Wide Pore Superficially Porous Particles with Various Bonded Phases For High Resolution Protein Chromatography

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Introduction

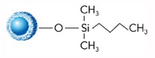
- The current work focuses on the effect of bonded phase chemistry and gradient elution variables in high resolution separations of proteins and monoclonal antibodies (mAb's).
- The creation of silica based SPP with a wide pore size (HALO® 1000Å) have helped address challenges in the reversed phase separations of large biomolecules (>50,000 MW).
- Separations run on the HALO 1000Å C4, ES-C18, and Diphenyl bonded phases are compared for chromatographic performance.
- Selectivity of protein separations can be manipulated using bonded phase options, temperature, and mobile phase composition in order to provide improved analytical solutions.

The improvements in performance of reverse-phase liquid chromatography (RP-LC) separations through the use of superficially porous particles (SPP) in packed columns have been well characterized. The recent (2017) introduction of HALO 1000Å, a wide pore SPP, provided similar improvements in peak widths and resolution for RP-LC separations of large proteins and mAb's. Further increases in chromatographic resolution require manipulation of the retention and selectivity for closely eluting biomolecules and their variants. Selectivity changes can be accomplished by varying the gradient elution variables or altering the particle's surface chemistry through different bonded phases. The HALO 1000Å surface chemistry has been modified with three complimentary bonded phases (C4, C18, and Diphenyl) to meet this need. This combination of SPP technology and gradient elution variables leads to selectivity manipulation and high resolution separations of large biomolecules, favoring correct structure assignment by LC/MS, including description of post translational and chemical modifications.

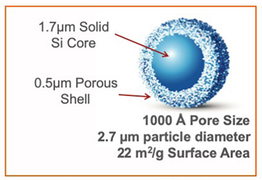
Experimental

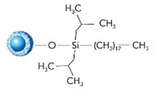
Columns: HALO 1000 Å C4/ES-C18/Diphenyl, 2.7µm, 2.1 x 50 and 150 mm produced at Advanced Materials Technology, Inc. (Wilmington, DE)
HPLC Column Dimensions: 2.1 x 150 mm **Instrument:** All chromatographic analysis performed on Shimadzu Nexera UHPLC system with LC-30 components including SPD-M30A PDA detector and 180µL mixer. **Chemicals and Reagents:** Trifluoroacetic acid (TFA) Pierce, Difluoroacetic Acid (DFA) Synquest Laboratories, Acetonitrile (ACN) and n-Propanol (nProp) HPLC Grade JT Baker **Samples:** Protein mixture samples MilliporeSigma, mAb's trastuzumab and denosumab were generous gifts of highly purified biotherapeutic grade products.
Protein Mixture Sample Preparation: Ribonuclease A 0.200mg/mL, cytochrome C 0.200mg/mL, lysozyme 0.100mg/mL, holotransferrin 0.200mg/mL, alpha-lactalbumin 0.080mg/mL, apomyoglobin 0.200mg/mL, enolase 0.300mg/mL, carbonic anhydrase 0.100mg/mL. Sample solvent aqueous 0.1% TFA. **mAb Sample Preparation:** Concentration for every sample was 2.0mg/mL in H2O + 0.1% TFA. **HPLC Column Flow Rate:** 0.40 mL/min unless specified otherwise. **UV Absorbance:** Protein mixture 220nm. IgG1 and IgG2 280nm with 350nm reference.

HALO 1000Å Bonded Phases

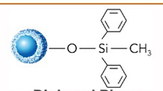


C4 Phase
- Traditional alkyl phase for protein separations
- 4.1 µmol/m^2 coverage



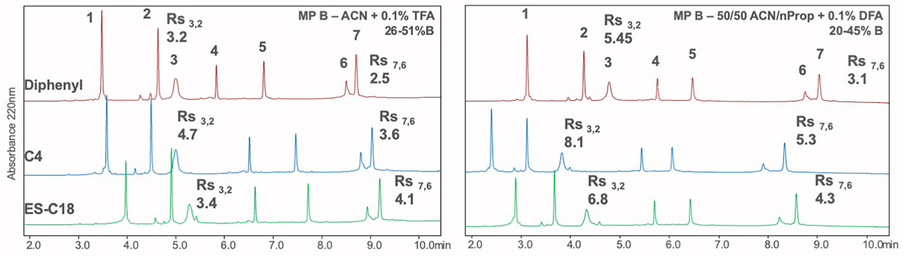


ES-C18 Phase
- Sterically-protected, long chain (C18) alkyl phase
- 1.9 µmol/m^2 coverage



Diphenyl Phase
- Aromatic phenyl phase
- Selectivity differs from alkyl phases
- 2.7 µmol/m^2 coverage

Comparison of Bonded Phases for Protein Mixture Separation



1. Effect of ACN and TFA Mobile Phase

2. Effect of ACN/nProp and DFA Mobile Phase

Protein peak identities (MW kDa): 1. Cytochrome C (12.3) 2. Lysozyme (14.3) 3. Holotransferrin (80) 4. α-Lactalbumin (14.2) 5. Apomyoglobin (17.0) 6. Enolase (46.5) 7.Carbonic Anhydrase (30.0), 60°C, 0.40mL/min, MP A- H_2O +0.1% TFA or DFA, Gradient time – 10 min, Initial pressures for Fig1. 175-250 bar; Fig2. 206-300bar

Seven proteins were separated by a fast gradient elution method at 60°C. Figure 1 compares the bonded phases under standard protein RP-LC conditions of ACN and 0.1% trifluoroacetic acid (TFA). In Figure 2 the organic eluent has been changed to a mixture of mixture of 50/50 ACN/nProp, and the acidic modifier has been changed to 0.1% difluoroacetic acid (DFA). A comparison of ACN + 0.1% DFA was run but not shown in this presentation. Similar results in regards to retention and selectivity were seen.

