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# Development of chemically stable solid phases for the target isolation with reduced nonspecific binding proteins

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**Abstract**—Poly(methacrylate) matrices for affinity resins were designed and synthesized based on our previous results that nonspecific protein absorption on affinity resins strongly depended on their hydrophobic property. The novel affinity resins bearing FK506 (**6a**, **6b**) captured specific binding protein, FKBP12, with a small amount of nonspecific binding proteins. The amount of nonspecific binding proteins on **6a**–**6b** was much reduced compared to that on commercially available poly(methacrylate) resins, Toyopearl™ (**8**), and was almost the same as that on one of the most popular resins, Affigel™ (**9**). Interestingly, **6a** and **6b** could isolate FKBP52 as a specific binding protein as well, although **8** and **9** could not.
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Affinity resins bearing bioactive compounds such as natural products, drugs, and toxins play an important role in the discovery of novel drug targets and the elucidation of drug mechanisms.[1,2] 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. Nonspecific binding of cellular proteins to affinity matrices is therefore a significant limitation to this approach.[3,4]

There are now some commercially available solid materials for preparation of affinity resins. Affigel™, agarose derivatives,[5] is one of the most popular matrices for this purpose. In our previous study, it was shown that the amount of nonspecific protein absorptions to Affigel™ was very small, which could be due to its high hydrophilic property.[3] However, Affigel™ is not suitable solid phases for organic synthesis, because it is easily denatured under organic synthesis conditions.[3] Thus, chemical approaches using this resin are often limited. In contrast, Toyopearl™, a poly(methacrylate) derivative,[6] is stable under most synthetic conditions, which allow the synthesis of more effective affinity resins. However, these methacrylate polymers bearing bioactive compounds often show high levels of nonspecific binding protein with the target protein.[3] Therefore, development of novel solid phases that are chemically stable as poly(methacrylate) derivatives and are hydrophilic enough for elimination of the nonspecific absorption as agarose ones is an important goal.

The phrase ‘nonspecific protein binding’ is usually used to represent proteins that bind to affinity resins based on physical adsorption.[7,8] In a previous paper,[3] we reported a linear relationship between hydrophobicity of ligands and the amount of nonspecific binding proteins, and effectiveness of introduction of hydrophilic spacers for reduction of those nonspecific binding proteins. However, it was difficult to eliminate such nonspecific binding proteins completely by introduction of them. We thought that it was difficult entirely to cover the hydrophobic surface by the introduction of hydrophilic spacers because those spacers were not always spatially close to the hydrophobic surfaces. Thus, we next attempted to introduce those hydrophilic spacers into monomers before polymerization reactions for improvement in reducing the nonspecific binding proteins because it could be effective to construct more hydrophilic surfaces on the solid phases comparing with introduction of hydrophilic spacers after polymerization reactions. We herein report the design and synthesis of novel methacrylate derivative bearing hydrophilic spacers and its polymers for the target isolation studies, and

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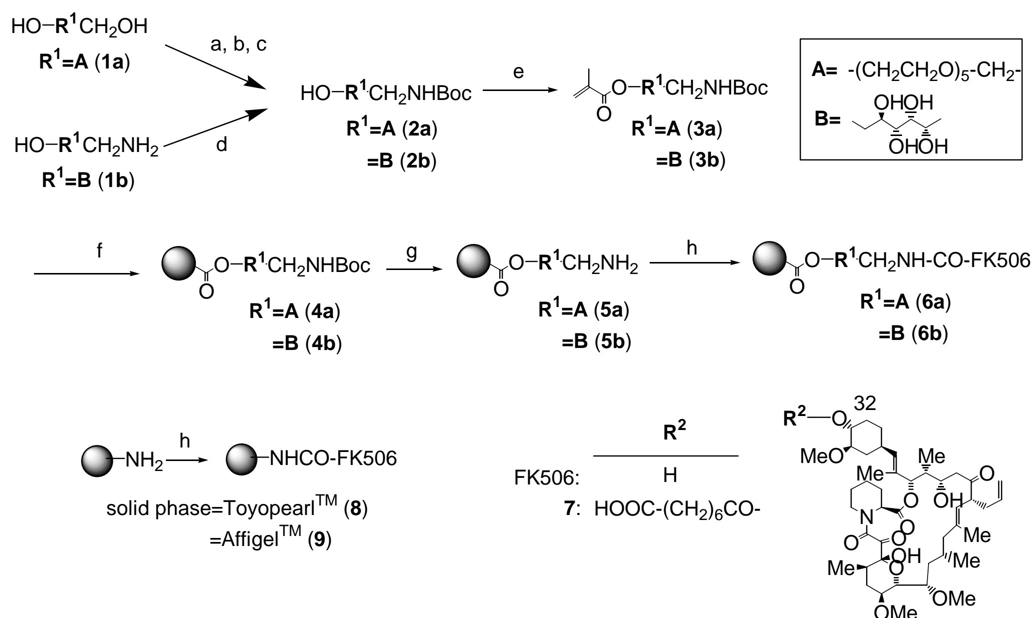
their effectiveness in reduction of nonspecific binding proteins maintaining the specific interactions.

