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Prototype sphere-on-sphere silica particles for the separation of large biomolecules

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ABSTRACT

The goal of this study was to evaluate the possibilities offered by a prototype HPLC column packed with ~2.5 μm narrow size distribution sphere-on-sphere (SOS) silica particles bonded with C4 alkyl chains, for the analytical characterization of large biomolecules. The kinetic performance of this material was evaluated in both isocratic and gradient modes using various model analytes. The data were compared to those obtained on other widepore state-of-the-art fully core–shell and fully porous materials commonly employed to separate proteins moreover to a reference 5 μm wide pore material that is still often used in QC labs. In isocratic mode, minimum reduced plate height values of h_{min} = 2.6, 3.3 and 3.3 were observed on butylparaben, decapeptide and glucagon, respectively. In gradient elution mode, the SOS column performs very high efficiency when working with fast gradients. This prototype column was also comparable (and sometimes superior) to other widepore stationary phases, whatever the gradient time and flow rate, when analyzing the largest model protein, namely BSA. These benefits may be attributed to the SOS particle morphology, minimizing the intra-particle mass transfer resistance.

Finally, the SOS column was also applied for the analytical characterization of commercial monoclonal antibody (mAb) and antibody-drug conjugate (ADC) samples. With these classes of proteins, the performance of SOS column was similar to the best widepore stationary phases available on the market.

1. Introduction

Highly efficient and faster separations have always been of great interest in high performance liquid chromatography (HPLC), and have become increasingly important in recent years, mainly driven by the challenges of analyzing more complex samples like therapeutic peptides and proteins. Thanks to its high resolving power, reversed phase liquid chromatography (RPLC) is an important and promising tool for the separation of biological macromolecules [1–3]. In such complex separations, the high kinetic performance offered by RPLC plays a key role. By using particulate (packed) columns, the efficiency of large molecule separations can mostly be improved by reducing the intra-particle mass transfer resistance. Since large molecules possess slow diffusivity they spend more time in the intra-particle pores, therefore their bands tend to broaden. To decrease this contribution, non-porous materials or partially porous materials can be applied. However, non-porous materials suffer from limited loading capacity and retention, therefore they have not became widespread in routine analytical labs. A better compromise between efficiency and loadability is the so-called core–shell particle technology.

Core–shell particles consist of a non-porous core surrounded by a porous shell presenting an elevated specific surface. This concept has been introduced in 1967 by Horvath et al. who worked on "pellicular particles" [4]. This material has been known over the years as "pellicular particles", "superficially porous particles", "controlled porosity material", "fused-core particles", "core–shell particles" or "shell particles". Guiochon and Gritti reviewed the applications of

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such particles to liquid–liquid and liquid-solid separations which was the starting point of two generations of core–shell packed columns with limited commercial success (Zipax, Corasil, Pellicosil or Poroshell) [5]. The real success of the core–shell technology came only in 2006 with the advent of core–shell columns offering remarkable separation performance and moderated backpressure. Indeed, core–shell particles present unique physical, chemical and mechanical characteristics responsible for the improvement of the van Deemter plot through the decrease of A, B and C terms (decreasing dispersion and resistance to mass transfer). In addition, core–shell particles are easily packed into columns, obtaining dense and homogeneous beds performing efficient separations and avoiding the increase of the backpressure and the need of instrumentation withstanding very high pressures.

The commercial success of this technology led to the commercialization of different column dimensions, chemistries and porosities, from numerous providers. The manufacture of core–shell particles is based on the production of non-porous cores which are subsequently enveloped by the porous shell. Special attention has to be paid to particle size and particle size distribution; pore dimension and porosity; as well as shell thickness and surface functionality [6].

Unger et al. used for the first time core–shell particles for the successful separation of complex proteins mixtures under gradient elution in 1986 [7]. More than 20 years later, a number of investigations demonstrated that the core–shell technology was particularly well suited for peptides and proteins analyses offering fast separations, excellent resolution and limited backpressure [8]. In this context, pore size was found to play a key role since large pores provided higher resolution [9].

Recently, an interesting alternative to core–shell particles was proposed [10,11]. The so called sphere-on-sphere (SOS) approach provides a simple and fast one-pot synthesis in which the thickness, porosity and chemical substituents of the shell can be controlled by using the appropriate reagents and conditions [12]. A study was carried out to find out how these particles were formed, by imaging the particles during the course of the reaction. Microscopic images suggested that a two stage nucleation process occurred. The first stage, not unlike core–shell synthesis, was the formation of the core microsphere. The second stage was nucleation of nanoparticles on the surface of these microspheres. SOS particles have been shown to be microporous with a pore diameter of less than 2 nm. However, while the surface of the material might not exhibit significant porosity, when packed into a HPLC column, the spaces between surface nanospheres provide superficial macroporosity. It has been proposed that for large molecules, larger pores as well as reduction of the shell thickness can be advantageous, due to the shorter diffusion distance and greater access to the surface area of the material [13,14]. Recently 2.9 μm SOS particles were demonstrated to have similar chromatographic performance than commercial core–shell materials (2.6 μm) when separating standard peptides and proteins of various sizes (e.g., lysozyme, myoglobin, ovalbumin. . .), while reducing the operating time and pressure [10].

