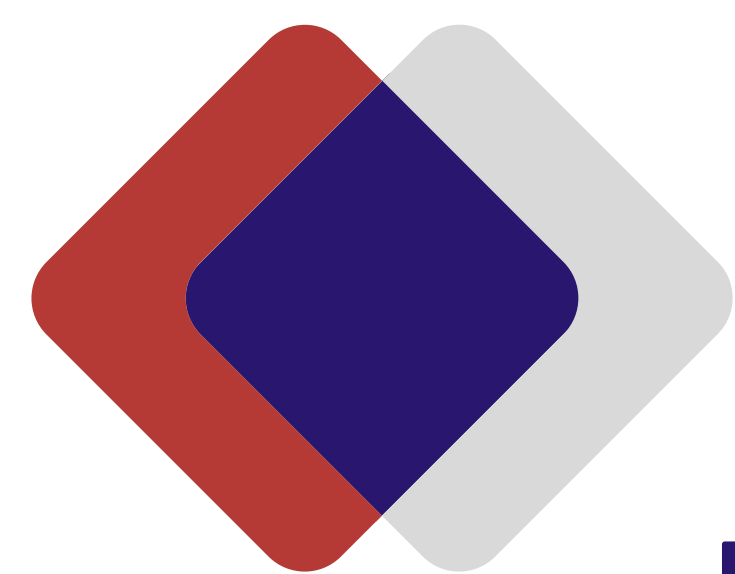




A Platform HPLC Strategy for Comprehensive Analytical Characterization of Bispecific Antibodies

Yinan Zhang, Linting Li, Xingrong Zhu, Xuyang Zhang, Minxin Wang and Ke Yang, Sepax Technologies, Inc., 5 Innovation Way, Newark, DE, 19711



BACKGROUND

Bispecific antibodies (BsAbs) present significant analytical challenges due to their increased structural complexity, molecular heterogeneity, and multiple degradation or misassembly pathways relative to conventional monoclonal antibodies. Rapid and reliable characterization of these attributes requires orthogonal analytical strategies that can be broadly applied across diverse BsAb formats. In this work, we present an integrated HPLC-based platform technology designed to support analytical method development and multi-attribute characterization of BsAbs. The platform combines size exclusion chromatography (SEC) for aggregate and fragment analysis, ion exchange chromatography (IEX) for charge heterogeneity profiling, hydrophobic interaction chromatography (HIC) for hydrophobicity-related variant analysis, and affinity chromatography for selective capture and enrichment of target subpopulations.

