Introduction

The construction of master cell lines for monoclonal antibody production and the research and development of manufacturing processes require methods to rapidly quantify, separate, and purify antibodies as well as a system for analyzing a large number of samples in a short period of time.\(^1\)–\(^5\) Protein A affinity chromatography has been used to extract and purify antibodies from a complex matrix.\(^6\)–\(^12\) Various particles, such as agarose, polymers, and silica, have been used as immobilization carriers for Protein A.\(^13\),\(^14\) Various spin columns, pretreatment methods, and particle types have also been studied; however, the treatment process is long due to the slow rate of antibody absorption by particles.\(^13\),\(^14\) Furthermore, the low pressure resistance of agarose gels is not suitable for high-speed processing.

Various studies have focused on columns with a fixed monolith for pretreatment.\(^15\),\(^16\) Polymer-type monoliths, such as polymethacrylate copolymer and styrene-divinylbenzene copolymer columns, are widely used for biochromatography owing to their structural stability and high solvent resistance.\(^17\),\(^18\) For polymer monoliths constructed with a single-pore structure (such as CIM monolith column, BIA Separations), it is difficult to improve liquid permeability while controlling target loading. The use of a monolith with a large through-pore to obtain a high processing speed often reduces the target adsorption.\(^19\)

Using silica monoliths, it is possible to independently control the through-pore, which controls the skeleton, and the mesopore, which controls the surface area of the target component, enabling high liquid permeability and high sampling loading.\(^20\),\(^21\) Namera and Saito have evaluated rapid pretreatment methods for biological samples using spin columns subjected to various chemical modifications.\(^22\),\(^23\)

However, monolithic silica causes non-specific protein adsorption, which decreases the recovery of target proteins from samples with low protein concentrations. An affinity column with monolithic silica has been prepared by using a silane reagent with a hydrophilic amino group or an epoxy group.\(^24\),\(^25\) However, silica was not uniformly coated by the silane reagent, and non-specific adsorption could not be reduced.

In this study, we prepared an immobilized Protein A-coated silica monolith with two hydrophilic polymers. In addition, the difference in the binding capacity was verified by changing the mesopore size of monolithic silica. We confirmed the liquid flow characteristics of the newly developed Protein A-immobilized spin column and constructed a rapid antibody purification method.

Experimental

Chemicals and reagents

Sodium dihydrogen phosphate, 2-hydrate, disodium hydrogen phosphate, anhydrous (Kanto Chemical Co., Inc., Tokyo, Japan), and water (Milli-Q system; Merck Millipore, Darmstadt, Germany) were used in preparing the mobile phase, 100 mM phosphate buffer (pH 7.0). Tetraethoxysilane (TEOS), 3-amino-propyltrimethoxysilane (APTES), 3-methacryloxypropyltrimethoxysilane (MPS), and 3-glycidyloxypropyltrimethoxysilane (GPTMS) were obtained from Shin-Etsu Chemical (Tokyo, Japan).
Japan). Polyethylene oxide, Immunoglobulin G human, and Protein A were provided by Sigma-Aldrich Co. (St. Louis, MO, USA). N-Disuccinimidyl carbonate (DSC), glycidyl methacrylate (HEMA), N,N-dimethylformamide (DMF), d-glucitol, ethylene diamine, and 2,2′-azobisobutyronitrile (AIBN) were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). A Chinese hamster ovary (CHO) cell culture medium containing monoclonal antibody was obtained from manufacturing technology association of pharmaceutical that asked for confidentiality. All other reagents were of analytical grade and were purchased from KISHIDA Chemicals (Tokyo, Japan).

**HPLC apparatus and conditions**

Chromatography was performed using a GL 7400 system (GL Sciences Inc., Tokyo, Japan). HPLC separation of immunoglobulin G was conducted on a Inertsil WP300 Diol column (4.6 × 250 mm, 5 μm) (GL Sciences Inc.) at 40°C, with a flow rate of 1 mL/min using a mobile phase of 100 mM sodium phosphate buffer (pH 7.0). The total run time was 10 min.

**Synthesis of a monolithic silica gel**

Tetraethoxysilane was added to a 1 M aqueous solution of nitric acid in the presence of polyethylene oxide with an average molecular weight of 100000 and d-glucitol. The mixture was stirred for 15 min at 25°C. The solution was kept at 40°C for gelation and aging for over 15 h. The aged gel was immersed in a 1.5 M aqueous urea solution at 110°C for 20 h. After drying at 40°C for 24 h, the gel was heat-treated at 600°C for 5 h. A silica rod of 4.2 mm in diameter was cut to a thickness of 1.5 mm. The mesopore and surface sizes were analyzed using the Autosorb-3B nitrogen absorption system (Quantachrome, Boynton Beach, FL, USA).

