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Characterization and Applications of the Dendrimer Monolayers to Bio-affinity Sensing for Development of Protein Chips

Mi-Young Hong and Hak-Sung Kim

Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), Taejon, Korea

The advance of proteomics has required the development of new technologies to allow the global analysis of protein functions and interactions in a massive and parallel manner. Along with proteomics tools such as yeast two-hybrid, liquid chromatography, affinity tag purification, and mass spectrometry, a protein chip technology has been of great interest because it is amenable to selective isolation of interacting partners on an affinity surface by a proper washing and the subsequent high-throughput interrogation of affinity-captured molecules.[1] In these aspects, the related approaches have primarily focused on the development of biomolecular interfaces to retain the activity and stability of immobilized biomolecules at high spatial densities, acquisition of relevant proteins on a proteome level, and detection methods for signal amplification with greater selectivity and sensitivity. In real applications of protein chips, well-built protein chips have accomplished the global analysis of protein interactions on a proteome-wide scale and the discovery of biomarkers through profiling of protein expression between sample and reference pools.[2] Recently, the protein chip technologies have broaden their utility towards a functional activity analysis of protein interactions/functions and post-translational modifications (PTMs) by handling the proteome directly on a genomic level.[3] In these areas, there still continues the request for technological improvement and novel applications of the protein chips.

The interface for immobilization of biomolecules on a solid surface should fulfill the necessities that the biomolecules are efficiently immobilized in a molecularly organized manner without loss of biological activity and that nonspecific adsorption of protein or cell debris is minimized. The surfaces immobilized or patterned with biomolecules have been widely applied to the simultaneous detection of diverse biospecific interactions in (multi-)biosensors and microarrays, complying with the growing demands from pharmaceutical, diagnostic and biomedical areas. To increase the efficiency and sensitivity of biospecific interactions, much attention has been paid to the development of interfacial layers enabling the immobilization of biomolecules with controlled orientation and minimized lateral steric hindrance.[4] These approaches have popularly employed the protein-ligand interaction of a streptavidin-biotin couple as the generic system to investigate the biospecific interaction on the surfaces including self-assembled monolayers (SAMs),[5] dendrimer monolayers,[6] and entangled polymeric layers.[7] By the manipulation of biotin-containing mixed SAMs on gold, the protein-ligand interactions have been extensively studied in

terms of the adsorption kinetics and surface coverage of streptavidin as a function of the surface density of biotin ligands. As a building unit of nano-structures and biosensing interfaces, a dendrimer as a monodisperse dendritic molecule has attracted great attention due to their physicochemical advantages; the precise molecular organization and the defined number of terminal groups for each generation, the radially emanated and regularly concentrated chain-end groups from and around the central core of the molecule, and the spherical shape at higher generations.[8] By our and other groups, it has been revealed that a variety of dendrimers having different chain-end groups are covalently attached to a chemically activated surface, commonly giving a molecularly organized layer.[9] Thus, the dendrimers have been extensively implemented as an interfacing molecule for DNA, protein, and antibody microarrays, proven the assets of the dendrimer-based surfaces such as the surface immobilization of biomolecules at high densities and a reliable detection with the high binding capacity of interacting partners.[6a, 10]

As a novel interface, the monolayers of poly(amidoamine) (PAMAM) dendrimers from first generation (G1) to fourth generation (G4) were characterized for protein-ligand interactions between streptavidin and biotin ligands. The formation of even and compact dendrimer monolayers on gold was confirmed by using FT-IR spectroscopy and ellipsometry. The surface fractional coverage of interacted streptavidin was analyzed by using surface plasmon resonance (SPR) spectroscopy. When the interfacial layer was functionalized with biotin and then subjected to the binding reaction of streptavidin, the biotinylated dendrimer monolayers resulted in much higher protein coverages (63 ~ 87%) compared with 11-mercaptoundecylamine SAMs (46%) and a poly-L-lysine layer (55%). At the G4 dendrimer layers, the observed streptavidin coverage (87%) approached the theoretical maximum level that can be attained given that protein molecules are closely packed in a hexagonal arrangement. In the binding process of streptavidin, the initial binding rate of streptavidin was two-fold higher in all dendrimer layers than the biotin-functionalized SAMs regardless of the biotin density. The dendrimer layers exhibited a much higher sticking probability, which is defined as the probability that the molecule adsorbs upon collision with a surface, at the fixed streptavidin coverage compared to other tested layers. Equilibrium binding analysis revealed that the binding of streptavidin onto the biotinylated G1 and G3layers fit to a Langmuir isotherm model. The kinetic and equilibrium analyses of streptavidin-biotin interactions revealed that the binding kinetics and surface coverages of streptavidin seem to be significantly affected by the structural characteristics of the dendrimer monolayers such as the surface orientation of biotin ligands and the surface corrugation, which provides the guidelines for construction of efficient interfacial layers for the biochip development.

