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Preparation of liposomes with glucose binding sites: liposomes containing di-branched amino acid derivatives

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Abstract

Di-branched amino acid derivatives, such as dihexadecyl-glutamate-glutamine (DHD-glu-gln), dihexadecyl-glutamate-asparagine (DHD-glu-asn) and dihexadecyl-glutamate-glutamic acid (DHD-glu-glu), were synthesized, and then incorporated into lipid vesicles (liposomes) using dipalmitoylphosphatidylcholine (DPPC). To form binding sites toward glucose, the liposomes containing amino acid derivatives were mixed with glucose above the phase transition temperature (PTT) of DPPC and subsequently the temperature was lowered below the PTT. The glucose-binding affinity of liposomes containing amino acid derivative with or without glucose imprinting was evaluated by surface plasmon resonance (SPR) and equilibrium dialysis technique. SPR of liposomes containing each amino acid derivative or three amino acid derivatives revealed that only the liposomes containing all three amino acid derivatives had glucose-binding affinity and that the glucose-imprinting process was essential to fix the amino acid derivatives into a glucose binding site on the liposomes. Equilibrium dialysis studies of glucose-imprinted liposomes produced curvilinear Scatchard plots, indicating that the amino acid derivatives play a role in glucose binding. Di-branched amino acid derivatives synthesized in this study are promising agent for the development of biocompatible synthetic glucose binding materials.

Keywords: Liposomes; Glucose; Glucose imprinting; Glucose binding; Surface plasmon resonance; Equilibrium dialysis

1. Introduction

In the last two decades, significant advances have been made in the development of self-regulated insulin delivery systems that can adjust insulin release in response to changes in the environmental glucose level. In self-regulated insulin delivery systems developed to date, the insulin release is controlled by using glucose oxidase (GOX)-based pH-sensitive membranes [1,2], GOX-based pH-sensitive erodible matrices [3], glucose-sensitive sol–gel reversible systems [4–8], or immobilized insulin systems [9,10]. Glucose-sensitive sol–gel reversible systems [7,8,11] and immobilized insulin systems [9,10] utilize concanavalin-A (Con-A) as a glucose-binding molecule to provide glucose-sensitivity. While Con-A has been widely used and highly effective in preparing self-regulated insulin delivery systems, it may not be suitable for clinical applications because it is known to be immunogenic [12,13]. Commonly used GOX also is not suitable for long-term applications of implantable insulin delivery devices due to loss of enzymatic activity over time. Polymers containing phenylboronic acid and/or poly(vinyl alcohol) [4,5] may also not be ideal due to its lack of glucose-specificity and biocompatibility information.

For development of clinically useful self-regulated insulin delivery systems for long-term implantation, it is necessary to obtain biocompatible glucose-sensitive molecules. For this reason, we have attempted to synthesize glucose-sensitive molecules. As a first step toward the synthesis, we analyzed in our previous studies 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 [14]. The analysis revealed that hydrogen bonding was the main interaction and multiple hydrogen bonds were formed between amino acid residues of the proteins and hydroxyl groups of glucose. The hydrogen bonding moieties were introduced into

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liposomes and molecular imprinting was done in the presence of glucose. The idea was that the liposomes above its phase transition temperature (PTT) would allow free rearrangement of amino acid derivatives to form binding sites toward glucose. The formed glucose-binding site can then be fixed by lowering the temperature below the PTT. This paper describes the synthesis of di-branched amino acid derivatives of phosphatidylcholine, preparation of liposomes, and characterization of glucose binding properties.

2. Material and methods

2.1. Synthesis of di-branched amino acid derivatives

Di-branched amino acid derivatives were synthesized following the reaction procedures shown in Fig. 1. Dihexadecyl-L-glutamate-*p*-toulene sulfate (DHD-glu·salt, **1**) was synthesized as a starting material for amino acid derivatives. In total, 120.0 mmole of L-glutamic acid (glu, Sigma, St. Louis, MO) and 24.0 mmole of *p*-toluenesulfonic acid monohydrate (Aldrich, Milwaukee, WI) were dissolved in benzene and stirred at room temperature for 1 h. Hexadecanol (44.0 mmole) (Aldrich, Milwaukee, WI) in benzene was added and reacted for 10 h with reflux. Water produced during the reaction period was removed using a Dean–Stark apparatus. After the reaction benzene was evaporated under vacuum and the residue was recrystallized in methanol to give **1** (Fig. 1A).

