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Protein Adsorption to 2D-arrayed microgels on SPR chip

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Microgels composed of poly(*N*-isopropylacrylamide-*co*-acrylic acid) with different acrylic acid contents were prepared. The dispersion of microgels with higher AAc content exhibited affinity with human immunoglobulin G (IgG). The maximum adsorption was observed around pH 5.0, while both low pH and basic conditions induced the rather low adsorption caused by electrostatic repulsion. Two-dimensional (2D) arrayed microgel was prepared and IgG adsorption on the array was compared with above mentioned adsorption in dispersion of microgels. Adsorptions of IgG on 2D particle array and in the dispersion exhibited similar dependence on both pH and temperature. Surface plasmon resonance (SPR) was used to investigate the kinetic parameters of protein binding onto 2D-arrayed microgels. The association and dissociation rate constants and the corresponding affinity at various pHs were obtained.

Key words: Protein adsorption, Surface Plasmon Resonance (SPR), Poly(N-isopropylacrylamide), Human immunoglobulin G

1. INTRODUCTION

Interest in stimuli-responsive or smart polymers has increased exponentially, because they display phase change or abrupt change in volume in respond to the external stimuli such as temperature, pH, ionic strength, and electric field. Our group [1-3] found that the poly(*N*-isopropylacrylamide) (PNIPAM) favored the adsorption of protein when the thermosensitive surface of microgel become hydrophobic above the lower critical solution temperature (LCST).

Surface plasmon resonance (SPR) allows for the direct visualization of these macromolecular interactions in real-time, and thus the data contain information on the rate and equilibrium binding constants that describe the interaction being investigated. During the last decade, the measurement of surface binding equilibrium and kinetics has become a very popular approach for the study of protein interactions [4-8]. There are many publications on protein adsorption onto polymer surfaces [9-11]; however, there have been no research reports on kinetic adsorption of protein onto temperature- and pH- sensitive microgel surface.

Here, a series of poly(*N*-isopropylacrylamide-*co*-acrylic acid) (poly(NIPAM-*co*-AAc) microgel with varying amounts of acrylic acid were synthesized. The particular microgel is of interest due to its charged surface and the similarities in the chemical functionality of the protein. It is worth designing two-dimensional (2D) arrayed microgel capable of differentiating the various interactions involved in the adsorption mechanism of protein on microgel surface. Therefore, the aim of this paper is to examine the influence of the pH and temperature of dispersion of microgels and 2D-arrayed microgel.

2. MATERIALS AND METHODS

2.1 Materials

N-isopropylacrylamide (NIPAM) was kindly given by Kojin Co. and recrystallized from hexane-toluene mixture (1/1 in volume basis). Acrylic acid (AAc) was purified by distillation under reduced pressure to remove hydroquinone inhibitor. potassium persulfate (KPS), N,N'-methylenebis(acrylamide) (BIS), and sodium dodecyl sulfate (SDS) were used without further purification. 11-amino-1-undecanethiol hydrochloride was purchased from Dojindo laboratories. SIA kit Au sensor chips were purchased from BIAcore company.

2.2 Microgel prepartion

Preparation of poly(*N*-isopropylacrylamide-*co*-acrylic acid) microgels was described in our previous reports [12,13] and the recipe was shown in Table 1.

2.3 Characterization

After polymerization, all prepared microgels were refined by dialysis followed by repetitive centrifugation decantation-redispersion and finally dispersed in each pH solution. Hydrodynamic diameters of particles were determined by dynamic light scattering (DLS) using a laser particles analyzer system (PAR-III, Otsuka Electronic Co.). The size and shape of dried particles were characterized by field emission transmission electron microscopy (TECNAI F20, Philips Electron Optics Co.).

2.4 Determination of protein loading of microgels.

The total protein loading on the microgel in dispersions were examined by BCA (Bicinchoninic Acid) analysis. Briefly, an amount of microparticles 5 mg was redispersed in 500 ul of 100 ppm IgG. The proteins were dissolved in buffer with different pHs at ionic strength 0.005. The mixtures were incubated at each fixed temperature (i.e., 20°C and 40°C) for a given time (1h). All mixtures were centrifuged to separate polymer particles from the supernatants after the adsorption process. The adsorbed amount of IgG adsorption was determined from the difference between the initial and the final concentration of IgG in the supernatant. The IgG

concentration in supernatant was measured using BCA protein assay.

