A Diphenyl Bonded Phase On Wide Pore Superficially Porous Particles For Efficient Separations of Proteins

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Objective

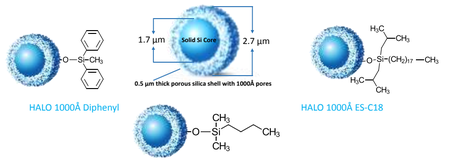
- The current work focuses on the characterization of a new Diphenyl bonded phase designed for high resolution separations of proteins.
- The creation of superficially porous particles (SPPs) of silica with a wide pore size (HALO® 1000 Å) have helped address challenges in the reversed phase separations of large biomolecules (>50,000 MW).
- The Diphenyl bonded phase protein separation performance is compared to separations performed with C4 and ES-C18 bonded phases.
- Selectivity of protein separations can be manipulated using bonded phase options, temperature, and mobile phase composition in order to provide improved analytical solutions.

Introduction

Packed columns of superficially porous particles (SPPs) of silica for use in reversed phase liquid-chromatography (RPLC) provide high performance capabilities. The recent introduction of HALO 1000 Å silica, a wide pore SPP, provides a packing material with adequate pore size for high performance RPLC separations of large proteins and monoclonal antibodies (mAb). To supply additional options for separations, various surface bonded phases for HALO 1000 Å have been examined, to complement the existing C4 and ES-C18 bonded phases previously developed for this particle. Since separations of proteins are conducted in a rather narrow range of mobile phase options, particular benefits would include changes in retention and selectivity for separations of larger proteins, particularly IgGs and component polypeptides, as well as chemical and post translation modifications of these molecules. Previous results identified that elevated column operating temperature and low mobile phase pH is often required to favor high recovery for RP separations of IgGs and other large proteins. Thus, hydrolytic resistance of bonded phases can be a concern, requiring assessment under aggressive operational conditions. Selectivity manipulation using bonded phases, combined with other operational parameters, leads to a high resolution separation of a therapeutic mAb.

HALO 1000Å Phases

Specifically Designed for Protein Separations

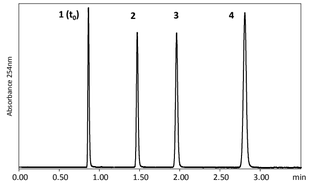


Experimental

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) **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:** All individual small molecule and protein samples included in mixtures MilliporeSigma, Monoclonal antibodies trastuzumab and denosumab were generous gifts of highly purified biotherapeutic grade products, and monoclonal antibody NISTmAb was purchased from NIST.

HPLC Samples: QC Small molecule concentrations ranged from 60 – 360 µg/mL. Protein Mixture concentrations were 0.22, 0.11, 0.11, and 0.48 mg/mL in H_2O + 0.1% TFA for Ribonuclease A, lysozyme, α-lactalbumin, and enolase, respectively. Monoclonal antibody concentration for every sample was 2.0 mg/mL in H_2O + 0.1% TFA. **HPLC Column Dimensions:** 2.1 x 50 mm used in acidic stability testing, all other columns were 2.1 x 150 mm. **HPLC Column Flow Rate:** All columns run at 0.40 mL/min unless specified otherwise. **UV Absorbance:** All protein and antibody chromatograms used 280 nm absorbance with 350 nm reference, all small molecule chromatograms used 254 nm. **Inj Volume** = 2µL for protein and antibodies, 0.2 µL for small molecules.

Comparisons of Retention and Selectivity for Small Molecule and Protein Mixtures



Example Small Molecule Separation on Diphenyl

25°C, 0.40mL/min, isocratic 50/50 ACN/H_2O, 0.2µL inj

Small Molecule Peaks

1 – Uracil (t_0) 2 – Hexanophenone
3 – Octanophenone 4 - Decanophenone

Comparison of Small Molecule Mixture

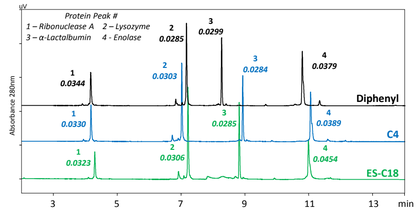
Bonded Phase	Retention Factor k'_4	Selectivity $\alpha_{4,3}$
Diphenyl	2.25	1.77
C4	4.95	2.13
ES-C18	17.92	2.81

All three bonded phases have measured efficiencies of ≥ 160,000 N/m under optimized conditions. No significant variance in column back pressure between phases.

