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Nanotexture Optimization by Oxygen Plasma of Mesoporous Silica Thin Film for Enrichment of Low Molecular Weight Peptides Captured from Human Serum

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Abstract

This study investigated the optimization of mesoporous silica thin films by nanotexturing using oxygen plasma versus thermal oxidation. Calcination in oxygen plasma provides superior control over pore formation with regard to the pore surface and higher fidelity to the structure of the polymer template. The resulting porous film offers an ideal substrate for the selective partitioning of peptides from complex mixtures. The improved chemico-physical characteristics of porous thin films (pore size distribution, nanostructure, surface properties and pore connectivity) were systematically characterized with XRD, Ellipsometry, FTIR, TEM and N₂ adsorption/desorption. The enrichment of low molecular weight proteins captured from human serum on mesoporous silica thin films fabricated by both methodologies were investigated by comparison of their MALDI-TOF MS profiles. This novel on-chip fractionation technology offers advantages in recovering the low molecular weight peptides from human serum, which has been recognized as an informative resource for early diagnosis of cancer and other diseases.

Keywords

Mesoporous silica thin film; Oxygen plasma; Low molecular weight proteome

Introduction

The identification of new biological markers represents a major challenge in early detection of diseases but the use of proteomic patterns for clinical diagnosis or prognosis offer a promising opportunity as suggested by the extensive research conducted in this area. 1–5 Many studies indicate that the circulating low molecular weight peptidome (LMWP), representing a mixture of small intact proteins plus fragments of larger proteins, is correlated to the pathological status of diseases. 6–12 The complexity of the proteome is an enormous challenge, as there are millions of proteins spanning 10 orders of magnitude in relative concentration and sizes up to hundreds of kDa. 10,13 The major barrier to overcome is the identification and detection of relatively small disease-related peptides present in trace amounts among a large background of very abundant and non-relevant proteins. Several strategies of sample treatment prior to analysis have been developed to resolve complex proteomes, including pre-fractionation processes 14–15, depletion of most abundant proteins 16–18, or more recently, an equalization bead approach to decrease the dynamic range of

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proteins in biological fluids¹⁹. In spite of these advances, the detection of low abundant markers and low molecular weight species in particular remains a critical challenge.

Innovations in nanodevices or nanomaterials, with precisely tunable characteristics at the nano-scale, greatly extend subcellular and molecular detection beyond the limits of conventional diagnostic modalities.^{20–23} Our group has developed a novel size-exclusion strategy based on mesoporous silica thin films for the efficient removal of the high molecular weight proteins and for the specific isolation and enrichment of low molecular weight species present in complex biological mixtures.^{24–28} The principle of this 3 step on-chip fractionation strategy is shown in Figure 1: a- The sample is spotted on the chip surface and LMW molecules partition into the pores during the incubation step; b- The larger protein species remain outside the pores and are removed during the washing steps; c- The captured small molecules are then eluted from the nanopores and submitted to MALDI-TOF MS (Matrix Assistant Laser Desorption/Ionization Time of Flight Mass Spectrometry) analysis.

Mesoporous silica thin films prepared by the evaporation-induced-self-assembly (EISA) of hydrolyzed silicate precursors with triblock copolymer as a structure-directing template has generated substantial interests.^{29–33} However, the conventional step to remove the organic template requires a very high temperature (> 400°C) for calcination and a time-consuming procedure to prevent thin film from cracking by gradually ramping the temperature during heating and cooling. Furthermore, film stress caused by thermal treatment results in a distort of porous nanostructure and a poor connectivity between pore channels, and consequently affects the accessibility of pores to guest peptides or small proteins in biological samples. Herein, a low-temperature oxygen plasma (OP) treatment is introduced to improve the nanotextures of mesoporous silica thin films and provide an ideal substrate to recover low mass peptides from human serum. Using a plasma source, the monatomic oxygen radical is generated and combines with the polymer to form ash which is removed with a vacuum pump. Compared to the conventional thermal calcination methods, the plasma treatment offers quicker fabrication, lower cost, facile control of pore size and an environmentally friendly process.^{34–36} More importantly for our interest, OP treatment preserves a well-defined porous nanostructure and a more hydrophilic surface while simultaneously removing the organic ligands and template. The improvement of features (pore size, film thickness, porosity, pore structure and connectivity) were subsequently investigated by combined characterization techniques, such as ellipsometry, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), Transmission electronic microscopy (TEM) and N₂ adsorption/desorption. Butanol (BuOH) with is utilized to enlarge the pore size with the superior suitability for O₂ plasma method than the conventional swelling agents. Their LMWP recovery was exhibited by MS profiles compared to chips prepared by thermal treatment.