2.5 Protein adsorption behavior onto microgel surface

Real-time kinetic interaction between IgG and microgel surface was analyzed using SPR Biacore2000. SIA kit Au glasses were cleaned with piranha solution (H_2O_2/H_2SO_4 = 1:3 by volume) and thoroughly rinsed with Milli-Q water and ethanol sequentially. The Au sensor chip was covered with amine-terminated self-assembled monolayer (SAM). SPR biacore 2000 was used to prepare microgel monolayer surface. Each 0.05% wt/wt of dispersed microgels at pH 3 was injected into SPR system. IgG (0.1mg/ml, pH 3) was injected onto microgel surface at 20°C. The flow rate of microgel and protein solution was 5 μl/s. To regenerate microgel surface, 100 mM NaOH aqueous solution were injected twice at the end of each protein adsorption, and then another buffer and protein (i.e., pH 5, 7, and 9, respectively) were injected and do the same procedure as pH 3.

Association and dissociation rate constants were determined by nonlinear fitting of individual sensorgram data using the BIA evaluation 3.1 software (Pharmacia Biotech, Uppsala, Sweden). The model fits the association phase of a 1:1 interaction (Langmuir) to the integrated rate equation: $R = (k_aCR_{max}[1\text{-}e\text{-}(k_aC + k_d)t])/(k_aC + k_d)$, where R is the SPR signal in RU at time t and C is the concentration of the analyte and Rmax is the maximum analyte binding capacity in RU [14].

The morphology of microgel monolayer before and after binding of protein was observed by atomic force microscopy (AFM) in contact mode with a SPR300/SPI3700 (SII Nano-Technology Inc.).

3. RESULTS AND DISCUSSIONS

3.1 Characterization of microgel particles

The TEM micrograph of the purified microgel is depicted in Fig. 1 showing that particles were highly monodisperse and displayed smooth spherical shape (under dehydration state). The particle sizes measured by dynamic light scattering (DLS) and by electron microscopy are showed in Table I. The size of microgels increases with increasing acrylic acid content. The introduced carboxyl groups have a strong effect on the swelling behavior in water of poly(NIPAM-*co*-AAc) hydrogels [15].

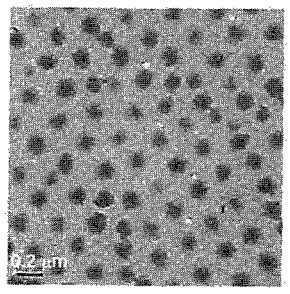

Fig.1. TEM of poly(NIPAM-*co*-AAc) microgel particles. The content of AAc is 5wt.% with presence of SDS.

3.2 Effect of environmental temperature and pH on adsorption of IgG protein onto poly(NIPAM-co-AAc) microgel in dispersion

Since the prepared microgels contained NIPAM segments, it is expected that the suspensions respond to temperature. In Fig. 2, the adsorbed amount of IgG onto microgel particles at different pHs and temperatures is shown. First, we noticed that there is a difference in IgG adsorption between 20°C and 40°C for poly(NIPAM). Basically, the binding amount of IgG increase by elevating temperature from 20°C to 40°C for all the cases. There is a remarkable dependence on the temperature for poly(NIPAM) microgel. The adsorption amount at 40°C is two times as much as that at 20°C for pH 5 and pH 7. This is that because poly(NIPAM) becomes more hydrophobic as temperature is higher than LCST. Therefore, the hydrophobic interaction leads to the higher binding amount of IgG. There is no different adsorption at pH 9 due to the same negatively charged in both of particles and IgG protein. However, with the AAc content increasing in the microgels, the effect of the temperature on the loaded amount of IgG becomes weak. This means that the adsorption of IgG to the poly(NIPAM-*co*-AAc) microgels is mainly govern by the electrostatic interaction and hydrogen bonding at the condition. In result, there is little effect of temperature on the IgG binding to the microgels with AAc segments. The difference in size between 20°C and 40°C becomes less with increasing AAc content (see Table I).

