The Next Generation of Proteomic Nanochips in Biomarker Discovery

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Abstract

As a potential source of diagnostic biomarkers for diseases at their early stage, the low-molecular weight (LMW) region of the blood proteome has gained increased interest recently. However, the presence of highly abundant proteins and the large dynamic range of serum/plasma proteins ultimately limit the sensitivity of the detection of low abundant species. In this study, we present a novel size-exclusion strategy based on nanoporous silica chips for the efficient removal of the high molecular weight proteins and for the specific isolation and enrichment of LMW species present in biological complex. We applied the Nanoporous Silica Chip Technology at the analysis of complex proteomic samples such as human serum and developed proteomic nanochips with different nanophase characteristics to specifically target the low molecular weight species present in the human circulating peptidome. Harvested peptides were analysed by MALDI-TOF and profiles consisting of more than 300 peaks in the range 800-20,000 m/z were generated. Tunable pore sizes, pore structure and surface chemistries were used as integrated “processors” for the recovery of LMW peptides and proteins. This approach will help in the selection of individualized therapeutic combinations that target the entire cancer-specific protein network, in the real-time assessment of therapeutic efficacy and toxicity, and in the rational modulation of therapy based on changes in the cancer protein network associated with prognosis and drug resistance.

Keywords low molecular weight proteome, nanoporous silica thin film

Introduction

For this study, we demonstrated the nanoporous silica thin film (NPSTF)-based approach in serum fractionation to harvest the low molecular weight components from human serum and investigated the connection between chemistry driven tailoring of nanophase characterizations in NPSTF with their specific efficacy on capturing the proteins of interest. There are two reasons for this study. First, it has been recognized that the collection of low molecular weight proteome (LMWP) in serum can extend our ability to obtain early warning information in the diagnosis of diseases, such as cancer and coronary artery disease. We have invented an “on-chip” fractionation to sort out and enrich LMWP from serum sample. With the assistance of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), we were able to detect and distinguish hundreds of LMW proteins and peptides in seconds. The conditions in chip fabrication were optimized in this study. Secondly, the tunable physico-chemical properties of nanoporous silica surfaces made this novel material a powerful tool in selectively gathering and concentrating LMWP in serum. In the synthetic chemistry of the triblock copolymer-templating approach to NPSTFs, we were able to control their morphologies, pore structure, and surface property. The multiformity improved the competence of our system to reach unprecedented limits-of-detection and to effectively select the desired part of the molecular spectrum.

Nanoporous silica thin films made by self-assembly of triblock copolymer (poly(ethylene oxide) (PEO)-poly(propylene oxide) (PPO)-poly(ethylene oxide) (PEO)) with hydrolyzed silicate precursors have aroused substantial interests in the past ten years due to their many potential applications on catalysis, separation, sensors, and opto-electric device. The current understanding of the synthetic pathways and related mechanisms are focused on evaporation induced self-assembly (EISA). Starting with homogeneous, hydro-alcoholic solutions of soluble silica sources (TEOS, tetraethoxysilane) and template polymer, the evaporation of solvent via spin-coating drove silica/copolymer self assembly into uniform thin-film nanophase. Nanoporous silica textures with uniform pore size in nano-scale and relatively large surface area were formed after removing organic template by calcinations. The characteristics of this novel material improve our ability to explore the low mass proteomes in serum, which have been regarded as a potential source of diagnostic biomarkers for diseases but largely neglected until now and embarrassed with current separation technologies.

We developed an easy and rapid approach to efficiently deplete large proteins and enrich LMWP with NPSTF base on size-exclusion, pore structure, and active surface characteristics to screen LMWP in human serum and provided the significant promise to explore the potential of our novel nanotechnology for proteomic-based biomarkers discovery. It was well known that the pore size and porosity of nanoporous silica mainly depend on the volume of hydrophobic component and the ratio of hydrophilic/hydrophobic chains of surfactants. We selected four typical representative triblock copolymers: PEOx-PPOy-PEOz (Pluronic L121), PEOx-PPOy-PEOz (Pluronic P123), PEOx-PPOy-PEOz (Pluronic F127), PEOx-PPOy-PEOz (Pluronic L64) as the synthetic templates to offer various pore size, pore structure and surface properties. The characterization of nanophase on NPSTFs was carried out by several techniques. The connection between the physico/chemical properties of various nanopores and the MS profiles of LMWP separated from human serum by the corresponding nanochips was investigated by statistic analysis.

