Identification of Human Plasma Proteins by Trypsin Immobilized Digestion Chip and Electrospray Ionization Tandem Mass Spectrometry

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Abstract

Self-assembled monolayers (SAMs) on coinage metal provide versatile modeling systems for studies of interfacial electron transfer, biological interactions, molecular recognition and other interfacial phenomena. Recently the bonding of enzyme to SAMs of alkanethiols onto Au electrode surfaces was exploited to produce a bio-sensing system. In this work, the attachment of trypsin to a SAMs surface of 11-mercaptoundecanoic acid was achieved using water soluble N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride and N-hydroxysuccinimide as coupling agent. Experimental results have revealed that the XPS C1s core levels at 286.3 and 286.5 eV (C with N), 288.1 eV (Amide bond) and 289.3 eV (Carboxylic acid) illustrate the immobilization of trypsin. These data are also in good agreement with FTIR-ATR spectra for the peaks valued at 1659.4 cm^{-1} (Amide I) and 1546.6 cm^{-1} (Amide II). Using nano-HPLC-ESI-MS/MS observations, analytical results have demonstrated the human plasma proteins digestion of the immobilized trypsin on the functionalized SAMs surface. For such surfaces, human plasma proteins were digested, which shows the enzyme digestion ability of the immobilized trypsin. The terminal groups of the SAMs structure can be further functionalized with biomolecules or antibodies to develop surface-base diagnostics, biosensors, or biomaterials.

Keywords: Self-Assembled Monolayers (SAMs), Human plasma proteins, Protein digestion, Surface analysis, nano-high performance liquid chromatography electrospray ionization tandem mass spectrometry (nano-HPLC-ESI-MS/MS)

Introduction

Proteomic characterization of human plasma for identification of disease-specific biomarkers promises to be a powerful diagnostic tool for defining the onset, progression and prognosis of human diseases. Plasma is the most important bio-fluid for general proteomic analysis and provides a rich sample for diagnostic analyses because of the expression and release of proteins (potential biomarkers) into the bloodstream in response to specific physiological states such as bacterial infections, cancer, and other disease to name a few. It is undoubtedly the fluid most general examined for protein levels, and clinical chemists around the analyses each day to quantitate proteinaceous disease markers (antibodies, metabolic enzyme, troponins, carcinoembryonic antigens, etc.) in addition to electrolytes and the usual small metabolites.

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Self-assembled monolayers (SAMs) have received a great deal of attention for their fascinating potential technical applications such as nonlinear optics and device patterning [1-3]. They were also used as an ideal model to investigate the effect of intermolecular interactions in the molecular assembly system [4,5]. It was very convenient to introduce functional structure as tail group on SAMs, and to investigate different molecular interactions with tail group on SAMs as induced by particular species [6,7].

SAMs formed by adsorption of either alkanethiols onto Au or alkylsilanes onto hydroxylated surfaces constitute an important class of model surfaces for fundamental studies of protein or enzyme adsorption. The process was assumed to occur with the loss of hydrogen, by the immersion of Au substrate in a dilute solution of the AT and the formation of well-ordered SAMs on Au surface [8-11]. The AT SAMs not only provide excellent model system to study fundamental aspects of surface properties such as wetting [12] and tribology [13], but also were promising candidates for potential

applications in the fields of biosensors [14], bio-mimetics [15] and corrosion inhibition [16].

In this study, *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-Hydroxysuccinimide (NHS) were utilized to activate the tail group of O=C-OH [17-19] and then immersed in the trypsin-contained solution to bind with $-NH_2$ in trypsin. For the $-NH_2$ tail group, water-soluble EDC and NHS were used to activate O=C-OH in trypsin to bind with $-NH_2$ on SAMs. As shown in the literatures [20-22], a variety of analytical techniques such as X-ray Photoelectron Spectroscopy (XPS), Infrared Reflection Absorption Spectroscopy (IR-RAS), Fourier-Transformed Infrared with Attenuated Total Reflection (FTIR-ATR), Ellipsometer and Contact angle measurements, the AT SAMs were densely packed films. A nano-HPLC-ESI- MS/MS system was used to obtain the proteins identification of tryptic peptides in this study.

