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Glucose binding to molecularly imprinted polymers

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Abstract—The main goal of this study was to prepare molecularly imprinted polymers (MIPs) with glucose recognition sites and to evaluate their glucose-binding properties for potential applications in glucose sensing and self-regulating insulin delivery devices. To mimic glucose-binding sites of natural proteins, monomers possessing functional groups similar to amino acids were used. Vinyl acetic acid (VAA), acrylamide (AAm), 4-pentenoic acid (PA), and allyl benzene (AB) were copolymerized with a cross-linking agent (*N,N′*-methylenebisacrylamide, BIS) in the presence of glucose as a template. The binding affinity of glucose to MIPs was examined by using an equilibrium dialysis technique. The dissociation constants of the MIPs were determined by Scatchard analysis. MIPs showed glucose-binding affinity, while polymers synthesized in the absence of glucose template did not show a glucose-binding property. MIPs composed of VAA, AAm, PA, and AB at optimized mole ratios of monomers and cross-linker showed the highest glucose-binding affinity, $K_D = 1.66$ mM, which is comparable to that of a well-known glucose binding protein, concanavalin A ($K_D = 1.84$ mM). The affinity between monomer and glucose was in the order VAA > AAm > AB > PA.

Key words: Glucose; glucose affinity; glucose-binding protein; glucose sensor; molecularly imprinted polymer; glucose imprinting.

INTRODUCTION

A self-regulated insulin delivery system is defined as a system that is capable of releasing insulin in response to changing blood glucose levels [1]. The increase of the glucose level triggers the system to modulate the release of insulin, i.e. release of insulin at the right time in the right amount. This requires glucose-sensitive or glucose-sensing ability in the system. A number of glucose-sensitive polymer and hydrogel systems have been developed. For convenience, they can be divided into systems utilizing glucose-sensitive swelling membranes [2, 3], glucose-sensitive erodible

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matrices [4–6], immobilized insulin [7], a glycosylated insulin–concanavalin A (Con-A) complex [8, 9], and glucose-sensitive phase-reversible hydrogels [10–12]. All of these systems utilize glucose oxygenase or Con-A as a glucose-sensing moiety. Since they are proteins, their stability is rather poor for long-term *in vivo* applications. Con-A is also known to be immunotoxic [13, 14] and for this reason, routine application in implantable devices may be prevented. Since at present there are no glucose-specific molecules other than several proteins (enzymes and lectins), we were interested in the synthesis of glucose-recognizing molecules that are biocompatible enough for repeated *in vivo* applications.

For the rational design of glucose-specific molecules, we conducted a comparative stereochemical analysis of specific interactions between glucose and five glucose-binding proteins, such as human β-cell glucokinase, d-xylose isomerase, lectins (*Lathyrus ochrus* isolectin I and Con-A), and glucose/galactose-binding protein [15]. The analysis revealed that the main interaction providing glucose specificity was hydrogen bonding between amino acids of the proteins and the hydroxyl groups of glucose. The most common amino acid residues involved in the hydrogen bonds were Asp, Glu, and Asn. Almost every hydroxyl group of the glucose molecule had at least one hydrogen bond with amino acid residues or water molecules. It was very common for each hydroxyl group to form multiple hydrogen bonds with many amino acid residues. Certain amino acid residues could form multiple hydrogen bonds with different hydroxyl groups of the glucose molecule. This network of hydrogen bonds in the right spatial arrangement was expected to provide glucose specificity. Hydrophobic interaction between the pyranose ring of glucose and aromatic rings of hydrophobic amino acid residues, such as Phe and Trp, also played an important role in the glucose specificity. This indicated that a certain spatial arrangement of those amino acid residues or their derivatives would result in glucose-specific binding sites. One approach to make such a spatially oriented amino acid derivatives is molecular imprinting.

Molecularly imprinted polymers (MIPs) are probably the most promising materials in the field of artificially generated molecular recognition [16–18]. The most common method of molecular imprinting is the polymerization of functional monomers and a cross-linking agent in the presence of a target molecule used as a template [19, 20]. Thermally or photochemically initiated polymerization results in a highly cross-linked insoluble polymer. Subsequent removal of the print molecules by extraction or hydrolysis leaves recognition sites that are complementary in size, shape, and chemical functionality to the template molecule. Molecular imprinting was also performed in aqueous solution using a polymer and cross-linkers rather than functional monomers [21]. MIPs have already been applied in several fields, such as ligand-binding assays [22], chromatographic separations of stereoisomers by using columns packed with MIPs [23, 24], selective sample enrichment by solid-phase extraction [25], and biomimetic sensors [26, 27]. In this work, we have prepared MIPs that recognize glucose as the first step towards preparing a modulated insulin delivery system that utilizes a synthetic glucose-binding mole-

cule. According to the analysis of glucose-binding sites of several proteins [15], the most common and essential amino acid residues involved in interactions with glucose are Asp and Asn for hydrogen bonding and Phe for hydrophobic interaction. For this reason, MIP systems were prepared using functional monomers that have functional similarities to the amino acid residues.

