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ORIGINAL PAPER

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Isolating the whole complex of target proteins of FK506 using affinity resins from novel solid phases

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Abstract The development of novel solid phases enabled us to create affinity resins that could be used to isolate the whole complex of target proteins responsible for the immunosuppressive effects of FK506 from rat brain lysate, whereas the affinity resins from commercially available matrices could not achieve this isolation. The results illustrate the enhanced effectiveness of the affinity resin made from this novel material at identifying the target protein of the bioactive compound compared to resins made from the well-known materials Affigel or Toyopearl. This effectiveness arises because the novel material is hydrophilic enough to reduce nonspecific binding proteins and because it has a higher density of ligands that capture the nonubiquitous target protein.

Keywords Affinity resins · Poly(methacrylate) · FK506 · Calcineurin · Calmodulin

Introduction

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]. These findings are essential components of modern pharmaceutical research since they often facilitate the development of novel drugs with greater selectivity and/or potency through the use of effective screening systems and structure-based design. The competition method is widely used to identify specific binding proteins. This technique is, however, difficult to use when large amounts of nonspecific binding proteins are found on the resins, which hide the other binding proteins. Therefore, the successful isolation of target proteins by affinity matrices often depends on the synthesis of polymeric resins that can capture the cellular target with maximum selectivity and efficiency. Indeed, there are commercially available solid materials that can be used to prepare affinity resins. Affigel [3], which consists of agarose derivatives, is among the most popular of these matrices. It is hydrophilic enough to reduce nonspecific protein absorption [4]. However, Affigel easily becomes denatured under the conditions of organic synthesis [4]. Thus, chemical approaches based on this material are often restricted. Toyopearl, a poly(methacrylate) derivative [5], is stable under most synthetic conditions, which permits the synthesis of more effective affinity resins. However, these methacrylate polymers bearing bioactive compounds often show high levels of nonspecific protein binding because of their hydrophobicicity [4]. Therefore, it is important to develop novel solid phases for creating affinity resins that are chemically stable and hydrophilic enough to eliminate nonspecific absorption.

In a previous paper [6], we reported on the design of two hydrophilic methacrylate monomers (**1**, **2**) used to create affinity resins. These monomers were designed with the aim of minimizing the amount of nonspecific binding proteins that bind to resins created from the polymers; the amount of nonspecific binding proteins that bound to the resulting resin was assessed in a quantitative analysis. These monomers were polymerized using 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, which was then followed by a deprotection reaction performed under

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acidic conditions, affording **3** and **4**, respectively (Scheme 1).

FK506 (**5**), an immunosuppressive drug, is often used as a standard compound in affinity chromatography assessment [4, 6, 7]. A FK506 derivative bearing a linker moiety (**6**, Scheme 1) was therefore designed to have the ability to bind to the target proteins based on the X-ray structure of the ternary complex of FK506, FKBP12, calcineurin A and B [4]. Affinity resins bearing **6** have the ability to capture FKBP12 [4, 6]. FKBP12, a FK506 binding protein (MW=12 kDa), is a peptidyl-prolyl *cis-trans* isomerase and is known to be a specific binding protein for FK506. It is usually easy to capture FKBP12 using affinity resins that bear FK506 since FKBP12 is an ubiquitous intracellular protein and its kd value with FK506 is quite low (kd=0.4 nM) [8].