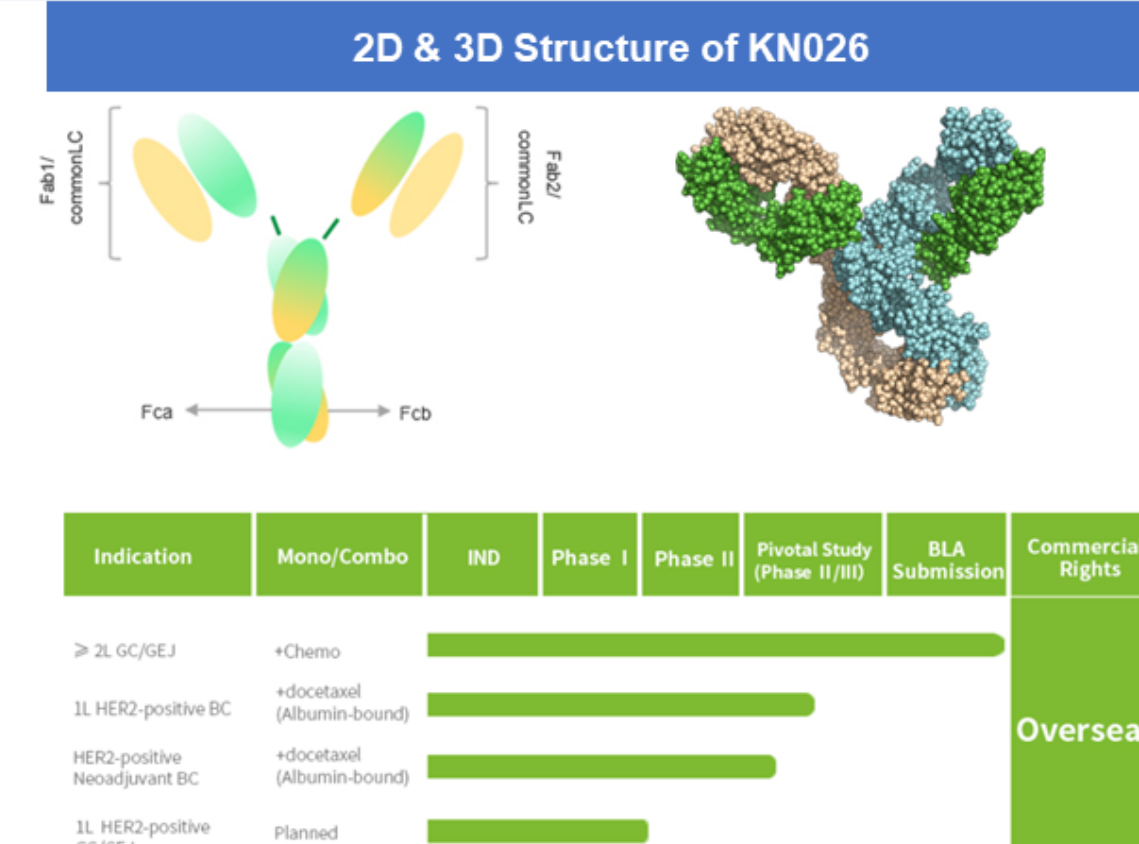
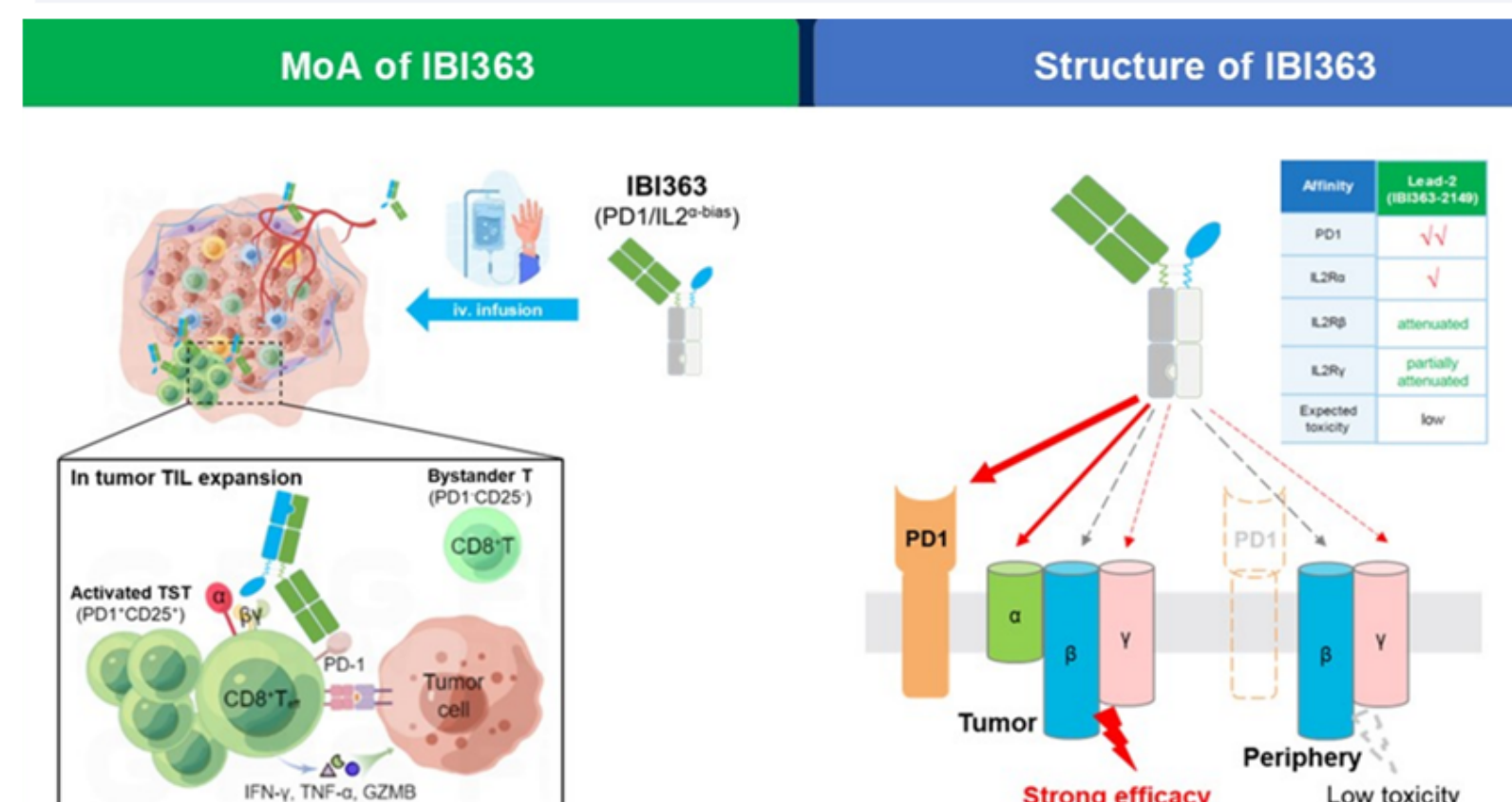
METHOD

