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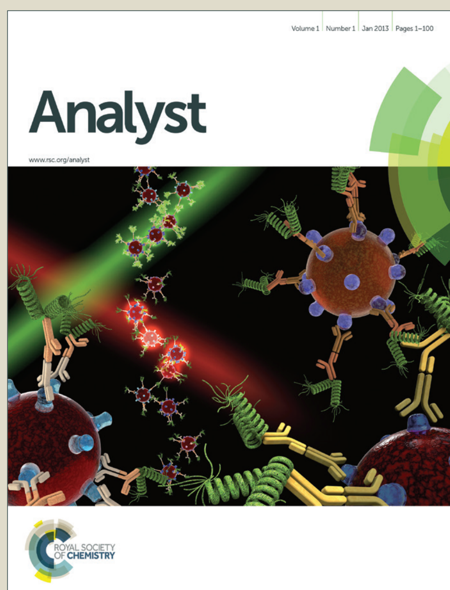

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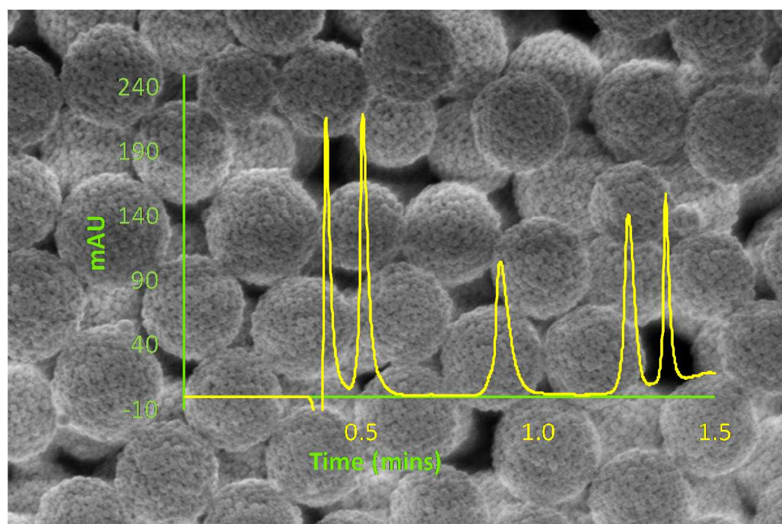
Title: Monodisperse spheres-on-sphere silica particles for fast HPLC separation of peptides and proteins

Authors: Richard Hayes, Peter Myers, Tony Edge and Haifei Zhang

One-pot synthesis of monodisperse spheres-on-sphere (SOS) silica microspheres is developed for fast separation of peptides and proteins mixtures by HPLC.



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COMMUNICATION

Monodisperse spheres-on-sphere silica particles for fast HPLC separation of peptides and proteins

Richard Hayes [a], Peter Myers [a], Tony Edge [b] and Haifei Zhang [a*]

Monodisperse spheres-on-sphere (SOS) silica particles are produced in a one-pot reaction, removing the need for time-consuming preparation and classification steps. Analysis of peptides and proteins using HPLC displays faster separation at lower operating pressure than commercially available fused core materials.

Fused core silica particles are comprised of a solid spherical core surrounded by many layers of silica nanoparticles. In recent years, fused core silica particles have been increasingly used for highly efficient separation with fast flow rate and relatively low back pressure.[1, 2] For example, a column packed with 2.7 μm fused core silica particles can provide efficiency comparable to sub-2 μm totally porous particles but as pressure is proportional to the particle size, fused core materials have the advantage of operating at pressures similar to that of 3 μm particles.[3, 4] The high efficiency is a product of the rough surface of the particle allowing better packing efficiency due to shear stress preventing bed expansion during the column packing process. This more stable column bed reduces the eddy dispersion (A-term in the van Deemter equation). The shorter diffusive path length due to the solid core can reduce the contribution of the C-term due to the fast mass transfer,[5-7] and the smaller pore volume also reduces the volume present for broadening from longitudinal diffusion (B-term) particularly for small molecules. Recently this type of particle has found widespread use in HPLC, for example in the separation of proteins and peptides,[8, 9] food analysis[10, 11] and environmental analysis.[12, 13]

