Spheres-on-sphere (SOS) Silica a Real Support for Separation of Large Biomolecules

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Review of upcoming challenges

The change in focus for the pharmaceutical industry over recent years to large protein therapeutics has resulted in a growth in the interest in the rapid separation of protein therapeutics, especially monoclonal antibodies and antibody-drug conjugates [1,2]. The changes in the types of analytes being monitored has had a significant impact on new stationary phase inventions where there has been a general drive by manufacturers to reduce particle size to improve the separation capability of the columns. However, there are limitations with the current particle technology, which presents a challenge for in the analysis of proteins [3].

There are two common silica particle types that are employed for the analysis of proteins;

- The first type of silica particle is non-porous and suffers from limited surface area, retentiveness and the ability to load the sample [4].
- The second type is a fully porous media. This has a high surface area, which can overcome the poor retentiveness and sample loading issues associated with the non-porous bead. Unfortunately, when looking at the physical dispersion processes that occur within the column, a larger analyte molecule will diffuse at a slower rate in the porous region of the stationary phase, which results in equilibration issues with the bulk flow [5]. This will result in a difference in retention times of individual analyte molecules and hence broader peaks.

A better compromise between efficiency and loadability is the core-shell technology. This technology overcomes the mass transfer issues while maintaining column performance and low back-pressure [6,7]. These particles were first developed in the late 1970s, and as a consequence several core-shell pellicular sorbents particles were commercialised. These original particles had a typically low surface area in the range of 5 – 15 m^2/g, which results in very low loading capacities and poor analyte retention. The new generation of core-shell particles offer better performance due to the many advances in silica sol-gel technology and in particular the control in the layer-by-layer (LbL) addition method used to generate the outer porous layer. This provided an increase in surface area reaching an optimum of 150 m^2/g and, using a uniform core, produced a very narrow particle size distribution. Despite the impressive progress in the LbL method [8,9], it remains a time-consuming process limited by the need for repeat deposition time, which takes several weeks. The initial offerings for the pore size were limited and did not meet the separation requirements for the increasing number of therapeutic proteins entering the pharmaceutical market. Thus, a particle with a wider pore system is needed due to the size of proteins, but the wider pore also needs to consider the various configurations that proteins can have, which results in an unpredictable behaviour within the porous system. The number of challenges encountered in the development of new biomolecules, has therefore necessitated the need for a new stationary phase to meet these demands.

Development of Spheres-on-Sphere silica microspheres

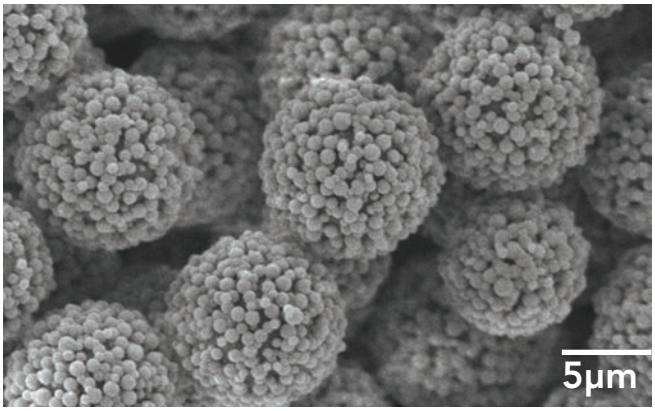
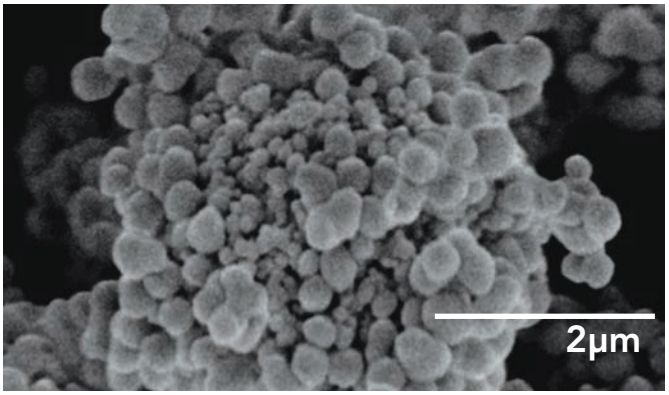