The study presented in this paper reveals the potential of O₂ plasma to generate a mesoporous silica thin film with advantageous physico-chemical properties, which provides a powerful substrate for LMWP enrichment from complex biological fluids. Because of the capability for the simplicity and rapidity of fabrication, and the greatly improved nanotexture of mesoporous films prepared by O₂ plasma, on-chip fractionation technology will be substantially improved.

Materials and Method

Materials

All reagents were of analytical grade or better. Tetraethyl orthosilicate (TEOS, 98%), trifluoroacetic acid (TFA, 99.8%), acetonitrile (99.8%), α -cyano-4-hydroxycinnamic acid

(CHCA, 99%) and Butanol (BuOH, 99%) were purchased from Sigma-Aldrich Co.. Ethanol (EtOH, 98%) and Hydrochloride acid (HCl, 12M) were purchased from Fisher Scientific Inc.. Pluronic F127 was gifted by BASF Co.. All these reagents were used as received without further purification. Deionized water (18.4 M Ω cm) used for all experiments from a Milli-Q system (Millipore, Bedford, MA, USA).

Fabrication of Mesoporous Silica Thin Films by High Temperature Calcination

In either case of using high temperature or oxygen plasma calcination, a typical preparation of the porous silica coating sol was carried out as follows: 14 ml of TEOS was dissolved in a mixture of 15 ml of ethanol, 6.5 ml of distilled water, and 0.5 ml of 6M HCl and stirred for 2 hours at 75°C to form a clear silicate sol. Separately, 1.8 g of Pluronic F127 was dissolved in 10 ml of ethanol by stirring at room temperature followed by addition of 0.5 ml of DI water to form a homogeneous polymer solution. In the case of enlarging the pore size by applying BuOH, we then added 3.5 ml of BuOH to the polymer solution under vigorous stirring. The coating solution was prepared by mixing 7.5 ml of the silicate sol into the F127 (or F127+BuOH) solution followed by stirring of this solution for 2 hours at room temperature. The pH of the mixture solution remained around 1.5. The coating sol was deposited on a silicon (1 0 0) wafer by spin-coating at a spin rate of 1500 rpm for 20 seconds. The thickness of the thin film could be controlled by adjusting the concentration of polymer in the precursor solution, while the porosity mainly depends on the molar ratio of polymer and silicate in the starting material. To increase the degree of polymerization of the silica framework in the films and to further improve their thermal stability, the as-deposited films were placed in an oven at 80 °C for 12 hours. For thermal calcination, the films were heated at 425°C in air to remove the organic surfactant. The temperature was raised at a heating rate of 1°C per min, and the furnace was maintained at 425°C for 5 hours. Afterwards the oven temperature was cooled to room temperature over 10 hours.

Fabrication of Mesoporous Silica Thin Films by O₂ Plasma Calcination

Spin-deposited thin films prepared with the same procedure as in Section 2.1 were dried at 100°C for 1 hour. Afterwards oxygen plasma treatment was performed in a Plasma Asher (March Plasma System) to hollow the pore by removal of the template. The treatment was carried out with an O₂ flow rate at 80 sccm and a power of 300 W for 10 minutes.

