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Chemical Physics Letters 421 (2006) 373–377
CHEMICAL PHYSICS LETTERS
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A novel immobilization technique using a poly(amino acid) multilayer designed for surface plasmon resonance sensing

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Received 24 December 2005; in final form 27 January 2006
Available online 21 February 2006

Abstract

The novel immobilization technique using a poly(amino acid) multilayer was designed for the SPR sensing platform. These poly(amino acid) multilayer was confirmed as a relatively hydrophilic surface with little tendency for nonspecific adsorption of biomolecules and a flat surface that the biomolecular interactions can occur uniformly on the sensing surface. The poly(amino acid) multilayer functionalized with biotin was investigated to control the surface density and to estimate the optimum space between ligands for effective SPR sensing. This multilayer study demonstrates the importance of surface density which is significantly sensitive for fabrication of surface functional group in the monitoring of kinetic phenomena.

Recently, a variety of attempts related to the biosensor development have been reported for the real-time analysis and monitoring of specific binding phenomena including antibody–antigen complex formation, receptor–ligand interactions, protein–DNA/RNA interactions as well as small molecule detection [1,2]. The surface plasmon resonance (SPR) biosensor has been widely used to investigate binding events occurring on biological surfaces by the detection of a refractive index change on a gold surface without the need to label molecules. The applications of SPR are diverse and ever growing including materials characterization, gas sensing, and most notably, biosensing [3,4]. Recently, we have used the SPR to evaluate the in vitro specific adsorption in the microparticles system such as lectin-conjugated alginate microparticles with pig mucin (PM) for oral insulin delivery [5] and biotin-conjugated microbubbles with avidin [6] for targeted ultrasound imaging. Recent progress of biochip has driven the commercial application in many areas, but the surface state of functional group has not been well controlled even if several methods were proposed such as using a dextran matrix [7], various SAMs (self-assembled monolayers) [8], poly(L-lysine) monolayers [9], dendrimers [10], and even lipid monolayer which resembles a cell membrane [11]. This report describes a novel immobilization technique of biomolecules at the sensing surface layer by using poly(amino acid) multilayer deposition on Au for effective SPR sensing as shown in Fig. 1.

For the development of an effective biosensor, the appropriate packing density of biomolecules on the sensing surface is necessarily required to readily detect a target molecule. However, the monitoring of kinetic phenomena such as association rate, dissociation rate, and affinity constant should be required the optimum surface density of functional group, not simply high packing density. This poly(amino acid) multilayer functionalized with functional group was investigated to control the surface density and to estimate the optimum space between functional groups (Fig. 1). This technique based on poly(amino acid) multilayer has some desirable characteristics for SPR sensing platform such as: (1) a simple and consecutive process,

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doi:10.1016/j.cplett.2006.01.098

