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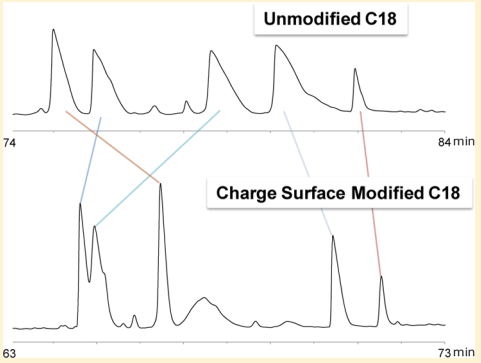
High-Resolution Peptide Mapping Separations with MS-Friendly Mobile Phases and Charge-Surface-Modified C18

Matthew A. Lauber,* Stephan M. Koza, Scott A. McCall, Bonnie A. Alden, Pamela C. Iraneta, and Kenneth J. Fountain

Waters Corporation, 34 Maple Street, Milford, Massachusetts 01757-3696, United States

Supporting Information

ABSTRACT: Ionic analytes, such as peptides, can be challenging to separate by reverse-phase chromatography with optimal efficiency. They tend, for instance, to exhibit poor peak shapes, particularly when eluted with mobile phases preferred for electrospray ionization mass spectrometry. We demonstrate that a novel charged-surface C18 stationary phase alleviates some of the challenges associated with reverse-phase peptide separations. This column chemistry, known as CSH (charged-surface hybrid) C18, improves upon an already robust organosilica hybrid stationary phase, BEH (ethylene-bridged hybrid) C18. Based on separations of a nine-peptide standard, CSH C18 was found to exhibit improved loadability, greater peak capacities, and unique selectivity compared to BEH C18. Its performance was also seen to be significantly less dependent on TFA-ion pairing, making it ideal for MS applications where high sensitivity is desired. These performance advantages were evaluated through application to peptide mapping, wherein CSH C18 was found to aid the development of a high-resolution, high-sensitivity LC-UV-MS peptide mapping method for the therapeutic antibody, trastuzumab. From these results, the use of a C18 stationary phase with a charged surface, such as CSH C18, holds significant promise for facilitating challenging peptide analyses.



The ability to effectively separate peptides is imperative to the success of many different analyses, ranging from the characterization and quality control of biotherapeutics[1−3] to the discovery of biomarkers through bottom-up proteomics.[4,5] Recent advances in separation technologies, including the commercialization of UPLC and robust sub-2-μm-particle stationary phases,[6−8] have facilitated such analyses by offering significant increases in the peak capacities of gradient reverse-phase separations. However, challenges persist in realizing the full potential of these tools, especially for separations of ionic analytes, such as peptides.

Ionic analytes have indeed proven challenging to separate by reversed-phase chromatography with optimal peak shape. Ionic analytes can exhibit characteristics of overloading, i.e., poor peak shape, on reversed-phase columns at mass loads orders of magnitude lower than neutral compounds.[9,10] This is particularly true when mobile phases preferred for electrospray ionization mass spectrometry (ESI-MS) are employed.[9] Because of the inherent complexity of peptide mixtures, such as proteolytic digests, there is, nevertheless, a need for detection by ESI-MS. It is unfortunate then that ionic analytes tend to be separated with the best peak shape and greatest peak capacity, using eluents that are detrimental to ESI-MS, for example, eluents that have high ionic strength or contain strong ion pairing agents.[11,12]

Active silanols have long been thought to be the cause of the poor peak shapes of positively charged analytes, such as peptides under acidic conditions. However, current particle technologies have near eliminated silanol activity at low pH.[13] It is unlikely then for simple interactions with deprotonated surface silanols to be the sole cause of the poor peak shape and loadability of ionic species encountered with highly inert, modern reversed-phase chemistries.[10] Additional hypotheses on the reverse-phase behavior of ionic analytes are currently being considered. Gritti and Guiochon have shown that ionic species, unlike their neutral analogs, adsorb to low-density, high-energy sites in addition to high-density, low-energy sites.[14,15] These disparate sites are believed to result from surface heterogeneity, with high-energy sites corresponding to regions deep within the bonded layer and close to the silica surface.[14,15] Although this phenomenon is consistent with the poor loadability of ionized species, the exact nature of the high-energy interaction sites that produce the poor peak shape remains to be elucidated. In yet another hypothesis, McCalley and co-workers have suggested that the poor loadability of ionized species is due to mutual Coulombic repulsion. Their "solute charge repulsion" theory[9,16] postulates that, as like-charged species are held on the hydrophobic C18 ligands, they exert repulsive forces upon each other. This results in limited use of the available surface area and, in turn, causes band broadening due to overload.

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The difference in the two theories is that Gritti and Guiochon propose that the tail of the overloaded peak is due to very low-density, high-energy sites on the sorbent while McCalley and co-workers propose that the front of the peak advances due to expulsion from pores already occupied by the like-charged analyte. As mentioned previously, the problematic peak shape for ionized analytes exists at much lower sample loads than for neutral analytes and its occurrence is worsened in the volatile, low-ionic-strength mobile phases that are desired for high-sensitivity MS analyses. High ionic strength eluents provide superior peak shape and loadability, because they are effective in shielding analytes from undesired interactions by reducing the Debye screening length, according to the electrical double layer (EDL) theory.[17]

Interestingly, most recent advances in reverse-phase chromatography have not aimed to address these challenges associated with peptide separations. Instead, a primary focus in the field has been on the investigation of different particle morphologies. The use of superficially porous particles has particularly garnered significant attention. In many studies, superficially porous particles have been found to exhibit noteworthy performance advantages, compared to fully porous particles.[18−21] It is widely appreciated, however, that there is a compromise among these particles in terms of efficiency, sample loading capacity, and analyte retention.[22] Fully porous particles tend to offer greater surface area and accordingly better loadability and analyte retention, while superficially porous particles tend to exhibit better kinetic efficiencies and amenability to faster flow rates/linear velocities.[22,23] There is clearly value in developing alternative particle morphologies for peptide separations. Nevertheless, manipulation of particle morphology does not address the issue of poor peak shape/asymmetry that results from secondary interactions. Other attributes of reverse-phase sorbents should be concomitantly considered to mitigate this problem, and likely these advances will be synergistic with different particle morphologies.

