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Detection and Kinetics of Mucosal Pathogenic Bacteria Binding with Polysaccharides

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Abstract The detection and kinetics of mucosal pathogenic bacteria binding on polysaccharide ligands were studied using a surface plasmon resonance biosensor. The kinetic model applied curve-fitting to the experimental surface plasmon resonance sensorgrams to evaluate the binding interactions. The kinetic parameters for the mucosal pathogenic bacteria (*Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Serratia marcescens*) with the alginate ligand were determined from a kinetic model. In addition, the binding interactions of the mucosal pathogenic bacteria with polysaccharide binding pairs (*Pseudomonas aeruginosa*/alginate, *Streptococcus pneumoniae*/pneumococcal polysaccharide, *Staphylococcus aureus*/pectin) were also compared with their kinetic parameters. The rate constants of association for *Pseudomonas aeruginosa* with the alginate ligand were higher than those for *Pseudomonas fluorescens*. *Serratia marcescens* had no detectable interaction with the alginate ligand. The adhesion affinity of *Pseudomonas aeruginosa* with alginate was higher than that for the other binding pairs. The binding affinities of the pathogenic bacteria with their own polysaccharide were higher than that of *Staphylococcus aureus* with pectin. Measuring the contact angle was found to be a feasible method for detecting binding interactions between analytes and ligands.

Keywords: Detection, kinetics, mucosal pathogenic bacteria, polysaccharide, surface plasmon resonance

Surface plasmon resonance (SPR) biosensors have a range of versatile applications in the drug discovery process, from target characterization, compound screening, and lead optimization to supporting clinical trials, regulatory approval, and biopharmaceutical manufacturing [3, 17–19]. SPR biosensors also allow direct real-time detection of the binding interactions of macromolecules without any chemical alteration of the ligands for signal generation. A captured molecule or ligand is covalently immobilized on the sensor chip, and a binding molecule captured by the immobilized ligand in a continuous-flow system [5, 6, 8, 12, 20]. In addition, a quantitative analysis of the molecular interaction and kinetic parameters can also be obtained from SPR biosensor systems [1, 10, 25].

The association and dissociation rates can be determined from SPR sensorgrams of reactions, such as extracellular matrix (ECM) proteins, hyaluronic acid, and chondroitin sulfate [14], revealing the binding properties of macromolecules with immobilized pathogen cell surfaces. Thus, various kinetic studies have already been published on the binding of macromolecule immobilized pathogens, such as collagen I on *Escherichia coli* O157:H7 [15], proteins on *Staphylococcus aureus* and *Staphylococcus epidermidis* [17], and ECM on *Salmonella typhimurium* [16].

In contrast, no studies have been reported on the kinetics of mucosal pathogenic bacteria binding with immobilized macromolecules, such as polysaccharides, using SPR biosensors, which could be meaningful for effectively evaluating the usability of macromolecule materials as immunizing agents and detecting reagents against mucosal pathogenic bacteria.

In common with higher living organisms, bacteria produce polysaccharides, which occur in a variety of locations in relation to the bacteria, and their precise disposition can be used as a means of classification [2, 9, 21]. Pneumococcal polysaccharides have already been extracted from *Streptococcus pneumoniae* and used as a vaccine against *Streptococcus pneumoniae* [22, 23]. Moreover, it is expected that *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*

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will specifically bind to a matrix covered with their own polysaccharides and possess the same polysaccharides on the cell, respectively. Among various polysaccharides, pectin exhibited high anti-adhesive properties towards *Staphylococcus aureus*, with a minimum inhibitory concentration (MIC) of 0.01 mg/ml [11], meaning that *Staphylococcus aureus* can strongly attach to pectin.

It has been previously suggested that effective antigenic properties could be derived from a high binding interaction between pathogenic bacteria and antigens [24]. Therefore, a kinetic study is needed to evaluate quantitatively the affinities of mucosal pathogenic bacteria towards specifically adhesive polysaccharides.