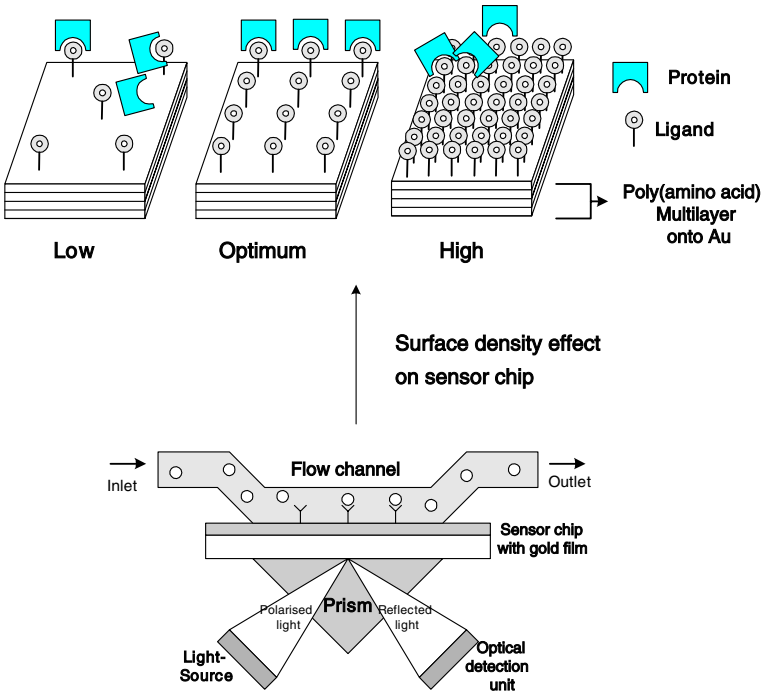

Fig. 1. Schematic representation of the controlling the density of biomolecules (biotin) onto gold surface for the surface plasmon resonance (SPR) sensing.

(2) a renewable sensing surface, (3) no denaturation of analyte on surface, (4) a minimized nonspecific binding due to relatively hydrophilic surface, and (5) a well-defined surface with precise control of ligands density on surface.

The poly(amino acid) multilayer having a well-defined composition and structures is obtained as follows. The multilayer was fabricated on a thin (47 nm) gold film that had been vapor-deposited onto silicon wafer. The gold films were cleaned by soaking and sonicating in cleaning solution (0.1 N NaOH, Triton X-100, 10 wt%) and subsequently water for 20 min at each time, followed by drying under high quality nitrogen gas. Alkanethiol monolayer of 11-mercaptoundecanoic acid (MUA; Aldrich) was formed on the cleaned gold films by immersing the surface into a 1.0 mM ethanolic solution for at least 10 h. Unbound MUA was removed by washing the films three times in absolute ethanol and in water, respectively. After forming the starting layer of MUA, the multilayer was deposited by electrostatic self-assembly of poly(L-lysine) (PLL, M_w 25700) as a cationic poly(amino acid) and poly(aspartic acid) (PAsp, M_w 25000) as an anionic poly(amino acid) (1.0 mg/mL, in PBS, pH 7.4) consecutively. PAsp is synthesized by thermal polymerization of L-aspartic acid (Sigma) followed by alkaline hydrolysis, where an intermediate, poly(succinimide) (PSI), is obtained and readily modified by grafting with various ligands having nucleophile [12–15]. The surface density of ligands on multilayer could be controlled by using the PAsp with various degree of substitution (DS). The PAsp with various DS are prepared prior to multilayer fabrication as shown in Fig. 2. The degree of substitution (DS) is the mole percentage of the grafted unit with ligands per total succinimide unit. As a model molecules, avidin–biotin couple ($K_A = 10^{15}\ M^{-1}$) was employed for the protein–ligand interaction on the poly(amino acid) multilayer as is widely applied in the biorelated fields. The PAsp of varying DS grafted with biotin were prepared in a similar synthetic manner to the previous paper [15]. The DS was calculated from the integral area of ^1H NMR peaks and the weight percentage of elemental analysis (EA). Biotins grafted (PAsp-g-biotin as shown in Fig. 2) with various DS (1.1%, 2.7%, 6.2%, 9.1%, and 18.4%) were used to functionalize the multilayer surface.

The poly(amino acid) multilayer formed by nonspecific electrostatic adsorption was relatively hydrophilic and thus biomolecules were hardly adsorbed nonspecifically on hydrophilic surface. The flatness of surface induced biomolecules uniformly adsorbed on the sensing surface by water contact angle, zeta-potential, and AFM measurements [15]. The complete cycle in refractive index over time for the avidin detection is shown in Fig. 3. When poly(amino acid) layers electrostatically adsorbed were exposed to a high salt solution (2.0 N NaCl), the deposited layer were completely desorbed [9]. In our previous work [15], the experimentally measured adsorption of the poly(amino acid) multilayer resulted in a shift in the SPR angle, 0.094 ± 0.009 (MUA layer), 0.054 ± 0.009 (PLL layer), and 0.042 ± 0.009 (PAsp layer). Generally, the assumption that 0.1° of angle shift and 1000 of RU (response unit) corresponds to 1.0 ng/mm^2 mass change at the sensing surface is accepted and used [16]. Then, it could be calculated, the observed SPR angle shift corresponds to a surface density (mass change/molecular weight) of 4.3×10^{-12} mol/mm^2 for the MUA layer, 3.7×10^{-12} mol/mm^2 for the lysine res-

