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New wide-pore superficially porous stationary phases with low hydrophobicity applied for the analysis of monoclonal antibodies

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ABSTRACT

The article describes the development of new stationary phases for the analysis of proteins in reversed phase liquid chromatography (RPLC). The goal was to have columns offering high recovery at low temperature, low hydrophobicity and novel selectivity. For this purpose, three different ligands bound onto the surface of superficially porous silica-based particles were compared, including trimethyl-silane (C1), ethyl-dimethyl-silane (C2) and N-(trifluoroacetomidyl)-propyl-diisopropylsilane (ES-LH). These three phases were compared with two commercial RPLC phases.

In terms of protein recovery, the new ES-LH stationary phase clearly outperforms the other phases for any type of biopharmaceutical sample, and can already be successfully used at a temperature of only 60°C. In terms of retention, the new ES-LH and C1 materials were the less retentive ones, requiring lower organic solvent in the mobile phase. However, it is important to mention that the stability of C1 phase was critical under acidic, high temperature conditions. Finally, some differences were observed in terms of selectivity, particularly for the ES-LH column. Besides the chemical nature of the stationary phase, it was found that the nature of organic modifier also plays a key role in selectivity.

1. Introduction

Since the introduction of the first commercial columns packed with sub-2 μm particles, continuous developments have taken place in the area of LC column technology [1]. Very small particles offer advantages in terms of throughput, while maintaining the separation efficiency. Besides particle size, the particle structure also plays a crucial role in separation efficiency. Particles consisting of an inner solid core and porous layer manifest the advantages of porous and nonporous particles. Superficially porous particles (SPP, often called “core–shell” or “shell” particles) have gained in popularity over the last 10 years [2,3,4,5]. Today, SPPs structure is considered as one of the most advantageous stationary phase morphologies for macromolecule separations, due to the shortened diffusion path inside the particles that needs to be travelled by the slowly diffusing large solutes [6,7,8,9,10,11,12]. Several wide-pore SPP particles (with 160 - 1000 Å average pore diameters and various shell thickness) are commercially available today and have been used for many applications in the field of macromolecule separations [13,14,15,16,17,18,19,20].

SPP technology is extending its application range through the introduction of alternative surface chemistries [21,22]. By using state-of-the-art wide-pore SPPs, superior efficiency can be achieved for proteins RP separations, however some issues might be observed such as the lack of selectivity between closely related protein species and/or the strong unwanted adsorption of large proteins on silica-based materials, thus resulting in low recovery [23,24]. In common practice, mostly alkyl modifications (C4, C8, C18) are applied for protein separations in reversed phase liquid chromatography (RPLC). The type of alkylsilane modification influences the retention of proteins and can therefore be used to manipulate the overall retention and, to a lesser extent, selectivity [25]. The relation between protein retention and surface chemistry is still not fully understood, and therefore stationary phase de-

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velopment remains mostly empirical. The relative hydrophobicity of the ligand, surface coverage, ligand density, carbon load, ligand flexibility, degree of exposure of the surface silanols and of course the type of ligand all influence protein retention, selectivity and recovery [25,26]. It was believed that shorter and less hydrophobic ligands (e.g. C3 or C4) provide higher recovery than longer alkyl ligands (C8, C18), and therefore the former phases were often preferred for protein separations. However, it seems that there is no direct dependence between protein recovery and the length of alkyl ligand, while ligand density seems to be more critical [23,25]. One needs to consider that large solutes cannot penetrate into the bonded-phase layer like small molecules, because the proteins are simply too large compared to the available space between ligands [27]. Beside alkyl chain modifications, phenyl surface chemistries have also been employed for protein separations. Diphenyl and polyphenyl phases show somewhat better recovery and alternative selectivity [21,22,28,29]. High surface coverage can be reached with phenyl-based bonded phase, thus limiting silanol interactions by masking the base particle. In addition, it can also provide alternative selectivity for proteins, through possible π-π interactions that are not present in common silica-based materials [28]. Using high coverage phenyl phase, milder conditions could be applied compared to traditional alkyl phases, thanks to the limited secondary interactions, [28]. Therefore, lower temperature (60-65°C instead of 80-90°C) and reduced amount of ion-pairing agent (e.g. 0.03% instead of 0.1% trifluoroacetic acid) are sufficient, which is beneficial since on-column protein degradation could be avoided and about 40% improvement in signal to noise ratio was observed with MS detection.