EXPERIMENTAL

Materials

The monomers used were vinyl acetic acid (VAA), acrylamide (AAm), allyl benzene (AB), and 4-pentenoic acid (PA). All of the monomers were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) and purified before use by sublimation under vacuum. *N,N′*-Methylenebisacrylamide (MBAAm), a cross-linking agent purchased from Aldrich Chemical Co., was used as received. *2,2′*-Azobisisobutyronitrile (AIBN) was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). All solvents were of analytical grade quality.

Polymer preparations

Glucose MIPs were synthesized by either free radical solution polymerization or UV polymerization. The composition of the monomers, cross-linker (MBAAm), template molecule (glucose), and AIBN for polymers prepared by free radical polymerization (polymers P1–P3) is shown in Table 1. The predetermined amounts of glucose and monomers were dissolved in 20 ml of dimethyl sulfoxide (DMSO) until a homogeneous solution was obtained. MBAAm and AIBN were dissolved in this solution. The solution was purged with nitrogen to remove oxygen, which acts as a free radical scavenger, and polymerized under a nitrogen atmosphere at 60°C for 4 h. For UV polymerization, the predetermined amounts of glucose and monomers were dissolved in 20 ml of DMSO. After MBAAm and AIBN were added, the solution was purged with nitrogen and polymerized under a UV source (366 nm) at 0°C for 12 h. The bulk polymers were washed with excess DMSO to remove any unreacted monomers, filtered, and dried in vacuum at 40°C for 18 h. The dried polymers were ground to particles of 50 μm diameter or smaller and the particles were separated by sedimentation from ethanol. Control polymers without glucose imprinting were prepared at the same time under identical conditions.

Glucose extraction

Glucose molecules used in imprinting were extracted by washing the MIPs with deionized distilled water (DDW). MIPs (0.5 g) were added to 10 ml of DDW and the solution was gently stirred at 25°C for 24 h. After decanting the DDW, the MIPs were dried under vacuum at 40°C for 18 h. Glucose extracted into DDW was assayed by the phenol–sulfuric acid assay method [28]. Glucose extractions were repeated until at least 97% of the imprinted glucose was removed from the MIPs.

Swelling studies

The dried MIPs (0.1 g) were placed into 20 ml of phosphate-buffered saline (PBS, pH 7.4) and the solution was kept at 25°C. Polymer samples were taken out of the buffer at timed intervals and the excess of buffer on the samples was removed. The weights of the swollen polymers were measured. The swelling ratio (S) was calculated by dividing the weight of the wet polymer by that of the dried polymer.

Evaluation of glucose-binding affinity to MIPs

Equilibrium dialysis between MIPs and glucose was run at 25 ± 0.5°C at pH 7.4, using a multi-sample microvolume dialyzer (EDM101B Equilibrium Dialyzer, Hoefer Pharmacia Biotech Inc., San Francisco, CA, USA). The dialyzer system consists of an eight-well dialysis module and a dialysis membrane with a molecular weight cut-off of 6000–8000. The membrane divided each well into two chambers of equal volume (0.5 ml). The module was attached to a dialysis mixer. Under gentle mixing, the glucose diffused into the chamber containing MIPs or non-imprinted control polymer samples. 0.5 ml aliquots of glucose solution (at concentrations of

Table 1.
Polymerization conditions of molecularly imprinted polymers[a]