Accordingly, this study focused on the detection and kinetics of mucosal pathogenic bacteria binding with polysaccharides to evaluate the binding interaction between bacteria and ligands using SPR with a BIAcore system. A kinetic model is also proposed based on applying curve-fitting to the experimental SPR sensorgrams using an integral rate equation. The kinetic parameters for *Pseudomonas aeruginosa* were compared with those for *Pseudomonas fluorescens* and *Serratia marcescens*. In addition, the binding interactions of mucosal pathogenic bacteria with highly attractive polysaccharide binding pairs (*Pseudomonas aeruginosa*/alginate, *Streptococcus pneumoniae*/pneumococcal polysaccharide, *Staphylococcus aureus*/pectin) were evaluated quantitatively in comparison with the kinetic parameters. Finally, the contact angles to the individual layers of the alginate self-assembled multilayer (SAM) were evaluated as a method of detecting the binding interactions between analytes and ligands.

MATERIALS AND METHODS

Microorganisms

The *Pseudomonas aeruginosa* (KCTC 2004), *Pseudomonas fluorescens* (KCTC 12348), and *Serratia marcescens* (KCTC 1299) were obtained from the Korean Collection of Type Cultures (KCTC), whereas the *Streptococcus pneumoniae* (ATCC 9163, type 1) and *Staphylococcus aureus* (ATCC 25923) were purchased from the American Type Culture Collection (ATCC). The cells were grown for 18 h at 37°C in a Luria-Bertani (LB) broth.

Reagents

Alginate (medium viscosity, 3,500 cps at 2% concentration) and pectin (from citrus fruits, 9000-69-5), purchased from Sigma (St. Louis, MO, U.S.A.), and pneumococcal polysaccharide (ATCC 164-X, type 1), purchased from ATCC, were used as surface-bound ligands to estimate SPR sensorgrams for the mucosal pathogenic bacteria. Chitosan (MW 50–80 K), with a degree of 85% deacetylation, was donated by Chitoful Co. (Yeosu, Korea), and glutaraldehyde, cysteamine, and chitosan were used as a self-assembled multilayer preparation. All other chemicals were of regent grade and used without further purification.

Preparation of Alginate SAM

Alginate attached to a SAM was prepared using the method described by Deng *et al.* [4], where the gold surface of the sensor chip was chemically activated with a "piranha" solution, consisting of sulfuric acid and an NaOH solution, and then immersed in a 0.2% (w/v) aqueous solution of cysteamine for 2 h. The resultant gold surface was rinsed with distilled water and dried with nitrogen. After immersing the gold surface in 2.5% of a glutaraldehyde solution for 1 h, the modified surface was then treated with 0.4% (w/v) chitosan dissolved in 2% acetic acid for 1 h, washed with distilled water to remove the adhesive chitosan on the chip surface, and then dried with nitrogen at room temperature. An alginate solution (1% [w/v] in distilled water) was dropped on the chitosan layer to form an alginate-chitosan multilayer by ionic interaction, rinsed with distilled water, and then dried at room temperature. The SAM formed on the gold surface of the biosensor was characterized through measuring the SPR responses at each preparation step. After each procedure, the changed refractive unit (RU) values were investigated using a BIAcore J system (BIAcore AB, Uppsala, Sweden).

Evaluation of Mucosal Pathogenic Bacteria Binding with Polysaccharides

The mucosal pathogenic bacteria were harvested by centrifugation after incubation in an LB medium for 18 h. The cells were washed once and resuspended at 1×10^{10} CFU/ml with a 0.5 M sodium acetate buffer. The prepared whole-cell suspension was injected into the BIAcore assay system fitted with the SPR chip at a flow rate of 17 μl/min for 7 min. The binding interactions between the mucosal pathogenic bacteria and the polysaccharide ligands on the sensor chip surface were determined by monitoring the changes in the RU values. *Pseudomonas fluorescens* and *Serratia marcescens* were employed as contrast groups for the same alginate ligand. The *Pseudomonas aeruginosa*/alginate pair, *Streptococcus pneumoniae*/pneumococcal polysaccharide pair, and *Staphylococcus aureus*/pectin pair, all with a highly attractive adhesion, were selected for a quantitative binding comparison.

