



An easy preparation of ‘monolithic type’ hydrophilic solid phase: Capability for affinity resin to isolate target proteins

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Abstract—An easy preparation method of monolithic type hydrophilic solid phase was discussed. Newly invented functional monomer with a hydrophilic cross-linking agent was co-polymerized to realize well-controlled monolithic co-continuous structure by use of diethylene glycol as porogenic solvent. We were able to control the content of the functional monomer up to 40 vol% without loss of monolithic structure. Those prepared were utilized as affinity resins after immobilization of FK506, an immunosuppressive drug as a ligand. It was found that the affinity resins prepared were hydrophilic enough to eliminate non-specific adsorption of proteins, while two of the target proteins of FK506 tested were successfully captured.

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1. Introduction

Affinity resins supporting bioactive compounds such as naturally occurring compounds, drugs, and even toxins play an important role in the discovery of novel drug targets and the elucidation of drug mechanisms.^{1,2} We have utilized some commercially available solid phases for preparation of affinity resins so far. Affigel™,³ agarose derivatives, is one of the most popular matrices for this purpose. This has highly hydrophilic property and affords excellent selectivity between specific protein binding and non-specific protein absorptions.⁴ However, Affigel™ is easily denatured under organic synthesis conditions.⁴ Thus, chemical approaches using this resin are often restricted. Toyopearl™, a poly(methacrylate) derivative,⁵ is stable under wide range of experimental conditions, which allows the preparations of more effective affinity resins. In spite of the chemical stability, these methacrylate polymers supporting bioactive compounds often show high levels of non-specific binding proteins with the target protein because of their relatively hydrophobic properties.⁴ Therefore, development of novel solid phases for affinity resins, which are chemical-

ly stable and hydrophilic enough for elimination of non-specific absorption, is now desired research target.

In a previous paper,⁶ we reported design of one possible hydrophilic methacrylate monomer (**1**) as shown in Figure 1 for the preparation of affinity resins based on quantitative analysis of the amount of non-specific binding proteins. This monomer was polymerized through a solution polymerization method with 2% glycerol dimethacrylate, a well-known cross-linking agent, in dioxane using azobisisobutyronitrile (AIBN) as a radical initiator. The co-polymerization resulted in *N*-Boc-protected solid phase (Fig. 1) and then followed by deprotection reaction under acidic conditions to realize an affinity resin. We have modified this affinity resin using FK506, an immunosuppressive drug as a ligand without loss of its bioactivity (Fig. 1). As previously reported, we were able to succeed in the first isolation of the whole target proteins of FK506 (calcineurin A and calcineurin B and with FKBP12, FKBP52, and calmodulin)⁷ with this affinity resin. In fact, FK506 has been often utilized as a standard compound for assessment of affinity chromatography.^{4,6–8} In addition, as expected, the amounts of non-specific binding proteins were quite little compared to those on Toyopearl™ based affinity resin.

Although, this affinity resin was successfully prepared, this resin had considerable swelling characteristics due

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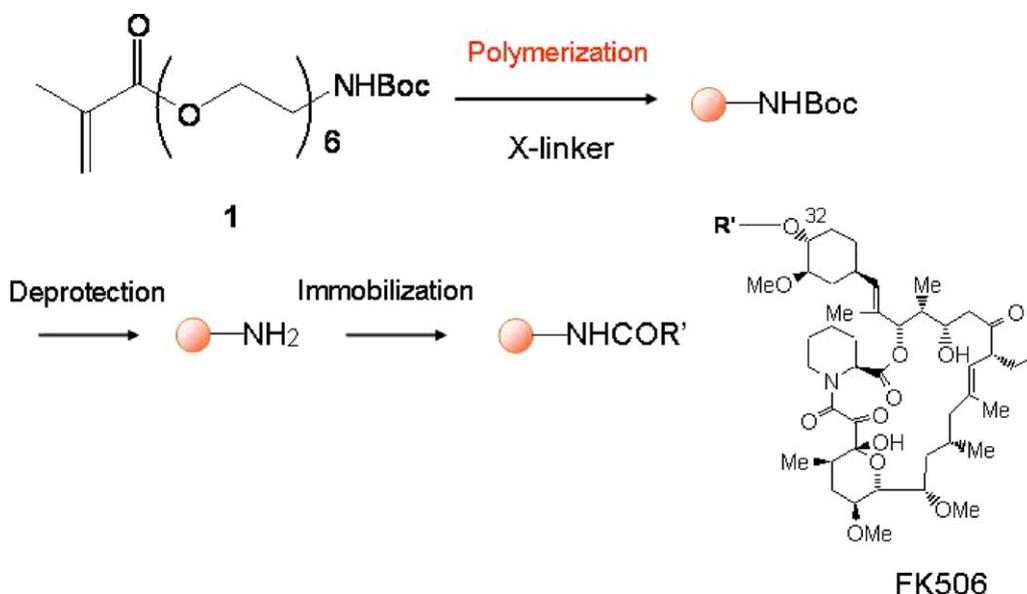