**Chemical modification of monolithic silica and immobilization of Protein A**

The supports were prepared by reacting the hydrophilic polymer with HEMA-GMA and immobilized Protein A, as shown in Fig. 1. Monolithic silica was first dried in a vacuum oven for 24 h. The dried monolithic silica was added to MPS in toluene and boiled for 5 h. The resulting MPS-modified monolithic silica was washed with methanol. This support was then dried under a vacuum at 70°C for 24 h. The MPS-modified monolithic silica was modified with HEMA, GMA, and AIBN as an initiator in DMF at 70°C for 6 h. The epoxy groups of GMA on monolithic silica were reacted with ethylene diamine via the epoxy ring opening reaction. The hydrophilic polymer-coated monolithic silica gel was put in a flask containing ethylene diamine in methanol. The reaction was carried out at 65°C for 6 h. The product was washed with methanol and dried at 70°C for 24 h. The amino-derivatized monolithic silica was modified by DSC in acetonitrile at 25°C for 24 h. Then, monolithic silica was washed with acetonitrile and dried at 25°C for 24 h. The carbon content of modified monolithic silica was determined by a thermo-gravimetric analysis using the WS002 Thermo-Analyzer (MAC Science Co., Yokohama, Japan).

Protein A was immobilized on a hydrophilic polymer-modified monolith by incubation at 5°C for 24 h. The Protein A ligand solution was dissolved in immobilization buffer (50 mM sodium phosphate buffer pH 7.4) to obtain a Protein A concentration of 10 mg/mL. Finally, the Protein A-immobilized silica monolith was washed with a 100 mM sodium phosphate buffer (pH 7.4) and remaining NHS activity was blocked with 500 mM ethanolamine in a 100 mM sodium phosphate buffer, pH 7.4. The amount of immobilized Protein A was estimated from the difference in peak areas before and after the reaction by SEC chromatography. The prepared monolithic silica disk was fixed on the spin column by supersonic adhesion.

**Purification procedure using the monolithic silica spin column**

The Protein A-immobilized extraction column was conditioned with 0.4 mL of PBS at 2300 × g for 30 s. Then, 0.2 mL of the CHO cell culture sample filtered using the 0.22-μm filter (Millex-GV syringe filter unit) was placed in the conditioned spin column, and the column was centrifuged at 2300 × g for 30 s. The column was subsequently washed with 0.5 mL of PBS for 30 s at 2300 × g. IgG was eluted by 0.2 mL of 100 mM...
citrate buffer (pH 3.5) for 30 s. Then, 5 μL of the extracted solution was injected into the HPLC system.

Results and Discussion

Synthesis of the monolithic silica disk and chemical modifications

As determined by SEM measurements, the monolithic silica gels with mesopore sizes of 8, 32, and 58 nm had nearly identical morphologies. Representative SEM images of samples with a mesopore size of 32 nm are shown in Fig. 2. A disc-type silica monolith with a uniform structure was prepared and fixed on the tip of a spin column.

Table 1 summarizes the carbon contents of the modified MPS and hydrophilic polymer on monolithic silica and the surface area of the synthesized materials. The mesopore of the monolith was adjusted by changing the alkaline treatment conditions during synthesis. Monolithic silica was coated with a hydrophilic polymer to reduce nonspecific protein binding by a modification of the anchor group (MPS), followed by the polymerization of HEMA on the monolithic silica surface. The amount of modified MPS added to the monolith with a large surface area was greater than that for a small surface area. On the other hand, a polymer coating on the monolith with a large surface area was less than that on the monolith with a small surface area. These results can be explained by a steric hindrance in monomer polymerization for monoliths with small mesopores.

Table 1 Poly-HEMA contents and pore structures of different silica monoliths

| Monolith  | Surface area/m² g⁻¹ | Pore size/nm | MPS, % | Poly-HEMA, % | Coated polymer, % |
|-----------|---------------------|--------------|--------|--------------|------------------|
| AF29-H10  | 390                 | 8            | 17.4   | 31.5         | 14.1             |
| AF30-H10  | 98                  | 32           | 5.4    | 26.2         | 20.8             |
| AF32-H10  | 36                  | 58           | 2.1    | 21           | 20               |

Amount of monomer in the reaction solution: 10% (v/wt) of the silica.

