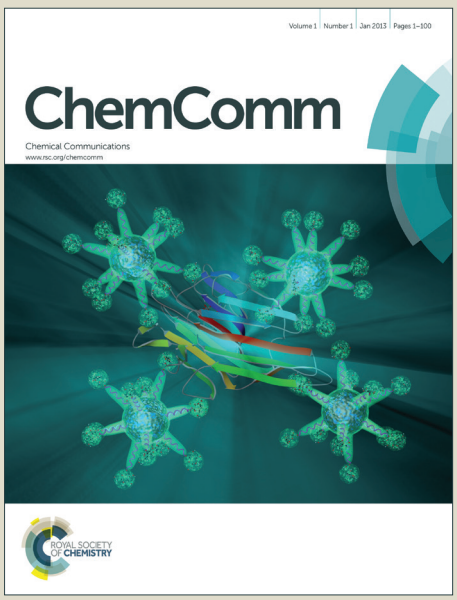
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

ARTICLE TYPE www.rsc.org/xxxxxx | XXXXXXXX

Storage of Serum Peptide Information in Nanoporous Silicon Microparticles

Jie Tan[a], Xiao Li[a], Guansheng Du[a], Aiwu Pan[b] and Jianmin Wu[a]*

Received (in XXX, XXX) Xth XXXXXXXXX 200X, Accepted Xth XXXXXXXXX 200X
First published on the web Xth XXXXXXXXX 200X
DOI: 10.1039/b00000000x

Serum peptides are becoming a rich source of disease biomarker, therefore, preserving serum peptide information after sample collection is of great importance. This work demonstrated that nanoporous silicon microparticles can be successfully applied in the storage of peptide information.

Serum is an ideal biological sample because it contains an archive of information due to the presence of a variety of proteins or peptides released by diseased tissue or cells[1-2]. While these components enhance the complexity of serum proteome, they also make serum an attractive sample for clinical studies[3]. Recently, new evidences have revealed that serum peptides in low-molecular weight (LMW) region are rich and untapped source of disease biomarkers[4]. Various researches have demonstrated that cancer can be detected and classified based on serum peptides probably due to the different protease activity between tumor and normal samples[5]. The pattern of serum peptides produced by various disease states holds a great deal of disease-specific diagnostic information.

Direct detection of low molecular weight peptides (LMWPs) in complex serum samples is still facing challenges since the high abundance and high molecular weight (HMW) components generally dominate the spectrum and tend to suppress the signal of peptides. Recently, approaches and materials that achieve selectively isolation of LMWPs from more abundant serum protein have been developed[6]. These new technologies will undoubtedly accelerate the discovery of new biomarker in the large pool of LMWPs. Nevertheless, several obstacles still exist in serum peptide-based biomarker discovery. Among them, the possible change of composition and relative abundance of serum peptides after sample collection might be a big problem that may hinder the application of the peptidomic-based technology. It has been reported that serum proteins may be degraded very quickly by proteases present in serum after blood sample was collected[7]. Similarly, fractions of peptides in serum sample may also further degrade into smaller fragments, resulting in the alteration of peptides pattern during the sample storage. Serum samples for proteomic analysis should be properly stored at -80 ℃ or lower immediately after separating from whole blood. However, in most of situation, the ultra-low temperature refrigerator may not be available in the process of sample collection outside a medical center or laboratory, especially when the collected serum samples need to be transported for several days, if they were collected from rural area that is long distance away from a laboratory. Therefore, storage of peptide information after serum sample collection is one of a big challenge facing the peptidomic research.

We have previously found that nanoporous Si microparticles (NPSMPs) with a suitable pore size could not only capture LMWPs from serum sample, but also effectively exclude protease (such as trypsin) and protect the captured peptides from degradation[8]. The goal of this study is to further elucidate, with the assistance of this nanotechnology, whether the peptide information in the original serum sample can be kept during different storage conditions.