Characterization Techniques

We utilized several characterization techniques to study the spin-coated mesoporous silica thin films. Through the use of a variable angle spectroscopic ellipsometer (J. A. Woollam Co. M-2000DI) and WVASE32 modeling software, the thickness of thin films and their porosities were measured using the Cauchy model and the Effective Medium Approximation (EMA) model, respectively. Ellipsometric optical quantities, the phase (Δ), and amplitude (ψ) were determined by acquiring spectra for 65°, 70°, and 75° angles of incidence using wavelengths from 300 to 1600 nm. Using Cauchy model, the top layer's thickness and reflective index were determined by fitting experimental data with the model and minimizing the mean square error. Using the EMA model, the films' porosities were calculated by assuming a certain volume of void in the pure silica and setting the top layer's thickness obtained by the Cauchy model. X-ray diffraction (XRD) patterns were obtained on Philips X'Pert-MPD system with Cu K α ray (45 kV, 40mA). θ -2 θ scans were recorded from all spin-coated films at 1s/0.001° steps over the angle range from 0.2° to 7°. Transmission electron microscopy (TEM; FEI Technai; FEI Co.) was used to obtain micrographs of the plane view of the porous silica thin films at a high tension of 200 kV. A Nicolet Magna 560 Spectrometer was applied in the transmission mode for FTIR measurement, with wavenumber resolution at 4 cm⁻¹, 256 scan, and the wavenumber range ~ 4000 cm⁻¹ – 520 cm⁻¹. During measurements, the FTIR chamber was purged with N₂ gas to remove vapor

moisture and carbon dioxide. N₂ adsorption/desorption analysis was applied for measuring surface area and pore size distribution using a Quantachrome Autosorb-3b BET Surface Analyzer. Quantachrome was used to record the N₂ adsorption/desorption isotherm at 77 K on the full range of relative P/P_0 pressures. Brunauer-Emmett-Teller (BET) surface areas were determined over a relative pressure range of 0.05 to 0.4. Nanopore size distributions were calculated from the desorption branch of the isotherms using Barrett-Joyner-Halenda (BJH) method.

Serum Fractionation

For each experiment, a 5 μ l sample of serum was transferred by automatic pipette onto the porous surface of the chip. The samples were incubated for 30 minutes at 25°C (room temperature) in a humidity chamber to prevent evaporation. The samples were washed 5 times with 10 μ l of sterile, deionized water to remove surface bound material. Peptides and proteins were eluted from the pores using a 1:1 (v/v) mixture of acetonitrile and 0.1 % TFA.

Mass Spectrometry

A matrix mixture (1:1) of 5 mg/ml CHCA and acetonitrile with 0.1% TFA was used for LMW peptide MALDI analysis. Each eluted sample was mixed in 2:3 ratio with matrix and spotted on MALDI plate in triplicate. All peptides and small proteins eluted from nanopores become positive charged during the interaction with TFA so positive mode was used to detect the signals of proteins. Mass spectra were acquired on an AB 4700 Proteomics TOF/TOF analyzer (Applied Biosystems, Framingham, MA) in both linear positive-ion and reflection modes, using 355 nm Nd-YAG laser. LMW proteins and peptides with m/z of 800–10000 Da and 700–5000 Da were selected for linear and reflection mode respectively. For linear mode, instrument setting were optimized at an acceleration voltage 20 kV, grid voltage of 18.8 kV, focus mass at 4000 Da, and low mass gate at 700. For reflector mode, instrument setting were optimized at an acceleration voltage 20 kV, grid voltage of 14 kV, focus mass at 2500 Da, and low mass gate at 700. Each spectrum was generated from 2000 (reflection) or 5000 (linear) laser shots per sample spot. External calibration was performed using 4700 Calibration standards mix of peptides and proteins (Applied Biosystems) in mass range of 800–10000 Da. Data Explorer software version 4.8 (Applied Biosystems) was used to process the raw spectra.

