

**Studies on Quality Evaluation of
Biopharmaceuticals
by Chromatographic and Electrophoretic Techniques**

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Chapter 1

General Introduction

1-1 Biopharmaceuticals

Biopharmaceuticals are macromolecules with a therapeutic effect, produced by the recombinant DNA technologies. They have emerged as important therapeutics for the treatment of various diseases including cancer, cardiovascular diseases, diabetes, infection, inflammatory, and autoimmune disorders.¹⁻² Biopharmaceuticals include monoclonal antibodies (mAbs), hormones, growth factors, fusion proteins, cytokines, therapeutic enzymes, blood factors, vaccines, and anticoagulants. These molecules have obvious benefits in terms of the safety and efficacy, therefore, about 250 products are approved for human use in the United States and European Union.¹ Especially, mAbs are considered as the fastest growing class of the therapeutics. Since the registration of the first mAb in 1986, the sales of the mAbs have grown every year. Their sales reached to 106.9 billion dollars in 2016.¹⁻⁵ In 2016, 42 biopharmaceuticals (25 mAbs) are called blockbuster, which sales are more than 1 billion dollars.⁵

The success of mAbs have triggered the development of various next generation formats such as bispecific mAbs, antibody-drug conjugates (ADCs), antibody fragments (nanobodies) and so on.^{1, 3, 6} In oncology, ADCs are particularly promising, since they synergistically combine a specific mAb linked to a biologically active cytotoxic drug via a stable linker.⁷⁻⁸ The promise of ADCs is that highly toxic drugs can selectively

be delivered to tumor cells thereby substantially lowering side effects as typically experiences with classical chemotherapy.⁹ Currently, two ADCs are marketed, brentuximab vedotin (Adcetris) and ado-trastuzumab emtansine (Kadcyla) and over 30 are in clinical trials.¹⁰⁻¹¹

1-2 Structure and Heterogeneity of Biopharmaceuticals

Biopharmaceuticals have a complexity far exceeding that of small molecule drugs. MAbs are large tetrameric immunoglobulin G (IgG) molecules of approximately 150 kDa, forming Y-like shapes.¹² They are structurally composed of four polypeptide chains, including two heavy chains (HC) of ~ 50 kDa and two light chains (LC) of ~ 25 kDa. These chains are connected through inter- and intrachain disulfide bonds. All the mAbs are glycoproteins having two conserved *N*-glycosylation sites.¹³ The *N*-glycans are usually complex biantennary oligosaccharides containing 0-2 nonreducing galactoses with or without fucose attached to the reducing end of *N*-acetylglucosamine. Sialic acids may be found as terminal sugars of glycan chains. From a functional point of view, mAbs consist of two regions, the crystallizable fragment (Fc) of ~ 50kDa and the antigen binding fragment (Fab) of ~50 kDa.¹⁴⁻¹⁶ Fc is responsible for the effector function, *i.e.*, antibody dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). Fab is primarily involved in antigen binding.

The majority of the currently approved full length human recombinant mAbs are produced using either Chinese hamster ovary (CHO) cells or mouse myeloma-derived cells. Since glycosylation patterns vary among species, the production system used for recombinant mAbs manufacturing affects their glycan profile.¹⁷⁻¹⁸ In addition, a variety of chemical and enzymatic modifications taking place during expression, purification, and long-term storage lead to a substantial heterogeneity.^{15-16, 19} Despite the fact that only a single molecule is cloned, thousands of possible variant combinations may exist for one given mAb. They all contribute to the safety and

efficacy of the product. The possible variants observed in mAbs are glycosylation, asparagine deamidation, aspartate isomerization, succinimide formation, *N*-terminal pyroglutamate formation, *C*-terminal lysine truncation, oxidation, glycation, cysteine variants, sequence variants, etc.¹⁵ The combination of these micro-heterogeneity sources in the peptide chains significantly increase the overall heterogeneity in an entire mAb. In comparison to naked mAbs, ADCs further add to the complexity due to the variability of the conjugation strategy.^{8, 20-21} The ADC products are indeed often heterogeneous, with respect to drug loading and its distribution on the mAb.

All these structural characteristics together with their stabilities have to be revealed during development and subsequently need to be closely monitored prior to clinical or commercial release. In assessing these characteristics also in demonstrating the comparability, a significant number of analytical tools need to be employed.^{14-16, 22}

1-3 Separation Methods for Production and Quality Evaluation of Biopharmaceuticals

Due to the increasing number of approved mAbs in the pharmaceutical area, the need for analytical techniques adapted for their detailed characterization has increased. As previously discussed, the intrinsic micro-heterogeneity is major concern with mAbs and should be critically evaluated because differences in impurities and/or degradation products could lead to serious health implications.²³

In general, identity, heterogeneity, impurity content and activity of each new batch of mAbs should be thoroughly investigated before release. This examination is achieved by using a wide range of analytical methods, including reversed-phase (RP) chromatography, size-exclusion chromatography (SEC), ion-exchange chromatography (IEX), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), capillary isoelectric focusing (CIEF), capillary zone electrophoresis (CZE), circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR), and mass spectrometry (MS). The goal of this multi-method strategy is to demonstrate the similarity between production batches of mAb by precisely determining the primary, secondary, and tertiary structures of mAbs.²⁴ The chromatographic and capillary electrophoretic methods are particularly well suited to this purpose.