Polymers		Monomers (mM)				Cross-linker (mM) MBAAm	Template (mM) α-D-glucose
		VA	AAm	PA	AB		
P1	1–4	2.0	2.0	—	2.0	15, 30, 45, 60	2.0
	5–8	2.0	2.0	—	2.0	60	0.5, 1.0, 1.5, 2.0
	9	4.0	1.0	—	1.0	60	2.0
	10	1.0	4.0	—	1.0	60	2.0
	11	1.0	1.0	—	4.0	60	2.0
P2	1–4	2.0	2.0	2.0	—	15, 30, 45, 60	2.0
	5–8	2.0	2.0	2.0	—	60	0.5, 1.0, 1.5, 2.0
	9	4.0	1.0	1.0	—	60	2.0
	10	1.0	4.0	1.0	—	60	2.0
	11	1.0	1.0	4.0	—	60	2.0
P3	1–4	2.0	2.0	2.0	2.0	20, 40, 60, 80	2.7
	5–8	2.0	2.0	2.0	2.0	80	0.7, 1.4, 2.0, 2.7
	9	3.5	1.5	1.5	1.5	80	2.7
	10	1.5	3.5	1.5	1.5	80	2.7
	11	1.5	1.5	3.5	1.5	80	2.7
	12	1.5	1.5	1.5	3.5	80	2.7

[a] Polymerization was initiated by heating at 60°C for 4 h. The initiator azobis(isobutyronitrile) was added to make 1 wt% with respect to the total amount of monomers and cross-linker. VAA = vinyl acetic acid; AAm = acrylamide; PA = 4-pentenoic acid; AB = allyl benzene; MBAAm = *N,N′*-methylene bisacrylamide.

25–200 μg/ml PBS) were placed in the donor chambers and 10 mg of the MIPs or non-imprinted control polymer samples were loaded into the receptor chambers. After loading PBS into the receptor chambers, the dialysis module was gently rotated at a speed of 20 rpm at $25 \pm 0.5^{\circ}$C. After reaching equilibrium (after 12 h), polymer samples were removed from the receptor chamber and dried in vacuum. Dried polymer samples were added to 2 ml of DDW and the solution was stirred at 25°C for 24 h to extract polymer-bound glucose molecules. The extracted glucose was assayed by the phenol–sulfuric acid assay method at 485 nm using a Beckerman DU-7 spectrophotometer [28].

RESULTS AND DISCUSSION

The binding affinity of MIPs is dependent on the stability of complexes between the template molecule and the functional monomers in the reaction mixture, as well as on preservation of the stability in the resulting polymers [29, 30]. Fixation of the template molecules within the selective cavities could be achieved either by utilizing non-covalent interactions, such as hydrogen bonding and ion-pair interaction (for non-covalent imprinting), or by reversible covalent interactions (for covalent imprinting) between the template molecule and the functional monomers. In the case of covalent imprinting, there is a stoichiometric relationship between the template molecule and the imprinted binding sites. In general, covalent imprinting is considered to be a less flexible method, since the interactions between the template molecule and functional monomers are limited to rapidly reversible covalent interactions. Non-covalent imprinting has no such restrictions, so the range of compounds that can be imprinted is much larger. However, since the association constant is relatively low, an excess of functional monomer is required to saturate the recognition sites and the removal of the template leaves a heterogeneous population of binding sites (Fig. 1). Though non-covalent imprinting has

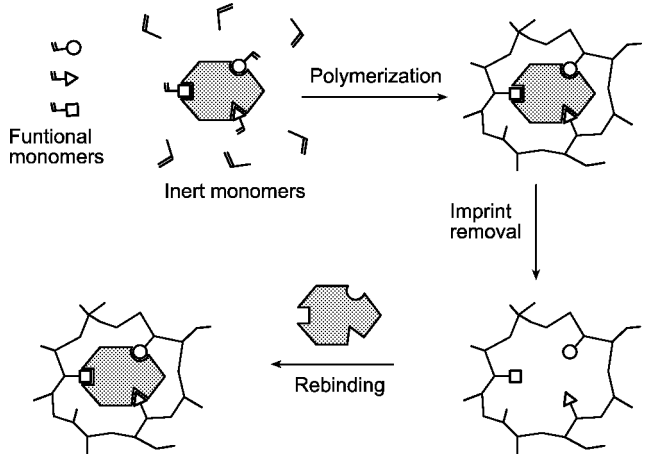
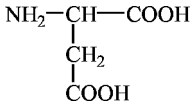
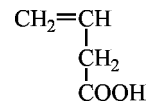
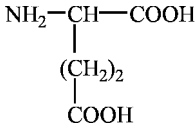
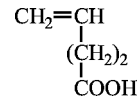
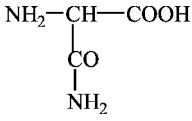
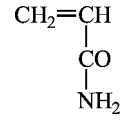
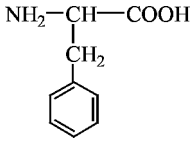
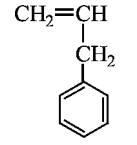


Figure 1. Schematic representation of molecular imprinting by non-covalent interaction.