The surface charge of silica sorbents has, for instance, been shown to have a significant effect on the chromatographic behavior of ionic analytes.[13,17,24−26] With this in mind, a novel charged surface hybrid (CSH) C18 stationary phase was developed. This sorbent was an evolution of the organosilica, ethylene-bridged hybrid (BEH) C18 fully porous stationary phase[27] in that its surface is modified with a low-level amount of basic moieties as well as the C18 bonded phase. Under acidic conditions, CSH C18 thus bears positive charge similar to basic analytes. Although not entirely understood, the surface charge of the particle appears to minimize undesired interactions involving like-charged analytes (possibly those mentioned above). CSH C18 has already been shown to significantly improve the peak shapes and loadability of basic, small molecules.[16,24−26,28]

In the present work, we have evaluated the use of CSH C18 for peptide separations, predicting similar improvements in chromatographic performance given that most all peptides bear at least one positive charge under the acidic conditions routinely used in reverse-phase chromatography. Peptide mixtures were separated with both CSH C18 and BEH C18 at analytical mass loads with mobile phases often used in LC-MS applications. From this work, CSH C18 was found to exhibit greater peak capacity, unique selectivity, and significantly less dependence, in terms of peak shape, on TFA ion pairing. Since CSH C18 requires little to no strong ion pairing agents for optimal performance, it is ideal for LC-MS applications or LC-UV applications that will need to be characterized by mass spectrometry. Recognizing this, CSH C18 was used to develop a high-resolution, Lys-C peptide map of the therapeutic antibody, trastuzumab. CSH C18 yielded a peptide map with minimal coelution, which, in turn, facilitated the characterization of disulfide linkages and charge-modifying deamidation.

EXPERIMENTAL SECTION

LC-UV-MS of Peptide Mixtures. Peptide mixtures (MassPREP Peptide Mixture; Waters, Milford, MA) were analyzed by LC-UV-MS with a Waters ACQUITY UPLC H-Class Bio chromatograph and a Waters Xevo G2 QTOF mass spectrometer (Milford, MA). The nine-peptide mixture defined in Table 1 was separated with two different narrow-bore columns: an ACQUITY UPLC CSH130 C18 column (2.1 mm × 150 mm, 1.7 μm, 130 Å, Waters, Milford, MA) and an ACQUITY UPLC BEH130 C18 column (2.1 mm × 150 mm, 1.7 μm, 130 Å, Waters, Milford, MA). For a given run, either a high (ca. 6 μg) or low (ca. 0.6 μg) mass load of the peptide mixture was separated at 40 °C with a flow rate of 0.3 mL/min and a gradient consisting of a 1-min hold of 98:2 water/ACN, followed by a 60-min linear ramp to 50:50 water/ACN. Acid modifier content in the mobile phase was maintained at 0.1% (v/v), but the composition was varied between separations via the quaternary mobile phase system. Mobile phases A (0.1% FA in water), B (0.1% FA in ACN), C (0.1% TFA in water), and D (0.1% TFA in ACN) were proportioned to deliver various ratios of FA to TFA. Eluting peptides were detected via absorbance at 214 nm with a TUV detector (500 nL flow cell, 10 Hz scan rate) before being infused into the ESI source of the Xevo G2 QTOF mass spectrometer. Settings for the QTOF included a capillary voltage of 3.0 kV, a source temperature of 120 °C, a desolvation temperature of 350 °C, and a cone voltage of 25 V. Mass spectra were acquired with a resolution of ∼20000 over a range of 50−1990 m/z at a rate of 10 Hz. Separations were analyzed in duplicate to evaluate the effect of each acid modifier composition investigated.

Table 1. Nine-Peptide Mixture

	peptide	sequence
1	RASG-1	RGDSPASSKP
2	Angiotensin 1−7	DRVYIHP
3	Bradykinin	RPPGFSPFR
4	Angiotensin II	DRVYIHPF
5	Angiotensin I	DRVYIHPFHL
6	Renin Substrate	DRVYIHPFHLLVYS
7	Enolase T35	WLTGPQLADLYHSLMK
8	Enolase T37	YPIVSIEDPFAEDDWEAWSHFFK
9	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ

Enzymatic Digestion of Trastuzumab. Trastuzumab (past expiry) was digested with *Achromobacter* protease I (Lys-C) under nonreducing conditions using an adaptation of a previously published procedure.[29] Reconstituted stocks of the antibody (21 mg/mL) were first denatured in the presence of iodoacetamide. This reagent alkylates free thiols, if present, and thereby minimizes disulfide scrambling. The antibody was diluted to 2.5 mg/mL into a buffer with a final composition of 6 M GuHCl, 0.5 mM iodoacetamide, and 0.1 M phosphate (pH 7.1) then incubated for 2 h at 37 °C. Denatured trastuzumab was then diluted to 0.4 mg/mL with a urea buffer and mixed