2.1.1. Synthesis of DHD-glu-gln

The mixture of **1** (1.0 mmole) and *N*-α-(*tert*-butoxycarbonyl)-L-glutamine (*N*-*t*-BOC-gln: 1.2 mmole, Sigma, St. Louis, MO) in 30 ml of chloroform was stirred at room temperature. The solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodimide·HCl (EDC: 1.2 mmole, Sigma, St. Louis, MO) and 4-dimethylaminopyridine (DMAP: 2.4 mmole, Aldrich, Milwaukee, WI) in chloroform was added to the mixture and reacted for 16 h at room temperature. After evaporation in vacuum, washing with distilled water, and filtration, the product was recrystallized in hot ethanol and separated by chromatography on a silica gel column with chloroform/methanol (95/5) to obtain the pure DHD-glu-BOC-gln. The BOC group was deprotected by trifluoroacetic acid. After treatment with 30% NH_4OH, the precipitates were collected, washed with cold water, and then dried under vacuum to give DHD-glu-gln.

2.1.2. Synthesis of DHD-glu-asn

The mixture of **1** (1.0 mmole) and *N*-α-*t*-BOC-L-asparagine (*N*-*t*-BOC-asn: 1.2 mmole, Sigma, St. Louis, MO) in 30 ml of chloroform were reacted at room temperature. EDC (1.2 mmole) and DMAP (2.4 mmole)

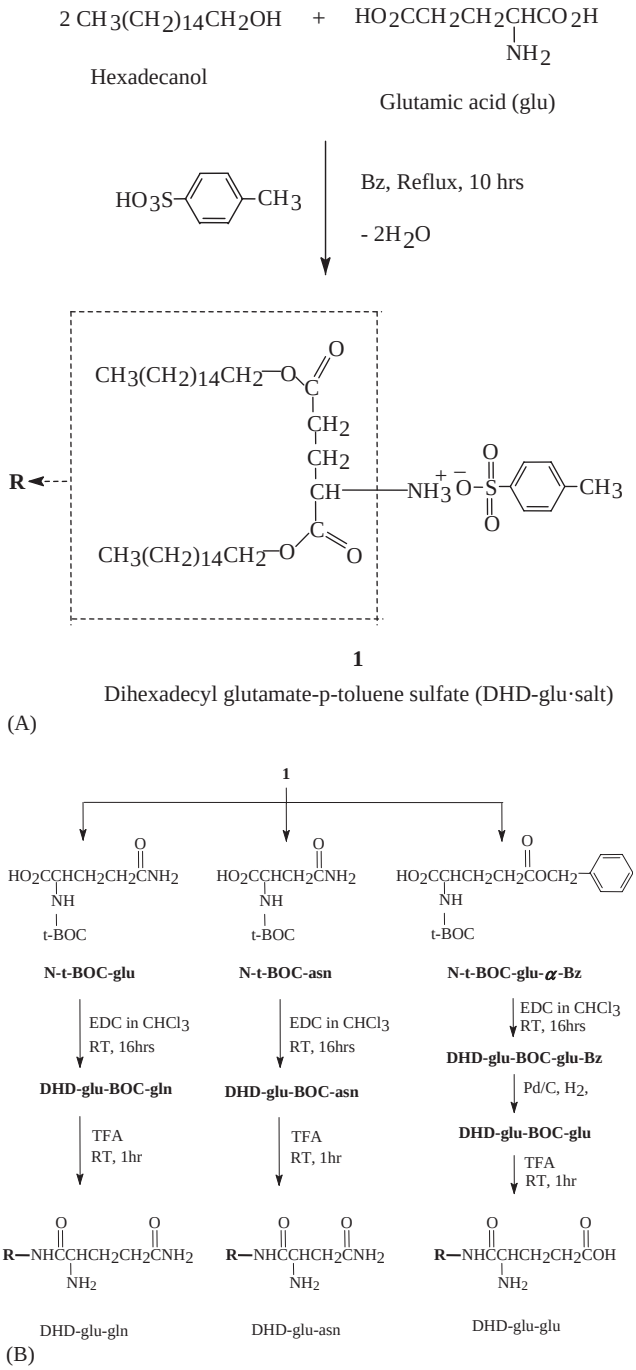

Fig. 1. Reaction procedure for dihexadecyl glutamate-*p*-toluene sulfate (A) and di-branched amino acid derivatives (B).

dissolved in chloroform were added to the reaction mixture. The other procedures of reaction and purification were the same as those described in synthesis of DHD-glu-gln.