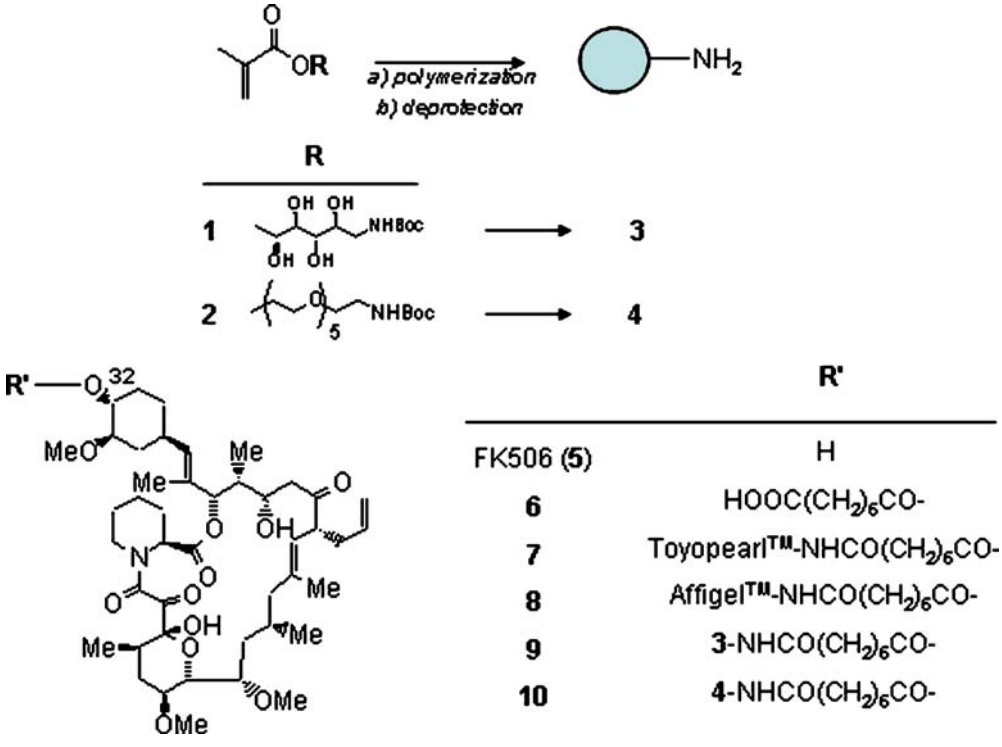
We reported on the isolation of FKBP12 and FKBP52 using the novel solid material (**3**, **4**) in a previous paper (see Electronic Supplementary Material (ESM), Fig. S1) [6]. FKBP52, a FK506 binding protein (MW=52 kDa), is also a peptidyl-prolyl *cis-trans* isomerase, and a specific binding protein for FK506 [9]. The amount of nonspecific binding proteins present on **9** and **10** was much less than that on **7** and similar to those present on the hydrophilic resin **8**. However, it is also known that neither FKBP12 nor FKBP52 were the target protein associated with the immunosuppressive effects of FK506; instead, the target proteins are the calcium ion and calmodulin-dependent phosphatases calcineurin A/B [10], although these target proteins were not identified in the previous study [6]. According to our knowledge, there is no report that describes the direct isolation of these target proteins by affinity resins. We believed that the reason for the failure of **9** and **10** to capture the targets was that the lysate preparation was not good enough to maintain the active forms. This prompted the present study, aimed at isolating the drug targets of FK506 using affinity resins.

We report here on the successfully isolation of the target proteins calcineurin A/B and calmodulin along with other specific binding proteins, FKBP12 and FKBP52, using the novel solid phases. The performances of the novel resins are also compared with those of commercially available resins.

Experimental

Reagents and materials

Goat polyclonal antibody IgG against FKBP52 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; cat. sc-1803), mouse monoclonal antibody IgG against calmodulin (Serologicals Corp., Norcross, GA, USA; cat. 05-173), rabbit polyclonal IgG against calcineurin A (Abcam Ltd., Cambridge, MA, USA; cat. ab12233), rabbit polyclonal IgG against calcineurin B (Serologicals Corp., cat. 07-069), horseradish peroxidase (HRP) conjugated anti-goat antibodies (Santa Cruz Biotechnology, Inc., cat. sc-2033), HRP conjugated anti-rabbit antibodies (Amersham Biosciences Corp., Piscataway, NJ, USA; cat. NA934V), and HRP conjugated anti-mouse antibodies (Amersham Biosciences Corp., cat. NA931V) were purchased and used without pretreatment, respectively. The specificities of the antibodies used were assessed via western blot analysis of the rat brain lysate (see ESM, Fig. S4).



Scheme 1 Synthesis of the novel solid phases (**3**, **4**) and the affinity resins bearing FK506 studied in this work (**7**–**10**)

AIBN was purchased from Wako Pure Chemicals Ltd. (Osaka, Japan), and purified using a recrystallization technique.

