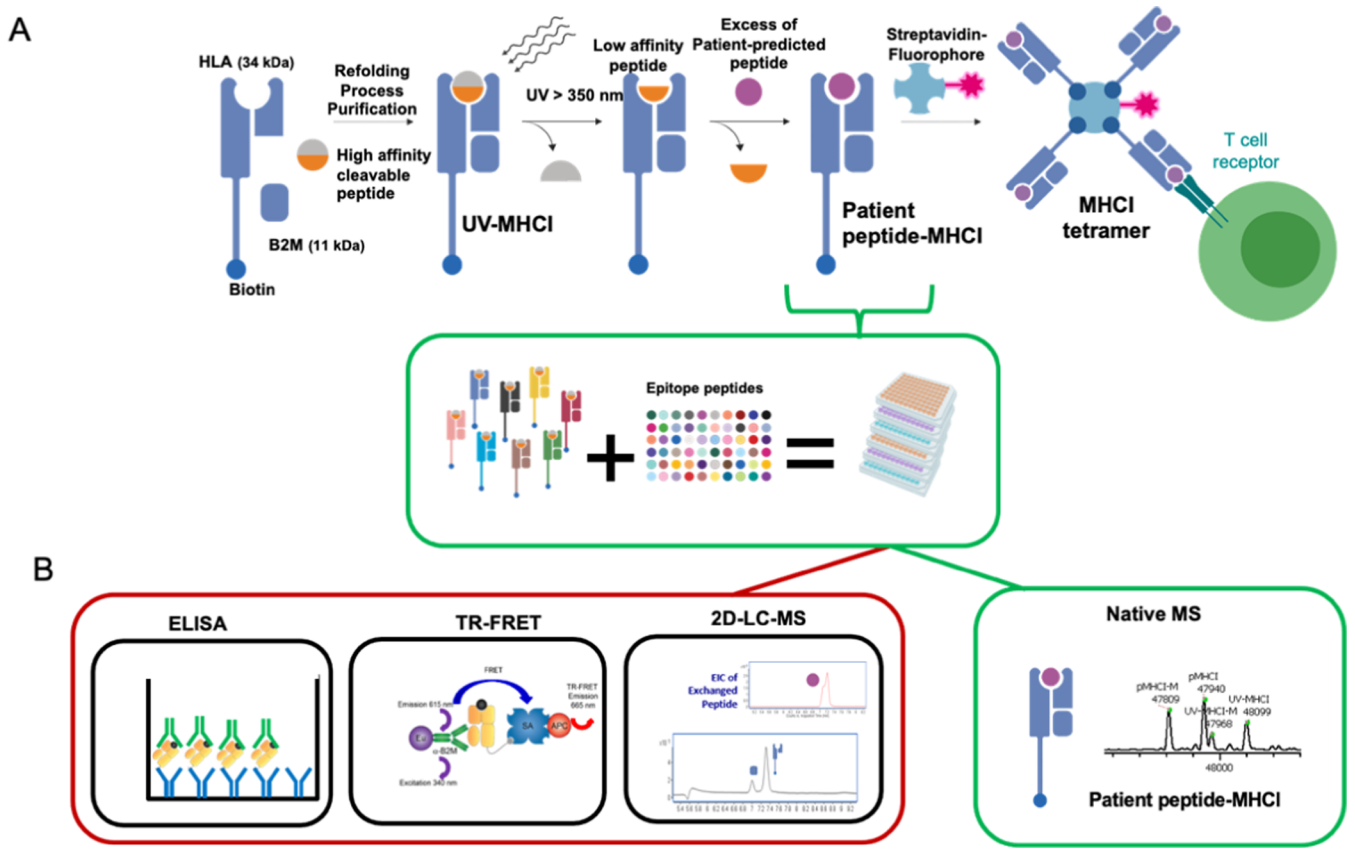


**Figure 1.** Schematic illustrating synthetic workflow to create (A) and validate (B) reagents for immune monitoring of cancer vaccines. (A) Recombinant HLA, B2M, and UV-peptide are reconstituted into the UV-MHCI complex. These are then irradiated with UV light in the presence of excess epitope/neoantigen peptide. Upon confirmation of complex formation (see B), pMHCI complexes are tetramerized using a streptavidin fluorophore for T-cell staining assays. (B) For informative immune monitoring of cancer vaccine patients, a large number of HLA alleles have to be tested for binding with many predicted neoantigen peptides, resulting in hundreds to thousands of pMHCI combinations. Current tools that assess pMHC formation are ELISA, time-resolved fluorescent resonance energy transfer (TR-FRET), and two-dimensional liquid chromatography−mass spectrometry (2D-LCMS) (red). This article introduces native mass spectrometry (green) as a novel assay to quantify and validate pMHCI complex formation.

antigenic peptides are presented, enabling the CD8+ T cells to recognize the neoantigen peptides presented on the MHCI and induce an anti-tumor response.[3] Given this anti-tumor role for neoantigen-specific T cells, there is great interest in leveraging and amplifying the cancer immunity cycle for therapeutic applications.

Broadly, this inspiration involves enabling a patient's T cells to mount an immune response against the cancer tumor. To enable this response, genomic sequencing of a patient's tumor sample in personalized medicine is coupled with computational algorithms to predict those peptides that have a high affinity for MHCI.[9,10] These methods in turn predict hundreds of mutant neoantigen candidates that may or may not be immunogenic or conducive to a therapeutic strategy.[11] Hence, the candidate peptides must first be experimentally tested for binding against hundreds of unique possible MHCI molecules, given the polymorphic nature of the various encoded human HLA alleles.[12] These *in vitro* "reconstituted" peptide−MHCI complexes are then used in T-cell staining assays that test for the immunogenicity of the peptide−MHCI complex, indirectly informing on the patient's ability to mount an immune response against the mutated cancer cells.[1] Thus, high-throughput "immune monitoring" requires methods to produce and characterize small quantities of the hundreds of peptide−MHC complexes (pMHCI) that can be tested for an individual patient's T-cell response.