The present study included 20 serum samples from 10 healthy volunteers and 10 rectal cancer patients, respectively. Among them, 10 samples were collected from men between 52 and 72 years old, and 10 samples were from women between 51 and 62 years old. All the samples were collected and clotted in 5 mL glass tubes at room temperature for up to 1 h. The clotted samples were then centrifuged at 4 ℃ for 5 min at 1000 rpm (1000 G). Sera (upper phase) were collected and stored at different conditions as described in Table 1.

Table 1 Storage conditions for different group of samples

Group	Temperature	Pretreatment	Time
Control	Fresh serum	--	0 day
a	25 ℃	No	1. 7 days
b	-20 ℃	No	2. 30 days
c	25 ℃	Yes	3. 60 days
d	-20 ℃	Yes	4. 90 days

Detail method and schematic workflow for the preparation and using of NPSMPs was provided in supporting information (Scheme S1, ESI†). Electrochemical etching a single-crystal silicon wafer yields mesoporous nanostructures with narrow distributed pore diameters and highly ordered straight pore channel (Fig. S1, ESI†). The pore diameter and porosity of NPSMPs used in this work is ~ 9 nm and ~26%, respectively, which can exclude proteins with MW larger than 14.4 kDa (Fig. S2, ESI†). Fourier transformed reflective interferometric spectroscopy was employed for establishing the relationship between cut-off MW and critical porosity of porous Si[8]. The surface of NPSMPs was modified with undecylenic acid, which can improve the stability of NPSMPs, as well as its affinity for peptides. Compared with nanoporous silica particles[9], mesoporous silica chip or film[10], mesoporous carbon[11], smart hydrogel[12] that have been employed for capturing LMWPs from serum samples, the pore diameter of pSi can be precisely tailored by electrochemical etching parameters. The well controlled pore diameter allows selectively trapping a subset of LMWPs and precluding the

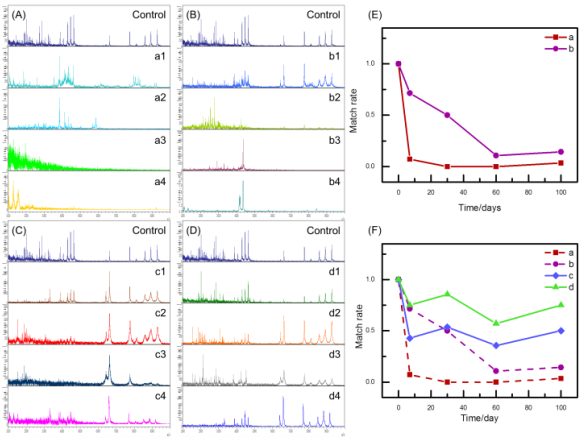


Figure 1. Example of peptide profiles of serum samples after they were stored for 7 days (1), 30 days (2), 60 days (3) and 90 days (4) at different store conditions listed in Table 1; The Control sample in (A)~(D) represents the fresh serum sample analyzed immediately after it was enriched with NPSMPs; (E) Match rate of (A) and (B); (F) Match rate of (A)~(D).

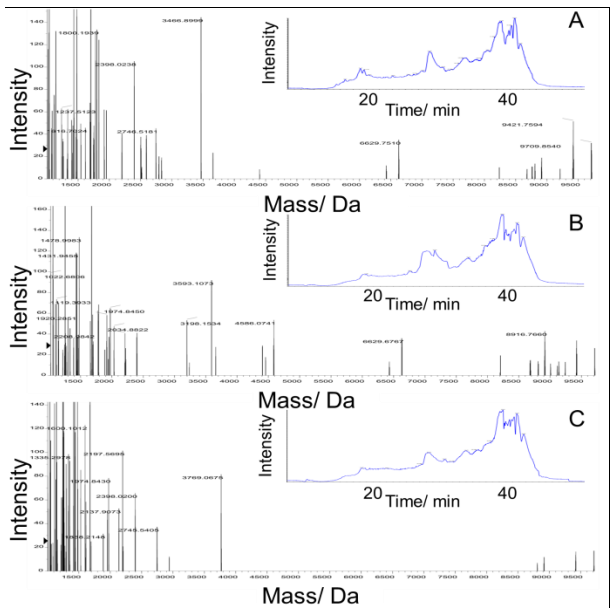


Figure 2 The representative LC-MS spectra of human serum peptides stored at -20 ℃ for 7 days. (A) The fresh serum sample analyzed immediately after it was enriched with NPSMPs; (B) The serum sample pre-treated by NPSMPs. (C) The serum sample without pretreatment. The insets in A, B and C are the total ion chromatogram of sample A, B and C.

