A Quantitative Analysis and Chemical Approach for the Reduction of Nonspecific Binding Proteins on Affinity Resins

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Tubulin and actin often bind nonspecifically to affinity chromatography resins, complicating research toward identifying the cellular targets of small molecules. Reduction of nonspecific binding proteins is important for the success of such biochemical approaches. To develop strategies to circumvent this problem, we quantitatively investigated the binding of tubulin and actin to a series of affinity resins bearing 15 variant ligands on 3 commercially available polymer supports. Nonspecific protein binding was proportional to the hydrophobicity of the affinity resins and could be quantitatively correlated to the CLOGP values of the ligands, which are a measure of compound hydrophobicity. When compounds had CLOGP values greater than 1.5, (amount of tubulin) = 0.73 × CLOGP − 1.1 ($n = 7$, $r = 0.97$), and (amount of actin) = 0.42 × CLOGP − 0.79 ($n = 7$, $r = 0.99$). On the basis of these studies, we designed a novel hydrophilic poly(ethylene glycol) (PEG) spacer (**26**) for the conjugation of ligands to chromatography resins. As predicted by our binding algorithm, introduction of this spacer reduced the amount of nonspecific protein binding in proportion to the number of ethylene glycol units.

INTRODUCTION

Affinity chromatography matrixes bearing bioactive compounds such as medicines, natural products, and toxins play an important role in the discovery of novel drug targets and the elucidation of drug mechanisms. Their effectiveness has been demonstrated by the discovery of FKBP12 (*1*), HDAC (*2*), and Ref-1 (*3*) as specific binding proteins of FK506 (**1**), Trapoxin, and E-3330, respectively. The successful isolation of target proteins by affinity chromatography depends on the synthesis of polymeric resins that can bind to the cellular target with maximum selectivity and efficiency. The nonspecific binding of cellular proteins to affinity matrixes is therefore a significant limitation to this biochemical approach. In particular, tubulin and actin often interfere with affinity chromatography studies, due to their high abundance and the similarity of their molecular weights (50 kDa and 42 kDa, respectively) to many putative target proteins.

Commercially available resins for the synthesis of affinity matrixes can be structurally classified as two types: one consisting of sugar derivatives such as agarose (*4*) or sepharose (*5*), and the other based upon methacrylate polymers (*6*). A wide variety of sugar-based resins are commonly used as bioseparators in molecular biology fields, but these supports are often irreversibly denatured in several synthetic conditions, such as organic solvents and strong acids and bases (*7*). These properties significantly restrict chemical approaches such as increasing the ligand density on the resin and synthesizing affinity resins bearing structurally complex compounds. In contrast, functional polymers such as poly(methacrylate) derivatives are stable under most synthetic conditions, which allow the synthesis of more effective affinity resins. This is attractive for organic chemists since the recent development of combinatorial chemistry allows us to synthesize a variety of compounds on functional polymers (*8*−*10*). However, methacrylate polymers bearing bioactive compounds often show high levels of nonspecific protein binding in comparison to agarose resins with the same ligands. Therefore, the reduction of nonspecific protein binding to methacrylate derivatives is now desired.

The phrase "nonspecific protein binding" is usually used to represent proteins that bind to affinity resins based on physical adsorption rather than specific binding such as "lock and key" interactions between ligands and receptors (*11*). They were usually thought to bind to the resin through hydrophobic interactions (*11*, *12*), and indeed methacrylate resins are more hydrophobic than agarose-derived supports. As there are no systematic studies of the relationship between nonspecific protein binding and the physical and chemical characteristics of affinity resins, we quantitatively investigated these interactions using 3 kinds of polymeric supports and 15 arbitrarily selected ligands. These studies demonstrated that (1) the profile of nonspecific binding proteins is approximately constant throughout the series of affinity

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[1] The following abbreviations were used: AcOEt, ethyl acetate; BB, Bromophenol Blue; buffer A, an aqueous solution of 25mM Tris-HCl (pH 7.4), 0.25 M sucrose; $CHCl_3$, chloroform; CH_2Cl_2, dichloromethane; DMAP, *N*,*N*-dimethylaminopyridine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDC, *N*-ethyl-*N*′-(3-(dimethylamino)propyl)carbodiimide; EDC HCl, *N*-ethyl-*N*′-(3-(dimethylamino)-propyl)carbodiimide hydrochloride; EtOH, ethanol; FK506, immunosuppressant tacrolimus; HOBt, 1-hydroxy-benzotriazole; 2-ME, 2-mercaptoethanol, MeOH, methanol; $MgSO_4$, magnesium sulfate; NMP, *N*-methyl-2-pyrrolidone; Pd−C, palladium on activated carbon; PEG, poly(ethylene glycol); PyBOP, bromotris(pyrrolidino)phosphonium hexafluorophosphate; rt, room temperature; sat. $NaHCO_3$ aq, saturated aqueous solution of sodium bicarbonate; SDS, sodium dodecyl sulfate; TBDPS, *tert*-butyldiphenylsilyl; TBS, *tert*-butyldimethylsiliyl; TBS-OTf, *tert*-butyldimethylsilyl trifluoromethanesulfonate; THF, tetrahydrofuran; TFA, trifluoroacetic acid; Tr, trityl.

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resins bearing different compounds, and (2) a linear relationship exists between amount of the nonspecific protein binding and ligand hydrophobicity, as measured by CLOGP values. Moreover, we determined that introduction of a hydrophilic spacer is effective for reduction of the nonspecific binding proteins.

EXPERIMENTAL PROCEDURES

Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F-254 plates. For normal chromatography, Merck silica gel type 60 (size 70−230) was used. All evaporation was performed with a rotary evaporator under reduced atmosphere. The structures of all compounds were confirmed by a LC-MS (Agilent 1100 Series LC/MSD) and 400 MHz proton nuclear magnetic resonance spectroscopy (Brucker, Advance-series 400). The chemical shift values are reported in parts per million on the δ scale from internal standard tetramethylsilane. No attempt was made to maximize the yields.

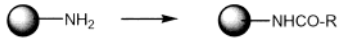
Mono-TBS-Protected FK506 (2) (*13*). FK506 (**1**, 1 g, 1.24 mmol) was dissolved with benzene, evaporated in vacuo, and then dried in vacuo for 1 h to remove water completely. CH_2Cl_2 (20 mL) was added and cooled under ice−water conditions, lutidine (0.58 mL, 4.97 mmol) and TBS-OTf (1.15 g, 4.35 mmol) were added thereto, and the mixture was stirred for 1 h. To the reaction mixture was added MeOH (0.2 mL), and then it was poured into a mixture of CH_2Cl_2 and sat. $NaHCO_3$. The separated organic layer was washed with water and brine and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo and then purified by chromatography on silica gel (eluted with 20% AcOEt in *n*-hexane). Fractions including the target compound were collected and evaporated in vacuo to give a 24,32-di-TBS protected FK506 derivative (0.62 g), which was used in the next reaction.