Successful *in vitro* loading of antigen peptides into MHCI molecules is essential to preparing these reagents. These complexes are inherently unstable and require an 8- to 10-amino acid peptide to be loaded in the MHCI groove to remain intact. Synthetic methods to generate recombinant pMHCI complexes from *Escherichia coli* have been achieved using a photocleavable peptide ultraviolet-MHCI (UV-MHCI) that is exchanged by the neoantigen peptide (made by *de novo* synthesis) under UV exposure (yielding the pMHCI).[13−15] After reconstitution, the pMHCI is tetramerized for use in T-cell staining assays (Figure 1A). Current analytical methods that validate the formation of pMHCI and that quantify the extent of peptide exchange (percent of UV-MHCI remaining) currently rely on indirect measurements, namely, enzyme-linked immunosorbent assay (ELISA)-based protocols, or denaturing mass spectrometry methods that disassemble the pMHCI complex prior to analysis (Figure 1B, red), providing limited information as to the degree of exchange and the quality of the resultant MHCI molecules.[1] There are thus academic and industrial opportunities to develop a sensitive and high-throughput platform for the direct detection and complete molecular characterization of the reagents used in immune monitoring (Figure 1B, green).


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In this report, we use native mass spectrometry (nMS), which preserves noncovalent interactions during MS analysis, to identify and validate the exact recombinant pMHC complexes relevant in cancer immunotherapy.[16,17] Clinical decisions are made based on T-cell staining data that use the pMHCI reagents analyzed in this report; hence, we demonstrate the assessment of both peptide affinity and the quality of the noncovalent complexes. The deployment of nMS platforms to characterize noncovalent complexes across a variety of biological and industrial systems speaks to a general trend that aims to preserve biomolecules in their "native" state during characterization,[18−21] namely, to preserve and learn information about the higher order organization and stoichiometry of protein complexes.[22] This is achieved using nondenaturing electrospray buffers, *e.g.*, ammonium acetate, and ionization conditions that preserve noncovalent interactions. Moreover, we detail the optimization of the platform in its sensitivity and throughput using capillary-zone electrophoresis,[23,24] size-exclusion chromatography,[25] and multiplexing strategies to ensure compatibility with the reagent and logistical requirements of downstream translational and clinical assays. Finally, with an eye toward complete molecular characterization, we demonstrate that modern nMS technology can perform a "controlled disassembly" of the complex, also known as top-down MS, enabling amino acid sequencing of the HLA allele and neoantigen peptide in a single experiment.[26]

## EXPERIMENTAL SECTION

**Native SEC-MS.** MHCI proteins were injected onto ACQUITY UPLC Protein BEH SEC column (200 Å, 1.7 μm, 4.6 mm × 150 mm, Waters Corporation) heated to 30 °C using an UltiMate 3000 RSLC system (ThermoFisher Scientific). A binary pump was used to deliver solvent A (water) and solvent B (100 mM ammonium acetate, pH 7.0) as an isocratic gradient of 50% solvent B at a flow rate of 300 μL/min for 10 min. Separated proteins were analyzed online *via* electrospray ionization into a Thermo Exactive Plus EMR Orbitrap instrument (ThermoFisher Scientific) using the following optimized parameters for data acquisition: sheath gas flow rate of 4 and auxiliary (AUX) gas flow rate of 0 in electrospray ionization (ESI) source; 4.0 kV spray voltage; 320 °C capillary temperature; 200 S-lens radio frequency (RF) level; 350−10,000 $m/z$ scan range; desolvation, in-source CID 0 eV, CE 0; resolution of 8,750 at $m/z$ 200; positive polarity; 10 microscans; 3 × 106 automatic gain control (AGC) target; fixed AGC mode; 0 averaging; 50 ms maximum IT; 25 V source direct current (DC) offset; 8 V injection flatapole DC; 7 V inter-flatapole lens; 6 V bent flatapole DC; 0 V transfer multipole DC tune offset; 0.8 V C-trap entrance lens tune offset; and trapping gas pressure setting of 3.

Acquired mass spectral data were analyzed using PMI Intact Mass software (Protein Metrics Inc.) under the following parameters: 1,500 to 6,000 $m/z$ range; 0.2 charge vectors spacing; 15 $m/z$ baseline radius; 0.02 $m/z$ smoothing sigma; 0.04 $m/z$ spacing; 3 mass smoothing sigma; 0.5 mass spacing; 10 iteration max; and 5−100 charge state range.

**Native CZE-MS.** MHCI proteins were buffer-exchanged using a Zeba Spin Desalting Plate, 96-well (Thermo Scientific) prior to native capillary zone electrophoresis (CZE)-MS analysis. The desalting plate was first equilibrated to room temperature and then centrifuged at 1,000*g* for 2 min to remove the storage buffer. The resin was washed 4 times with 250 μL of 50 mM ammonium acetate, pH 7.0 by centrifuging at 1,000*g* for 2 min. The wash plate was emptied after each spin and then replaced with a sample collection plate. Samples were added on the resin and centrifuged at 1,000*g* for 2 min.