high abundance large proteins such as serum albumin, immunoglobulin and protease in blood sample[13-19] (Fig. S3, S4, ESI†). Moreover, without the need for any elution step, the LMWPs captured in the pore channel of NPSMPs can be directly ionized by UV laser with the assistance of α-cyano-4-hydroxycinnamic acid (CHCA), probably owing to the well aligned pore structure of the material, and the charge separation capability of the porous semiconductor under the radiation of laser energy. Therefore, the protein-laden microparticles can be directly spotted onto a MALDI plate for mass spectroscopic analysis[8]. This nanotechnology provides a powerful tool that may accelerate the peptidomic research. In the present work, the capability of NPSMPs for storing serum peptides information was evaluated. The collected serum samples (group c and d) were immediately mixed with NPSMPs and shaken for 5 min. The microparticles were collected by centrifugation after they were washed with water, and then stored at different temperature for further testing. For comparison, serum samples (group a and b) without pre-treatment with NPSMPs were also stored at conditions as indicated in Table 1.

Before peptide fingerprint was analyzed with matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS), the samples (group a and b) were also enriched with the same NPSMPs, since the peptide profile cannot be acquired when the serum sample was directly spotted on a MALDI plate. To quantify the change of peptides in comparison with original control sample (analyzed immediately after sample collection), match rate (M%) was calculated according to the following equation:

$$M\% = P_m/P_c$$

Here, P_c denotes the number of peptide peak found in the control sample, and P_m is the number of peaks matched with the control sample after the test sample has been stored in a specific condition. Serum sample without NPSMPs protection displayed a severe degradation during short-term storage (< 8 h), whereas the peptide profile could be well preserved in the sample protected with NPSMPs (Fig. S5, ESI†). As to the long-term storage test, irrespective of what temperature the samples were stored at, the pattern of peptide fingerprint measured in the group (a) and (b) changed dramatically after 7 days (Fig. 1A), and the match rate decreased rapidly (Fig. 1E) during the whole storage time. After 7 days, the match rate in group (a) decreased to 30%. If samples were stored at lower temperature (-20 ℃), the decreasing of match rate is significantly slower (Fig. 1B, E). However, the match rate still decreased to ~50% after 30 days. In contrast, alteration in the mass spectral profile measured in group (c) and (d) reduced significantly (Fig. 1C and D) during sample storage. The overall match rate of serum peptide stored in NPSMPs is remarkably higher than that of samples without protection (Fig. 1F). The variation of peptide profile was also confirmed by LC-MS/MS technology. The results (Fig. 2) also indicate that the peptide profile of the NPSMPs treated sample have a high match rate with that of fresh serum sample. While the match rate between the non-protected serum sample and the fresh sample significantly decreased especially in the HMW range. The protection effect may come from the size exclusion capability of the NPSMPs. As reported in our previous work[8], the pore diameter of the porous silicon can be strictly controlled by the electrochemical etch condition. The porous Si with porosity of 26% can selectively capture LMWPs (MW< 14.4KDa) and exclude proteases (Fig. S2B, ESI†), preventing the peptides from further degradation. Furthermore, the peaks shown in LC-MS/MS spectra were identified (Table S1, S2, ESI†). Most of identified peptides have the same mass value with the peaks found in MALDI-TOF spectra (Table S1, ESI†).