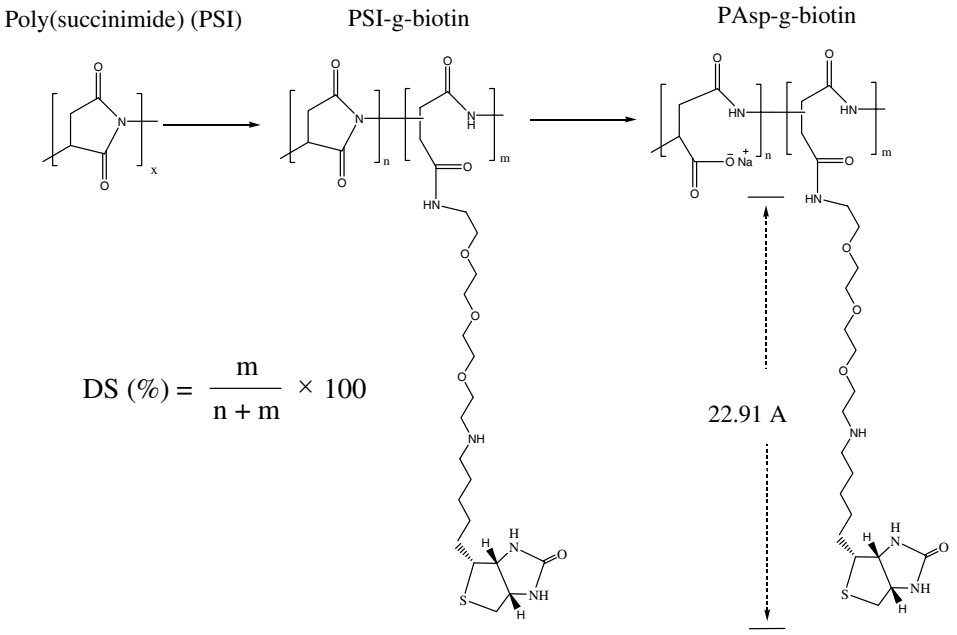

Fig. 2. Molecular structures of Poly(succinimide) (PSI), Poly(succinimide)-g-biotin (PSI-g-biotin), and Poly(aspartic acid)-g-biotin (PAsp-g-biotin) which are key molecules in the fabrication of functionalized poly(amino acid) multilayers.

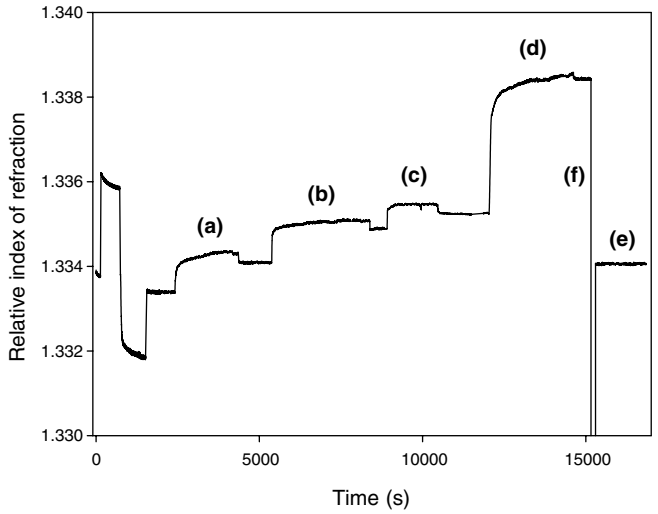

Fig. 3. The complete cycle in refractive index over time during exposure of a gold surface to the cleaning solution and then to the PBS solution of: (a) MUA (1 mM in ethanolic solution), (b) Poly-L-lysine (0.5 mg/ml), (c) poly(aspartic acid) (0.5 mg/ml) modified, and finally (d) binding of avidin (1.0 μM) on poly(amino acid) multilayers. The flow of PBS 7.4 for washing (e) and 2.0 M NaCl for renewable surface (f).

idue, and 3.16×10^{-12} mol/mm^2 for the aspartic acid residue. Therefore, it is estimated that MUA molecules are oriented nearby perpendicular to the surface and that the poly(amino acid) backbones are aligned parallel to the gold surface.