Buffer-exchanged MHCI proteins were injected onto a HSB chip (908 Devices Inc.) using a ZipChip system (908 Devices Inc.). A ZipChip autosampler was used to deliver a protein complex background electrolyte (BGE) solution, pH 6.5, containing isopropyl alcohol, histidine, ammonium acetate, and dimethyl sulfoxide. The final ZipChip method was optimized with the following parameters: 500 V/cm field strength; 3 nL injection volume; 0.5 min pressure assist start time; 2 min replicate delay; and 3 min analysis time. Separated proteins were analyzed online *via* electrospray ionization into a Thermo Exactive Plus EMR Orbitrap instrument (ThermoFisher Scientific) using the following parameters for data acquisition: sheath gas flow rate of 2 and AUX gas flow rate of 0 in ESI source; 0 kV spray voltage; 250 °C capillary temperature; 200 S-lens RF level; 1,500 to 6,000 $m/z$ scan range; desolvation, in-source CID 75 eV, CE 0; resolution of 17,500 at $m/z$ 200; positive polarity; 3 microscans; $3 \times 10^6$ AGC target; fixed AGC mode; 0 averaging; 20 ms maximum IT; 15 V source DC offset; 9 V injection flatapole DC; 8 V inter-flatapole lens; 10 V bent flatapole DC; 0 V transfer multipole DC tune offset; 0 V C-trap entrance lens tune offset; and trapping gas pressure setting of 2.

Acquired mass spectral data were analyzed using PMI Intact Mass software (Protein Metrics Inc.) under the following parameters: 1,500−6,000 $m/z$ range; 0.2 charge vectors spacing; 15 $m/z$ baseline radius; 0.02 $m/z$ smoothing sigma; 0.04 $m/z$ spacing; 3 mass smoothing sigma; 0.5 mass spacing; 10 iteration max; and 5−100 charge state range.

**Native Top-Down MS.** Analyses were performed on a modified Orbitrap Eclipse Tribrid Mass Spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with PTCR and an extended mass range to $m/z$ 8000. pMHCI complexes at 10 μM were buffer-exchanged to 100 mM ammonium acetate and introduced to the mass spectrometer through static nano-electrospray. Complexes were subject to increasing source collision energy (from 0 to 125 V) to dissociate the components, and then, ions from either the HLA subunit or the dissociated peptide were selected and isolated for MS/MS fragmentation. Top-down spectra were analyzed using ProSight Lite (http://prosightlite.northwestern.edu/)

**Generation of Synthetic UV-MHC and pMHC Complexes.** Detailed methods to produce synthetic UV-MHC and pMHC complexes at small and large scales are reported elsewhere.[1] Briefly, recombinant HLA alleles and β2M were overexpressed in *E. coli*, purified from inclusion bodies, and stored under denaturing conditions (6 M Guanidine HCl, 25 mM Tris pH 8) at −80 °C. In a 200 μL reaction, the peptide (0.01 mM, per well), oxidized and reduced glutathione (0.5 and 4.0 mM, respectively), recombinant HLA alleles (0.03 mg/mL), and β2M (0.01 mg/mL) were combined in a 96-well plate. Refolding was performed with each experimental peptide and for each HLA of interest. The MHCI complex was incubated at 4 °C for 3−5 days for refolding. Peptides were also designed with a UV-cleavable amino acid (denoted "J") at different positions along the peptide sequence. Formation of stable conditional MHCI complexes upon refolding with the redesigned UV-peptides was identified by ELISA.[1] All peptides used here were purchased from JPT (https://www.jpt.com) or ELIM Biopharm (www.elimbio.com).


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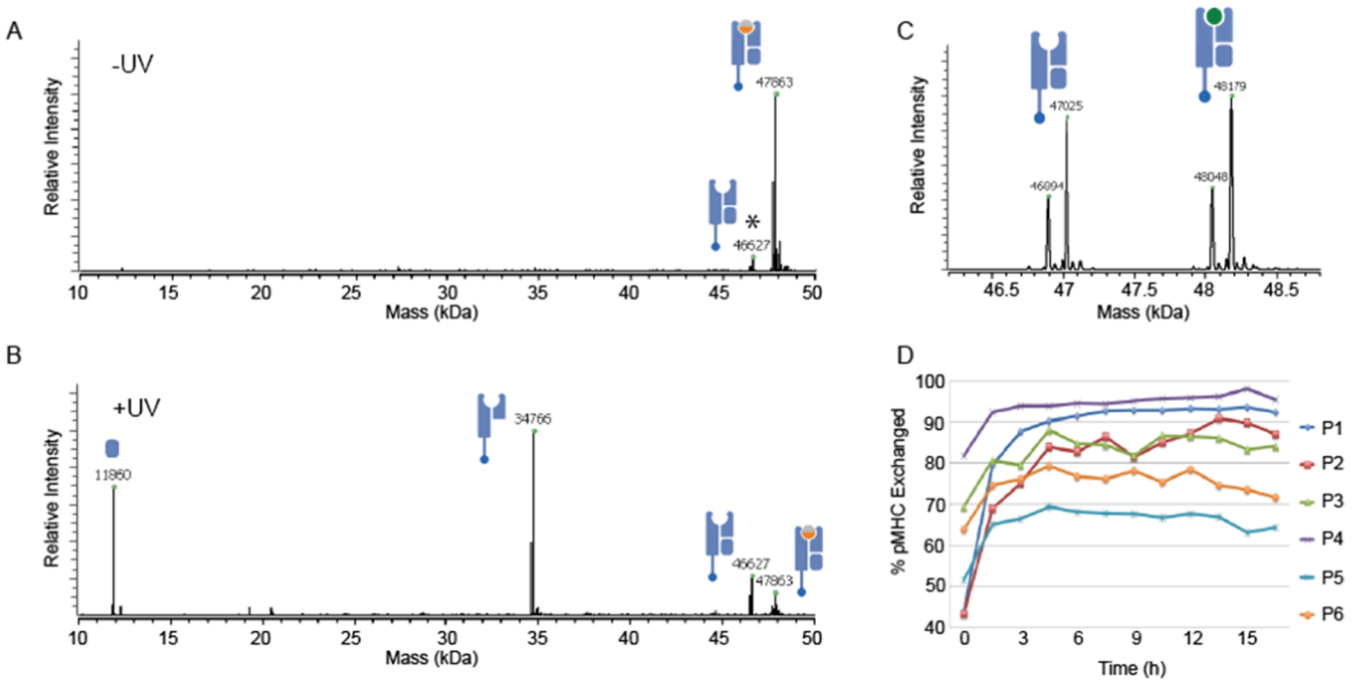


**Figure 2.** Native MS spectra before (A) and after (B) UV exposure of the UV-MHC complex using HLA A02:05. (C) Successful detection of the fully exchanged pMHCI complex using HLA A02:01. The spectrum shows HLA proteoforms with and without the initiator Methionine (±131 Da). (D) Time-resolved quantitation of pMHC exchange (reported as percentage) for six peptides on HLA A02:01. A list of peptide sequences is provided in Suppporting Table S1.