Figure 1. Preparation strategy of affinity resin with FK506 as supporting drug.

to only 2% cross-linked structure. In fact, depending on the solvents utilized, the resin was swollen to suppress easy handling. Therefore, with the monomer prepared **1**, we had to prepare stable format of the affinity resins. If we pay attention to chemically stable Toyopearl™, it is beaded format and there are relatively large pores in it, which may contribute to facile handling as well as easy transfer of polypeptides through the pores. Therefore, first we prepared macroporous polymer beads having up to 50% cross-linked density using the functional monomer **1**, but the beads did not work as good as that on Toyopearl™ for the capture of FKBP12. This may be due to even small pores of the prepared beads.⁹

If we notice the entire isolation process of the target proteins by the affinity resin, where deprotection reaction of *t*-BOC group, immobilization reaction of drug molecule, and isolation process of target proteins should be involved after preparation of the solid phase, flow-through type monolith, which has co-continuous macroporous structures, will be clearly effective. The monolithic media have been usually utilized as stationary phases for high performance liquid chromatography (HPLC), therefore those reported so far tended to have some hydrophobic surface properties due to majority of separation mode being reversed-phase mode. In this paper, we wish to report a simple and easy preparation method of hydrophilic and stable polymer monolithic affinity resin and attempt to isolate some of the target proteins of FK506.

2. Results and discussions

Conventionally, polymer monoliths have been prepared by in situ polymerization of functional monomers, cross-linking agent, and initiator in porogenic solvent (pore forming diluent). As porogenic solvent, binary or ternary mixtures of poor and/or good solvents are occasionally used for controlling a variety of pore sizes

and their structures.¹⁰ The porogenic solvents dominantly selected for the preparation of monolith type media are usually poor solvents of the monomers utilized to form macro through-pores required for liquid flow. In the case of the porogenic poor solvents, the growing polymer chains tend to aggregate each other because van der Waals attraction surmounts the steric hindrance mutually expelling the polymer chains.¹¹ Thus, in the case of the ordinary polymer monolith preparations, the phase separation between growing polymer chains and porogenic solvent proceeds so fast and the coarsening of monolithic structure inherently leads to heterogeneous macroporous structures composed of tiny micron size globular particles.

According to the above-mentioned knowledge, first we tried to prepare monolithic affinity resins using mixture of good and poor solvents for the monomers utilized in polymerization system. However, irregular structures were obtained as expected even if we employed additional polymer porogen, too. Therefore, we switched the preparation method to simplify the whole preparation strategy. Although we utilized a quite simple polymerization system with radical initiator as well as relatively low molecular weight porogenic solvent, nicely controlled, co-continuous structures were observed except for gel 'e' as shown in Figure 2. These results were rather interesting because usually the polymerization system employed in this study tends to afford rather particle agglomerated structures as mentioned above.

