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Microcolumns with self-assembled particle frits for proteomics

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Abstract

LC–MS–MS experiments in proteomics are usually performed with packed microcolumns employing frits or outlets smaller than the particle diameter to retain the packing material. We have developed packed microcolumns using self-assembled particles (SAPs) as frits that are smaller than the size of the outlet. A five to one ratio of outlet size to particle diameter appears to be the upper maximum. In these situations the particles assembled into an arch over the outlet like the stones in a stone bridge. When 3 μm particles were packed into a tapered column with an 8 μm outlet, two particles bridged the outlet with 0.3 pl dead volume and perfect success rate. In peptide analysis by LC–MS, the peak width at half height was normally less than 6 s, compared to 12 s without SAPs. The LC–MS–MS system provided 37% sequence coverage (21 matched peptides) for a tryptically-digested sample of 10 fmol bovine serum albumin. We also describe application of the SAP principle to make disposable pipette tip columns with short pieces of fused-silica capillary as the outlet.

Keywords: Frits; Self-assembled particles; Capillary columns; Instrumentation; Proteomics; Proteins

1. Introduction

One of the essential tools for proteome-scale protein identification is nano-scale liquid chromatography combined with electrospray ionization tandem mass spectrometry (nanoLC–ESI-MS–MS) [1–3]. Typical microcolumns for this system utilize reversed-phase materials with 3–10 μm diameter packed into fused-silica capillaries with 12–100 μm diameter, where sintered silica particles have been used as frits [4–7]. However, it is difficult to prepare these frits with high reproducibility and the frits are a potential cause for peak broadening due to post-column mixing and the interaction between the bare silica and solutes such as peptides and proteins. An alternative approach is to use a fritless column with a tapered outlet [8–10]. In this case, the tapered end is used as an ESI emitter as well as a restrictor for packed particles. This approach is quite attractive for LC–MS because the post-column dead volume can be minimized. In our experience, however, column production according to reported procedures was not stable and the column was easily blocked during the packing process. This was because the particle size was almost equal to that of the outlet, and a single particle often blocked the outlet completely.

While current approaches almost always use particle sizes equal to the outlet diameter, it is interesting to note that, Kennedy and Jorgenson reported that 5 μm particles were easily lodged at some point in the column with 15–25 μm I.D. during the slurry packing process and the resulting aggregate of

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particles could not be moved even if the pressure was increased to over 300 bar [4]. Lord et al. also reported that 3 μm particles were retained by 10–20 μm outlets lacking any frit in a capillary electrochromatography (CEC) column [11], while Mayer et al. showed that CEC does not necessitate this narrow outlet because of the electrophoretic attraction of particles towards the anode [12].

In this study, we produced self-assembled particles (SAPs) as the frit at the outlet of the tapered column, based on the principle of the stone bridge arch, to improve the column production as well as to minimize the post-column dead volume. In this construction, an ''arch'' of particles above the outlet serves as a stable structure retaining the particles. The same principle was applied to a disposable pipette tip column containing a short piece of fused-silica capillary as the narrow outlet and both column constructions were applied to peptide analysis for proteomics.

2. Experimental

2.1. Materials

Fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). Packing materials [Vydac 218MSB3 (C_{18}, 3 μm), Vydac 218MSB5 (C_{18}, 5 μm)] were generously donated by Grace Vydac (Hesperia, CA, USA). Inertsil ODS-3 (C_{18}, 3 μm) was obtained from a packed column (GL Sciences, Tokyo, Japan) and bulk materials of Poros 10 R2 and 20 R2 were purchased from Applied Biosystems (Foster, CA, USA). Tapered electrospray needles, PicoTip, were purchased from New Objective (Woburn, MA, USA). GELoader tips with 1–10 μl capacity were from Eppendorf (Hamburg, Germany). Bovine serum albumin (BSA) and bombesin were from Sigma–Aldrich Denmark (Copenhagen, Denmark). HPLC-grade acetonitrile (ACN) and water were from Fisher Scientific (Schwerte, Germany) and Sigma–Aldrich, respectively. Heptafluorobutyric acid (HFBA) was purchased from Pierce (Rockford, IL, USA). All other reagents were also of analytical grade.