We designed such that poly(amino acid) multilayers could be used as a sensing platform for the functionalization with various ligand molecules and biospecific interactions. First, avidin–biotin interactions as a typical protein–ligand model were investigated on this biotinylated poly(amino acid) multilayer surface. The interaction between avidin and the functionalized poly(amino acid) multilayer with biotin is shown in Fig. 4. The binding level of avidin increases in response unit change (ΔRU) as the surface density of biotin increases. Table 1 shows the ΔRU with respect to each layer having the different DS. Also, Fig. 5 shows the number density of avidin on multilayer surface versus the DS of biotin. As DS increases, the number density of avidin increases, but the efficiency

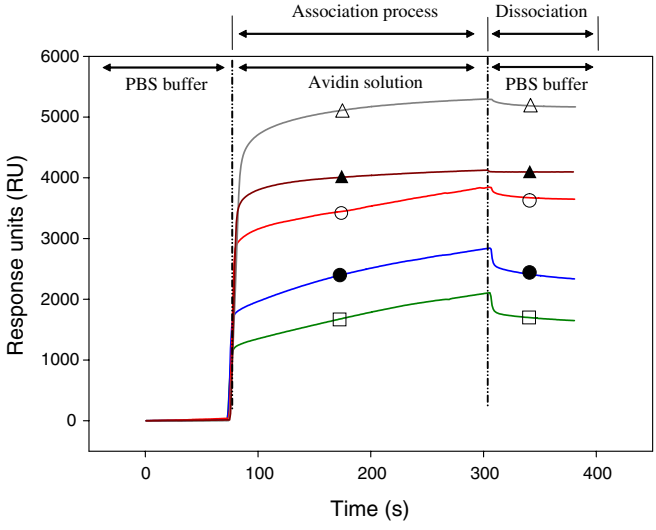

Fig. 4. SPR responses for binding of avidin to poly(amino acid) multilayer surfaces. The avidin solution was injected at a flow rate of 20 μl/min over the sensor chip. ((□), PAsp-g-biotin-1 layer; (●), PAsp-g-biotin-2 layer; (○), PAsp-g-biotin-3 layer; (▲), PAsp-g-biotin-4 layer; (△), PAsp-g-biotin-5 layer outwards, respectively.)

Table 1
SPR response unit changes (ΔRU) and calculated surface density as the functionalized poly(amino acid) multilayers series

Functionalized poly(amino acid) multilayers	DS[a] (%) of PAsp-g-biotin	Response unit (ΔRU)[b]	Surface density (ng/mm^2)[c]	Surface avidin number density (number/mm^2)[d]
1	1.1	1685	1.68	15.3×10^9
2	2.7	2389	2.39	21.8×10^9
3	6.2	3664	3.67	33.5×10^9
4	9.1	4095	4.10	37.4×10^9
5	18.4	5180	5.18	47.2×10^9

[a] The mole percentage of the grafted biotin per total poly(amino acid) backbone unit.
[b] Response unit changes (ΔRU) from the binding of avidin on the poly(amino acid) layers.
[c] Calculated from the response unit (RU).
[d] Calculated from the RU and M_w of avidin (66000 Da) on the multilayer surface.