Measurement of Contact Angle

The contact angles of the self-assembling materials were measured for each self-assembling step (cysteamine-gluteraldehyde-chitosan-alginate) using a Contact Angle Meter (G-1, Erma, Tokyo, Japan). The variations in the contact angle for the self-assembling steps were compared with the variation in the RU value obtained from the SPR sensorgrams.

Theory of Kinetic Analysis

The association and dissociation rate constants were calculated by curve-fitting to the SPR sensorgrams using an analytical solution of the rate equation for a complex formation, and the general rate equation for a binary complex formation is written as

$$[A]+[B]\underset{k_{dis}}{\overset{k_{ass}}{\rightleftarrows}}[AB]$$
$$\frac{d[AB]}{dt}=k_{ass}[A][B]-k_{dis}[AB] \quad (1)$$

where [A] and [B] mean the analyte and surface-bound ligand, respectively, k_{ass} is the association rate constant, and k_{dis} is the dissociation rate constant. Masuda *et al.* [13] suggested a solution equation for the rate equation of a complex formation as follows:

$$\frac{dR}{dt}=k_{ass}C(R_{max}-R)-k_{dis}R \quad (2)$$

and also as

$$\frac{dR}{dt}=k_{ass}CR_{max}-(k_{ass}C+k_{dis})R \quad (3)$$

where dR/dt is the rate of the surface complex formation, R is the RU value reflecting the amount of analyte bound to the immobilized ligand on the sensorchip, R_{max} is the RU value when the binding sites of the immobilized ligand have been saturated by the analyte, and C is the concentration of analyte in the free solution.

However, the present study used a solution method based on the integration of Eq. (3), where Eq. (3) is rewritten as

$$\frac{dR}{dt}=k_{ass}CR_{max}\left(1-\frac{k_{ass}C+k_{dis}}{k_{ass}CR_{max}}R\right) \quad (4)$$

Eq. (4) can be expressed as an integral equation, as in Eq. (5). The integral equation can then be solved with the variation of R as a function of t, as in Eq. (6)

$$\int_0^R \frac{1}{1-\frac{k_{ass}C+k_{dis}}{k_{ass}CR_{max}}R}dR=k_{ass}CR_{max}\int_0^t dt \quad (5)$$

$$R=\frac{k_{ass}CR_{max}}{k_{ass}C+k_{dis}}[1-\exp(-k_{ass}C-k_{dis})t] \quad (6)$$

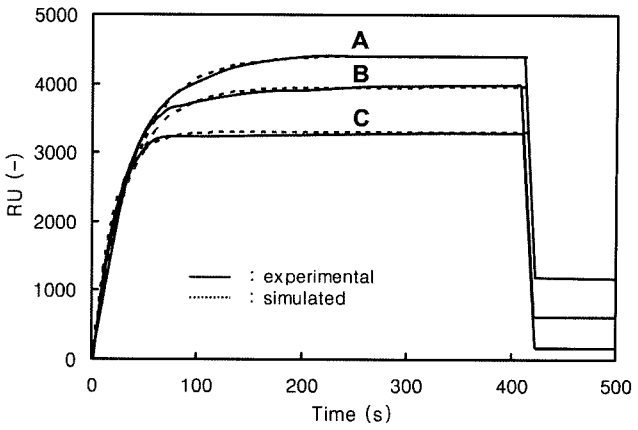

Fig. 1. Experimental and simulated SPR sensorgrams of *Pseudomonas aeruginosa* and alginate ligand with different injected cell concentrations: (**A**) 1×10^9 CFU/ml, (**B**) 1×10^8 CFU/ml, and (**C**) 1×10^7 CFU/ml.

