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Properties of flaky affinity resin with co-continuous structure

Tomoko Mori,[a] Akito Tanaka,[b] Takuya Kubo,[a] Kunimitsu Kaya,[a] Mari Sakamoto[a] and Ken Hosoya[a,*]

[a]*Graduate School of Environmental Studies, Tohoku University, Aoba 6-6-20, Aramaki, Aoba-ku, Sendai 980-8579, Japan*
[b]*Department of Pharmacy, School of Pharmacy, Hyogo University of Health Sciences, 1-3-6 Minatojima, Chuo-ku, Kobe 650-8530, Japan*

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Abstract—A new type of flaky affinity resin for capture of the target proteins was prepared to discuss its properties compared with those of a particulate affinity resin. The resin prepared had totally co-continuous structure (monolith) and was utilized in the shape of flake. The concentration of surface amino groups for immobilization of ligand was determined to be 22.3 μmol/ml. Immobilizations of ligand such as Sulfonamide, Ketoprofen, Captopril, or Methotrexate (MTX) on the affinity resin were quantitatively proceeded to afford fully covered (100%) affinity resins. Control of the immobilization rate of affinity resin using Sulfonamide or Ketoprofen was successfully achieved with the calculated immobilization rate. The flaked shape of affinity resin (100–400 μm) presumably simplified affinity experimental procedures and the affinity resin immobilizing Sulfonamide effectively captured one of the target proteins, CAII, without non-specifically bound proteins. The observed properties of the flaky affinity resin as well as ease in handling are really useful for capture of the target proteins of possible rare ligands.

1. Introduction

Research on search of the target proteins using ligand-immobilized affinity resin is a classic yet new method, which is getting much attention for drug discovery, because the resin immobilizing bioactive compounds as ligand is able to capture its target proteins directly from a protein lysate prepared using possibly affected organs and/or cells.[1,2]

Traditionally the solid supports immobilizing ligand were packed into columns to capture the target proteins in fluid, but this column method tends to require rather large volume of materials including the gels as well as protein lysate, while handling of particulate affinity resins might be simplified. This presumably results in loss of valuable items (ligand as well as protein lysate). Therefore, it can be hardly utilized for very rare ligand such as naturally occurring compounds or toxic compounds. In addition, proteins are possibly denatured through this column method due to relatively large volume in the column.

To avoid the disadvantages of column method, tiny amount of affinity resins might be directly added into protein lysate to capture the target proteins. In comparison to the column method, experimental procedures using dispersed affinity resins should be complicated, because collection of affinity resin is essential. Therefore, some devisal should be required for preparation of the affinity resins. In addition to the devisal, quantitative immobilization of ligand to the affinity gel, simplified experimental procedures including washing and elution steps, and effective binding of target proteins without non-specifically bound proteins will be required.

Now we have some commercially available affinity gels for capture of target proteins. However, as mentioned in our previous papers,[3–5] these have both merits and demerits. Actually, in our previous papers,[3–5] we have prepared new affinity gels using newly synthesized monomers, which realized the first capture of target proteins including secondary bound proteins to an immunosuppressive agent, FK-506, through one affinity experiment by the use of one affinity resin. The affinity gels prepared in our works were found to have acceptable chemical stability and negligibly small amount of non-specifically bound proteins. In addition, we have succeeded to create morphologically co-continuous

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* Corresponding author. Tel.: +81 22795 6333; fax: +81 22795 4543; e-mail: hosoya@mail.kanakyo.tohoku.ac.jp

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affinity gels. Although 'monolithic' affinity gels have been reported so far, affinity gels having totally and well-controlled co-continuous structure were the first case. In fact, the affinity resin having better co-continuous structure tended to afford better capture of target proteins to FK-506.

In this report, we wish to propose a new flaky shape of affinity gel having totally co-continuous structure. We call this gel as *Moli-gel*, which might image an affinity gel having '*mo*no*li*thic' structure. Moli-gel is in fact comminute monolith, but the flaky shape will afford several advantages as described later. We wish to reveal the amount of surface amino groups and immobilization rate, utilizing variety of ligands under fully covered or the controlled condition.

2. Results and discussion

2.1. Chemical yield of Moli-gels and their morphology

Moli-gels were prepared with the prescribed feed as summarized in Table 1 to certify the reproducibility. The chemical yields were plotted against the polymerization time as shown in Figure 1. All the plots were average of three to six times polymerizations and batch-to-batch reproducibility was really excellent. Interestingly, quantitative yield was obtained only after 1 h polymerization time, while thermal decomposition of the gels was negligible after 48 h.

As revealed in Figure 2, morphologies of Moli-gels prepared through different polymerization time showed all well-controlled, co-continuous structures and completely the same morphologies, having 1 μm size skeletons and 2–3 μm size through pores. These observations suggest that polymerization of Moli-gel proceeds evenly to afford the good co-continuous structure. This also suggests that the amino groups on the surface of Moli-gel are also homogeneously dispersed, because further polymerization by Lig-m must afford thicker skeletons after long polymerization time.

2.2. Determination of amount of surface amino groups

Titration method afforded the total amount of surface amino groups by the following calculation equations.