Bispecific Antibody IBI363

❖ IBI363 is a first-in-class PD-1/IL-2 α -bias bispecific antibody fusion protein to block PD-1 checkpoint and rejuvenate exhausted tumor-specific T cells by cis-activating α -bias IL-2. IBI363 utilizes a structural interlocking Knob-into-Hole (KiH) technology to ensure precise heterodimerization and superior molecular stability. It has potential to address the unmet clinical need of patients (pts) with immunotherapy-resistant and cold tumors.

Bispecific Antibody KN026

❖ Anbenitamab (KN026) is an anti-HER2 bispecific antibody developed using the proprietary Fc-based heterodimer bispecific platform technology called CRIB (Charge Repulsion Induced Bispecific). Anbenitamab can simultaneously bind two non-overlapping epitopes of HER2, resulting in HER2 signal blockade. Through antibody-induced receptor clustering, it enhances ADCC and CDC effects while promoting the down-regulation of HER2 receptors on the cell surface.



To demonstrate the versatility of the platform, two structurally distinct BsAbs^[1,2] were evaluated as representative case studies. These examples were selected to reflect differing molecular architectures and physicochemical behaviors, thereby challenging method robustness and transferability across formats.

Affinity: Targeted Capture & Enrichment

Affinity Chromatography acts as the primary gatekeeper, before deep characterization can occur. By utilizing specific ligands (such as Protein A, G, or L), the Sepax platform enables the selective capture of the BsAbs from complex cell culture supernatants, ensuring that downstream analysis is performed on clean, relevant samples.

IEX: Mapping Charge Variants

Ion Exchange Chromatography (IEX) is employed to resolve charge variants resulting from deamidation, C-terminal lysine clipping, or sialylation. Because BsAbs often combine different heavy or light chains, subtle shifts in the isoelectric point (pI) require the high-efficiency salt or pH gradients provided by Sepax resins to ensure purity.

SEC: Monitoring Size Heterogeneity

Size Exclusion Chromatography (SEC) is the gold standard for detecting and quantifying aggregates (HMWS) and fragments (LMWS). Given the increased propensity for BsAbs to form dimers and higher-order aggregates, the high-resolution resins in the Sepax portfolio ensure accurate separation of these species from the monomeric therapeutic.

HIC: Assessing Hydrophobicity Variants

Hydrophobic Interaction Chromatography (HIC) is critical for identifying variants that differ in surface hydrophobicity, often caused by misfolding or post-translational modifications. In BsAbs analysis, HIC is particularly useful for separating "product-related impurities" such as mispaired homodimers from the desired heterodimeric bispecific molecule.