High-performance liquid chromatography (HPLC) is one of the key techniques for the characterization of biopharmaceuticals.^{14, 25} Among various modes of HPLC, IEX, SEC, hydrophobic interaction liquid chromatography (HIC), hydrophilic interaction liquid chromatography (HILIC), and RP liquid chromatography are often used for the

characterization of biopharmaceuticals. Capillary electrophoresis (CE) has been widely applied for the analysis of biopharmaceuticals, because of its high resolving power and miniaturized format.^{14, 25} In the different electrophoretic modes that can be employed, a high electrical field is always applied to separate molecules based on differences in charge, size, or hydrophobic properties. Three CE modes are commonly used in the analysis of mAbs; capillary gel electrophoresis (CGE), CIEF, and CZE. The former two techniques are the capillary counterparts of the traditional gel format techniques or SDS-PAGE and isoelectric focusing (IEF), in which several drawbacks, *e.g.*, including extended analysis time, low efficiency, limited reproducibility, and use of toxic reagents, are pointed out. The capillary format enables automation of the experiments and provides better resolution. In contrast to conventional gel format electrophoretic techniques, CE also has the potential to be directly coupled with MS to obtain an improved identification of the compounds by resolving the co-migrated analytes which have different mass-to-charge ratios in each other.

Size distribution or mono-dispersity of a mAb product is important for both safety and efficacy. Components smaller than the intact mAb are often generated by an enzymatic or non-enzymatic cleavage. Components larger than the individual antibody is often generated by molecular association, aggregation, or even precipitation. A full spectrum of species, from molecular dimer to oligomer to higher-order aggregates, may be present in a mAb preparation.²⁶

SEC commonly used to determine the size-related heterogeneity, for example, protein aggregation and fragmentation.^{14, 27} The main advantage of SEC is the mild mobile phase conditions that permit the characterization of proteins with minimal impact on the

conformational structure and local environment. SEC is also useful in determining if cleaved components are incorporated into monomeric molecules under native conditions. In this technique, a gel suspended in an aqueous buffer solution is packed into a chromatographic column. The gel consists of spherical porous particles with a carefully-controlled pore size, through which the biomolecules diffuse based on their molecular size differences. The separation power of an SEC column increases in direct proportion to the square root of the column length, so the separation of heterogeneous samples requires long columns that can be obtained by joining multiple columns in a series.

As well as SEC, SDS-PAGE has been used for several decades for size-based separation of proteins. When SDS completely reacts with proteins, the reaction produces SDS-protein complexes of the same charge. Then, the mobilities of these complexes under electrophoretic conditions are only dependent on their hydrodynamic sizes in a sieving matrix, and smaller proteins have higher mobility. However, SDS-PAGE is a labor-intensive and time-consuming method. Especially, manual operations are sources of irreproducibility. This is the reason why CGE is now recognized as an important analytical tool in the biopharmaceutical industry. CGE is used to determine the apparent molecular weight of molecules. In the field of mAbs, CGE allows assessment of product-size heterogeneity, purity, and stability. Sample preparation consists of heating the sample in the presence of a high concentration of SDS, which denatures the secondary and tertiary structures without affecting the disulfide bonds, thus resulting in uniformly charged proteins. The capillary is subsequently filled with a sieving matrix composed of polymers, leading to a separation based solely on the hydrodynamic radius of the protein.

Charge heterogeneity is a very important characteristic in mAbs and ADCs because it relates to their quality, stability, and efficacy.

In IEX, charge variants are separated by differential interactions on a charged support. The number of potential charge variants depends on the primary sequence of proteins. In addition, changes in charge may be additive or subtractive, depending on any modifications. Thus, IEX profiles become more complex, and the overall resolution of individual variants may be lost. This property is particularly apparent for mAbs with molecular weights of ~ 150 kDa.

Based on *pI* differences, CIEF is used to characterize the charge heterogeneity of proteins. A pH gradient is formed inside the capillary, and the proteins migrate under the electric field until their global charge becomes zero (the pH is equal to their *pI*).