Methods
Fabrication of nanoporous silica thin films

A typical preparation of the coating sol was carried out as follows: the required amount of TEOS was dissolved in the mixture of ethanol, distilled water, and 2 M HCl and stirred for 1 hour at 75°C to form a clear silicate sol. Separately, a portion of surfactant was dissolved in ethanol by stirring at room temperature. In the case of applying the swelling agent, the amount of PPG solution was put into the surfactant solution with vigorous stirring at room temperature. The coating solution was prepared by mixing the silicate sol into the triblock co-polymer solution followed by stirring of the resulting sol for 2 h at room temperature. The pH of the mixture solution remained around 1.5. The coating sol was deposited on a Si (1 0 0) wafter by spin-coating at the spin rate of 2500 rpm for 20 seconds. 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 heated at 80 °C for 12 hrs. The films were calcinated at 425°C to remove the organic surfactant. The temperature was raised at a heating rate of 1°C per min, and the furnace was heated at 425°C for 5 h. The films produced were transparent and crackless.

Serum Fractionation

Various silica wafers were cut, using a diamond-tip pen, into square pieces roughly 1cm x 1cm. As shown in Figure 1, each experiment, a 10µl was pipetted onto the porous surface of the wafter square. The samples were then incubated for 30 minutes in 25 degrees (room temperature) in a wet chamber (100% humidity) to prevent sample evaporation. The samples were washed 5 times with 10µl of DI water, allowing the droplets to rest on the surfaces for 0.5 min after each wash. Peptides and proteins were eluted from the pores by using a 1:1 (volume ratio) mixture of acetonitrile and 0.1 % trifluoroacetic acid (TFA) and pipetting the solution up and down for approximately 1 minute. Sequential elutions were performed with MALDI-TOF mass spectroscopy to achieve complete recovery of the proteins contained in the nanoporous silica layer.

Results and Discussion

The key to select a triblock copolymer was the hydrophilic(PEO)/hydrophobic(PPO) ratio, which was the main factor to determine the pore size and pore periodicity. Only a few polymers with the longer hydrophilic (PEO) chain with a given PPO block length offer assembling ordered nanoporous structure, like P123 and F127. Although L-type copolymers (e.g., L64 or L121) were not able to stabilize the ordered nanophase due to their short PEO block chain, we observed that they exhibited greater flexibility to tune pore size and pore accessibility, consequently improving protein recovery in MS profile. It was necessary to control the pH value in the range of 1 to 2 in order to avoid the precipitation of silicate and achieve equilibrium between the condensation of silicate and its hydrolysis in solution. In addition, adjusting the molar ratios of certain types of polymers to silicate directly affected porosity and nanostructure. Thickness, reflective indices and porosities of nanoporous silica thin films prepared by the specific polymer were deduced from ellipsometry. Surface area, pore volume, and pore size distribution were carried out by N2 gas adsorption/desorption. These results are given with the certain molar ratio of starting materials in Table 1.

Table 1. Molar ratio of starting materials to fabricate nanoporous silica thin films with different template polymers and their physical properties calculated by Ellipsometry and N2 adsorption/desorption isotherms for nanoporous silica thin films.

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>Thickness (nm)</th>
<th>Reflective Index</th>
<th>Pore Volume (μl/mg)</th>
<th>Average pore size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P123</td>
<td>505.5 ± 0.2</td>
<td>1.255 ± 0.001</td>
<td>46.6 ± 0.2</td>
<td>40.1 ± 0.1</td>
</tr>
<tr>
<td>L64</td>
<td>803.3 ± 1.6</td>
<td>1.189 ± 0.002</td>
<td>56.0 ± 0.2</td>
<td>360 ± 3</td>
</tr>
<tr>
<td>L121</td>
<td>857.0 ± 0.3</td>
<td>1.169 ± 0.001</td>
<td>55.0 ± 0.4</td>
<td>287 ± 2</td>
</tr>
<tr>
<td>L121-50%PPG</td>
<td>571.5 ± 1.0</td>
<td>1.161 ± 0.002</td>
<td>60.0 ± 0.0</td>
<td>499.3 ± 7</td>
</tr>
<tr>
<td>L121-75%PPG</td>
<td>940.3 ± 5.5</td>
<td>1.156 ± 0.000</td>
<td>60.0 ± 0.2</td>
<td>604.7 ± 0</td>
</tr>
<tr>
<td>F127</td>
<td>884.7 ± 1.7</td>
<td>1.261 ± 0.002</td>
<td>54.0 ± 0.1</td>
<td>528.6 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.124 ± 3.71</td>
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Scanning transmission electron microscopy (STEM) analysis was performed on the plant view of nanoporous silica thin films. Figure 2 displayed STEM images of NPSTFs prepared with different copolymers. As shown in Figure 2a, the 2D-hexagonally arranged nanostructure was formed parallel to the chip surface by preparing with P123 as template. Figure 2b displayed a hexagonal honeycomb structure of nanoporous silica thin film using F127. L-type block copolymer (Pluronic L64 or L121) could not provide the periodic nanotexture with silicate. Figure 2c and d are the STEM images of the top view of NPSTFs prepared by L64 and L121 with the formation of worm-like nanopores.

