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Separation of peptides and intact proteins by electrostatic repulsion reversed phase liquid chromatography

Fabrice Gritti*, Georges Guiochon

Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1600, USA

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ABSTRACT

A new brand of BEH-C_{18} hybrid particles chemically bonded to a leash carrying an amine group permits the implementation of electrostatic repulsive interactions chromatography. Using columns packed with this material, the influence of the concentration of positive charges bonded to the BEH-C_{18} surface on the overloaded band profiles of a few positively charged peptides and proteins was investigated in the gradient elution mode. Three columns packed with endcapped BEH-C_{18} particles bonded with three different surface-charge densities (LOW, MEDIUM and HIGH) were used and compared with those provided by a column packed with non-doped, endcapped BEH-C_{18} particles. The surface concentrations of fixed charges in the LOW, MEDIUM and HIGH columns were estimated at 0.029, 0.050, and 0.064 μmol/m^2, for example, about two orders of magnitude smaller than the surface density of bonded C_{18} chains (2.1 μmol/m^2). Three different mobile phase additives (0.1% v/v of trifluoro-acetic, phosphoric, and formic acid) were used to optimize the purification levels of proteins under different loading conditions. The weak ion-pairing ions (formate and phosphate) generate smaller retention but broader, more fronting band profiles than those eluted with a stronger ion-pairing ion (trifluoroactate). This effect is worse in the presence of fixed charges at the surface of the BEH-C_{18} particles. This was explained by an enhanced anti-Langmuirian adsorption behavior of the charged proteins in the presence of fixed surface charges. As the protein concentration increases in the bulk, so does the internal ionic strength, the electrostatic repulsive interactions weaken, and retention increases. Band fronting is mostly eliminated by replacing weak ion-pairing acids with TFA with which the adsorption isotherm remains weakly langmuirian. Faster but still complete gradient separation of insulin and myoglobin were achieved with the HIGH column than with the reference neutral column, despite a measurable loss in selectivity.

1. Introduction

Surface charged, organic/inorganic hybrid (CSH) packing materials were recently introduced as a promising alternative packing material to conventional neutral hybrid particles for the analysis of small basic compounds in RPLC [1–3]. The remarkable advantages of this new mixed mode (electrostatic repulsive interactions combined with RPLC-like dispersive interactions or ER-RPLC) are the reduction of peak tailing of protonated bases under acidic pHs and shorter analysis times. A similar combination of intermolecular interactions is also provided in hydrophilic interaction chromatography (HILIC) by so-called electrostatic repulsion HILIC (ERLIC) stationary phases used for isocratic separations of charged solutes, phosphopeptides [4] and in proteomic research [5].

In ER-RPLC, the adsorption behavior of positively charged basic compounds on CSH-C_{18} particles remains heterogeneous [6–9] and is similar to the well-known adsorption behavior of ionizable compounds on neutral RPLC-C_{18} columns as studied in the past by accurate frontal analysis data or inverse chromatographic methods [10–25]. The main differences involve the effect of the charged surface on the chemical equilibrium constants which become smaller electro-chemical equilibrium constants. A complete thermodynamic study of the overloaded band profiles of nortriptylinium hydrochloride measured on a BEH-C_{18} (no surface charges) and three CSH-C_{18} columns (with surface densities of bonded charges of 0.024, 0.05 and 0.064 μmol/m^2) refined quantitatively the adsorption model for small protonated bases in ER-RPLC [26]. Irrespective of the loading factor used, the agreement between experimental and calculated band profiles was always excellent for a Langmuir-Moreau adsorption isotherm model. In

* Corresponding author. Fax: +1 865 974 2667.
E-mail addresses: gritti@ion.chem.utk.edu (F. Gritti), guiochon@utk.edu (G. Guiochon).

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contrast, the classical electrostatically modified Langmuir (EML) isotherm [27] could not satisfactorily account for the observations because it ignores the surface heterogeneity and the adsorbate-adsorbate interaction at high loading factors.

The use of the Langmuir–Moreau isotherm provided a consistent adsorption mechanism. The most abundant adsorption sites are the weak ones and are located at the interface between the C_{18}-bonded layer and the internal eluent. Strong adsorbate–adsorbate interactions take place onto these weak sites at high concentrations in the presence of counter-ions (Moreau term). The few strong adsorption sites are located deep amidst the C_{18} chains where either isolated silanols or fixed positive charges are attached to the surface of the BEH particles (Langmuir term), offering a larger inter-chain space for a partition-like retention mechanism. The analysis of the best isotherm parameters showed that the electro-chemical equilibrium constants on the weak and strong adsorption sites decrease by factors 2 and 10, respectively, with increasing the surface charge density from 0 to 0.064 μmol/m^2. Due to their proximity to the active sites, the fixed charges and their surface density impact directly on the tailing of protonated bases: the larger the density of the fixed charges, the lesser peaks tail because the electro-chemical equilibrium constant for adsorption onto active sites drops rapidly.