CZE is the most straightforward separation method among electro-driven separation techniques. The capillary is filled with a background electrolyte and the separation proceeds according to differences in the analyte electrophoretic mobilities depending on their charge-to-size ratio. CZE is a technique perfectly adapted for the separation of proteins with post-translational modifications or degradations that affect the charge of the molecules, such as deamidation, sialylation, C-terminal lysine truncation, or N-terminal pyroglutamate formation. CZE, as a charge-based separation technique, is used to confirm the identity of a therapeutic protein, impurities, and charge heterogeneity. Because the molecular weights of the protein variants are of comparable size, the separation selectivity in CZE is predominantly governed by charge differences.²⁸

Fc *N*-glycosylation of mAbs has significant impact on effector function²⁹⁻³⁰, clearance³¹⁻³², and immunogenicity.³³⁻³⁴ For example, removal of core fucose can significantly enhance the binding affinity to Fcγ receptors, resulting in increased ADCC, while mAbs containing high levels of high-mannose glycans show faster clearances than other glycoforms.³¹⁻³²

HILIC is an important technique to analyze *N*-glycans originating from biopharmaceuticals.¹⁶ The HILIC separation is based on the differential partitioning of the solutes between an acetonitrile-enriched mobile phase and a water-enriched solvent layer adsorbed onto the surface of the column support. Electrostatic interactions also exist depending on the stationary phase, buffer, and pH. Combined with 2-aminobenzamide (2-AB) labeling and fluorescence detection, HILIC is the gold standard for glycan analysis.

Also, CGE-laser induced fluorescence (LIF) of 1-aminopyrene-3,6,8-trisulfonic acid (APTS) labeled *N*-glycans is widely and routinely applied in biopharmaceutical industries to determine the *N*-glycan profiles.³⁵⁻³⁷ APTS introduces three negative charges and a fluorophoric group into the glycans allowing highly efficient, fast, and sensitive separations. Since CGE is not MS compatible, glycan structures were needed to be identified through the use of glycan standards or a battery of exoglycosidases. Compared to CGE, CZE buffers are typically MS-friendly. Therefore, CZE has also been widely used to separate both labeled and unlabeled *N*-glycans.

During the production of mAbs, chromatographic methods are commonly used. Industrially, recombinant mAbs are bioproduced in living cells, such as CHO cells.³⁸ Once produced, the mAb must be isolated from the cellular and media components used

for its production. This primary isolation process begins after the cells are harvested for the mAb. Once the mAb is harvested, the product pool contains the mAb as well as cellular components (media components, proteins, and DNA) and viruses that may be present during the production process. In the first chromatography step, the product pool is run through a protein A affinity column. The purpose of the protein A affinity step is to remove media components, cellular debris, and putative viruses. The product pool is further purified over an ion exchange column and viral filtration to remove additional contaminants, such as aggregates, DNA, virus, and residual CHO proteins. After purification, ultrafiltration/diafiltration is performed to concentrate the mAb. Protein A affinity chromatography is a key purification step used during the purification of mAbs harvested from cell culture fluid. The use of protein A affinity chromatography in industrial mAb purification is commonplace as it is efficient, scalable, and reproducible.³⁹

1-4 Purpose and Contents of the Thesis

Separation techniques, or chromatography and electrophoresis-based methods, are important tools for the characterization of biopharmaceuticals. The aims of this thesis are the development of the separation methods for biopharmaceutical related compounds, such as charge variants, size variants, and glycans, with improving the analytical performances, *i.e.*, efficiency, resolution, selectivity and applicability in LC and CE.

In Chapter 2, the application of CZE to the evaluation of charge variants of mAbs and ADCs is described. The charge profiles of ADCs are changeable during production and storage, because the antibody itself suffers from degradation, leading to charge heterogeneity as described above. However, the analysis of charge variants for ADCs has so far rarely been reported.⁴⁰ This is challenging due to (1) the hydrophobicity of low-molecular weight drugs, which causes an undesirable hydrophobic interaction during the analysis (*i.e.*, with cation exchange chromatography columns), (2) the complexity caused by the charge heterogeneity of naked antibodies and low-molecular weight drugs themselves, and (3) the variation of drug distribution. To solve these problems, the author developed a CZE method, because CZE requires no denaturants or solid-phase interfaces as separation media. CZE enabled to analyze the inherent heterogeneity of ADCs at close to their native states. The author successfully demonstrated the method validation for the use with an identity and purity test for mAbs and ADCs.

In Chapter 3, the analytical procedures to identify the fragment confirmed in SDS-CGE were described. Analysis of fragments is conducted mainly by size-based separation methods, such as SEC, SDS-PAGE, and SDS-CGE.^{14, 25, 41} SDS-CGE is now widely used for the precise evaluation of mAb fragmentation, because SDS-CGE show a superior separation efficiency, automated operation, shorter separation time, and capability of accurate protein quantification.⁴²⁻⁵⁰ However, the identification of fragments observed in SDS-CGE is challenging to carry out. Consequently, few reports have been published regarding approaches to identifying observed fragments in SDS-CGE.⁵¹ Thus, further development of methodologies that allow the identification of SDS-CGE peaks is needed. To identify the fragment of SDS-CGE, the author conducted and compared following procedures: (1) in-gel digestion peptide mapping, (2) RP liquid chromatography (RPLC) coupled with MS (RPLC-MS), and (3) the application of a Gelfree 8100 fractionation system following RPLC-MS.