Table 2.
Structural comparison of amino acids with monomers used in the preparation of MIPs

Amino acids	Structure	Monomers	Structure
Aspartic acid (Asp)		Vinyl acetic acid (VAA)	
Glutamic acid (Glu)		4-Pentenoic acid (PA)	
Asparagine (Asn)		Acrylamide (Aam)	
Phenyl alanine (Phe)		Allyl benzene (AB)	

such a limitation, it can still mimic interactions between glucose and the amino acid residues in glucose-binding proteins, which are non-covalent. Four types of interactions involved in glucose binding are hydrogen bonding, hydrophobic interaction, van der Waals interaction, and ionic coordination [31]. In the design of synthetic glucose-binding polymers, monomers that can interact with glucose by either hydrogen bonding or hydrophobic interaction were the first choices. Monomers that have the same functional groups as those of amino acids were chosen to prepare the MIPs (Table 2). The molar ratio of cross-linker (MBAAm) to total functional monomers was varied from 2.5 : 1 to 10 : 1 for optimization of the physical stability of MIPs. The ratio of total functional monomers to glucose was in the range of 3.0 : 1 to 12.0 : 1, as determined by the solubility of glucose.

Properties of MIPs

Complete extraction of template molecules from MIPs is a prerequisite for the precise evaluation of the binding affinity of MIPs. To investigate the effect of the degree of cross-linking on the glucose extraction, the amounts of glucose extracted from polymer 1 or polymer 4 of the P1, P2, and P3 series (see Table 1) were measured by the phenol–sulfuric acid assay method. The amounts of glucose remaining in different MIPs after each extraction and drying process are shown in Fig. 2. Although more than 97% of bound glucose molecules were extracted after five consecutive extractions, the amount of glucose extracted from highly

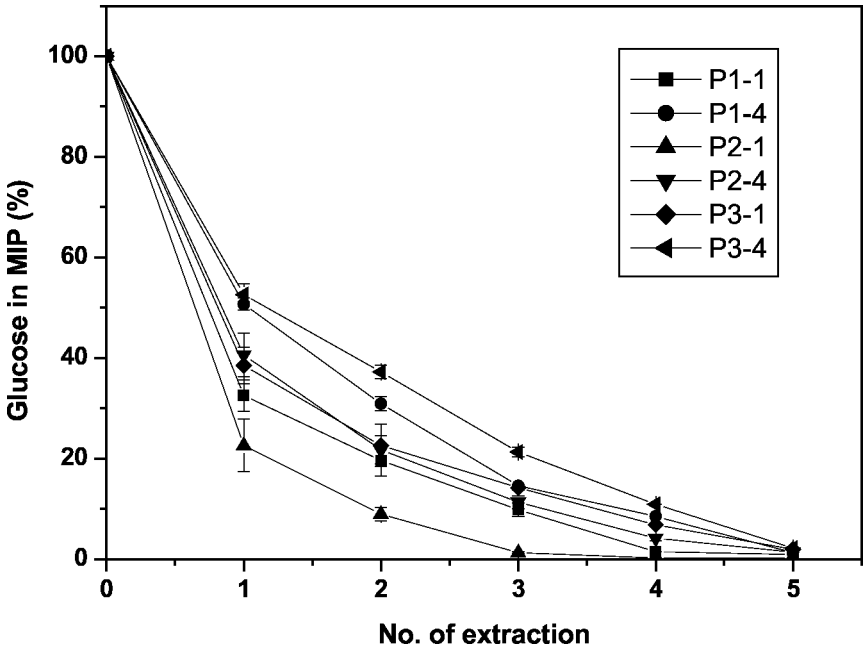


Figure 2. Amount of glucose remaining in MIPs with different cross-linking densities after repeated extraction in deionized distilled water at 25 °C. Each extraction lasted for 24 h ($n = 3$).

cross-linked polymers (P1-4, P2-4, and P3-4) after each extraction in the early cycles was much smaller than the amounts from the less cross-linked polymers (P1-1, P2-1, and P3-1). This may be because the high degree of cross-linking can restrict the mobility of glucose and/or of functional monomers in MIPs [20]. When the polymers prepared with the same amount of cross-linker were compared, the amount of glucose remaining in MIPs was in the order P3-4 > P1-4 > P2-4 or P3-1 > P1-1 > P2-1. This indicates that the interaction between glucose and functional monomers is influenced by the composition of the functional monomers. Figure 3 shows the amount of glucose remaining in MIPs after each extraction. MIPs were synthesized using the same cross-linkers, but in the presence of different amounts of glucose. The amount of glucose used during the imprinting process does not appear to affect the glucose binding to the MIPs. For example, no significant differences were observed between P1-5 and P1-8. The amount of glucose remaining in MIPs was in the order P3-8 > P1-8 > P2-8 or P3-5 > P1-5 > P2-5. These results suggest that monomers in P3 interact with glucose with a higher affinity than those in P1 or P2.