When the concentration of resulting HCl is X (N),

$$X \times 3/1000 = 0.1 \times f_{\mathrm{NaOH}} \times \mathrm{NaOH}\ (\mathrm{ml})/1000 \quad (1)$$

Therefore,

$$X\ (\mathrm{N}) = (0.1 \times f_{\mathrm{NaOH}} \times \mathrm{NaOH}\ (\mathrm{ml}))/3 \quad (2)$$

When the amount of quaterized amino groups is Y (mol),

$$Y\ (\mathrm{mol}) = (0.1 \times f_{\mathrm{HCl}} - X) \times 3/1000 \quad (3)$$

When the amount of the surface amino groups is Z (μmol/ml) and the volume of gels utilized is v (ml),

$$Z\ (\mu\mathrm{mol/ml}) = Y \times 10^6/v \quad (4)$$

The amounts of surface amino groups were summarized in Figure 3. Regardless of mixing time with the HCl solution, the amounts of surface amino groups were nearly constant. The average value for Toyopearl was found to be 88.8 μmol/ml. This value is just comparable to that mentioned in company's brochure (94 μmol/ml); therefore, this titration method might be acceptable as a measurement method.

Moli-gels afforded almost constant amount of amino groups regardless of the mixing time and pyridine treatment of the gels. The average value was calculated as much as 22.3 μmol/ml, which is almost one-fourth of that of Toyopearl. Based on the feed ratio (Table 1), 70 μmol/g should be observed. Since elemental analysis and chemical yield suggested nearly quantitative introduction of the monomer, Lig-m for immobilization of ligand, surface amino groups were presumably limited, due to characteristics of monolithic media. In fact, pore size analyses afforded ca. 20 m^2/g of pore surface area. This value is generally one-tenth of average microporous polymer particles. In addition, total porosity of Moli-gel was observed as high as ca. 51%; therefore, the observed amount of the surface amino groups on Moli-gel (22.3 μmol/ml) might be reasonably acceptable.

2.3. Immobilization rate of ligands on Moli-gel and Toyopearl

Figures 4 and 5 indicated immobilization rate of Moli-gel and Toyopearl using ligands Sulfonamide and Ketoprofen, respectively. On Moli-gel, both ligands were introduced with nearly calculated equivalent for 0.1, 0.5, and 1.0 of the feed equivalent. In addition, Moli-gel treated with pyridine also afforded quantitatively corresponding immobilization rate. This quantitative introduction of ligand will be very important for novel affinity resins because circumstance and/or density of immobilized ligand must affect capture of proteins.

On the other hand, the amount of ligands immobilized was found to double based on the feed equivalent on Toyopearl. As mentioned before, usually affinity resins are supplied as water or aqueous alcohol dispersion; therefore, the real volume of Toyopearl might be below the measured volume even after centrifugal sedimentation. Once Toyopearl is dried for measurement of its weight, the reactivity of the amino groups is found to be much lowered. Therefore, the measurement of solvent dispersion of affinity resin could be essential for this ligand immobilization experiment.

In Figure 6, immobilization of Captopril or Methotrexate: MTX was indicated with those of Sulfonamide and Ketoprofen. In these experiments, 4 equivalents of each ligand were utilized for the fully covered immobilization, but the immobilization rate by Captopril and Methotrexate: MTX tends to be lowered by 40% after 48 h reaction on Toyopearl. At this moment, the reason for lower immobilization rate has not been clear, but steric hindrance of the ligands as well as reactivity of surface amino groups should be possible reasons.

Table 1. Feed composition of Moli-gel

Sample	9G (mg)	DEG-p (mg)	ADVN (mg)	DEG-m (mg)	Lig-m (mg)
A	2597.3	5082.2	59.8	97.3	231.2
B	2599.2	5084.3	60.0	98.4	231.4
C	2174.5	4222.0	60.0	81.5	192.7
D	2579.0	5018.2	60.1	97.3	231.2
E	2572.1	5029.8	59.9	97.6	232.5
F	2581.9	5034.8	60.0	97.9	231.8

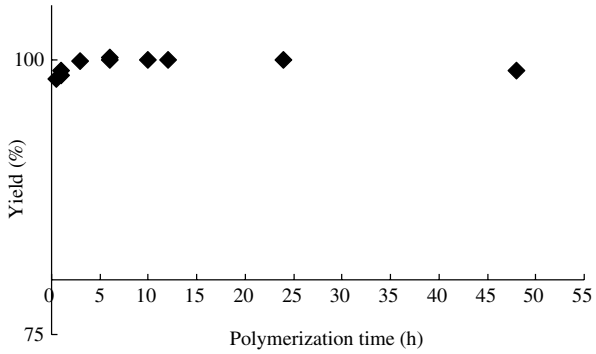

Figure 1. Yield of Moli-gel at each polymerization time.

Other possible reasons are differences in their micro-structures. As shown in Figure 7, macroporous structure of Toyopearl was found to be rather different from that of Moli-gel. Due to a monolithic, co-continuous structure of Moli-gel, micron-size through pores were formed, therefore mass-transfer is expected to be greater within Moli-gel compared with that within Toyopearl having sub-micro-size pores.[6] As shown later, this is a reason why Moli-gel can be utilized in a flaky shape. One of the required properties of novel affinity gels will be easy handling and high reactivity; therefore, the flaky shape of Moli-gel is one of the best solutions as described later.