2.1.3. Synthesis of DHD-glu-glu

The mixture of **1** (1.0 mmole) and *N*-α-*t*-BOC-L-glutamic acid-α-benzyl ester (*N*-*t*-BOC-glu-Bz: 1.2 mmole, Sigma, St. Louis, MO) in 30 ml of chloroform

was stirred at room temperature. EDC (1.2 mmole) and DMAP (2.4 mmole) in chloroform were added to the reaction mixture. The Bz moiety in DHD-glu-BOC-glu-Bz (200 mg) was deprotected using 10% palladium on activated carbon (Pd/C) under N_2. The BOC group in DHD-glu-BOC-glu was deprotected using the same method as described in the synthesis of DHD-glu-gln to give DHD-glu-glu.

The synthesized amino acid derivatives were identified by plasma desorption mass spectrometry (PDMS) and ^{1}H-NMR spectrometry. Mass spectra were obtained on a BioIon 20R mass spectrometer. ^{1}H-NMR spectra were taken at 300 MHz with a Bruker ARX-300 spectrometer in $CDCl_3$. Chemical shifts are expressed in δ (ppm) values with tetramethylsilane as the internal standard.

2.2. Preparation of glucose-imprinted liposomes

Liposomes were prepared by the modified membrane-hydration method [15]. 40 mg (54.4 μmole) of L-α-phosphatidylcholine, dipalmitoyl (DPPC, Sigma, St. Louis, MO) was dissolved in 2 ml of methanol/chloroform (1/1). 2.6 mg (3.63 μmole) of DHD-glu-gln, 2.4 mg (3.63 μmole) of DHD-glu-asn, and 2.5 mg (3.63 μmole) of DHD-glu-glu were dissolved in 2 ml of methanol/chloroform (1/1). The DPPC solution and the amino acids solution were mixed in a 50 ml round bottom flask. To make a thin film the mixed solution was evaporated under vacuum at 50°C with a rotary evaporator. 6 ml of phosphate-buffered saline (PBS, pH 7.4) was poured onto the film and the film was hydrated at 50°C by whirling the flask. The contents in the flask were rapidly vortexed for removal of lipid from the sides of the vessel. The lipid was sonicated with the titanium probe 4 mm below the surface of the lipid suspension. Nitrogen gas was gently passed through the vessel as a continuous flow during sonication. After sonication, the liposomes were left to stand above their transition temperature for 30 min to allow the annealing process to come to composition. The liposomes suspension was centrifuged at $1 \times 10^5 g$ for 20 min to sediment undispersed lipid and remove titanium particles released from the probe.

The liposomes having the dibranched amino acids were incubated together with free glucose to imprint glucose in liposomes above PTT of DPPC for 3 h, and then cooled down the suspension to 4°C using an ice-water bath [16]. After incubation, the glucose-imprinted liposomes were dialyzed to remove free glucose or binding glucose by using dialysis cassettes (Slide-A-Lyzer®, MWCO; 10,000, Pierce Chemical Company, Rockford, IL).

2.3. Evaluation of glucose affinity

2.3.1. Surface plasmon resonance analysis

In total, 2 mM DHD-glu-gln, DHD-glu-asn, and DHD-glu-glu were prepared with PBS, pH 7.4, respectively, and the mole ratio of DPPC to each amino acid derivative was adjusted to 7:3. Liposomes containing each amino acid derivative or three amino acid derivatives were coated for 50 min on the gold surface of a miniaturized and integrated surface plasmon resonance (SPR) sensor (TISPR-1, Texas Instruments, Dallas, TX). Refractive index (RI) was monitored while adding glucose solution to the surface on which liposomes were adsorbed. Integration time of the signal and the intensity of the light-emitting diode were adjusted 2.22 ms and 5-medium, respectively. Before measurement, sensors were equilibrated with PBS, pH 7.4 at least 10 min at room temperature. A flow rate of 40 μl/min was used for all of SPR experiments.