A mixture of the di-TBS protected FK506 (1.87 g, 1.81 mmol), toluenesulfonic acid monohydrate (86.1 mg, 0.453 mmol), CH_2Cl_2 (20 mL), and MeOH (20 mL) was stirred at rt for 1 h. The reaction mixture was poured into a mixture of AcOEt and sat. $NaHCO_3$. Separated organic layer was washed with water and brine and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo and then purified by chromatography on silica gel (eluted with 15% acetone in *n*-hexane). Fractions including the target compound were collected and evaporated in vacuo to give **2** (1.33 g, 80%). MS (*m*/*z*): 918 (M+), 940 (M + Na).

FK506 with a Linker Moiety (3). A mixture of **2** (138 mg, 0.15 mmol), suberic acid mono-TBDPS ester (86.7 mg, 0.218 mmol), DMAP, (16.5 mg, 0.098 mmol), EDC·HCl (69.1 mg, 0.261 mmol), and CH_2Cl_2 (1 mL) was stirred at room temperature for 1.5 h. This mixture was poured into a mixture of AcOEt and an aqueous solution of ammonium chloride. The organic layer was washed with water and brine and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo and then purified by chromatography on silica gel (eluted with 20% ACOET in *n*-hexane). Fractions including the target compound were collected and evaporated in vacuo to give a crude protected intermediate (44 mg, 24.6%). ^{1}H NMR ($CDCl_3$) δ: −0.1−0.1 (6H, m), 0.70−2.65 (71H, m), 2.69−3.96 (16H, m), 3.89−5.69 (10H, m), 7.35−7.69 (10H, m).

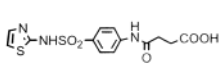
A mixture of the protected intermediate (44 mg, 0.037 mmol), 48% HF aqueous solution (0.12 mL), and acetonitrile was stirred at room-temperature overnight. This mixture was poured into a mixture of AcOEt and an aqueous solution of ammonium chloride. The organic layer was washed with water and brine and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo and then purified by chromatography on silica gel (eluted with 5% MeOH in $CHCl_3$). Fractions including the target compound were collected and evaporated in vacuo to give **3** (14.2 mg, 40%). ^{1}H NMR ($CDCl_3$) δ: 0.82 and 0.85 (3H, d, J = 6.5 Hz, Me), 0.91 and 0.94 (3H, d, J = 6.4 Hz, Me), 0.96 and 1.00 (3H, d, J = 6.5 Hz, Me), 0.8−1.2 (3H, m) 1.3−2.4 (39H, m), 2.46 (1H, m), 2.72 and 2.79 (1H, dd, J = 16, 2.4 Hz), 3.02 (1H, m), 2.95−4.0 (14 H), 3.76 and 4.42 (1H, m), 4.22 and 4.86 (1H, brs, OH), 4.69 (1H, dt, Jt = 9.2 Hz, Jd = 4.4 Hz, C*H*-OOC-linker), 4.61 and 5.02 (1H, m), 4.95−5.10 (4H, m), 5.19 and 5.32 (1H, each s, C*H*), 5.71 (1H, m, C*H*═CH_2). MS (*m*/*z*): 960 (M^+)

Table 1. Structure of Ligands Studied in This Work and Their CLOGP Values

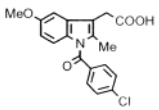


	R-COOH	CLOGP *
fatty acids		
5	CH_3COOH	-0.07
6	$CH_3(CH_2)_2COOH$	0.85
7	$CH_3(CH_2)_4COOH$	1.77
8	$CH_3(CH_2)_6COOH$	2.67
9	$CH_3(CH_2)_9COOH$	4.04
10	$CH_3(CH_2)_{12}COOH$	5.41
11	$CH_3(CH_2)_{14}COOH$	6.33
12	$CH_3(CH_2)_{16}COOH$	7.69
bioactive and related compounds		
13	$Ph(CH_2)_2COOH$	1.41
14	$PheNHCO(CH_2)_3COOH$	1.32
15	3-pyridylacetic acid	0.30
16	succinyl sulfathiazole	0.74
17	indomethacin	3.69
18	Ac-L-Trp-OH	0.13
4b	$_{32}$-FK506-COOH	7.48

succinyl sulfathiazole(**16**)



indomethacin(**17**)



Affinity Resin Bearing FK506 on Toyopearl (4b). A mixture of **3** (38.4 mg, 0.04 mmol), Toyopearl (Tosho, AF-Amino-650M, cat. 08002, 100 μL, 0.01 mmol), EDC·HCl (9.2 mg, 0.048 mmol), HOBt (6.5 mg, 0.048 mmol), and DMF (1 mL) was shaken at room temperature for 6 h. After removal of solvents by filtration, the resin was washed with DMF. The reaction ratio was determined by the ninhydrin test (82%). The resin was mixed with a 20% DMF solution of acetic anhydride at rt for 1 h and was washed with DMF and 20% aqueous solution of EtOH.

The synthesis of affinity resins bearing FK506 on AffiGel (**4a**, BIO-RAD, AffiGel 102 Gel, cat. 153−2401) and TentaGel (**4c**, Fluka, TentaGel S-NH_2, cat. 86364) were carried out according to a similar manner. AffiGel was used after washed with DMF five times. Other resins (**5**−**18**, Table 1) were prepared according to a similar manner.

Mono-*O*-Trityl-Protected Pentaethylene Glycol (20). To a mixture of pentaethylene glycol (**19**, 10 g, 42.0 mmol), DMAP (0.9 g, 7.4 mmol), and pyridine (100 mL)

was added triphenylmethyl chloride (11.6 g, 41.6 mmol) at rt, and the mixture was stirred at 35 °C overnight. After evaporation in vacuo, the resulting mixture was poured into a mixture of $CHCl_3$ and water. The organic layer was washed with sat. $NaHCO_3$ aq and brine and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo and then purified by chromatography on silica gel (eluted with 1.6% MeOH in $CHCl_3$). Fractions including the target compound were collected and evaporated in vacuo to give **20** (10.4 g, 51.2%). ^{1}H NMR ($CDCl_3$) δ: 3.16 (2H, t, J = 5.3 Hz, CH_2OTr), 3.49 (2H, m, CH_2OH), 3.55−3.66 (16H, m), 7.14−7.41 (15H, m).