If poor solvent to the monomers, chloropentane (Mw 107) or xylene (Mw 106), was utilized as the porogenic solvent instead of diethylene glycol (Mw 106), as shown in Figure 3, only continuous polymers (micro gels) were obtained as reported so far. The detailed polymerization mechanism has not been elucidated; however, this preparation method is practically useful, because only water was utilized to wash the porogen away. In these cases,

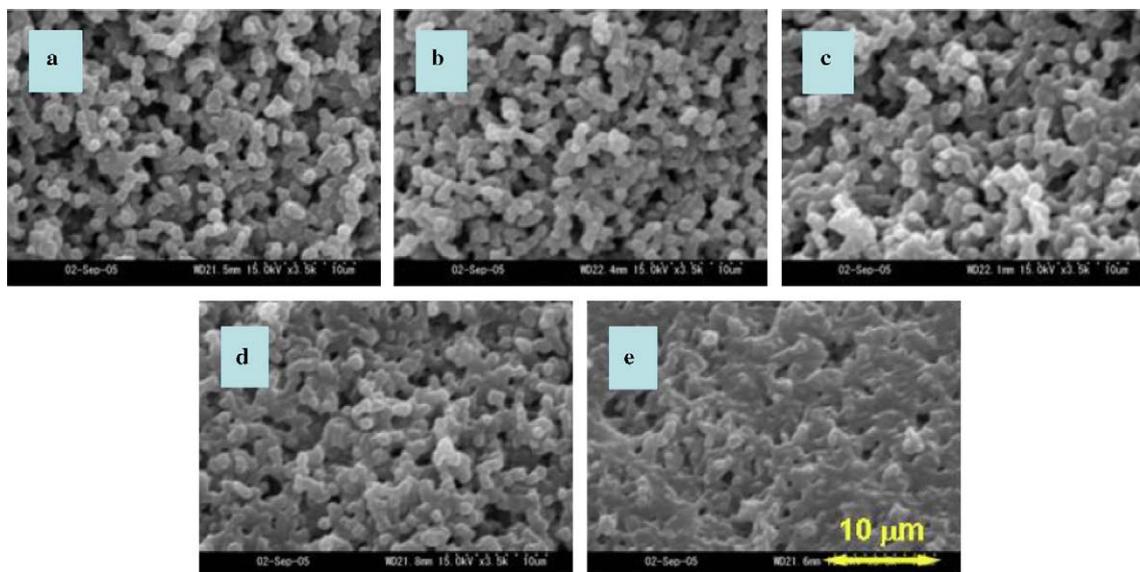


Figure 2. Scanning electromicrographs (SEM) and the feed compositions of the resins (a–e) prepared with diethylene glycol (DEG) as porogenic solvent.

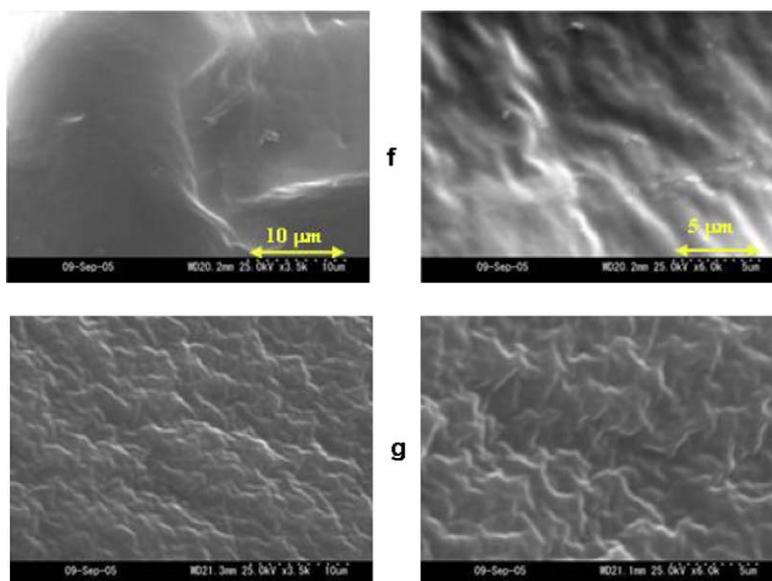


Figure 3. SEM of the resins prepared using chloropentane (f) and xylene (g).

up to 40 vol% of the functional monomer (**1**) is introduced to the final monolithic resins.