3.3 Protein adsorption onto 2D-arrayed microgels

A series of the microgels contained different AAc contents are used for study of protein adsorption. 2D-arrayed microgel surface was prepared by injection of dispersed microgel [12,13]. After binding the microgels onto SAM surface, there are some spaces between microgels remaining with amine group of SAM surface. To avoid the effect of charge from SAM surface, the amine group was masked by acetylation reaction [16]. Finally, the protein was injected into flow cell and then bound onto microgel array.

In order to discuss the influence of the charge of protein on adsorption behavior, the amount of adsorbed IgG onto the poly(NIPAM) surface was studied by using SPR at different pHs. Fig. 3 shows SPR response arising from the IgG binding onto the microgel surface (NA5) after exposure to a IgG solution with different pHs. The adsorption of IgG is dependent on the charged state both of the microgel and IgG molecule at each pH. The adsorption is lowest at pH 9 due to the same sign charge between the microgels surface and IgG.

At pH 3, although there are opposite charged between IgG and microgel surface, the lowest adsorption could obtained. It is due to that the electrostatic repulsion among IgG molecules. The results obtained here indicate that the electrostatic repulsion among IgG molecules is significant factor to how the IgG molecules adsorbed at this surface. Both pH 5 and pH 7 gave the same affinity of IgG binding onto the microgel surface. However, there was difference in rate of adsorption between pH 5 and pH 7. The details will be discussed in the kinetic section. The adsorption of IgG onto microgel surfaces was studied as a function of pH and temperature, shown in Fig. 4. The significant temperature dependence

Table I. Feed amount of monomers used in the microgel preparation and diameter of particles

Sample no.	Monomer feeds				Hydrodynamic diameters (nm)		Diameters of dry particle (nm)
	NIPAM (g)	AAc(g)	BIS (g)	C_{AAc} (wt.%)	20 °C	40 °C	
N	1.44	-	0.17	0	150.2	-	117.0
NA5	1.44	0.072	0.17	5	196.7	163.5	150.3
NA10	1.44	0.144	0.17	10	245.3	223.1	218.7
NA15	1.44	0.216	0.17	15	315.1	295.3	287.2

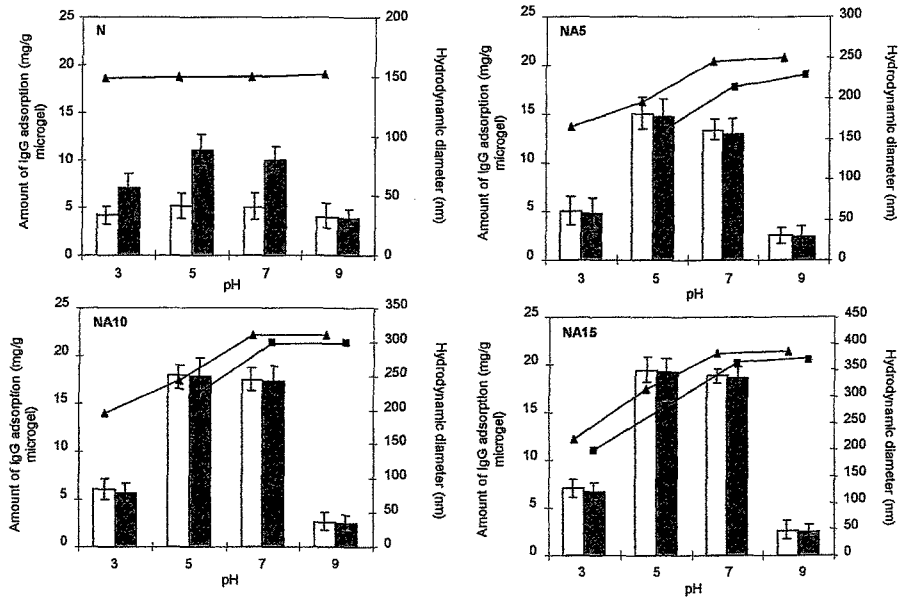

Fig. 2. Amounts of IgG adsorption onto poly(NIPAM-*co*-AAc) microgel in dispersions at temperature of 20°C (□) and 40°C (■) at various pHs . Initial concentration of IgG was 0.1 mg/ml. The values are the mean of three experiments; error bars indicate the standard deviation. Hydrodynamic sizes presented with plots and lines