In this study, new stationary phase chemistries such as monomeric trimethyl-silane (C1), ethyl-dimethyl-silane (C2) and N-(trifluoroacetomidyl)-propyl-diisopropylsilane (ES-LH) have been prepared and bounded on wide-pore silica-based SPP materials (Halo 1000 Å). The selectivity and retentivity of these new phases were compared to commercially available materials, namely the Halo 1000 Å Protein C4 and Diphenyl columns. The aim of this study was to find new stationary phase chemistries, allowing to decrease hydrophobicity, limit unwanted secondary interactions and provide alternative selectivity for protein separations, compared to commonly used alkyl- and phenyl-phases. Low hydrophobicity was of major interest, since a less retentive stationary phase requires less organic solvent and expectedly lower operating temperature. Therefore, milder (less-denaturing) conditions were expected to be set on these new phases to maintain similar retention as on common alkyl- and phenyl-phases. Lower operational temperatures could benefit artefactual protein modifications that increase in rate with temperature. The recovery, retentivity and selectivity of the three new phases were systematically compared to commercially available phases by analyzing various mAb samples. The impact of organic modifier nature was also evaluated.

2. Experimental

2.1. Chemicals and samples

Acetonitrile (AcN), methanol (MeOH), isopropanol (iPrOH) and water were purchased from Fisher Scientific (Reinach, Switzerland). Trifluoroacetic acid (TFA) and dithiothreitol (DTT) were purchased from Sigma-Aldrich (Buchs, Switzerland). IdeS (FabRICATOR®) was purchased from Genovis AB (Lund, Sweden). FDA and EMA approved monoclonal antibody (eculizumab, ipilimumab, nivolumab, obinutuzumab, ofatumumab, pembrolizumab, ramucirumab, reslizumab, rituximab, trastuzumab), and a bispecific antibody (emicizumab) sample were obtained as European Union pharmaceutical-grade drug products from their respective manufacturers.

2.2. Chromatographic system

Measurements were performed on a Waters Acquity UPLC I-Class system (Waters, Milford, MA, USA) equipped with a binary solvent delivery pump, an autosampler, and fluorescence (FL) detector. The system includes a flow through needle (FTN) injection system with 15 µL needle and a 2 µL FL flow-cell. For all measurements, a sample volume of 0.5 µL was injected and FL detection (excitation at 280 nm, emission at 360 nm, 20 Hz) was applied. Data acquisition and instrument control were performed by Empower Pro 3 software. Method development was assisted by DryLab software (Molnar Institute, Berlin, Germany). Data was treated in Excel (Microsoft).

2.3. Columns

New chromatographic materials were produced by bonding various organosilanes to fully hydroxylated superficially porous silica particles previously described as 1000Å pore size, 2.7 µm particle size Halo® FusedCore materials, with a 0.5 µm shell thickness, resulting in a specific surface area of approximately 21 m^2/g of silica [19]. The various chemical moiety on the surface include the C1 bonding, which is a monomeric trimethyl-silane (TMS), the C2 bonding, which is ethyl-dimethyl-silane, the ES-LH bonding, which is the N-(trifluoroacetomidyl)-propyl-diisopropylsilane [26], and two commercially available materials, namely the Halo 1000 Å Protein C4 and Diphenyl (DP) columns. The C1, C2 and ES-LH phases are expected to provide lower retentivity, better recovery and altered selectivity vs. the commercial C4 or diphenyl phases. All the materials were provided by Advanced Materials Technology (Wilmington, DE, USA) and packed into 50 × 2.1 mm columns.

2.4. Sample and mobile phase preparation

To evaluate and compare the recovery and selectivity of the different stationary phases, mAbs were analyzed at intact and subunit levels. Intact therapeutic proteins were diluted to 1 mg/mL with water and injected without further preparation. Preparation of mAb subunits was performed using the 1 mg/mL solution of intact samples. The disulfide bridges of intact proteins were reduced to 25 kDa light chain (LC) and 50 kDa heavy chain (HC) fragments, with DTT. The 25 kDa subunits (Fc/2, LC and Fd fragments) were created by digesting the intact mAbs with IdeS enzyme and then by further reduction with DTT. Sample preparation was performed according to the protocols described elsewhere [30].