Selectivity changes in specific protein pairs achieved through bonded phase and mobile phase manipulation (See peaks 4,3 and 6,5). In Figure 2 n-Prop and DFA decreased overall retention time of proteins and the gradient was adjusted to ensure retention in middle to end of gradient.

Avg. Protein Rt (min)
Figure 1: ES-C18 (6.7) > C4 (6.4) > **Diphenyl (6.1)**
Figure 2: **Diphenyl (6.0)** > ES-C18 (5.7) > C4 (5.3)

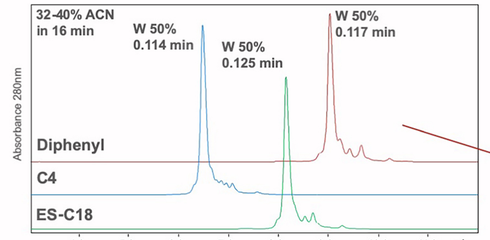
Differences in selectivity and resolution for proteins seen across bonded phases and mobile phases. Mobile Phase of ACN/nProp + 0.1% DFA is LC/MS friendly and provides full resolution of all peaks.

Comparison of Bonded Phases and Temperatures for Intact IgG1 (trastuzumab) Separation

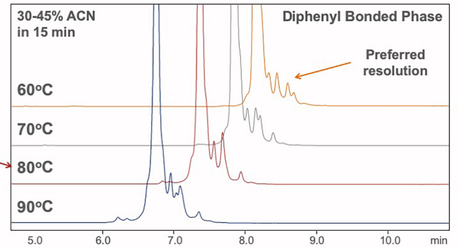
Separations of IgG1's are challenging due to biomolecule's size (~150 kDa), and complexity in confirmation and disulfide bridging. The HALO 1000Å particle was developed to help solve these separation problems, and the chromatograms in this section present examples of high performance separations. Figure 3 reveals the effect of bonded phase chemistry on the separation of the therapeutic drug trastuzumab. All three phases are able to separate and resolve some of the minor variants found in this drug. Each phase shows a different selectivity of minor variant peaks. Figure 4 exhibits the effect of temperature on minor variant selectivity using the Diphenyl phase. Higher resolution can be obtained through optimizing the bonded phase, temperature, organic eluent, and other gradient elution variables.

IgG1 and IgG2 Retention Trend
ACN: Diphenyl ≥ ES-C18 > C4 (3 of 4)
ACN/nProp: Diphenyl > ES-C18 > C4 (4 of 4)

Diphenyl phase provided increased retention and subtle selectivity changes for minor variants of intact IgG1. This phase was used to study the effect of temperature on IgG1 minor variant selectivity, with the preferred selectivity at 60°C.



3. Bonded Phase Comparison of Intact IgG1
80°C, 0.40mL/min, MP A- H_2O+0.1% TFA, MP B- ACN+0.1% TFA



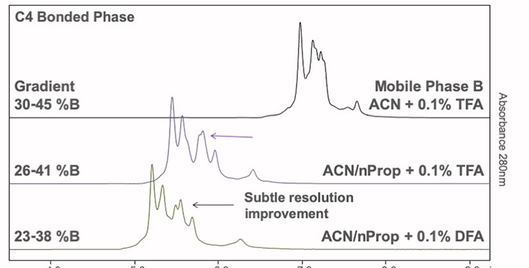
4. Effect of Temperature on IgG1 Separation Using Diphenyl Phase
0.40mL/min, MP A- H_2O+0.1% TFA, MP B- ACN+0.1% TFA

Intact IgG2 (denosumab) Selectivity Manipulation

Similar to IgG1's, the separation of intact IgG2's is made more challenging due the higher abundance of closely related isoforms. There are three main isoforms (B, A/B, and A) and each has its own variants. The three isoforms differ in the location of their disulfide bridges in the F_{ab} region.

Figure 5 provides an example of a method improvement scheme for an intact IgG2 separation, using the therapeutic drug denosumab. Previous research indicated that the C4 bonded phase had favorable surface chemistry for this separation, and was thus used in this example. For this high temperature (80°C) gradient separation the organic eluent was varied, along with the acidic modifier. The gradient start and end points were adjusted to help match retention times. The final chromatogram uses mobile phase conditions which are LC/MS friendly.

Using the favorable surface chemistry of the C4 phase, the resolution of the IgG2 isoforms were significantly improved by the addition of nProp. Subtle but useful selectivity changes are noticed when varying the acidic modifier to the LC/MS friendly DFA.



5. Effect of Mobile Phase on IgG2 Separation Using C4 Phase
80°C, 0.40mL/min, MP A- H_2O + specified acid, MP B- specified, gradient time 15 minutes,

Conclusions

- The HALO 1000Å bonded phases demonstrated subtle, but useful differences in selectivity. All three phases were highly effective in typical RP-LC separations of proteins and IgG's, with comparable peak widths.
- Trends in protein retention were effected by the bonded phase and organic eluent. In general IgG's are more retained on the Diphenyl phase.
- Gradient elution variables like organic eluent, temperature, and acidic modifier have a significant effect on retention and mAb variant resolution.
- Selectivity differences between the bonded phase 1000Å SPP materials and gradient elution variables can be usefully employed for high resolution protein separations.
- The resolution gained by these new technologies provides a greater level of detail in the structural differences of well characterized biotechnology products.

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