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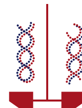

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Characterization of particulate matter binding peptides screened from phage display

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Particulate matter (PM), especially particulates with diameters of less than 2.5 μm, can penetrate the alveolar region and increase the risk of respiratory diseases. This has stimulated research efforts to develop detection methods so that counter measures can be taken. In this study, four PM binding peptides were obtained by phage display and binding characteristics of these peptides were investigated using the peptide array. The strongest binding peptide, WQDFGAVRSTRS, displayed a binding property, measured in terms of spot intensity, 11.4 times higher than that of the negative control, AAAAA. Inductively coupled plasma mass spectrometry (ICPMS) analysis of the transition metal compounds in the PM bound to the peptide spots was performed, and two peptides showed higher binding towards Cu and Zn compounds in PM. These results suggest that the screened peptides could serve as an indicator of transition metal compounds, which are related to adverse health effects, contained in PM.

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[**Key words:** Particulate matter; Phage display; Peptide array; Transition metal; Metal binding peptide]

Particulate matter (PM) is defined as fine solid or liquid matter suspended in the Earth's atmosphere (1). Both natural and man-made sources contribute to airborne PM. Natural sources include smoke from forest fires and volcanic eruptions while man-made sources include automobile exhaust and smoke from power generation activities (2). PM is a complex mixture of elemental and organic carbon (EC and OC), inorganic compounds such as sulfates and nitrates, as well as metal compounds (3,4). Airborne PM is an important metric of air quality and a major source of health concern in urban areas (5). Long-term exposure to fine PM with diameters of less than 2.5 μm is associated with increased risk of lung cancer and cardiovascular diseases (6) while short-term exposure is associated with cardiopulmonary diseases (7). The proven adverse health effects of PM have stimulated various research efforts to develop methods for its detection, so that appropriate counter measures can be taken.

Typical detection methods employ the use of air filters to collect PM, followed by physical and/or chemical characterization of the sample. Physical characterization methods such as dynamic light scattering provides particulate size distribution and particulate mass (8) while chemical characterization methods, such as gas chromatography–mass spectrometry, provides composition data of the PM sample (9). Although the detection is comprehensive, this method suffers from the drawbacks of long processing time and decreased accuracy of the chemical analyses due to incomplete extraction of insoluble components of PM (10).

To overcome these drawbacks, a high flow rate aerosol-into-liquid collector was developed to directly collect PM as concentrated slurries (11). By coupling this sampling technique with analysis techniques such as X-ray fluorescence (12) and instrumental neutron activation (13), rapid detection and chemical characterization of ambient PM can be achieved. Trace metals and elements can also be detected with reasonable accuracy since the collected slurry samples are highly concentrated. However, this system involves the use of highly complex and expensive equipment, which limits its application and versatility (10). Biomolecules may present a viable solution to the cost and complexity issues present in the aforementioned system. By combining a suitable transducing system with biomolecules, the chemical interaction between the biomolecules and the analyte can be converted into a quantifiable signal (14). Among various biomolecules, peptides have relatively simple structures, making them easier to synthesize and more stable to environmental fluctuations. Furthermore, peptides can bind not only to other biomolecules but also metal particulates (15,16). Our group has also found peptides capable of binding to metal particulates such as Ag (17), Au (18) and ZnO (19,20), making them promising candidates for PM detection.

In this study, PM binding peptides were screened using the phage display technique (21) and their binding properties were confirmed by performing a PM binding assay on a peptide array (22). Given the multicomponent nature of PM, chemical characterization of the bound PM was also performed using ICPMS to evaluate the binding characteristics of the peptides. Emphasis was

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placed on the measurement of transition metals and their derivatives because the adverse health effects of PM are believed to be caused by oxidative stress resulting from the formation of reactive oxygen species (ROS) (23) and several toxicological studies have established a positive correlation between transition metals and ROS activity (24,25). The purpose of this study is to screen for peptides capable of binding to PM and characterize its binding preferences to metal containing compounds in PM. The data from this study shall serve as a basis for future peptide screening and design to enhance their binding properties. To the best of our knowledge, our work is the first attempt at screening and designing peptides for PM recognition.