For the peptide exchange reaction, purified UV-MHCIs were incubated in the presence of 100-fold molar excess of synthetic peptides of interest. UV-MHCI was present at a concentration of 50 $\mu$g/mL in 25 mM TRIS pH 8.0, 150 mM NaCl, 2 mM EDTA, and 5% ethylene glycol. The peptide exchange reaction mixture was incubated for 25 min under a UV lamp (UVP 3 UV Lamp, Analytik Jena) set to 365 nm to induce cleavage of the UV-sensitive amino acid "J," which is 3-amino-3-(2-nitro)phenyl-propionic acid. Upon cleavage of the conditional peptide ligand, synthetic peptides with suitable properties (affinity, solubility) exchanged into the complex displacing any fragments of the cleaved conditional ligand. Samples were incubated at room temperature, overnight, to allow for peptide exchange to occur.

**ELISA.** An ELISA method for characterization of peptide-stabilized MHCI after peptide exchange was adapted from Rodenko et al. (Generation of peptide−MHC class I complexes through UV-mediated ligand exchange. Nat Protoc 1, 1120−1132 (2006)) and described elsewhere.[1] Briefly, a 96-well Nunc Maxisorp plate (ThermoFisher Scientific, Waltham, MA) was coated with 100 $\mu$L of 2 $\mu$g/mL streptavidin (ThermoFisher Scientific, Waltham, MA) in PBS. After incubation for 2 h at 37 °C, the plate was washed four times with 300 $\mu$L of wash buffer, 0.05% Tween 20 in PBS. The plate was then blocked with 300 $\mu$L of blocking buffer, 2% BSA in PBS, and incubated for 30 min at room temperature. After incubation, the blocking buffer was tipped out, and 100 $\mu$L of 5 nM pMHCI solutions were added to each well. The plate was then incubated for 1 h at 37 °C, followed by washing four times with 300 $\mu$L of wash buffer. An HRP-conjugated anti-$\beta$2m antibody (clone 2M2, Biolegend, item number 280303) diluted to 1 $\mu$g/mL in blocking buffer was added to each well at a volume of 100 $\mu$L per well. The plate was then incubated for 1 h at 37 °C, followed by washing four times with 300 $\mu$L of wash buffer. The color reaction was developed with ABTS substrate solution (ThermoFisher Scientific, Waltham, MA) with 150 $\mu$L of solution added per well. The plate was incubated for 10−15 min at room temperature, and the color development was monitored by eye. The reaction was stopped with 100 $\mu$L of stop buffer, 1% SDS, and then absorbance at 405 nm was measured by a plate reader (BioTek Epoch 2 Microplate Scpectrophotometer; Agilent, Santa Clara, CA).

**2D LC-MS.** A two-dimensional liquid chromatography mass spectrometry (2D LC-MS) method was used to characterize peptide binding to MHCI complexes.[1] Briefly, 2−5 $\mu$g of MHCI−peptide mixtures were injected on the instrument and sent to the first dimension column. The first dimension LC method employed an analytical size-exclusion column (SEC) (Agilent AdvanceBio SEC, 300Å, 2.7 $\mu$m, 4.6 mm × 15 mm) to separate the intact complex from excess peptide run at an isocratic flow of 0.7 mL/min in 25 mM TRIS pH 8.0, 150 mM NaCl for 10 min with signals acquired at 280 nm. A sampling valve collected the entirety of the complex peak that eluted from 1.90 to 2.13 min in a volume of 160 $\mu$L and injected it onto the second dimension reversed phase column (Agilent PLRP-S 1000 Å, 8 $\mu$m, 50 mm × 2.1 mm). The second dimension column was exposed to a gradient of 5−50% mobile phase B in 4.7 min at 0.55 mL/min with the column heated to 80 °C. Mobile phase A was 0.05% TFA, and mobile phase B was 0.05% TFA in acetonitrile. The column eluent was sent to an Agilent 6224 TOF LCMS for mass spectrometry data acquisition.

The peak area of the MHCI complex in the first dimension and mass spec detection of the peptide in the second dimension are used to determine successful peptide binding. Successful binding of a peptide into the complex after cleavage of the conditional ligand during the peptide exchange reaction stabilizes the complex and results in nearly complete recovery