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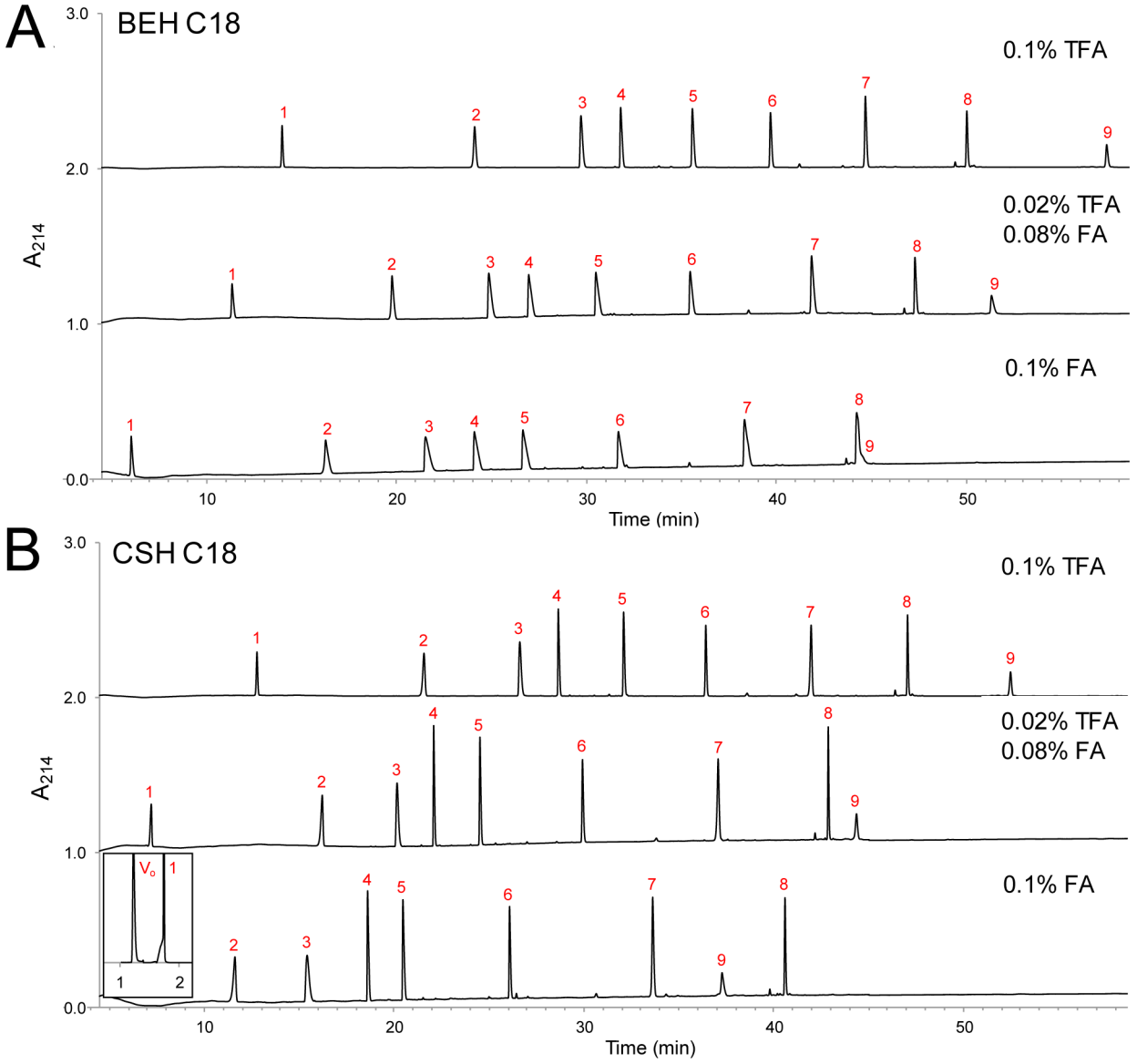

Figure 1. Separations of a nine-peptide mixture with (A) BEH C18 and (B) CSH C18 at high mass loads (ca. 6 μg of mixture). Chromatograms obtained with mobile phases containing several different acid modifier compositions are shown. Peaks are labeled according to Table 1.

with Lys-C at an 8:1 w/w ratio. The final buffer composition during digestion was 2.9 M urea, 1.0 M GuHCl, 0.04 M NH_2OH, and 0.08 mM iodoacetamide (pH 7.1). Lys-C digestions were incubated at 37 °C for 16 h, then quenched by acidification with TFA and stored at 4 °C.

Reduced Lys-C digests of trastuzumab were prepared from the nonreduced digests. The above procedure was followed. However, after acidification, samples were reduced by the addition of a 1 M solution of TCEP to a final concentration of 40 mM. The pH for this step was ~2. After 1 h at room temperature, the reduced digest was stored at 4 °C.

Peptide Mapping of Trastuzumab. Digests of trastuzumab were also analyzed by LC-UV-MS with a Waters ACQUITY UPLC H-Class Bio and Xevo G2 QTOF (Milford, MA). Nonreduced Lys-C digests of trastuzumab were separated on both an ACQUITY UPLC CSH130 C18 column (2.1 mm × 150 mm, 1.7 μm, 130 Å, Waters, Milford, MA) and an ACQUITY UPLC BEH130 C18 column (2.1 mm × 150 mm, 1.7 μm, 130 Å, Waters, Milford, MA). In each analysis, 36 μg of digest was separated using an elevated column temperature of 60 °C, a flow rate of 0.3 mL/min, and a gradient consisting of a 5 min hold of 99.5:0.5 water/ACN followed by a linear ramp to 60:40 water/ACN over 120 min. The acid modifier in the mobile phase was 0.02% TFA/0.08% FA, delivered by proportioning mobile phases as mentioned above. Eluting peptides were detected by UV absorbance prior to being infused into the ESI source of the QTOF. A valve was used between the flow cell and the mass spectrometer in order to avoid contaminating the ESI source with the nonvolatile components of the digestion buffer. Effluent was diverted from waste to the ESI source after 3 min into the run. The same detector settings mentioned before were employed; except the wavelength of absorbance was 210 nm and the scan rate for MS acquisition was 2 Hz. In addition, lockspray was employed to obtain higher mass accuracy. A solution of 0.1 μM Glu-fibrinopeptide B in 50:50 water/ACN, 0.1% FA was infused at a flow rate of 20 μL/min and sampled every 1 min to correct the mass calibration.