For recovery studies, mobile phase A was 0.1% TFA (v/v) in water, mobile phase B was 0.1 % TFA (v/v) in AcN. For selectivity and retentivity study, three different organic modifiers were tried (AcN, MeOH and iPrOH).

2.5. Recovery and the effect of mobile phase temperature

On-column adsorption of intact mAbs was evaluated in a systematic way. Short gradient runs (8 min) were carried out on the prototype C1, C2 and ES-LH as well as on the commercial C4 and diphenyl columns at different temperatures, namely $T = 60$, 65, 70, 75, 80, 85 and 90 °C. The flow rate was set to $F = 0.5$ mL/min. The gradient was set as 25 – 45 % B on all columns. All the species eluted with appropriate retention at all temperatures on each column.

To compare the adsorption of mAbs on the different stationary phases, the recovery of intact ipilimumab, ofatumumab, ramucirumab, reslizumab and rituximab was assessed by comparisons of integrated area counts. The mAbs were selected to cover a wide range of behaviour (e.g. rituximab is known to strongly adsorb on RP phases, while ofatumumab is known to be less critical in

terms of unwanted adsorption) [24]. The peak areas corresponding to a given concentration at different temperatures were plotted as a function of peak areas obtained at the same concentration and the highest temperature. Since the largest peak areas were systematically observed at the highest temperature, the relative recovery could be determined and its temperature dependence could be shown. The relative peak areas (expressed as recovery percentage) related to the values observed at the highest temperature on the ES-LH column (as reference) were plotted as a function of temperature. Polynomial functions were fitted on the experimental peak area values to show the recovery trend.

3. Results and discussion

Using bonded phases with sufficiently low hydrophobicity in RPLC would be interesting for several reasons. First, lower hydrophobicity would enable decreasing the amount of organic modifier in the mobile phase, thus applying less denaturing conditions. Second, better recovery is also expected due to weaker hydrophobic interactions between hydrophobic protein residues and the stationary phase (if the coverage and/or ligand density are high enough to mask residual silanols).

In addition, such low hydrophobicity columns could be good "starting columns" for multi-column protein separations, or for online protein fractioning by serially coupling columns in their order of increasing retentivity, as recently suggested [31,32]. Indeed, one limitation of this new protein column coupling approach is the relatively narrow range of retentivity observed with commercially available wide pore RP columns. By introducing new, less retentive phases, the boundaries of the column coupling approach could be extended.

Last but not least, new stationary phase chemistries can also affect selectivity. This is an important aspect, since the selectivity of commercially available columns for protein samples are quite similar under RPLC conditions [21]. When analyzing proteins, the possible secondary interactions with the residual silanols also have to be considered (which is in practice very important because of the high number of charges on the protein surface, compared to small solutes). The ligand density of columns bonded with shorter alkyl chains can be higher than that of longer chains (less steric hindrance), thus the accessible hydrophobic surface area may be even larger for phases bonded with short alkyl ligands. Furthermore, if there are residual unbounded silanols present on the silica surface, they will be more accessible in cases where the stationary phase is composed of short alkyl chain ligands or made with lower ligand surface coverage [31]. Therefore, it is not obvious for large solutes how ligand density and chain length impact the overall retention and selectivity. In a former study, we tested some phases made of C4 and DP and possessing different coverages (10%, 50% and nominal 100%). The stationary phases with 50% partial coverage showed similar retention than the commercial phases having the same chemistry, however mAbs eluted in somewhat broader peaks. Such behaviour might be explained by the more accessible surface silanols, which probably promote additional electrostatic interactions. The less covered phases (10%) showed slightly lower retention, but proteins eluted in broad and asymmetrical peaks. This observation suggested that too much accessible surface silanols broaden the peaks (possibly through ion-exchange or coulombic interactions), while on the other hand, the lower retention was probably due to the much lower ligand density, and thus lower hydrophobicity of the phase.