The commercially available solid phases, Affigel (Bio-Rad, Hercules, CA, USA; AffiGel102, cat.153-2401) and Toyopearl (Tosoh, Tokyo, Japan; AF-Amino-650M, cat. 08002), were purchased and were used after they had been washed with NMP.

Synthesis of the affinity resins

A FK506 derivative (**6**) was synthesized in the same manner as described previously [4].

A mixture of **1** (300 mg, 0.86 mmol), glycerol dimethacrylate (4 μL, 0.017 mmol), azobisisobutyronitrile (AIBN, 1.41 mg, 0.0086 mmol) and dioxane (0.75 mL) was heated at 80 °C for 15 h. The resulting polymer was ground using a mortar grinder, and was washed successively with methanol (MeOH) and 2-methyl-*N*-pyrrolidone (NMP) to give N-Boc protected **3** (280 mg, 93%). This N-Boc protected polymer (230 mg) was treated with a mixture of TFA, CH_2Cl_2, dioxane and water (10 mL, 50:39:10:1) at room temperature (rt) for 15 h, and then washed successively with dioxane, NMP, water, MeOH and ether to give **3** (200 mg). A mixture of **3** (9.5 mg, 25 μmol), **6** (2.4 mg, 2.5 μmol), 1-hydroxybenzotriazole (HOBt, 0.7 mg, 5 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC HCl, 1 mg, 5 mmol) and NMP (1.5 mL) was stirred at rt for 15 h. After it had been collected by filtration, the solid phase was washed with 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 successively washed with NMP and acetonitrile to give **9** (30 μL). Other affinity matrices bearing **6** (**7**, **8** and **10**) were prepared in a similar manner.

Preparation of lysate

Fresh rat brain was homogenized (1:10, wt/vol) in buffer A (0.25 M sucrose, 0.3 mM *N*,*N*-diethylthiocarbamate, 50 mM Tris-HCl, pH=7.5, 2 mM $CaCl_2$, 2 mM $MgCl_2$). The homogenate was centrifuged at 8,000 g for 30 min. The supernatant obtained was used as the lysate and kept at −80 °C before use.

Capture of specific binding proteins by affinity resins

The lysate was gently stirred with affinity resin (10 μL) at 4 °C for about 40 min to capture the binding proteins. The resins were precipitated by centrifugation in a microcentrifuge at 12,000 rpm for 1 min. The resins were then washed five times with 1.0 mL of buffer A. The washed beads were then resuspended in 20 μL of SDS sample buffer solution (Nacalai Tesque Inc., Kyoto, Japan; Sample Buffer Solution with 2-ME(2x) 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–HCl, pH=6.8), shaken at 25 °C for 10 min, and centrifuged for 1 min. The supernatant was subjected to SDS-PAGE followed by siliver staining. Each protein studied in this work was identified using a MS/MS ion search method based on ESI ion trap mass spectrometry (Thermoelectron, Waltham, MA, USA; LTQ) after in-gel digestion. A commercially available apparatus (Dainippon Seiki Co. Ltd., Kyoto, Japan; cat. code: 1D-SDS) was used to cut off strips including the protein.

Western blot analysis

Western blot analysis was carried out on the proteins in the following way. The proteins were subjected to SDS-PAGE followed by electroblotting onto a PVDF membrane (Invitrogen, Carlsbad, CA, USA; cat. LC2002) 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 the antibody IgG against each protein for 1 h at rt. Following washing, the membrane was incubated with HRP-conjugated antibodies 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).

Dilution endpoint of **6** on **9**

Affinity resins bearing 1/10, 1/100, 1/1000, and 1/10000 of **6** were prepared in a similar manner to **9**. The capture of specific binding proteins and western blot analysis involving these resins were also carried out as described above.