In this experiment, it tends to complete a preliminary study for a potential biomedical application of functionalized SAMs to immobilize with enzyme as a protein mixture digestion biochip. A mass spectrometry system could use the biochip for a protein mixture digestion and identification. We present proteomic profiling data of human plasma protein identification using nano-HPLC-ESI-MS/MS.

Materials and methods

Formation of SAMs

A 200 nm thick Au film was prepared by electron beam evaporation onto Si (111) surface (*Silicon Sense*) primed with an adhesion layer of 20 nm Ti. The Au (111) substrates were cleaned by H_2O_2 solution for 15 sec. followed rinsing by high-purity ethanol (RDH 32205, Riedel-deHaën), and then immersed into 0.5 mM ethanolic alkanethiol solution at room temperature for 12 hrs [22-23]. Two kinds of chemicals for SAMs preparation were used: 1-Dodecanethiol: $C_{12}H_{26}S$ (44130, Fluka), and 11-mercaptoundecanoic acid: $C_{11}H_{22}O_2S$ (450561, Aldrich).

Immobilisation of trypsin onto SAMs

To immobilize trypsin, the 11-mercaptoundecanoic acid /Au surface was immersed in the coupling agent: 75mM, EDC (E-6383, Sigma) and 15mM, NHS (H-7377, Sigma) at 4°C for 30 min [24,25]. Water-soluble EDC and NHS were used for activating O=C-OH [18,19] and then the EDC-NHS buffer was removed and replaced by the 0.2 μg/μl (w/v) trypsin (V511A, Promega) at 4°C for 24 hrs. The SAMs wafer was thereafter washed by DI water and dried out mildly at 4°C.

The amounts of immobilized trypsin on SAMs surface was measured by an estimation of:

$$\textit{Immobilized trypsin } (\mu g/cm^2) = (Wt - W)/A$$

Where A was the dimensional area of the SAMs surface, W and Wt were the weights of SAMs surface before and after trypsin immobile process.

Ellipsometric measurement

The thickness of the SAMs monolayers was determined by an optical ellipsometer (LPS-400, J. A. Woollam Co., Inc.), equipped with a He/Ne laser of ?=632.8 nm as the light source [21].

Contact angle measurement

The contact angles (*?*) were measured in air using a goniometer (Krüss apparatus). A Milli-Q grade water (Millipore Co., Inc.) was used to contact with the sampling dimension by the sessile drop method [26].

X-ray Photoelectron Spectroscopy measurement

XPS spectra were acquired with a Physical Electronics PHI 1600 ESCA photoelectron spectrometer with a magnesium anode at 400W and 15kV-27mA (Mg K_a 1253.6 eV, type 10-360 spherical capacitor analyzer). The specimens were analyzed at an electron take-off angle of 70°, measured with respect to the surface plane. The operating conditions were as follows: pass energy 23.4 eV, base pressure in the chamber below 2×10^{-8} Pa, step size 0.05, total scan number 20, scan range 10 eV (for multiplex scan).

Fourier-Transformed Infrared Reflection-Absorption and Attenuated Total Reflection Spectrosocopies

All infrared (IR) spectroscopy optical benches were acquired with a conventional Fourier-transformed (FT) Spectrometer (FTS-175C, Bio-Rad) equipped with a KBr beam splitter and a high-temperature ceramic source. Win-IR, Win-IR Pro (Bio-Rad) and Origin 6.0 (Microcal Software, Inc.) were used for the data acquisition and analysis. The spectra were recorded with a resolution of 4 cm^{-1} using about 500 scans and an optical modulation of 15 kHz filter.

Human plasma preparation

Venous blood was obtained from six normal young individuals, ages in the ranging of 20-25. The blood was collected in a glass vacutainer (5 ml) and mixed with the anticoagulant 3.8% (w/v) sodium citrate (9 vol. blood: 1 vol. citrate) in tubes. Platelet pool plasma (PPP) were prepared by centrifugation of whole blood at 5000g for 20 min. at 4°C. The protein concentration of the plasma samples were measured by Bio-Rad Bradford total protein assay kit (Bio-Rad Laboratories, Inc.), and adjusted to 1 mg/mL by 25 mM ammonium bicarbonate.