2-(2-Tritylhydroxyethoxy-(2-ethoxy-(2-ethoxy-(2-ethoxy-(2-ethoxy)))))acetic Acid (21). To a mixture of **20** (10.2 g, 21.2 mmol), THF (200 mL), and DMF (50 mL) was added 60% sodium hydride (3.1 g, 77.4 mmol) under nitrogen atmosphere over 10 min at 0 °C, and the mixture was stirred at the same temperature for 30 min. After 2-bromoacetic acid (6.5 g, 46.8 mmol) was added thereto, the mixture was stirred at rt for 1 h and then cooled at 0 °C, and sodium hydride (11.6 g, 290 mmol) was added. The mixture was stirred at rt for 1 h and was poured into a mixture of AcOEt and water. This mixture was adjusted to pH 6 by addition of 2 M potassium hydrogen sulfate. The separated organic layer was washed with brine and dried over $MgSO_4$. After the filtration, filtrate was evaporated in vacuo and then purified by chromatography on silica gel (eluted with 15% MeOH in $CHCl_3$). Fractions including the target compound were collected and evaporated in vacuo to give crude **21** (12.4 g). ^{1}H NMR ($CDCl_3$) δ: 3.34 (2H, t, J = 5.2 Hz), 3.76−3.84 (18H, m), 4.13 (2H, s, CH_2COOH), 7.30−7.83 (15H, m).

2-(2-Tritylhydroxyethoxy-(2-ethoxy-(2-ethoxy-(2-ethoxy-(2-ethoxy)))))acetic Acid Benzyl Ester (22). A mixture of the above crude **21** (12.4 g), DMAP (0.29 g, 2.4 mmol), benzyl alcohol (3.1 mL, 30.0 mmol), EDC·HCl (4.5 g, 23.5 mmol), and CH_2Cl_2 (100 mL) was stirred at rt overnight. The reaction mixture was poured into a mixture of $CHCl_3$ and water. The separated organic layer was washed with sat. $NaHCO_3$ aq and brine and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo and then purified by chromatography on silica gel (eluted with 50% AcOEt in *n*-hexane). Fractions including the target compound were collected and evaporated in vacuo to give crude **22** (12.0 g, 90.1%). ^{1}H NMR ($CDCl_3$) δ: 3.16 (2H, t, J = 5.4 Hz), 3.55−3.65 (18H, m), 4.11 (2H, s), 5.11 (2H, s, CH_2Ph), 7.15−7.40 (20H, m).

2-(2-Hydroxyethoxy-(2-ethoxy-(2-ethoxy-(2-ethoxy-(2-ethoxy))))) acetic Acid Benzyl Ester (23). A mixture of **22** (12.0 g, 1.59 mmol), 5% TFA in CH_2Cl_2 (150 mL), and water (150 mL) was stirred at 0 °C for 10 min. The reaction mixture was poured into sat. $NaHCO_3$ aq. The separated organic layer was washed with brine and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo and then purified by chromatography on silica gel (eluted with 1.5% MeOH in $CHCl_3$). Fractions including the target compound were collected and evaporated in vacuo to give crude **23** (7.0 g, 95.0%). ^{1}H NMR ($CDCl_3$) δ: 3.30 (2H, t, J = 5.2 Hz, CH_2OH, 3.62−3.76 (18H, m), 4.12 (2H, s), 5.20 (2H, s), 7.36−7.41 (5H, m).

2-(2-Azidoethoxy-(2-ethoxy-(2-ethoxy-(2-ethoxy-(2-ethoxy)))))acetic Acid Benzyl Ester (24). To a mixture of **23** (7.0 g, 18.1 mmol), DMAP (0.4 g, 3.3 mmol), and pyridine (45 mL) was added *p*-toluenesufonyl chloride (5.2 g, 27.2 mmol) at 0 °C. After the mixture was stirred at rt overnight, *p*-toluenesufonyl chloride (5.2 g, 27.2 mmol) and DMAP (120 mg, 0.98 mol) were added thereto at 0 °C. The reaction mixture was stirred at rt for 2 h and poured into a mixture of EA and water. The separated organic layer was washed with sat. $NaHCO_3$ aq, water, and brine and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo to give a crude tosylate intermediate, which was used in the following reaction.

A mixture of this crude intermediate, NaN_3 (11.8 g, 0.18 mol), and DMF was stirred at 60 °C for 1 h. The reaction mixture was stirred at rt for 2 h and then poured into a mixture of AcOEt and water. The separated organic layer was washed with sat. $NaHCO_3$ aq, water, and brine and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo, and the resulting residue was purified by chromatography on silica gel (eluted with 25% AcOEt in *n*-hexane). Fractions including the target compound were collected and evaporated in vacuo to give crude **24** (3.3 g, 44.3%). ^{1}H NMR ($CDCl_3$) δ: 3.31 (2H, t, J = 5.2 Hz, CH_2N_3), 3.54−3.87 (18H, m), 4.13 (2H, s), 5.12 (2H, s), 7.20−7.30 (5H, m).

2-(2-Aminoethoxy-(2-ethoxy-(2-ethoxy-(2-ethoxy-(2-ethoxy)))))acetic Acid Benzyl Ester (25). A mixture of **24** (1.94 g, 4.72 mmol), 10% Pd−C (0.5 g), and MeOH (50 mL) was stirred under hydrogen atmosphere at rt for 2.5 h. After filtration, the filtrate was evaporated in vacuo, to give crude **25** (1.4 g, 100%) which was used in the next reaction without further purifications. MS (*m*/*z*): 296 (M^+)

2-(2-*N*-Fmoc-aminoethoxy-(2-ethoxy-(2-ethoxy-(2-ethoxy-(2-ethoxy)))))acetic Acid Benzyl Ester (26). To a mixture of **25** (1.25 g, 4.23 mmol) and 10% aqueous solution of $NaHCO_3$ was added a mixture of 9-fluorenylmethoxycarbonyloxysuccinimide (2.15 g, 6.37 mmol) and dimethoxyethane (14 mL) at rt, and the mixture was stirred at rt overnight. After filtration, the filtrate was washed with 2 M $NaHSO_4$ and brine and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo and then purified by chromatography on silica gel (eluted with 0.7% MeOH in $CHCl_3$). Fractions including the target compound were collected and evaporated in vacuo to give **13** (1.38 g, 63.0%). ^{1}H NMR ($CDCl_3$) δ: 3.34 (2H, t, J = 5.0 Hz, FmocNHCH_2), 3.50−3.71 (18H, m), 4.05 (2H, s), 4.12 (1H, t, J = 7.0 Hz), 4.33 (2H, d, J = 7.0 Hz), 5.57 (1H, s, FmocN*H*), 7.22−7.95 (8H, m). Anal. Calcd for $C_{27}H_{37}NO_{10}$ (monohydrate): C,60.55; H 6.96; N 2.62. Found: C, 60.72; H 6.92; N 2.42.

Representative Procedure for Introduction of the Hydrophilic Spacer on Resins. A mixture of **13** (21 mg, 0.04 mmol), Toyopearl (100 μL, 0.01 mmol), PyBOP (26 mg, 0.05 mmol), *N*,*N*-diisopropylethylamine (17 μL, 0.10 mmol), CH_2Cl_2 (0.4 mL), and NMP (0.1 mL) was shaken at rt for 4 h. After filtration, the resin was washed with DMF five times. The reaction ratio was determined by the ninhydrin test (81%). After the resin was washed with DMF carefully, 0.5 mL of a mixture (acetic anhydride:CH_2Cl_2:NMP = 1:8:2) was added for acetyl capping of remained amines. The reaction mixture was shaken at rt for 3 h and then washed with DMF more than five times. The capping reaction was confirmed by the ninhydrin test.