As revealed in Figure 8, after several chemical reactions including immobilization of ligands in organic solvent, morphology of Moli-gel did not change, where co-continuous structure was kept unchanged without any swelling in the organic solvents utilized. This is simply because Moli-gel is highly cross-linked.

2.4. Capture of CAII with Sulfonamide as ligand

In this study, we utilized Sulfonamide as a ligand, because this is commercially available and one of the target proteins is Carbonic anhydrase-II (CAII), which is known as dominant protein in rat-brain protein lysate. For fair comparison, the amount of surface ligand should be similarly controlled; however, the detailed

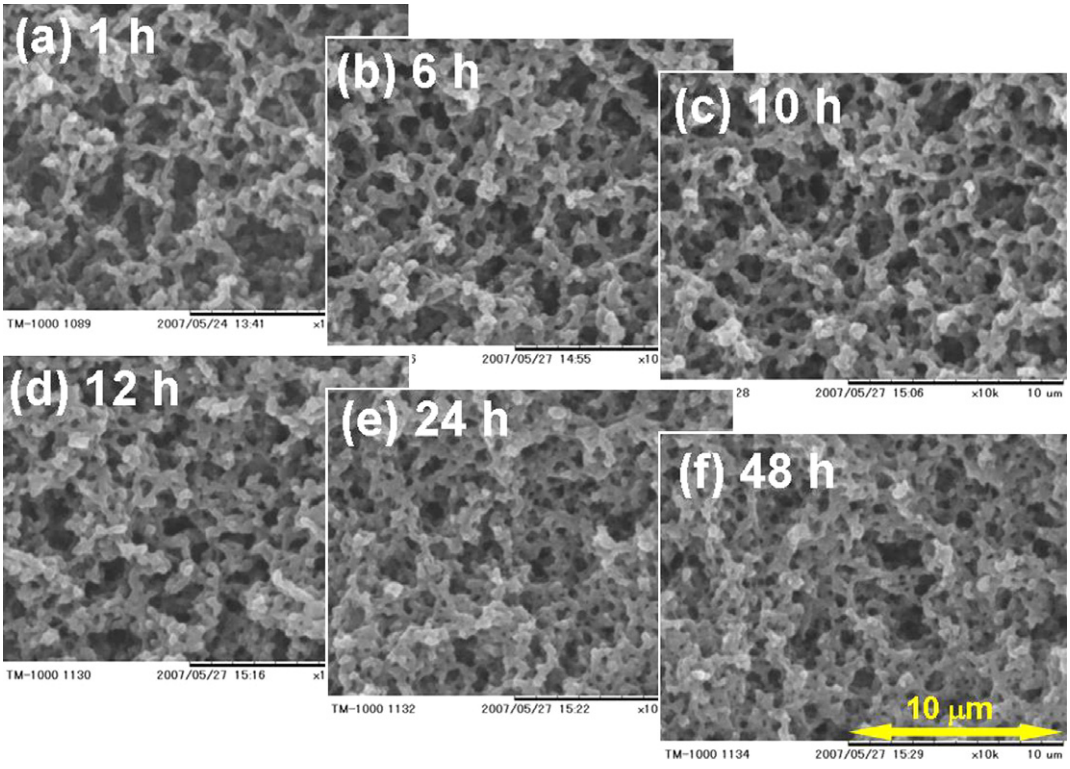

Figure 2. SEM image of morphology of Moli-gels (polymerization time; (a) 1 h, (b) 6 h, (c) 10 h, (d) 12 h, (e) 24 h, (f) 48 h).

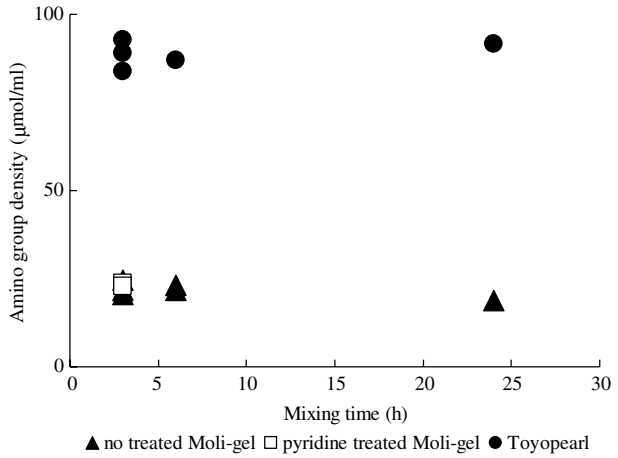

Figure 3. Amount of surface amino groups of Moli-gel and Toyopearl.

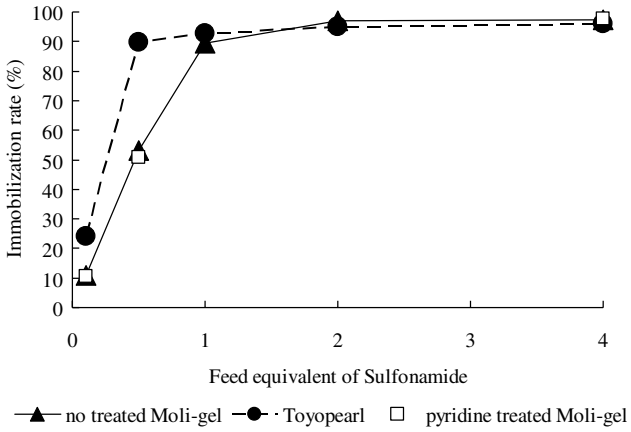

Figure 4. Sulfonamide immobilization rate.