Results and Discussion

Tailoring of nanotexture in mesoporous silica thin films fabricated by different methods (oxygen plasma vs. high temperature)

One key characteristics of Pluronic F127 (PEO₁₀₆-PPO₇₀-PEO₁₀₆) is its ability to form an ordered periodic nanostructure due to its higher molecular weight compared to other PEO-PPO triblock copolymers. The well-defined porous architecture prepared with F127 offers a more homogenous nanostructured film for recovering proteins of interest.²⁴ In this study, O₂ plasma calcination was investigated as a potential method to hollow the pores and preserve the well-ordered mesostructure by eliminating mechanical deformations caused by thermal treatment, consequently impacting the capability of mesoporous thin films for harvesting LMWP. As summarized in Table 1, the physical properties of the thin films prepared with F127 or F127+BuOH are listed to illustrate the superiority of using O₂ plasma instead of high temperature calcination. The physical parameters of the porous thin film produced by O₂ plasma demonstrate that the low temperature route maintains the original framework of precursor micelle better than thermal treatment. In addition, there is a significant reduction in the fabrication time with OP treatment by avoiding a time-intensive processing in the conventional thermal calcination process.

The structural characterization is carried out by XRD pattern at small angle range ($2\theta = 0.5 \sim 6^\circ$) in order to observe the spacing of the pore walls. Figure 2 exhibits the changes in the nanostructure ordering of mesoporous thin film prepared by F127 before and after O_2 plasma and thermal treatment respectively. The black curve, representing the pattern of as-deposited thin film, indicated a hexagonal nanostructure before calcination with the appearance of a high intense (100) reflection peak at $2\theta = 1.026$ ($d_{100} = 8.604$ nm) and a lower intensity peaked at (200) with $d_{200} = 4.600$ nm. Both XRD patterns of OP-treated thin film (blue curve) and HT-treated thin film (red curve) also demonstrate the formation of hexagonally arranged periodic silica nano-composite, with differential shifts of the (100) peak from that of the as-deposited film. A slight change in peak intensity (d_{100} , 8.448 nm) of OP-treated thin film indicates a well-preserved nanostructure after the low temperature plasma treatment. Thermal calcination results in a reduction of peak intensity and a decreased d_{100} (7.362 nm), implying that the nanostructure and its periodicity were affected by the shrinkage caused by the higher temperature process. Similar XRD patterns for F127+BuOH system under the O_2 plasma treatment illustrate well-preserved hexagonal nanostructure, and superiority to the method of high temperature calcination.

In Figure 3, TEM imaging of thin film (plane view) further confirms the hexagonal arrangement of nanopores synthesized by F127 or F127+BuOH for all conditions we studied. Compared to using F127 alone (Fig. 3 a and b), the addition of BuOH in the precursor solution results in enlarged pore size in the final products, which are displayed in Fig. 3 c and d. As shown in Fig. 3 b and d, the plane view of the thin film prepared with F127 or F127+BuOH and calcinated by O_2 plasma exhibits a more ordered meso-structure than the product after thermal treatment (Fig. 3a and b).

The variety of adsorption/desorption isotherms between different calcination methods are exhibited in Figure 4 and the main morphological parameters were determined and correlated to the calcination processing conditions. Comparisons of pore size distribution for each set of using F127 and F127+BuOH and between films treated by the different methods (O_2 plasma vs. high temperature) are shown. The BJH method for calculating pore size distributions is based on a model of the adsorbent as a collection of cylindrical pores. As depicted in fig. 4a and c, the pore size distributions on those films calcined by O_2 plasma display a more symmetric and sharper single peak respectively at a pore diameter of 3.72 nm and 6.02 nm, which strongly points to a silica framework with uniform distribution of nanotexture. The adsorption and desorption isotherms in both systems are shown in Figure 4 b and d. All systems show hysteresis in the isotherms, clearly indicating a first-order capillary condensation transition and a nanoporous structure with interconnecting channels. However, the adsorption/desorption isotherms for both systems prepared with O_2 plasma describe a typical Type IV isotherm with a H2 hysteresis loop, which possess a more sloping adsorption branch and nearly vertical desorption branch, indicating increased internal pore connectivity.