With those affinity resins, we tried to capture the target proteins of FK506. The experimental conditions and methods as well as reagents utilized were carefully copied as described in our previous work.^{6,7,12} As shown in Figure 4, all the prepared resins including e isolated significant amount of a target protein FKBP12 compared with that on commercially available Toyopearl™ based affinity resin. Two resins prepared using chloropentane or xylene did not afford meaning isolation of proteins. It is notable that although large amount of non-specific proteins were also captured on Toyopearl™, the prepared affinity resins showed quite small amount of non-specific pro-

teins. It is because we utilized the monomers consisting of ethylene oxide (EO) units. Careful observation realized the amount of captured FKBP12 on the affinity resin e seemed to be smaller than those on other monolithic type resins as shown in Figure 4. This is probably due to even worse monolithic structure of the affinity resin e, while the amount of FK506 was presumably larger on e, which suppresses effective transfer of proteins within the resins. This result is consistently correlating those on the resins prepared using chloropentane or xylene as porogenic solvent. CBB method also supported the result described above as shown in top of Figure 5.

Interestingly, the existence of calcineurin A on Toyopearl™ was obviously shown by the Western blot

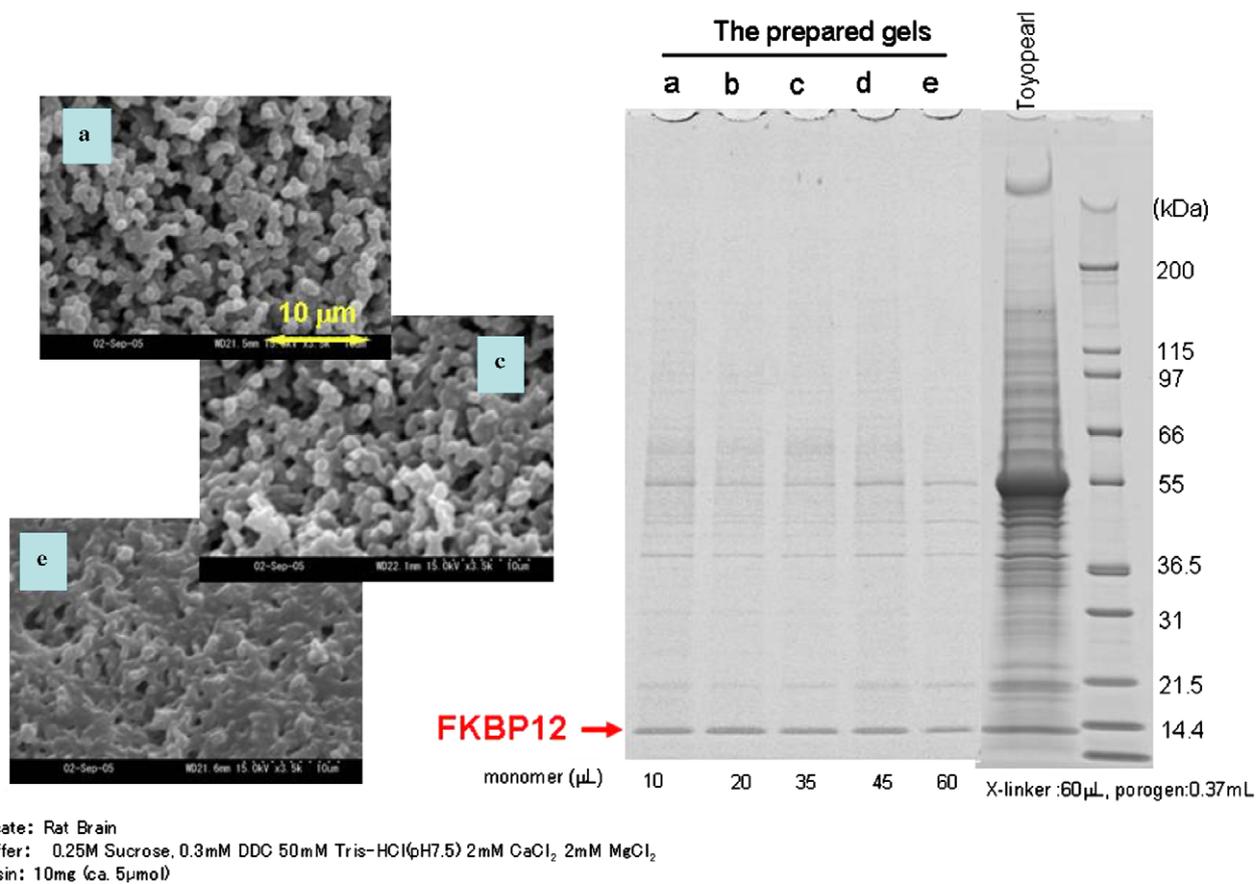


Figure 4. Isolation of the target proteins with affinity resins as well as Toyopearl™.