This Fmoc resin was mixed with 0.5 mL of a mixture (piperidine:DMF:CH_2Cl_2 = 1:4:4) at rt for 3 h. After filtration, the resin was five times washed with DMF to afford the objective resins bearing the hydrophilic spacer **13**. The amount of amine was estimated by the ninhydrin (79 μmol/mL).

Preparation of Rat Brain Lysate. Preparation of tissue extracts of rat brain. Fresh rat brain were homogenized (1:10, wt/vol) in buffer A. The homogenate was

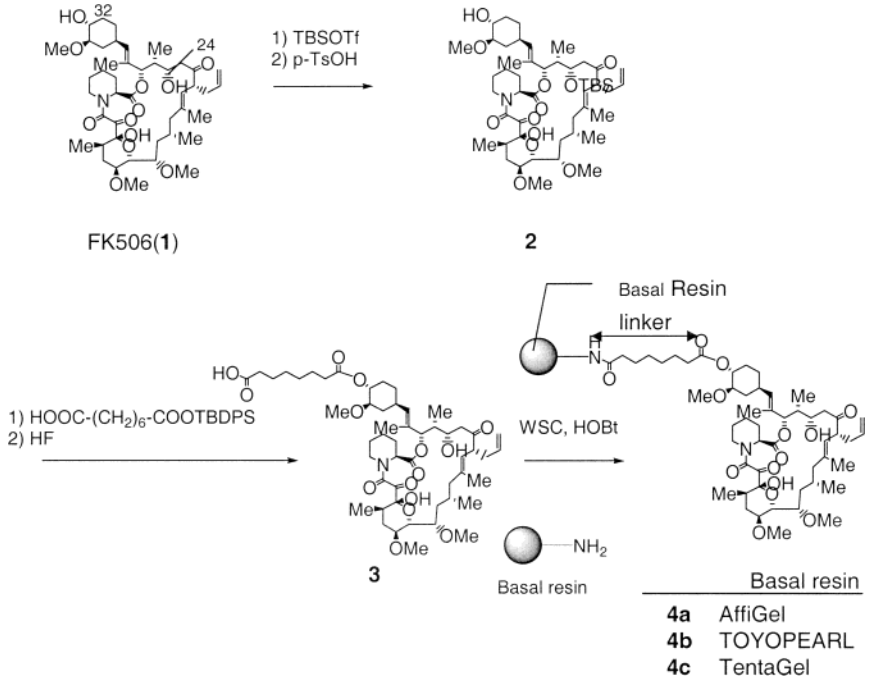


Figure 1. Synthesis of affinity resins bearing FK506.

centrifuged at 9500 rpm for 10 min. After supernatant was separated, it was centrifuged at 50 000 rpm for 30 min again. Obtained supernatant was used for lysate and kept at −80 °C before use.

Binding Assay on Affinity Resins. The lysate as crude tissue extracts were diluted by buffer A and total protein concentration was prepared about 7 mg/mL. This lysate was stirred calmly with affinity resin at 4 °C for about 15 h to adsorb the nonspecific binding proteins or specific binding proteins. A typical mixture has a total volume of 1.0 mL, consisting of buffer A, 10 μL of beads that is previously equilibrated by buffer A, and 0.5 mL of tissue extract. After incubation, the resins were precipitated by centrifugation in a microcentrifuge at 12 000 rpm for 1 min. The resins were washed five times with 1.0 mL of buffer A. The washed beads were then resuspended in 20 μL of SDS sample buffer solution (nakalai, sample buffer solution with 2-ME(2×) for SDS−PAGE, cat. 30566−22, including 4% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) BB, 10% (v/v) 2-mercaptoethanol, 0.125 M Tris pH 6.8), shaken at 25 °C for 10 min, and centrifuged for 1 min. The supernatant was subjected to SDS−PAGE followed by CBB staining.

RESULTS AND DISCUSSION

Nonspecific Protein Binding on Commercially Available Resins Bearing FK506. To understand the effect of polymer structure on the amount of nonspecific protein binding, we first synthesized affinity resins bearing a common ligand, FK506 (**1**, Figure 1), on three types of commercially available and widely used resins, AffiGel, Toyopearl, and TentaGel. AffiGel, a polymer of sugar derivatives, is thought to have hydrophilic surface properties and is now one of the most popular resins for preparation of affinity matrixes (*14*). AffiGel is not suitable for organic synthesis, because it is easily denatured in organic synthesis conditions and becomes denatured even in DMF (*15*). Thus, chemical approaches using this resin are limited. Toyopearl consists of poly(glycidyl methacrylate) whose epoxy moieties have been chemically converted to a hydrophilic spacer after the initial polymerization reaction (its detailed structure is not published) (*16*). TentaGel is a polystyrene polymer with long PEG spacers and is often used as resin for the synthesis peptide libraries (*17*).

FK506 (**1**) is an immunosuppressive drug that targets to FKBP12 (FK506 binding protein) with a K_d of 0.4 nM (*18*). Structure and functions of the complex of FK506 (**1**) and FKBP12 have been well-characterized at molecular level by S. L. Schreiber et al. (*19*−*21*). Since the molecular weight of FKBP12 (12 kDa) differs significantly from that of actin (42 kDa) and tubulin (50 kDa), it is easy to observe and compare the amounts of specific and nonspecific protein binding. Introduction of a linker moiety onto FK506 (**1**) to connect it to the resins was carried out on a hydroxyl group at the 32 position of FK506 (**1**, Figure 1) because crystal structure studies indicate that this position is not involved in the binding of FK506 with FKBP12 (*21*). The synthesis of affinity resins bearing FK506 (**1**) is shown in Figure 1. Selective protection of a hydroxyl group at the 24 position of FK506 (**1**) was carried out by two steps, and the linker moiety was introduced by a standard esterification protocol, followed by deprotection of TBS and TBDPS in acidic conditions, giving the FK506 derivative (**3**). Conjugation of **3** to the base resins, AffiGel, Toyopearl, and TentaGel, was performed by EDC and HOBt in DMF to give the affinity resins (**4a**−**c**). Although ligand immobilization onto AffiGel is usually carried out in an aqueous solution using excess amount of ligand and condensation reagents such as EDC and HOBt, or preactivated ligand, **3** was conjugated to AffiGel in DMF to conserve compound.

