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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 peptides 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.

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Keywords

Biomarker discovery; Nanoporous silica film; Peptide; MALDI-TOF MS; Hepsidin

Introduction

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 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 participate 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 regards 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 1A, 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 6M HCl, and stirred for 2 h at 75 °C to form a clear silicate solution. Separately, certain amounts of triblock copolymer (2.4g Pluronic F127 for preparing 2-dimensional hexagonal structures, 1.5g Pluronic F127 for preparing 3-dimensional cubic structures, 1.0g Pluronic L64 for L64 chips and 1.2g 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 O₂ 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 200 kV. N₂ adsorption/desorption analysis was applied for the measurement of surface area and pore size distribution. Quantachrome was used to record the N₂ adsorption/desorption isotherm at 77 K on the full range of relative P/P₀ 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 the Barrett-Joyner-Halenda (BJH) method.²¹

5 μ M of hepcidin dissolved in 100mM NaCl were incubated on F127 2D-hex & 3D-cubic NSCs and then washed with deionized water. The NSCs were incubated in vacuum chamber overnight before XPS measurement. PHI Quantera XPS equipped with Ar⁺ ion gun was used to construct concentration depth profile. The Ar⁺ ion sputtered NSCs at accelerating voltage 3kV in the area 2 $\times$ 2mm. Because the thickness of silica film was determined by ellipsometer, the etching rate on porous silica can be calibrated by sputtering until oxygen (O1s) signal vanishing. 9sec of sputtering time interval was used to reach 5.25nm depth spacing at 35nm/min of Ar⁺ ion etching rate. Nitrogen (N1s) spectra were observed to identify the amount of hepcidin trapped at different depths.

Sample preparation

Blood and urine samples were obtained from informed and signed consent healthy volunteers and patients at Chinese PLA General Hospital, Beijing, China. Blood samples were collected without anticoagulant healthy human volunteers and patients with inflammation in the morning. They were allowed to stand for 15 minutes to allow the blood to clot, then centrifuged at 3500g for 25 min. Urine samples were collected from healthy human volunteers in the morning, and were centrifuged at 3000g for 10 min. Serum and urine samples were aliquoted and then frozen at -80°C until required. 5 μ l serum or urine was pipetted into each well, ensuring that no precipitate in the base of a tube was placed on the chip. The samples were allowed to incubate at room temperature in a humidified chamber for 30 min. After incubation, the serum samples were removed and the wells were washed four times with 10 μ l deionized water. For elution a 50% ACN and 0.1% TFA solution was prepared in deionized water, and 5 μ l was applied into each well and left in it for 90 seconds to extract the peptides from nanopores. Then the elution solution was pipetted and transferred into a fresh microcentrifuge tube, which were stored at -80°C until further analysis.

MALDI-TOF MS analysis

0.5 μ l of each sample was spotted on the target plate and allowed to dry completely, while preparing the matrix solution (5g/l of α -cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN and 0.1% TFA). Following, 0.5 μ l of matrix solution was spotted on the target plate and also allowed to dry. A matrix solution of 4g/L of HCCA in a mixture of ACN and 0.2% TFA (1:1, v/v) was used for MALDI-TOF-MS analysis. MALDI-TOF spectra were acquired in the positive reflectron mode in the range of 1500–4000 Da. All testing was performed

under high vacuum at or below 10^{-7} Torr. The acceleration voltage, electron voltage, and lens voltage were 19kV, 20kV, and 9.75kV, respectively, with a delay time of 100ns. Low mass deflection was set at 1000Da. For each sample, the mass spectra were acquired from 500 laser shots. The spectra were calibrated externally using a peptide calibration standard and raw spectra were processed with flexAnalysis 3.0 (Bruker Daltonics). Peptides were analyzed using a MALDI-TOF.