In the current study, very short alkyl chains (weak hydrophobic interactions) have been examined to assess hydrophobicity of the surface. Thus, C1 and C2 bonded stationary phases were prepared and tested. Besides reducing the alkyl chain length, we also tried the N-(trifluoroacetomidyl)-propyl-diisopropylsilane (ES-LH) bonding which has already been mentioned in an early work as potentially less retentive RP phase than alkyl modified phases [26]. Since fluorine has an electronegative character, it can interact electrostatically with the hydrogen atoms of the amine- or carboxyl groups of protein backbone (H-bonding) and can attract the partial positive charges of proteins (ion-dipole or dipole-dipole interaction). The fluorine groups on the ES-LH phase probably play an important role in both stabilizing the trifluoracetylamido- from hydrolysis, as well as contributing a London dispersion force to the retention processes. However, the polarity of the propylamide and the effect of fluorine are hard to measure (or estimate). This ES-LH phase is assumed to be highly stable under low pH and high temperature conditions [26].

3.1. On-column protein adsorption, impact of temperature

In general, mAbs are analyzed at elevated temperatures (e.g. $T = 80$ - 90°C) under RPLC conditions, to achieve appropriate protein recovery, especially when they are analyzed at intact level [23,24]. At low temperature, most proteins may strongly adsorb (through mixed-mode mechanism, involving hydrophobic and ionic interactions) and stick onto the surface of the stationary phase. Therefore, mAbs often elute as broad and asymmetric peaks and suffer from incomplete elution (low recovery). On the other hand, the use of lower mobile phase temperature can be beneficial since it could decrease the risk of on-column protein degradation and might extend column lifetime, but adsorption and recovery have to be kept reasonable.

The recovery of ipilimumab, ofatumumab, ramucirumab, reslizumab and rituximab were measured at various temperatures. Based on our experience [23,24], rituximab and ramucirumab are considered as the most critical ones since they tend to form strong non-desired secondary interactions and used to show poor recovery at lower temperature. Reslizumab and ofatumumab are not really problematic in terms of recovery, while ipilimumab is an average sample from the recovery point of view.

All of the tested mAbs showed excellent recovery (above 90%) on the new ES-LH column within the entire temperature range, from 60 to 90°C. Fig. 1 shows the recovery plots obtained for rituximab, ipilimumab and reslizumab, as representative examples. For the worst mAb, rituximab (Fig. 1A), the lowest recovery was observed with the C4 phase, and the ranking of columns from recovery point of view – from worst to best - was: C4, C2, C1, DP and ES-LH. For ipilimumab (average mAb), the DP and ES-LH columns resulted in the highest recovery (> 90% already at $T = 60$°C) (Fig. 1 B). The C2 phase also offered an acceptable recovery in the entire temperature range. On the C1 and C4 phases, recovery dropped significantly when working at $T < 75$ - 80°C. Finally, for the less critical mAb, reslizumab (Fig. 1 C), the ES-LH, C1, C4 and DP phases always showed recovery values higher than 80% at $T \geq 60$°C, only the C2 phase resulted in slightly lower recovery.

To conclude on recovery, the new wide-pore ES-LH phase seems to be very promising, as it does not necessitate the use of very high temperature. Appropriate recovery can already be reached at $T = 60$°C. (Please note that if peak resolution is important then higher temperature is still beneficial, as intact mAbs elute in sharper peaks at higher temperature due to the improved mass transfer kinetics.) Among the commercial phases, the phenyl bonding often provided higher recovery than alkyl bonding (this is in agreement with data reported earlier for a polyphenyl phase compared to alkyl phases [21,22]).

3.2. Column retentivity and selectivity

The retentivity and selectivity of the different stationary phases were compared by injecting separately intact rituximab, ipili-