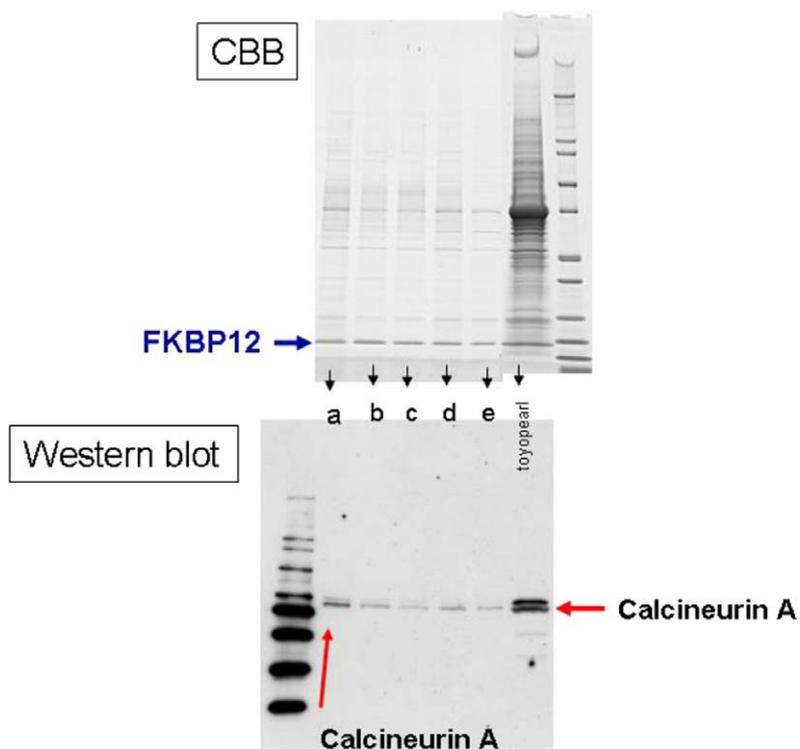


Figure 5. CBB and Western blot analyses of the isolated proteins.

analyses (bottom of Fig. 5) although this protein was hardly identified by means of silver stain method because of the predominance of non-specific binding proteins. These results exhibited that reduction of non-specific binding proteins was critical for identification of target protein.

In comparison to the amount of isolated calcineurin A on Toyopearl™, those on our newly prepared affinity resins were small. Interestingly, that on affinity resin 'a' was found to be much larger than those on other b to e. At this moment, the reason is not clear, but experimental method including capture time as well as washing method might affect those results. These observations clearly suggest that not only the gel morphology but also surface chemistry are important to manage the performance of affinity resins. Further studies of monolithic type affinity resins are now under progress and we will have to manage the surface properties including surface chemistry.

3. Conclusion

Monolithic type solid phases were prepared using a quite simple and easy preparation method. The monolithic affinity resins afforded significant amount of one of the target proteins of FK506, FKBP12 and monolithic structure meaningfully affected the performance of the affinity resins. In addition, another target protein, calcineurin A, was successfully isolated well-controlled monolithic type affinity resin.