MATERIALS AND METHODS

Phage display screening The initial screening of PM binding peptides using phage display was performed according to the protocol written by New England Biolabs. An aliquot of Ph.D.-12 phage displayed random peptide library, diluted in 100 μl of TBST (50 mM Tris−HCl (pH 7.5), 150 mM NaCl, 0.1% v/v Tween-20), was incubated in a microtiter well coated with the PM sample (NIES CRM No. 28 Urban Aerosols) at room temperature for 1 h. The well was washed 10 times with TBST to remove unbound phages. After washing, bound phages were eluted using a PM suspension [100 μg PM/ml TBS (50 mM Tris−HCl (pH 7.5), 150 mM NaCl)]. The eluate was amplified using *E. coli* ER2738 strains and the panning process was repeated three times. Recovered phages were titered after every round of panning to determine the input titer for the next round of panning. DNA sequence analysis was performed for eluted phages (10 clones) from the third round of panning.

Construction of peptide array A peptide array containing the peptide sequences screened from phage display and polyalanine (AAAAA) as negative control was constructed using the SPOT-synthesis technique invented by Frank (22). Activated Fmoc amino acids (0.5 M) were spotted on a cellulose membrane (grade 542; Whatman, Little Chalfont, UK) with a diameter of 6 mm using an automated peptide spotter (MultiPep RSi; Intavis AG, Köln, Germany), followed by Fmoc deprotection using 20% piperidine. This cycle was repeated with *N,N′*-dimethylformamide (DMF) and methanol washing steps performed in between the spotting and deprotection steps until the entire peptide was synthesized.

Binding assay Deprotection of side chains was performed using a reagent mixture containing water, triisopropylsilane and trifluoroacetic acid in a volume ratio of 2:3:95. After the removal of the protecting groups, the membrane was washed using dichloromethane, followed by DMF and methanol. The membrane was dried and washed again using ethanol. Prior to conducting the binding assay, the membrane was equilibrated in TBST buffer containing 15 mM NaCl two times for five minutes each. A particulate suspension (0.05% w/w) was prepared by mixing particulates in TBST buffer containing 15 mM NaCl. The particulates used in this study include PM (NIES CRM No.28 Urban Aerosols), Fe (Wako Pure Chemicals Industries, Osaka, Japan), Fe (II, III) oxide (Sigma Aldrich, Tokyo, Japan), Zn (Sigma Aldrich) and ZnO (Sigma Aldrich). This suspension was added to the membrane and the set-up was incubated at room temperature for 16 h with gentle shaking. At the end of the binding assay, the membrane was washed in TBST containing 15 mM NaCl for 35 min, with replacement of TBST buffer after 15 min and 25 min to remove nonspecific binding. The membrane was scanned and the spot intensities were measured using ImageQuant TL 7.0 (GE Healthcare, Uppsala, Sweden).

SEM imaging A semicircular area with a diameter of 5 mm was punched out from the peptide spot. The specimen was coated using an osmium coater (Meiwafosis, Tokyo, Japan) for 15 s at 10 mA. SEM images were obtained using VE-9800 (Keyence, Osaka, Japan) operated at 5 kV.

ICPMS analysis To analyze the bound PM components in the peptide spots, a circular area with a diameter of 5 mm was punched out from the peptide spot. A total of 10 peptide spots corresponding to each peptide sequence was placed in an airtight container and a reagent mixture comprising of 250 μl of deionized water, 500 μl of aqua regia and 500 μl of hydrogen peroxide was added. The samples were incubated in a thermostatic tank maintained at 120°C for 3.5 h. After the spots were acid dissolved, the samples were cooled to room temperature and 3750 μl of deionized water was added to make up a fixed volume of 5 ml for each sample. The samples were passed through a filter ((D-2) 25HP type). Counts per second (CPS) of each element was measured using the 7700× ICPMS system (Agilent Technologies, Tokyo, Japan) and the concentration of the element was determined using calibration curves. A total of 11 representative transition metals (Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, Y, La, Pb) were analyzed.