where k_{ass}, k_{dis}, C, and R_{max} are constants. The analyte concentration (C) is determined before measuring the SPR sensorgram, and R_{max} can be obtained from the result after estimating the SPR sensorgram. Therefore, the SPR sensorgrams obtained from the experimental results were simulated using Eq. (6) with an optimized k_{ass} and k_{dis}. The rate constants, k_{ass} and k_{dis}, were determined from the results of the best curve-fitting to the experimental SPR sensorgrams. The affinity (K_A) was then determined from k_{ass}/k_{dis}.

Results and Discussion

Detection of Mucosal Pathogenic Bacteria with Polysaccharide Using SPR Sensorgrams

The experimental and simulated SPR sensorgrams of *Pseudomonas aeruginosa* and the alginate ligand with various cell concentrations are shown in Fig. 1. The RU values in the SPR sensorgrams initially rapidly increased with the binding of *Pseudomonas aeruginosa* to the alginate ligand at the different cell concentrations (1×10^7 CFU/ml to 1×10^9 CFU/ml), and then reached an equilibrium within 5 min. The bound cell masses were calculated by converting the equilibrium RU values according to the calibration factor supplied from the BIAcore biosensor.

Table 1. Rate constants and affinity of mucosal pathogenic bacteria (analytes) binding with alginate ligand.

Analyte	Concentration of analyte (CFU/ml)	k_{ass} (ml/(CFU·s))	k_{dis} (s^{-1})	K_A (ml/CFU)
Pseudomonas aeruginosa	1×10^9	4.2×10^{-11}	1.3×10^{-15}	3.2×10^4
	1×10^8	5.1×10^{-10}	1.5×10^{-14}	3.4×10^4
	1×10^7	5.2×10^{-9}	1.6×10^{-13}	3.3×10^4
Pseudomonas fluorescens	1×10^9	1.2×10^{-11}	5.0×10^{-15}	2.4×10^3
Serratia marcescens	1×10^7–1×10^9	-*	-	-

* Not detected.

Table 2. Rate constants and affinity of binding pairs of mucosal pathogenic bacteria (analytes) with polysaccharide ligands.

Mucosal pathogenic bacteria	Polysaccharide ligand	Concentration of analyte (CFU/ml)	k_{ass} (ml/(CFU·s))	k_{dis} (s^{-1})	K_A (ml/CFU)
Pseudomonas aeruginosa	Alginate on chitosan SAM	1×10^9	4.2×10^{-11}	1.3×10^{-15}	3.2×10^4
		1×10^8	5.1×10^{-10}	1.5×10^{-14}	3.4×10^4
		1×10^7	5.2×10^{-9}	1.6×10^{-13}	3.3×10^4
Streptococcus pneumoniae	Streptococcal polysaccharide	1×10^9	2.6×10^{-11}	1.2×10^{-15}	2.2×10^4
		1×10^8	3.0×10^{-10}	1.3×10^{-14}	2.3×10^4
		1×10^7	3.7×10^{-9}	1.7×10^{-13}	2.2×10^4
Staphylococcus aureus	Pectin	1×10^9	3.5×10^{-11}	1.8×10^{-15}	1.9×10^4
		1×10^8	4.4×10^{-10}	2.4×10^{-14}	1.8×10^4

The bound cell masses obtained were 93.3, 53.1, and 1.4 ng/mm^2 for the injected cell concentrations of 1×10^9, 1×10^8, and 1×10^7 CFU/ml, respectively.

The simulated SPR curves obtained from best curve-fitting based on the kinetic model matched well with the experimental SPR sensorgrams. The rate constants of association and dissociation determined from the simulation results are summarized in Tables 1 and 2.