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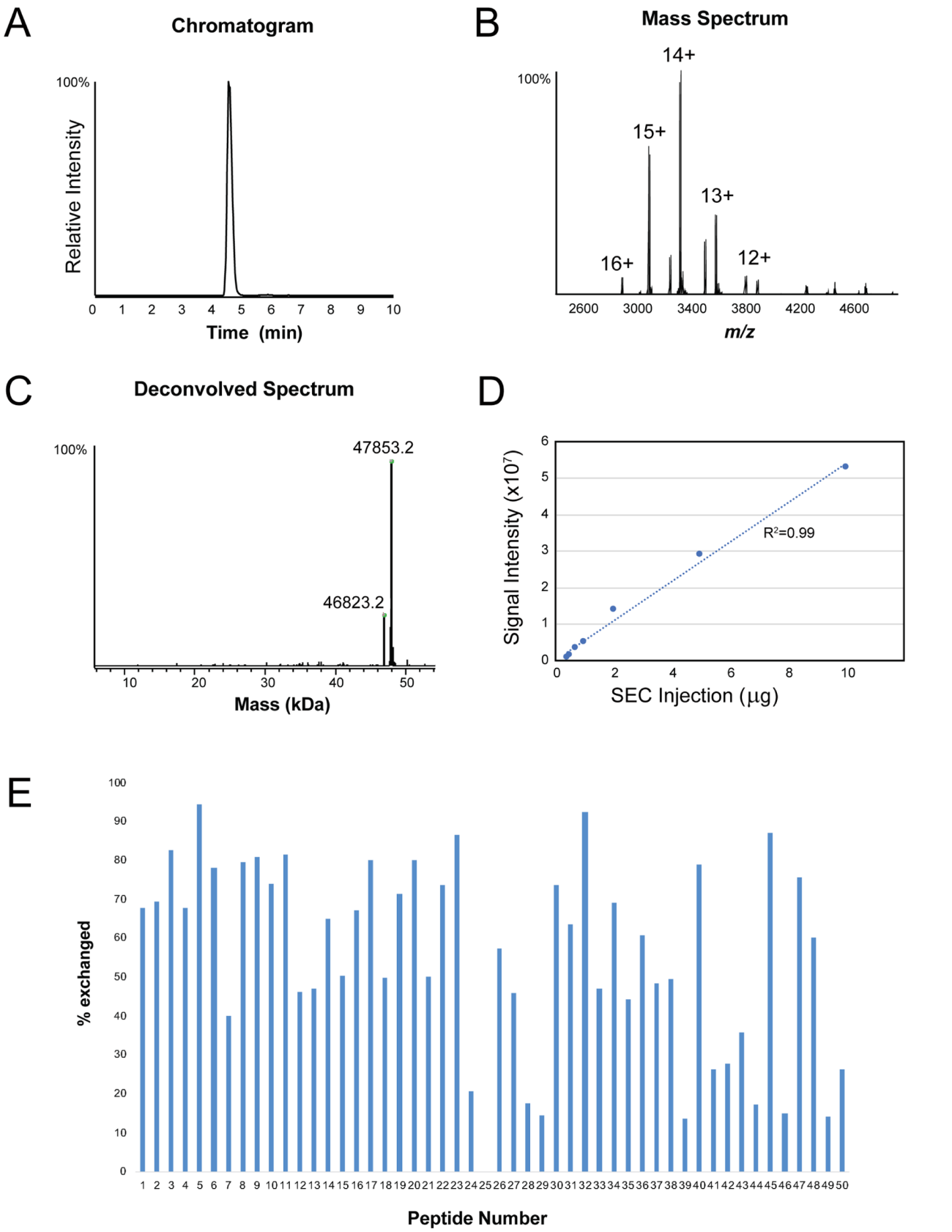


**Figure 3.** Analysis of UV-MHCI by native MS coupled to size-exclusion chromatography (SEC), reflected as (A) chromatogram, (B) broadband mass spectrum, and (C) deconvolved spectrum. (D) Limit of detection analysis using SEC-MS for the UV-MHCI complex. (E) 50 neoantigen peptides exchanged on HLA A02:01 using the SEC-nMS method. A list of peptide sequences is provided in the Supporting Table S2.



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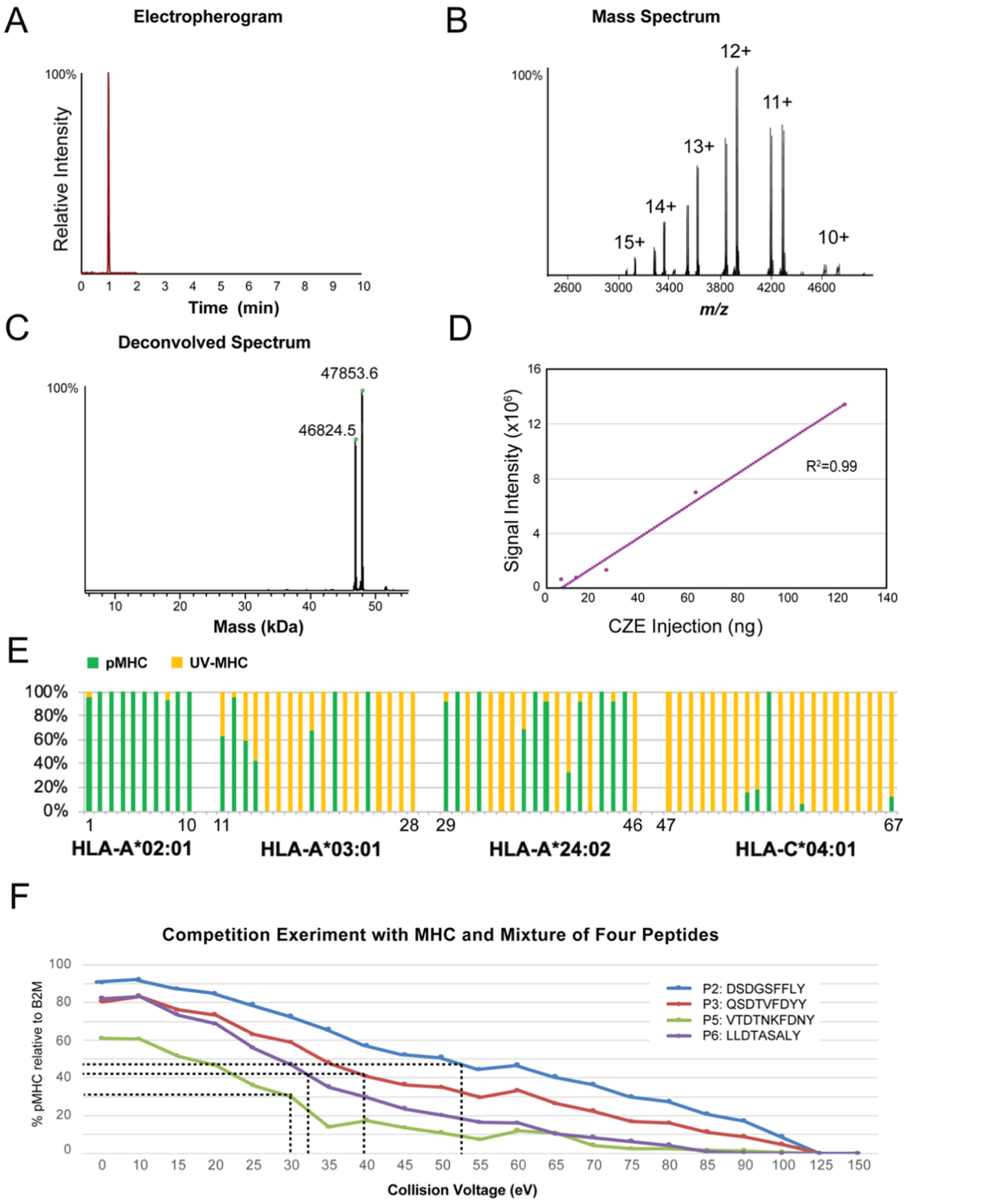