2.3.2. Equilibrium dialysis

Equilibrium dialyzer (EDM101B Equilibrium Dialyzer, Hoefer Pharmacia Biotech Inc., San Francisco, CA) with 8-well dialysis module and a dialysis membrane with a MWCO of 6000–8000 were used. The dialysis membrane divided each well into two chambers and the volume of each chamber was 0.5 ml. Glucose solutions (50–200 μg/ml) as donors were placed in one side and the glucose-imprinted liposomes as receptors were loaded into the other side. The dialysis module was gently rotated at a speed of 20 rpm at room temperature for 36 h. At the end of experiment, aliquots were taken from both sides of the dialysis chambers. The glucose concentration after dialysis was determined by phenol–sulfuric acid assay method [17].

3. Results and discussion

3.1. Identification of di-branched amino acids

The synthesized amino acid derivatives were identified by PDMS and ^{1}H-NMR spectrometry. Values of mass-to-charge ratio (m/z) and chemical shift (δ) of the derivatives are reported in Table 1. Mass-to-charge ratios of amino acid derivatives were in good agreement with their calculated mass. DHD-glu · salt was identified by peaks of toluene sulfate group at 7.7 and 7.1 ppm, and peaks of methylene and methyl at 1.2 and 0.8 ppm, respectively. DHD-glu-gln and DHD-glu-asn were identified by the characteristic methylene and methyl peaks at 1.2 and 0.8 ppm, together with the disappearance of peaks at 7.7 and 7.1 ppm of the toluene sulfate group and the disappearance of the 1.4 ppm of BOC group peak of DHD-glu · salt. DHD-glu-glu was identified by the appearance of methylene and methyl group peaks and by the disappearance of several peaks of toluene sulfate, benzyl, and BOC groups at 7.3, 5.1, and 1.4 ppm, respectively.

Table 1
Mass-to-charge ratio (m/z) and chemical shift (δ) of di-branched amino acid derivatives

Sample	m/z (MH^+)		δ (ppm)[a]
	Calculated	Found	
DHD-glu · salt	597.1	597.4	7.7, 7.1, 1.2, 0.8
DHD-glu-gln	724.1	725.2	1.2, 0.8, 7.7[b], 7.1[b], 1.4[b]
DHD-glu-asn	710.1	711.0	1.2, 0.8, 7.7[b], 7.1[b], 1.4[b]
DHD-glu-glu	725.1	726.1	1.2, 0.8, 7.3[b], 5.1[b], 1.4[b]

[a] Solvent was $CDCl_3$ and internal standard was tetramethylsilane.
[b] Peaks disappeared as a result of reaction or deprotection.

3.2. Evaluation of glucose affinity by using SPR analysis

SPR is an optically based transducer, which is extremely sensitive to minute variation in RI, and, in general, change of RI occurs within about 100 nm of the surface of the transducer [18]. The SPR transduction layer is typically composed of a gold surface because gold is quite inert, which makes its use in biochemical analysis attractive based on stability considerations. The attachment of the interfacial chemical layers, the immobilization or subsequent binding of proteins, and the other biological materials, all cause effective RI changes, which can be measured by the SPR transducer. The expression for reflectivity is given by $R_N = (R_S - R_B)/(R_A - R_B)$, where R_N is normalized reflectivity, R_S is the reflectivity for the test sample, R_A is reflectivity in air, and R_B is reflectivity with the light emitting diode off [19].

To investigate the glucose binding affinity qualitatively, liposomes having each amino acid derivative or three amino acid derivatives with or without glucose imprinting were analyzed using SPR liquid sensing system [20,21]. Glucose-imprinted liposomes having amino acid derivatives were adsorbed onto the gold surface and then unadsorbed liposomes were washed with PBS, pH 7.4. Glucose dissolved in PBS, pH 7.4 (100 μg/ml) was pumped through the flow cell at a flow rate of 40 μl/min and RI was monitored as a function of time. Fig. 2 shows the RI of the glucose-imprinted liposomes having three amino acid derivatives. The RI value increased from 1.337314 to1.338935 for 2 h, indicating glucose was bound to the glucose-imprinted liposomes. The RI change (ΔRI) of each sample is summarized in Table 2. Compared to the glucose-imprinted liposomes (E), other liposomes containing each amino acid derivative (A, B, and C) and three amino acid derivatives without glucose imprinting (D) show almost negligible RI change. The difference of ΔRI values implies that glucose imprinting is essential to impart glucose affinity to amino acid derivatives.