Serum and urine hepcidin identification

To verify the correspondence of the 2788 m/z peak to Hep-25, dithiothreitol (DTT) was added to a portion of the serum samples to reduce the four disulfide bridges associated with its cysteines to eight sulfhydryl groups (-SH). Such a reaction would be expected to add 8 Da to Hep-25's molecular mass based on the uptake of eight additional protons. Further verification was obtained by the addition of iodoacetamide (IAA). Given the presence of eight -SH groups in fully reduced Hep-25, a second peak shift from 2796 m/z to 3252 m/z accurately reflected the addition of four IAA molecules.

LC-MS/MS was used to further prove the fragment at m/z 2788 showing the same sequence with hepcidin. Reversed phase chromatography was performed on an Agilent 1200 series HPLC with gradient solvent (A, 0.1% formic acid in water; B, 0.1 % formic acid in acetonitrile), the samples were first injected into HPLC and then analyzed LTQ-Orbitrap XL. 1 μ L (100 μ M) synthetic human hepcidin in 1% formic acid, 5 mM NH_4OAc buffer was injected as a standard to optimize the parameters. All the other human serum and urine samples followed with the same acquisition method.

Method validation

Rabbit serum was used for the preparation of the serum standard curve because rabbit hepcidin has a different molecular weight from human peptide such that no interference from mass spectrometry.⁸⁻¹⁰ For urine, the hepcidin standard curve was obtained with a 200mL urine sample that was stripped with 200mg charcoal for 2 h.¹⁹ Both the stripped urine and the rabbit serum were processed on the nanoporous silica chips as described above, and then analyzed by MALDI-TOF-MS. No matrix interference was observed. Synthetic human hepcidin (DTHFPICIFCCGCCHRSKCG-MCCKT, average mass 2789.4 Da and monoisotopic mass 2787.1 Da) was used for calibration curve assay. ACTH fragments 18-39 (RPVKVYPNGAEDESAEAFPLEF, monoisotopic mass 2464.2 Da) was used as internal standard. The lower limit of quantification (LLOQ) is the lowest amount of hepcidin which can be quantified with acceptable precision and accuracy. A series of hepcidin standards were prepared in both rabbit serum (0, 1, 2.5, 5, 10, 20, 50, 80, 100 nM; n=6) and urine (0, 1.56, 3.125, 6.25, 12.5, 25, 50, 100 nM; n=6) to prepare a standard curves for each. The samples were processed on NPS thin films, and eluted by 5 μ L of ACN and 0.1% TFA (1:1, v/v) containing 50 nM ACTH, and then analyzed by MALDI-TOF-MS. A subset of the rabbit serum standards (5, 20, 50 nM) were analyzed and measured six times to evaluate the precision and accuracy of this measurement method (ideal versus measured concentration). Samples from two healthy human volunteers were also analyzed on three consecutive days to obtain intra- and inter-day reproducibility; each sample was measured 3 times.

Statistic analysis

Statistical significance (p values) was calculated by unpaired two-tailed Student's t-test using GraphPad Prism 5. Scatter plots were generated by GraphPad Prism 5 and presented as mean $\pm$ s.d. SPSS was used to analyze correlation significance (r values) and p values were calculated by two-tailed Student's t test using Spearman's rho. Median, P2.5 and P97.5 were calculated from original values by SPSS. Excel was used to analyze other data sets unless otherwise specified.

Results

NPS thin films fabrication

The NPS thin films were fabricated by modification of our previously reported protocols with nanotextures specifically and precisely tailored for hepcidin enrichment.²⁰ This method is based on Evaporation Induced Self-Assembly of silicate sol-gel solution with triblock-copolymer templates (Figure 1B): During the spin-coating process, the hydrophobic chains of the triblock-copolymer aggregate to form micelles initially with increasing surfactant concentration and that eventually form an organized organic-inorganic mesostructure. After removing the polymer template by calcination, NPS films with high surface area-to-volume ratios remain.²² Various nanoporous structures can be fabricated by a variety of polymer. The nanopores are influenced by the composition of the precursor solution that drives the cooperative assembly of the polymer template with the silicate species. To optimize Hep-25 harvesting from complex bodily fluids, three triblock copolymers (Pluronic L64, L121, and F127) were investigated as the structure-directing agents. The NPS thin films using L64 exhibit smaller pore size (3.2 nm). L121 with a longer hydrophobic chain resulted in larger pore (6.0 nm, Figure 2A). The addition of swelling agent polypropylene glycol (PPG), the pore size was enlarged to 6.5 nm (L121+PPG, Figure 2B). L121 and L64 produce chaotic worm-like nanostructures, while F127 with longer hydrophilic chains leads to formation of highly ordered periodic nanostructures. Further modulation of the pore structure of NPS films prepared with F127 was achieved by tuning the ratio of surfactant to silicate molecule. This ratio determines the interfacial curvature among the water, block copolymer, and silicate phases, leading to a structural progression between 3-dimensional cubic (F127-3D, Figure 2C, pore size 3.9 nm) and 2-dimensional hexagonal (cylindrical pores, F127-2D, Figure 2D, pore size 3.7 nm) structures.²⁰