**Figure 4.** High-throughput analysis of peptide−MHCI complexes using CZE-MS, reflected as (A) electropherogram, (B) broadband mass spectrum, and (C) deconvolved spectrum. (D) Limit of detection analysis using CZE-MS for the UV-MHCI complex. (E) Quantitation of peptide exchange, with green signal corresponding to pMHCI and yellow to nonexchanged UV-MHCI. (F) MHCI HLA allele A01:01 was incubated with four peptides simultaneously. The native MS platform was used to quantify the extent of peptide exchange. Binding dynamics probed with collision energy (eV), with lower percentage reflecting disassembly of the MHCI complex into subunits. The dashed lines reflect the $Vc_{50}$ calculation defined as the collision voltage required to dissociate 50% of the peptide from the pMHCI complex (P2: 52.5 eV; P3: 40 eV; P5: 30 eV; P6: 32.5 eV). A list of peptide sequences is provided in Supporting Table S3.

of the starting complex measured in the first dimension SEC analysis. The peptide that has exchanged into the complex can then be detected in the second dimension, where the complex is run under denaturing conditions with mass spectral analysis allowing for direct detection of the peptide of interest. Unsuccessful peptide exchange reactions result in destabilized complex after the cleavage of the conditional ligand when a peptide fails to bind to and stabilize the complex. This is


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measured as a reduction in the A280 peak area of the complex on SEC and an absence of peptide in the second dimension. In some cases, no reduction in peak area is observed; however, the peptide is not detected by mass spectrometry. A small number of peptides, due to their properties, are not captured by the second dimension chromatography column and method. In these cases, the peak area recovery is enough to determine successful exchange when positive and negative controls for peptide binding are used.

## RESULTS AND DISCUSSION

For the analysis of neoantigen binding, the recombinant MHCI complex is first incubated with a photocleavable peptide that, upon UV exposure, loses affinity for the MHCI complex, enabling competitive binding with a neoantigen. MHCI is a glycoprotein with one N-linked glycosylation site at Asn86;[27−29] however, recombinant constructs produced in *E. coli* do not contain glycans, simplifying the generation of reagents for T-cell assays and streamlining the assessment of peptide exchange by MS analysis.

The native MS readout of the MHCI-photocleavable peptide complex using an HLA allele A02:05 (UV-MHCI) prior to UV exposure reports masses consistent with the UV-MHCI complex. Close inspection of the spectrum reveals that there are populations in the peptide-bound and -unbound states (marked with an *, Figure 2A). We hypothesized that the peptide-unbound population of the MHCI complex most likely resulted from the gas-phase dissociation of the UV peptide from the UV-MHCI. To test this, we exposed the UV-MHCI complex to UV light. The MS readout showed that after exposure, the majority of the MHCI complex disassembled into free peptide, B2M, and HLA subunits. Given that no neoantigen (*i.e.*, "exchange" peptide) was added prior to UV exposure, complex disassembly is consistent with reports that the peptide is critical to the stability of the MHCI complex *in vitro* (Figure 2B). Therefore, an "empty" MHCI complex in the gas phase due to dissociation inside the MS instrument is representative of the peptide-bound population in solution.

Next, the UV-MHCI complex with HLA A02:05 was incubated with a neoantigen and exposed to UV light overnight. The MS readout successfully detected the peptide-bound MHCI complex (pMHCI) and a population of the "empty" MHCI complex (Figures 2C and S1). Given that no masses were detected for UV-MHCI and that there are no MS or chromatographic signals for the disassembled complex, we reason that there is in fact complete peptide exchange.

To test the quantitative nature of the platform and assess the length of time necessary for peptide exchange, we quantified the exchange of six standard peptides with known MHCI-binding affinities on HLA A02:01 (Figures 2D and S2 and Suppporting Table S1). The quantitative results demonstrate that the MS readout is representative of MHCI occupancy in solution. Moreover, the experiment showed that peptide exchange plateaus after the first two hours were independent of binding affinities, enabling us to shorten peptide and MHCI incubation time for future assay development and translational applications.