4. Experimental

4.1. Monomers and other materials

We utilized polyethylene glycol 400 dimethacrylate as a cross-linking agent through this study because of its effectively long hydrophilic linkage. The cross-linking agent utilized in our previous works was glycerol dimethacrylate, but this monomer involves –OH functional group in the middle of its structure. This –OH group is not chemically inert, therefore we utilized **9G** in this study. This cross-linking agent (**9G**) was kindly donated by Shin-Nakamura Chemical Co. (Wakayama, Japan) and utilized without further purification. Newly prepared monomer (**1**) was utilized as comonomer to immobilize drug FK506 as described previously.⁶

We utilized 2,2'-azobis(2,4-dimethylvaleronitrile) (ADVN) (Wako Pure Chemical, Co., Osaka, Japan) and diethylene glycol (DEG) (Wako Pure Chemical, Co., Osaka, Japan) as an initiator and porogenic solvent, respectively.

Rabbit polyclonal IgG against calcineurin A (Abcam Ltd., Cat. No. ab12233) and HRP-conjugated anti-rabbit antibodies (Amersham Biosciences Corp. Cat. No. NA934V) were purchased and used without pre-treatments, respectively.

Table 1. Feed composition of prepared resins

| | PEG type monomer 1 (μl) | X-linker 9G (μl) | DEG (ml) |
|---|--------------------------------|-------------------------|----------|
| a | 10 | 60 | 0.37 |
| b | 20 | 60 | 0.37 |
| c | 35 | 60 | 0.37 |
| d | 45 | 60 | 0.37 |
| e | 60 | 60 | 0.37 |

4.2. Preparation of monolithic resins

First of all, 4 mg of ADVN was dissolved in 60 μl of the cross-linking agent **9G** followed by addition of the prescribed amount of the monomer **1** (Table 1) and 0.37 ml of diethylene glycol. This clear solution was polymerized at 60 °C for 1 h. The gel obtained was washed with water repeatedly to remove the porogenic solvent and other impurities. Chloropentane as well as xylene were also utilized as reference porogens. We just utilized SEM to observe the surface morphology. By changing the amount of the monomer **1** as described in Table 1, we prepared five kinds of gels, where the porosity was changed with regard to each other due to the amount of added monomer **1**.

After preparations of the monolithic resins, we immobilized FK506 as a drug ligand as described previously.⁶ Commercially available solid phases, Affigel™ (BIORAD, AffiGel102, Cat. No. 153-2401) and Toyopearl™ (TOSHO, AF-Amino-650M, Cat. No. 08002), were purchased to be utilized as reference solid phases.⁷

4.3. 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 CaCl₂, and 2 mM MgCl₂). The homogenate was centrifuged at 9500 rpm for 10 min. After the 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.

4.4. Capture of specific binding proteins by affinity resins

The lysate was stirred calmly with affinity resin (10 mg) at 4 °C for about 40 min to capture binding proteins. The resins were completely removed by centrifugation in a microcentrifuge at 12,000 rpm for 1 min. The resins were washed five times with 1.0 mL buffer A. The washed resins were then re-suspended in 20 μL SDS sample buffer solution (Nacalai, Sample Buffer Solution with 2-ME(2x) for SDS-PAGE, Cat. No. 30566-22, including 4%(w/v)-SDS, 20%(v/v)-glycerol, 0.01%(w/v)-BB, 10%(v/v)-2-mercaptoethanol, and 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 silver staining. Each protein studied in this study was identified by MS/MS ions search method using an ESI ion trap mass spectrometry (Thermolectron, LTQ) after in-gel digestion. A commercially available apparatus (Dainippon

Seiki Co., Ltd., Cat. code: 1D-SDS) was used for cutting off each strip including the protein.

4.5. Western blot analysis

Traditionally utilized CBB method was carried out as reported previously,⁶ while Western blot analysis on protein was carried out as the following way. The protein was subjected to SDS-PAGE followed by electroblotting onto PVDF membrane (Invitrogen, Cat. No. LC2002) using the Invitrogen XCell II™ blot module. After blocking with Blocking one™ (Nacalai Tesque Inc., Cat. No. 03953-95) for 30 min at rt, the membrane was incubated with antibodies IgG against the protein for 1 h at rt. Following washing, the membrane was incubated with HRP-conjugated antibody 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. No. RPN2132). The resulting light was detected on Hyperfilm ECL (Amersham Biosciences Corp. Cat. No. RPN1674K).

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