We mixed the FK506 bearing resins (**4a**−**c**) with lysate prepared from rat brain using a simple buffer (buffer A; 0.25 M sucrose, 25 mM Tris-HCl, pH 7.4). Rat brain lysate was thought to be suitable since it includes both the specific binding protein, FKBP12, and the nonspecific binding proteins such as tubulin and actin. The binding proteins to each affinity resin were completely eluted by a SDS sample buffer solution, after extensive washes using buffer A. Eluted proteins were resolved by SDS−polyacrylamide gel electrophoresis, and the identification of tubulin, actin, and FKBP12 were performed by MALDI-TOF mass spectrometry peptide mass fingerprinting after

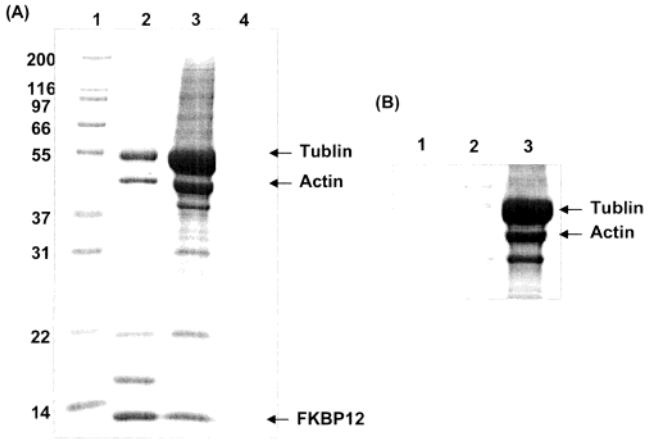


Figure 2. Binding proteins on the resins (A) eluted by SDS sample buffer, (B) eluted proteins by three different buffers. (A) Lane 1, molecular weight ladder; lane 2, eluted proteins from AffiGel bearing FK506; lane 3, that from Toyoperal bearing FK506, lane 4, that from TentaGel bearing FK506. (B) Lane 1, eluted proteins by 1.4 M NaCl; lane 2, that by 8 M urea; lane 3, that by the SDS sample buffer.

in-gel digestion. As determined by binding competition with soluble FK506, we confirmed that only FKBP12 specifically interacts with the FK506-bearing resins and that other proteins such as tubulin and actin are nonspecific binding proteins.

A comparison of the protein binding profiles using the three affinity resins is shown in Figure 2A. AffiGel resin (**4a**, lane 2) and Toyopearl resin (**4b**, lane 3) could capture the target protein, FKBP12. However, TentaGel (**4c**, lane 4) failed to sequester significant amounts of proteins including FKBP12 even though it is often used as a resin for peptide libraries. The reason for this result with TentaGel was not clearl (*22*). With respect to nonspecific protein binding, Toyopearl resin (lane 3, Figure 2A) bound several proteins with high efficiency, whereas the AffiGel support exhibited much less nonspecific binding (lane 2, Figure 2A). Nonspecifically bound tubulin and actin were observed in particularly large quantities, obscuring any specific protein binding in the 40−55 kDa molecular weight range. To understand the origins of actin and tubulin binding to the Toyopearl resin, we attempted to elute the proteins by three kinds of buffers (Figure 2B); that is, we first washed the Toyopearl resin by 1.4 M NaCl and 8 M urea, which are thought to be able to disturb ionic interaction between proteins and resins, and then washed by the SDS sample buffer solution. The SDS sample buffer is thought to disturb hydrophobic interaction as well, because it includes a high concentration (4%) of the surface-active agent, SDS. As shown in Figure 2B, the nonspecific binding proteins such as actin and tubulin were eluted only by this sample buffer, which indicated that the binding to Toyopearl resins come from hydrophobic interaction not hydrogen bonding, which coincides with Hofstee's result in which several proteins nonspecifically bind to *n*-octyl-agarose through hydrophobic interaction (*11*).

Assuming that binding of nonspecific protein binding to affinity resins is due to hydrophobic interactions, it is logical that much more nonspecific binding is observed with the Toyopearl resin than the AffiGel support, because the matrix surface of AffiGel is more hydrophilic than that of Toyopearl.

Quantitative Study on Relationship between Nonspecific Protein Binding and Ligand Hydrophobicity. Aiming to reduce the nonspecific binding of proteins to the Toyopearl resins, we attempted to quantify the relationship between amount of tubulin and actin binding and hydrophobic property of affinity resins. For this purpose, we synthesized fifteen affinity resins bearing various ligands on Toyopearl and profiled the proteins bound to these resins after the supports were mixed with the rat brain lysate. These ligands were arbitrarily selected, and their structures were shown in Table 1 with their CLOGP values. CLOGP values are a representative descriptor of a compound's hydrophobicity and are often used in QSAR (quantitative structure−activity relationships) studies were calculated by CLOGP program (Version 4.72, Daylight Chemical Information Systems Inc.). All ligands have a carboxylic acid which was used to immobilize the compounds to the amino group of Toyopearl resin as shown in Table 1. Compounds **5**−**12**, saturated fatty acids, were selected to discuss about effect of simple hydrophobicity of ligands. We also selected some compounds to address the effects of several chemical moieties such as phenyl ring (**13**), amide bond (**14**), pyridine ring (**15**), and amino acid (**18**), which often appear in bioactive compounds and drugs. Some drugs, such as succinyl sulfathiazole (**16**) and indomethacin (**17**), were also selected with FK506 (**4b**) to investigate the effects of drug structure.

Protein binding to each affinity resin was shown in Figure 3A. Proteins binding to the resins were obtained by elution using the SDS sample buffer solution. Proteins from resins bearing hydrophilic ligands such as acetic acid (lane 5), butyric acid (lane 6), 3-phenylpropionic acid (lane13), glutaralinic acid (lane 14), 3-pyridylacetic acid (lane 15), and *N*-acetyltryptophan (lane 18) were almost undetectable. Proteins were observed from other resins while the amount was different each other. Amounts of proteins in lane 7 and 8 were very low compared with others. The large difference in amounts between lane 8 and lane 9 was particularly noteworthy because their structural difference is only three methylenes, indicating that this difference arose from nonstructural distinction. Visible protein species in Figure 3A were similar to each other and almost the same as that from the FK506 resin except for two unknown proteins (A, B) in lane 16 and FKBP12 in lane 4b, while the structures of each ligand on the resin were very diverse. Proteins at 36 KDa in lane 10, 11, 12, and 17 were identified as glyceraldehydes-3-phosphate dehydrogenase [EC 1.2.1.12] by mass spectrum analysis; however, we have not determined whether the 36 kDa bands in lane 9 and 4b are also the same enzyme or not. We also do not know the physiological relevance of the binding of this enzyme to fatty acid-containing resins. On the other hand, the total amount of nonspecific binding differed in a manner that correlated with ligand hydrophobicity. For example, resins bearing less hydrophobic ligands (CLOGP < 1.5) such as acetic acid (**5**), butyric acid (**6**), phenylpropionic acid (**13**), glutaralinic acid (**14**), 3-pyridylacetic acid (**15**), and succinyl sulfathiazole (**16**) exhibited little nonspecific binding, while those bearing hydrophobic ligands (CLOGP > 1.5) sequestered much more nonspecific protein. To address this relationship quantitatively, we measured the amount of the largest proteins, tubulin and actin, and plotted them with CLOGP values of ligands (Figure 3B).