Non-specific adsorption of IgG by poly-HEMA-coated monolithic silica

To achieve the minimum nonspecific binding of IgG to monolithic silica, we investigated the effects of a modification using a silane reagent and the monomer ratio, which indirectly reflected the polymer density on the monolithic silica, as summarized in Table 2. The monolithic silica with 32 nm mesopore, which has the highest capacity to modify hydrophilic polymers compared to that with 8 and 58 nm mesopores, was used for chemical modifications. Several methods for protein immobilization, such as coupling using an amino group or using thiol or carboxyl groups, have been reported. Modification using a single kind of silane reagent can immobilize proteins to the silica surface; however, in areas where proteins are not immobilized, the functional group is hydrolyzed and remains as a non-specific adsorption site. Furthermore, it is well known that the negatively charged surface of silica gels confers an ionic behavior, a partially hydrophobic surface, and silanol groups. Therefore, the silica gel material is expected to show nonspecific binding to proteins via ionic attraction and non-polar and hydrogen-bonding interactions.

A method in which the silica surface is coated with a hydrophilic polymer to reduce nonspecific adsorption has been

Table 2 Effect of the modification reagent on the non-specific adsorption of IgG by modified monolithic silica

| Monolith | Modification       | Ratio of reagent:silica, % | Carbon content, % | Non-specific absorption to monolithic silica (n = 4) |
|----------|--------------------|-----------------------------|-------------------|-----------------------------------------------|
|          |                    |                             |                   | IgG absorption, % | CV, % |
| AF30-H1  | MPS-poly HEMA      | 1                           | 9.4               | 38.3                                           | 5.2   |
| AF30-H10 | MPS-poly HEMA      | 10                          | 26.2              | 8.3                                            | 3.1   |
| AF30-H20 | MPS-poly HEMA      | 20                          | 30.2              | 10.1                                           | 4.0   |
| AF30-H40 | MPS-poly HEMA      | 40                          | 28.5              | 15.5                                           | 6.4   |
| AF30-M   | Metacrylate silane | 10                          | 5.4               | 91.6                                           | 3.4   |
| AF30-E   | Epoxy silane       | 10                          | 6.2               | 75.8                                           | 6.1   |
| AF30-A   | Amino silane       | 10                          | 7.1               | 72.8                                           | 5.3   |

Mesopore size of monolithic silica: 32 nm. IgG solution; 0.1 mg/mL. 500 μL in 10 mM potassium phosphate buffer (pH 7.0).
Effect of the eluent volume on the elution efficiency was evaluated in the range of 20 – 800 μL of citric acid buffer (pH 3.5); n = 4.

Table 3 Effect of the ratio of monomer HEMA to GMA on Protein A immobilization

| Monolith   | Ratio of monomer HEMA:GMA, % | Carbon content, % | Amount of immobilized Protein A (μg/monolith disk) CV, % |
|------------|------------------------------|-------------------|--------------------------------------------------------|
| AF30-HG10  | 10:1                         | 26.5              | 121, 7                                                  |
| AF30-HG10  | 10:1                         | 27.6              | 234, 8                                                 |
| AF30-HG5   | 5:1                          | 29.4              | 247, 12                                                |
| AF30-HG2   | 2:1                          | 27.6              | 253, 13                                                |
| AF30-HG1   | 1:1                          | 25.5              | 262, 15                                                 |

Amount of monomer in the reaction solution: 10% (v/wt) of the silica.

reported. In particular, 2-hydroxyethylacrylate was used as a monomer to coat the monolithic silica surface in order to reduce nonspecific protein adsorption.

In the case of the monolithic silica fixed to the spin column, modification with 3-aminopropylsilane, 3-glycidoxypropylsilane (derivatized to diol), or 3-methacryloxypropylsilane caused high IgG adsorption. Using the monolith modified with a hydrophilic polymer, the nonspecific adsorption of IgG was greatly reduced (Table 2). These results suggested that the formation of the hydrophilic polymer phase on the surface of the monolithic silica made the surface inaccessible to the protein, thereby reducing nonspecific adsorption. The amount of the polymer on the surface increased depending on the concentration of the introduced monomer. Non-specific adsorption was significantly reduced using 10% HEMA monomer in the reaction solution.

Immobilization of Protein A to the developed monolithic silica

Next, we investigated methods to immobilize proteins while reducing any non-specific adsorption by the copolymerization of GMA, a functional monomer, with a hydrophilic polymer on the silica monolith. By the copolymerization of a hydroxyl group-containing monomer and epoxy-containing monomer, the protein can be immobilized at a certain distance on the support. In this study, glycidyl methacrylate was used as a functional monomer for the immobilization of Protein A, and 2-hydroxyethylacrylate was used to reduce any nonspecific adsorption. The ratio of HEMA to GMA, which serves as a scaffold for immobilizing proteins, was examined (Table 3). Irrespective of the ratio of HEMA to GMA, the total amount of modified polymer remained constant.