Results and discussion

Capture of the target proteins by the affinity resins

If the target proteins are to be captured by the affinity resins, it is vital that the lysate is prepared such that the ability of the target protein to bind with the bioactive compound is retained. It has been reported that calcineurin A and B form a complex with FK506 and calmodulin in the presence of calcium ion and cannot bind with them in the absence of this ion [10]. Thus, we isolated them using lysate prepared in the presence of calcium ion, since we failed to capture them without calcium ion in a previous study (see ESM, Fig. S1) [6]. There were many binding proteins on each affinity resin, as shown in Fig. 1a, so 1 μmol of FK506 was added into the lysate as a competitor in order to identify specific binding proteins. The amount of target protein was believed to be small because FK506 has shown potent bioactivity at low dosages than this [11].

A careful search for specific binding proteins with the expected molecular weights of calcineurin A (58.6 kDa), calcineurin B (19.2 kDa), and calmodulin (16.7 kDa) in silver-stained SDS gels (Fig. 1a) showed that the target proteins, calcineurin B (D) and calcineurin A (E), were successfully identified as specific binding proteins by **9** along with previously isolated proteins, such as FKBP12 (A) and FKBP52 (B) (Fig. 1a). The proteins discussed in this study were identified using an MS/MS ion search method based on ESI ion trap mass spectrometry after in-gel digestion. The amount of calcineurin A was very small, but was clearly identified through careful observation. Calmodulin (C) was also isolated as a specific binding protein with the same molecular weight as calcineurin B. This result was intriguing, because X-ray structural analysis of the ternary complex of the calcineurin A, calcineurin B, FKBP12 and FK506 showed that calmodulin was indirectly bound to the complex of FKBP12 and FK506 through calcineurin A and B [12], which indicated that the whole protein complex binds to **9** in its active form. According to our knowledge, this is the first example of the direct isolation of the whole target protein complex associated with the immunosuppressive activity of FK506 using affinity resins bearing FK506. The existence of these proteins on **9** was also confirmed by the western blot method (Fig. 1b). On the other hand, only calcineurin B was found on **10** using the MS/MS ion search method, while calcineurin B should form stable complexes with calcineurin A in the mixture. We thought that the amounts of calcineurin A and calmodulin on **10** were not enough to be identified using the MS/MS ions search method, since they had only just been detected in the western blot experiment (Fig. 1b).

The target proteins were not found on the commercially available resins (**7**, **8**) at the silver stain level. This result was consistent with the fact that target proteins such as calcineurin A/B have not been identified by agarose-type affinity resins bearing FK506. Liu et al. originally identified them using an indirect method: they identified the proteins using glutathione S-transferase (GST)-affinity chromatography and a GST-FKBP12 fusion protein in the presence of calcium ion and FK506 [10].

Interestingly, the existence of calcineurin A and calcineurin B on **7** is shown up by the western blot method (Fig. 1b) even though these proteins were barely identified using the silver stain method because of the predominance of nonspecific binding proteins. These results show that a reduction of the amount of nonspecific binding protein is critical to the identification of the target protein. On the other hand, these target proteins were barely identifiable on **8**, even by western blot analysis, while it did show the ability to capture FKBP12 (Fig. 1b).

Critical density of FK506 on 9 needed to capture each protein

It is notable that there was difference between **8** and **9** in terms of their ability to capture the target proteins, because these polymers have similar sugar-like structures (see ESM, Fig. S2) and have given similar results in a previous study [6]. We thought there were two potential reasons for this difference; one was the different densities of FK506 on