In order to clarify the change of peptides profile in different mass range, the total peak intensity in MALDI-TOF spectra within mass range from 2kDa ~6kDa (LMW range), and 6kDa

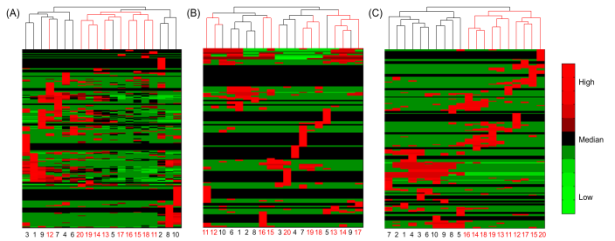


Figure 3. Cluster analysis and heat map view of mass spectra data on serum peptides from colorectal cancer patients and from healthy controls. 10 serum samples were collected from cancer patients (No. 11~20) and 10 samples were from healthy volunteers (No. 1~10). **(A)** Samples were processed and analyzed immediately after collection. **(B)** Samples were stored at -20 ℃ for 7 days without pre-treatment. **(C)** Samples were pre-treated with NPSMPs and stored at -20 ℃ for 7 days. The red, black and green color in the heat map represents high, medium, and low intensity/absent peak intensity, respectively. Branches and clusters in the cluster tree are color-coded: colorectal cancer patients in red; healthy person controls in black.

~ 10 kDa (HMW range) were calculated, respectively. Without NPSMPs protection, the total peak intensity in the LMW range increased remarkably during the first 7 days (Fig. S6A, ESI†). The phenomenon should be ascribed to the degradation of HMW peptides in the initial storage periods. That will result in the increasing of total peak intensity in the LMW range and decreasing of total peak intensity in HMW range (Fig. S6B, ESI†). In contrast, when goes to the samples pretreated with NPSMPs, the variation of total peak intensity both in the LMW and HMW range was not so significant. LC-MS/MS results also proved that the peptide profile in HMW range obviously changed if the sample was not protected by NPSMPs. The sequence of the lost peptides during sample storage was listed in supporting information (Table S1, ESI†).

Severe changes in serum peptide pattern may lead to a rapid loss of disease information contained in the peptide fingerprint. Cluster analysis was performed to elucidate whether the samples from cancer patient and health person can be classified after the samples were stored in different conditions. The detail method was described in supporting information. Data in Fig. 3A show that the original serum samples from the colorectal cancer patients and those from the healthy controls appear in separate clusters with only one exception(shown in red and green on the dendrogram, respectively). However, after 7 day's storage at -20 ℃ without NPSMPs protection, the peptide fingerprint between the cancer patients and healthy persons cannot be well classified (Fig. 3B), indicating the losing of disease information during sample storage even at low temperature. Accordingly, the statistical data further support the conclusion that storage of serum sample with a proper manner is necessary. In the present work, we resort to the nanotechnology to resolve the problem. Due to the effective protection by NPSMPs, the original serum peptide information has been well kept during sample storage. Fig. 3C illustrates the map of cluster analysis using the mass spectral data obtained from samples pre-treated with NPSMPs and stored for 7 days at -20 ℃. The statistical analyses show that the colorectal cancer patients and those from the healthy controls can be fully separated and discriminated. The result of cluster analysis in Fig. 3C is even better than that obtained from the original samples, probably because the original samples contain too much redundant peptide information. In summary, this work has provided solid evidences that the porous nanostructure can successfully serve as a storage material for preserving peptide information. If coupled with tandem MS technology, quantitation and identification of serum peptides with high fidelity can be achieved. The material may find wide application in the storage of various biofluid samples for disease diagnosis.

This research is supported by the National Science Foundation of China (No. 21075111, 21275218) and program of S & T in Zhejiang province, China (No. 2013C37083)

Notes and references

[a] *Institute of Microanalytical System(IMAS), Department of Chemistry, Zhejiang University, Hangzhou, China.. Fax: 86 571 88273572; Tel: 86 57188273496; E-mail: wjm-st1@zju.edu.cn*
[b] *Hospital of Zhejiang University, Hangzhou, China.Tel: 86 571 88206009 E-mail: awpan818@hotmail.com*
† Electronic Supplementary Information (ESI) available: See
DOI: 10.1039/b000000x/