Selectivity Values of Protein Separation

Bonded Phase	$\alpha_{2,1}$	$\alpha_{3,2}$	$\alpha_{4,3}$
Diphenyl	1.91	1.18	1.34
C4	1.85	1.31	1.26
ES-C18	1.84	1.25	1.27

Aromatic and alkyl bonded phases exhibit shifts in selectivity for proteins. These shifts in selectivity provide ability to alter biomolecule retention and resolution through changes in bonded phase chemistry.

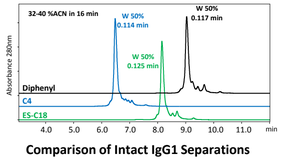


Bonded Phase Comparison of Protein Mixture Separation

60°C, 0.40mL/min, MP A- H_2O+0.1% TFA, MP B- ACN +0.1% TFA, 20-55%B in 15min
Peak widths (min) at half-height are displayed below the peak #.

Protein retention not predicted from retention of small molecule mixture.
Diphenyl phase has similar overall protein retention to alkyl phases. Selectivity differences are observed between the three phases, which exhibit similar PW for each protein.

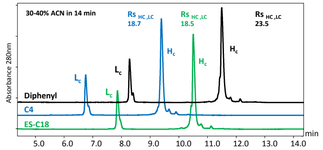
Comparisons of Retention and Selectivity for IgG1 (trastuzumab) and IgG2 (denosumab) Separations



Comparison of Intact IgG1 Separations

80°C, 0.40mL/min, MP A- H_2O+0.1% TFA, MP B- ACN+0.1% TFA

The Diphenyl phase has the greatest retention for separations of intact IgG1 and its reduced heavy and light chains. Increased retention is complimented by changes in selectivity for minor variants of intact IgG1.
The Diphenyl phase has an increased (25%) resolution of the heavy and light chain, under the selected separation conditions.



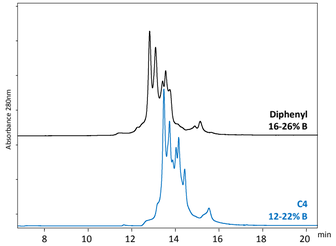
Reduced IgG1, Heavy and Light Chain Separation

80°C, 0.40mL/min, MP A- H_2O+0.1% TFA, MP B- ACN + 0.1% TFA
Reduction 2mg/mL trastuzumab w/ 20mM DTT in 6M Guanidine

Depiction of IgG2 Isoforms

The chromatogram to the right compares the Diphenyl and C4 phase, in which the gradient range for the C4 method has been adjusted to match retention times. In this example the C4 phase better resolves the main cluster of IgG2 isoforms, while the Diphenyl better resolves the tail peaks.

When compared under the same gradient method, the Diphenyl phase has greater retention than both the C4 and ES-C18 phase for denosumab.

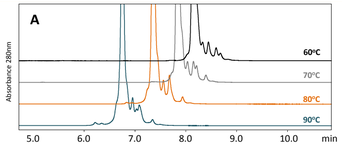


Retention Time Adjusted Separation of Intact IgG2

80°C, 0.20mL/min, MP A- 88/10/2 H_2O/ACN/nProp +0.1% DFA, MP B- 70/20/10 nProp/ACN/H_2O + 0.1% DFA, 20min gradient

Diphenyl phase provides increased retention and subtle selectivity changes for the results above, and three additional IgG samples.

Manipulation of Selectivity for Intact IgG1 (trastuzumab) Separation using Diphenyl 1000 Å



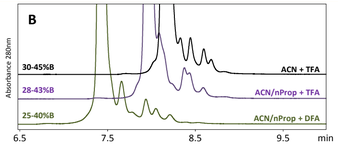
Effect of Temperature

Temp listed, 0.40mL/min, MP A – H_2O+ 0.1% TFA, MP B- ACN +0.1% TFA, 30-45%B in 15min

Chromatogram A

A standard gradient separation of trastuzumab (IgG1) was run over multiple temperatures to assess the effect on selectivity of the minor variant peaks eluting after the main peak.

The separation at 60°C displayed favorable separation of the minor variants from the main IgG peak, thus this temperature was selected for study of mobile phase modifiers.