Current methods of producing core shell particles involve multiple steps. The first step is synthesis of the core, followed by the formation of the porous shell. The outer layers can be applied in a number of ways: sol-gel synthesis,[14] layer-by-layer (LbL) coating,[15] spraying,[16] or dry impacting.[17] Particles with very tight size distributions are achievable by the commonly used LbL method for HPLC packing materials. However, this method is highly time-consuming which can take days or even more than one week to complete. Recent research has found that modifying the Stöber method[18] with tetraethyl orthosilicate (TEOS) as the silica precursor and adding polyvinyl alcohol (PVA) plus cetyltrimethylammonium bromide (CTAB) leads to the production of monodisperse, porous silica microspheres.[19] If 3-mercaptopropyltrimethoxysilane (MPTMS) is used in place of TEOS, silica microspheres are produced with a single layer of nanospheres coating the surface. These are described as spheres-on-sphere (SOS) particles.[20]

A study was carried out to find out how these particles were formed, by imaging the particles at regular time intervals during the course of the reaction. SEM images at each interval suggested that a two stage nucleation process occurred. The first stage, not unlike fused core synthesis, was the formation of the core microsphere. The second stage was nucleation of nanoparticles on the surface of these microspheres. No further growth was observed after 3 hours. The morphology of the prepared particles can be seen in Fig. 1. The mean particle diameter was 5.3 μm with $d_{90/10}$ (an indication of dispersity, where a smaller value indicates a tighter size distribution) of 2.41. For comparison, a typical 2.7 μm fused core product has $d_{90/10}$ of less than 1.15.

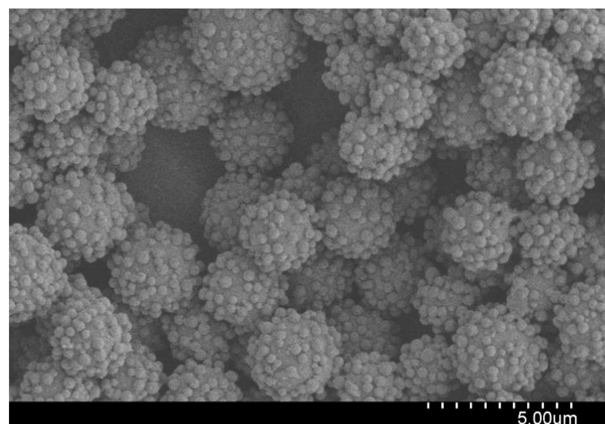

Fig. 1 SEM image of standard SOS silica particles by PVA and CTAB.

When analysed using nitrogen adsorption, all calcined 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 plus decreasing the thickness of the shell can be advantageous due to the shorter diffusion distance and greater access to the surface area of the material.[21, 22] Indeed, after functionalising the surface with a butyl group the material was shown to be effective in the fast separation of proteins and large molecules in HPLC with very low back pressure.[23]

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By modifying the reaction conditions it is possible to control the morphology of the spheres produced. For example reducing the concentration of ammonium hydroxide in the reaction leads to slower particle formation and greater nucleation on the surface. It has been found that the use of polyvinylpyrrolidone (PVP) in place of PVA produces monodisperse particles with a smooth or bumpy surface depending on whether CTAB is present or not. Example SEM images can be seen in Fig. S1 and Fig. S2. By altering the reaction condition further with the addition of cetyltrimethylammonium chloride (CTAC) (to replace CTAB) leads to the formation of monodisperse SOS particles with a single shell of nanospheres covering the entire core. The morphology can be seen in Fig. 2. The mean particle diameter was 2.90 µm with $d_{90/10}$ of 1.36, showing it to be similar in terms of size and distribution to current fused core materials. A summary of the physical properties of the two packing materials can be seen in Table 1.

