Suppression of Hydrophobicity and Optimizations of a Ligand-Immobilization for Effective Affinity Chromatography Using a Spongy Monolith

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Abstract
In this study, we reveal the suppression of non-specific hydrophobic interaction in poly(ethylene-co-glycidylmethacrylate) (PEGM) based spongy monolith (SPM), PEGM-SPM, which has recently been reported as a new platform of separation medium for affinity chromatography in our previous study, by a simple acidic treatment. Additionally, the immobilization procedures of protein-A toward the PEGM-SPM and the separation conditions for immunoglobulin G (IgG) were optimized for further effective affinity separations. As a result of treatment by a mixture of trifluoroacetic acid and acetonitrile, the hydrophobicity was dramatically suppressed in the PEGM-SPM. The optimizations for the density of PEGM in the PEGM-SPM, the protein A immobilization, and the binding/releasing conditions showed that variety of proteins and peptides were not retained on the protein A immobilized spongy column at all while IgG was absolutely separated by a simple stepwise pH gradient condition.

Keywords: Spongy monolith; Poly(ethylene-co-glycidylmethacrylate); Affinity chromatography; Hydrophobicity; Protein A; IgG

1. Introduction
Since antibody-based medicines have attracted attentions due to their high selectivity toward their target antigens, relatively low levels of side effects, and stability in vivo, a variety of antibody-based medicines have been approved in recent years [1,2]. These medicines can be produced using standard cell culture procedures [3-7]. To obtain a high-quality antibody medicine at low cost, it is necessary to select highly productive cells, optimize the culture conditions, and develop an efficient purification method. To evaluate the productivity of a system for biosynthesis of an antibody, especially of the immunoglobulin G (IgG) subtype, a chromatographic system using a protein A immobilized column is often employed for selection and optimization of the cell culture. In order to process a large number of samples, it is necessary to perform a rapid optimization using high-throughput chromatography [8,9]. For certain antibodies, more than 100 kg is required at the clinical investigation stage [10-12]. The separation media in which protein A is immobilized onto a crosslinked-agarose adsorbent are used for the analysis and purification of an antibody [13-15] as a suitable separation antibodies [16-20]. However, as for currently available separation media, elution throughput is often limited, resulting in an inefficient optimization of purification and productivity. Furthermore, the expense of such adsorbents (30-fold higher than other typical adsorbents) and the difficulty of column packing contribute to the high final price of antibody-based medicines [21-23]. Therefore, there is an urgent demand for new separation media that can facilitate higher throughput and lower cost.

To achieve high throughput and low cost, monolithic materials with continuous three-dimensional (3D) structures are advantageous [24-26]. For purification of biomolecules, the monolithic structure is suitable for rapid reactions because the flow-through pores are integrated with the skeleton [27-33]. In a typical monolith, silica- or polymer-based materials are prepared by sol–gel reaction and/or phase separation. Accordingly, the control of pore size, especially for larger pore (> 10 μm), scale up in column size, and packing to columnar tubes are not easy. Instead of these typical monoliths, we proposed using a
sponge-like material, spongy monolith (SPM), as a novel separation medium [34,35]. Furthermore, we recently reported the novel SPM consisted of poly(ethylene-co-glycidylmethacrylate) (PEGM). The monolith allowed to immobilize certain proteins due to the reaction between epoxy groups in PEGM and amino groups in proteins. In case of a protein A-immobilized SPM, we successfully achieved a high throughput affinity separation of IgG. Additionally, an online digestion of an antibody was also revealed by a pepsin-immobilized SPM under high flow rate [36]. Although we suggested the possibility of PEGM-SPM as a new platform for the protein-based reaction, the fundamental studies, such as the hydrophobicity of the PEGM-SPM and density of the immobilized proteins were not satisfied in the previous report. In order to apply the SPM for practical uses, we carry out the fundamental examinations regarding a suppression of hydrophobicity by simple acidic treatment and an optimization for the immobilization procedures using protein A as a ligand.