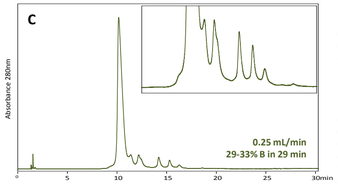


Effect of Mobile Phase Modifiers

60°C, 0.40mL/min, MP A- H_2O + 0.1% TFA or DFA, MP B – specified above, Gradient time 15min

Chromatogram B

ACN was compared to a 50/50 ACN/nProp eluent mixture. nProp has previously been shown to alter biomolecule selectivity.
Comparisons of acidic modifiers 0.1% TFA and 0.1% DFA are shown using the 50/50 ACN/nProp organic eluent.
The combination of ACN/nProp and DFA yielded the preferred separation of the minor variants.



High Resolution Separation of Intact IgG1

60°C, MP A- H_2O+ 0.1% DFA, MP B- 50/50 ACN/nProp+ 0.1% DFA

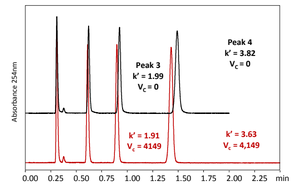
Chromatogram C

Using the preferred combination of column temperature and mobile phase modifiers examined in Chromatograms A and B, leads to an improved separation of trastuzumab.

The initial gains in minor variant selectivity and resolution were emphasized by developing the gradient conditions. The gradient slope was decreased from 0.50 to 0.14 %B per minute. To further resolve these variants, the flow rate was decreased from 0.40 to 0.25 mL/min.

Selectivity modifications of an intact IgG1 separation were achieved through the use of changes in method conditions. These conditions combined with the Diphenyl bonded phase created a high resolution separation of the IgG1.

Acidic Stability of Diphenyl Bonded Phase



Retention Results of Stability Test

25 °C, isocratic 45/55 ACN/H_2O, 100 Hz, 0.2µL inj

Stability Testing Regime

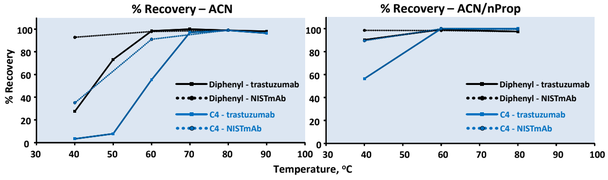
- Temp. – 90 °C
- Flow Rate – 0.70 mL/min
- Mobile Phase A – aq. 0.2% TFA *(pH = 1.60)*
- Mobile Phase B – ACN + 0.2% TFA
- Gradient - 5-40% B in 10 min + 5 min equil.

Simulates aggressive gradient method

Blank gradients were followed by small molecule retention measurement following every 10 injections. Retention and peak shape changes were minimal during the course of this column challenge of low pH and high temperature.

A decrease of 5% in the retention factor for small molecules was observed for the Diphenyl after 4,000 column volumes using highly aggressive stability challenge (high T and low pH). This result supports that the Diphenyl phase shows increased resistance to hydrolytic attack at the siloxane bond to the silica surface.

Temperature Dependence of IgG Recovery



Left figureMP A – H_2O + 0.1% TFA, MP B – ACN + 0.1% TFA -> 30-45%B in15min,
Right figure MP A – H_2O + 0.1% TFA, MP B- 50/50 ACN/nProp + 0.1% TFA -> 28-43%B in 15min

Diphenyl increases recovery of IgG1s and IgG2 (data for denosumab not shown) at lower temperatures compared to C4 and ES-C18 (data not shown). Different IgG analytes require different temperatures for high recovery, even IgGs of the same isotope. Simple changes to the mobile phase like the addition of n-propanol can shift high recovery to lower operating temperatures than observed for ACN. NISTmAb shows an artifact lower k' peak at T>70°C, but high overall recovery.

Conclusions

- The new HALO 1000 Å Diphenyl bonded phase was highly effective for separations of large proteins using typical reversed phase conditions, with comparable peak widths and shapes to those observed for alkyl bonded phases (C4 and ES-C18).
- The Diphenyl bonded phase is noted to be highly stable and resistant to low pH catalyzed high temperature hydrolysis of the surface.
- Selectivity differences between the bonded phase 1000 Å SPP materials can be usefully employed for high resolution protein separations. Thus, a strategy for varying bonded phase, temperature and mobile phase composition was demonstrated for resolution of closely related mAb IgG variants.

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