We designed methacrylate monomers bearing poly(ethyleneglycol) (**3a**) and D-glucamine (**3b**) moieties because introductions of those moieties to Toyopearl™ as hydrophilic spacers resulted in much reduction of the nonspecific binding proteins maintaining binding with the specific binding protein in our previous studies.[3,4] These novel monomers were synthesized as shown in Scheme 1.[9] Replacement of a hydroxyl group of hexaethylene glycol (**1a**) with an amino group was carried out via a phthalimide derivative, followed by introduction of a *tert*-butoxycarbonyl (Boc) group on primary amine to give **2a**. A Boc group was introduced on amino group of D-glucamine (**1b**) which afforded **2b**. **2a** and **2b** were coupled with methacryloyl chloride in the presence of *N*,*N*,*N*′, *N*′-tetramethyl-1,3-diaminopropane to afford the methacrylate monomers **3a** and **3b**, respectively. These monomers were polymerized by a solution polymerization method with 2% glycerol dimethacrylate, a well-known crosslinker reagent, in dioxane using azobisisopropyronitrile (AIBN) as an initiator, which gave N-Boc protected solid phases (**4a**–**4b**). AIBN was purchased from Wako Pure Chemical and purified through re-crystallization technique. After grinding by a mortar grinder, Boc groups were removed by treatment with trifluoroacetic acid (TFA) to give desired solid phases **5a** and **5b**. A FK506 derivative with a linker moiety (**7**) was immobilized on **5a** and **5b** to give **6a** and **6b**, respectively. FK506, an immunosuppressive drug, was specifically bound to FKBP12, a FK506 binding protein 12 kDa, with a $K_d$ of 0.4 nM,[9] and was often used as standard compound for assessment of affinity chromatography.[3,4,10] The design of **7** was performed based on an X-ray structure of FK506 and FKBP12 that exhibited a hydroxyl group at the 32 position was not involved in the interaction.[3] FK506-affinity resins using the commercially available matrices, Toyopearl™ (**8**) and Affigel™ (**9**), were also prepared.

To assess capacity of the FK506-affinity resins (**6a**, **6b**, **8**, and **9**) to capture the specific binding protein, FKBP12, specifically, these resins were mixed with lysate obtained from rat brain, and proteins bound on them were comparatively analyzed.[11] This lysate was known to include FKBP12 with other miscellaneous proteins.[3,4] After extensive washes with lysate buffer, binding proteins were completely eluted by SDS sample buffer solution and analyzed by SDS–polyacrylamide gel electrophoresis (Fig. 1). As shown in Figure 1, all FK506 affinity resins successfully captured FKBP12. However, a large amount of nonspecific binding proteins such as tubulin (Fig. 1A), actin (panel B), and glyceraldehyde-3-phosphate dehydrogenase (panel C) were found on FK506-Toyopearl resins (**8**). On the other hand, these nonspecific binding proteins were little observed on **6a** and **6b**, while these resins consisted of the same poly(methacrylate) polymers as well as **8**. The amount of nonspecific binding proteins was almost the same as that on the hydrophilic polymer, Affigel™ (**9**). These results supported the speculations that the amounts of nonspecific binding proteins strongly depends on hydrophobicity of affinity resins. Specificity of binding proteins to FK506-affinity resins was confirmed by the competition method.[11] Interestingly, another specific binding protein (D, Fig. 1A) was observed on **6a** and **6b**. Analysis by LC–MS/MS ions search method using an ESI ion trap mass spectrometry (Thermoelectron, LTQ) after in-gel digestion exhibited that this band was another FK506