T-cell staining assays and other immune monitoring screens downstream of the MS analysis require small amounts (nanograms) of many different and unique MHC-peptide complexes. For example, immune monitoring of a cancer vaccine patient may involve testing >60 neoantigen peptides for six HLA alleles, resulting in more than 360 individual and low-nanogram samples per patient.[30] Thus, to ensure compatibility with these translational applications, we first developed the MS platform coupled to size-exclusion chromatography (Figure 3A−C). The SEC-MS method successfully detected the UV-MHC complex from 4 $\mu$L injections containing serial dilutions ranging from 10 to 0.3 $\mu$g, or 2500 to 100 $\mu$g/mL, in the span of a 10 min chromatography run (Figures 3D and S3). We further quantified the exchange of 50 peptides on HLA A02:01 using the native SEC-nMS platform, demonstrating the feasibility of acquiring medium to large-size quantitative data sets (Figure 3E). Additionally, we found that the nMS results are comparable to the assessment of the same 50 peptides using ELISA and 2D-LCMS (Supporting Figure S4A). A comparison of the various methods in terms of cost, run time, and throughput is offered (Supporting Figure S4B).

We hypothesized that a microchipCZE-based method (*i.e.*, ZipChip from 908 Devices) would provide additional sensitivity and throughput due to low flow, nanoliter injection volumes, and shorter run times (<2 min). However, existing methods for native protein analysis on the ZipChip platform had been developed for proteins with high isoelectric point (pI), requiring electrophoretic migration of analytes as cations in solution. To analyze MHCI complexes with low pI (5.1), a new ZipChip method was required. Whereas the existing ZipChip protein analysis methods utilize a neutral polymer surface chemistry, this new method utilized a bare glass surface chemistry. The bare glass surface has a strong negative charge at neutral pH, which repels negatively charged analytes and imparts a strong electroosmotic flow to force all analytes to migrate toward the ESI orifice of the chip. This new method enabled successful detection of the MHCI complexes from 3 nL injections down to 6 ng, sensitivity below 2 $\mu$g/mL, and <2 min per analysis (Figures 4A−D and S5). Moreover, we challenged the reproducibility of the CZE-based method by measuring a UV-MHC signal for HLA A01:01 at two different concentrations (Supporting Figure S6).

We deployed the high-throughput CZE-nMS method to quantify the UV-MHCI complex formation (Supporting Figure S7) and peptide exchange for 67 peptides across four HLA isotypes (Figure 4E and Supporting Table S3). To further expand the throughput of the assay, we experimented with a "multiplex" modality of the quantitative MS platform (Figure 4F). By incubating up to four nonisobaric neoantigens simultaneously with the UV-MHCI complex, we were able to detect their exchange and binding to the MHCI in a single experiment. The peptide−MHC affinities measured in the "competition experiment" are lower than those calculated in single-peptide incubations (Supporting Figure S8). We reason that because the MHC dimer is the limiting reagent in the "competition experiment," the peptides reach an equilibrium that is representative of their affinities relative to each other. Moreover, stepwise increases in collision energy resulting in pMHCI disassembly enable the multiplex assay to approximate the $Vc_{50}$—defined as the collision voltage required to dissociate 50% of the complex—for all four pMHCI complexes simultaneously (P2: 52.5 eV; P3: 40 eV; P5: 30 eV; P6: 32.5 eV). While this additional experiment involves individual injections for each collision voltage setting, the $Vc_{50}$ measure of gas-phase stability can be used to further differentiate peptide−MHC affinities as needed.


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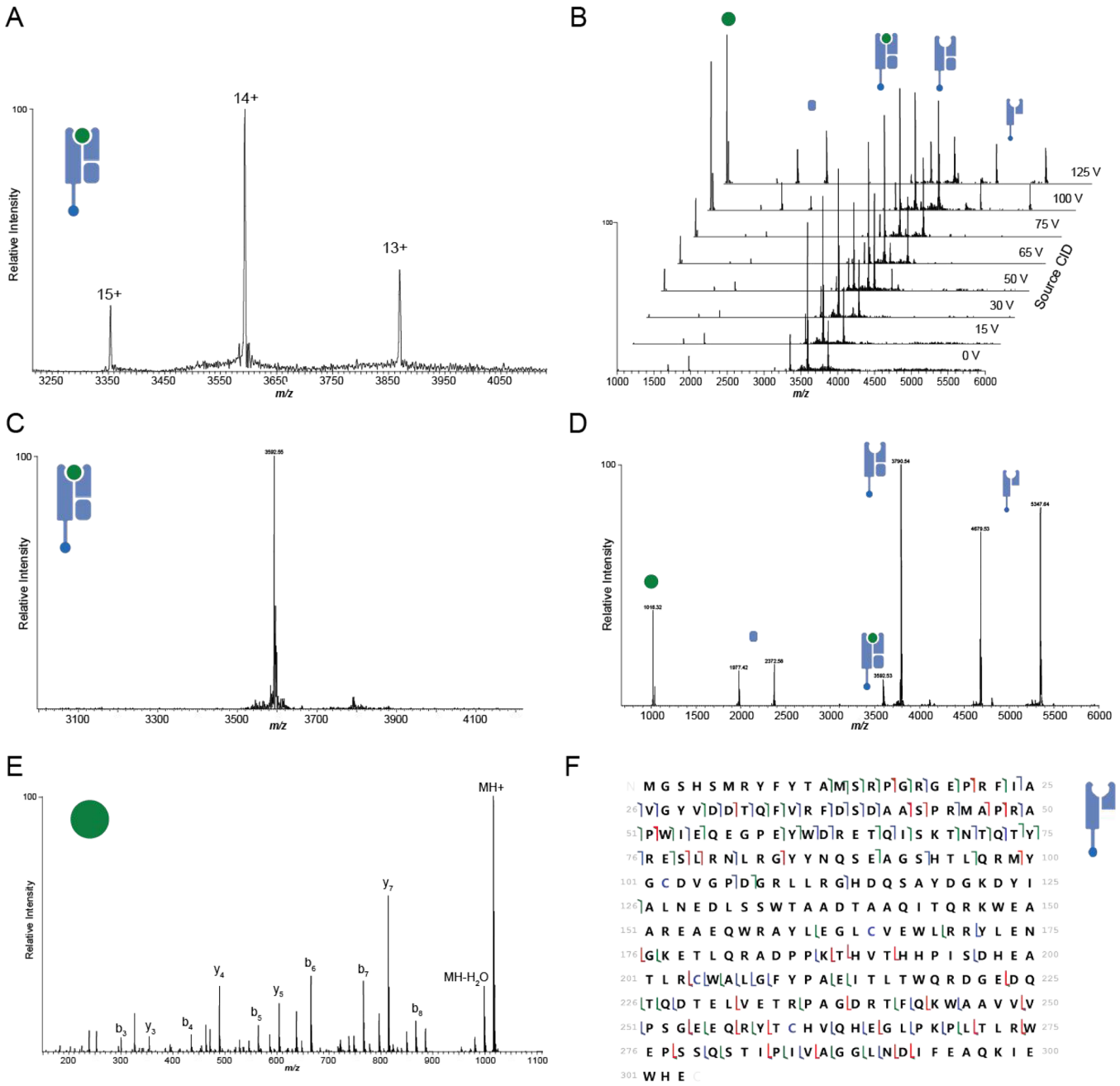