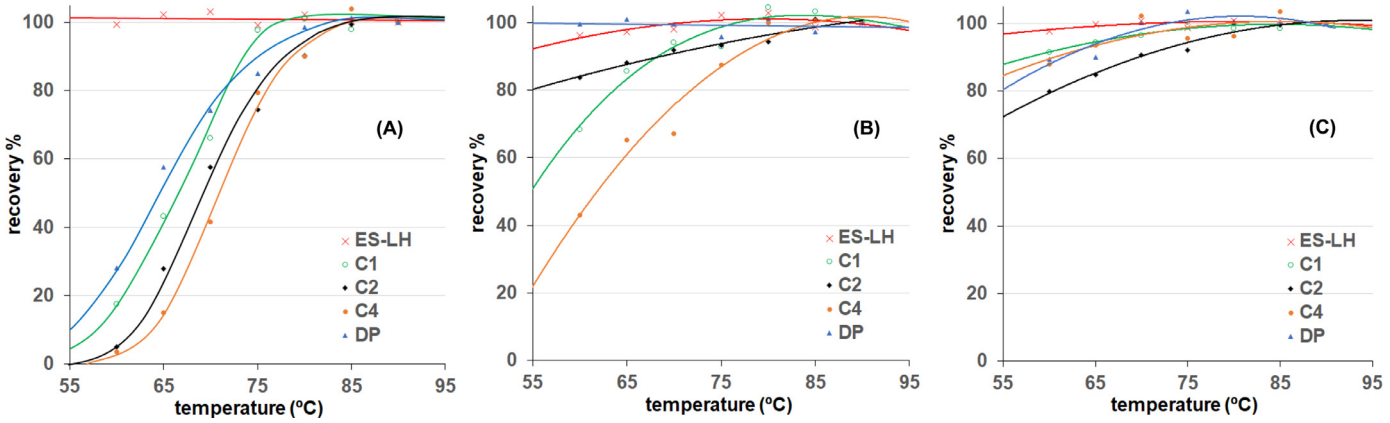

Fig. 1. Comparison of intact mAbs recovery: rituximab (A), ipilimumab (B), and reslizumab (C). Columns: 50 × 2.1 mm, 2.7 µm 1000Å, stationary phases: ES-LH (red), C1 (green), C2 (black), C4 (orange) and DP (blue). Mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in AcN, gradient: 25 – 45%*B* in 8 min, $F = 0.5$ mL/min, $T = 90$°C.

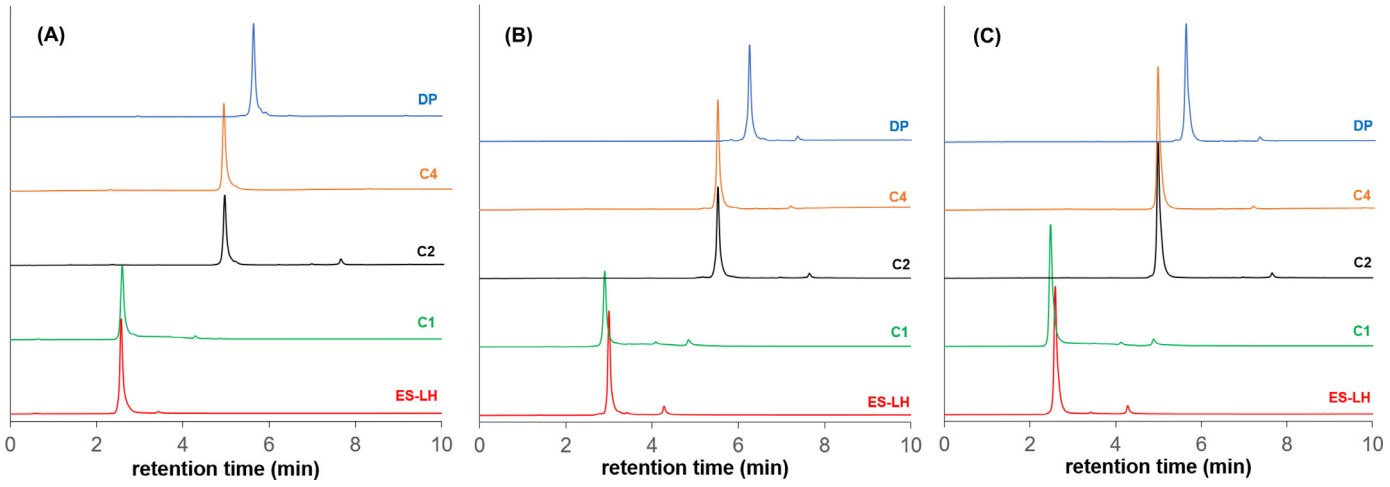

Fig. 2. Comparison of columns retentivity for intact mAbs: rituximab (A), ipilimumab (B), and ofatumumab (C). Columns: 50 × 2.1 mm, 2.7 µm 1000Å, stationary phases: ES-LH (red), C1 (green), C2 (black), C4 (orange) and DP (blue). Mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in AcN, gradient: 25 – 45%*B* in 8 min, $F = 0.5$ mL/min, $T = 90$°C.