RESULTS

Affinity: Targeted Capture & Enrichment

The ProAqa Excel phase enables rapid, accurate mAb titer quantification for cell line screening, upstream optimization, and QC. Built on rigid, monosized PS/DVB beads with a recombinant Protein A ligand, it selectively captures Fc-containing immunoglobulins except IgG3 and supports high-flow, high-pressure, high-throughput analysis.

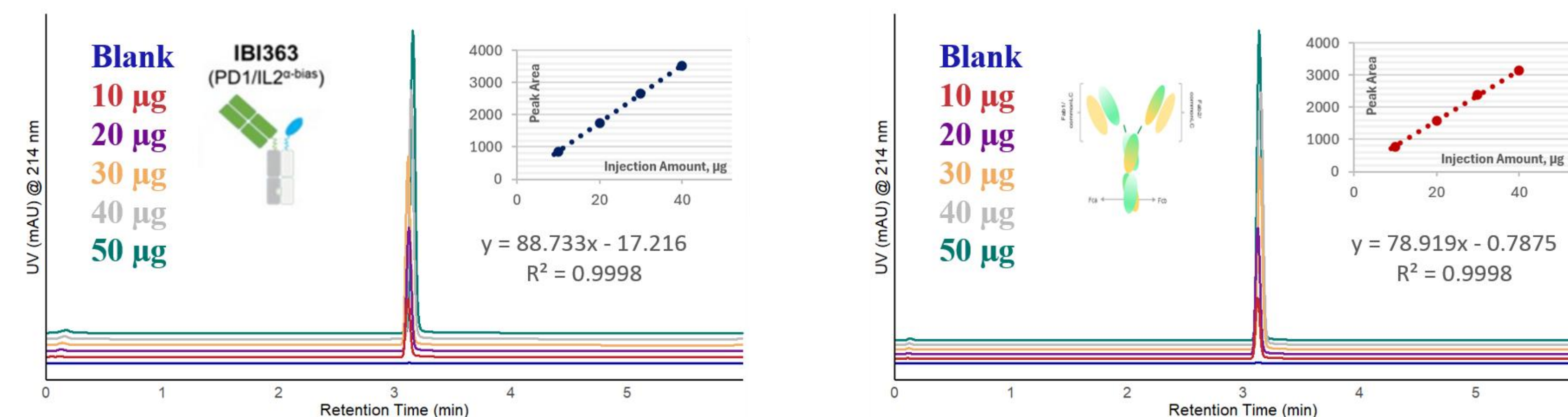


Figure 1. Loading capacity assessment of two selected BsAbs on a 2.1 × 30 mm ProAqa Excel column. Both BsAbs exhibited excellent linearity over an injection range of 10–50 µg at a flow rate of 1.0 mL/min. The mobile phases: loading: 50 mM phosphate buffer with 150 mM NaCl, pH 7.0; and eluting: 100 mM glycine, pH 2.5. Detection was performed at 214 nm.

SEC: Monitoring Size Heterogeneity

Sepax SEC phases separate analytes based on the hydrodynamic size relative to the pore size of the stationary phase. Smaller particles improve separation efficiency and provide higher resolution.