The SPR sensorgrams of *Pseudomonas fluorescens* and *Serratia marcescens* attached to the alginate ligand were compared with those of *Pseudomonas aeruginosa* as regards the extent of binding. The experimental and simulated SPR sensorgrams of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* and the alginate ligand with an injected concentration of 1×10^9 CFU/ml are shown in Fig. 2. The increments of the RU value variation between *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* differed significantly. The change in the RU value for the *Pseudomonas fluorescens* cells was weak with an injected concentration of 1×10^9 CFU/ml, and there was no change with an injected concentration below 1×10^8 CFU/ml. Thus, the minimum concentration for the detection of *Pseudomonas fluorescens* by the SPR biosensor was found to be *ca.* 1×10^9 CFU/ml. The RU value did not increase for *Serratia marcescens* and the alginate ligand with the same injected concentration range, indicating that *Serratia marcescens* had no detectable binding interaction with the alginate ligand.

The experimental and simulated SPR sensorgrams of *Streptococcus pneumoniae* attached to the pneumococcal polysaccharide with various injected cell concentrations are represented in Fig. 3. The RU values in the SPR sensorgrams rapidly increased because of the binding of the cells to the surface of the pneumococcal polysaccharide ligand as soon as the analyte was injected, and then also reached an equilibrium after a few minutes. The highest RU value was obtained at 1×10^9 CFU/ml, yet this was lower than the value obtained for *Pseudomonas aeruginosa* and the alginate ligand.

Fig. 4 shows the experimental and simulated SPR sensorgrams of *Staphylococcus aureus* bound to the surface of the pectin ligand with different injected cell concentrations. The RU value did not change with a cell concentration below 1×10^7 CFU/ml. The RU value with 1×10^9 CFU/ml was also small compared with that for the *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*

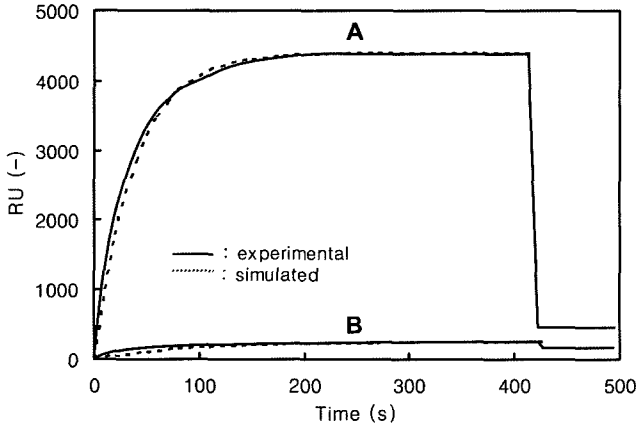

Fig. 2. Experimental and simulated SPR sensorgrams of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and alginate ligand with 1×10^9 CFU/ml of cells: (**A**) *Pseudomonas aeruginosa* and (**B**) *Pseudomonas fluorescens*.

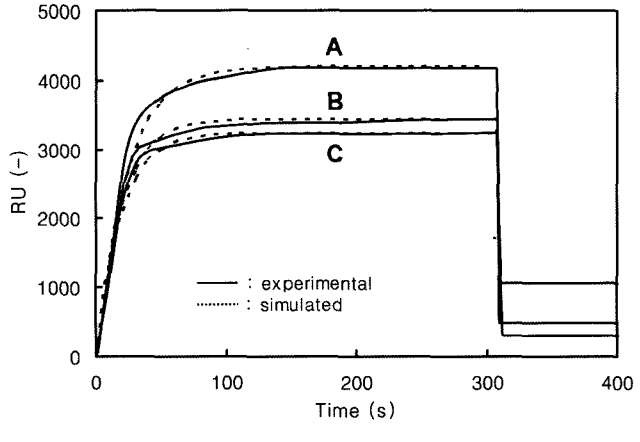

Fig. 3. Experimental and simulated SPR sensorgrams of the *Streptococcus pneumoniae*/pneumococcal polysaccharide binding pair with different injected cell concentrations: (**A**) 1×10^9 CFU/ml, (**B**) 1×10^8 CFU/ml, and (**C**) 1×10^7 CFU/ml.