**Figure 5.** (A) Native mass spectrum of pMHC using the Orbitrap Eclipse "Tribrid" MS platform, showing minimal baseline dissociation of a peptide. (B) Controlled disassembly of the pMHC complex as a function of the source collision energy (Source CID, V). (C) Isolation of the 14+ charge state of the pMHC in preparation for selective disassembly using HCD. (D) Ejection of pMHC subunits after isolation using HCD. (E) Fragmentation of an isolated peptide, MAPIDHTTM, showing sequencing capability of the platform. (F) Fragmentation map of the mouse H2-Db allele after top-down sequencing using HCD (blue flags), EThcD (red flags), and UVPD (green flags).

We were interested in the correlation between the quantitative native MS results and the NetMHCpan 4.0 *in silico* predictions for peptide binding affinities to the various alleles[10] (Supporting Figure S9). We found that, when excluding the MS results for the 0 or 100% pMHC complex, there is a statistically significant correlation ($p$-value = 0.0009).

Finally, with an eye toward complete molecular characterization, we leveraged native top-down MS technology to detect pMHCI formation and to sequence subunits in the same MS experiment. Using a ThermoFisher Orbitrap Eclipse Tribrid mass spectrometer with the extended mass range, we first detected the intact mass of the MHCI−peptide complex with minimal gas-phase baseline dissociation of the peptide relative to the aforementioned experiments (Figure 5A). Given this finding, we measured the ejection of peptides and disassembly of the pMHC complex as a function of collision voltage (Figure 5B), confirming the platform's ability to interrogate the protein composition of the pMHC complex in a single run. Next, we isolated the HLA and peptide subunits ejected from the complex, subjected them to collisions with neutral gas, and fragmented for sequence characterization (Figure 5C−F). This crucial step validates the HLA allele isoform and clarifies the sequence of the peptide, including any potential post-translational modifications (PTMs) that could affect MHCI-


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binding affinity or recognition by T-cell receptors. While the top-down sequencing step or MS-based compositional analysis is not always necessary, prominent PTMs such as disulfide-bonded peptide dimers or oxidation go undetected by widespread assays like ELISA, complicating the validation of clinical pMHCI reagents (Supporting Figure S10). Hence, structural interrogation of MHCI reagents in addition to peptide exchange assessment using high-resolution, high-sensitivity, and high-throughput native MS can bolster confidence in clinical immune monitoring.

## CONCLUSIONS

State-of-the-art mass spectrometry now enables direct detection and characterization of neoantigen binding to recombinant MHCI complexes with performance comparable to established assays and *in silico* prediction tools. Coupled to capillary-zone electrophoresis and multiplexing strategies, the nMS platform can be automated to quantify peptide exchange at high sensitivity and throughput, to calculate the $Vc_{50}$ as a measure of gas-phase stability of the pMHCI, and, importantly, to validate the integrity of the reagents used in clinical T-cell assays. We have also shown that incorporating a top-down sequencing workflow can identify the HLA allele and bound peptide in the same experiment, enabling the deployment of the native top-down MS platform to characterize the atomic composition of recombinant pMHCI complexes.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c02423.

Deconvolved native mass spectrum reflecting detected signals for pMHCI and UV-MHCI complexes; six standard peptides incubated overnight with HLA allele A02:01; chromatograms and deconvolved mass spectra for UV-MHCL complexes; peptide exchange results for 50 peptides on HLA A02:01, table comparing the three methods in terms of advantages and disadvantages; electropherogram and deconvolved mass spectra for UV-MHCL complexes; detection of UV-MHC complexes by CZE-MS in two technical replicates at two different concentrations; measure of pMHC complex formation to validate integrity of reagents in the CZE-*n*MS high-throughput screen; multi-plex vs single peptide affinity data as measured by CZE-*n*MS; plot of adjusted NetMHCpan 4.0 rank; peptides used to generate pMHC reagents can be modified in ways that alter affinity for the MHC complex (Figures S1−S10); peptide sequences for main text Figures 2D, 3E, and 4E (Tables S1−S3) (PDF)

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### Author Contributions

#L.F.S. and W.P. are contributed equally to this work.


### Notes

The authors declare the following competing financial interest(s): LS, WP, MD, CB and WS are employed by Genentech, Inc., a for-profit company that produces and markets therapeutics.AB and SM are employed by 908Devices, GH by BGI, and KS and RH by Thermo Fisher Scientific, for-profit companies that manufacture and sell mass spectrometry equipment.

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