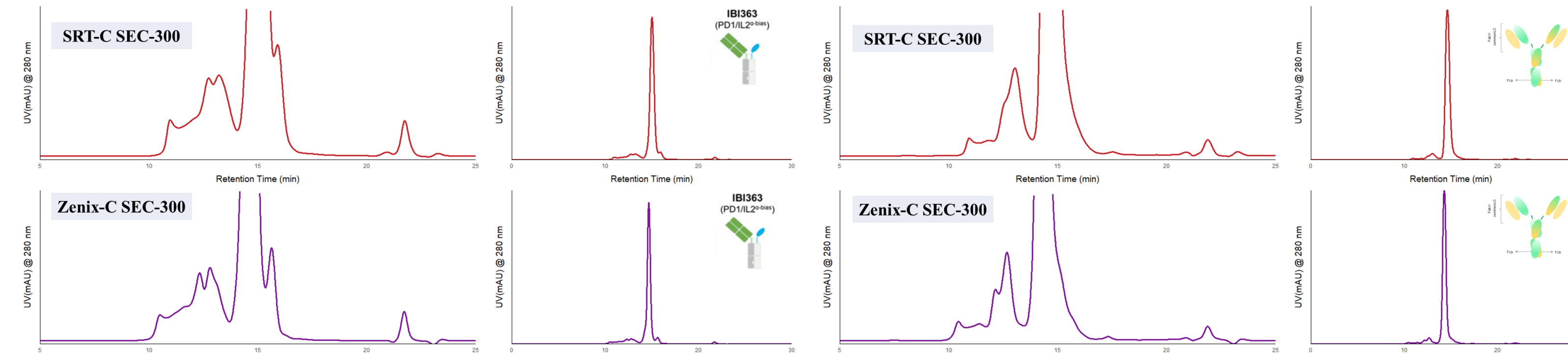


Figure 2. Aggregation analysis of two BsAbs on Sepax SRT-C 300 Å and Zenix-C 300 Å columns (7.8 × 300 mm) showed good resolution at 1.0 mL/min, with the 3 µm Zenix phase delivering superior performance. The mobile phase, 50 mM phosphate buffer with 250 mM KCl at pH 7.0, was optimized to enhance recovery and minimize non-specific interactions. Detection was monitored at 280 nm.

Mispairing Analysis of BsAbs – BioMix SEC-300 Column

For bispecific antibody mispairing analysis, mixed-mode SEC^[3] offers dual selectivity and superior resolving power by combining hydrophobic selectivity with size-exclusion effects.

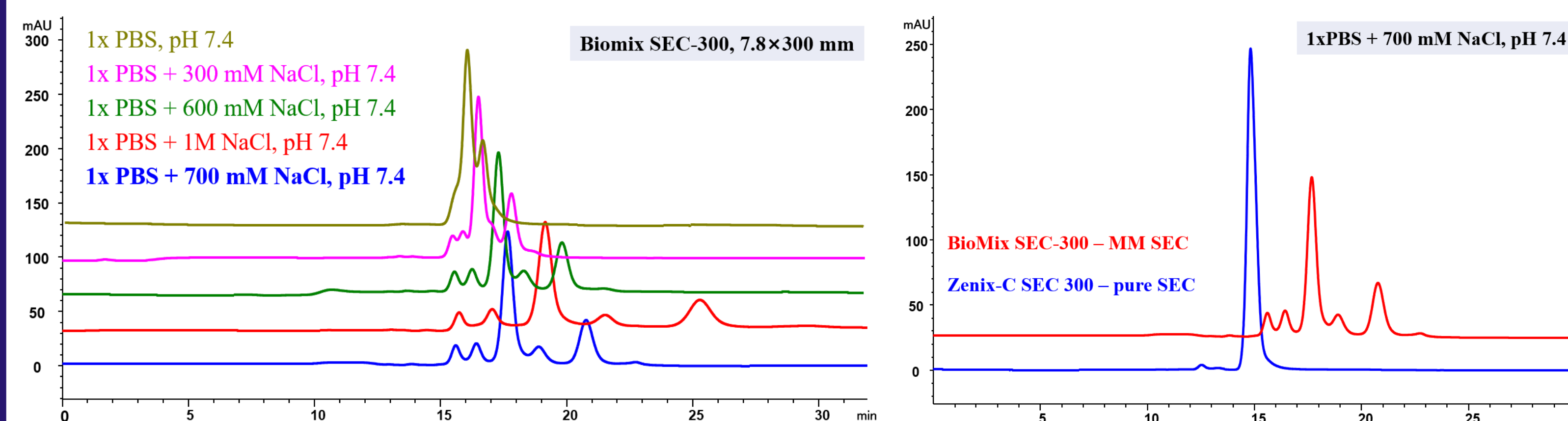


Figure 3. Bispecific antibody mispairing analysis on Sepax BioMix SEC 300 Å columns (7.8 × 300 mm). After optimization, MM SEC demonstrated effective selectivity for the separation of mispaired species.