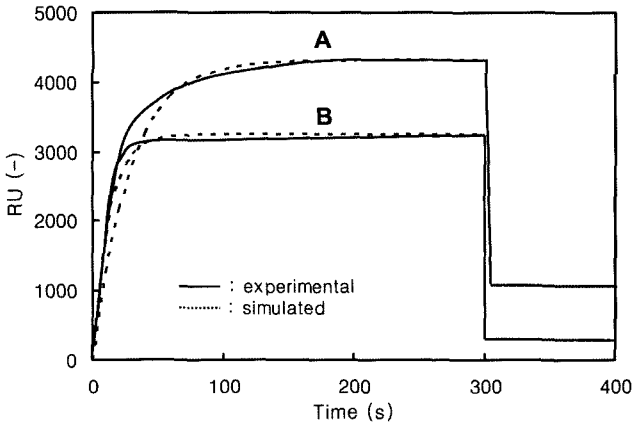

Fig. 4. Experimental and simulated SPR sensorgrams of the *Staphylococcus aureus*/pectin binding pair with different injected cell concentrations: (**A**) 1×10^9 CFU/ml and (**B**) 1×10^8 CFU/ml.

binding pairs, indicating that the binding interaction of the *Staphylococcus aureus*/pectin binding pair was weaker than that of the other binding pairs.

Kinetics of Mucosal Pathogenic Bacteria Binding with Polysaccharide Ligands

The rate constants and affinity of the mucosal pathogenic bacteria and alginate ligand are summarized in Table 1. The constants were determined from best curve-fitting to the experimental SPR sensorgrams. The kinetic model explained above was applied to the simulation of the experimental SPR sensorgrams, and rate constants of association were obtained from about 10^{-11} ml/(CFU·s) to 10^{-9} ml/(CFU·s) for the mucosal pathogenic bacteria binding with the polysaccharide ligands with an injected concentration from 1×10^7 CFU/ml to 1×10^9 CFU/ml. The magnitude of the rate constants was mainly governed by the injected cell concentration and became smaller when increasing the injected concentration. The reason for this conclusion was because the value of the injected concentration was much larger than any of the other constants in the kinetic model. Nonetheless, the binding mass of *Pseudomonas aeruginosa* increased when increasing the injected cell concentration. The concentration limit for the detection of *Pseudomonas aeruginosa* with the alginate ligand using the SPR biosensor was *ca.* 1×10^7 CFU/ml.

The rate constants of association for *Pseudomonas aeruginosa* with the surface of the alginate ligand were higher than those for *Pseudomonas fluorescens*. No rate constants were obtained for *Serratia marcescens* on the alginate ligand, as no binding occurred with the injected concentration range, meaning that the cells had no detectable interaction with the alginate ligand.

The affinity of *Pseudomonas aeruginosa* was *ca.* 50 times higher than that of *Pseudomonas fluorescens*, implying that *Pseudomonas aeruginosa* was more attracted to bind with the alginate ligand than the other cells. Furthermore, for the binding of *Pseudomonas aeruginosa* with the alginate ligand, not only was the association rate faster, but the amount of cells attached was also much higher than that for *Pseudomonas fluorescens* and *Serratia marcescens*. The binding interaction of *Pseudomonas aeruginosa* with the alginate ligand was also stronger than that for *Pseudomonas fluorescens* and *Serratia marcescens*.

The rate constants for the binding pairs of mucosal pathogenic bacteria with polysaccharide ligands were estimated to compare their binding interaction. The binding pairs were already ascertained as highly adhesive pairs of pathogenic bacteria and polysaccharides. Table 2 presents the kinetic parameters for the mucosal pathogenic bacteria binding with the polysaccharide ligands, where the rate constants of association were *ca.* 10^{-11} ml/(CFU·s) to 10^{-9} ml/(CFU·s) for the injected cell concentration. In contrast, the rate constants of dissociation were *ca.* 10^5 times smaller than those of association.