In the nano-HPLC-ESI-MS/MS analysis of human plasma proteins, 20 μl of the protein suspension sample described above was deposited on the trypsin-immobilization surface. The samples were allowed to shake slowly at 37ºC, 30 min using an incubator for digestion and then transferred into sample vials.

Protein identification by reverse phase nano-HPLC-ESI -MS/MS

The protein tryptic digests were fractionated using a C18 microcapillary column (75 μm i.d. × 15cm) at a flow rate of 200 nL/min with a nano-HPLC system (LC Packings, Netherlands) coupled to an ion trap mass spectrometer (LCQ DECA XP Plus, ThermoFinnigan, San Jose, CA) equipped with an electrospray ionization source. The RP separation was performed using a linear acetonitrile gradient from 100% buffer A (5% acetonitrile/0.1% formic acid) to 60% buffer B (80% acetonitrile/0.1% formic acid) in 450 min using the micro pump [27].

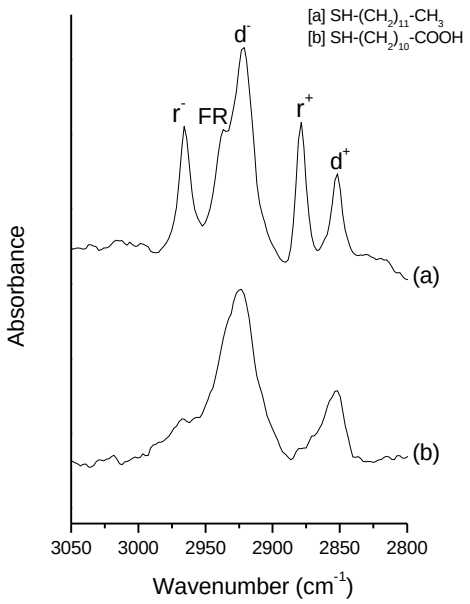

Figure 1. FTIR-RAS spectra show the high-frequency region: 3050-2800 cm^{-1} of the SAMs (a) 1-Dodecanethiol, (b) 11-mercaptoundecanoic acid.

Tandem mass spectrometry data was analyzed by the database search software SEQUEST (Bioworks 3.1, TheroFinnigan, San Jose, CA) to interpret MS/MS spectra. DTA files were generated from product ion scan data (threshold intensity set at 10,000), and used to search against human FASTA protein sequence database. SEQUEST results were filtered with criteria similar to those developed by Yates and co-workers [28]. Proteins were initially annotated by similarity searches using NCBI PubMed (http://www.ncbi.nlm.nih.gov/), SWISS-Port/ TrEMBL (http://www.expasy.org/), and Bioinformatic Harvester EMBL (http://harvester.embl.de/) databases.

Result and Discussion

Surface characterization: film thickness and contact angles

Ellipsometry was an invaluable technique for measuring film thickness of alkanethiols adsorbed on SAMs surface [29]. The optical ellipsometric characterization obtains a film thickness of 16.57±0.55 Å for 11-mercaptoundecanoic acid/Au, and 14.67±0.46 Å for 1-Dodecanethiol/Au, consistent with the formation of films of one molecular layer.

Contact angle for 1-Dodecanethiol/Au surface was indicative of a well-ordered and homogeneous layer with methyl group at the monolayers/ambient interface (contact angle was 97.00°±0.29). Measurement of 11-mercaptoundecanoic acid/Au using water as probe liquid give advancing contact angles of less than 15° (14.67°±0.46), with the carboxyl or the hydroxyl tail group were therefore polarizable and hydrophilic [30,31]. The SAMs surfaces consistent as a high free energy surface.