Measurement of actin and tubulin on the gel were carried out by a GS-710 Calibrated Imaging Densitometer (BIO-RAD, software; Quantity One-4.1.0). In lane 16, there were three visible and unidentified proteins (11, 34, and 36 kDa proteins); however, tublin and actin were not detected. This plot demonstrated that the amount of nonspecific protein binding has a linear relationship with

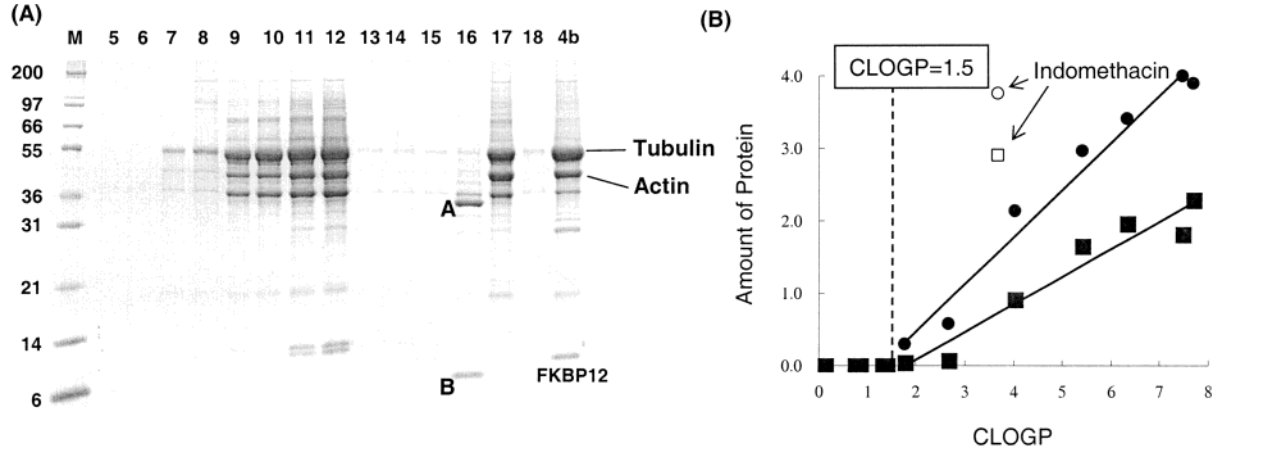


Figure 3. (A) Binding proteins on resins bearing various ligands. (B) A plot of estimated amount of tubulin and actin with CLOGP values of each ligands on the resins. (A) Lanes 4b, 5−18 showed binding proteins on each resin bearing compounds **4b**, **5**−**18** (Table 1), respectively. (B) The amount of actin and tubulin was measured by a GS-710 Calibrated Imaging Densitometer (BIO-RAD, software; Quantity One-4.1.0). The linear equations were obtained by Microsoft Excel 2002. CLOGP values were calculated by CLOGP program (Version 4.72, Daylight Chemical Information Systems Inc.).

a simple descriptor for a compound's hydrophobicity, CLOGP. Indomethacin-containing resin (**17**) is the only exception for this trend, demonstrating much more nonspecific binding than would be expected for its CLOGP value. A statistical analysis of the other 14 affinity matrixes resulted in the following algorithms:

$$\text{(amount of tubulin)} = 0.73 \times \text{CLOGP} - 1.1 \ (n = 7, r = 0.97) \ \text{(A)}$$

$$\text{(amount of actin)} = 0.42 \times \text{CLOGP} - 0.79 \ (n = 7, r = 0.99) \ \text{(B)}$$

when CLOGP values of compounds were over 1.5, except for that bearing indomethacin (Figure 3B). The reason for the unusually high levels of nonspecific binding observed with the indomethacin-containing resin is not clear.

To our knowledge, this is the first report to quantitatively describe the relationship between nonspecific protein binding and a single descriptor of ligand character with excellent regression factors, 0.97 for tubulin (eq A) and 0.99 for actin (eq B), respectively.

Toyopearl resin bearing ligands whose CLOGP value is less than 1.5 in this work exhibit little nonspecific protein binding. In this study, we found the relationship between ligand hydrophobicity and the amount of actin and tubulin capture; however, we observed the relationship using only a series of affinity resins which consist of Toyopearl as the base resin and the 15 ligands shown in Table 1. Thus, now we actually do not have enough data to discuss whether this relationship can be generally observed in other series of affinity resins or if the 1.5 CLOGP value is universal or variable. We believe that the critical CLOGP value will be more than 1.5 when a hydrophilic basal resin such as AffiGel is used since the hydrophobicity of the resin−ligand conjugate could be a vital factor in addition to that of ligand alone.

These results indicated the representative nonspecific binding proteins, actin and tubulin, bind to hydrophobic affinity resins without regard to molecular shapes of ligands. High-affinity binding to the resin was indicated because these proteins were not eluted by washing with 1.4 M NaCl and 8 M urea, as shown in Figure 2B. These results are surprising because high-affinity protein−ligand interactions are generally believed to involve a "lock and key" relationship (*20*). It is difficult to imagine a common pharamacophore of stearic acid and FK506, yet their affinity resins gave very similar proteins as shown in Figure 3A. We believe that the reason for this unusual binding could be that the proteins denature to variable degrees (*21*) upon contact with hydrophobic ligands in an aqueous solution. Such protein denaturation would likely involve the binding of aromatic and aliphatic amino acids by the resin-bound ligands, disrupting the core hydrophobic interactions that maintain protein stability.

Chemical Approach To Reduce Nonspecific Protein Binding. The results in Figure 3B indicate that an increase of ligand hydrophilicity on Toyopearl resins can decrease the amount of nonspecific protein binding. Therefore, we next attempted to reduce nonspecific protein binding by introduction of a hydrophilic spacer between the base resin and ligands. We designed a hydrophilic spacer based upon poly(ethylene glycol) (PEG) (**26**, Figure 4). PEG derivatives are known to be hydrophilic and are sometimes used as hydrophilic moieties in surface-active agents (*25*). PEG derivatives are also known to have few unfavorable biological effects. These properties of PEG derivatives were thought to be suitable for a spacer moiety in affinity resins, as seen with TentaGel (*17*). The hydrophilic spacer (**26**) has an amino acid-like structure that allows study of its repetition effects while usual PEG derivatives are synthesized by the polymerization process and their exact structure is not known (*26*). The amino group was protected by an Fmoc (9-fluorenylmethoxycarbonyl) to facilitate the synthesis of polymer on resin by the Fmoc strategy. FK506 was also used as a ligand on the affinity resin to compare to that in Figure 1B.