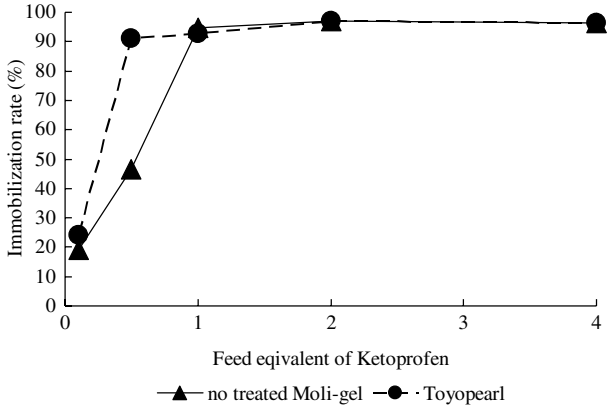

Figure 5. Ketoprofen immobilization rate.

properties of Toyopearl have been unknown. Therefore, in this study, we utilized the gels fully covered with Sulfonamide.

As shown in Figure 9a, capture of CAII on Moli-gel was confirmed to be comparably achieved without non-specifically bound proteins compared with that on Toyopearl. This is the previously reported advantage of Moli-gel.[4] Using silver stained SDS gel (Fig. 9b), possible specifically bound proteins were faintly observed on Moli-gel just below the band of CAII as well as in much higher Mw region indicated by blue dotted arrows. On Toyopearl, those bands could be hardly detected. Again, we applied serial affinity chromatography.[5] This is of course not only due to non-specifically bound proteins but also through the experimental procedures, the protein lysate could not be completely removed from dispersion of Toyopearl even after the centrifugation. The residuum of lysate proteins must be one reason for lots of 'non-specifically bound' proteins with Toyopearl on the SES-PAGE.

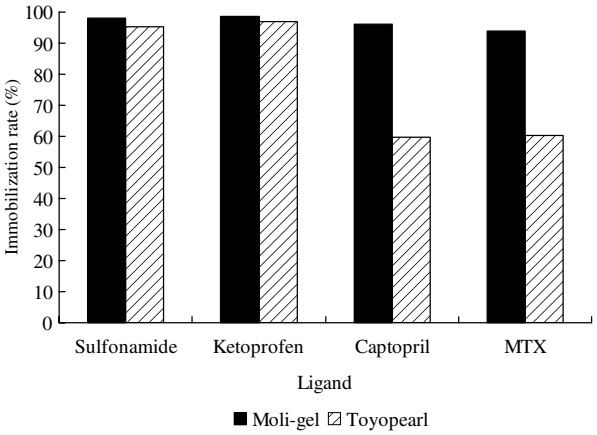

Figure 6. Difference in immobilization rate of Moli-gel and Toyopearl for four different ligands.

As mentioned previously, Moli-gel has flaky shape with 100–400 μm size, while Toyopearl has spherical shape with 40–90 μm particle size. By the use of Toyopearl, the particles tended to be spontaneously re-dispersed after sedimentation; therefore, complete removal of lysate as well as another solutions cannot been achieved without loss of the particles when micro-pipets were utilized through experiments. In contrast, complete removal of protein lysate can be easily achieved when we utilized Moli-gel based affinity resin.

Usually, several times of washing procedure take place through the affinity experiments in capture of proteins, but the target proteins having relatively small binding constant will be also removed through several washing processes. Therefore, washing procedure as few as possible will be desired for search of really unknown target proteins.

Easy handling of Moli-gel was performed as illustrated in Figure 10. Due to relatively large flaky shape of Moli-gel, no spin-down (centrifugation) was found to be essential, while Toyopearl needed spin-down for complete sedimentation of the particles. After the protein capture experiment, Moli-gel based affinity resins spontaneously afforded sedimentation, while Toyopearl showed even dispersed particles in the experimental tube. Although Moli-gel had relatively large size, the treatment time for capture of proteins was short enough compared with that of Toyopearl.[7] This is also due to co-continuous structure of Moli-gel.