2.2. Preparation of columns

A glass reservoir filled with a slurry of the particles in methanol (10–30 mg/100 μl) was placed in an air-pressure pump (Protana Engineering, Odense, Denmark) connected to a helium gas bomb. A tapered needle (PicoTip, see above) was connected to a fused-silica transfer capillary (15 cm×100 μm I.D.×365 μm O.D.) via a PTFE sleeve (LC Packings, Amsterdam, The Netherlands). The end of the transfer capillary was immersed in the slurry solution and pressure was applied very slowly. The speed of the first aggregate of particles was controlled to approximately 15 cm/30 s until the particles reached the end of the needle (pressure was normally less than 5 bar) in order to avoid destroying the opening structure as discussed below. Subsequently, pressure was gradually increased to 50 bar. During packing, vibration was applied to the column by a Shavy electric shaker (Model PS151, Seiko, Tokyo, Japan). After packing, the column was connected to a HPLC pump and washed with LC–MS mobile phases at 100–150 bar. For storage, an Innova Quartz capillary cap (Phoenix, AZ, USA) was attached to the end of the columns and the whole columns were immersed in a solution of water–methanol (1:1).

In the cases where the SAP structures were formed with the help of a narrow outlet capillary, the fused-silica capillary for the column (75–100 μm I.D.×365 μm O.D.) was connected to an outlet fused-silica capillary (2 cm×15 or 20 μm I.D.×365 μm O.D.) via an LC Packings PTFE sleeve or an Innova Quartz glass sleeve without any dead volume. Following this the slurry was packed as in the case of the tapered column. In this instance, a constant pressure of 50 bar was applied for packing the whole column.

For the disposable microcolumn, a slurry of Poros R2 was prepared according to the protocol from the manufacturer. The slurry was introduced into a GELoader pipette tip where a short piece of fused-silica capillary (1–2 mm×50 μm I.D.×190 or 280 μm O.D.) prepared by a capillary cutter (FS315, Upchurch, Oak Harbor, WA, USA) was placed in the middle point (see also Fig. 4, below). Then, the column was washed with methanol dispensed by a disposable syringe according to the reported procedure [13,14].

2.3. Microscopy

A Leica DMRA microscope (Leica Microsystems, Wetzlar, Germany) with Leica IM500 software was used to take magnified images of columns and to measure the size distribution of particles. A hundred particles were selected at random and their diameters were manually measured using the measurement function of the software.

2.4. LC–MS

A QSTAR Pulsar quadrupole time-of-flight (TOF) tandem mass spectrometer (MDS-Sciex, Toronto, Canada) with an Agilent HP1100 binary capillary pump (Palo Alto, CA, USA) and a Valco Cheminert C2 10-port injection valve (150 μm inner pore, Houston, TX, USA) was used as the LC–MS system throughout this study. A nanoelectrospray ion source (Protana Engineering) was used to hold the packed ESI needles via a Valco titanium union, and a spray voltage of 1800 V was applied. The mobile phases A and B consisted of 0.02% HFBA, 0.5% acetic acid in water and in water–acetonitrile (1:4), respectively. The flow-rate of 200 nl/min was accomplished by dividing the original flow (10–20 μl/min) using a flow splitter tee with a restrictor column (150×0.5 mm I.D.).

BSA was digested at 10 pmol/μl and diluted to 10 fmol/μl. For LC–MS–MS experiments, new columns were packed, bombesin was run and the BSA analysis was performed after a blank run. Database searches were performed against an NCBI (National Center for Biotechnology Information) non-redundant database using the MASCOT software package (Matrix Sciences, London, UK).

3. Results and discussion

At first, 10 μm Poros particles were packed into the tapered capillaries (8 and 15 μm outlets) according to the reported procedure [8,10]. However, these columns were often blocked during the packing process even when columns with the 15 μm outlet were used. Examination with a microscope revealed that the particle size can vary considerably and thus particles with a diameter greater than 15 μm were blocking the outlet completely (Fig. 1a). The mechanism of the success and the failure in column preparation is illustrated in Fig. 1b and c. When one of the particles leaks from the aggregate and the particle is large enough to occupy the outlet, the column is blocked (Fig. 1b). When two or more particles aggregate and go to the outlet simultaneously, the particles stop in the middle point of the tapered region (Fig. 1c). The success rate was 60–70% in our case, even when packing parameters such as the applied pressure, the slurry concentration and the slurry solvent were varied. To improve the success rate, two types of columns were investigated as shown in Fig. 2. One was a tapered column packed with particles of a diameter much smaller than the outlet, e.g., 3 μm particles and 15 μm outlet (Fig. 2a). Instead of being flushed out of the column because of their smaller size, the assembled particles were retained at the outlet like stones of a ''keystone'' arch in the stone-bridge (Fig. 2, left picture) [11]. This structure is mechanically very stable as evidenced by the survival of stone bridges and Roman buildings. This concept was also applicable to a column connected to another capillary with smaller inner diameter (Fig. 2b). Another type of column is shown in Fig. 2c. In this case, the column does not contain any keystone particle and only two assembled particles supported the packing of the column. This construction resembles a ''double-stone'' arch in a different style of stone bridge (Fig.