In Chapter 4, a new material, spongy monolith immobilized with protein A and/or pepsin, was developed to achieve affinity reaction effectively. For purification of biomolecules, the protein A column is commonly used, however, its cost and throughput become a bottleneck to reduce the cost of biopharmaceuticals. The monolithic structure is suitable for rapid reactions because the flow-through pores are integrated with the skeleton.⁵²⁻⁵⁷ 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 ($>10\ \mu\text{m}$), scale up in column size, and packing to columnar tubes are not easy. Instead of these typical monoliths, the authors proposed using a sponge-like material or spongy monolith as a novel separation medium.⁵⁸⁻⁵⁹

The spongy monolith is prepared simply by blending a thermoplastic resin above its melting point with water-soluble pore templates. The author demonstrated the immobilization of protein A with epoxy-functionalized spongy monolith column, and its functionality including capture amount of mAbs under high flow rate.

In Chapter 5, universal media for the efficient separation of DNA and/or glycans based on the molecular size in CGE was described. In general, agarose gels and polyacrylamide (PAA) gels are usually utilized for gel electrophoresis (GE).⁶⁰⁻⁶¹ The agarose gel can be easily prepared. The agarose gel allows the effective separation of DNAs in the range of 0.1–60 k base pair by controlling the concentration of agarose. However, the agarose gel is not suitable for the separation of small size differences because of its larger pores. On the other hand, the pore sizes of a PAA gel are controllable, so that the smaller DNAs can be separated. Meanwhile, the range of the suitable molecular size is limited in the PAA gels. Additionally, the toxicity of the acrylamide monomer and the non-specific interactions by amide groups toward biomolecules are also problematic in using PAA.⁶² Instead of these gels, poly(ethylene glycol) (PEG) has attracted attention as another separation medium in GE. In this study, the author prepared a variety of PEG-based hydrogels with PEG dimethacrylate by changing the concentration and ethylene oxide unit in a capillary to control the polymer network. The separable ranges of the molecular weight for glucose and DNA ladders were evaluated with the prepared capillaries by CGE. Additionally, the author demonstrated the separation of sugars carved out from mAbs as a practical application in the CGE analysis.

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Chapter 2

Validation of Capillary Zone Electrophoretic Method for Evaluating Monoclonal Antibodies and Antibody-Drug Conjugates

2-1 Introduction

Monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs) have emerged as promising classes of therapeutics in the biopharmaceutical industry because of their advantages of having highly specific targeting towards a wider range of indications. More than 40 mAbs have been approved for use in indications such as cancer and inflammatory diseases.¹⁻⁵ ADCs combine the potency of cytotoxic drugs with the high specificity of mAbs and have become increasingly important as new targeted therapies in oncology. The primary sites used for protein-directed conjugation are the amino groups of lysine residues or the sulfhydryl groups of inter-chain cysteine residues. Two ADCs have been approved by the FDA for the treatment of late-stage metastatic breast cancer and relapsed Hodgkin's lymphoma.⁶⁻¹³

Charge heterogeneity is a very important characteristic in mAbs and ADCs because it relates to their quality, stability, and efficacy. Every product has its own primary structure, thus showing its specific *pI*. Changes of charge profile suggests several post translational modifications, such as C-terminal variants (lysine truncation¹⁴ and proline amidation¹⁵), deamidation¹⁶⁻¹⁷, glycation¹⁸⁻¹⁹, and pyroglutamic acid cyclization.²⁰⁻²¹

These modifications are generated during production and storage of products.²² Some modifications of amino acids located in complementarity determining regions reduce the binding activity of antigens.²³ Therefore, an evaluation of charge heterogeneity is significant to the identification of the products and the monitoring of the quality of the products based on their charge profiles.

To evaluate charge variants of mAbs, cation exchange chromatography (CEX)²⁴⁻³⁰, isoelectrophoretic focusing (IEF)³¹, capillary IEF (cIEF)³²⁻³⁴ and imaged cIEF (icIEF)³⁵⁻³⁸ are usually used. However, these methods require many experimental optimizations, which take a long time for measurement, and the obtained data is usually less quantitative and less reproducible. Capillary zone electrophoresis (CZE) has become increasingly accepted as an attractive alternative to IEF and CEX for assessment of charge heterogeneity of mAbs, because CZE allows simpler to conduct experiments, faster to obtain results, and high reproducibility.³⁹⁻⁴⁷

The charge profiles of ADCs are changeable during production and storage, because the antibody itself suffers from degradation, leading to charge heterogeneity as described above. Therefore, it is desirable to monitor charge variants of ADCs to assure their quality is maintained. However, charge-based development methods and validation for ADCs has so far rarely been reported.⁴⁸ Charge variant analysis of ADCs is challenging due to (1) the hydrophobicity of low-molecular weight drugs, which causes an undesirable hydrophobic interaction during the analysis (*i.e.*, with CEX columns), (2) the complexity caused by the charge heterogeneity of naked antibodies and low-molecular weight drugs themselves, and (3) the variation of drug distribution. CZE require no denaturants or solid-phase interfaces as separation media, enabling the inherent heterogeneity of ADCs to be analyzed at close to their native states.

Therefore, CZE is suitable for separation method for detecting the charge heterogeneity of ADCs.