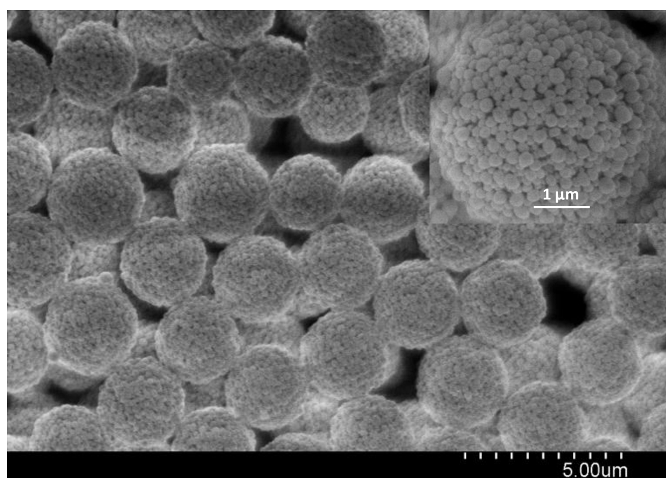

Fig. 2 Monodisperse SOS particles prepared under modified reaction conditions with PVP and CTAC. The inset image shows a close-up look of a SOS particle.

Modification of SOS with an alkyl group was performed by heating within a sealed vessel using microwave irradiation. The reaction was analogous to using conventional heating techniques - SOS particles were dispersed in toluene, followed by the addition of imidazole and butyl(chloro)dimethylsilane (C4 ligand), before heating at 110 °C. The major advantage of microwave bonding is the fast reduction in time for the reaction to reach completion, in this case 20 minutes compared to 8 hours on a heating mantle. Endcapping was performed using the same experimental procedure, using 1-(trimethylsilyl)imidazole as the endcapping reagent. Carbon content was found to be 0.13%, equating to a coverage of 4.20 µmol/m^2 using the Berendsen-de Galan equation.[24] The carbon content appears to be very low compared to the fused core material, however due to the small surface area the coverage is actually greater for the SOS material.

C4 functionalised SOS particles were packed into a stainless steel HPLC column with dimensions of 100 × 2.1 mm before testing with analytes ranging from dipeptides to large proteins. The first test was a standard peptide mixture comprised of five small molecules. The chromatogram can be seen in Fig. 3A. Full separation of all analytes was observed within 3 minutes with a maximum back pressure of 259 bar. In comparison, a Thermo Scientific Accucore 150-C4 column (2.6 µm, 100 × 2.1 mm) with a 15 nm pore size suited to biomolecules completed the separation in 4 minutes. This also displayed a higher operating pressure of 290 bar. The chromatogram for the Accucore material can be seen in Fig. 3B. Peak capacities were calculated for both columns, the Accucore column performing slightly better with an average value of 72 compared to 56 for the SOS material. Modifying the gradient to a two-step method at faster flow rate allowed full separation in less than 1.4 minutes on the SOS column (Fig. 4).

Table 1 Physical properties of bonded SOS and Accucore C4 materials. Accucore data obtained from Thermo Scientific Phase Overview brochure.

	SA (m^2/g)	Particle size (µm)	$d_{90/10}$	% carbon	Coverage (µmol/m^2)
SOS C4	4.3	2.90	1.36	0.13	4.20
Accucore 150-C4	80	2.6	1.12	2	3.57