2. Experimental

2.1. Chemicals and reagents

Methanol of HPLC grade, sodium hydroxide (NaOH), hydrochloric acid (HCl), ethylene glycol, acetonitrile, trifluoroacetic acid (TFA), avidin from egg white, acetone, toluene, ethylbenzene, di-sodium hydrogen phosphate, sodium dihydrogenphosphate dehydrate, and α-amylase were purchased from Nacalai Tesque (Kyoto, Japan), protein A, albumin from bovine serum crystallized (BSA), lysozyme, hexamethylenediamine, and trypsin were from Wako Pure Chemical Industries (Osaka Japan), n-propylbenzene, butylbenzene, and n-hexylbenzene were from Tokyo Chemical Industry (Tokyo, Japan), poly(ethylene-co-glycidyl) methacrylate, PEGM, poly-L-arginine, β-lactoglobulin, Tyr-Tyr-Tyr, fetuin, IgG1 kappa from human myeloma plasma, and α1-acid glycoprotein were from Sigma Aldrich Japan (Tokyo, Japan), respectively. Deionized water was obtained from a Milli-Q Direct-Q 3UV system (Merck Millipore, Darmstadt, Germany).

2.2. Instruments

A Scanning electron microscopy was carried out by a TM-1000 (Hitachi High-Technologies, Hitachi, Japan), LC analyses with photo diode array detection by an LC-20 Prominence (Shimadzu Co., Kyoto, Japan), and Fourier transform infrared spectroscopy (FTIR) by an FT-IR Nicolet iS5 ATR (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3. Preparation of a spongy monolith

PEGM of 35 or 15wt%, in which glycidyl methacrylate of 8% is contained, 65 or 85wt% of pore templates (pentaerythritol), whose particle size in diameter was classified around 10 μm, and 7wt% of auxiliary of pore templates (poly(oxyethylene, oxypropylene)) triol were melted at 130 °C and homogeneously kneading. The resulting material was extruded as a columnar shape at 130 °C. The columnar material was immediately cooled in water to obtain the stick like material. After cooling, the material was washed in water under an ultrasonication to remove water-soluble compounds. At this stage, water-soluble compounds functioned as the pore templates (PEGM-SPM).

2.4. Packing of a spongy monolith

For packing spongy monoliths in a stainless steel column, we utilized an empty column with an internal diameter of 4.6 mm. The diameter of the spongy monolithic (4.8 mm) was greater than the internal diameter of the empty column. Nevertheless, the elasticity of the spongy monolith material facilitated the packing. The procedure for packing was as follows: One end of the spongy monolith was compressed with a thermal shrinkage tube at 120 °C. After cooling, the shrinkage tube was removed; and the diameter of the compressed end of the spongy monolith was reduced less than 4.6 mm. After macerating the spongy monolith into ethylene glycol as a lubricity agent, the shrunk portion of the spongy monolith was inserted into the empty column and pulled from the other end, until the non-shrunk portion completely filled the column. Finally, the excess portion of the spongy monolith was cut and the column end module was connected. At this point, the shrunk end of the spongy monolith was completely cut and only the portion of the material with the initial diameter was packed into the column. Then, the prepared column was connected to a pump of liquid chromatography for continuous elution. The mixture of methanol/water was eluted to the column for further washing to remove the pore templates and the homogenization of the packing condition.

2.5. Preparation of a protein A immobilized column

Phosphate buffered salts (PBS) solution was prepared with a PBS tablet into pure water of 100 mL (9.57 mM, pH 7.5). Pierce Recombinant protein A of 5 mg was dissolved in the PBS solution of 5 mL. For conditioning the column, acetonitrile and pure water were passed through the PEGM-SPM at 37 °C for 5 mL in each solvent. The protein A solution (1.0 or 2.0 mg mL⁻¹) was fulfilled into the PEGM-SPM completely, and then the column was incubated at 37°C for 8 to 24 h. The completed column was washed with pure water for 1 h at 1 mL min⁻¹ (ProA-SPM).