A gradient optimized particularly for separations with the CSH130 C18 column was employed for more-detailed analyses,

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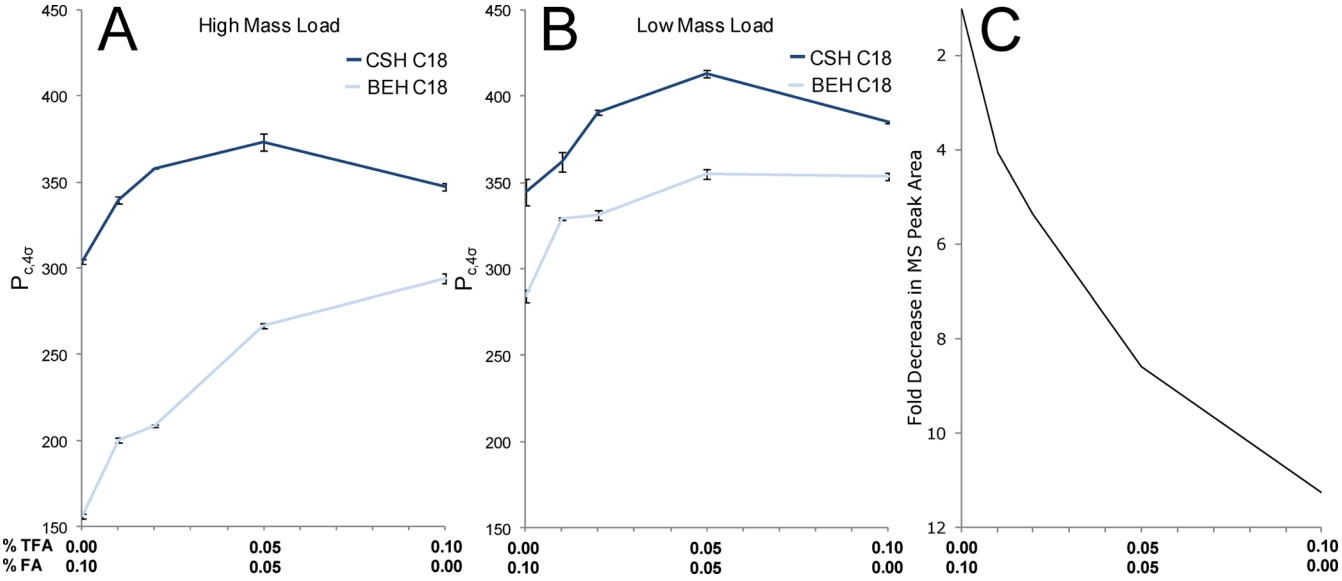

Figure 2. LC and MS performance as a function of acid modifier composition. Peak capacity at (A) high (ca. 6 μg) and (B) low (ca. 0.6 μg) mass load. (C) Fold decrease in MS peak area.

including separations of reduced Lys-C digests as well as investigations of site-specific modifications. In contrast to the nonoptimized gradient (above), the optimized gradient gradient contained multiple breaks, several linear segments, and a final ramp to a lower concentration of ACN (68:32 water/ACN). Both these gradients are tabulated in the Supporting Information (Table S1).

UV-MS data acquired during these peptide mapping separations were analyzed through automated interpretation with BiopharmaLynx (V 1.3.2), as well as manual interpretation with MassLynx (V4.1). Peptide identifications were made through matching experimental masses to the theoretical masses of peptides derived from the published sequence of trastuzumab.[30] The Lys-C peptides discussed in this work, including disulfide-linked and deamidated peptides, were identified with mass errors of <15 ppm.

RESULTS AND DISCUSSION

A new C18 stationary phase for peptide separations would ideally offer improvements in peak capacities and be less dependent on strong ion pairing agents, to be more compatible with ESI-MS. In search of such a column chemistry, we chose to investigate the performance of charged surface hybrid (CSH) C18 for peptide separations. BEH C18 is an organo-silica hybrid stationary phase with alkyl end-capping and trifunctional bonding of C18.[27] CSH C18 is based on the same BEH particle as BEH C18 with similar end-capping and trifunctional bonding, but is also bonded with a low-level amount of basic functional groups. These surface-charge-modifying moieties are expected, and empirically determined,[26] to have a pK_a near 5. As a result, the CSH particle exhibits a positive surface charge at acidic pH (<5), depending, to some extent, on the amount and type of organic solvent in the mobile phase.[31] Differences observed in the application of these two column chemistries (BEH C18 and CSH C18) report on the effects of adding a positive charge to the surface of a C18 sorbent.