The synthesis of hydrophilic spacer (**26**) is shown in Figure 4A. Single protection of a pentaethylene glycol (**19**) was carried out by using trityl chloride, followed by alkylation of the remaining hydroxyl group to introduce the acetic acid moiety, to give **21**. After introduction of a benzyl group at the carboxylic acid, the trityl group was removed under acidic conditions to afford **23.** The hydroxyl group was converted to an azide group via a *p*-toluenesulfonyl group (**24**). Both the conversion of azide group to amino group and the removal of the benzyl group were performed by hydrogenation. Finally, an Fmoc group was introduced to the amino group to give the desired Fmoc-protected hydrophilic spacer monomer (**26**). The synthesis of FK506 affinity resins bearing the

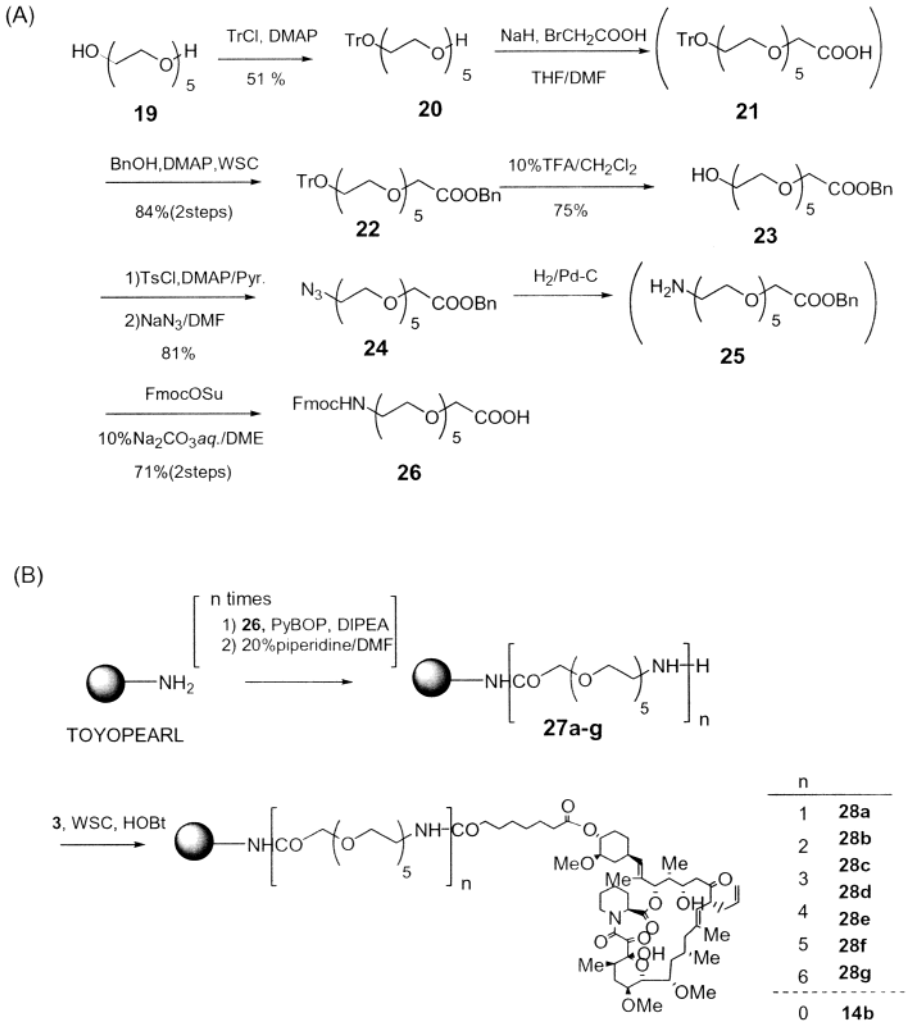


Figure 4. (A) Synthesis of a hydrophilic spacer (**26**). (B) Synthesis of FK506 affinity resins bearing *n* mers of the hydrophilic spacer between solid phase and FK506.

hydrophilic spacer between solid phase and FK506 is shown in Figure 4B. Toyopearl resin was used as basal base similar to that of Lane 3 in Figure 1B. Repetitions of the spacer monomer (**26**) on resins were carried out by the Fmoc strategy using PyBOP as a condensation reagent. Finally, introduction of FK506 derivative with the linker moiety at the end of the PEG moiety was performed by using EDC and HOBt to give the desired resin (**28a**−**g**).

We analyzed the protein binding to each resin after mixing with rat brain lysate as shown in Figure 1B (Figure 5). As shown in Figure 5A, introduction of the hydrophilic spacer was very effective for reduction of the nonspecific protein binding, as expected, and the repetition effect is also obvious while the binding of the target protein, FKBP12, is almost constant throughout the resin series (Figure 5B). The affinity resin bearing a hexamer of the hydrophilic spacer ($n = 6$, lane 8 in Figure 5A) yields the same amount of FKBP12 to that without any spacer ($n = 0$, lane 2 in Figure 5A) while giving over four times less amount of nonspecific binding proteins. These results reinforce our observations shown in Figure 3B and demonstrate the effectiveness of the introduction of hydrophilic spacer. The effectiveness by introduction of the spacer in Figure 5A was obvious, but the estimated effect in Figure 5B did not seem to be consistent with that in Figure 5A; that is, the amount of tubulin and actin in lane 2 appear to be less. We thought the amount of protein was underestimated by the imaging densitometer because the shape of the tubulin band was irregular due to an excessive amount on the gel.

The unfavorable proteins were still observed even in lane 8, and Figure 5B indicated that the effect appeared to reach near the maximum, but their amounts were still greater than that of the target protein, FKBP12. We thought the most important factor suggested by Figure 3A is that reduction of the hydrophobicity near the resin surface is vital to reduce the amount of proteins. Thus, the first introduction is very drastic (lane 3 compared with lane 2 in Figure 5A), because it causes introduction of hydrophilicity near the surface. However, addition of the hydrophilic spacer causes introduction of hydrophilicity at an area far from the surface because of repetition, so that the effectiveness decreased. This prompted our next project in which hydrophilicity was introduced near the surface to reduce the unfavorable proteins, which will be reported in the future.

CONCLUSION

Tubulin and actin are often found as nonspecific binding proteins in affinity chromatography studies, interfering with research toward the identification of drug targets. Reduction of nonspecific protein binding is important for the success of such biochemical approaches. Although it is known that nonspecific binding proteins tend to stick to hydrophobic affinity resins, there is no systematic and quantitative study on the relationship