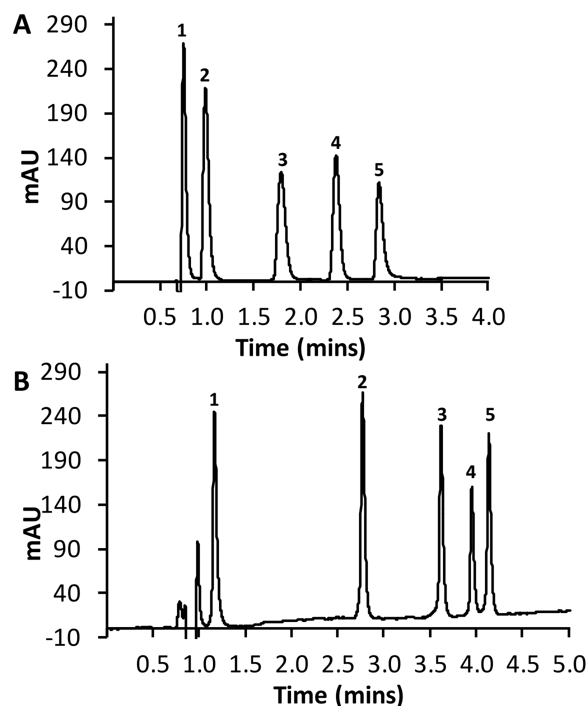

Fig. 3 Chromatograms showing separation of peptide standard test mix on (A) SOS C4 column, gradient: 10–40% B in 4 mins; (B) Accucore 150-C4 column, gradient: 10–47.5% B in 5 mins. Mobile phase: A: 0.02 M KH_2PO_4, pH2.70 + 0.1% TFA; B: acetonitrile + 0.1% TFA. Flow rate: 300 µL/min; temperature: 40 °C. The order of analyte elution is same for both chromatograms: 1, Gly-Tyr; 2, Val-Tyr-Val; 3, met-enk; 4, leu-enk; 4, angiotensin II.

A selection of proteins ranging in size from 6 to 45 kDa were also analysed on both columns. The overlaid chromatogram for SOS C4 can be seen in Fig. 5A. All analytes were retained within 6 minutes with maximum back pressure of 400 bars. This is comparable in performance to the Accucore 150-C4 column from

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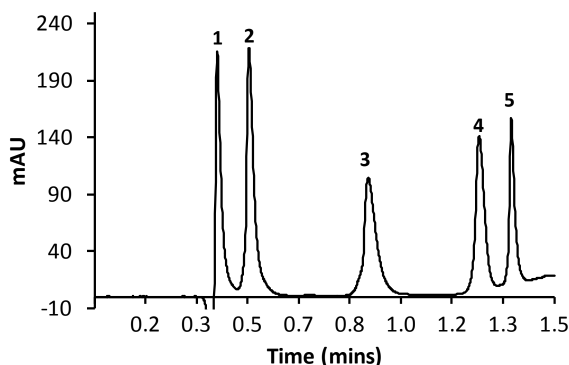

Fig. 4 Chromatogram showing separation of peptide standard test mix on SOS C4 column with modified gradient. Mobile phase: A: 0.02 M KH_2PO4, pH2.70 + 0.1% TFA; B: acetonitrile + 0.1% TFA; gradient: 10–14% B in 0.5 mins, 14-60% B in 0.5 mins, held at 60% B for 0.5 mins; flow rate: 600 μL/min; temperature: 40 °C. Analytes of elution: 1, Gly-Tyr; 2,, Val-Tyr-Val; 3, met-enk; 4, leu-enk; 5, angiotensin II.

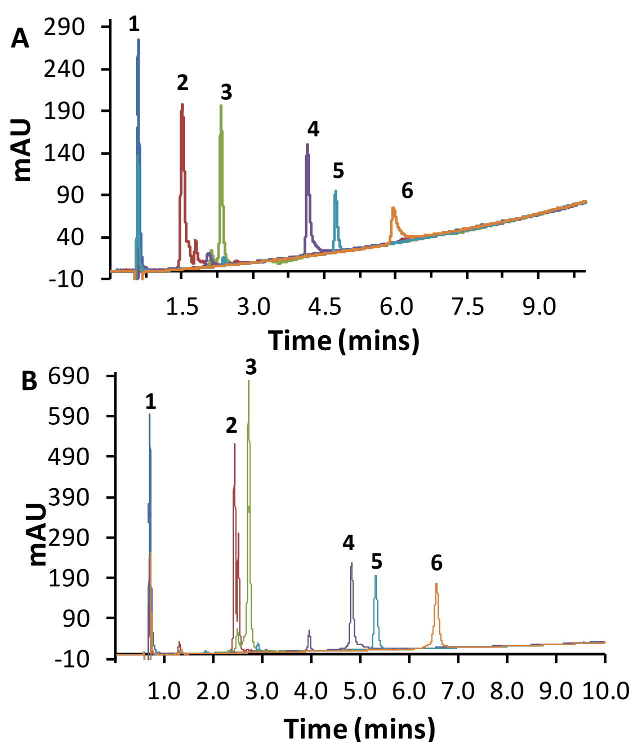

Fig. 5 Overlaid chromatograms showing protein separation on (A) SOS C4 column and (B) Accucore 150-C4 column. Mobile phase: A: water + 0.2% TFA; B: acetonitrile + 0.2% TFA; gradient: 30–75% B in 10 mins; flow rate: 400 μL/min; temperature: 50 °C. The order of analyte elution is same for both chromatograms: 1, ribonuclease A (14 kDa); 2, insulin (6 kDa); 3, lysozyme (14 kDa); 4, myoglobin (17 kDa); 5, carbonic anhydrase (30 kDa); 6, ovalbumin (45 kDa).