2.6. Conditions for liquid chromatography (LC)

For reversed-phase LC (RPLC) evaluations, an isocratic mode was employed using water / acetonitrile = 2/8 (v/v) at
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40 °C under 1.0 mL min⁻¹. For an affinity separation of the ProA-SPM, a 50 nM phosphate buffer with 150 mM NaCl (pH 7.3) (A) and pH 2.5 or 4.0 (B) was employed at 25 °C by a stepwise gradient utilized at 100% A (0 to 5 min) and 100% A to 100% B (5 to 30 min). For separation of peptides and proteins, a 0.1% aqueous TFA (A) and 0.1% TFA in acetonitrile was employed at 25 °C by a gradient utilized at 100% A (0 to 5 min), 0 to 100% B linear gradient (5 to 15 min), and 100% B (15 to 30 min).

3. Results and discussions
3.1. Effect of the hardness on PEGM-SPM for column packing
In our previous study, we employed relatively hard PEGM-SPM with 35wt% of PEGM (PEGM-SPM-35) as a platform for the affinity chromatography. However, in this case, the reproducibility for column packing is not satisfied because the operability of PEGM-SPM was not good due to its hardness. Therefore, we expected that the softer sponge is prefer to easiness for the packing procedures and provide better reproducibility. Then, we prepared another PEGM-SPM with 15wt% PEGM (PEGM-SPM-15). As results, the newly prepared monolith, PEGM-SPM-15 showed significantly better operability due to its softness. In brief, the column packing by the procedures described in Experimental section allowed easiness for the packing without any physical stress. The morphological images by SEM of PEGM-SPM-35 and PEGM-SPM-15 are shown in Fig. 1. Although PEGM-SPM-15 shows slightly wider pores compared to PEGM-SPM-35, the almost similar structure was obtained even if we use 15wt% PEGM. Then, we examined the reproducibility of the PEGM-SPM-15 packed column by simple reverse-phase LC. As shown in Table 1, the retentions of alkylbenzenes by hydrophobicity due to PEGM on three kind columns prepared with PEGM-SPM-15 were almost similar and the relative standard deviations of each retention factor were fallen within 6.0%. Therefore, we concluded that the softer PEGM-SPM is prefer for the column based on the better operability.

![Fig. 1. SEM images of the PEGM-SPM. (a) 35wt% PEGM, (b) 15wt% PEGM.](image)

Table 1. Retention factors and RSDs of alkylbenzenes on PEGM-SPM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>column 1</th>
<th>column 2</th>
<th>column 3</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>toluene</td>
<td>0.49</td>
<td>0.44</td>
<td>0.45</td>
<td>5.8</td>
</tr>
<tr>
<td>ethylbenzene</td>
<td>0.58</td>
<td>0.58</td>
<td>0.61</td>
<td>2.9</td>
</tr>
<tr>
<td>propylbenzene</td>
<td>0.81</td>
<td>0.81</td>
<td>0.78</td>
<td>2.2</td>
</tr>
<tr>
<td>butylbenzene</td>
<td>1.15</td>
<td>1.17</td>
<td>1.10</td>
<td>3.2</td>
</tr>
<tr>
<td>hexylbenzene</td>
<td>2.42</td>
<td>2.43</td>
<td>2.54</td>
<td>2.7</td>
</tr>
</tbody>
</table>

t₀, acetone (column 1, 1.41; column 2, 1.39; column 3, 1.39)

3.2. Hydrophobicity of the protein-immobilized PEGM-SPM
In case of protein A immobilized PEGM-SPM (ProA-SPM) described in our previous report, we revealed that hydrophobicity was successfully suppressed by covering the monolithic skeleton with proteins, briefly the most of proteins were not retained on ProA-SPM in LC. However, we wondered the possibility that small part of the original surface based on PEGM is still remained and contribute to hydrophobic interaction even after the protein modification. To confirm the presence of hydrophobicity, typical reverse-phase LC was carried out with protein-immobilized SPM column. As a reference of a protein-immobilized SPM, avidin was also immobilized on the monolith. In both of protein A and avidin, PEGM-SPM-15 was employed for the immobilization. Firstly, the chromatograms for typical proteins containing BSA and lysozyme with on an original PEGM-SPM-15, ProA-SPM, and Avidin-SPM are indicated in Fig. 2. BSA (66.0 kDa, pI 4.7) and lysozyme (14.4 kDa, pI 10.8) have different Mw/pI and show no affinity to protein A and avidin, so that these proteins are suitable for the typical proteins to evaluate the non-specific hydrophobic interaction. According to these chromatograms, only PEGM-SMP-15 showed the hydrophobic retentions under the linear-gradient reverse-phase condition. The results suggested that there were no enough surface area for hydrophobic interaction with biomolecules such as the proteins in the protein-immobilized SPMs. On the other hand, Table 2 showed the retention of alkylbenzenes on the protein-immobilized SPMs. Obviously, the retention of alkylbenzenes on the protein-immobilized SPMs was almost same as PEGM-SMP-15. Therefore, we assumed that small parts of hydrophobic surface due to PEGM was still remained even after the protein immobilization.