LMW harvesting using nanoporous silica chips. The fabricated chips were used for isolating LMW peptides from human serum. Figure 3 shows the example of MALDI-TOF spectra of serum sample before and after treating with nanoporous silica chips synthesized by the template of L121. In
figure 3.a, most signals from LMW region were very weak and undistinguishable. After being treated by nanoporous silica chips, as shown in figure 3.b, the majority of large molecules in the serum sample were depleted by washing and thereby the low molecular-weight component can be enriched to become identifiable. We also applied the same serum sample on the nonporous and pure silica surface to evaluate the efficiency of NPSTF on LMWP recovery. In figure 3.c, there was not any significant peak to indicate that the simple silica surface was able to bound either large molecules or low mass peptides and proteins.

Reproducibility and reliability of the approach are crucial for any clinical applications. We assessed the consistency of our on-chip fractionation assay by reproducing the same experiment in 6 replicates with 6 aliquots of the same human serum sample. After fractionation, the spectra of the replicates showed a highly reproducible MS signal (Figure 4.a for low mass range and Figure 4.b for high mass range). The reproducibility of the procedure was statistically evaluated for the relative intensities of the detected peaks by calculating coefficient of variation (CV). As shown in Figure 4.c, the histogram exhibited the general variability of the peak signals measured by the average CV estimated at 13.10% for replicate samples after fractionation.

In this study the NPSTFs prepared with different polymers exhibited similar functions in separating large carrier molecules, but displayed different enrichment of low mass protein based on their specific pore size, pore structure, and surface affinity. The distinguished difference in profiling of the captured LMW peptides can be observed in figure 5.a for chips prepared with P123, F127 and L121+100%PPG. The chips prepared with P123 have relatively higher hydrophobic surface, smaller pore volume and 2D hexagonal nanostructure that has a poor accessibility to proteins. So they harvested the least peptides among the chips we studied, though it shared the similar pore size with the one using F127 owing 3D honeycomb hexagonal nanstructure. With the largest average pore size and the best surface hydrophilicity, the nanoporous silica chip from L121+PPG captured a great number of peptides within a wide molecular weight region. The chips prepared with F127 preferred to enrich the peptides in low mass range due to its periodic pore structure with the uniform small pore size. To assess the ability of nanoporous silica chips that were developed to capture LMWP, we exhibited a hierachical clustering analysis of peptides extracted from four different nanoporous silica chips with various pore size in figure 5.b. Based on the intensity of detected proteomic features after fractionation on the different chips, the red or green color indicated the relative peptide and protein concentration, i.e., higher than or lower than the median value, respectively (black was the median value). Each row represented an individual proteomic signal (MALDI MS profile) and each column represented an individual sample (Chip). The clustering algorithm clearly separated the samples in two major clusters representing specific proteomic patterns for smallest pores and biggest pores chips. The high intensity of smaller LMWP was obtained by the chips prepared with L64 and F127 due to their preferred pore size, 3.50nm and 3.71nm. The nanoporous silica chips with large pores (prepared by L121+PPG) offer the intensive signal for the peptides and proteins with higher molecular weight. Meanwhile the increase of PPG in affecting LMWP’s profile.
was also investigated from the clustering analysis. With the pore size enlarged by the swelling agent from 50% to 100% in molar ratio to template polymer, the peptides and proteins recovered showed the more intensive color in red, which indicate that the NPSTF with large pore size facilitated the enrichment of peptides and proteins with high molecular weight.

Conclusions

Much evidence indicated that the low molecular-weight region of circulatory proteomes is a rich resource of diagnostic biomarkers for the early detection of diseases. In this study, we explored a series of nanoporous silica chips and refined their use in selective capturing and enriching the LMW peptides and proteins through investigating various nanophase characteristics of chips’ surface. By utilizing four Pluronic surfactants (F127, P123, L64 and L121) as templates in synthesis, the periodic 2D hexagonal, 3D cubic and non-ordered nanoporous silica thin films were obtained and used in protein recovery by offering tunable pore size, pore volume and different surface property. Based on the MALDI-TOF MS profiles, we have demonstrated that different repertoire of peptides and proteins were recovered from the same serum sample by our nanoporous silica chips via size/shape exclusion and surface electrostatic interaction. Further improvement in LMWP capturing and identification can be achieved by studying the interaction between specific proteins and the nanophase properties of the chips’ surface and the effect of chemical functional groups with various charge and polarity conjugated on nanoporous silica chip surface on selective LMWP recovery. Through the individual or integral uses of nanoporous silica chips with various characteristics, the novel technology may provide a platform which offers a rapid and efficient sequence for analyzing LMWP in clinical diagnosis of diseases.

References

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