Peptide Mixture: Peak Shape and Peak Capacity. A mixture comprised of nine different peptides (Table 1) ranging in both size and polarity were analyzed by LC-UV-MS with two different narrow-bore columns, one packed with 1.7 μm BEH C18 and another packed with 1.7 μm CSH C18. Mass loads of the mixture that are relatively high (ca. 6 μg), yet common for peptide mapping,[2,29,32] were first investigated. Figure 1A shows a set of UV chromatograms resulting from separations with the BEH C18 column. The top chromatogram was obtained with 0.1% TFA in the mobile phase, while the other two chromatograms were obtained with a blend of either 0.02% TFA/0.08% FA or just 0.1% FA. The limitations of C18 sorbent without a modified surface charge, so-called conventional C18, are exemplified by this example. With TFA present for ion pairing, peaks were both narrow and symmetrical. However, with exchange of the strong ion pairing agent (TFA) for the weaker ion pairing agent (FA), peak shapes broadened and took on right angle shapes that are demonstrative of overloading effects.[33,34] Remarkably different separations were observed with CSH C18 (Figure 1B). Unlike BEH C18, CSH C18 yielded excellent peak shapes that were not as sensitive to changes in acid modifier composition.

Improved peak shapes result in greater peak capacity separations and higher signal-to-noise ratios. Therefore, a comparison of BEH C18 and CSH C18 was evaluated quantitatively by means of their respective peak capacities. Peak widths were measured from UV chromatograms at peak half-height, averaged, and subsequently converted to 4σ peak capacities (see Table S2 in the Supporting Information). Peptides 1, 8, and 9 were excluded from the calculations. With the 0.1% FA mobile phase, peptide 1 (RASG-1) eluted from the CSH C18 column near the void volume with the peak artificially narrowed by the injection concentration and peptides 8 (enolase T37) and 9 (melittin) coeluted from the BEH C18 column.

Peak capacities calculated for both columns are plotted in Figure 2A, as a function of acid modifier composition. Values progressing from the left to the right of the figure correspond to decreasing concentrations of FA and increasing concentrations of TFA. This figure highlights a key performance advantage of CSH C18. With 0.1% TFA, CSH C18 produced an ~20% greater peak capacity than BEH C18. With 0.1% FA, the difference was far more substantial. CSH C18 yielded an

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astounding 90% greater peak capacity. In addition to producing greater peak capacity peptide separations than BEH C18, CSH C18 proved to be far less dependent on TFA ion pairing. Peak capacities were also determined where possible from all nine peptides in the mixture (Table S2 in the Supporting Information). These values presented very similar trends in column performance (see Figure S1 in the Supporting Information).

Understanding that the poor peak shape of ionic analytes is generally a loading phenomenon, we repeated the aforementioned separations at 10-fold lower mass loads (ca. 0.6 μg). Peak widths observed in these separations are tabulated in the Supporting Information (Table S3) and calculated peak capacities are displayed in Figure 2B. Peak capacities for both BEH C18 and CSH C18 increased with the lower mass load, as would be expected. Increases were largely incremental for most conditions. Separations with BEH C18, wherein little to no TFA was present in the eluent, were an exception. In which case, sizable increases in peak capacity were observed by lowering mass load. This is consistent with the fact that TFA is generally needed to improve the loadability of conventional C18 stationary phases. In all, CSH C18 still produced greater peak capacities than BEH C18, though the increases were less pronounced (10%−20% greater, depending on the acid modifier composition). The performance advantage of CSH C18 is indeed related to improved loading capacity. The improved loadability of CSH C18 is an excellent asset for challenging separations, particularly those that involve mixtures comprised of species present at vastly different concentrations. Conventional C18 may not be ideal for these separations, because dynamic range can be impaired by the band broadening of highly abundant species when mass loads are increased in order to detect low-abundance species.

Peptide Mixture: MS Sensitivity. The greatest performance increase of CSH C18 over BEH C18 was observed when using mobile phases with little to no strong ion pairing agent, such as TFA. A profound consequence of this is that CSH C18 is an excellent candidate for LC-ESI-MS. It is widely understood that strong ion pairing agents, most notoriously TFA, cause significant ion suppression during electrospray ionization.[35,36] The impact of acid modifier composition on the detection of the peptides in these experiments was investigated. Peak areas from the total ion chromatograms obtained during the high mass load separations were summed for all peptides and for both columns. The total counts for a given eluent were then normalized to the signal obtained with 0.1% FA. These data are summarized in Figure 2C as fold decrease in MS signal. In transition from 0.1% FA to 0.1% TFA, MS signal decreased 11-fold. That CSH C18 does not require much, if any, TFA to obtain optimal loadability/peak capacity makes it ideal for LC-MS applications in which high sensitivity is desired.

Peptide Mixture: Retention and Selectivity. Separations with CSH C18 did not differ from BEH C18 in only peak shape and peak capacity. Retention and selectivity differences were similarly prominent. Changes in retention are immediately apparent in comparison of Figures 1A and 1B. CSH C18 is slightly less retentive than BEH C18. Indeed, peptides were found to elute from the CSH C18 column with less ACN, when compared to the BEH C18 column: 4% less ACN in 0.1% FA and 2% less ACN in 0.1% TFA. A positive surface charge has been predicted to result in partial exclusion of positively charged analytes from the pore network of particles.[17] It would, in turn, be expected for there to be an excluded volume and reduced retention with CSH C18. Presumably, the repulsive effects of a positive surface charge would also weaken any affinity of positively charged analytes for high-energy adsorption sites.[24] Retention differences between BEH C18 and CSH C18 were least pronounced with separations involving 0.1% TFA. These conditions yield the greatest extent of ion pairing/charge screening, thus indicating that ionic effects are a cause of reduced retention with CSH C18. In terms of application, these observations suggest it is necessary to alter gradient methods developed for conventional C18 in order to make them suitable for use with charged surface C18.