Table 2. Retention of alkylbenzenes on PEGM-SPM and protein-immobilized SMPs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PEGM-SPM-15</th>
<th>ProA-SPM</th>
<th>Avidin-SPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>toluene</td>
<td>0.49</td>
<td>0.47</td>
<td>0.41</td>
</tr>
<tr>
<td>ethylbenzene</td>
<td>0.58</td>
<td>0.62</td>
<td>0.56</td>
</tr>
<tr>
<td>propylbenzene</td>
<td>0.81</td>
<td>0.85</td>
<td>0.81</td>
</tr>
<tr>
<td>butylbenzene</td>
<td>1.15</td>
<td>1.25</td>
<td>1.16</td>
</tr>
<tr>
<td>hexylbenzene</td>
<td>2.42</td>
<td>2.55</td>
<td>2.62</td>
</tr>
</tbody>
</table>
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Fig. 2. Chromatograms of the proteins in each column. (a) for BSA, (b) lysozyme. LC conditions: column size, 100 mm × 4.6 mm i.d.; flow rate, 1.0 mL min⁻¹; mobile phase, (A) 0.1% aqueous TFA (A) and (B) 0.1% TFA in acetonitrile utilized at 100% A (0 to 5 min), 0 to 100% B linear gradient (5 to 15 min), and 100% B (15 to 30 min); temperature, 25 °C; detection, UV 220 nm.

To examine the possibility of suppression for hydrophobicity of the monolith, hydrolysis reactions toward PEGM-SPM-15 were carried out with aqueous HCl, NaOH, and TFA solutions. Acetonitrile was also added to the solutions because the hydrophobic surface of PEGM-SPM was hard to be wetted only by aqueous solutions. After hydrolysis, only TFA solutions were worked for the ring opening reaction of epoxy groups in PEGM, which contributes increase of hydrophilicity. Then, the reaction conditions with TFA regarding concentration of TFA and the reaction time were optimized. In brief, the concentration of TFA and the reaction time were controlled from 0.1% to 5.0% and from 1 to 7 days, respectively.

Table 3. Retention of alkylbenzenes on PEGM-SPM before and after hydrolysis.

<table>
<thead>
<tr>
<th>Alkylbenzene</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>toluene</td>
<td>0.44</td>
<td>0.01</td>
</tr>
<tr>
<td>ethylbenzene</td>
<td>0.58</td>
<td>0.03</td>
</tr>
<tr>
<td>propylbenzene</td>
<td>0.81</td>
<td>------</td>
</tr>
<tr>
<td>butylbenzene</td>
<td>1.17</td>
<td>0.04</td>
</tr>
<tr>
<td>hexylbenzene</td>
<td>2.43</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Finally, the condition using 5.0% TFA in acetonitrile for 24 h was effectively functionalized for the hydrolysis. In fact, the specific absorption around 3500 cm⁻¹ in FTIR and the decrease of hydrophobic retention in reverse-phase LC were observed as shown in Fig. 3 and Table 3, respectively. These results suggested that hydrophobicity of PEGM-SPM is easily suppressed by simple treatment with a TFA solution.