1. S. Hu, J. A. Loo and D. T. Wong, *Proteomics*, 2006, **6**, 6326-6353.
2. S. Linder, A. M. Havelka, T. Ueno and M. C. Shoshan, *Cancer lett.*, 2004, **214**, 1-9.
3. J. N. Adkins, S. M. Varnum, K. J. Auberry, R. J. Moore, N. H. Angell, R. D. Smith, D. L. Springer and J. G. Pounds, *Mol.Cell. Proteomics*, 2002, **1**, 947-955.
4. L. A. Liotta, M. Ferrari and E. Petricoin, *Nature*, 2003, **425**, 905.
5. J. Villanueva, D. R. Shaffer, J. Philip, C. A. Chaparro, H. Erdjument-Bromage, A. B. Olshen, M. Fleisher, H. Lilja, E. Brogi, J. Boyd, M. Sanchez-Carbayo, E. C. Holland, C. Cordon-Cardo, H. I. Scher and P. Tempst, *J clin.invest.*, 2006, **116**, 271-284.
6. M. M. C. Cheng, G. Cuda, Y. L. Bunimovich, M. Gaspari, J. R. Heath, H. D. Hill, C. A. Mirkin, A. J. Nijdam, R. Terracciano, T. Thundat and M. Ferrari, *Curr Opin Chem Biol*, 2006, **10**, 11-19.
7. S. Ayache, M. Panelli, F. M. Marincola and D. F. Stroncek, *Am. J. Clin. Pathology*, 2006, **126**, 174-184.
8. J. Tan, W. J. Zhao, J. K. Yu, S. Ma, M. J. Sailor and J. M. Wu, *Adv. Healthcare Mater.*, 2012, **1**, 742-750
9. (a) R. Terracciano, M. Gaspari, F. Testa, L. Pasqua, P. Tagliaferri, M. M. Cheng, A. J. Nijdam, E. F. Petricoin, L. A. Liotta, G. Cuda, M. Ferrari and S. Venuta, *Proteomics*, 2006, **6**, 3243-3250. (b) R. J. Tian, H. Zhang, M. L. Ye, X. G. Jiang, L. H. Hu, X. Li, X. H. Bao and H. F. Zou, *Angew. Chem, Int. Ed.* ,2007,**46**,962-965.
10. Y. Hu, A. Bouamrani, E. Tasciotti, L. Li, X. W. Liu and M. Ferrari, *ACS Nano*, 2010, **4**, 439-451.
11. H. Q. Qin, P. Gao, F. J. Wang, L. Zhao, J. Zhu, A. Q. Wang, T. Zhang, R. Wu, H. F. Zou, *Angew. Chem, Int. Ed.*, 2011, **50**, 12216-12221.
12. A. Luchini, D. H. Geho, B. Bishop, D. Tran, C. Xia, R. L. Dufour, C. D. Jones, V. Espina, A. Patanarut, W. Zhou, M. M. Ross, A. Tessitore, E. F. Petricoin and L. A. Liotta, *Nano Lett*, 2008, **8**, 350-361.
13. M. M. Orosco, C. Pacholski and M. J. Sailor, *Nature nanotechnology*, 2009, **4**, 255-258.
14. C. Pacholski, M. Sartor, M. J. Sailor, F. Cunin and G. M. Miskelly, *J Am Chem Soc*, 2005, **127**, 11636-11645.
15. B. E. Collins, K. P. S. Dancil, G. Abbi and M. J. Sailor, *Adv. Funct. Mater.*, 2002, **12**, 187-191.
16. L. M. Karlsson, M. Schubert, N. Ashkenov and H. Arwin, *Thin Solid Films*, 2004, **455-456**, 726-730.
17. L. M. Karlsson, P. Tengvall, I. Lundström and H. Arwin, *J. Colloid Interface Sci.*, 2003, **266**, 40-47.
18. I. Rea, A. Lamberti, I. Rendina, G. Coppola, M. Gioffrè, M. Iodice, M. Casalino, E. De Tommasi and L. De Stefano, *J. Appl. Phys.*, 2010, **107**, 014513.
19. L. D. Stefano, I. Rendina, A. Mario Rossi, M. Rossi, L. Rotiroti and S. D'Auria, *J. Phys: Condens Matter*, 2007, **19**, 395007..