When comparing the affinity among the binding pairs, the affinity of *Pseudomonas aeruginosa* with the alginate ligand exhibited the highest values for all the injected concentrations. The association rate constants and affinity of *Streptococcus pneumoniae* with the pneumococcal polysaccharide were higher than those of *Staphylococcus aureus* with pectin. Although *Staphylococcus aureus* exhibited a significantly attractive adhesion to pectin, the kinetic parameters were lower than those for the binding pairs, where the pathogen bacteria were coupled with their own polysaccharides. *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* attached more strongly to the ligands covered with their own polysaccharide, respectively, plus their association rate constants and affinities were also higher than those for *Staphylococcus aureus* with pectin.

Detection of Binding Layer Using Contact Angle Variation

The variations in the contact angles for the self-assembling materials are shown in Fig. 5. The variation of the contact

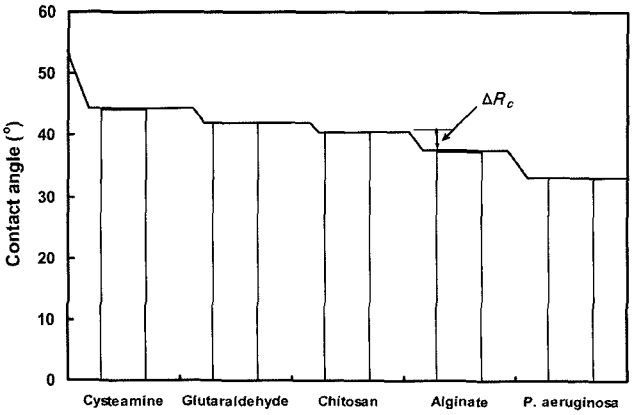

Fig. 5. Variations in the contact angle of the individual layer during binding of self-assembling materials and *Pseudomonas aeruginosa*.

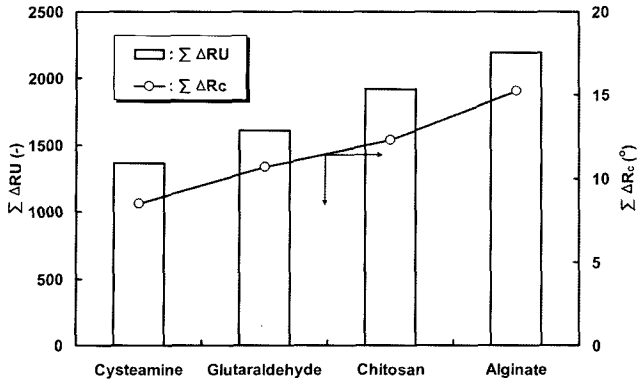

Fig. 6. Summation of contact angle variation ($\Sigma\Delta R_c$) and summation of RU value ($\Sigma\Delta RU$) during binding of self-assembling materials.

angle to the individual self-assembled layer was expressed as ΔR_c and decreased as the number of layers increased. The decrement of the contact angle differed according to the binding interaction with individual layers.

The variations in the contact angle were measured during the preparation of the alginate SAM and binding of *Pseudomonas aeruginosa* with the SAM. The contact angle changed with the individual binding layers of the SAM. The summation of the contact angle variation ($\Sigma\Delta R_c$) with each individual layer and summation of the RU variation ($\Sigma\Delta RU$) under the same conditions are represented in Fig. 6. The variation patterns of the contact angle were very similar to the variation of the RU value measured by the BIAcore biosensor, although the values of the two methods could not be quantitatively matched. Nonetheless, measuring the contact angle was found to be a feasible method for detecting the binding interaction between analytes and ligands.

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