The adsorption of hepcidin on NPS thin films

A standard solution containing Hep-25 was used to test the adsorption capacity of NPS films with four typical different pore sizes, and the amounts of Hep-25 recovered was scaled with the pore size (Figure 3A). For NPS thin films prepared using L64, L121, and L121+PPG that possess similar pore structure, the amount of recovered Hep-25 increased along with pore size extension. The NPS films prepared using F127-2D with a pore size of 3.7 nm and significantly different pore structure, showed substantial enrichment. To further investigate the difference of hepcidin enrichment between NPS thin films F127-2D and -3D, the depth of hepcidin adsorption of 2D and 3D NPS thin films was also measured by XPS. Figure 3B shows the depth profiling of the nitrogen amount of Hep-25 enriched with 2D-hex and 3D-cubic NPS thin films. The higher nitrogen signal was observed in F127 2D-hex NPS thin

film, illustrating that Hep-25 can penetrate deeper in this NPS morphology design. The detailed XPS spectroscopy was shown in Figure S1. The thickness of F127 2D-hex and 3D-cubic thin films were both around 750nm that were measured by ellipsometry and also confirmed by SEM (Figure S2). Ar⁺ ion took around 22min to completely remove 750nm of NPS thin films, and the N1s signal were completely disappeared after 14mins of Ar⁺ ion sputtering.

Surface charge is also an important factor to be considered in the fractionation process. Most of the surfaces of Hep-25 are positively charged at pH 7, as calculated by Swiss PDB viewer (SPDBV) software (Figure 3C and D). As a negative control experiment, the surface charge of NPS films was modulated from negative to positive through surface modification with an amino group-containing molecule. As expected, the efficiency of Hep-25 loading decreased significantly (Figure S3).

Method validation in serum and urine samples

To better understand the hepcidin adsorption capacity of F127-2D NPS films, we compared the urine and serum MALDI-TOF MS spectra obtained with and without on-chip enrichment. No peak related to hepcidin or its derivatives were observed without NPS films enrichment (Figure 4A). In contrast, after on-chip fractionation, Hep-25 (2788.17 Da, [M+H]⁺) was clearly observed within serum and urine mass spectra (Figure 4B and C). The sequence and the four disulfide bonds of Hep-25 extracted from HBF were confirmed by both the reduction reaction and MS/MS sequence measurement (Figure S4-S6). Hep-20 (2191.78 Da, [M+H]⁺) and Hep-22 (2434.94 Da, [M+H]⁺) were also detected from urine (Figure 4C). To assess the sensitivity, the standard curves for Hep-25 in both serum and urine samples were generated (Figure S7) and the lower limit of quantification (LLOQ) is 1.56 nM. The precision and accuracy were also evaluated. The coefficient of variation (%CV) did not exceed 21%, and relative error (%RE) was within 18% (Table S1). In addition, two individual serum samples were analyzed to evaluate intra- and inter-day assay variability (3 replicates per subject per time point). The intra-day CV values fell within a range of 2–6%, while the inter-day CV values did not exceed 16% (Table S2).