The swelling behavior of MIPs was examined by measuring the swelling ratios (S) in PBS. The swelling behavior of MIPs prepared with different cross-linker amounts is shown in Fig. 4. While there were no significant differences in the S values among the highly cross-linked MIPs (P1-4, P2-4, and P3-4) irrespective of their monomer compositions, there was a pronounced difference in the S values among MIPs with

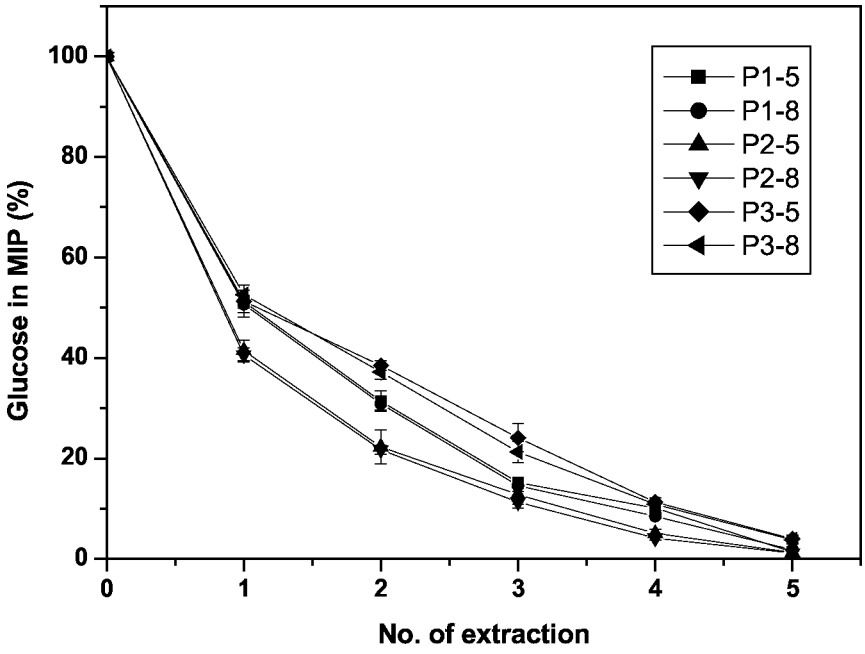


Figure 3. Amount of glucose remaining in MIPs after repeated extraction in deionized distilled water at 25 °C. Each extraction lasted for 24 h. MIPs were synthesized in the presence of different amounts of glucose as template. The cross-linking densities of the MIPs were the same ($n = 3$).

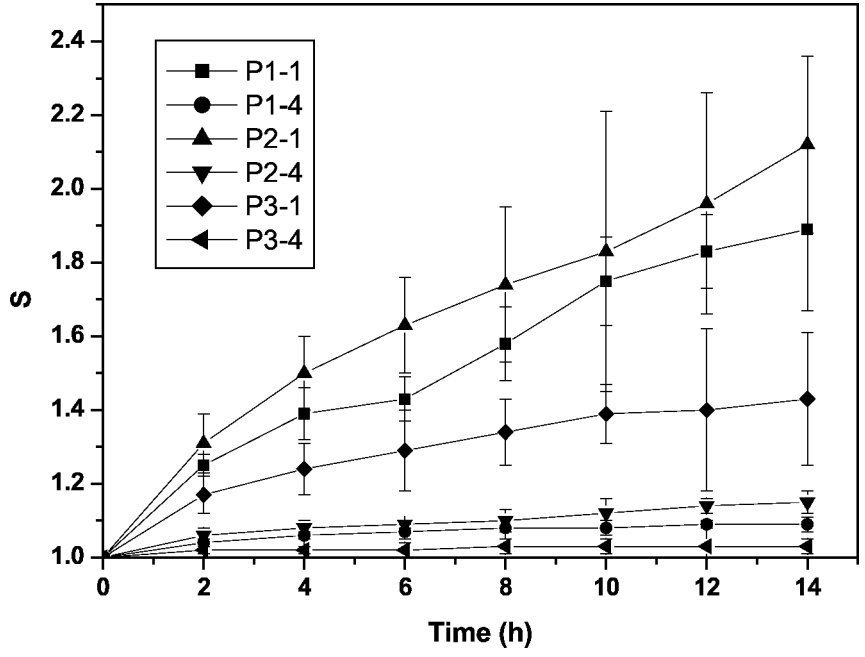


Figure 4. Swelling ratios (S) of MIPs having different cross-linking densities. Swelling was measured at 25 °C in PBS ($n = 3$).

lower cross-linking densities ((P1-1, P2-1, and P3-1). In addition, the degree of swelling was in the order P2 > P1 > P3, indicating that the physical stability of MIPs in aqueous solution was dependent on the degree of cross-linking [30].