Investigation of film structure by FTIR-RAS

Figure 1 shows that the FTIR-RAS spectra of the SAMs of the alkane and carboxylic acid. The position of the C-H stretching bands of the methylene groups of the alkyl chains indicates the order of the alkyl chains within SAMs. In the spectrum of the SAMs, two absorption bands at 2918 and 2850 cm^{-1} were assigned to asymmetric (d^-) and symmetric (d^+) C-H stretching bands of the methylene groups, respectively. The peak positions of CH_3 stretching modes were consistent with the presence of a dense crystalline-like phase: r^+, 2876 cm^{-1}; FR, 2935 cm^{-1}; r^-, 2963 cm^{-1}. The band positions of 11-mercaptoundecanoic acid/Au indicated that the band frequencies at 1705 cm^{-1} and 1400 cm^{-1} were assigned to residual carboxylic acid stretch, ?(C=O) and symmetric carboxylate stretch, $?_s(COO^-)$, respectively [20].

Structural confirmation of trypsin-immobilized surfaces

In this study, water-soluble EDC and NHS were used for activating O=C-OH and immobilizing trypsin. The amounts of immobilized trypsin on SAMs surface was controlled to ca. 0.05 μg/cm^2. In the FTIR spectra, the peak at 1407.2 cm^{-1} was usually assigned to carboxylate stretch (COO^-). The peak at 1659.4 cm^{-1} was usually assigned to amide I (R-CONHR', C=O stretching) and the peak at 1546.6 cm^{-1} to amide II (R-NHR', NH deformation, N-H bending and C-N stretching). Thus, the poly-complex between trypsin and 11-mercaptoundecanoic acid was formed; amino groups in trypsin form complexes with carboxyl groups in 11-mercaptoundecanoic acid. Surface analyses used XPS to measure the binding structure on the SAMs metal surface. The binding energy of C1s core level at 289.3 eV (O=C-O) and of O1s core level at 532.0 eV and 533.3 eV examined by XPS, could be assigned to the O=C-O structure, which was the characteristic group of 11-mercaptoundecanoic acid.

The XPS C1s core level spectra for the trypsin-immobilized SAMs was deconvoluted into six peaks: 284.6 and 285.4 eV (hydrocarbon and carbon), 286.3 and 286.5 eV (C with N, amine), 288.1 eV (C with O, carbonyl or amide bond), and 289.3 eV (C with O, carboxylic acid), respectively. The trypsin-immobilized SAMs surface displayed a significant increase of C-N and amide group. The groups were corresponding with trypsin molecules (C-N) or a complex between trypsin and 11-mercaptoundecanoic acid (amide group). The binding energy at 286.3 and 286.5 eV was assigned to C-N binding in trypsin. The O=C-OH group altered notably owing to the participation of functional group of the 11-mercaptoundecanoic acid binding with trypsin as amide group.

In the XPS measurements, the variations of O1s and N1s with respect to C1s signal ratios were correlated with the significant presence of chemical species at the trypsin-immobilized SAMs surfaces, respectively.

Cellular location and known function of the identified proteins

A nano-HPLC-ESI-MS/MS system was used to obtain the fragmentation patterns of tryptic peptides in this study. This analysis resulted in the identification of 440 unique proteins in the human plasma proteome (Protein list not shown, please contact with the corresponding author). Human serum albumin