3.3. Optimizations of the protein A-immobilization

To obtain an effective ProA-SPM, the effect of the immobilization procedures containing the concentration of protein A and the reaction time were examined. In brief, we employed the concentrations including 0.5, 0.7, and 1.0 mg mL⁻¹, and the reaction time including 1, 4, 16, and 24 h. The results of reverse-phase separations with BSA are summarized in Fig. 4. According to these chromatograms, the protein A of 1.0 mg mL⁻¹ as a concentration and 24 h for the reaction are at least needed to suppress the hydrophobic interaction to BSA.

3.4. Conditions and selectivity of affinity separations on ProA-SPM

After determination of the preparation procedures of a ProA-SPM, we optimized the chromatographic condition on the affinity separation of IgG. In our previous study, we utilized a strong acidic solution (pH 2.5) for the elution of IgG. In this case, the elution was obviously achieved as a clear-cut peak.
However, in fact IgG might be denatured such by generating dimer under strong acidic condition. In general, the affinity between protein A and IgG is cleaved from pH 3.9 to pH 4.6, so that we employed milder conditions for the elution. Briefly, as shown in Fig. 5, the chromatogram under pH 2.5 showed the split peak, which might be caused by denaturing. On the other hand, when we used pH 4.0 for the elution, a clear one peak was obtained without any splitting. Consequently, we decided to employ the solution at pH 4.0 as an elution condition. Furthermore, the durability of the ProA-SPM was evaluated by 100 times repeated separations of IgG using the buffer at pH 4.0, and then we confirmed the durability without the denaturation of protein A: the RSDs of the retention time and recovery of IgG were estimated as 0.45% and 0.41%, respectively.

Finally, by using the optimized ProA-SPM and affinity chromatographic conditions, the selectivity for IgG was examined. As shown in Table 4, a variety of proteins and peptides, which have several molecular weight and isoelectric point (pl), were analyzed with ProA-SPM under the optimized affinity separation condition. The chromatograms for each solutes are summarized in Fig. 6. All the tiny peaks at 7.0 min in these chromatograms are shock peak due to the stepwise gradient from neutral to acidic. According to these chromatograms, only IgG was clearly separated by simple step-wise pH gradient, while any other proteins and peptides were immediately eluted without any interactions toward ProA-SPM. Regardless of any molecular weight and pl of proteins (the result of lysozyme was indicated above), the affinity separation was successfully achieved. The results strongly suggested that the optimized ProA-SPM and its chromatographic condition contributed the effective affinity separation of IgG. We believe that the ProA-SPM will allow to explore of new antibody-based drugs and their purifications.

![Fig. 4. Chromatograms of BSA on ProA-SPMs.](image)

| Table 4. Proteins and peptides used for the affinity separation. |
|-----------------------------|----------|----------|
| lysozyme                   | 14307    | 10.8     |
| β-lactoglobulin            | 18400 (monomer) | 36800 (dimer) | 5.2 |
| α-amylase                  | 54000    | 6.0      |
| fetuin                     | 48400    | 3.3      |
| α₁-acid glycoprotein       | 44100    | 2.7      |
| Pro-Leu-Gly                | 284      | 2.7      |
| Phe-Ala                    | 236      | 5.3      |

4. Conclusion

In this study, we carried out the fundamental examinations for PEGM-based spongy monoliths. We revealed the suppression of hydrophobicity of the PEGM-SPM due to original property of PEGM by a simple treatment using a TFA solution. After optimizations of the protein A immobilization by suitable reaction time/concentration of protein A and affinity chromatographic conditions by using a milder pH elution, we successfully demonstrated the abundantly selective separation of IgG with the ProA-SPM without any non-selective adsorption for the proteins and peptides. These fundamental results will contribute the development of discovering new antibody-based drugs and their analysis/purification techniques.
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Fig. 6. Affinity separation of proteins and peptides with ProA-SMP. Chromatographic condition: column, ProA-SMP (100 mm × 4.6 mm i.d.); flow rate, 1.0 mg mL\(^{-1}\); mobile phase, 50 mM phosphate buffer with 150 mM NaCl pH 7.3 (A) and pH 4.0 (B) stepwise gradient at 100% A (0 to 5 min) and 100% A to 100% B (5 to 30 min); temperature, 25 °C; detection, UV 220 nm.

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