For constitution of the dendrimer layer resistant to nonspecific adsorption of protein in biological samples, a tri(ethylene oxide)-attached, G4 PAMAM dendrimer (EO_3- dendrimer) was synthesized and its layer on gold was characterized. NMR analysis and MALDI-TOF mass spectrometry revealed that ~ 61 amine groups of a G4 PAMAM dendrimer were covalently conjugated with EO_3 units, accounting for a 95% modification level. The layer of the EO_3-dendrimer was formed on gold and the resulting surface was characterized by FT-IR spectroscopy, ellipsometry, and contact angle goniometry. The EO_3-dendrimer resulted in more hydrophilic and less compact layers with no substantial deformation of the molecule

during layer formation by virtue of the EO_3 units, compared to a G4 PAMAM dendrimer. At the biotinylated surface of the EO_3-dendrimer layer, the specific binding of avidin approached the surface density of 5.2 ± 0.2 ng·mm^{-2}, showing ~ 92% of full surface coverage. The EO_3-dendrimer was found to be considerably effective in lowering the nonspecific adsorption of bovine serum albumin (BSA) and exhibited a slightly increased resistance to adsorption of serum proteins.

Next, the dendrimer monolayer was implemented as the interfacing layer for chip-based analyses of protein interactions and PTMs. The monolayer of a G4 PAMAM dendrimer was adopted to construct the immuno-affinity surface of an antibody layer. The antibody layer as a bait on the dendrimer monolayer was found to result in high binding capacity of antigenic proteins and a reliable detection. The performance of the on-chip digestion procedure was investigated with respect to the ratio of trypsin to protein, digestion time, composition of a reaction buffer, and the amount of affinity-captured protein on a surface. As a result, the maximum efficiency was obtained when the digestion was conducted with the trypsin-to-protein ratio of 5:1 for 3h at 37 °C. The addition of a water-miscible organic solvent to a reaction buffer had no significant effect on the digestion efficiency under the optimized digestion conditions. The on-chip digestion method identified the affinity-captured BSA, lysozyme, and ferritin at the level of around 100 fmol. Interestingly, the detected number of peptide hits through the on-chip digestion was almost similar regardless of the amount of captured protein ranging from low- to high-femtomole levels, whereas the efficiency of in-solution digestion decreased significantly as the amount of protein decreased to low-femtomole levels. The structural alignment of the peptide fragments from on-chip-digested BSA revealed that the limited exterior of the captured protein is subjected to attack by trypsin. The established detection procedures enabled the identification of BSA in the biological mixtures at the level of 0.1 ng/mL. The use of antibodies against the proteins involved in the metabolic pathway of *L*-threonine in *Escherichia coli* also led to discrimination of the respective target proteins from cell lysates.

In addition, we established a chip-based analysis of sumoylation, the PTM process that involves the covalent attachment of the small ubiquitin-like modifier (SUMO) family protein to a target protein in eukaryotic cells. Substrate proteins were arrayed onto a glass surface at high spatial densities followed by the addition of the reaction mixture for sumoylation, and the SUMO conjugation was readily detected by using fluorescent dye-labeled antibody. Under optimized conditions, the on-chip sumoylation of Ran GTPase-activating protein 1 (RanGAP1) domain resulted in at least 7-fold higher fluorescence intensity than that of its mutant (K524A) irrelevant to SUMO conjugation. The on-chip sumoylation was also verified and quantified by using SPR spectroscopy. The microarray-based analysis developed in this study seems to provide an approach that can be used for identification of SUMO target proteins from preexisting protein pools and proteome arrays as well as for screening of inhibitors in a high throughput manner.

In conclusion, the dendrimer monolayers as an interfacial layer rendered the high spatial density for immobilization of probe molecules as well as the consequent high capturing capacity of target proteins. From a wide range of biochip applications, these assets of the dendrimer-coated surface have broadened its implementation to the interfacing molecule for preparation of DNA, protein, and antibody microarrays, enabling the signal amplification for detection of DNA hybridization and the quantitative assay with

substantially lowered detection limit and signal variation. In these approaches, the utility of the dendrimer monolayers was extended to an interfacial layer for direct on-chip identification of target proteins and a microarray-based analysis of SUMO modification on cellular proteins.

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