Quantification of hepcidin levels in HBF

To evaluate our approach in clinical application, 119 healthy volunteers (n=60 male, n=59 female) and 19 patients clinically diagnosed with a variety of inflammatory conditions (n=11 male, n=8 female) were recruited for this study and classified according to age as well as several important hemoglobin and iron indices, including total serum iron, serum ferritin, total iron binding capacity (TIBC), and transferrin saturation (TS%) (Table S1). The median of each serum and urine Hep-25 level in the male and female healthy volunteers indicated gender-dependency (Figure 5A and Table S1). The patients suffering from inflammation showed significantly elevated Hep-25 levels as compared to healthy volunteers (Figure 5B). For women younger than 55 years of age, Hep-25 levels in both serum and urine were found to be close to or below the detection limit, while significantly higher Hep-25 levels were observed in post-menopausal women (age ≥ 55; Figure 5C). We also evaluated the correlation between serum Hep-25 and serum ferritin, which is commonly used to clinically evaluate

iron storage. Hep-25 levels in healthy volunteers correlated well with serum ferritin ($r=0.65$, $P<0.001$, Figure 5D).

Discussion

In this study, we developed a hepcidin enrichment approach based on nanoporous silica thin film and investigated the mechanism of hepcidin adsorption among different chips. This new approach has a great potential for clinic translation. The adsorption of the target peptide in NPS films is determined by the cooperative function of multiple parameters of nanopores, including pore diameter, surface area, pore volume, surface charge and pore morphology. First, matching the size of Hep-25 to the pore diameter is a key factor to achieve an appropriate molecular cut-off and high efficiency enrichment. Previous reports have demonstrated that the pore size-exclusion mechanism by mesoporous silica and carbon particles.^{23–26} Larger pores, offer more space to capture more peptides, however, may also enrich larger and more abundant proteins reducing the sensitivity of Hep-25 in MALDI-TOF MS. Hep-25 possesses a hairpin molecular structure with dimensions of $2.6\text{nm} \times 1\text{nm}$.² Therefore, the optimization of pore size is necessary to improve the sensitivity of this assay. In our study, the harvesting efficiency was significantly enhanced when the ratio of pore size (3.7 nm) to protein size to be around 1.5.

Recovery of Hep-25 is also influenced by pore morphology and pore volume. For NPS thin films produced with F127, the 3D cubic pore structure recovered less Hep-25 than the 2D hexagonal (hex) structure, despite the similarity in their pore sizes. In previous studies, the 3D-cubic pore structure exhibited better pore connectivity resulting in better absorption capacity and a higher release rate in drug delivery systems where the loaded molecule was much smaller than the pore size.^{27, 28} In addition to pore morphology, the pore volume is also an essential factor. From N_2 adsorption analysis, we observed strong differences in pore volume between the 3D and 2D films (0.427 & $0.742\text{ cm}^3/\text{g}$ for 3D & 2D, respectively).²⁰ Larger pore volume should exhibit higher absorption capacity, and thus, better enrichment²³. The difference between the adsorption capacity of 3D and 2D NPS films was also measured by XPS which showed that molecules of hepcidin can go deeper in 2D NPS films. The difference between the enrichment may therefore be attributable to a combination of parameters, including averaged pore volume, and pore morphology.

The electrostatic interaction between hepcidin and silica thin film surface was also considerate in our study. The pH of the solution below isoelectric point of proteins results in an electrostatic attraction between positively charged peptides and the negatively charged nanoporous silica surface.²⁹ Electrostatic interactions between the cationic hepcidin and the negatively charged silica surface drive of the binding of hepcidin to the silica. Patwardhan, et. al., have also reported the use of this mechanism in binding peptides to silica nanoparticles.³⁰ As shown in Figure 1A, size-exclusion and the electrostatic ion pairing largely contribute to the enrichment of hepcidin. The low molecular weight proteins and peptides diffuse into silica nanopores, while the large proteins are excluded by molecular weight cutoff that is dictated by pore size. Furthermore, the diffusion depth of hepcidin is mainly associated with the morphology and inter-connectivity of the silica nanopore. The larger pore surface area can offer more binding sites. Therefore, optimal pore morphology

and the larger pore surface area can increase the selectivity and efficiency of hepcidin enrichment. After testing the effect of pore size, pore structure, and surface charge, F127-2D-hex nanoporous silica thin films exhibited the most loading capacity and were selected for further studies.