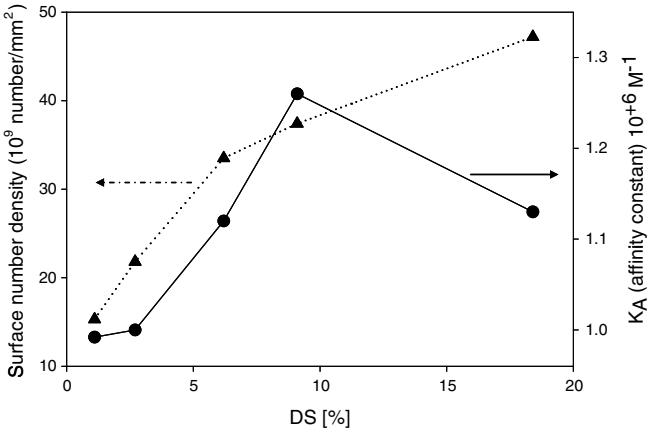

Fig. 5. The surface number density of avidin on poly(amino acid) multilayer and affinity constants (K_A) of avidin to poly(amino acid) multilayer as a function of DS. The triangle represents surface number density and circle represents K_A.

decreases. Such a reduction of efficiency would be attributed to the sterically hindered binding of avidin. At the high coverage, the interaction of biotin and avidin would be significantly restricted on the biotinylated surface partially covered with a significant amount of avidin. Also, there is a possibility that avidin occupies two biotin groups or more, increases as the surface density of biotin groups increase because avidin is tetrameric when it binds to biotin [17].

The SPR spectrum reveals both the association and dissociation process of analyte when it flows through the sensor chip. In this experiment, the flow rate of analyte (avidin) was 20 μL/min, and the flow time was about 5 min, at which the dissociation process begins, representing kinetic changes to PBS solution. The affinity constant ($K_A = \frac{k_a}{k_d}$) between avidin and biotinylated multilayer was calculated according to the following equation:

$$\frac{dRU}{dt} = k_a[A]RU_{max} - (k_a[A] + k_d)RU$$

where RU_{max} is the RU when the sensing surface is saturated with Avidin, $[A]$ is the concentrations of the Avidin, k_a and k_d are the association and dissociation rate constants, respectively [18].

Fig. 5 shows the K_A of avidin on biotinylated multilayer calculated with above equation using the BIAcore software. The K_A of functionalized multilayer were different against expectation. Interestingly, PAsp-g-biotin-4 (DS 9.1) outwards layer shows the largest K_A due to a lower k_d. This results strongly suggest that the optimum space (or density) of biotin on the surface is required for the specific binding of avidin. In fact, the high packing density of biomolecules on the sensing surface would be required to readily detect a target molecule. However, the optimum surface density should be considered in case of the monitoring of kinetics in specific binding phenomena such as association, dissociation, and affinity constant.

Let the side groups, grafted biotin to poly(amino acid), be uniformly distributed and all of the grafted biotin groups face outside. Then, the molecular alignment of biotinylated poly(amino acid) could be evaluated with respect to DS, by taking the bond length of fully extended *trans* peptide, $n \times 3.8$ Å [19]. At DS = 9, the extended distance of the amino chain between grafted biotins ($n = 100/9 = 11$ or 12) is 4.2 nm or 4.6 nm. At DS = 6, and 18, the extended distance is 6.1 nm or 6.5 nm and 1.9 nm or 2.3 nm, respectively. The dimension of avidin molecule is approximately $4.5 \times 4.5 \times 5.8$ nm [17]. The chain distance between grafted points would not be the same as the estimated value from the conformation of amino acid, two-dimensional packing of amino acid monomer on the multilayer surface.

In conclusion, the poly(amino acid) multilayer surface could be used as a simple and effective sensing platform for SPR sensing. Also, the interaction of avidin with biotinylated multilayer surface showed the maximum affinity constant indicating the existence of optimum spacing between neighboring biotin. The SPR measurement showed the optimum spacing, roughly estimated between DS 18.4 and DS 6.2 in this study.

Acknowledgements

The authors are thankful for financial support from Ministry of Science and Technology through the strategic national R&D Program and partially the BK 21 project.

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