Ionic effects also appear to impart CSH C18 with unique selectivity. As with retentivity, the biggest selectivity differences between CSH C18 and BEH C18 were observed in separations with little to no TFA ion pairing agent. Separations in which 0.1% TFA was present for ion pairing appeared fairly similar in terms of selectivity (Figures 1A and 1B). Peptides eluted from both columns, under these conditions, successively from species 1−9. However, in separations with just 0.1% FA, a change in selectivity among the two sorbents was readily apparent. Under such conditions, enolase T37 (8) and melittin (9) coeluted when using BEH C18, yet were well-resolved for CSH C18. Melittin (9) actually eluted from the CSH C18 column 4 min *earlier* than enolase T37 (8), which resulted in a change in elution order, compared to the BEH C18 column. Melittin contains six basic functional groups, whereas enolase T37 contains only three. Since melittin consequently bears more positive charge at acidic pH, it is not surprising that its retention can be affected to a greater degree by the positive surface charge of CSH C18. This observation suggests that the retention of peptides on CSH C18 is correlated, to some extent, with their charge. The charged stationary phase can thereby exhibit unique selectivity, compared to conventional C18.

Peptide Mapping of a Therapeutic mAb. The observed properties of CSH C18 lend themselves to demanding separations, such as the peptide mapping separations routinely employed to characterize biopharmaceuticals. Peptide mapping is used in both the development and quality control of therapeutic proteins, such as monoclonal antibodies (mAbs).[37−40] Proteins are digested enzymatically, separated by reverse-phase chromatography and detected by UV absorbance and/or mass spectrometry. The acquired chromatograms and data inherent in them serve to identify the therapeutic proteins along with their sequences and modifications. Peptide mapping is applied not only as a general identification tool but also to characterize and monitor the stability as well as impurities of the protein drug product.[38] These objectives represent a chromatographic challenge given that there is a need to resolve each of the tens to hundreds of unique peptide species in a proteolytic digest across a wide dynamic range.

We have evaluated the suitability of CSH C18 for peptide mapping by applying it to the analysis of one of the most successful therapeutic mAbs, trastuzumab. Trastuzumab is a humanized IgG1 that binds to and disrupts the function of the HER2 receptor, overexpressed in 20%−30% of invasive breast carcinomas.[41] In peptide mapping, it is desirable to monitor a digest with as minimal complexity as possible in order to minimize coelution and improve the chance of detecting modifications. For this reason, Lys-C is sometimes preferred over trypsin, as it cleaves after only lysine and not both lysine and arginine residues.[42,43] Lys-C thereby produces fewer but,