Figure 1. Spheres-on-sphere particles prepared via one-pot synthesis method

Recently, a unique type of core–shell particle, nanospheres-on-microsphere or known as spheres-on-sphere (SOS) silica, has been prepared in a one-pot synthesis from a single precursor 3-mercaptopropyltrimethoxysilane (MPTMS) [10], Figure 1. They offer an interesting alternative to the mainstream approach of producing solid core-shell silica particles which uses time-consuming LbL approach. They are made via a simple and fast one-pot synthesis which is highly advantageous, offering potential benefits on reaction time, easier quality control, materials costs, and process simplicity for facile scale-up. There have been limited reports on the one-pot synthesis of core-shell silica microspheres which are suitable for HPLC [10,11], but these approaches including spheres-on-sphere have not yet been employed for commercial use. The shell thickness, porosity and chemical substituents of the shell can be controlled by using the appropriate reagents and conditions. A time 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. This can be controlled by solution pH and solvent



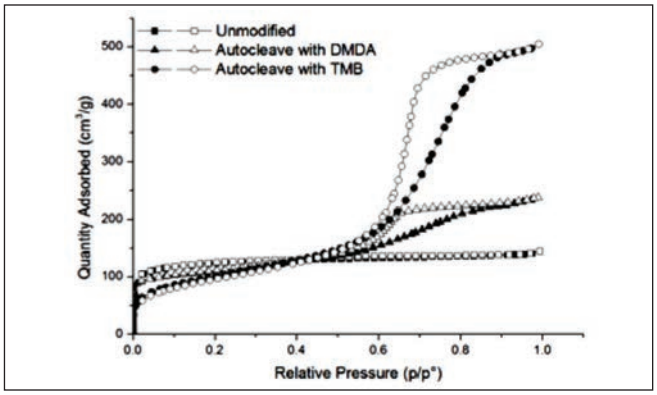
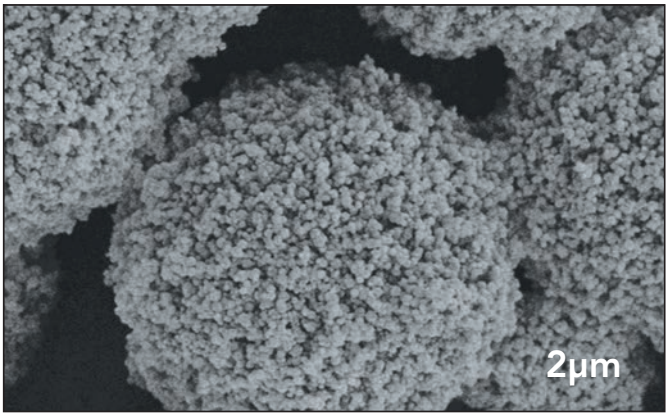


Figure 2. (A) SOS particles formed with the co-condensation of TMOS and (B) N2 adsorption isotherm profile generated after CTAB templating and hydrothermal treatment.

ratios. For the SOS particles, both the nanospheres and microspheres are nearly free of mesopores, but does exhibit some microporosity generated from the organic part of the silane. The interstitial porosity generated from the packing of nanospheres provides surface macroporosity, which is the platform for liquid phase separation.

This was the initial principles of this mechanism, but it was further utilised in order to introduce mesoporosity into the shell of the particles. Although MPTMS has been used for the preparation, MPTMS was required to co-condense with TEOS or TMOS to form surfactant-templated mesopores or covalently attached to preformed mesoporous silica with interesting surface morphology [12] (Figure 2). The resulting particles exhibited higher surface area reaching up to 680 m^2/g. Other precursors were co-condensed with MPTMS and this has resulted in a change in shell morphology and porosity. This method was further explored to generate more complex structures that are analogous to the structure of a fractal [13], where the porosity of the spheres is removed, resulting in the surface area being attributable to the morphology of the particle rather than the pore diameter. The type of pores within the particles is critically important for certain applications, especially bio-separations, as it can greatly affect mass transfer effects between the bulk flow and pore regions.

Use of Spheres-on-Sphere silica microspheres in bio-separation



The SOS particles were functionalised with different chemistries and used for the separation of small molecules such as nitroaniline, acetophenone, biphenyl and sugars [10,12,14]. It was interesting to observe that these particles were capable of separating such small molecules. Other new chemistries were also explored such as the entrapment of a crystalline phase by forming a layer of metal–organic framework (MOF) nanocrystals within the porous shell [15,16]. MOFs are a type of crystalline porous materials via the linkage of metal