In order to evaluate whether our assay was providing biologically and clinically meaningful measurements, the hepcidin levels in 119 healthy volunteers and 19 inflammation patients were tested by our approach. The average and range of the measured Hep-25 levels in both urine and serum are comparable to the literature.^{8, 9, 11, 13} Significant demographic variation, in terms of gender and age, were observed with consistently higher levels of both serum and urinary hepcidin in men over of all ages, and post menopausal women over age 55 as compared to menstruating women under age 55.¹⁴ These gender differences likely reflect the low iron storage in women, and correlated with the lower levels of serum ferritin, serum iron, hemoglobin, transferrin saturation, and higher total iron binding capacity, when compared to the male samples (Table S1). The menopausal dependent of hepcidin levels is consistent with previous reports.^{7, 14} Our data highlight the strong correlation between inflammation and induction of hepcidin overexpression in serum. During inflammation, increased IL-6 induces overexpression of Hep-25.⁴ As expected, the patients suffering from inflammation showed significantly elevated levels of Hep-25 as compared to those of the healthy volunteers (Figure 5B). Furthermore, after biostatistics analysis of the peptide profiles by ClinProTools, another 19 peptides were also found to exhibit significant changes in inflammation patients, and 4 peptides were identified as the fragments degraded from acute phase proteins (Figure S8 and Table 3). As a preclinical validation of this technique, our findings indicate that this nanopore-based method is sensitive, reliable, and can accurately reflect iron status in the body.

By integrating the size-exclusion mechanism of nanopore film and the electrostatic interactions between the cationic biomarker and the negatively charged silica surface, we successfully enriched and quantitatively detected low abundance and low molecular weight peptide biomarkers hepcidin from non-pretreated complex biological samples (human bodily fluids). The pore size, pore volume, morphology, and surface potential could be modified to fit with physicochemical properties of low abundance and low molecular weight peptide biomarkers to achieve the selective capture. The reliability we further certified by the assay of more than 100 clinical samples. Our finding presents a simplified, reliable, and one-step high throughput approach for the label-free quantification of the single biomarker, Hep-25, from non-pretreated human bodily fluids. The application of and proof-of-concept for this new approach could help not only to improve our understanding of iron disorder diseases, but also consequently enable hepcidin to become a viable diagnostic biomarker for clinical uses.

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Figure 1.

The schemes of peptides fractionation and NPS thin films fabrication. (A) The schematic of peptide fractionation from human bodily fluids using NPS thin films. (B) The route of surfactant-directed formation of NPS thin films.

Figure 2.
TEM images of NPS thin films: (A and B) L121 and L121+PPG NPS films with 6.0 nm and 6.5 nm pore size; (C and D) F127-3D and F127-2D NPS films with 3.9 nm and 3.7 nm pore size.

Figure 3.

The effects of physicochemical characteristics of NPS thin films for loading hepcidin. (A) Measurement of purified hepcidin mixture after fractionation on nanoporous chips with different pore sizes and morphologies. (B) The depth profiles of hepcidin enriched inside nanopores of F127-2D & 3D NPS thin films. The amount of hepcidin was determined from N1s spectra collected by XPS. (C and D) The electrostatic surface potential of hepcidin (red negative, blue positive) at pH 7 (PDB code: 2KEF, pI=8.22).

Figure 4. Hepcidin detection by MALDI-TOF MS with and without enrichment on NPS thin films. (A) Mass spectrum of serum without fractionation. (B) and (C) Mass spectrums of serum and urine samples after selective enrichment.

Figure 5.

Clinical assay of hepcidin levels in healthy volunteers and inflammation patients. These scatter plots allow for comparisons between (A) genders, (B) healthy volunteers to individuals suffering from acute or chronic inflammation and (C) pre- and post-menopausal women. The scatter plot in (D) shows the good correlation between serum hepcidin and serum ferritin.