IEX: Mapping Charge Variants

Proteomix IEX phases are designed to achieve high ion-exchange capacity and performance, resolving charge variants resulting from deamidation, C-terminal clipping, or sialylation.

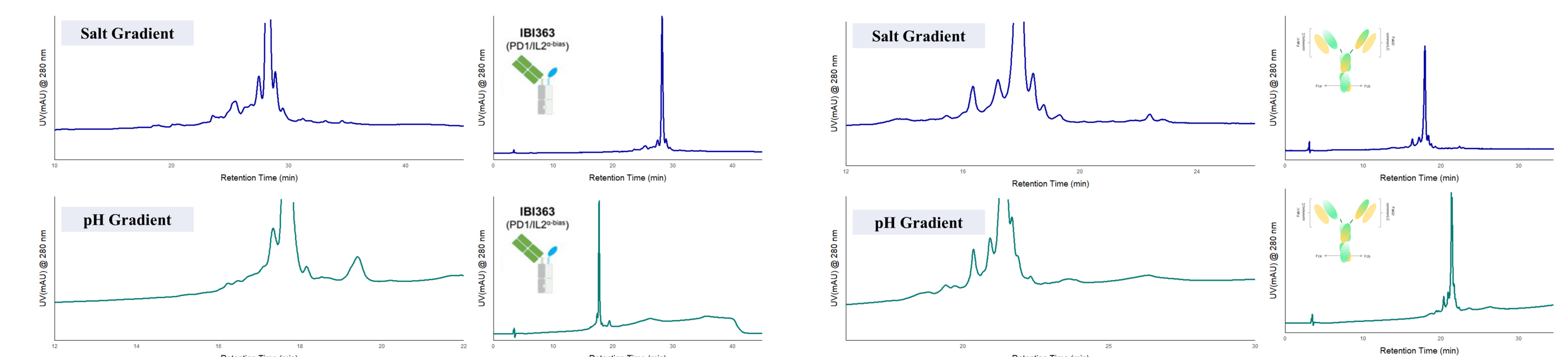
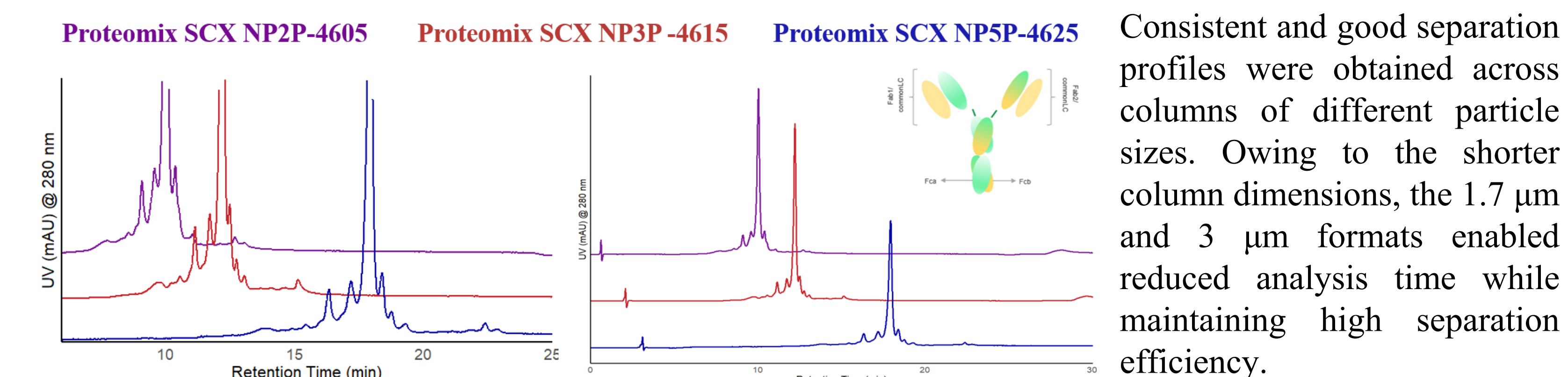