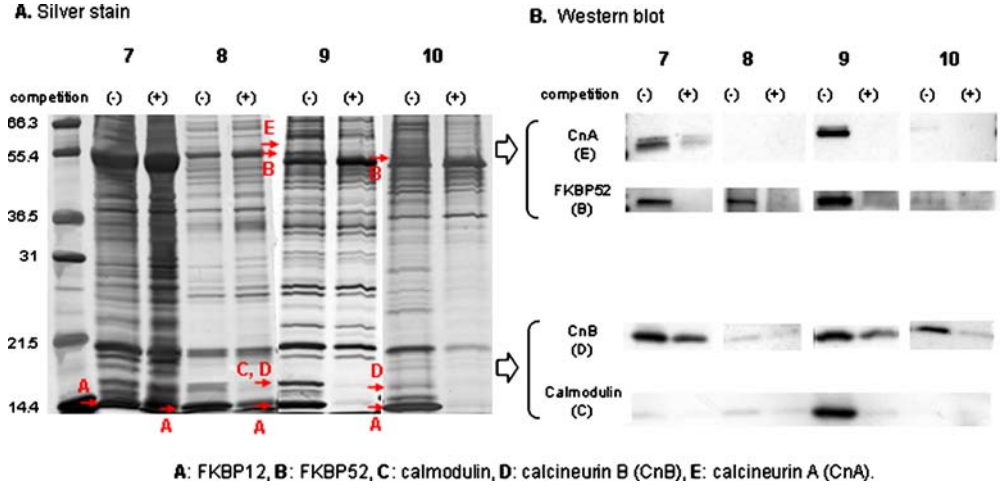


Fig. 1a Binding proteins on affinity matrices bearing FK506 (**7**–**10**) in the presence of calcium ion.Ten microliters of each resin were mixed with 1 mL of lysate obtained from rat brain with or without the competitor (**5**) at 4 °C for 1 h. After separation of the resins by centrifugation and extensive washes with lysate buffer (50 mM Tris–HCl, pH=7.5, 0.25 M sucrose, 0.3 mM *N*,*N*-diethylthiocarbamate, 2 mM $CaCl_2$, 2 mM $MgCl_2$), the binding proteins were completely eluted by an SDS sample buffer solution and analyzed via SDS–polyacrylamide gel electrophoresis. The proteins mentioned in this study were identified using an MS/MS ions search method using the MASCOT database (ver. 2.1.0, Matrix Science) and ESI ion trap mass spectrometry (Thermoelectron, LTQ) after in-gel digestion, respectively. A summary of the database search results for the proteins identified on **9** is shown in (see ESM, Table S1). A commercially available apparatus (Dainippon Seiki Co., Ltd., cat. code: 1D-SDS) was used to cut off strips including the desired protein from the silver-stained SDS gels. One micromole of FK506 was added for the competition experiment. Western blot studies were carried out using anti-calcineurin A, FKBP52, calcineurin B and calmodulin antibodies, respectively

the affinity resins, and the other was their different physicochemical properties. The density of the ligand on **8** was almost 12 μmol/mL [3], while that on **9** was almost 100–200 μmol/mL according to ninhydrin tests (data not shown). Accordingly, the amount of FK506 on **8** was almost 1/10 of that that on **9**, which could critically affect the identification of target proteins. In order to assess this possibility, we examined the dilution endpoint of FK506 on **9** (Fig. 2), which showed that most of the target proteins could be identified by the silver stain method after the amount of FK506 was diluted 1:10, but that they could not be identified at 1:100 dilution. This experimental correlation between the results from the dilution endpoint experiment and the difference in ligand density indicated that the reason for the difference is the density of FK506 ligands on the polymers. These results illustrate the importance of synthesizing affinity resins with high ligand densities if we wish to capture small amounts of target proteins. **9** has a hydrophobic alkyl chain in its structure (see ESM, Fig. S2), while all moieties of **8** are hydrophilic. Therefore, it is thought that **9** is more hydrophobic than **8**. However, the influence of this difference in physicochemical properties is yet to be deduced.

In this study, **10** failed to capture the whole complex of target proteins. As yet we cannot explain this difference, since they have similar ligand densities (both **3** and **4** gave 100–200 μmol/mL of amine groups by the ninhydrin test; data not shown) and putative structures (see ESM, Fig. S2).

We are currently in the process of polymerizing other matrices using the monomers (**1** and **2**) by other methods because **9** and **10**, which were powdered by a mortar grinder after solution polymerization, had morphologically heterogeneous distributions of particles (see ESM, Fig. S3). Target identification results for these novel solid phases will be reported in the future.