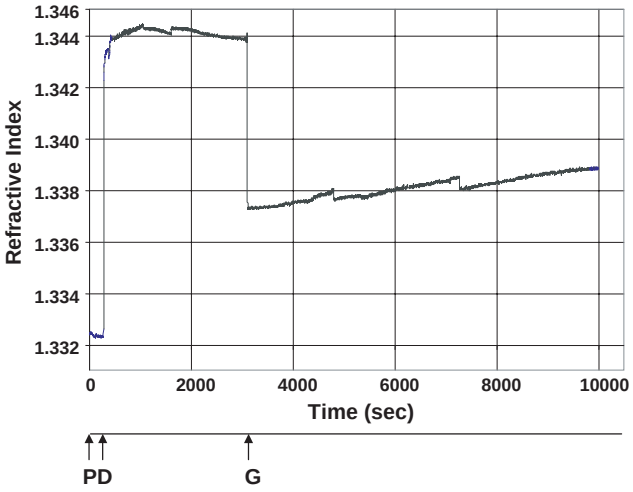

Fig. 2. RI change of glucose-imprinted liposomes containing amino acid derivatives: P; PBS buffer, pH 7.4, D; DPPC liposomes, G; glucose solution.

Table 2
Refractive index change (ΔRI) of liposomes by SPR

Sample	ΔRI ($\times 10^{-3}$)
DHD-glu-gln (A)	0.010
DHD-glu-asn (B)	0.037
DHD-glu-glu (C)	0.019
A + B + C (D)[a]	0.094
A + B + C (E)[b]	1.621

[a] Liposomes prepared without glucose-imprinting.
[b] Liposomes prepared with glucose-imprinting.

3.3. Evaluation of glucose-affinity by using equilibrium dialysis

Quantitative glucose-binding affinities of liposomes containing amino acid derivatives were evaluated by using an equilibrium dialysis technique. Glucose concentrations were assayed by using the phenol–sulfuric acid assay method [17], in which λ_{max} of the assayed glucose solution was 485 nm. Absorbance values for calibration increased linearly with increasing concentration of glucose from 5 to 200 μg/ml, giving a correlation coefficient (R^2) of 0.9995. To determine the glucose-affinity of DPPC liposomes as a control, liposomes without amino acid derivatives were dialyzed against glucose at room temperature. Glucose concentrations in the donor or receptor after dialysis are shown in Fig. 3A. The data of equilibrium dialysis were analyzed according to the Scatchard plot of $r/c = Kn - Kr$ [22]. In the equation, r is the ratio of the moles of bound ligand (i.e., glucose) per mole of binding compound (amino acid derivative), c is the molar concentration of free ligand, n is the number of binding sites on the binding compound, and K is the association constant

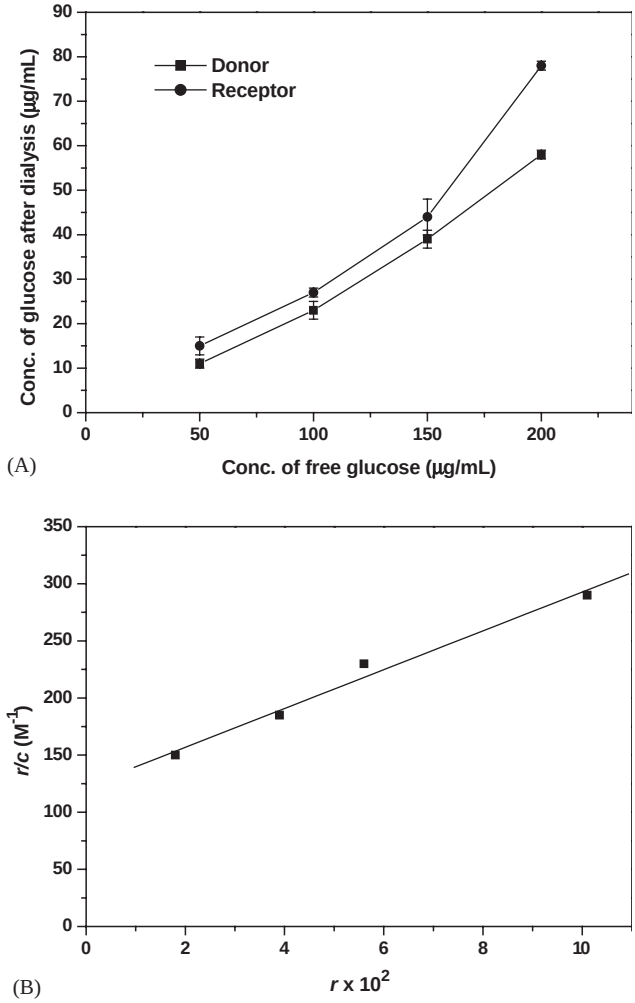

Fig. 3. Concentration of glucose after equilibrium dialysis (A) and Scatchard plot (B) of liposomes prepared with DPPC only.