Despite the unavoidable loss of selectivity with increasing surface charge density of CSH-C_{18} particles [26], higher purity levels can be achieved in the preparative chromatography of the small protonated bases, which have access to the active sites between the C_{18} chains. However, it is questionable whether this advantage of CSH-C_{18} particles extends to the more voluminous molecules of peptides and proteins. It is an important problem because the concentration and purification of proteins is one of the most intensively studied separation process in the biotechnology, biomedicine and food production industries. In order to dissolve large amounts of these bio-molecules, acidic pHs are often required. The net charge of peptides and proteins is positive and they will inevitably experience electrostatic repulsion under ER-RPLC conditions. Yet, the literature dealing with the use of charged RPLC stationary phases for protein purification is scarce. New positively charged hybrid ultrafiltration membranes were recently synthesized for the separation of proteins [28–30]. So, it is worth investigating the potential of these new CSH-C_{18} stationary phases for the separation and purification of large biomolecules. At the same time, it is important to minimize the cost of the purification process by selecting the most appropriate acidic mobile phase additive since its removal from the collected fractions can be necessary due to its possible *in vivo* toxicity). This could involve further expensive chemical steps [31].

The retention times under analytical conditions by gradient elution and the overloaded band profiles at high loading factors of two peptides (bradykinin and β-lipotropin) and six proteins (insulin, lyzozyme, myoglobin, β-lactoglobulin, albumin, and apo-transferrin) were measured, using four columns, three packed with 1.7 μm CSH-C_{18} particles having three levels (LOW, MEDIUM, and HIGH) of surface density of fixed positive charges and one with reference, neutral 1.7 μm BEH-C_{18}. The dimensions of these four columns are the same (2.1 mm × 150 mm) and they were all operated at the same flow rate of 0.2 mL/min. The gradient ramp lasted 10 min, the initial and final volume fractions of acetonitrile in water were set at 5% and 75%, respectively, and the wash step was 2 min long. The columns were all re-equilibrated during 8 min before the next run. The impact of the nature of the acidic mobile phase additive (0.1% of trifluoroacetic, formic, or phosphoric acid) on the retention factor and the shape of the overloaded band profiles measured for small and large loading factors will be discussed in depth. The present work does not investigate the biological activity of the purified proteins after their separation in presence of acidic additives and organic solvent in the aqueous eluent. This issue is definitely relevant in practice when purifying large proteins because their structure can be modified in such solvent conditions. The function of the original protein can then be affected and its therapeutic effects lost. The purification process of biomolecules presented in this work applies essentially to peptides and small proteins. Finally, this work reports on the potential advantages and downsides of the new CSH-C_{18} stationary phase for the purification of intact proteins.

2. Experimental

2.1. Chemicals

The mobile phase used was a mixture of acetonitrile and water, both HPLC grade, from Fisher Scientific (Fair Lawn, NJ, USA). The acidic pH of the mobile phase was obtained by adding 0.1% in volume of trifluoroacetic acid (TFA, > 99%, Across Organics, NJ, USA), phosphoric acid (H_3PO_4, 85%, Aldrich, Milwaukee, WI, USA), or formic acid (HCOOH, > 95%, Aldrich, Milwaukee, WI, USA) to the water and acetonitrile bottle reservoirs. Acetonitrile was filtered before use on a surfactant-free cellulose acetate filter membrane, 0.2 μm pore size (Suwannee, GA, USA). The peptides β-lipotropin (Glu-Leu-Ala-Gly-Ala-Pro-Pro-Glu-Pro-Ala, porcine, TFA counter ion) and bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, acetate counter ion) were both purchased from American Peptide (purity > 98 %, Sunnyvale, CA, USA). Insulin (human) was a generous gift from Eli Lilly (Indianapolis, IN, USA). All the other intact proteins, lyzozyme (chicken eggs), myoglobin (horse skeletal muscle), β-Lactoglobulin (bovine milk), albumin (turkey eggs), and apo-transferrin (human, iron-poor) were all purchased from Aldrich.

2.2. Materials

The four 2.1 mm × 150 mm BEH-C_{18} research columns used in this work were a gift from their manufacturer (Waters, Milford, MA, USA). Each one was packed with one of the four different batches of BEH-C_{18} fully porous particles described below:

1. The first research batch was obtained by endcapping a standard batch of 1.7 μm BEH-C_{18} particles.
2. The second batch was prepared by bonding a small amount of charge groups to the surface of a BEH material, then C_{18} derivatizing and endcapping it. The corresponding column is named "LOW" in the rest of this work. Note that the C_{18} surface coverage is only 2.2 μmol/m^2 for this research batch instead of 3.2 μmol/m^2 for the commercial BEH-C_{18} product.
3. The third batch was similarly made by bonding a medium amount of charge groups. The corresponding column is named "MEDIUM".
4. The fourth batch was prepared with a high amount of charge groups. The corresponding column is called "HIGH".

The three charge-modified BEH-C_{18} particles were prepared by modifying the surface of bare BEH particles with a low concentration of a charged groups, then devivatizing and endcapping the product with a trimethylsilane reagent. Table 1 lists the important physico-chemical properties (specific surface, specific pore volume, average pore diameter, C_{18} surface coverage, and fixed charge density) of these four batches of BEH-C_{18} particles along with the total porosity of the corresponding packed columns.

2.3. Apparatus

All the measurements were performed on a new prototype 1290 Infinity HPLC system (Agilent Technologies, Waldbroen, Germany)