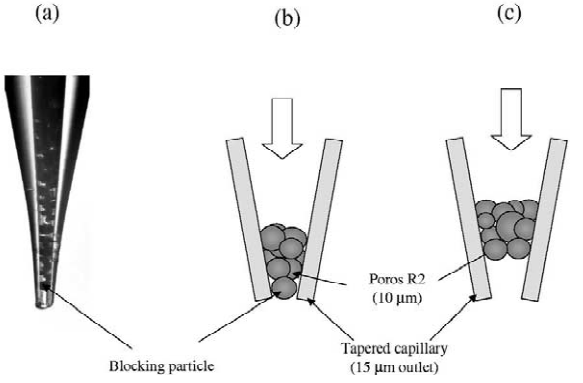

Fig. 1. Mechanism of the success and failure in the column preparation. (a) Close-up image of a blocked column. Particles: Poros R2 (10 μm), column, PicoTip (75 μm I.D., 15 μm outlet). (b) Failure case. (c) Success case.

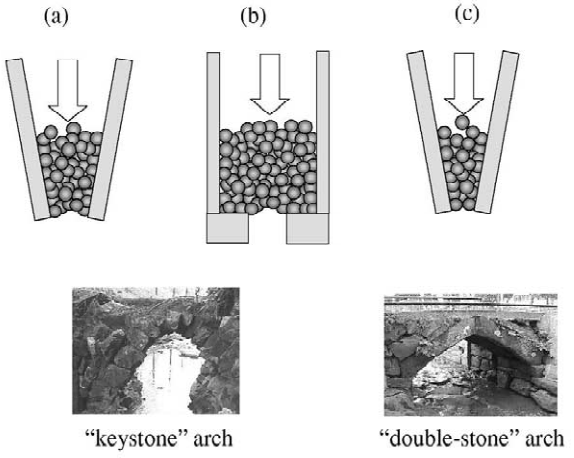

Fig. 2. Columns utilizing arch types found in stone-bridges. (a) Tapered column with ''keystone'' arch, (b) column with ''keystone'' arch on narrow outlet capillary, (c) tapered column with ''double-stone'' arch, (left picture) a stone-bridge with keystone arch, (right picture) a stone-bridge with double-stone arch. Both pictures from http://www.kumamotokokufu-h.ed.jp/kumamoto/isibasi.

2, right picture). Both the keystone and double-stone tapered columns were superior to the conventional fritless columns (Fig. 1) because it was possible to achieve a higher column preparation success rate as well as to minimize post-column dead volume. The double-stone type has the advantage over the keystone type in that a smaller outlet size can be realized, which is better suited to stable electrosprays at lower flow-rates. To minimize the post-column dead volume of the double-stone tapered column, the outlet size was investigated theoretically (Fig. 3a). From simple geometric considerations, the minimum length for double-particle arch, L, was calculated as:

$$L = d(\cos\theta + 1) \quad (1)$$

where d is the diameter of the particle and θ is the angle of the tapered region. In our case, θ was 5–10°, i.e., cos θ was approximately 0.99, and L is approximately 2d. The particle diameter d depended on the size and distribution of the particles. According to measurements by microscope, the average size of Inertsil ODS-3 was 3.9 μm, 86% of the particles were less than 5 μm, and 99% of the particles were less than 6 μm. The tapered needles have a specification of ±1 μm. Therefore, when the outlet was