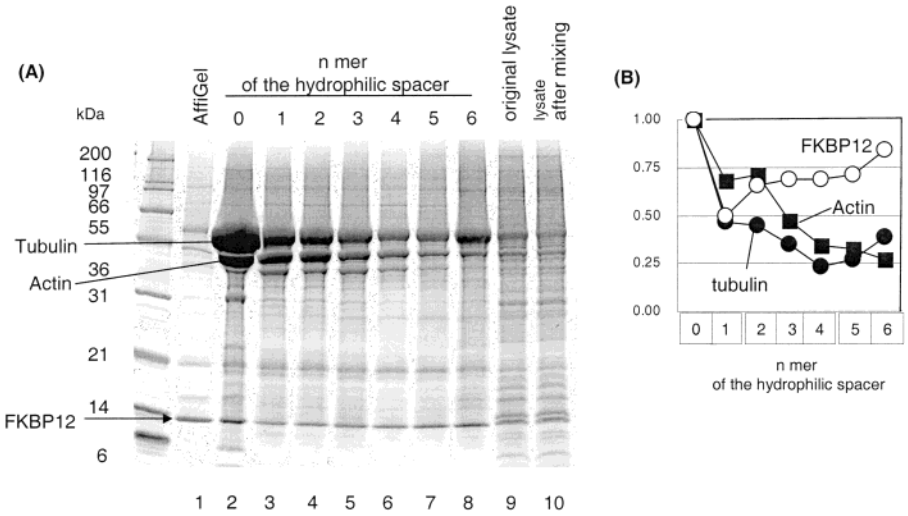


Figure 5. (A) Reduction effect on introduction of the hydrophilic spacer. (B) A plot of the estimated amount of nonspecific binding proteins (tubulin and actin) and the specific binding protein (FKBP12) with repetition numbers of the spacer. (A) Lane 1, molecular weight maker; lane 2, AffiGel bearing FK506 (**4a**); lane 3, Toyopearl bearing FK506 without the hydrophilic spacer ($n = 0$, **4b**), lane 4−9, that with n mer of the hydrophilic spacer ($n = 1$ to 6, **28a**−**g**); lane 10, original lysate prepared from rat brain; lane 11, lysate after mixed with **4b**. (B) The amount of actin and tubulin was measured by a GS-710 Calibrated Imaging Densitometer (BIO-RAD, software; Quantity One-4.1.0), and all were represented as a ratio compared to the value of lane 2 ($n = 0$).

between affinity resin hydrophobicity and amount of the nonspecific protein binding. We therefore first compared nonspecific binding proteins on three kinds of affinity resins, consisting of commercially available and widely used base resins AffiGel, Toyopearl, and TentaGel, with a common ligand, FK506 (Figure 2). This study showed that the hydrophilic resin, AffiGel, has low levels of nonspecific protein binding while Toyopearl exhibits much higher levels. Unfortunately, AffiGel is not chemically stable while functional polymers, Toyopearl and TentaGel, are stable in most chemical conditions, which restricts chemical modification and synthesis of desired affinity resins. Thus, the development of novel methods to reduce nonspecific protein binding to functional polymers such as Toyopearl is needed.

To reduce the unfavorable proteins, we first scrutinized the relationship between ligand hydrophobicity and the amount of the nonspecific protein binding using a series of 15 affinity resins bearing variant ligands on Toyopearl (Table 1, Figure 3A). These results showed that the amount increased in proportion to a simple descriptor of hydrophobicity of ligands, CLOGP (Figure 3B). A statistical calculation on the amount of representative nonspecific binding proteins, actin and tubulin, with CLOGP of ligands exhibited a strong correlation; that is, (amount of tubulin) $= 0.73 \times$ CLOGP $- 1.1$ ($n = 7$, $r = 0.97$), (amount of actin) $= 0.42 \times$ CLOGP $- 0.79$ ($n = 7$, $r = 0.99$) when CLOGP values of compounds were over 1.5, with the exception of Toyopearl conjugated to indomethacin. On the other hand, resins bearing hydrophilic ligands, whose CLOGP is less than 1.5, showed small amounts of actin and tubulin. According to our knowledge, this is the first report that shows the a quantitative relationship between the amount of nonspecific protein binding and affinity resin hydrophobicity.

Since this relationship suggested that reducing ligand hydrophobicity will be effective for the minimizing of the nonspecific protein binding to affinity matrixes, we next attempted to introduce hydrophilic spacer (**26**) between the ligand and base resin (Figure 5A and 5B). The amount of nonspecific protein binding proteins decreased in proportion to the number of spacer monomers while that of the target protein, FKBP12, is essentially constant. These results exhibited the effectiveness of the introduction of a hydrophilic spacer to reduce the unfavorable proteins on Toyopearl resin.

It is known that the addition of surface-active agents into affinity chromatography buffers is effective for reducing nonspecific protein binding, because such reagents can noncovalently convert hydrophobic surfaces to hydrophilic ones. But the use of surface-active agents is not always useful because their addition sometimes causes the denaturation of the target proteins while the introduction of a hydrophilic spacer is not thought to affect the activities of proteins. Dextran, a sugar derivative and similar to agarose, has been used as a hydrophilic spacer to reduce nonspecific binding as well (*27*); however, it is chemically unstable like that of AffiGel. On the other hand, the PEG type of hydrophilic spacer (**26**) is chemically stable, making it suitable for a variety of applications. We believe that the utility of the PEG type spacer is not restricted to affinity resins but that these moieties could also have useful applications in the reduction of nonspecific protein binding on artificial materials in life science. We also apply the spacer on gold surfaces for SPR (surface plasmon resonance) chemistry to reduce noise in the BIACORE experiments and observed similar results described in this paper (data will be reported). Therefore, results described in this work could be effective for reduction of nonspecific protein binding in a wide range of experimental studies involving native proteins.

The nonspecific binding proteins were still observed even after repeated introduction of the spacer (Figure 5A), which indicated that the effect reaches a maximum and that it may be difficult to remove them completely only by introduction of the PEG spacer. We are now developing other hydrophilic and chemically stable spacers and other methods to introduce hydrophilicity on the surface of affinity resins to completely eliminate the nonspecific proteins. Their synthesis and application will be reported in the future.

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(15) As described in the text, AffiGel is stable in aqueous solution and usually used in aqueous solutions. However, it is impossible to use an excess amount of ligand for immobilization since we usually synthesize the compound with linker moiety via more than several synthetic steps, and only small amounts are available. Thus, the immobilization reaction, such as amide formation, needed to be carried out in organic solvent to avoid the presence of water. But AffiGel is sometimes irreversibly denatured in organic solvent and sometimes gives false-positive targets; for example, we found a novel nonspecific binding protein when we synthesized affinity material bearing FK506 in acetonitrile while it was not detected when prepared in an aqueous solution.

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(24) It was known that enzyme activities were still observed after absorption on *n*-octyl-agarose (*11*). These results indicated that the conformation of proteins was not unique; that is, some of them were completely denatured and others were partially denatured; moreover, native proteins can also bind on the surface that are occupied by various conformational states of the enzymes.

(25) There are many surface-active agents that include PEG in their structures as a hydrophilic moiety; for examples, polyoxyethylene(23) lauryl ether ("Brij 35") and other "Brij" series, polyoxyethylene branched monocyclohexyl ether ("Triton") and other "Triton" series, and polyoxyethylene(20) sorbitan monolaurate ("Tween20") and others in the "Tween" series.

(26) For example, the average repetition number of oxyethylene unit of TentaGel's PEG moiety is 68, and the number is not unique.

(27) Dextran is used as hydrophilic spacer in the Biacore chips. Dexatran plays as a spacer between gold foil and ligands in the Biacore system. We have observed nonspecific binding proteins after treatment of the gold foil bearing a ligand via the Dextran spacer with acetonitrile (data not shown).

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