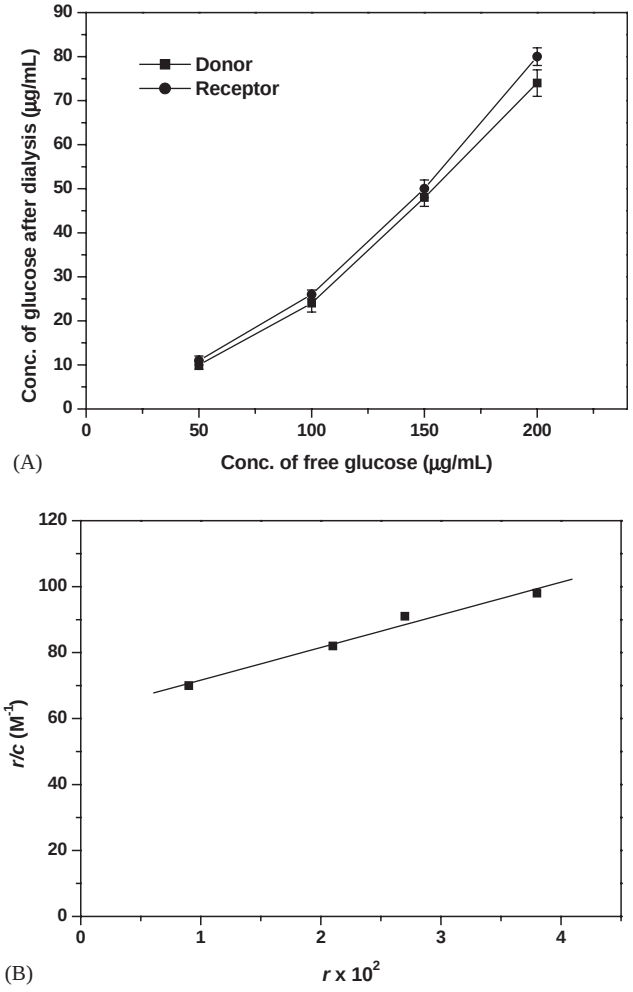

Fig. 4. Concentration of glucose after equilibrium dialysis (A) and Scatchard plot (B) of liposomes having amino acid derivatives without glucose imprinting.

for the ligand-binding compound complex. In the plots, the data points were fitted to straight lines by linear regression analyses using the values on the x-axis as the independent variable, r, and the values on the y-axis as the dependent variables, r/c. The straight line gave the values of Kn (intercept on the y-axis), n (intercept on the x-axis), and K (negative slope). The Scatchard plot of binding of glucose to DPPC liposomes only is shown in Fig. 3B. If a compound has affinity for the ligand, the slope of the plot should be negative. However, the slope obtained in the plot was positive and thus, it was thought that DPPC liposomes alone have no glucose binding affinity.

Liposomes containing amino acid derivatives without glucose imprinting were dialyzed against glucose as another control. Figs. 4A and B show concentration of glucose in donor or receptor after equilibrium dialysis and the Scatchard plot, respectively. Positive K values in this Scatchard plot mean that these liposomes also have no glucose-binding affinity. These liposomes did not experience the glucose imprinting process of incubation and quenching through the liposome phase transition, thus allowing for rearrangement of the amino acid derivatives. Therefore, lack of the glucose binding affinity of these liposomes may be explained by the mobility of both the DPPC molecules and amino acid derivatives in the liposome membrane.