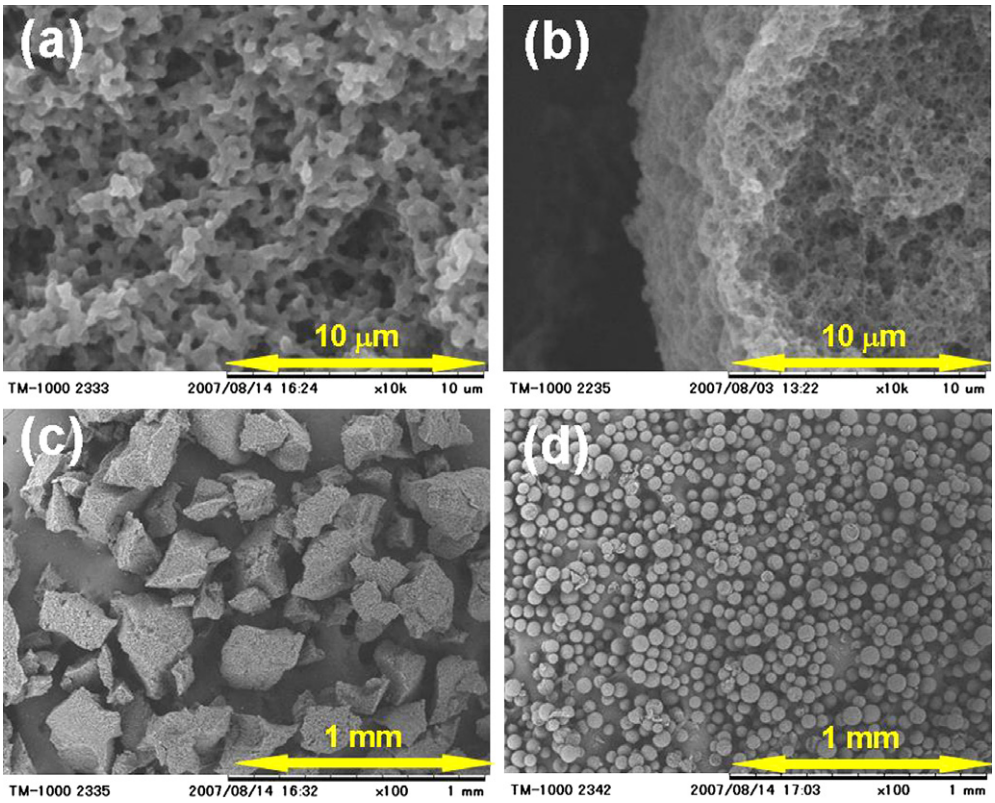

Figure 7. SEM image of Moli-gel and Toyopearl (a) inside structure of Moli-gel, (b) inside structure of Toyopearl, (c) shape of Moli-gel, (d) shape of Toyopearl.

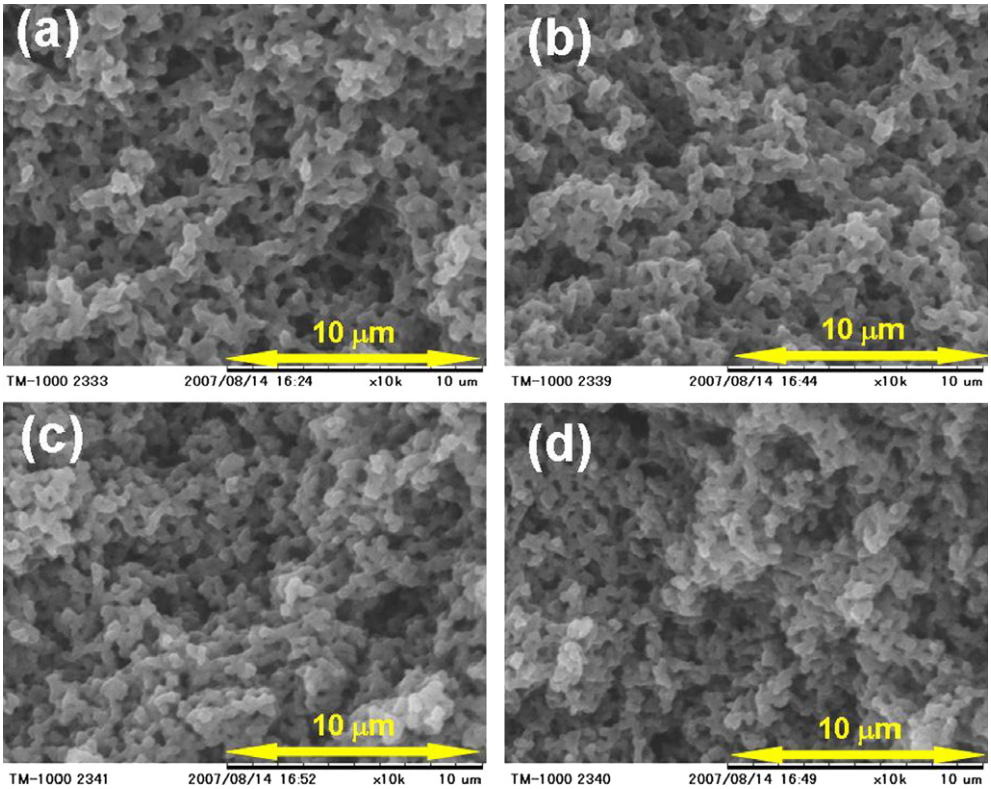

Figure 8. SEM image of Moli-gels about stability of structure (a) before deprotection, (b) after deprotection, (c) sulfonamide affinity resin, (d) ketoprofen affinity resin.