Glucose-binding affinity to MIPs

The binding affinities between glucose and MIPs were determined by equilibrium dialysis techniques. Each MIP was dialyzed against glucose and the amount of glucose bound to the MIP was calculated using the predetermined calibration curve of the glucose solution. Information on the equilibrium, glucose + MIP $\leftrightarrow$ glucose–MIP, was obtained using the following Scatchard plot, a tool already applied in MIP work [25, 32]:

$$B/[\mathrm{F}] = B_{\max}/K_{\mathrm{D}} - B/K_{\mathrm{D}},$$

where B is the amount of glucose bound to the polymer, [F] is the concentration of free glucose (approximated by the analytical concentration of glucose), K_{D} is the dissociation constant of the glucose–MIP complex, and $B_{\max}$ is the apparent maximum number of binding sites. Figure 5 shows the amount of glucose bound to P1-4 polymer against free glucose (a) and the Scatchard plot of the data (b) after the equilibrium dialysis test. Linear regression of the Scatchard plot ($R^2 = 0.997$) gave a K_{D} value of 1.94 mM [association constant (K_{A}) $= 1/K_{\mathrm{D}} = 5.13 \times 10^2$ M^{-1}]. The linearity of the Scatchard plot indicates that the binding sites are identical and independent. Con-A was also dialyzed against glucose to determine the K_{D} value, which was then compared with those of the MIPs. The K_{D} value of Con-A was found to be 1.57 mM ($K_{\mathrm{A}} = 6.35 \times 10^2$ M^{-1}), which is in agreement with the value reported in the literature [33]. The K_{D} or K_{A} value of P1-4 suggests that MIP has glucose-binding affinity and thus, MIPs having higher glucose-binding affinity than Con-A could be achieved by an optimized composition of monomers and cross-linker. On the other hand, the K_{D} values of glucose-non-imprinted polymers were in the range of 32.6–49.1 mM ($K_{\mathrm{A}} = 30.7$–20.4 M^{-1}) and no relationship between K_{D} and the polymerization conditions was found. The results of Scatchard analyses for MIPs prepared using different amounts of cross-linker or glucose are summarized in Table 3. With an increase in the cross-linker amount (1–4 of P1, P2, and P3 polymers), the K_{D} value decreased in all series of polymers, indicating that glucose bound with higher affinity to MIPs with a higher cross-linking density at the fixed ratio of monomers. Once glucose-binding sites are formed in MIPs by non-covalent interactions between glucose and functional monomers, the stability of the binding sites would determine the subsequent binding affinity of MIPs. The stability of a binding site is dependent on the degree of cross-linking of the functional monomers. Thus, the higher glucose-binding affinity of highly cross-linked MIPs may be due to the increased stability of glucose-binding sites in the MIPs. In addition, the K_{D} values of the MIPs decreased in all series of polymers as the amount of glucose for imprinting was increased (5–8 of P1, P2, and P3