mumab, ofatumumab, a mixture of mAbs (including trastuzumab, nivolumab, pembrolizumab, eculizumab and obinutuzumab) and reduced cys-linked ADC (brentuximab vedotin). For each individual mAb, the initial and final mobile phase compositions were adjusted to elute the species in the retention time window comprised between 2 x t_0 (twice column dead time) and the final gradient time (t_G) – whilst maintaining the gradient steepness (ΔB% range was kept at 20%).

When injecting the three individual mAbs, AcN was used as modifier. With such aprotic organic solvent, the lowest retentivity was observed on the ES-LH and C1 columns (comparable retentivity, whatever the sample), then the C2 and C4 columns showed higher - and more or less similar – retention, and finally the DP column provides the highest retention (Fig. 2). The peak shapes and symmetry values were acceptable on all phases.

The impact of organic modifier was studied by injecting a mixture of five different mAbs (Fig. 3). With AcN, the retentivity of the columns increased in the following order: ES-LH, C1, C2, C4 and DP (Fig. 3 A). AcN gradient of 25 – 45% resulted in appropriate retention on all the five stationary phases. The less retained trastuzumab and nivolumab were poorly separated on the DP and C2 columns, while pembrolizumab and eculizumab co-eluted on the C4 phase. It is interesting to notice that the elution order of eculizumab and obinutuzumab was reversed on the ES-LH column compared to the other columns. In this example, the best overall separation was obtained with the ES-LH column.

When using MeOH as organic solvent, the retentivity order of the different columns was quite comparable, however the selectivity changed to a greater extent (Fig. 3 B). A MeOH gradient of 40 – 60% resulted in appropriate retention on the ES-LH, C1 and C2 columns but gave too high retention on the C4 and DP columns. Thus, on the latter two phases, the gradient was adjusted to 45 – 65%. With MeOH, the C1 column showed altered selectivity with a reversed elution order for eculizumab and obinutuzumab. On the ES-LH column, trastuzumab eluted as a broad peak, which could be attributed to a partial separation of variants. With MeOH, we clearly highlight the lower retentivity of the ES-LH phase, compared to the other phases.

With iPrOH, the retentivity ranking of the different stationary phases was changed (Fig. 3 C). Surprisingly, the ES-LH column was found to be more retentive with iPrOH compared to C1, C2 and C4 phases. The retentivity order of the columns was: C1, C2, C4, ES-LH and DP. On the C1 and C2 columns, a 15 – 35% gradient worked well, while on the C4, ES-LH and DP columns a 20 – 40% iPrOH gradient yielded appropriate retention. The C1 column showed poor peak shape and low recovery. On the C2 and C4 columns, pembrolizumab and ecolizumab eluted in reversed order compared to the other three columns.