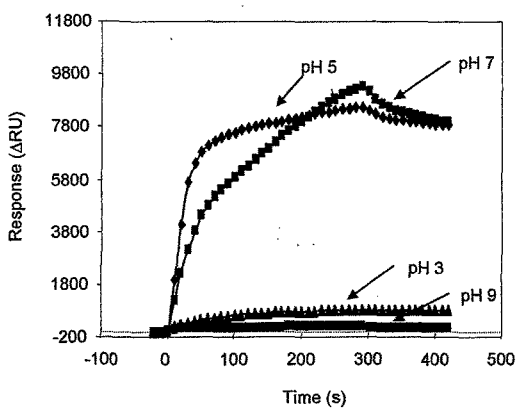

Fig. 3. Sensorgrams of the IgG adsorptions onto 2D-arrayed poly(NIPAM-*co*-AAc) microgel surface (NA5) in various pH buffers. 25μl of 0.1 mg/ml of IgG solution was injected at 20°C with a flow rate of 5 μl/min in SPR running buffer.

was obtained on poly(NIPAM) surface. The adsorbed amount of IgG increased by raising temperature from 20°C to 40°C for all pH ranges. It is noted that the temperature dependence for poly(NIPAM) microgel as mentioned above. The thermosensitive behavior of poly(NIPAM) is governed by hydrophilic to hydrophobic character change when temperature rises from values below to above the LCST. Therefore, hydrophobic interactions are likely to have a contribution coming from the increase in the hydrophobic character of the polymer at temperature above LCST especially near pI of IgG [1]. The different responses result from differently charged poly(NIPAM) surface without AAc segment having the same negative charge all pH ranges adsorb the different IgG charges. The effect of temperature on the IgG adsorption becomes lower with the presence of AAc. It is due to the loss of temperature sensitive property at higher AAc content. The hydrodynamic size of microgel is also used to confirm this point. With the higher AAc content the difference in size between 20°C and 40°C becomes lower (see Table 1). The microgel is possible to be dissociated and disappeared in thermosensitive property even low pH. IgG adsorption increases as the amount of AAc increases. The small IgG adsorption was also observed at pH 3 even the opposite sign charge. the maximum adsorption was observed at around pH 5.0. At this pH, the charges of microgel surface and IgG molecule are opposite. The electrostatic interaction between microgel surface and IgG molecule resulted in the high adsorption in both pH 5 and pH 7. These results are the same tendency compared to the dispersion (BCA analysis). It revealed that protein adsorption onto 2D-arrayed microgels could