Conclusions

We reported in a previous paper the development of two novel poly(methacrylate) derivatives (**3**, **4**). These solid phases bearing a FK506 derivative (**6**) could isolate not only FKBP12 but also FKBP52, both specific binding proteins to FK506, along with only small amounts of nonspecific binding proteins from lysate prepared from rat brain, while commercially available resins afforded only FKBP12 (see ESM, Fig. S1). In this study, we attempted to isolate the whole complex of target proteins responsible for the immunosuppressive effects of FK506 using these novel resins, since FKBP12 and FKBP52 specifically bind to FK506 but not the target proteins. As a result, the known target proteins, calcineurin A, calcineurin B, FKBP12, FKBP52 and calmodulin, were successfully isolated by **3** bearing **6** (**9**) from rat brain lysate in the presence of calcium ion (Fig. 1a). The specificities of these proteins were assessed in competition experiments. These proteins were not identified using affinity matrices based on **4** or the commercially available resins Toyopearl and Affigel bearing the same ligand under the same conditions (Fig. 1a).

Western blot analysis showed that calcineurin A/B and FKBP52 were captured on **7** (Fig. 1b), while these proteins were hardly identified by the silver stain method due to the the predominance of nonspecific binding proteins. This shows that reducing the amount of nonspecific binding protein is critical to target protein identification. A dilution

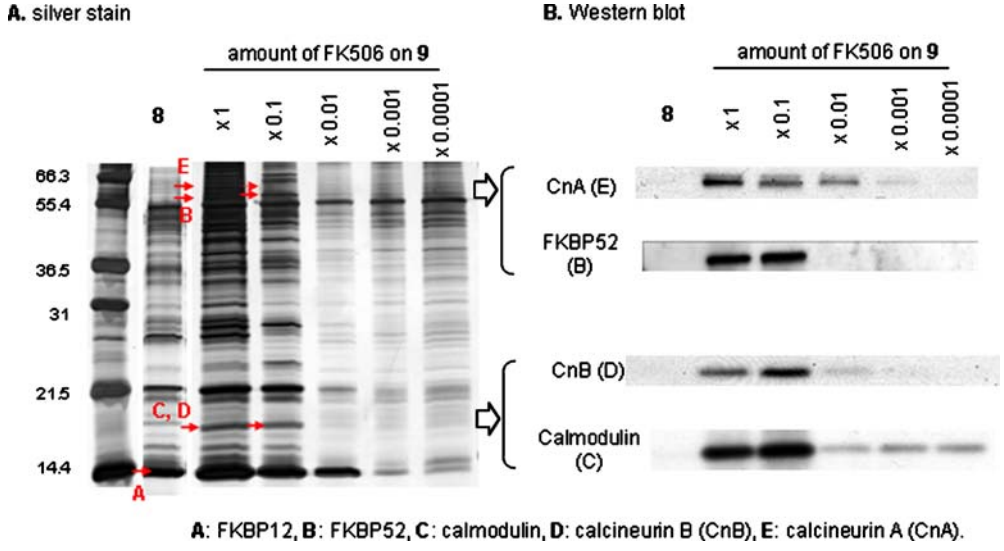


Fig. 2a–b Dilution endpoints for the identification of calcineurin A, FKBP52, calcineurin B, and calmodulin on **9** by silver stain (**a**) and western blot methods (**b**). The novel solid materials bearing 1/1 (**9**), 1/10, 1/100, 1/1000, and 1/10000 of the FK506 derivative (**6**) and the FK06-Affigel resins (**8**) were mixed with 1 mL of lysate obtained from rat brain at 4 °C for 1 h, respectively. The binding proteins on each resin were analyzed by silver stain and western blotting methods. These experimental dilution endpoints show that the amount of FK506 in our material is the critical factor that influences the capture of the specific binding proteins, and the poor performance of Affigel is due to its low density. The experimental procedure is described in the Experimental section

endpoint experiment investigating the amount of FK506 on **9** was carried out to address the difference between **8** and **9**, and this indicated that the inability of **8** to capture the target proteins could arise from its low ligand density. This result shows the importance of synthesizing affinity resins that have high ligand densities, which allow them to capture target protein with a low kd value or that is present in only small amounts.

So far, affinity resins bearing bioactive compounds have been widely used with the excellent results [1, 2]. However, it was not easy to identify the target protein using commercially available solid materials (Fig. 1). We believe that our development of the novel solid phase **3** could pave the way to new research into identifying various target proteins using bioactive compounds on affinity resins.

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