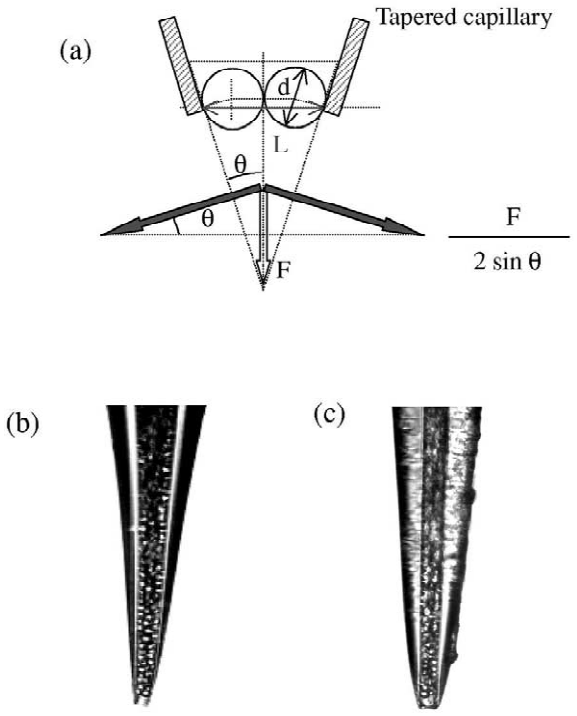

Fig. 3. Scheme and close-up images of tapered columns with ''double-stone'' arches. (a) Geometric illustration of ''double-stone'' arch, (b) close-up image of packed column (75 μm I.D., 8 μm outlet) packed with Inertsil ODS-3 (3 μm), (c) close-up image of packed column (20 μm I.D., 10 μm outlet) packed with Inertsil ODS-3 (3 μm).

more than 7 μm, the percentage of failure should be less than 1%. Fig. 3b and c shows typical close-up images of the packed needles with Inertsil ODS-3. Two particles arched the outlets of both 8 μm and 10 μm columns with the minimum post-column dead volume, as expected. The dead volume was less than 1 pl in both cases (0.3 and 0.6 pl for Fig. 3b and c, respectively). For 8 μm outlet columns, however, the success rate was not perfect because 1% of the particles were larger than 6 μm and blocked the outlet. Smaller particles such as Vydac 3 μm C_{18} (average 3.3 μm, ±2 μm, 99%) have provided perfect results. Further improvement could be achieved by filtering the particles to remove the larger ones [6].

Fig. 3a also shows that the force to pack particles was amplified by a factor of 1/2sin θ perpendicular to the capillary wall (3.5 times with the parameters

Table 1
Stability of the SAP arches of the different columns

Outlet size (μm)	Outlet type	Vydac, 3 μm (3.3 μm)	Inertsil, 3 μm (3.9 μm)	Vydac, 5 μm	Poros R2, 10 μm	Poros R2, 20 μm
8	Taper	O	O	X	X	–
10	Taper	O	O	–	–	–
15	Taper	O	O	O	X	–
15	Narrow tube	O	O	O	X	–
20	Narrow tube	X	X	O	O	X
50	Narrow tube	X	X	X	O	O
75	Narrow tube	–	–	–	X	O

Particles leaking from the arch were collected by pipette tips or in-line filters during the following steps: solvent A (10 min)—solvent B (10 min)—pressure release—solvent A (10 min)—pressure release—solvent B (10 min). O; No leak of particle, X; leak or block, –: not tested. Triplicate analyses were performed for each condition.

given above). Actually, when the pressure applied was over 10 bar at the beginning of the packing process, the outlet was destroyed by the first aggregate of particles. To avoid this problem, it was critical to set the initial pressure at a minimum. On the other hand, for the columns with a narrow outlet capillary (Fig. 2b), no attention to pressure has to be paid during packing.

Next, the stability of the SAP arch was investigated for various columns with different sizes of particles and outlets. Duplicate gradient elution and sudden pressure release were applied and all solvents from these columns were collected to check for leak particles. The results are summarized in Table 1. Although Lord et al. reported that 3 μm particles were retained by a 20 μm outlet in the CEC column [11], a few 3 μm particles leaked from the 20 μm outlet in our experiments, presumably because HPLC requires a stronger assembled structure than CEC. However, when the outlet was smaller, no leaking particles were observed, e.g., in the case of 15 μm outlet with 3 μm particles. Similar trends were observed for larger particles. As a consequence, the outlet required for the arches should be at most five-times larger than the particle size for 3 to 20 μm particles. However, when these columns were dried during storage, rearrangement of the particles was often observed in the next use, and a few particles leaked from the columns in some cases. Therefore, these columns were stored in the methanolic solution as described in the Experimental section.