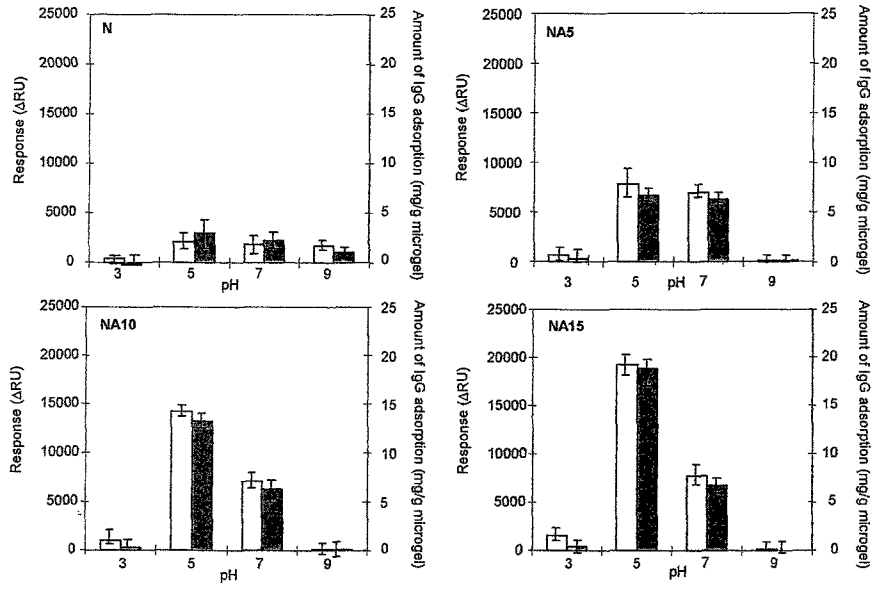

Fig. 4. Amounts of IgG adsorption onto 2D-arrayed poly(NIPAM-*co*-AAc) microgel particles at temperature of 20°C (□) and 40°C (■) in various pHs by SPR. Initial concentration of IgG was 0.1 mg/ml. The values are the mean of three experiments; error bars indicate the standard deviation.

given us the information about the general adsorption on dispersed particle. Thus, the kinetic study of IgG adsorption onto 2D-microgel surface could also investigate by using SPR.

3.4 Kinetic study of IgG adsorption onto 2D-arrayed microgel (NA5).

Table II. Values of the binding and dissociation rate constants for interaction of IgG and microgel surface (NA5) at various pHs.

pH	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_A (M^{-1})	K_D (M)
3	2.37×10^4	9.47×10^{-5}	2.50×10^8	4.00×10^{-9}
5	5.04×10^4	3.46×10^{-4}	1.45×10^8	6.88×10^{-9}
7	2.38×10^4	7.12×10^{-4}	3.35×10^7	2.99×10^{-8}
9	1.78×10^4	6.11×10^{-4}	2.92×10^7	3.43×10^{-8}

The values of the binding rate coefficient k_a, the dissociation rate coefficient k_d are given in Table II. Association and dissociation rate constant for the binding of IgG onto microgel surface in various pH solutions. As above mentioned, IgG binding onto microgel surface (NA5) gave the highest response in both pH 5 and pH 7. However, there is difference in the binding rate coefficient k_a and the dissociation constant k_d. The k_a of pH 5 is 2.1 times faster than the association rate constant of pH 7. It is due to the less charge of IgG at pH 7, which is near to pI of IgG. The electrostatic interaction between IgG and microgel was less than that of pH 5. The affinity constant (K_A) of pH 5 was also higher than that of pH 7. The affinity constant (K_A) is the highest at pH 3. It was implied that the IgG molecules which can avoid the electrostatic repulsion among IgG molecules strongly adsorb onto microgel surface. It is due to the strongest positive charge of IgG compared with other pHs. However, only small amount of IgG molecules can pass the electrostatic interaction and adsorb strongly onto microgel surface. At pH 9, the k_a and K_A are the lowest among other pHs, the response of binding is also the lowest compared to others. Due to electrostatic repulsion between IgG molecule and microgel surface, the IgG adsorb onto microgel slowly including weak interaction at pH 9.

4. CONCLUSION

A series of poly(N-isopropylacrylamide-*co*-acrylic acid) with different acrylic acid contents were prepared for IgG adsorption analysis. Two-dimensional (2D) arrayed microgel was prepared and IgG adsorption on the array at different pHs and temperatures was investigated using SPR Bicore2000. The pH dependent adsorption was explained by the contribution of electrostatic force. IgG adsorption onto microgel dispersion also measured using BCA protein analysis. The results of SPR analysis after eliminating the influence of chip surface gave the same tendency to those in the dispersion. The kinetic study of IgG adsorption could study from SPR. Although the IgG can adsorb onto microgel surface including microgel dispersion in the same amount at pH 5, there are differences in kinetic adsorption. The IgG adsorption at pH 5.0 provided the rate which is faster than that of pH 7.0 and also the stronger binding. The data from kinetic study represent the essential information for understanding how the binding event of IgG onto microgel system.

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