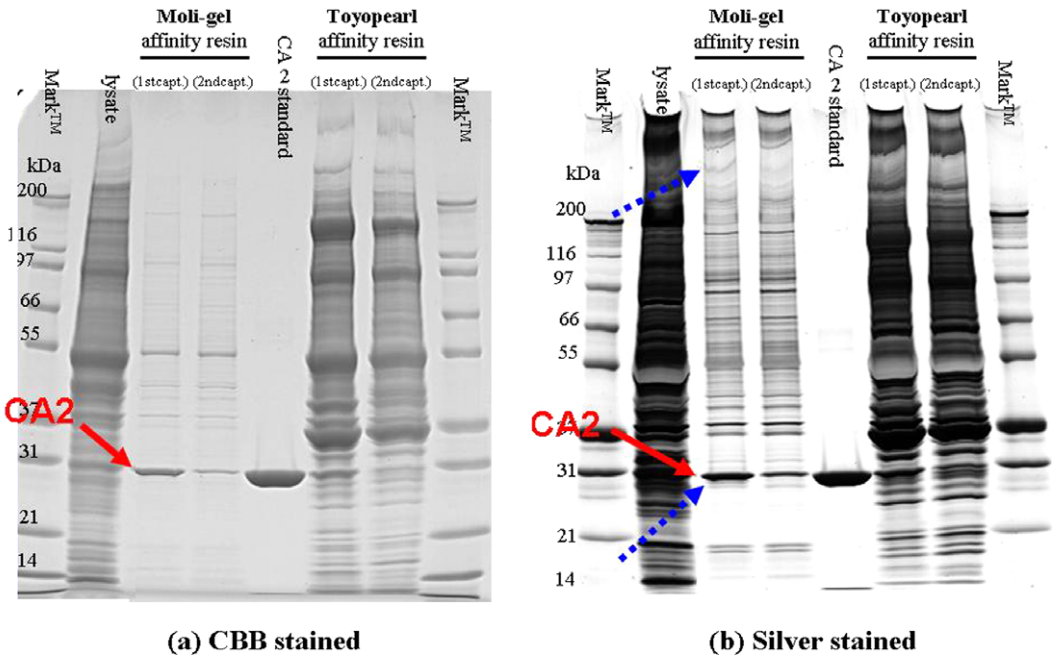

Figure 9. SDS–PAGE analysis by sulfonamide affinity resin (a) CBB stained, (b) silver stained.

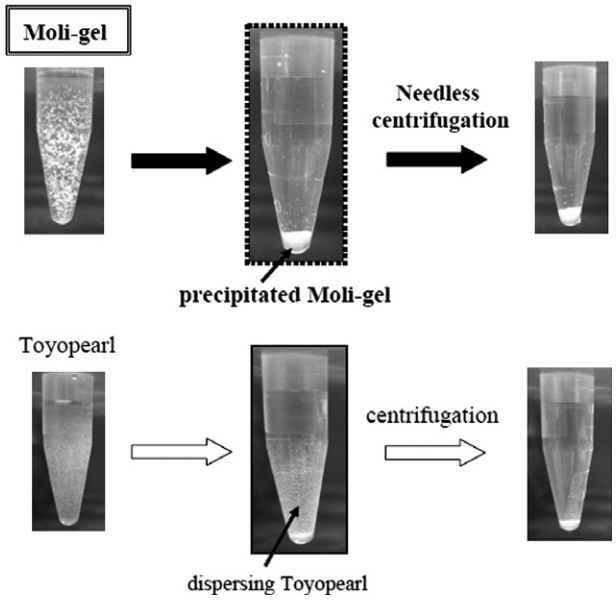

Figure 10. Picture of washed Moli-gel and Toyopearl before conditioning and after proteins combining test.

3. Conclusion

The affinity gel prepared had monolithic, co-continuous structures, therefore we called this gel as Moli-gel. Moli-gel was found to have even, totally co-continuous structure and have 22.3 μmol/ml of the amount of surface amino groups. Several ligands were immobilized quantitatively or the controlled coverage, and capture of protein was comparably achieved without non-specifically bound proteins. In this study, Moli-gel was utilized as flaky shape having 100–400 μm size, therefore, no centrifugal sedimentation was required. Removal of protein lysate was completely achieved, which might avoid lots of proteins on SDS–PAGE. A flaky Moli-gel based affinity resin will have some advantages for search of the target proteins of rare ligands, such as naturally occurring drugs as well as toxins.

4. Experimental

4.1. Solvents and reagents

Solvents and reagents were utilized without further purification, unless it was particularly mentioned. Monomers, porogen, and ligands are structurally illustrated in Figure 11.

A monomer for immobilization of ligand *N-tert*-butoxy-17-amino-3,6,9,12,15-pentaoxaheptadecane-1-yl methacrylate (*Lig-m*) was kindly supplied by *Reverse Proteomics Research Institute* (Kisarazu, Japan). A hydrophilic–lipophilic control monomer, 2-(2-methoxyethoxy) ethyl methacrylate (*DEG-m*), was purchased from TCI (Tokyo, Japan), while a cross-linking monomer, polyethylene glycol #400 dimethacrylate, NK ESTER 9G (*9G*) was donated from SHIN-NAKAMURA Chemical Co., Ltd (Wakayama, Japan).

Reagents for determination of surface amino groups, 76% w/w phenol/EtOH (*phenol/EtOH*) and 0.0002 M KCN/pyridine, were both purchased from Applied Biosystems (Tokyo, Japan). A commercially available affinity gel, Toyopearl™ (*Toyopearl*), was purchased from Tosoh Bioscience (Pennsylvania, USA, AF-Amino-650M, http://www.toyopearl.com).