The effectiveness of the treatment was also evaluated by the degree of organic removal and the preservation of active silanol group formed on the pore surface. FTIR analyses of silica thin films made by F127 before and after calcination for different methods are shown in Figure 5. The FTIR provides a characterization of the bonding configuration and molecular structure of the specific groups within the porous framework. For all FTIR spectra collected in Figure 5, a baseline correction was carried out using a series of zero points and spline fitting. The first significant change between thin films prepared under different conditions is observed in the stretching modes of O-H bonds ($-OH/H_2O$) in $3100 \sim 3800$ cm^{-1} . The increased absorbance for OP-treated thin film (blue curve) in this broad band indicates the formation of hydrophilic silanol group (Si-OH), which adsorb moisture and act as convenient anchoring points for the enrichment of proteins from biological complex. While

for the high temperature calcination process, represented by red curve, a very low spectral intensity is seen in the same region, indicates a portion of surface silanol groups to be extensively dehydrated, resulting in a substrate not perfectly compatible with conjugating guest molecules. The second marker of showing the preservation of organic group on OP-treated thin films can be attributed to the slight decrease of C-H₃ bond at ~2960 cm⁻¹ after O₂ plasma treatment, illustrating the calcination replaced Si-CH₃ group with various molecular species, including Si-OH, Si-OH-OH₂ and Si-CH=O (another hydrophilic bond at 1740 cm⁻¹). The curve in red represents thermal calcination method and illustrates a aggressive depletion of all organic groups regardless of their remaining surface activity. In addition, the FTIR peaks at 1074 cm⁻¹ and 793 cm⁻¹ indicate that the silica networks are composed of Si-O-Si backbones. A slight shift of the main peak towards higher wave number in the case of using O₂ plasma may be indicative of higher bond energies in the silica network caused by the higher degree of cross linking.³⁷⁻³⁸ Similar FTIR results were acquired for mesoporous silica thin films synthesized using F127 with BuOH. It can be concluded that O₂ plasma, as a alternative to thermal calcination in the step for removing organic additives, can not only generate mesoporous films, but also retain the silica surface affinity that constitutes the predominant factor in the enrichment of LMWP.

Serum fractionation and MS analysis

The dimensionality of the mesoporous silica thin film is of crucial importance in regards to the fractionation effectiveness. With the nanoscale morphology characterized as above, it is possible to correlate mesoporous silica thin films fabricated using different calcination methods with their fractionation efficacy. Figure 6 a shows the MS spectrum of the unprocessed serum sample for peptides in the range of 800 to 10,000 Da. The spectrum illustrates the signal suppression in the LMW region due to the presence of highly abundant, high molecular weight (HMW) proteins such as Albumin and IgG.¹⁷ Figures 6 b and c show the MS spectra of the recovered peptides from serum sample in the same m/z range from the mesoporous silica thin films calcined by F127/High Temperature and F127/Oxygen Plasma respectively. Their MS profiles demonstrate the capability of mesoporous silica thin film to enrichment of the LWM components, leading a significant improvement of MS detection in the low mass range, and depletion of the large molecules.²⁴ In figure 6 d, the peptides fractionated on the mesoporous silica thin film prepared with F127+BuOH/Oxygen Plasma, which possesses an increased pore size, shows the MS profile with the increased selectivity for higher molecular weight species. From this we conclude that the thin films calcined by O₂ plasma offer a protein recovery with ideal resolution and sensitivity due to its superior control of homogeneous nanotextures.

As shown in Figure 7, to evaluate the calcination methods-dependent of the mesoporous silica chips to capture the LMW peptides, a hierarchical clustering analysis of peptides extracted from separating unprocessed serum sample (F-) and fractionated peptides from the four different thin films into two major clusters, which represents F127/High Temperature (HT), F127+BuOH/High temperature (HT+B), F127/Oxygen Plasma (OP) and F127+BuOH/Oxygen Plasma (OP+B). Comparison of the clusters shows the enrichment and higher intensities of LMW peptides for certain area, and that the thin films of OP and OP+B have high intensity of peptides capture in a broader range than HT and HT+B treated chips. From this we conclude that the thin films calcined by O₂ plasma offer a protein recovery with improved resolution and sensitivity versus those thermally prepared due to its superior control of homogeneous nanotextures.

