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Nanopore film based enrichment and quantification of low abundance hepcidin from human bodily fluids

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Abstract

Endogenous peptides that represent biological and pathological information of disease have attracted interest for diagnosis. However, the extraction of those low abundance peptides is still a challenge because of the complexity of human bodily fluids (HBF). Hepcidin, a peptide hormone, has been recognized as a biomarker for iron-related diseases. There is no rapid and reliable way to enrich them from HBF. Here we describe a peptide extraction approach based on nanoporous silica thin films to successfully detect hepcidin from HBF. Cooperative functions of nanopore to biomolecule, including capillary adsorption, size-exclusion and electrostatic interaction, were systematically investigated to immobilize the target peptide. To promote this new approach to clinical practices, we further applied it to successfully assay the hepcidin levels in HBF provided by healthy volunteers and patients suffering from inflammation. Our finding provides a high-throughput, rapid, label-free and cost-effective detection method for capturing and quantifying low abundance peptides from HBF.

Key words: Biomarker discovery; Nanoporous silica film; Peptide; MALDI-TOF MS; Hepcidin

Endogenous serum peptides that carry important information of disease are considered to be great potential biomarkers for clinical diagnosis. However, due to the extremely high dynamic range of protein concentration in serum and the interference of highly abundant and large proteins, the detection of the serum peptide biomarkers remains a challenge. Herein, we

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developed a silica nanopore-based assay to selectively enrich and quantify a low-abundance peptide, hepcidin, from human body fluids (HBF).

Hepcidin, a hormone peptide, has been recognized as a potential biomarker for iron-related disease.[1–3] The bioactive form of hepcidin consists of 25 amino acids (Hep-25) that binds to the iron export protein ferroportin on the plasma membrane of target cells and promotes its internalization and degradation, thereby down-regulating cellular iron exchange.[4] Pathological excess or deficiency of hepcidin could lead to a variety of iron disorders and be used as a diagnostic tool in clinic. For example, both anemia of chronic disease (ACD) and iron deficiency anemia (IDA) present similar clinical indicators, such as decreased serum iron level and transferrin saturation. However, the fact that hepcidin levels in circulation are elevated in ACD, but lowered in IDA, can aid in a more accurate diagnosis.[5] Considering that hepcidin participates in pathogenesis of many iron-related disorders, we believe the measurement of hepcidin levels in HBF is urgently needed to facilitate the personalized medicine. Unfortunately, no assay is currently approved by the U.S. Food and Drug Administration for hepcidin detection due to technical limitations. Several methods have been developed for quantifying hepcidin, including antibody-based[6,7] and mass spectrometry (MS)-based methods.[8–13] However, only a few antibodies have been generated, and these lack the selectivity to differentiate Hep-25 from the other two N-terminal truncated hepcidin isoforms,[14] Hep-20 and Hep-22, which are not expected to play significant roles in iron metabolism.[15,16] In regard to the MS-based methods, surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS measurements are challenged by a lack of isotopic resolution that impairs accurate quantification that uses peak area, while LC-MS/MS methods provide high sensitivity but low throughput.[17] Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS benefits from good isotopic resolution, but still requires large volume sample and time-consuming pre-treatment and its ability in serum was not demonstrated.[18,19]

Considering all of the above conceptual and technical obstacles posed by current methods for detecting hepcidin in human bodily fluids, we developed a high-throughput peptides extraction approach based on nanoporous silica (NPS) thin films with nanotextures (pore size, surface, and structure) specifically and precisely tailored for hepcidin enrichment. We further investigated the mechanisms of hepcidin enrichment in nanopores, including size-exclusion, surface charge, and pore morphology effects, and provide a basis for understanding the interaction of the target peptide with NPS thin films, which is highly useful for adapting this material for a variety of biomedical and clinical applications by using chemical functionalization of nanotextured surfaces. As illustrated in Figure 1, *A*, the silicone masks were placed on top of the NPS films to normalize the area of sample exposure. Serum and urine samples were first spotted into each well and then incubated at room temperature. Only small proteins and peptides can diffuse into the nanopores, while large proteins are excluded and subsequently removed by washing. The small peptide fractions were extracted by elution buffer. Using this procedure, Hep-25 can be enriched in the optimized nanopores, and then analyzed by MALDI-TOF MS. Our method requires only microliter sample volume and eliminates time-consuming sample pretreatment, while still maintaining a high degree of precision, accuracy, and sensitivity. In a clinical validation of our technique, Hep-25 levels were quantified in both serum and urine from 119 healthy volunteers and 19 patients suffering from inflammation. The levels of hepcidin were found to be gender, menopausal, and inflammation status dependent.

Methods

Materials

Pluronic L121 (PEO5-PPO70-PEO5), Pluronic L64 (PEO13-PPO30-PEO13) and Pluronic F127 (PEO106-PPO70-PEO106) are gift from BASF Co. All the other chemicals utilized in the study were purchased from Sigma-Aldrich Co. Synthetic human hepcidin was obtained from Peptides Institute Inc. (Osaka, Japan). Rabbit serum was obtained from Solarbio Science & Technology Co. (Beijing, China). Peptide analysis was carried out using a Bruker Daltonics' MicroflexTM LRF MALDI-TOF (Billerica, MA, USA). LC-MS/MS was performed using HPLC (Agilent 1200 series) and LTQ-Orbitrap XL (Thermo, San Jose, CA).

Fabrication of NPS thin films

The NPS thin films were fabricated by modification of protocols previously reported by our group.[20] Briefly, 14 ml of tetraethyl orthosilicate (TEOS) was dissolved in a mixture of 15 ml of ethanol, 6.5 ml of distilled water and 0.5 ml of 6 M HCl, and stirred for 2 h at 75 °C to form a clear silicate solution. Separately, certain amounts of triblock copolymer (2.4 g Pluronic F127 for preparing 2-dimensional hexagonal structures, 1.5 g Pluronic F127 for preparing 3-dimensional cubic structures, 1.0 g Pluronic L64 for L64 chips and 1.2 g Pluronic L121 for all L121 chips, respectively) were dissolved in 10 ml of ethanol by stirring at room temperature. This polymer solution was mixed with 10 ml of the silicate solution and stirred for 2 hours at room temperature to obtain the coating solution, with the pH around 1.45. The final coating solution was spin coated on a 4" silicon wafer at a rate of 1500 rpm for 20 s. The resulted films were aged in an oven at 80 °C for 12 hours. Then the temperature was raised to 425 °C at a rate of 1 °C per min, and maintained at the final temperature for 5 h to remove the polymer template. Thereafter, the oven was cooled to room temperature over 10 hours. The NPS thin films were pre-treated by oxygen plasma to establish a saturated hydroxyl-terminated surface. The treatment was performed in a Plasma Asher (March Plasma System) with an O_2 flow rate of 80 sccm and a power of 300 W for 10 min.

Characterization of NPS thin films

We utilized several techniques to characterize the spin-coated NPS thin films. Transmission electron microscopy (TEM; FEI Technai; FEI Co.) was used to obtain micrographs of the plane view of the nanoporous silica thin films at a high tension of