The aim of this study was to evaluate the possibilities of prototype SOS columns for the separation of biologics. Column performance was studied in both isocratic and gradient elution modes with several model solutes. Our purpose was to compare the achievable peak capacity of the SOS column to other widepore state-of-the-art fully porous and core–shell materials commonly employed for protein separations. Real life samples of native, reduced and digested monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs) were analyzed using the SOS column, to demonstrate its possibilities. To the best of our knowledge, no data on the applicability of such materials have been reported for mAbs and ADCs.

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_{ec}^2 ~1–3 μL^2. The UV detector operated with a 500 nL flow cell, set to 240 nm (for butylparaben) and 280 nm (for peptides and proteins) and 40 Hz sampling rate. Data acquisition, data handling and instrument control were performed by Empower Pro 2 (Waters, Milford, MA, USA) software. Then, data were exported and edited using MS Excel.

2.2. Chemicals and columns

Water was obtained from a MilliQ Purification System from Millipore (Bedford, MA, USA). Acetonitrile (gradient grade), trifluoroacetic acid (TFA, puriss p.a.), dithiothreitol (DTT, ≥99.0%), uracil, parabens (methyl-, ethyl-, propyl- and butyl-) and protein standards such as glucagon (MW~3.5 kDa), insulin (from bovine pancreas, MW~5.7 kDa), myoglobin (from equine skeletal muscle, MW~17.7 kDa) and albumin (BSA from bovine serum, MW~69.3 kDa), cetyltrimethylammonium bromide (≥98%), 3-mercaptopropyl trimethoxysilane (95%), ammonium hydroxide (28–30% ammonia basis, ACS reagent), imidazole (≥99%, ACS reagent) and trimethylsilylimidazole (≥98%) were purchased from Sigma–Aldrich (Buchs, Switzerland). Butyl(chloro) dimethyl silane (>97%) was purchased from Tokyo Chemical Industry. Model peptides, including CH-866 (MW = 1311.5 g/mol), CH-868 (MW = 1311.5 g/mol), CH-869 (MW = 1277.5 g/mol) and CH-870 (1295.5 g/mol) decapeptides were purchased from ChinaPeptides Co., Ltd (Shanghai, China). These model peptides are analogs of the commercial therapeutic peptide, triptorelin in which only one amino acid was altered. Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved therapeutic IgG1 monoclonal antibody (rituximab, mabthera), and antibody drug conjugate (ADC) brentuximab-vedotin were kindly provided by Alain Beck from the Center of Immunology Pierre Fabre (Saint-Julien en Genevois, France).

Prototype SOS (sphere-on-sphere) C4 (100 mm × 2.1 mm, ~2.5 μm) columns were kindly provided by the University of Liverpool and Thermo Fisher Scientific, Runcorn, UK. Aeris Widepore C18 (100 mm × 2.1 mm, 3.6 μm) column were purchased from Phenomenex Inc (Torrance, CA, USA). Halo Protein C4 (150 × 2.1 mm, 3.4 μm) column was a generous gift from Stephanie Schuster, Advanced Materials Technology (Wilmington, DE, USA). Agilent Zorbax 300SB-C18 5 μm (150 × 2.1 mm) column was obtained from Agilent Technologies (Santa Clara, CA, USA). Acquity BEH-300C18 column with a particle size of 1.7 μm (150 × 2.1 mm, 300 Å) was purchased from Waters (Milford, MA, USA).

2.2.1. Synthesis of SOS particles

Polyvinylpyrrolidone (PVP, 10 k MW, 2.5 g) and cetyltrimethylammonium bromide (CTAB, 0.125 g) were dissolved in distilled water (50 mL). Methanol (80 mL) was added with stirring, followed by ammonium hydroxide (1.4%, 20 mL). Solution stirred for 15 mins before addition of 3-mercaptopropyl trimethoxysilane (MPTMS, 4 mL) in 0.5 mL portions 1 min apart. Reaction was stirred overnight.

Narrow size distribution SOS particles were collected by centrifugation and washed with distilled water (3 × 50 mL), then methanol (3 × 50 mL) before drying under vacuum at 60 °C. Par-