**Scheme 1.** Synthesis of affinity resins bearing FK506.[13] Reagents and conditions: (a) phthalimide, triphenylphosphine, and diisopropyl azodicarboxylate/THF; (b) $NH_2NH_2$/MeOH; (c) $Boc_2O$, $K_2CO_3$/MeOH; (d) $Boc_2O$/MeOH; (e) methacryloyl chloride, *N*,*N*,*N*′,*N*′-tetramethyl-1,3-diaminopropane/acetonitrile; (f) AIBN, 2% glycerol dimethacrylate/dioxane, 100 °C; (g) TFA/$CH_2Cl_2$/dioxane/$H_2O$ = 50:39:10:1; (h) **7**, EDC HCl, HOBt/NMP.

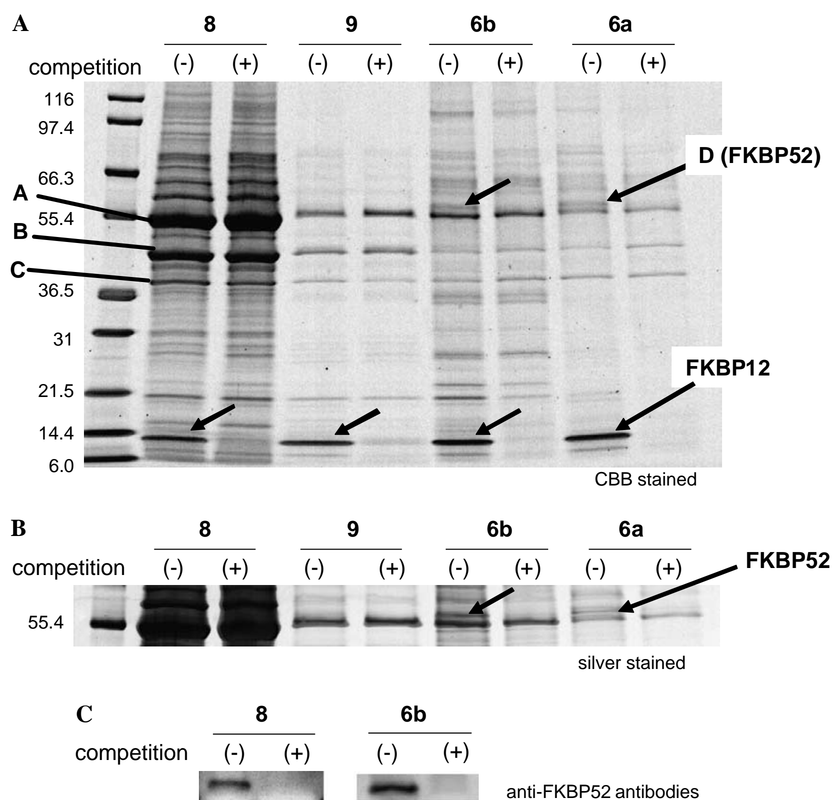



**Figure 1.** Binding proteins on affinity matrices bearing FK506 (**6a**, **6b**, **8**, and **9**). (A) Each resin (10 µL, 1 µmol FK506 on resins) was mixed with 1 mL of lysate obtained from rat brain at 4 °C for 1 h.[12] After separation of the resins by centrifugation and extensive washes with lysate buffer (25 mM Tris–HCl, pH 7.4, 0.25 M sucrose, 0.3 mM *N*,*N*-diethylthiocarbamate, 2 mM $CaCl_2$, and 2 mM $MgCl_2$), binding proteins were completely eluted by SDS sample buffer solution and analyzed by SDS–polyacrylamide gel electrophoresis (stained by Coomassie brilliant blue (CBB)). FKBP12 and FKBP52 (D) are known as specific binding proteins to FK506. Other proteins such as tubulin (A), actin (B), and glyceraldehyde-3-phosphate dehydrogenase (C) are known to be nonspecific binding proteins. Identification of proteins was performed by MS/MS ions search method using an ESI ion trap mass spectrometry (Thermoelectron, LTQ) after in-gel digestion, respectively. A commercially available apparatus (Dainippon Seiki Co., Ltd., cat. code: 1D-SDS) was used for cutting off each strip including the desired protein from CBB or silver-stained SDS-gels. One micromole of FK506 was added for the competition experiment. (B) FKBP52 was successfully isolated by **6a** and **6b**, not by **8** and **9**. The same SDS gel to the above was stained by silver after decolorization of CBB stain. (C) Western blot studies using anti-FKBP52 antibodies on **8** and **6a**.