In this study, the method validation of CZE was conducted for use with an identity and purity test for mAbs and ADCs. The validation items consisted of specificity, linearity, quantitation limit, precision (repeatability and intermediate precision), accuracy, range and robustness. We believe that the validation of CZE for assessing ADCs was successfully demonstrated for the first time, and CZE is applicable not only for mAbs but also ADCs.

2-2 Experimental Section

2-2-1 *Materials and reagents*

The mAbs and ADCs were all manufactured at Daiichi Sankyo Co., Ltd. (Tokyo, Japan). 6-Aminocaproic acid (EACA), hydroxypropyl methyl cellulose (HPMC) and triethylenetetramine (TETA) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Acetic acid, 0.1 M hydrochloric acid, sodium chloride, 2 M sodium hydroxide and urea were purchased from Wako Pure Chemical Industries (Osaka, Japan). A neutral capillary was purchased from Beckman Coulter, Inc. (Brea, CA, USA). An analytical ProPac WCX-10 column (250 × 4 mm i.d.) was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). 2-Morpholinoethanesulfonic acid, monohydrate (MES) was purchased from Dojindo Laboratories (Kumamoto, Japan).

2-2-2 *CEX*

The CEX analysis was conducted on a Shimadzu Prominence LC20 HPLC system equipped with a UV detector. A ProPac WCX-10 column and mobile phase A (20 mM phosphate buffer pH 6.6) and mobile phase B (0.5 M sodium chloride in mobile phase A) were used with a gradient of 0% to 40% mobile phase B for 39 min at a flow rate of 0.5 mL min⁻¹. The sample was not diluted with mobile phase A, and 5 µL of the sample was injected into the column. The column temperature was 40 °C with detection at 280 nm.

2-2-3 *CZE*

The CZE analysis was performed on a Beckman PA800 plus system equipped with a

UV detector. The capillary was thermostated at 25°C, and detection was performed at 214 nm. The capillary (50 µm id, total length 50 cm, effective length 40 cm) was used with a constant voltage of 30 kV for all analyses. Before each sample injection, the capillary was rinsed with 0.1 M hydrochloric acid, running buffer of 0.05% HPMC, 380 mM EACA, and 1.9 mM TETA, pH 5.7. Samples were diluted with water (for mAbs) or their formulation buffer (for ADCs) to 2 mg mL⁻¹ and injected at 0.5 psi for 10 s.

2-2-4 Method validation

The validations consisted of the specificity (measurement of formulation buffer, degraded sample, and other samples), linearity, quantitation limit, precision (repeatability and intermediate precision), accuracy, range, and robustness. As model samples, two different mAbs and two different ADCs were selected: mAb-A, of which theoretical *pI* was 9.0; mAb-B, of which theoretical *pI* was 7.4; ADC-C, of which drug antibody ratio (DAR) was relatively low (DAR 4); and ADC-D, of which DAR was relatively high (DAR 8). For the specificity test, samples were incubated in chambers for a specific period of time to prepare degraded samples. Detailed information for each test is described below.

2-2-4-1 Specificity

The formulation buffer, degraded samples, and other mAb samples were measured to confirm the specificity of the method.

2-2-4-2 Linearity

Seven test samples, of which concentrations were 1, 5, 10, 25, 50, 100, and 200% of

the target concentration (2 mg mL⁻¹), were measured. The correlation coefficients and Area/Conc. (%) for main and total peak areas were calculated as a function of sample concentrations.

2-2-4-3 Quantitation limit

The concentration providing $S/N \geq 10$ and Area/Conc. (%) of the main peak within $\pm 30\%$ of that of the 100% concentration was determined as the limit of quantitation (LOQ) of this method. The relative standard deviation (RSD) of the main peak area in this concentration was calculated. The measurement was repeated six times.

2-2-4-4 Precision: Repeatability and Intermediate precision

Repeatability was evaluated by six consecutive analysis of the samples. The %RSD of the peak area of the main peak in the target concentration was calculated. In addition, the RSD of the migration time of the main peak was calculated. Intermediate precision was demonstrated following with the design of the experiment summarized in Table 2-1. Experimental days, analysts, capillary lots and instruments were set as the experimental factors. The %RSD of the peak area of the main peak in the target concentration was calculated.

Table 2-1. Design of experiment for intermediate precision.

Day	Analyst	Capillary lot	Instrument	Repetition
1	A	X	α	2
2	B	Y	α	2
3	B	Z	α	2
4	B	X	β	2
5	A	Y	β	2
6	A	Z	β	2

2-2-4-5 Accuracy

Accuracy was evaluated on the basis of the specificity, linearity and precision studies.

2-2-4-6 Range

Range was determined from the basis of the linearity, precision and accuracy studies.

2-2-4-7 Robustness (*Running buffer components*)

The samples at the target concentration were measured by using running buffers with different pH and HPMC concentrations (0.05% HPMC pH 5.7 ± 0.1 , 0.05% $\pm 0.005\%$ HPMC pH 5.7).

