Journal of Pharmaceutical and Biomedical Analysis 104 (2015) 130–136

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Evaluation of new superficially porous particles with carbon core and nanodiamond–polymer shell for proteins characterization

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ARTICLE INFO

Article history:
Received 16 September 2014
Received in revised form
18 November 2014
Accepted 20 November 2014
Available online 27 November 2014

Keywords:
Superficially porous
Peak capacity
FLARE®
Aeris®
Protein separation

ABSTRACT

A new superficially porous material possessing a carbon core and nanodiamond–polymer shell and pore size of 180 Å was evaluated for the analysis of large proteins. Because the stationary phase on this new support contains a certain amount of protonated amino groups within the shell structure, the resulting retention mechanism is most probably a mix between reversed phase and anion exchange. However, under the applied conditions (0.1–0.5% TFA in the mobile phase), it seemed that the main retention mechanism for proteins was hydrophobic interaction with the C18 alkylchains on this carbon based material.

In this study, we demonstrated that there was no need to increase mobile phase temperature, as the peak capacity was not modified considerably between 30 and 80 °C for model proteins. Thus, the risk of thermal on-column degradation or denaturation of large proteins is not relevant. Another important difference compared to silica-based materials is that this carbon-based column requires larger amount of TFA, comprised between 0.2 and 0.5%.

Finally, it is important to mention that selectivity between closely related proteins (oxidized, native and reduced forms of Interferon α-2A variants) could be changed mostly through mobile phase temperature.

1. Introduction

Thanks to its high resolving power, reversed phase liquid chromatography (RPLC) is an important and promising technique in the separation of biological macromolecules [1–3]. Due to the development of instruments [4,5] and column technology [6–10], the past decade served significant improvements in the separation of proteins under reversed phase conditions. Sub-2 μm fully porous particles and core–shell materials possessing large pore size in the range of 170–300 Å provide outstanding performance when analyzing proteins larger than 10–15 kDa [9]. This is explained by the excellent kinetic performance produced by small fully porous particles and by the so-called core–shell advantages [11] when using superficially porous materials. Except for kinetic performance improvements, recently packed column developments focused on the introduction of various stationary phase chemistries (e.g. C3—C12, pentafluoro-phenyl, phenyl-hexyl, biphenyl, etc.), assumed to serve different selectivity from the conventional C18 phases. Surface modifications have been executed on the traditional silica or hybrid-based materials, which are the workhorses of liquid chromatography [12]. Recently, an alternative, carbon–nanodiamond based C18 superficially porous material has been introduced to the market under the trademark FLARE® from Diamond Analytics. This new column technology could be particularly interesting in the separation of large analytes, since it possesses an average pore size of 180 Å and offers the benefits of core–shell particle technology.

Natural diamond microparticles have been first reported as chromatographic packing in 1973 [13]. However, the low surface area and inhomogeneous adsorption properties of the particles (due to their natural origin) resulted in poor efficiency. Besides efficiency issues, limited availability and high costs prevented their spread in liquid chromatography. Thanks to continuous developments in diamond production technology over the last 40 years, high purity synthetic particles of controlled size and shape became easily accessible at relatively low costs [14]. The physical and chemical properties of these high quality particles renewed interest for chromatographic applications. Diamond particles possess excellent thermal conductivity [14], pH stability [15] and are biocompatible [16], which could make them useful for protein separations. The core of the commercial state-of-the-art FLARE®

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http://dx.doi.org/10.1016/j.jpba.2014.11.039
0731-7085/

material is a carbonized poly(divinylbenzene) particle with a diameter of ca. 3.4 μm. Poly(allylamine)–nanodiamond hetero-layers are deposited onto the surface of the carbonized core by a modified layer-by-layer method [17]. The resulting core–shell is synthesized to a shell thickness of ca. 0.1 μm and a finished particle size of 3.6 μm with a surface area around 23 m^2/g. The particles are then functionalized, cross-linked and sieved. The surface area is somewhat lower than on silica-based or graphitic phases but provides sufficient retentivity. Studies in the past few years showed the applicability of carbon/nanodiamond/polymer based shell particles both in normal and reversed phase conditions [18]. Thanks to the improvement in the manufacturing technology of these particles and columns, a continuous increase of kinetic efficiency in the separation of low molecular weight solutes has been reported [14]. Latest results showed promising 100,000–120,000 N/m values for the separation of alkyl benzenes which corresponds to a reduced plate height (h) value of ~2.3 [17]. These efficiencies are outstanding compared to earlier carbon/nanodiamond/polymer based shell particles, but are still below the efficiencies reported for silica based shell materials. The 3.6 μm wide pore shell silica particles showed 150,000–200,000 N/m [9], while sub-3 μm shell silica particles dedicated for macromolecular separations provided 200,000–260,000 N/m [8], measured by low molecular weight analytes. This clearly reflects that further improvement in the manufacturing technology of carbon/nanodiamond/polymer material is needed. On the other hand, thanks to its mixed-mode hydrophobic-anion exchange surface, it could provide alternative selectivity and retention mechanism, compared to traditional reversed phases.