[14]

specific binding protein, FK506-binding protein 52 (FKBP52, MW = 51.7 kDa).[12] FKBP52 could not be detected on Affigel™ bearing the same ligand (**9**). The reason for this difference between our resins (**6a**–**6b**) and agarose ones (**9**) is not clear now. FKBP52 was not detected on Toyopearl™ resins bearing FK506 (**8**) (Fig. 1B) because of the existence of large amounts of nonspecific binding proteins such as tubulin while FKBP52 was detected on it by Western blot analysis on FKBP52 (Fig. 1C). These results demonstrated the importance of reduction of the nonspecific binding proteins for identifications target finding.

Since **6a** and **6b** are poly(methacrylate) polymers, they are thought to be stable in several synthetic conditions in contrast to Affigel™ and other sugar polymers. For example, the poly(methacrylate) polymers (**4**–**6**) were stable under the synthetic conditions studied in this work. Among them, the stability of **4b**, **5b**, and **6b** was notable because these polymers had a sugar-like structure that was similar to that of Affigel™.

These results demonstrated the effectiveness of our novel solid phases for affinity resins. To assess generality of the resins, isolation studies using other ligands are in process and those results are going to be reported. Polymerization of other matrices using the monomers (**3a**–**3b**) by other methods is in progress because **6a** and **6b** were powdered by a mortar grinder after a solution polymerization and had heterogeneous distributions of particles (Fig. S1). Results on these novel solid phases for target identification will be reported in the future.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.09.011.