2-3 Results and Discussion

2-3-1 CEX method development for ADCs

The conjugation of drugs to mAbs increases the structural complexity of a product, which triggers the need for improved separation methods. However, charge-based methods for evaluating ADCs have rarely been reported. First, we demonstrated a CEX method for charge variant evaluation of ADC-D (which had a DAR 8). Figure 2-1 shows an example of CEX chromatograms of ADC-D, indicating low separation efficiency and low reproducibility. The separation of ADC charge variants was not improved by changing sample preparation (with or without dilution by its formulation buffer), mobile phase pH or gradient programs. However, when the naked antibody (without the low-molecular weight drug of ADC-D) and other mAbs were analyzed, the method showed robust and reproducible results. These results indicate that the low robustness of the CEX method for the ADC was caused by some ADC specific characteristics, such as the hydrophobicity of a low-molecular weight drug. The validation of the CEX method for assessing ADCs was difficult as long as we solved the undesirable interaction of the low-molecular drug with the CEX column and confirmed reproducible results. Therefore, we concluded that the CEX method was not suitable for evaluating charge variants of ADCs qualitatively, and developed another alternative method, CZE, to obtain reproducible separation profile of ADCs.

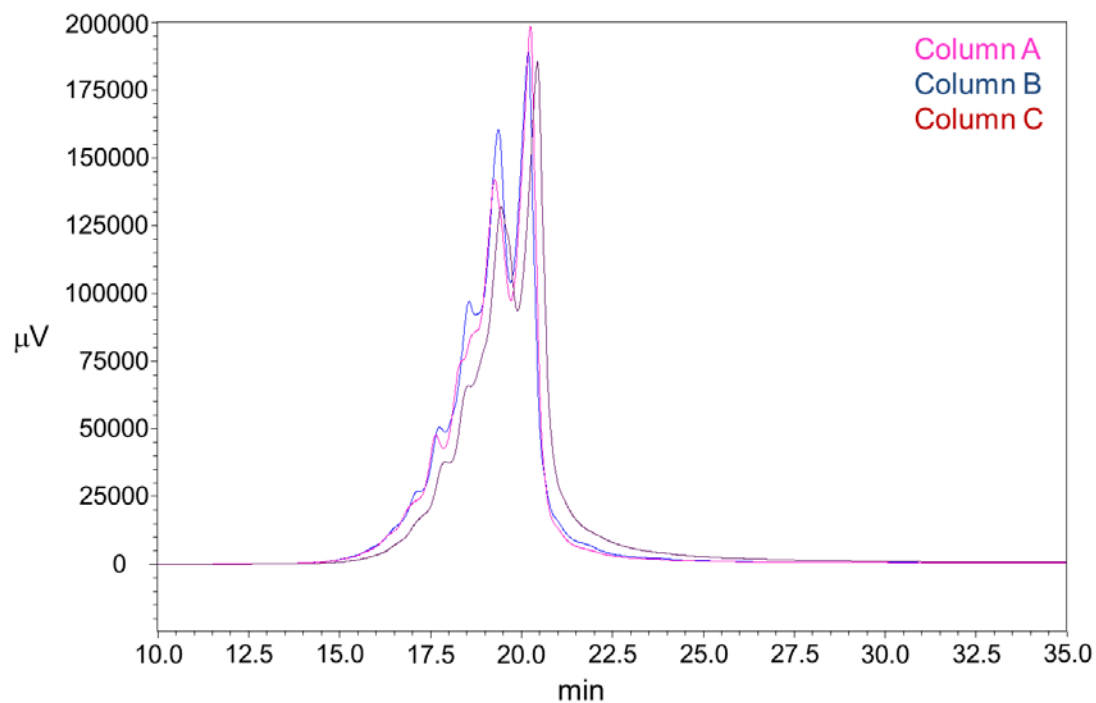


Figure 2-1. Chromatograms of ADC-D using three lots of CEX columns. Analytical conditions: analytical column, ProPac WCX-10 column (250 × 4 mm i.d.); mobile phase A, 20 mM phosphate buffer pH 6.6, mobile phase B, 0.5 M sodium chloride in mobile phase A; flow rate, 0.5 mL min⁻¹; gradient program, 0% to 40% mobile phase B for 39 min; column temperature, 40 °C; detection, 280 nm; injection, 5 μ L.

2-3-2 Summary of method validation of CZE for mAbs and ADCs

A CZE method for assessing mAbs and ADCs was demonstrated and validated. The principle of the method was the same as the method reported by He et al.³⁵ Briefly, we modified some experimental conditions, such as the use of a neutral capillary to reduce sample adsorption to the capillary inner-wall and a sample preparation to ensure a lower LOQ and longer sample solution stability. The samples were diluted with water (for mAbs) or their formulation buffer (for ADCs) to the 2 mg mL⁻¹ sample concentration as a standard condition. Two mAb samples, mAb-A (pI 9) and mAb-B (pI 7), and two ADC samples, ADC-C (having a lower drug to antibody ratio [DAR] 4) and ADC-D (having a higher DAR 8), were used to ensure the wide range of the method applicability. The validation of the method, including the specificity, linearity, quantitation limit, precision, accuracy and robustness, was conducted. The summary of the validation results is listed in Table 2-2. The method was applicable to more than 10 products, including ADCs without any modification, and showed specific migration times and separation profiles. Therefore, the CZE method is useful to identify and quantitate the charge variants of mAbs and ADCs in the manufacturing process and quality evaluation of biopharmaceuticals. Detailed results are described in following sections.