Compound utilized as ligand in this study, 4-carboxybenzenesulfonamide (*Sulfonamide*), was purchased from Sigma–Aldrich, Japan (Tokyo, Japan), while 2-

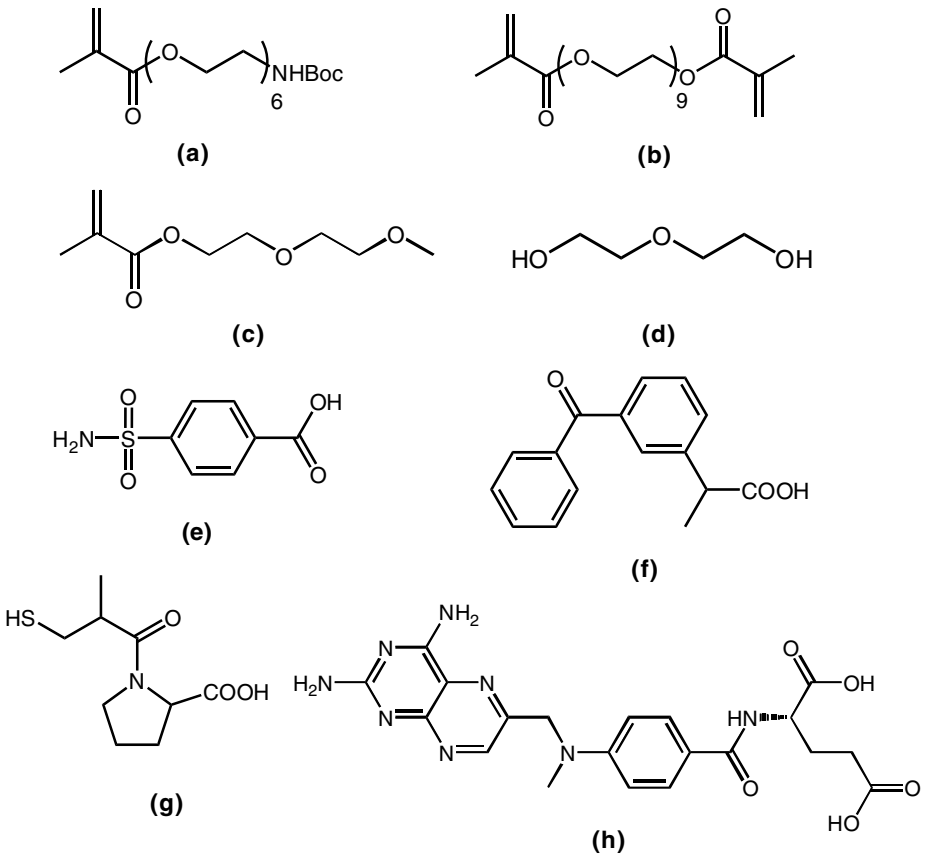

Figure 11. Structure of monomer, porogen, and ligand. (a) *N-tert*-butoxy-17-amino-3,6,9,12,15-pentaoxaheptadecane-1-yl methacrylate (Lig-m), (b) 2-(2-methoxyethoxy)ethyl methacrylate (DEG-m), (c) polyethyleneglycoldimethacrylate (9G), (d) 2,2′-oxydiethanol (DEG-p), (e) 4-carboxybenzenesulfonamide (Sulfonamide), (f) 2-(3-benzoyl phenyl)-propionicacid (Ketoprofen), (g) 1-(mercapo-2-methylpropanoyl)pyrrolidine-2-carboxylic acid (Captopril), (h) (*S*)-2-(4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzamido)pentane dioic acid (Methotrexate: MTX).

(3-benzoyl phenyl)-propionic acid (*Ketoprofen*) was obtained from TCI (Tokyo, Japan). A solvent for immobilization reaction of the ligand, *N*-methyl-2-pyrrolidinone, dehydrated (*dry-NMP*), was purchased from KANTO Chemical Co., Inc. (Tokyo, Japan). Reagents for condensation reaction, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (*water-soluble arbodiimide: WSCD*) and 1-hydroxy benzotriazole (*HOBt*), were purchased from PEPTIDE Institute, Inc. (Osaka, Japan).

Poly-acrylamide gel was supplied from Bio-Rad Laboratories (Tokyo, Japan) as Ready Gels J 7.5–15% 161J381V, while protein standards for SDS–PAGE were purchased from Invitrogen (Tokyo, Japan, Mark12™).

The following materials were all purchased from Wako Pure Chemical, Co., (Osaka, Japan): 2,2′-oxydiethanol (*DEG-p*), 2,2′-azobis(2,4-dimethylvaleronitrile) (*ADVN*), methanol, acetone, ethanol, oxalic acid, 1 mol/l sodium hydroxide solution, 1 mol/l hydrochloric acid, 1.0 w/v% phenolphthalein ethanol (90%) solution, 1-(mercapo-2-methylpropanoyl)pyrrolidine-2-carboxylic acid (*Captopril*), (*S*)-2-(4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzamide) pentanedioic acid (*Methotrexate: MTX*), acetonitrile, and 0.3 mM sodium *N*,*N*-diethyldithio carbamate trihydrate.