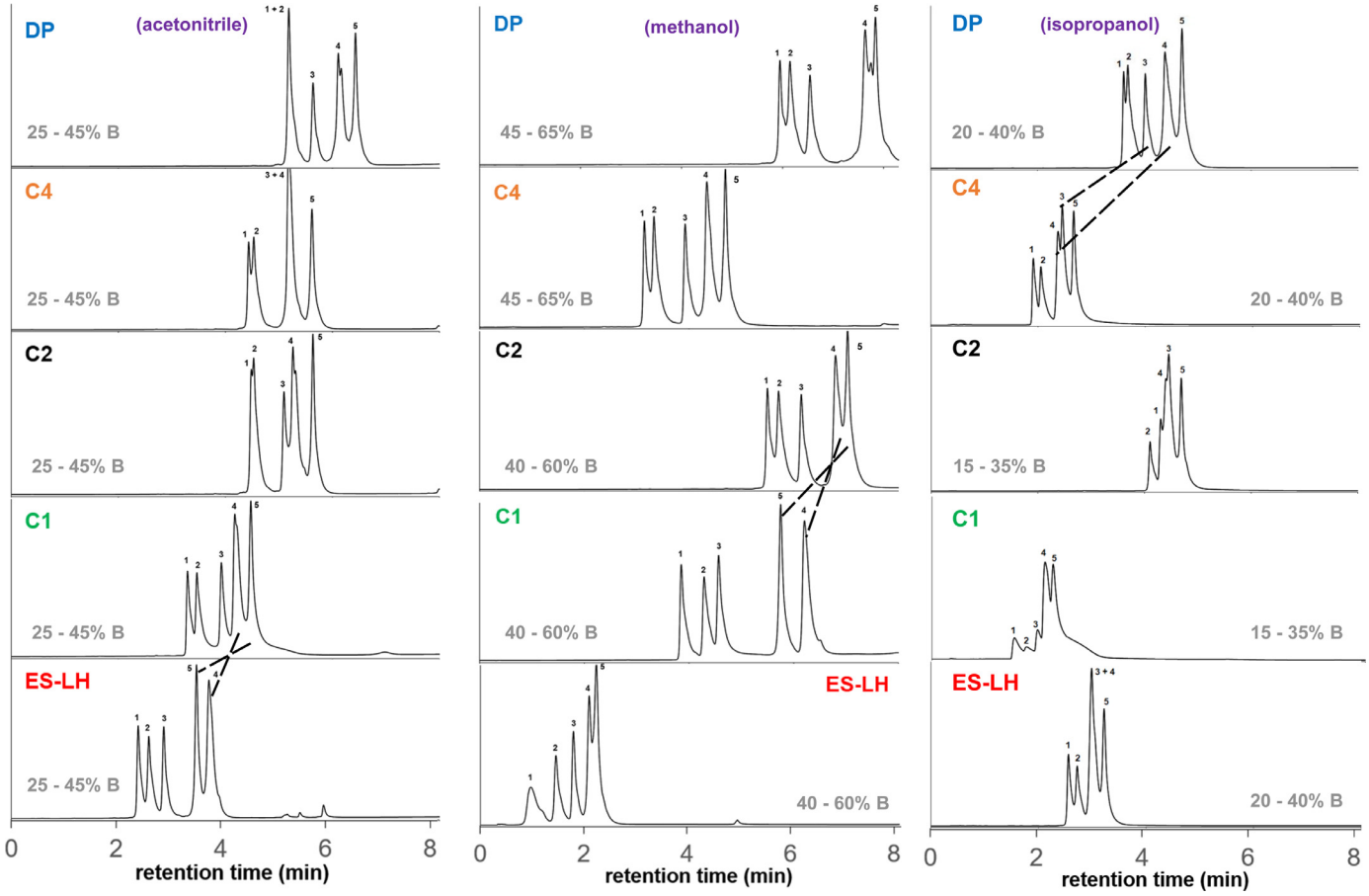

Fig. 3. Comparison of selectivity for intact mAb mixture using acetonitrile (left panel), methanol (middle panel) and isopropanol (right panel) as organic modifier and 0.1% TFA as mobile phase additive. Sample: trastuzumab (1), nivolumab (2), pembrolizumab (3), eculizumab (4) and obinutuzumab (5). Dashed lines indicate elution order changes.

Please note, that the relative retentivity of the ES-LH phase compared to the other columns was the lowest with MeOH and the highest with iPrOH. This behaviour is not yet fully understood, but may involve polar interactions and solvation energies. Further works are planned to better understand the retention mechanism of the ES-LH phase.

3.3. Fast separation of emicizumab subunits on the new ES-LH column

As a final example, a fast separation was developed on the new ES-LH column to illustrate its potential for bispecific antibody separations. Emicizumab is an asymmetric bsAb consisting of two different HCs and one LC, therefore after reduction and digestion, two different Fd and Fc/2 species are expected. As initial measurements, two gradients were run (t_{G1} = 4 and t_{G2} = 12 min, 20 – 40 %B) at two temperatures (T_1 = 70 and T_2 = 90°C). Then a retention model was created in DryLab and a two dimensional resolution map was plotted. The gradient steepness, initial-, final mobile phase composition and temperature were optimized *in silico*. It was found that a linear gradient from 26 to 35% AcN in 4 min (F = 0.5 mL/min) and at T = 90°C provides good separation for all subunit species. Then, an experimental run was performed and excellent agreement was found between predicted and experimental chromatograms. Fig. 4 shows the corresponding chromatograms for the separation of the LC, Fc/2, Fd and HC fragments. As expected, two peaks of Fd and two peaks of Fc/2 fragments could be separated with this fast method. This method can be a useful tool to check the heterogeneity and mispairing of bsAb samples.