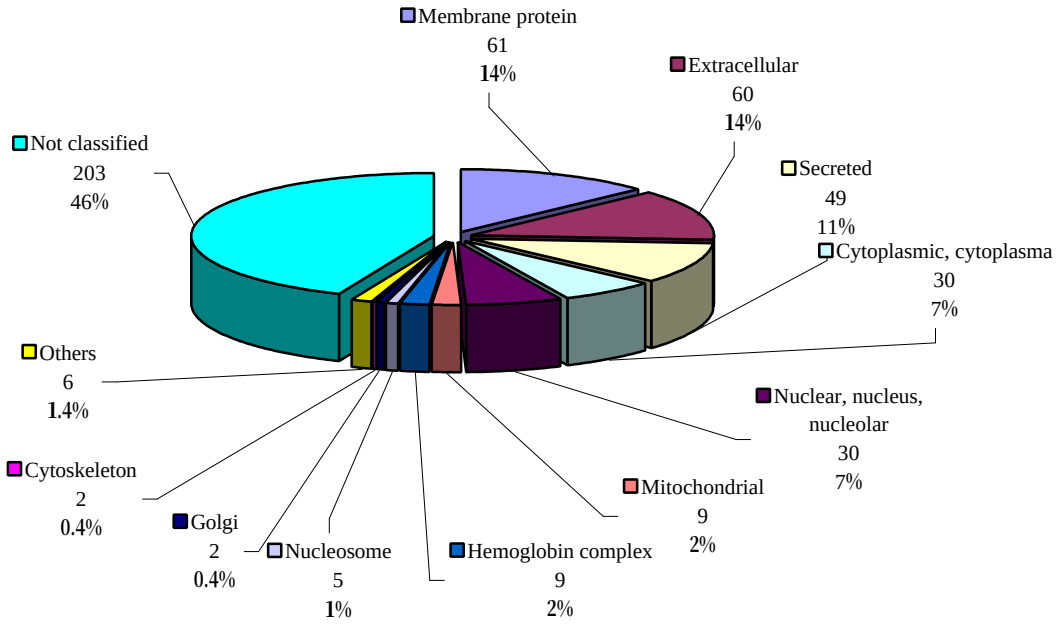

Figure 2. Distribution of cellular locations of 440 proteins identified in this study.