We also found that we could use the stone arch principle to prepare disposable microcolumns (Fig. 4). Previously, the frits of these columns, which are used in similar applications as ZipTips from Millipore, were prepared by flattening using the pair of flat pliers [13] or heated tweezers [14]. In our experience, however, this process needed some expertise and did not work every time. In our new method, the frit was prepared simply by pushing a short piece of fused-silica capillary into the pipette tip, which does not require any expertise. The success rate of the preparation was 100% ($n=50$, three persons) even when those who had not done the procedure before prepared them. The desalting and concentration efficiency of trypsin-digested peptide samples for matrix-assisted laser desorption ionization (MALDI) and off-line nanoESI was equivalent to the disposable columns we have used before (data not shown).

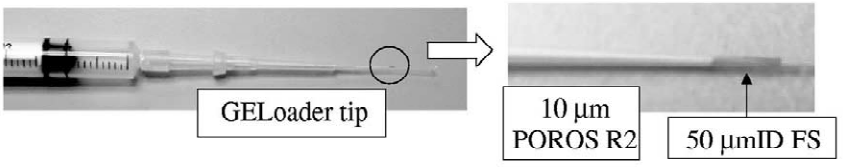

Fig. 4. Disposable pipette tip column. Pipette tip, GELoader 1–10 μl; packing material, Poros 10R2; outlet capillary, 1 mm×50 μm I.D.×190 μm O.D.

Finally, the double-stone tapered columns were applied to LC–MS analysis. On a 20 min gradient with 10 to 60% B the obtained peak width at half height was normally less than 6 s, as shown in Fig. 5. This system was also used for high-sensitivity peptide sequencing by LC–MS–MS. As shown in Fig. 6, there were 21 matched peptides from 10 fmol trypsin-digested BSA peptides and 37% of the sequence (gi|1351907, serum albumin precursor) was covered. The conventional column (10 cm×75 μm I.D.) connected to an unpacked ESI needle resulted in peak-widths of 12–14 s and 7 matched peptides from 50 fmol of trypsin-digested BSA.

In conclusion, we have developed microcolumns with SAPs as frits, based on the principle of the stone-bridge arch. The success rate of the preparation was significantly improved over columns prepared using conventional procedures. In particular, tapered double-stone columns were prepared to minimize post-column dead volume and provided high efficiency in LC–MS analysis. The minimum outlet size in our approach depends on the particle size. To make SAP frits for very small outlet diameters (smaller than 5 μm) would therefore require particles of 1 μm or smaller. These particles could be employed for the arch structure whereas the rest of the column could be packed with larger diameter particles.

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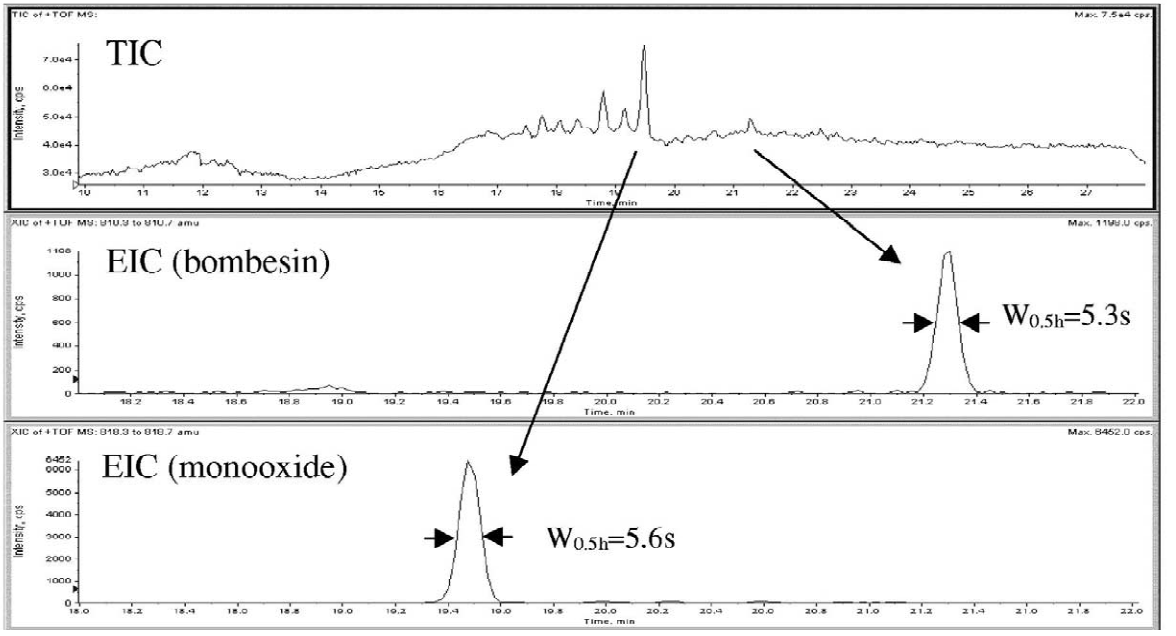