Conclusions

Mesoporous silica thin films have been prepared by oxygen plasma treatment at low temperature and used in harvesting peptides and low molecular weight proteins from human

serum, which provide an unprecedented opportunity for clinical diagnosis. Compared to conventional thermal calcination, the significant reduction of processing time makes this method valuable in many potential applications. Moreover, the optimized oxygen plasma treatment could lead to very satisfactory results, both in the preservation of a well-defined pore architecture, and a surface compatible to enrich LMWP. The addition of BuOH allows us to precisely tune the pore size of mesoporous silica thin film by using O₂ plasma and the larger pore size results in a more efficient recovery as illustrated by the higher intensity of the enrichment pattern observed MS. The combination profile provide a platform to selectively enrich LMWP at different mass range for potential proteomics study. We investigated the efficacy of LMWP recovery on the substrate treated by different methods by comparing MS profiles. The results demonstrate that the advantageous properties of the O₂ plasma process has the potential to promote proteomic biomarker discovery.

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Figure 1.
The protocol of serum fractionation on Mesoporous silica thin films

Figure 2.

XRD patterns displaying change in pore wall spacing before and after different calcination methods. XRD patterns for as-deposited thin film before removal of organic components (black curve), the mesoporous silica thin films after O₂ plasma (blue curve) and after high temperature (red curve).

Figure 3. TEM imaging of the plan view of mesoporous silica coating. (a) prepared by F127 and high temperature calcination; (b) prepared by F127 and O₂ plasma calcination; (c) prepared by F127+BuOH and high temperature calcination and (d) prepared by F127+BuOH and O₂ plasma.

Figure 4.

N₂ adsorption/desorption analysis (pore size distribution (a and c) and isotherms (b and d)) of mesoporous thin films prepared using Pluronic F127 or F127+BuOH under different calcination conditions. Pore size distribution of the thin film prepared by F127 (a) and F127+BuOH (c) between O₂ plasma and high temperature treatments; adsorption/desorption isotherms of the thin film prepared by F127 (b) and F127+BuOH (d) between O₂ plasma and high temperature process, respectively.

Figure 5. Comparison of the FTIR spectra for the thin films synthesized by F127 before (black curve) and after conventional thermal calcination (red curve) and O₂ plasma treatment (blue curve).

Figure 6.

MS spectra of serum peptides in the m/z range of 800 ~ 10,000 Da. (a) MS profile for serum sample without on-chip fractionation; (b) MS profile for the peptides enriched using the mesoporous silica thin film chip fabricated by F127 with high temperature calcination; (c) MS profile for the peptide enriched using the mesoporous silica thin film chip fabricated by F127 with oxygen plasma treatment; (d) MS profile for the peptide enriched using the mesoporous silica thin film chip fabricated by F127+BuOH with oxygen plasma treatment.

Figure 7.

Effect of different production methods on LMW peptides recovery. The unsupervised hierarchical clustering analysis of peak intensities for serum samples fractionated by chips produced by F127/High Temperature, F127+BuOH/High Temperature, F127/Oxygen Plasma and F127+BuOH/Oxygen Plasma, while unprocessed serum sample as a negative control, represented by F-. Red indicates peak intensities higher than the median value, green indicates that peak intensities is lower than the median value, black represents peak intensities equals to the median values. Each row represents an individual MALDI MS mass peak and each column represents a type of mesoporous silica thin film.

Table 1

Physical properties of porous thin films

Polymer used	Calcination method	Thickness (nm)	Porosity (%)	Average Pore Size (nm)	Surface Area (m ² /g)	Pore Volume (cc/g)	Fabrication Duration (hr)
F127	High Temp	949.97 ± 4.20	54.911 ± 0.407	3.71	563.5	0.431	43 hrs
F127	O ₂ Plasma	1126.88 ± 10.55	62.621 ± 0.529	3.72	594.9	0.700	3 hrs
F127 + BuOH	High Temp	524.46 ± 5.91	60.390 ± 0.492	6.19	602.2	0.518	44 hrs
F127 + BuOH	O ₂ Plasma	566.71 ± 13.04	63.469 ± 0.557	6.27	625.4	0.822	3.5 hrs