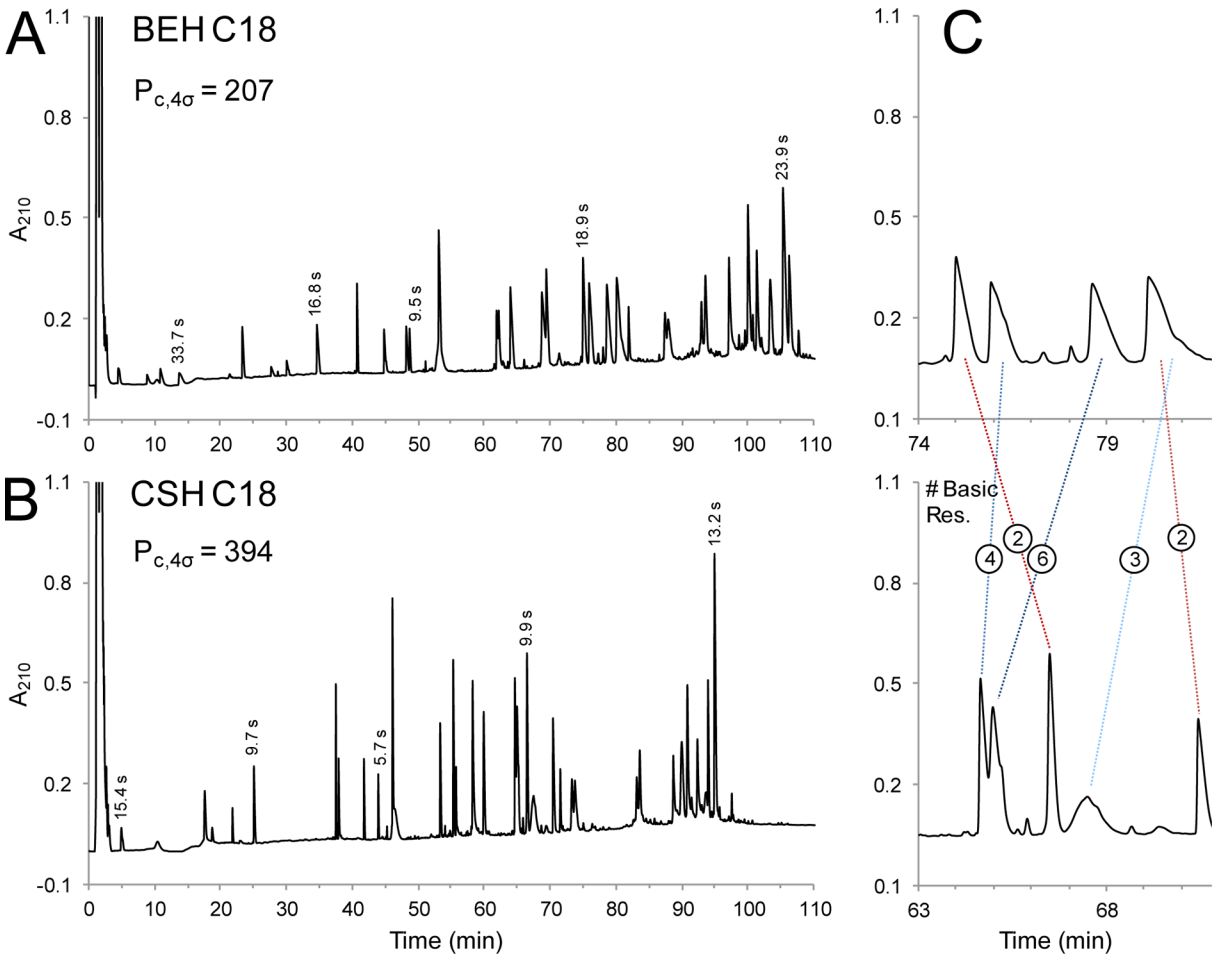

Figure 3. Lys-C peptide maps of trastuzumab obtained with (A) BEH C18 and (B) CSH C18. Peak widths at half height ($w_{0.5}$) are shown for five peptides spread across the separations. Peak capacities (4σ) were calculated from the averages of these values. (C) Corresponding retention windows from each peptide map. Colored lines indicate the change in elution order of the different peptide species, as detected by ESI-MS. The number of basic residues for each identified species is provided.

on average, larger peptides. Lys-C also maintains higher activity than trypsin in harsh, denaturing conditions, which are needed to achieve complete digestion of certain samples.[42,43] In the following work, CSH C18 was employed in Lys-C peptide mapping, although it could be applied with similar effect to tryptic peptide mapping. Nonreduced Lys-C digests of trastuzumab were separated at mass loads common to peptide mapping experiments (20−50 μg)[2,29,32] using the same two narrow-bore columns from the preceding work. Two-hour gradients and mobile phases containing a blend of 0.02% TFA and 0.08% FA were used to optimize peak capacity. In addition, elevated column temperatures of 60 °C were employed to improve the recoveries and peak shapes of the large, up to 11 kDa, peptides common in Lys-C digests (see Figure S2 in the Supporting Information).

Chromatograms obtained with BEH C18 and CSH C18 are provided in Figure 3. Similar to previous comparisons, it is apparent that CSH C18 yielded superior peak shapes. The peak capacity for each separation was assessed to confirm this observation. Five MS-identified species across the separations were subjected to measurements, and their peak widths at half height are marked (see Table S4 in the Supporting Information, as well as Figure 3). These values approximate 4σ peak capacities of 207 for BEH C18 and 394 for CSH C18. As expected based on the results with the peptide standards, the charged surface stationary phase offered a marked performance advantage. The impact of the increased peak capacity can be seen in Figure 3C, wherein corresponding retention windows from the two peptide maps are presented. Where the BEH C18 chromatogram displayed significant coelution, the CSH C18 chromatogram displayed reasonable resolution. As previously observed, selectivity differences were apparent between BEH C18 and CSH C18. Five different species were identified by ESI-MS and mapped from the BEH C18 to the CSH C18 separation in Figure 3C. The two chromatograms were aligned according to the average retention shift between the chromatograms of ∼9 min. Patterns in the elution order are thus indicative of changes in selectivity. Interestingly, peptides in this example were affected to varying degrees in going from BEH C18 to CSH C18. Some eluted relatively earlier (marked with blue lines), while others eluted relatively later (red lines). As it turns out, the peptides that eluted relatively earlier contained three or more basic residues, and those that eluted relatively later contained only two basic residues. This observation corroborates the previous suggestion that the charge of a peptide is correlated with its retention on CSH C18.

The fact that a high peak capacity peptide map could be obtained with CSH C18 using a low TFA eluent was beneficial, with respect to MS sensitivity. Figure 4 presents a chromatographic window from the middle of the UV peptide map.

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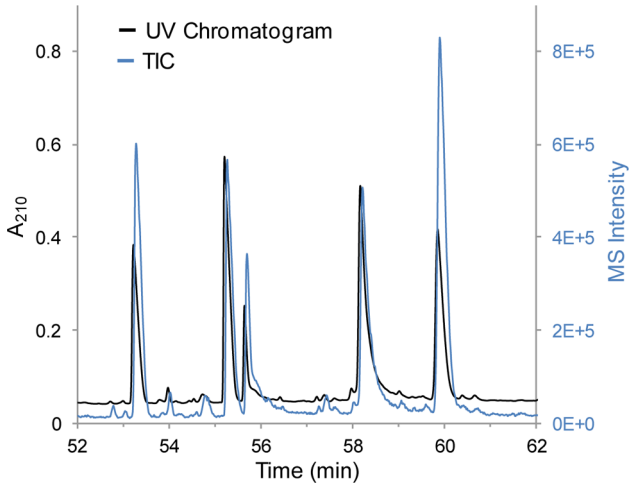

Figure 4. LC-UV-MS peptide mapping with CSH C18. Overlaid UV chromatogram and total ion chromatogram (TIC) for a retention time window from the nonreduced Lys-C peptide map.

Overlaid onto this trace is the TIC that was obtained in series with a QTOF mass spectrometer. With the blended 0.02% TFA/0.08% FA mobile phase, the acquired ESI TOF MS signal was equal to or greater than the UV signal. When relying on higher concentrations of TFA for peak shape, MS signal can be suppressed to levels lower than the UV signal. Notice that if 0.1% TFA had been employed, MS signal would have been decreased by greater than 2-fold (refer to Figure 2C), which would have resulted in TIC signal below that of the UV signal. With the conditions presented here, we achieved an optimized solution for peptide mapping that provides excellent transferability between peptide map characterization by ESI-MS and quality control separations, which are typically based solely on UV. However, the sensitivity of mass spectrometers is ever increasing, for instance, with the recent introduction of off-axis ion guides.[44,45] LC-UV-MS analyses with future generation MS instrumentation may not be challenged with matching the signals of TIC and UV chromatograms. Regardless, the amenability of charge surface modified C18 to efficiently separating peptides with MS-friendly mobile phases is likely to remain beneficial to future peptide mapping approaches.