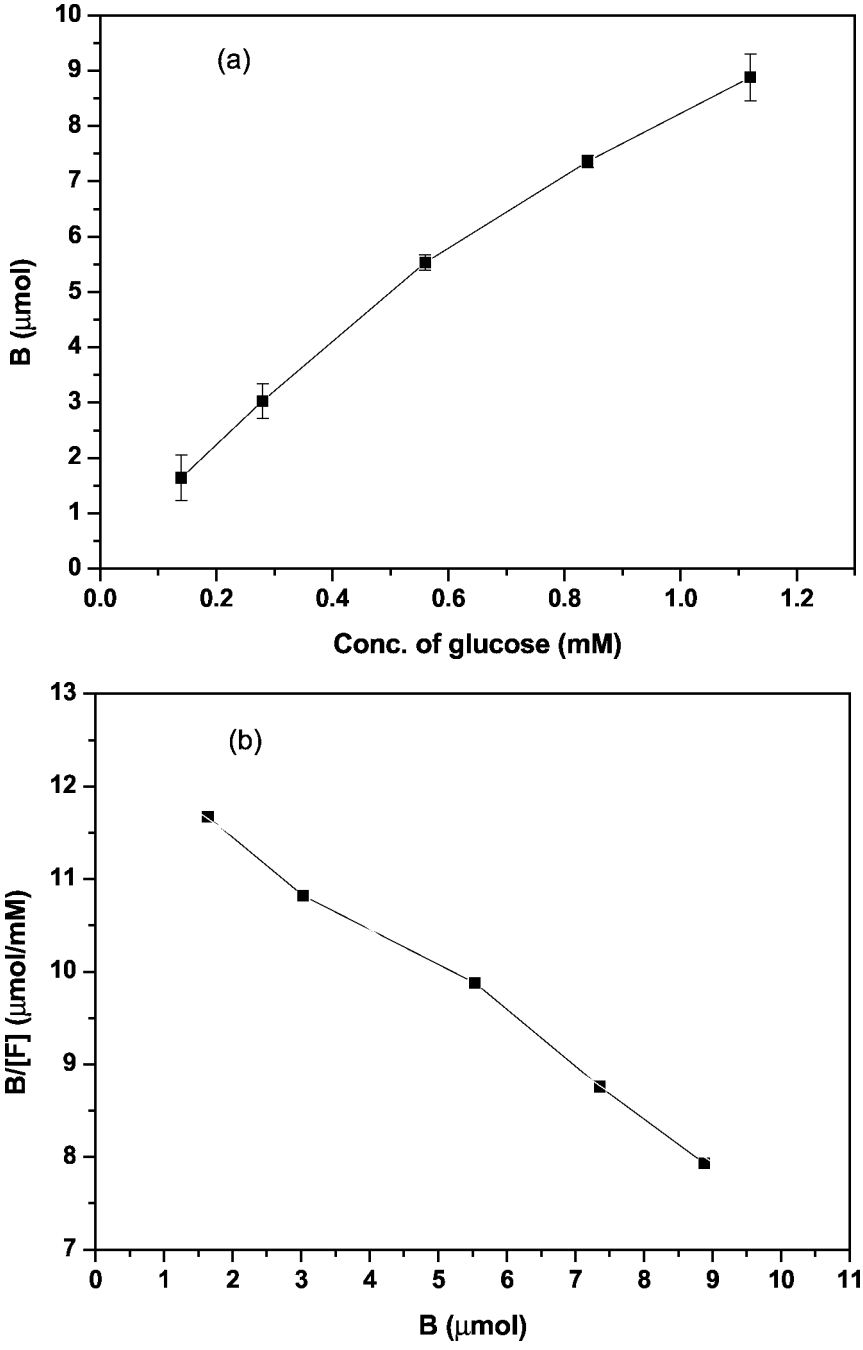


Figure 5. Amount of glucose bound to P1-4 at pH 7.4 (a) and the Scatchard plot for the bound glucose (b). B is the amount of glucose bound to polymer and [F] is the concentration of free glucose ($n = 3$).

Table 3.
Dissociation constants (K_D) of MIPs having different amounts of cross-linker[a] or glucose as a template molecule[b]

Polymer	$K_D \pm$ SD (mM)	Polymer	$K_D \pm$ SD (mM)	Polymer	$K_D \pm$ SD (mM)
P1-1	6.48 ± 0.07	P2-1	8.09 ± 0.38	P3-1	4.27 ± 1.31
P1-2	4.21 ± 0.09	P2-2	5.93 ± 0.17	P3-2	2.84 ± 0.55
P1-3	2.93 ± 0.11	P2-3	3.37 ± 0.04	P3-3	2.14 ± 0.19
P1-4	1.94 ± 0.21	P2-4	2.32 ± 0.29	P3-4	1.66 ± 0.03
P1-5	11.59 ± 0.01	P2-5	13.05 ± 0.05	P3-5	9.31 ± 0.02
P1-6	8.24 ± 0.09	P2-6	10.37 ± 0.22	P3-6	6.64 ± 0.06
P1-7	2.49 ± 0.01	P2-7	5.29 ± 0.06	P3-7	2.05 ± 0.16
P1-8	1.94 ± 0.21	P2-8	2.32 ± 0.29	P3-8	1.66 ± 0.03

[a] 1–4 of P1, P2, and P3.
[b] 5–8 of P1, P2, and P3.

polymers). In these polymers, the same amounts of functional monomers and cross-linker were used. Therefore, the higher glucose-binding affinity by a higher glucose concentration is most likely due to the fact that more functional monomers would participate in the formation of a binding site. When the K_D values were compared with respect to the monomer composition, P1 polymers composed of VA, AAm, and AB had lower K_D values than those of P2 polymers composed of VA, AAm, and PA. In addition, P3 polymers composed of VA, AAm, PA, and AB had lower K_D values than those of P1 polymers. These results support the finding that interactions between glucose and functional monomers can be achieved by hydrogen bonding (P2) and that additional hydrophobic interaction can increase the glucose-binding affinity of MIPs.