The following materials were purchased from Nacalai Tesque, Inc. (Kyoto, Japan): trifluoroacetic acid (*TFA*), sodium hydrogen carbonate, ninhydrin, pyridine, *N*-methyl-2-pyrrolidone, acetic anhydride, *N*,*N*-dimethylformamide (*DMF*), sucrose, 1 M Tris–HCl buffer solution (pH 7.6), sample buffer solution with 2-ME (2×) for SDS–PAGE, including 4% (w/v)-SDS, 20% (v/v)-glycerol, 0.01% (w/v)-CBB, 10% (v/v)-2-mercaptoethanol, 0.125 M Tris–HCl, pH 6.8, running buffer solution (10×) for SDS–PAGE, Rapid Stain CBB Kit, and Sil-Best Stain-neo for Protein and Nucleic Acid/PAGE.

4.2. Apparatus

The gels prepared were observed through a Miniscope TM-1000 (*SEM*) (HITACHI). A UV-mini 1240 UV–vis spectrophotometer (SHIMADZU) was utilized for ninhydrin method. A Beckman centrifuge, Avanti J-25, was utilized with a JLA-10500. GS-800 Calibrated Imaging Densitometer (Bio-Rad) recorded images of SDS–PAGE.

4.3. Preparation of affinity gel (Moli-gel)

The prescribed amount of Lig-m, DEG-m, and 9G was carefully dissolved in the porogen, DEG-p with a radical initiator, ADVN, and polymerized at 60 °C for the pre-

Table 2. Amount of ligand for immobilization

	Ligand (mg)				Condensing agent	
	Sulfonamide	Ketoprofen	Captopril	MTX	WSCD (ml)	HOBt (mg)
Moli-gel	15.9	20.3	17.4	36.4	18.6	13.0
Toyoparl	40.6	30.8	26.3	27.2	28.3	19.7

scribed polymerization time. The resulting gels were washed with methanol, acetone, followed by pure water, then comminuted to become flaky shapes (100–400 μm) by a stainless steel mesh (425 μm) and a spatula.

Moli-gel flaked was then washed with 10% aqueous TFA, de-BOC reaction was carried out at room temperature for 3 h in 95% TFA (10 ml). After the reaction, de-BOC Moli-gel was washed with water repeatedly, then neutralized with saturated sodium hydrogen carbonate solution. The resulting Moli-gel was stored in pure water. Chemical yields of Moli-gel were calculated based on the feed amount of monomers. SEM observation took place after platinum–palladium spatter. Toyopearl was also observed with SEM.

4.4. Determination of amount of surface amino groups

4.4.1. Titration method. NaOH solution (0.1 N) was approved by a standard 0.1 N oxalic acid aqueous solution. 0.1 N HCl solution was then standardized by the 0.1 N NaOH standard solution.

Dispersion of Moli-gel or Toyopearl was poured into a graduated vial to measure the accurate volume of the gels after their sedimentation. Gels were treated with 3 ml of 0.1 N aqueous HCl for 3, 6, or 24 h with stirring. After removal and recovery of resulting aqueous HCl by filtration, the gels were washed by pure water and the water was combined with the recovered HCl. This HCl solution was titrated with the standard 0.1 N NaOH aqueous solution with phenolphthalein as the indicator. This titration was also applied for pyridine treated Moli-gel. This titration took place at least three times and the reproducibility was good (within 8%).

4.5. Ninhydrin method

As described in Refs. 8–10 ninhydrin method was also applied to determine relative concentration of the amino group.

Twenty microliters of aqueous dispersion of Moli-gel was washed with 20% aqueous ethanol, then 5 μl of phenol/EtOH, 100 μl of KCN/Py, and 20 μl of Ni/EtOH were added followed by the reaction at 95 °C for 5 min. Then, 870 μl of 20% aqueous ethanol was added into the reaction mixture to remove the 'color' from the gels. The colored supernatant was spectroscopically measured at 570 nm using the UV spectrometer.

4.6. Immobilization of ligand

Due to the quite small amount of gels utilized, we have prepared dry-NMP solution of each ligand by prescribed amount (Table 2). After washing of de-BOC gels with acetonitrile followed by dry-NMP, the prepared solution of ligand was added to the gels with 1.2 equiv of WSCD and/or HOBt. The equivalent of added ligand was 0.1, 0.5, 1.0, 2.0, or 4.0 to the amino groups. The reaction was carried out at ambient temperature for usually 48 h. After the reaction, the amount of residual amino groups was measured using the ninhydrin method in comparison to that of original gel. After the immobilization reaction, residual amino groups were covered by the treatment of acetic anhydride in DMF.

4.7. Capture of proteins

Protein lysate was prepared using reported method from rat brains[5] and stored at −80 °C. The affinity resins were treated with protein lysate at 4 °C for 4 h by the reported method[5] to capture the target proteins. In this work, after the removal of protein lysate from the gels, only one-time washing process took place. The captured proteins were recovered using SDS sample buffer solution (30 μl), then a SDS–PAGE was examined. In this work, the experiment of competition method was carried out using '*serial affinity chromatography*'.[5]

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.10.099.

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