which the last analyte eluted at 6.5 minutes with maximum back pressure of 460 bar (Fig. 5B). Initial tests using 0.1% trifluoroacetic acid (TFA) content in each mobile phase resulted in excessive peak tailing indicating there is some residual silanol activity on the silica surface. Increasing the amount of TFA to 0.2% reduces the influence of these silanol groups, providing better resolution and sharper peaks. A common problem with protein analysis is the carry-over between injections. A blank injection was run after each analyte with no traces of carry-over observed, most likely due to the small surface area and limited pore structure for compounds to become trapped.

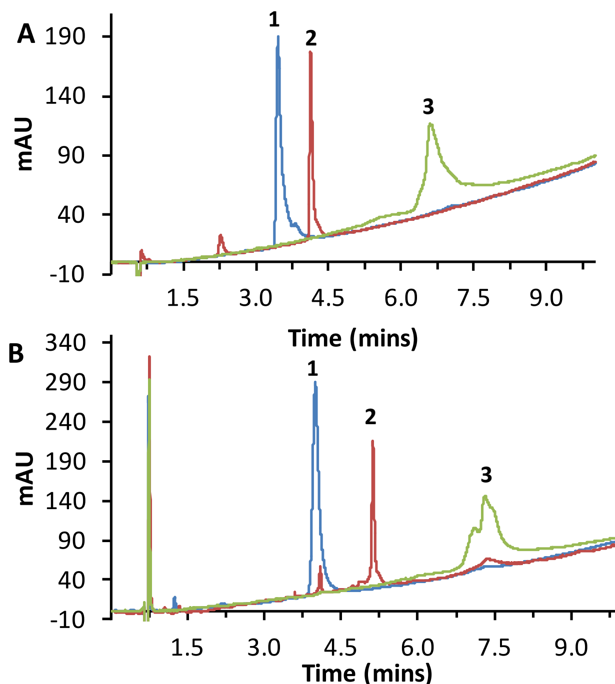

Fig. 6 Overlaid chromatogram showing large protein separation on (A) SOS C4 column and (B) Accucore 150-C4 column. Mobile phase: A: water + 0.2% TFA; B: acetonitrile + 0.2% TFA; gradient: 30–75% B in 10 mins; flow rate: 400 μL/min; temperature: 50 °C. The order of analyte elution is same for both chromatograms: 1, BSA (66 kDa); 2, myoglobin (17 kDa); 3, thyroglobulin (670 kDa).

Finally, two large proteins – bovine serum albumin (BSA, 66 kDa) and thyroglobulin (670 kDa) were analysed under the same conditions on the SOS C4 column. Myoglobin was also included to ensure that the elution time remained consistent with previous analyses. The overlaid chromatogram is given in Fig. 6A. Peak tailing is observed, especially in the case of larger analytes. A second endcapping stage to reduce the number of residual silanol groups may improve this. The thyroglobulin peak is also quite broad with a long elution time indicating it is held quite strongly by the stationary phase. Increasing the temperature may help to sharpen this peak by reducing the elution time, or by altering the gradient condition to a higher organic content mobile phase earlier in the run. Similar retention times were seen on the Accucore column, chromatogram seen in Fig. 6B, however the thyroglobulin peak was poorly defined suggesting the pores are too small for the molecule (radius of ~100 Å) to fully access.

Conclusions

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A fast, efficient synthesis of monodisperse SOS particles suitable for use in HPLC has been developed. C4 functionalised SOS particles have been shown to be effective in the fast separation of a wide size range of peptides and proteins with comparable performance to commercial fused core materials. A reduction in operating pressure of up to 13% and a 30% reduction in run time has been achieved. The size distribution of SOS particles could be improved by classification, but this does not appear necessary given the narrow particle size distribution and the performance shown in these HPLC applications.

Notes and References

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