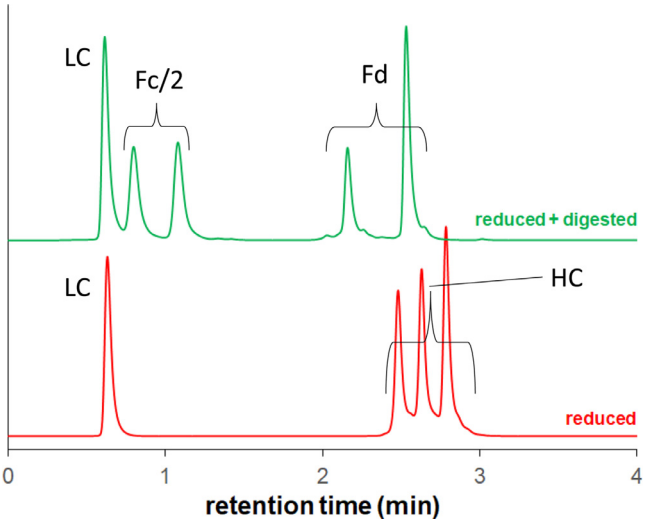

Fig. 4. Optimized fast separation of emicizumab subunits. Column: prototype 50 × 2.1 mm, 2.7 µm 1000Å ES-LH. Mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in AcN, gradient: 26 – 35%B in 4 min, F = 0.5 mL/min, T = 90°C. Peaks: light chain (LC), single chain Fc fragment (Fc/2), heavy chain (HC) and the heavy chain fragment of the Fab unit (Fd).

4. Conclusion

Our purpose was to develop new phases offering low hydrophobicity, limited secondary interactions and alternative selectivity

compared to commonly applied RP phases. Therefore, trimethyl-silane (C1), ethyl-dimethyl-silane (C2) and N-(trifluoroacetomidyl)-propyl-diisopropylsilane (ES-LH) ligands were bound onto the surface of superficially porous silica-based particles. These three new phases were applied for mAb separations and compared to commercial phases.

When studying protein recovery, the new ES-LH stationary phase was exceptionally good compared to all materials bonded with common alkyl or phenyl ligands. For most mAbs, it appears that acceptable recoveries might be achieved even at $T = 60^{\circ}C$ versus the 80 or 90°C being routinely used for such applications. The C1 and C2 phases showed similar recovery to phenyl phase but outperformed the commercial C4 phase. Indeed, the ES-LH phase provided recovery which has not been seen yet and therefore is very promising. It will be of future interest to see if this effect on recovery is shown for other classes of proteins.

Regarding retentivity, the new ES-LH and C1 materials exhibited significantly lower protein retention than the C2, C4 and DP phases. That is an important feature, since with the ES-LH and C1 phases, less organic solvent is required in the mobile phase to attain appropriate retention. In addition, these two phases can extend the potential of the recently suggested multi-column protein separation approach [31,32]. However, it is important to mention that lifetime of a column packed with the C1 phase was shorter than for the other phases (faster hydrolysis of the very short chain at high temperature and low pH, due to higher accessibility).

In terms of selectivity, all the three new phases showed some differences. For intact mAbs, the retention order was changed in some conditions. Another important finding was that the nature of organic modifier also plays an important role in protein separation selectivity, revealing significant differences amongst the bonded phases.

When considering all these features, it seems that the new ES-LH phase is a very good candidate to extend RP phases applied for protein separations. Due to its unique surface chemistry, this new phase exhibits high recovery at low temperature, low hydrophobicity and novel selectivity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Szabolcs Fekete: Writing – original draft, Methodology, Investigation. **Amarande Murisier:** Investigation. **Alain Beck:** Resources. **Jason Lawhorn:** Resources. **Harry Ritchie:** Resources. **Barry Boyes:** Writing – review & editing. **Davy Guillarme:** Supervision, Writing – review & editing.

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