To compare the contribution of each monomer to glucose binding, MIPs having different monomer ratios (9–11 of P1 and P2 polymers, or 9–12 of P3 polymers) were dialyzed against glucose and the data were analyzed by a Scatchard plot. The results are summarized in Table 4. When the data of 9–11 in P1 polymers were compared, the contribution of functional monomers to the glucose binding affinity was in the order VAA > AAm > AB. This implies that hydrogen bonding of the carboxyl group in VAA is stronger than that of the amine group in AAm and also that the hydrophobic interaction of AB is weaker than the hydrogen bonding of VAA or AAm. The K_D values of 9–11 in P2 polymers were in the range of 2.3–4.1 mM, lower than those of P1 polymers. In addition, the highest K_D value of P2-11 polymer, representing the lowest glucose-binding affinity, indicates that PA has weaker hydrogen bonding than that of VAA or AAm. As can be seen in Table 4, the K_D values of 9–12 of P3 polymers were in the range of 2.0–2.9 mM. The lowest K_D value of P3-9 indicates that the carboxyl group of VAA serves as the main functional group for glucose binding. In addition, the relatively low K_D value of P3-12 suggests that hydrophobic interaction of AB with glucose can increase the glucose-binding affinity of MIPs which already have glucose-binding affinity by hydrogen bonding [34].

Table 4.
Dissociation constants (K_D) of MIPs having different molar ratios of monomers

Polymer	$K_D \pm$ SD (mM)	Polymer	$K_D \pm$ SD (mM)	Polymer	$K_D \pm$ SD (mM)
P1-9	1.99 ± 0.03	P2-9	2.26 ± 0.09	P3-9	1.99 ± 0.11
P1-10	2.24 ± 0.01	P2-10	2.95 ± 0.02	P3-10	2.23 ± 0.06
P1-11	3.58 ± 0.22	P2-11	4.11 ± 0.06	P3-11	2.84 ± 0.01
—	—	—	—	P3-12	2.35 ± 0.05

Table 5.
Dissociation constants (K_D) of MIPs prepared by UV polymerization and thermal polymerization

Polymer	$K_D \pm$ SD (mM)[a]	$K_D \pm$ SD (mM)[b]
P1-8	2.49 ± 0.06	1.94 ± 0.21
P2-9	3.83 ± 0.09	2.26 ± 0.09
P3-8	2.72 ± 0.01	1.66 ± 0.03

[a] K_D of UV-polymerized polymers.
[b] K_D of thermally polymerized polymers.

UV polymerization is preferred since it has been demonstrated that polymers made at low temperature exhibit higher recognition abilities [35]. Therefore, the polymers having the highest glucose-binding affinity in each polymer series (P1-8, P2-9, and P3-8) were also prepared by UV polymerization at 0°C. The K_D values of UV-polymerized polymers and those of thermally polymerized polymers are summarized in Table 5. In general, it is believed that weak non-covalent interactions, such as hydrogen bonding, essential for imprint formation and subsequent recognition, are stronger at lower temperatures due to a favorable entropy term [36]. However, the K_D values of UV-polymerized polymers were higher than those of thermally polymerized polymers. The lower binding affinity of UV-polymerized MIPs may be explained by the lower degree of cross-linking, because of less initiation of AIBN at low temperature.

CONCLUSIONS

MIPs having glucose-binding affinity can be synthesized by copolymerization of mixtures of amino acid-mimicking functional monomers, excess cross-linker, and glucose as a template. Glucose-binding affinity evaluated by the Scatchard analysis was dependent on the composition of the functional monomers, as well as on the degree of cross-linking. The MIPs composed of vinyl acetic acid (VAA), acryl amide (AAm), 4-pentenoic acid (PA), and allyl benzene (AB) had the lowest K_D value of 1.66 mM, which is comparable to the K_D value of Con-A (1.84 mM). Furthermore, the results of the glucose-binding affinity of MIPs having different

molar ratios of functional monomers demonstrate that the strength of interaction with glucose is in the order VAA > AAm > AB > PA.

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