After the monolith was coated with the hydrophilic polymer at each ratio, the amino group and the NHS group were modified and Protein A was immobilized. The amount of immobilized Protein A increased as the ratio of GMA increased from 1 to 10%. The amount of Protein A on the silica monolith did not increase even for GMA ratios exceeding 10%.

Effect of elution volume on the recovery of IgG

To ensure that IgG was completely eluted from the sorbent, the effect of the eluent volume on the elution efficiency was evaluated in the range of 20 – 800 μL (at 800 μL: 400 μL twice). The IgG recovery relative to the volume of acidic solvent is shown in Fig. 3. The results showed that the recovery of IgG increased as the volume of citric buffer increased from 20 to 50 μL. To ensure complete recovery, 100 μL of citric acid was used to elute the loaded spin column. When an agarose gel column was used, a large amount of eluate (over 0.8 mL) was required for antibody recovery from the spin column.

Effect of the mesopore size on the maximum IgG adsorption

Macropores provide good permeability, while mesopores play an important role in determining the total surface area. Table 4 summarizes a comparison on the Protein A contents of silica monoliths with different of mesopore sizes prepared using the same immobilization method. The total Protein A content per unit mass of support for 8-nm mesopores was 1.7-fold higher than that obtained with a 58-nm pore size.

Next, we investigated the binding capacity of IgG to monolithic silica. The maximum adsorption capacity of IgG for 32-nm mesopores was 3-fold higher than that obtained with a 8-nm pore size (Fig. 4). Access by the antibody (MW 150000, size 10 to 15 nm) was likely limited by the small size of the monolith pore. The lower IgG recovery for the 58-nm mesopore monolith than for the 32-nm mesopore monolith may be due to the lower amount of immobilized Protein A. Using the 32-nm mesopore monolith, the linear calibration curve (for each antibody concentration over 10 - 300 μg/mL) had a correlation coefficient of >0.999.

We examined the rotation speed and time required to pass a sample solution (0.5 mL) through the column. The time required decreased as the rotational speed increased, and the amount of antibody adsorbed from a biological sample depended on the time that the antibody is in contact with Protein A. Although the optimal centrifugation speed for recovery was less than 2300 × g (5000 rpm), the extraction process was not time-consuming. In the case of a spin column filled with the Protein A-immobilized agarose gel, the sample and resin are mixed, requiring 5 to 10 min each for adsorption and desorption steps, and antibody purification is completed in 5 min. In addition, there is a risk of antibody denaturation due to prolonged exposure to acid solutions during desorption. When the developed column was used, high quality antibody can be recovered because the time of exposure to acid solution was very short.

In some cases, the Protein A-immobilized spin column requires stability in alkali conditions for regeneration. The stability of the newly developed spin column was investigated by examining the recovery of IgG using 0.5 mL of a 0.1 N
NaOH solution. The developed spin column showed a recovery rate of more than 90%, even after being used 10 times.

**Purification of IgG from the CHO cell culture medium**

CHO cells were harvested by centrifugation, and the culture medium was collected and used as a source for the purification of IgG using the newly prepared spin column. Monoclonal antibodies in the CHO cell culture were analyzed and quantified by reference to a standard curve. Typical chromatograms obtained for purified IgG are shown in Fig. 5. The purity of the sample obtained using monolithic silica spin column was 98%, calculated from the total peak area without the peak area from elution buffer. The antibody concentrations in samples collected from the medium were 1.2 and 0.5 mg/mL.

In screening of the master cell line for antibody production, it is necessary to evaluate small amounts and large numbers of culture solution; however, when a conventional agarose gel column (ex. Protein A HP Spin trap) is used, a large amount of the eluate (over 0.8 mL) is necessary for antibody recovery from the spin column (Fig. 3). A diluted antibody solution is recovered from the spin column, which reduces the sensitivity of HPLC measurements. The newly developed spin column showed a high recovery rate for 100 μL of eluate, suggesting that purification and detection using the monolithic silica column is suitable for analyses of small solution volumes and low concentrations of IgG.

**Conclusions**

The proposed monolithic spin column for purification provides a rapid and efficient tool for the extraction and determination of IgG in CHO cell culture medium. The efficient and rapid purification of IgG is important for high-throughput screening aimed at master cell development. Monolithic silica was modified with hydrophilic polymers and used immobilized Protein A to prevent protein adsorption, resulting in rapid purification and high antibody recovery rates. IgG was separated within 15 min by purification with the monolithic spin column in conjunction with HPLC-SEC. The method exhibits a high-throughput capability owing to the short time required for purification.

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