## References and notes


1. Harding, M. W.; Galat, A.; Uehling, D. E.; Schreiber, S. L. *Nature* **1989**, *341*, 758.
2. Taunton, J.; Hassig, C. A.; Schreiber, S. L. *Science* **1996**, *272*, 408.
3. Tamura, T.; Terada, T.; Tanaka, A. *Bioconjugate Chem.* **2003**, *14*, 1222.
4. Shiyama, T.; Furuya, M.; Yamazaki, A.; Terada, T.; Tanaka, A. *Bioorg. Med. Chem.* **2004**, *12*, 2831.
5. http://www.bio-rad.com/.;
6. http://www.tosoh.com/EnglishHomePage/tchome.htm.;
7. Hofstee, B. H. J. *Adv. Exp. Med. Biol.* **1974**, *42*, 43.
8. Hofstee, B. H. J. *Biochem. Biophys. Res. Commun.* **1973**, *53*, 1137.
9. Siekierka, J. J.; Hung, S. H.; Poe, M.; Lin, C. S.; Sigal, N. H. *Nature* **1989**, *341*, 755.
10. Shimizu, N.; Sugimoto, K.; Tang, J.; Nishi, T.; Sato, I.; Hiramoto, M.; Aizawa, S.; Hatakeyama, M.; Ohba, R.; Hatori, H.; Yoshikawa, T.; Suzuki, F.; Oomori, A.; Tanaka, H.; Kawaguchi, H.; Watanabe, H.; Handa, H. *Nat. Biotechnol.* **2000**, *18*, 877.
11. The preparation of lysate, binding experimental for capturing the binding proteins, and analysis of proteins were carried out in similar ways to that described in Ref. 3.
12. Peattie, D. A.; Harding, M. W.; Fleming, M. A.; DeCenzo, M. T.; Lippke, J. A.; Livingston, D. J.; Benasutti, M. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 10974.
13. A 40% toluene solution of diisopropyldiazocarboxylate (93.5 mL, 0.19 mmol) was added to a mixture of hexaethylene glycol (**1a**, 50 g, 0.18 mmol), phthalimide (29.1 g, 0.2 mmol), triphenylphosphine (52.4 g, 0.2 mmol), and tetrahydrofuran (THF, 500 mL) over 15 min at 0 °C, and was stirred at room temperature (rt) for 15 h. After evaporation in vacuo, the resulting residue was dissolved with a mixture of chloroform ($CHCl_3$) and water. The separated organic layer was dried over $Na_2SO_4$. After filtration, the filtrate was evaporated in vacuo to give crude mono-phthalimide derivative, which was used for the next reaction without further purification. This crude mono-phthalimide derivative was mixed with hydrazine (17 mL, 0.36 mmol) and methanol (MeOH, 300 mL), and was heated at reflux for 3 h. After evaporation in vacuo, the resulting residue was dissolved by chloroform. After removal of insoluble matters by filtration, the filtrate was dried over $Na_2SO_4$. After filtration, the filtrate was evaporated in vacuo. The residue was applied to the column chromatography (silica gel, 200 g), and was eluted with $CHCl_3$ and 50% MeOH/$CHCl_3$. Fractions including the objective were collected and evaporated in vacuo, to give a mono-amino derivative (21.4 g) A mixture of the mono-amino derivative (20.7 g, 73.5 mmol), di-*tert*-butyl dicarbonate (16.1 g, 73.5 mmol), potassium carbonate (20.3 g, 147 mmol), and MeOH (50 mL) was stirred at rt. After 15 h, the reaction was poured into a mixture of a saturated aqueous sodium bicarbonate and $CHCl_3$. The organic layer was washed with saturated sodium chloride (300 mL) and dried over $Na_2SO_4$. After filtration, the filtrate was evaporated in vacuo to afford **2a** (28 g, 42%). $^1$H NMR ($CDCl_3$) $\delta$ : 1.44 (9H, s, *t*Bu), 3.32 (2H, m, –NHC$H_2$CH$_2$), 3.53–3.74 (22H, m, OC$H_2$C$H_2$O–), 5.22 (1H, br s, N*H*). ESI-MS (*m*/*z*): 382.2 (382.24 Calcd for $C_{17}H_{36}NO_8$, $M^+$+H). Methacryloyl chroride (0.91 mL, 9.4 mmol) was added to a mixture of **2a** (1.5 g, 3.9 mmol), *N*,*N*,*N*′,*N*′-tetramethyl-1,3-diaminopropane (0.99 mL, 5.9 mmol), and acetonitrile (50 mL), and was stirred at rt for 2 h. The reaction was poured into a mixture of a saturated aqueous solution of sodium bicarbonate and $CHCl_3$. The organic layer was washed with brine, dried over $Na_2SO_4$, and filtered. The filtrate was evaporated in vacuo and was purified by column chromatography (silica gel 50 g, eluted with 50% ethyl acetate (EA)/*n*-hexane, 67% EA/*n*-hexane, and EA) to give **3a** (0.9 g, 51%). $^1$H NMR ($CDCl_3$) $\delta$: 1.44 (9H, s, *t*Bu), 1.95 (3H, dd, $J$ = 1.3, 6.6 Hz, CH$_2$=C(C$H_3$)CO–), 3.31 (2H, m,–NHC$H_2$CH$_2$), 3.53–3.78 (20H, m, OC$H_2$C$H_2$O–), 4.30 (2H, m, –COOC$H_2$-CH$_2$O–), 5.05 (1H, br s, N*H*), 5.57 (1H, m, C$H_2$=C(CH$_3$)CO–), 6.13 (1H, m, C$H_2$=C(CH$_3$)CO–). Anal. Calcd for $C_{21}H_{39}NO_9$/0. 4$H_2O$: C, 55.2; N, 3.07; O, 32.9. Found: C, 55.2; N, 3.03; O, 33.1. **3b** were prepared by a similar manner to that of **3a**, $^1$H NMR (DMSO-$d_6$) $\delta$: 1.38 (9H, s, *t*Bu), 1.89 (3H, s, CH$_2$=C(C$H_3$)CO–), 2.94 (1H, m, –NHC$H_2$–), 3.13 (1H, m, –NHC$H_2$–), 3.45 (1H, m, 4-C*H*), 3.59 (2H, m, 2-C*H* and 3-C*H*), 3.75 (1H, m, 5-C*H*), 4.04 (5H, dd, $J$ = 6.6, 11.3Hz, –COOC$H_2$CH), 4.26–4.30 (2H, m, –COOC$H_2$CH and 2- or 3-O*H*), 4.53 (1H, d, $J$ = 6.3 Hz, 4-O*H*), 4.73 (1H, d, $J$ = 4.7 Hz, 2-or 3-O*H*), 4.90 (1H, d, $J$ = 6.1 Hz, 5-O*H*), 5.66 (1H, m, C$H_2$=C(CH$_3$)CO–), 6.08 (1H, s, C$H_2$=C(CH$_3$)CO–), 6.52 (1H, t, $J$ = 5.4Hz, N*H*). Anal. Calcd for $C_{15}H_{27}NO_8$: C, 51.6; H, 7.79; N, 4.01. Found: C, 51.4; H, 7.85; N, 3.90. A mixture of **3a** (399 mg, 0.89 mmol), glycerol dimethacrylate (4.3 µL, 0.018 mmol), azobisisobutyronitrile (1.46 mg, 0.0089 mmol), and dioxane (0.259 mL) was heated at 80 °C for 15 h. The resulting polymer was ground by a mortar grinder and washed successively with MeOH, 2-methyl-*N*-pyrrolidone (NMP) to give **4a** (210 mg, 53%). **4a** (170 mg) was treated with a mixture of TFA, $CH_2Cl_2$, dioxane, and water (10 mL, 50:39:10:1) at rt for 15 h, and then washed successively with dioxane, NMP, water, MeOH, and ether, to give **5a** (125 mg). The amount of amino group of **5a** was determined by the Ninhydrin test and was 2.27 µmol/mg. A mixture of **5a** (11 mg, 25 µmol), **7** (2.4 mg, 2.5 µmol), 1-hydroxybenzotriazole (HOBt, 0.7 mg, 5 µmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC HCl, 1.0 mg, 5 µmol), and NMP (1.5 mL) was stirred at rt for 15 h. After collection by filtration, the solid phase was washed by NMP and was treated with a mixture of water, NMP, and acetyl anhydride (10 mL, 1:7:2) at rt for 1 h. The resulting resin was washed with NMP and acetonitrile to give **6a** (80 µL). **6b** were prepared by a similar manner.
14. Western blot analysis on FKBP52 was carried out the following way. The proteins were subjected to SDS–PAGE followed by electroblotting onto PVDF membrane using the Invitrogen XCell II™ blot module. After blocking with Blocking one™ (Nacalai Tesque Inc., cat. 03953-95) for 30 min at rt, the membrane was incubated with anti-FKBP52 IgG (Santa Cruz Biotechnology, Inc., cat. sc-1803) for 1 h at rt. Following washing, the membrane was incubated with HRP-conjugated anti-goat antibodies (Santa Cruz Biotechnology, Inc., cat. sc-2033) for 1 h at rt and washed again. The membrane was soaked for 5 min in the detection reagent ECL plus (Amersham Biosciences Corp. cat. RPN2132). The resulting light was detected on Hyperfilm ECL (Amersham Biosciences Corp. cat. RPN1674K).