Table 2-2. Summary of CZE validation results.

Parameter	mAb-A	mAb-B	ADC-C	ADC-D
Specificity				
Formulation buffer	Separated from formulation buffer components.			
Degradation sample	Detected the degraded changes.			
Other mAbs/ADCs	Observed specific migration times and separation profiles.			
Linearity				
peak area	R = 1.000	R = 1.000	R = 0.999	R = 0.999
Area/Conc. (%)	92–107%	82–102%	93–123%	92–121%
LOQ	1.0%	1.0%	1.0%	1.0%
RSD of peak area	8.7%	7.2%	7.2%	3.9%
Precision; Repeatability				
RSD of peak area%	0.6%	0.8%	2.5%	0.8%
RSD of migration time	0.4%	0.1%	0.1%	0.1%
Precision; Intermediate precision				
RSD of peak area%	1.4%	1.4%	1.2%	2.3%
Accuracy	Pass	Pass	Pass	Pass
Range	1–200%	1–200%	1–200%	1–200%
Robustness (Running buffer components)	Robust for pH changes (pH 5.7 ± 0.1) and HPMC concentrations (0.05% ± 0.005% HPMC)			

2-3-2-1 Specificity

Electropherograms of the samples and their formulation buffers are shown in Figure 2-2. No interference peaks were observed from the formulation buffer around sample derived peaks. One peak was detected around 6 min in mAb-B, ADC-C and ADC-D. The peak was identified as L-histidine, which has a UV absorbance at 214 nm and is positively charged at pH 5.7. The peak was sufficiently separated from the samples and thus, had a negligible impact on the evaluation of the purity of the samples. Therefore, the method specifically detect the sample and quantify its charge variants.

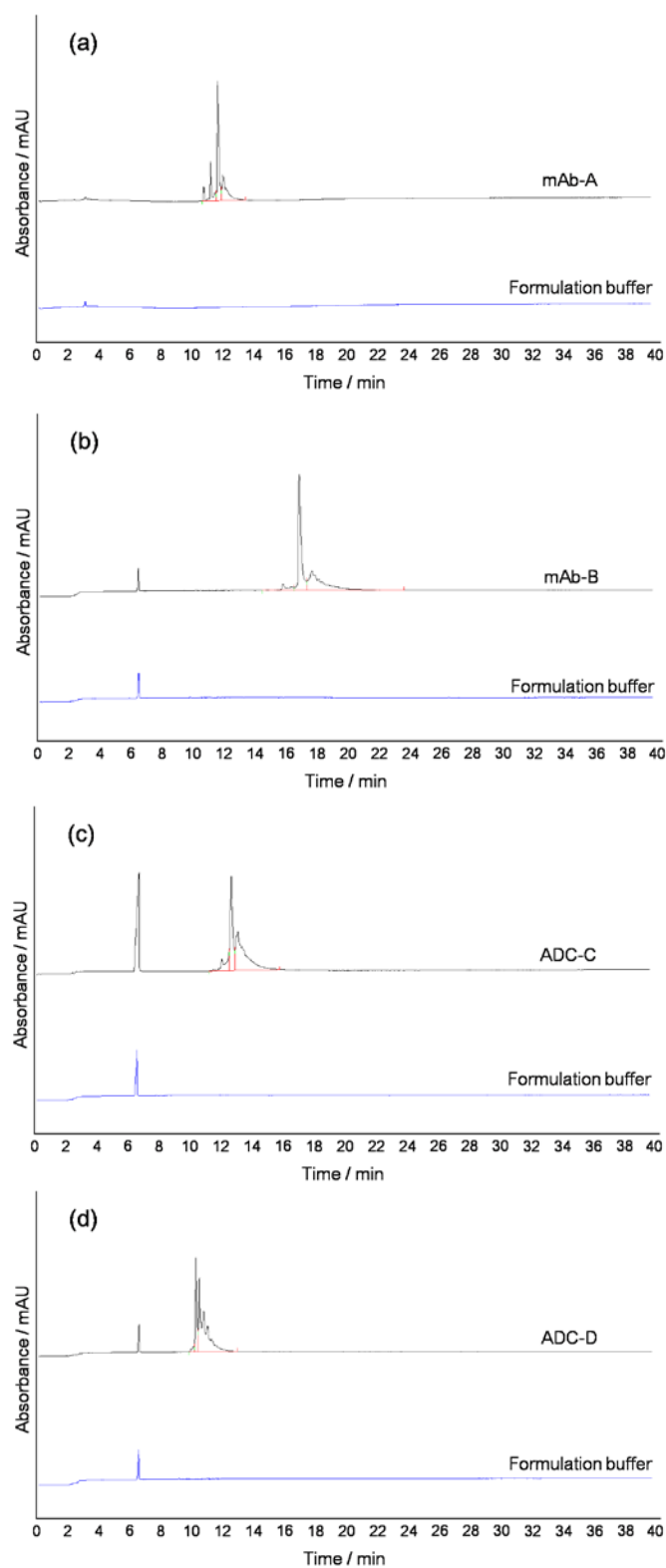


Figure 2-2. Electropherograms of (a) mAb-A, (b) mAb-B, (c) ADC-C, (d) ADC-D and their formulation buffers. The formulation buffers of mAb-B, ADC-C and ADC-D