In these experiments, MS sensitivity was indeed more than sufficient to make the identification of peptides straightforward. By assigning fully proteolyzed Lys-C peptides, sequence coverages of 97.1% and 96.7% were obtained from the BEH C18 and CSH C18 separations, respectively. The charge states of each peptide species were easily resolved, and mass errors for all assignments were <15 ppm. Mass spectrometric data supporting these assignments, along with the labeled peptide maps, can be found in the Supporting Information (see Table S5 and Figure S3). The difference in coverage between the two column chemistries was in the identification of a single tripeptide (EYK) from the constant region of the heavy chain, H:321−323. This peptide eluted very early from the BEH C18 column at 4.5 min, and likely eluted even earlier from the CSH C18 column, in the void volume along with the hydrophilic buffer components of the digest. The remaining unassigned sequence coverage corresponded to peptides not expected to be retained by C18; these were peptides all less than 500 Da in mass and primarily comprised of hydrophilic residues. Moreover, most of the unassigned sequence coverage corresponded to the constant regions of the antibody. There was, for instance, no compromise with either column chemistry, in terms of the ability to detect and monitor peptides from the highly significant complementarity-determining regions (CDRs),[30] which are responsible for antigen binding and closely linked to drug efficacy.

With the high peak capacity, high sensitivity LC-UV-MS analyses facilitated by CSH C18, we aimed to study several aspects of trastuzumab in greater detail. To this end, a gradient optimized specifically for the Lys-C peptide mapping of trastuzumab with CSH C18 was developed and applied to the characterization of disulfide linkages and deamidation. Discussion of these analyses is provided in the Supporting Information.

CONCLUSIONS

Ionic analytes, such as peptides, can be challenging to separate via reverse-phase chromatography with optimal efficiency. For example, they tend to exhibit poor peak shapes with mobile phases preferred for electrospray ionization mass spectrometry (ESI-MS). We have demonstrated that a novel charged surface C18 stationary phase alleviates some of the challenges associated with reverse-phase peptide separations. This column chemistry, known as CSH (charged surface hybrid) C18, expands upon an already robust organosilica hybrid stationary phase, BEH (ethylene-bridged hybrid) C18. Its surface is modified with a low-level amount of basic functional groups, along with the C18 bonded phase. At the low pH common to most reverse-phase separations, it thus bears a positive charge, just like most peptides. Although not entirely understood, this positive surface charge appears to minimize undesired interactions involving the peptide analytes. Whether those affected interactions are primarily adsorbate−adsorbent or adsorbate−adsorbate in nature, or some combination of the two, is still unclear. It seems most plausible, however, that the positive charge helps to minimize undesired adsorbate−adsorbent interactions. Conceivably, the positive surface charge could do so by repelling like-charged adsorbate away from the base particle such that adsorbate−adsorbent interactions are mediated exclusively via the outermost regions of the bonded phase, that is the high-density, low-affinity sites described by Gritti and Guiochon.[14,15] There is also the possibility that the moieties bearing the positive surface charge interact with and thereby effectively neutralize deeply seated, nonend-capped silanols, which have been postulated to interact with basic analytes through long-range water bridges.[46]

No matter the underlying theory, the effects of a sorbent with a positive surface charge are noteworthy. For the peptide separations investigated in this work, CSH C18 was found to exhibit improved loadability and greater peak capacities than BEH C18. Its performance was also seen to be significantly less dependent on TFA ion pairing, making it ideal for MS applications where high sensitivity is desired. With this in mind, CSH C18 was used to develop a high-resolution, high-sensitivity LC-UV-MS peptide mapping method for the therapeutic antibody, trastuzumab. Characterization of trastuzumab, including its disulfide bonds and deamidation, was facilitated with CSH C18. High peak capacity separations with minimal coelution were obtained, even with the relatively high mass loads needed to detect low abundance modifications and the low TFA eluent needed to match ESI-MS and UV sensitivity. In this regard, CSH C18 facilitates the use of a single, high-resolution peptide mapping method for both routine, UV-based analysis as well as in-depth, LC-MS

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characterization. Lastly, the work presented here has shown that the positive surface charge of CSH C18 imparts it with unique selectivity compared to BEH C18, albeit at the expense of retentivity. For these reasons, it seems that a C18 stationary phase with a modified surface charge, like CSH C18, makes for an exceptional companion to conventional C18.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental experimental information, supplemental results and discussion on mapping disulfides and deamidation, a table containing gradient conditions, tables providing chromatographic data for separations of the nine peptide mixture, a figure showing the peak capacities based on peak width data of all nine peptides from the test mixture, a figure presenting the effect of elevated column temperature on peptide mapping, a table containing mass spectral data for species assigned in Figure 3, fully assigned nonreduced peptide maps of trastuzumab along with supporting MS data, a figure highlighting the development of a gradient optimized for peptide mapping of trastuzumab with CSH C18, chromatograms of both nonreduced and reduced peptide maps of trastuzumab along with corresponding MS data, figures demonstrating the analysis of scrambled disulfides, a figure highlighting the detection of deamidated Asn55, and a table containing MS and UV data used in the relative quantitation of deamidation at Asn55. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel.: (508) 482 3017. E-mail: Matthew_Lauber@Waters.com.

Notes

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