Figure 4. Charge-variant analysis on Sepax Proteomix SCX columns (4.6 × 250 mm) was performed using both pH-gradient and salt-gradient methods, which showed different selectivities for BsAb charge heterogeneity. A pH-gradient screen was first conducted for a sample with unknown pI, followed by salt-gradient optimization. Final conditions were: mobile phase A, 20 mM MES, pH 6.5; mobile phase B, 20 mM MES + 0.3 M NaCl, pH 6.5; flow rate 0.5 mL/min; detection at 280 nm.



HIC: Assessing Hydrophobicity Variants

Proteomix HIC phases separate analytes according to hydrophobicity and their interaction strength with the hydrophobic stationary phase.

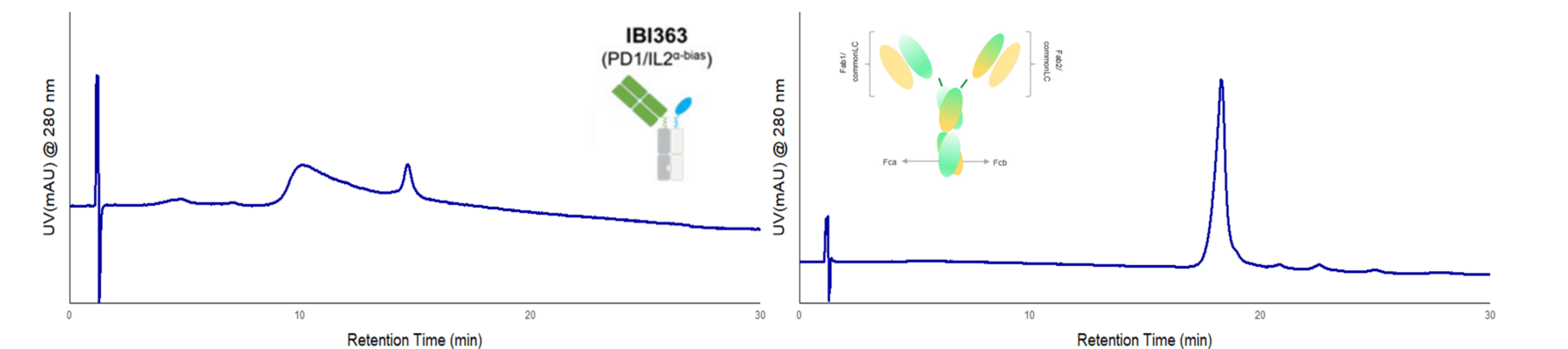


Figure 5. Hydrophobicity analysis was performed on a Proteomix HIC NP1.7 Phenyl column (4.6 × 100 mm) using 2.0 M ammonium sulfate/20 mM KH₂PO₄, pH 7.0 as MPA and 20 mM KH₂PO₄, pH 7.0, as MPB at 0.5 mL/min with UV detection at 280 nm. Modified heavy-chain and light-chain species showed distinct HIC selectivity, and separation performance was optimized by column temperature.

Reference:
[1] Zhou J, et al. MA11.04 first-in-class PD-1/IL-2 bispecific antibody IBI363 in patients with advanced non-small cell lung cancer in a phase I study[J]. Journal of Thoracic Oncology, 2024, 19(10): S97.
[2] Liu, D., et al. Efficacy and safety of KN026, a bispecific anti-HER2 antibody, in combination with KN046, an anti-CTLA4/PD-L1 antibody, in patients with advanced HER2-positive nonbreast cancer: a combined analysis of a phase Ib and a phase II study. Sig Transduct Target Ther 10, 104 (2025).
[3] Jiang, H., Xu, W., Liu, R., Gupta, B., Kilgore, B., Du, Z., & Yang, X. (2020). Characterization of bispecific antibody production in cell cultures by unique mixed mode size exclusion chromatography. Analytical chemistry, 92(13), 9312-9321.