Fig. 5. LC–MS analysis of bombesin and the oxidized products. Sample; bombesin and the oxidized products; total 100 fmol; column, 10 cm×75 μm I.D., 8 μm outlet (Inertsil ODS-3); MS scan, m/z 400–1600/1.5 s; gradient, B 10–60% (0–20 min); flow-rate, 200 nl/min.

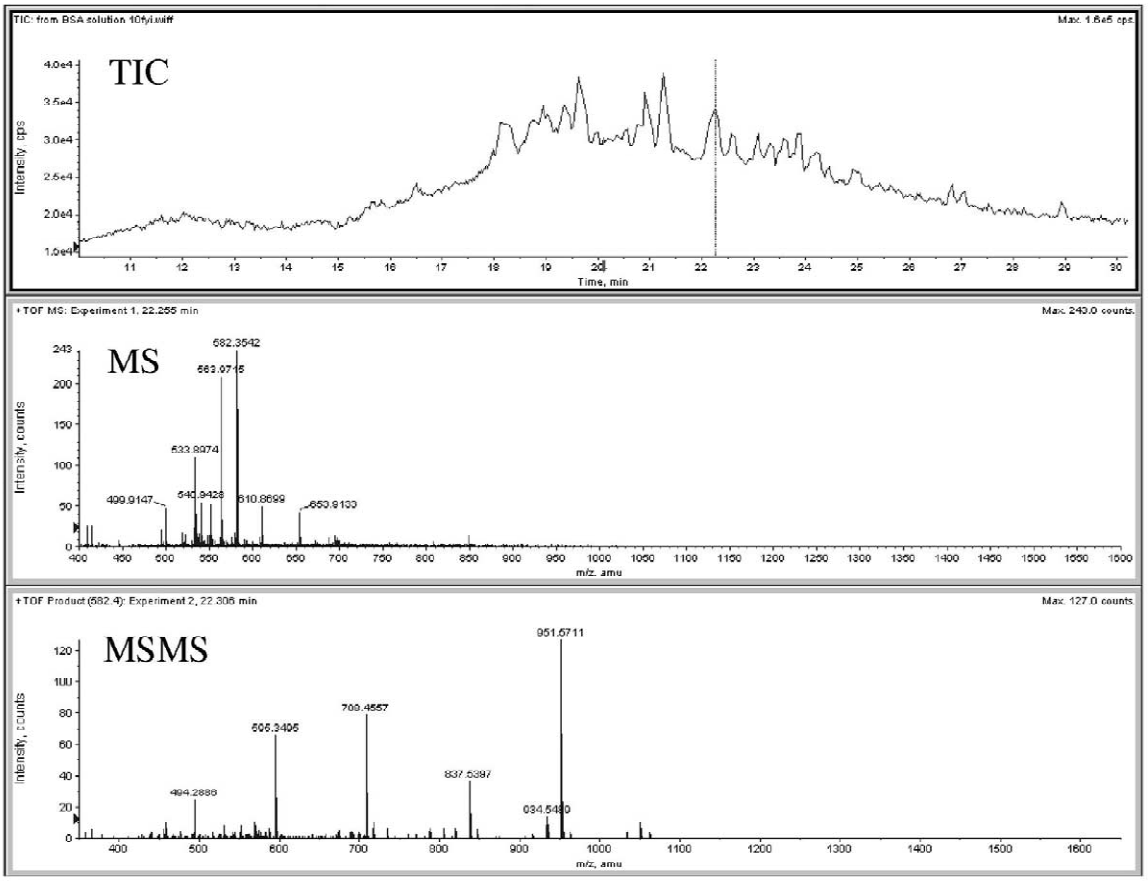

Fig. 6. LC–MS–MS analysis of trypsin-digested peptides from 10 fmol BSA. Column, 10 cm×75 μm I.D., 8 μm outlet (Inertsil ODS-3); MS scan, m/z 400–1600/1.5 s; MS–MS scan, m/z 350–1650/3 s for four parent ions; gradient, B 10–60% (0–30 min); flow-rate, 200 nl/min.

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