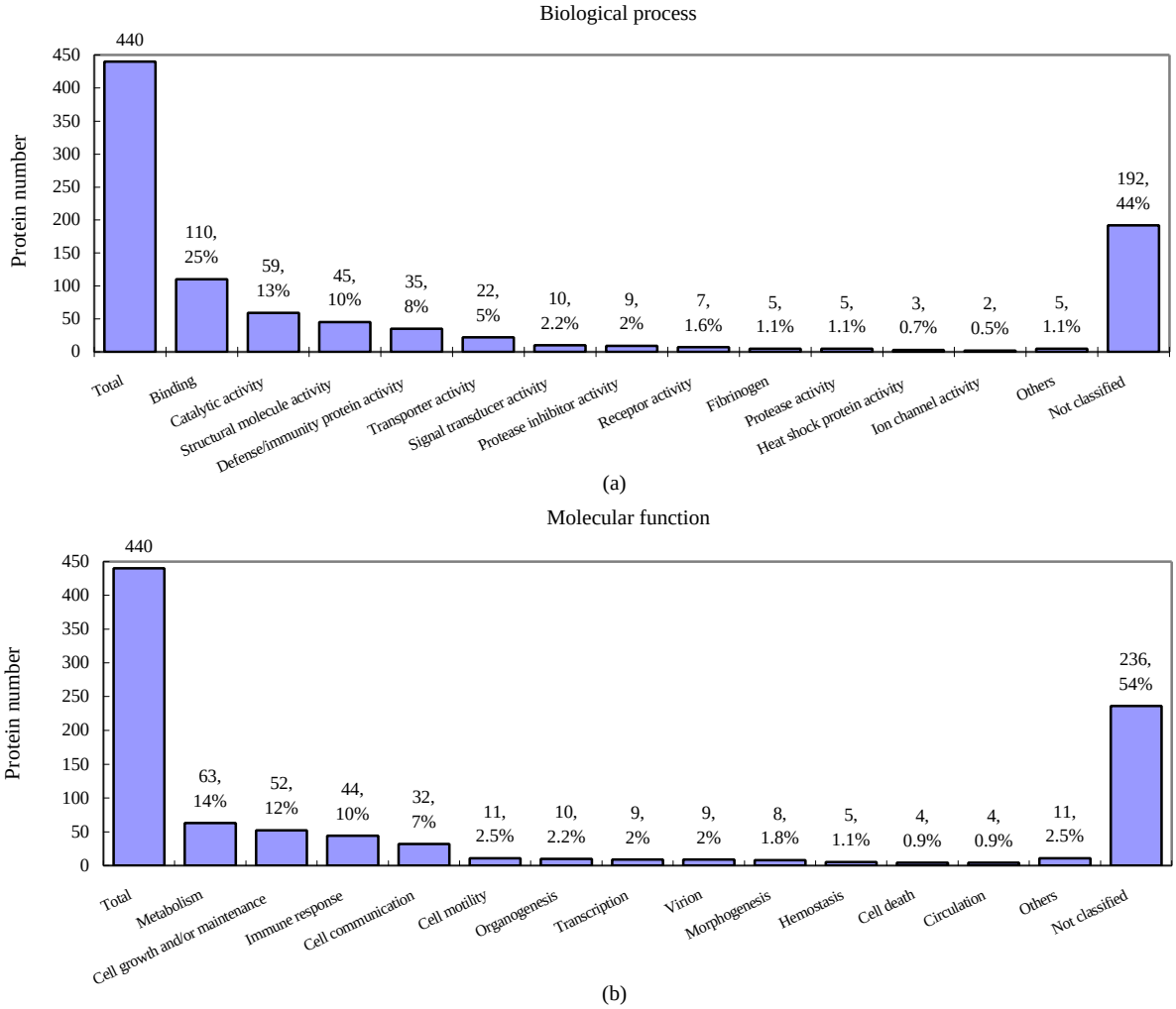

Figure 3. The number and percentage of proteins with certain reported known biological process and molecular functions. (a)Biological process; (b)Molecular function.

is by far the most abundant protein in plasma, responsible for more than 50% of the protein mass in normal individuals. Together with immunoglobulins, transferring, fibrinogen, complement components, apolipoproteins and a few other proteins, the top 20 or so proteins were responsible for about 99% of the protein mass in plasma. Figure 2 showed the distribution of cellular locations of proteins identified in this study. Among 440 proteins identified, 109 proteins (25%) were known to be secreted into extracellular space. Sixty-one proteins (14%) were known to be membrane protein. Thirty proteins (7%) were known to be cytoplasma proteins. Thirty proteins (7%) were known to be nuclear proteins. A few mitochondrial, hemoglobin complex, nucleosome, golgi and cytoskeleton proteins were also identified. A considerable portion of the identified proteins (46%) has not been reported for their cellular locations.

We used the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) to explore what known functions of the identified proteins had been reported in the literature. Swiss-Prot/TrEMBL (http://www.expasy.org) [32] was used to obtain relevant information regarding to the functions of the 440 proteins. Figure 3 shows the number and percentage of proteins with certain reported known biological process and molecular functions. Among 440 proteins, 110 proteins were binding proteins. Sixty-three proteins were about metabolism. Fifty-nine proteins have been known to be associated with catalytic activity, and fifty-two proteins have been known to be associated with cell growth and/or maintenance. Protein functions related to structure molecule activity, immune response, defense/immunity protein activity and cell communication were also surveyed, and these functions were linked to considerable portions of 440 proteins identified in this study. Around 152 proteins had no prior functional information reported.

Conclusion

Industrial-scale proteomics involves large sample volumes and so requires extensive separation prior to final analysis by mass spectrometry. The results presented provide an example of the 11-mercaptoundecanoic acid self-assembled monolayers (SAMs) applications for the enzyme digestion chip. SAMs formation provides an easy way to prepare the structure that can be further functionalized with biomolecules to yield biorecognition surfaces for use in medical devices. The carboxyl functional thiol monolayer gives an excellent way to immobilize enzyme, protein or other biomolecules for selective sensing of different analyses. The application of SAMs for the immobilization of enzymes to Au surfaces has considerable potential to produce reproducible enzyme biochips. In summary, we have presented the modification of Au interface via 11-mercaptoundecanoic acid SAMs and proved that the SAMs on Au can be a trypsin digestion chip for human plasma proteins analysis by nano-HPLC-ESI-MS/MS. The data form a database for the diversity and relative abundance of various proteins found in the human plasma proteins. We have assembled an enlarge list of proteins observed in human plasma by combining the proteomics approaches. The database provides not only information on the nature of protein contents in human plasma proteins but also potential protein diagnostic biomarkers to be examined in further investigations. The materials of SAMs were easy to be obtained, and this method was simple and easy to develop surface-base diagnostics, biosensors, or biomaterials. This paper illustrates a preliminary investigation of the application of SAMs of alkanethiols to the preparation of covalently immobilized enzyme biosensors.

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