To our knowledge, no data on the applicability of these materials for protein separations have been reported to date. The aim of this study was to evaluate the kinetic efficiency and selectivity of this novel stationary phase for protein characterization. Model proteins and real life samples of native, oxidative stressed and reduced therapeutic proteins were analyzed using the FLARE® column to demonstrate its possibilities and limitations.

2. Experimental

2.1. Instrumentation

Chromatographic experiments were performed on a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA). The instrument was equipped with a binary solvent manager, autosampler, thermostated column compartment, and UV detector. The autosampler was equipped with a flow through needle injection system. The average extra-column peak variance of our system was found to be around σ^2_{ec}~0.5–2.5 μL^2. The UV detector operated with a 500 nL flow cell, set to 280 nm and 40 Hz sampling rate. Data acquisition, data handling and instrument control were performed by Empower Pro 2 (Waters, Milford, MA, USA) software.

2.2. Chemicals and columns

Acetonitrile (gradient grade), trifluoroacetic acid (TFA, puriss p.a.), hydrogen peroxide (30 wt.%, ACS reag. grade), dithiothreitol (DTT, ≥99.0%), methionine (≥98.0%) and protein standards such as transferrin (human, MW ~ 77.0 kDa), cytochrome-c (from horse heart, MW ~ 12.4 kDa) and albumin (BSA from bovine serum, MW ~ 69.3 kDa) were purchased from Sigma–Aldrich (Buchs, Switzerland). Water was obtained from a MilliQ Purification System from Millipore (Bedford, MA, USA). Recombinant interferon alfa-2A (MW ~ 19.2 kDa, Roferon) was obtained from Roche Pharma (Switzerland).

FLARE® C18 mixed-mode (100 mm × 2.1 mm 3.6 μm, 180 Å) column was purchased from Diamond Analytics (Orem, UT, USA), and Aeris Widepore XB-C18 (100 mm × 2.1 mm, 3.6 μm, 200 Å) column was purchased from Phenomenex (Torrance, CA, USA).

2.3. Methodology

2.3.1. Sample preparation

For the kinetic evaluation, protein standard mixture of transferrin, BSA and cytochrome-c was prepared containing 0.3 mg/mL of each protein in water. For the selectivity studies Interferon alfa-2A was injected as received. Reduced and oxidized proteins have been prepared from 100 μL of the Interferon solutions. A small amount of DTT was added to reduce the protein, then the sample was incubated at 30 °C for 30 min in dark. Oxidation was carried out by adding 1 μL of hydrogen-peroxide solution and incubation at 30 °C for 60 min. Oxidation was quenched by adding a small amount of methionine to the sample.

2.3.2. Chromatographic methods

For the gradient separation of the proteins, mobile phase "A" was prepared from water and mobile phase "B" was prepared from acetonitrile.

Peak capacity was measured applying gradient elution of 20–45%B on the FLARE® column, and 30–55%B on the Aeris WP column (in order to keep the apparent retention factors in the same range). Gradient times were set to 4, 12, 20, 28, 36, and 44 min, followed by 1.5 min equilibration. Flow rate was set to 0.2 mL/min and 0.4 mL/min. Mobile phases contained 0.1, 0.2, 0.3 and 0.5% TFA. Columns were thermostated at 30 °C and 50 °C. 2 μL of the samples were injected in full loop injection mode.

Selectivity studies on Interferon variants included gradient runs of 25–50%B on the FLARE® column. Gradient times were set to 7 min, flow rate was fixed at 0.3 mL/min in all cases. Mobile phases contained 0.3 and 0.5% TFA, and temperature was set at 30, 50 and 90 °C. Detection has been carried out using fluorescence detection (λ_{ex}: 280 nm, λ_{em}: 360 nm, 20 Hz sampling rate). 2 μL of the samples were injected in full loop injection mode.

2.3.3. Determining peak capacity

Peak capacity is a concept first described by Giddings [19] and soon put to good use by Horvath for gradient chromatography [20]. It is a measure of the separation power that includes the entire chromatographic space together with the variability of the peak width over the chromatogram. In this study, peak capacities were experimentally determined from the gradient time (t_g) and the average measured peak width at 50% height ($W_{50\%}$). The following equation was used to estimate the peak capacity based on peak width at 4σ, corresponding to a resolution of R_s = 1 between consecutive peaks:

$$P = 1 + \frac{t_g}{1.7 \cdot w_{50\%}} \quad (1)$$

In order to avoid the imprecision associated with the measurement of peak widths at base for proteins often containing closely related variants (i.e. for a heterogeneous sample) the peak width at half height was preferred in this study. This way, the impurities present in the sample, and partially resolved from the main component, did not confuse the measurement.

Peak capacity was plotted as a function of gradient time.

3. Results and discussion

When high molecular weight solutes have to be separated, the column performance is mostly determined by the mass transfer resistance because of the low diffusivity of proteins. Recent work suggests that the improvement in mass transfer of the new generation of superficially porous particles can mostly be explained by the reduction in external film mass transfer resistance (c_f term in