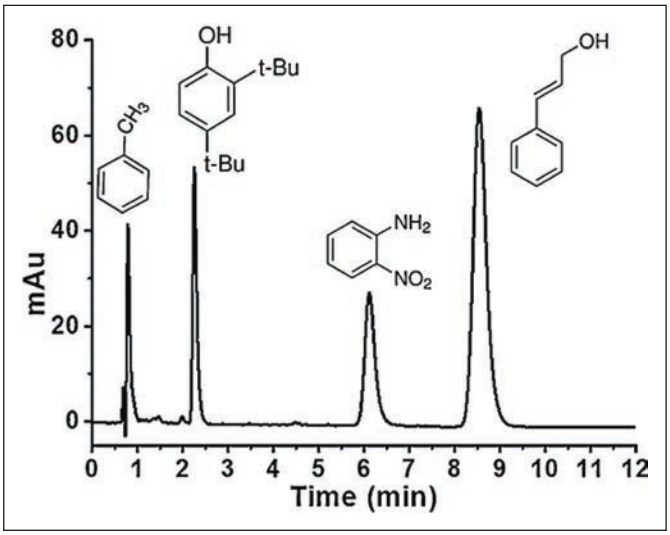


Figure 3. (A) SEM images of SOS particles modified with ZIF-8 MOF. (B) The diol-functionalized SOS particles show improved efficiency for separation of toluene, 2,4-di-tert-butylphenol, o-nitroaniline, and cinnamyl alcohol mixture. Mobile phase: heptane:dioxane (90:10, v/v), 10 µL injection, flow rate 1 cm3/min, back pressure 14 bars, column dimension 4.6 mm I.D. X 50 mm L.

ions and organic ligands. Most MOFs exhibit microporosity of varied morphologies although great effort (e.g., by ligand extension, combining the synthesis with surfactant templating) has been made to prepare mesoporous MOFs. The pore size, pore shape, and pore surface functionality are well defined in MOFs, which are suitable for highly selective separation of gas molecules or small molecule liquids [17]. MOFs can be difficult to pack into columns, which presents a major issue [18]. However, columns that are packed with MOFs demonstrate low separation efficiency and column stability. This is because MOFs are normally prepared as irregularly shaped micro-particles. The porous nature of the SOS silica particles combined with surface functionality enabled a unique entrapment of MOFs, such as HKUST-1 and ZIF-8, and exhibited some control over the crystal shape growth with enhanced separation efficiency. It was demonstrated that the SOS particles can be combined with other materials to produce unique separations. However, the major focus of this material is the use of standard SOS silica particle as a stationary phase for biomolecules separation.

The morphology of the porous structure within SOS silica is inherently stochastic, but it has been determined that it has a fractional

dimension [19]. However, the random nature of the pore morphology means that there is no scale dilation associated with traditional silica's. In such complex separations, the high kinetic performance dramatically effects the mass transfer of large molecules into and out of the pores, where the diffusion affects the rate of equilibration of the analyte concentration between the pressure driven regime and the diffusional regime. By using particulate (packed) columns, the efficiency of large molecule separations can be improved significantly 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 lessen 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 become widespread in routine analytical labs. It has been proposed that for large molecules, larger partially porous materials coupled with the reduction of the shell thickness can be advantageous, due to the shorter diffusion distance and greater access to the surface area of the material [20,21].

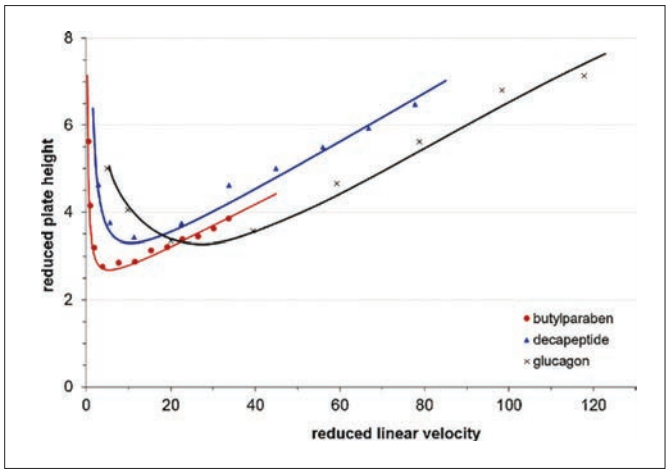


Figure 4. h–υplots, observed on sphere-on-sphere column with butylparaben, decapaptide (CH-869) and glucagon. Mobile phase consisted of 83/17 (v/v%) water/acetonitrile for butylparaben. Mixtures of water (0.1% TFA)/acetonitrile (0.1%TFA) 83/17 (v/v%) and 75/25 (v/v%) were used as mobile phases for the deca-peptide and glucagon, respectively. Temperature was set to 30°C.