RESULTS AND DISCUSSION

PM binding peptides obtained from phage display From phage display, four peptide sequences were found from 10 sequenced plaques as PM binding candidates (Table 1). Out of 10 plaques, seven of them gave the sequence GLHTSATNLYLH. The three remaining plaques gave unique sequences of AGYPLSENFYYP, WQDFGAVRSTRS, and FHPRLQQDHWLH. Interestingly, all four sequences are hydrophilic and contain aromatic amino acids such as Histidine (H), Tyrosine (Y), Phenylalanine (F) and Tryptophan (W), which are underlined in Table 1. An Alanine (A) scan (stepwise replacement of amino acids with A from N to C terminus) was performed on the sequence having the highest frequency, GLHTSATNLYLH. Among the 12 peptide sequences, six sequences had reduced PM binding compared to the base sequence. In particular, spot intensities i.e., PM binding were reduced by approximately 25% when the aromatic residues, H and Y, were replaced with A (Table S1). On the other hand, the peptide sequences displayed isoelectric points ranging from 4.00 to 9.60. These results indicate that hydrophilicity and aromatic amino acid residues (H, Y) are important for PM binding.

Binding assay and evaluation of overall PM binding property A representative part of the peptide array after the completion of the binding assay is shown in Fig. 1A. Peptides obtained from phage display gave gray spots, which is an indication of PM binding. The measured spot intensities are shown in Fig. 1B. All peptide sequences showed higher spot intensities, with statistical significance, than the negative control, AAAAA. SEM images of the peptide spots are shown in Fig. 1C. PM appeared as white, spherical-like particles in these images and all observed particles had a diameter of less than 2 μm. Some PM was observed in the negative controls (panels B and 1 in Fig. 1C). This is likely due to nonspecific binding where PM is physically entrapped by the fibers of the cellulose membrane. In the case of spots corresponding to phage display peptide sequences (panels 2−5 in Fig. 1C), the number and density of bound PM were higher compared to the negative controls, suggesting the presence of chemical interactions between peptide synthesized membrane and PM.

Composition analysis using ICPMS In order to evaluate the composition of PM bound to each peptide, the peptide spots from the PM binding assay were subjected to ICPMS analysis. The ICPMS results for Cu and Zn are shown in Fig. 2. Sequences having the H residue, FHPRLQQDHWLH and GLHTSATNLYLH, displayed higher binding properties to Cu and Zn compounds in PM. The reverse is true for sequences lacking the H residue, WQDFGAVRSTRS and AGYPLSENFYYP. This observation is in line with the results of previous studies suggesting the importance of H for binding to Cu and Zn compounds (18,25). In a study conducted by Xue and Dong, imidazoles are able to react with metal surfaces such as Cu and form a protective layer on the metal surface (26). This reaction mechanism is a possible reason for the binding property of H, which contains an imidazole group, towards Cu compounds. On the other hand, in a study conducted by our group (19), the binding affinity of ZnO binding peptides increased with increasing H substitution. The binding characteristic of H residues towards metals and metal oxides was also demonstrated by PM binding peptides towards metal compounds contained in PM.

TABLE 1. Peptide sequences obtained from phage display and their respective frequencies.

Sequence[a]	Frequency	Isoelectric point	Average hydropathy[b]
GLHTSATNLYLH	7/10	6.92	−0.050
AGYPLSENFYYP	1/10	4.00	−0.575
WQDFGAVRSTRS	1/10	9.60	−0.900
FHPRLQQDHWLH	1/10	7.02	−1.392

[a] Underlined amino acid residues are aromatic.
[b] A negative average hydropathy value indicates that the peptide is hydrophilic.

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