Glucose-imprinted DPPC liposomes containing amino acid derivatives were dialyzed against glucose and the results were shown in Figs. 5A and B. The glucose-imprinted liposomes produced a Scatchard plot with curvilinear lines. According to literature precedent, curvilinear Scatchard plots represent the summation of two straight lines that form the curve [22,23]. Interpretation for this type of plot is that there are at least two different and independent binding sites each with its own independent association constant. Fitting of the equation for the Scatchard plot ($r/c = Kn - Kr$) to the high-r values produced binding parameters ($K = 587/\mathrm{M}$, $n = 0.531$), indicating modest binding properties of the

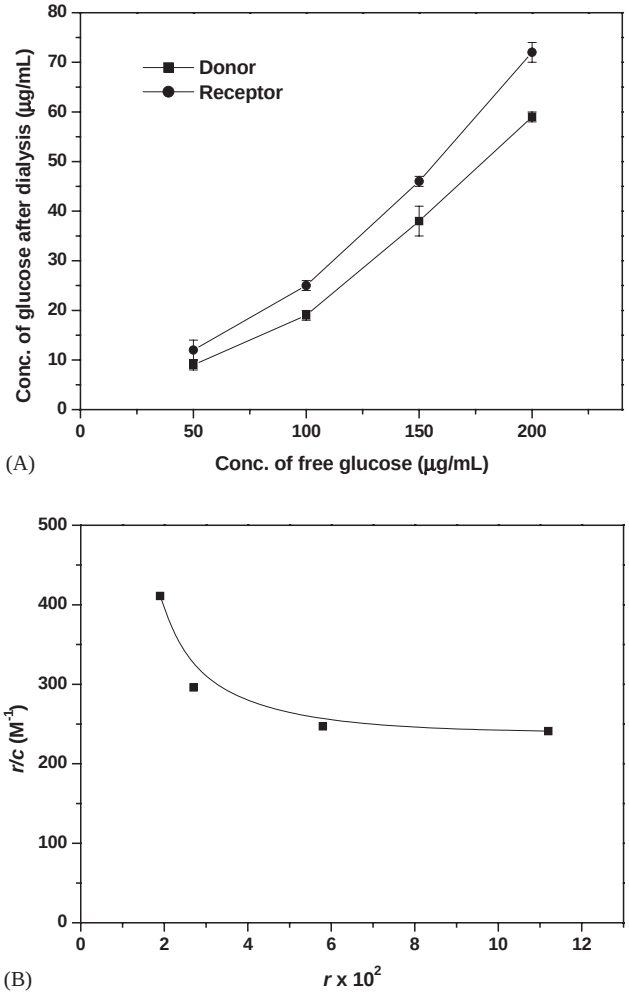

Fig. 5. Concentration of glucose after equilibrium dialysis (A) and curvilinear Scatchard plot (B) of DPPC liposomes containing amino acid derivatives with glucose imprinting.

glucose-imprinted liposomes. Low-r values seem to involve a second group of binding sites. The binding parameters ($K = 13{,}819/\mathrm{M}$, $n = 0.048$) for this second group of sites indicate stronger binding to glucose. In the glucose-imprinted liposomes, the binding sites are formed by amino acid derivatives having chemically different groups, such as amino, carbonyl, and carboxyl groups. In addition, certain functional groups in amino acid derivatives are expected to form hydrogen bonding with glucose molecules in the right spatial arrangements. Those interactions could not be obtained with one specific amino acid derivative as shown in Table 2. Therefore, this curvilinear Scatchard plot may be due to the independent binding properties of two binding sites formed with spatially oriented amino acid derivatives. Although these glucose-imprinted liposomes showed modest to good glucose affinity, more detailed studies using other amino acid derivatives should be conducted to elucidate the glucose binding motif and to obtain higher glucose binding affinity.

4. Conclusions

As non-proteinous glucose binding molecules, dihexadecyl-glu-gln, dihexdecyl-glu-asn, and dihexadecyl-glu-glu synthesized in this study provide binding affinity to glucose. Glucose was imprinted into liposomes made by DPPC and amino acid derivatives above the PTT of DPPC. Qualitative glucose-binding affinity evaluated by SPR technique revealed that the glucose-imprinting process is essential to impart glucose-binding properties to the liposomes containing amino acid derivatives. Quantitative glucose-binding affinity was evaluated by equilibrium dialysis producing non-linear Scatchard plots, which indicates each amino acid derivative provides independent binding site for glucose molecule. Glucose-binding affinity of di-branched amino acid derivatives shows promise as a basis for the development of biocompatible synthetic glucose binding materials.

Acknowledgements

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