The SOS particles have been used for the successful separation of complex proteins mixtures under gradient elution with good peak shapes [10,12,22]. Further studies have shown that the prototype particles with standard C4 bonding have similar chromatographic performance to commercial core–shell materials (2.6 µm) when separating standard peptides and proteins of various sizes (e.g., lysozyme, myoglobin, ovalbumin...etc), whilst reducing the operating time and pressure which is advantageous for high flow chromatography [22,23]. The kinetic performance of this material was also evaluated in both isocratic and gradient modes using various model analytes. The data were compared to those obtained on other wide pore state-of-the-art 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 hmin= 2.6, 3.3 and 3.3 were observed for butylparaben, deca-peptide and glucagon, respectively (Figure 4). In gradient elution mode, the SOS column performed with very high peak capacity when working with fast gradients. This prototype column was also comparable (and sometimes superior) to other wide pore stationary phases, regardless of the gradient time and flow rate, when analysing the largest model protein, such as BSA. These benefits may be attributed to the SOS particle porous shell morphology, minimising the intra-particle mass transfer resistance [10].

The SOS column was also applied for the analytical characterisation of commercial mono-clonal antibody (mAb) and antibody-drug conjugate (ADC) samples [23] (Figure 5). Characterisation of a bio-pharmaceutical product, performed with appropriate analytical techniques, provides useful information on purity and/or protein stability in its formulation. Antibody heterogeneity is related to conformational isoforms. The reduction of the disulfide bonds of IgGs and then the RPLC analysis of the reduced fragments is a commonly used test to determine whether the conformational variants are disulfide-related or not. With these classes of proteins, the performance of SOS column was similar to the best wide pore stationary phases available on the market. There is still some way to go with the concept of SOS particles, as only a few examples have been investigated and substantial more work has to be done in applying the technology for more complex separations

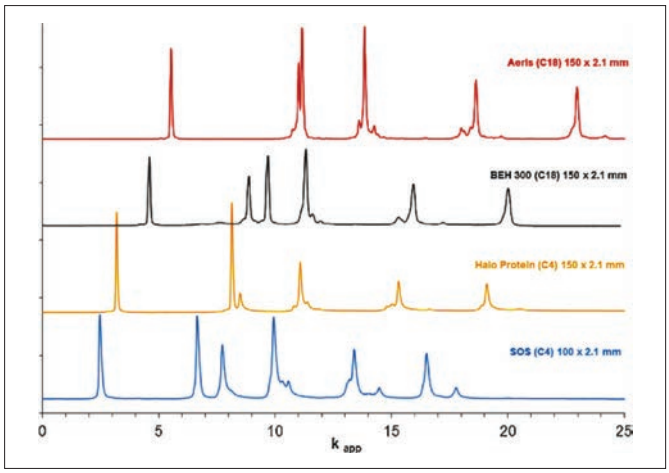


Figure 5. Representative chromatogram of reduced ADC (brentuximab-vedotin). Columns: Prototype SOS (sphere-on-sphere) C4 (100 mm × 2.1 mm, ~2.5 µm), HaloProtein C4 (150 × 2.1 mm, 3.4 µm), BEH300C18 (150 × 2.1 mm, 1.7 µm) and Aeris Widepore C18 (150 × 2.1 mm, 3.6 µm). Mobile phase A: 0.1% TFA, mobile phase B: 0.1% TFA in acetonitrile. Flow-rate of 0.4 mL/min, gradient: 27 – 42%B in 12 min on the SOS column and 30 – 45% B on the other columns, temperature: 80°C, UV detection was carried out at 280 nm.

Conclusion and outlook

Spheres-on-Spheres particles can have different structures and morphologies depending on the reaction conditions. It has been widely exploited for a very wide range of applications. However, for chromatography, SOS microspheres are utilised for protein separation. The relevant preparation methods are explained and its advantages over the current LbL approach. We have focused on the method of synthesis and explained the route the particles take to form the desired morphology via a one-pot synthesis method to form a type of core–shell structure. The method was utilised to produce mesoporous shell with high surface area and more complex structures that are more analogous to the structure of a fractal.

One type of SOS particles was assessed for small molecules separation and entrapment of new crystalline phases. This demonstrated the potential of this material as a new stationary phase for chromatographic application. With the unique core-shell property and the superficial macroporosity of the shell, the packed

SOS particles were deemed to be a suitable stationary phase to embark upon the new challenges in the field of biomolecules. The Initial evaluation of this material (without optimisation) have shown equivalent or better performance in separation of certain mixtures, compared to the conventional core–shell and wide pore silica particles. This prototype material was successfully applied for the analysis of model proteins and for therapeutic monoclonal antibody (mAb) and antibody-drug conjugate (ADC) samples. The kinetic performance of the SOS material was also evaluated and compared to that of other wide pore state-of-the-art fully porous and core–shell materials commonly employed to separate proteins. As illustrated, the performance achieved with the SOS column was comparable and sometimes better than other columns dedicated to proteins analysis and packed with particles ranging between 1.7 and 5 µm. The results were very promising for a prototype material and this clearly demonstrates there is a still lot of opportunity for optimisation.

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