contain l-histidine as a buffering salt. Analytical conditions: neutral capillary, i.d./o.d. 50/360 $\mu\text{m}/\mu\text{m}$ and effective/total length of 40/50 cm/cm; separation voltage, +30 kV; detection, 214 nm; capillary temperature, 25 °C; sample storage temperature, 15 °C; injection, 0.5 psi for 10 s; 0.05% HPMC, 380 mM EACA, 1.9 mM TETA, pH 5.7; 2.0 mg mL⁻¹ samples.

Electropherograms of the initial and degraded samples are shown in Figure 2-3. Changes in the electropherograms between the initial and degraded samples were observed. Especially, acidic peak group (APG) migrating after the main peak was increased and the main peak was decreased in the degradation samples. It is well known that thermal degradation causes deamidation and leads to the increase of APG, which has been confirmed in IEF and CEX.^{16, 22, 29} The increase of the APG in CZE agreed well with these results. Therefore, CZE demonstrated its ability to monitor the degradation of samples, and will be useful for stability testing of biopharmaceuticals.

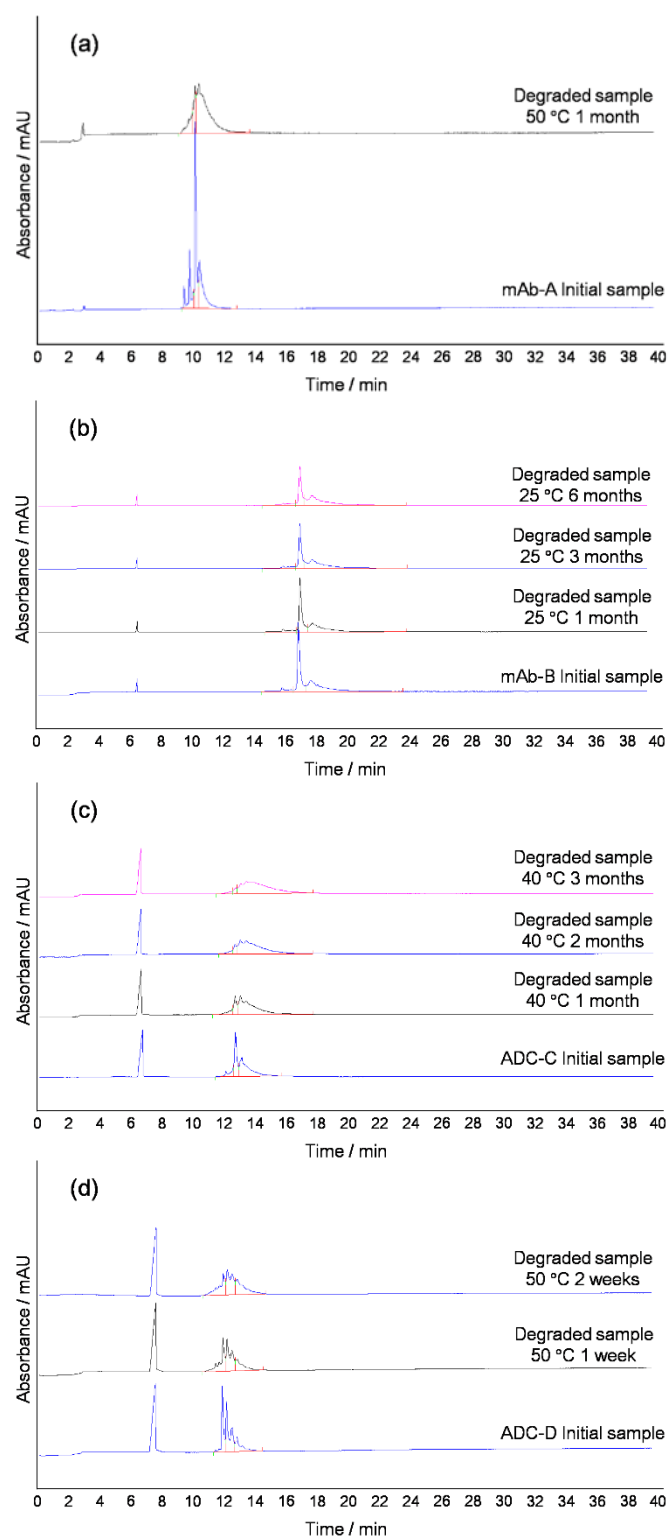


Figure 2-3. Electropherograms of (a) mAb-A, (b) mAb-B, (c) ADC-C, (d) ADC-D and their degraded samples. Experimental conditions were the same as those in Figure 2-2.

Electropherograms of 11 samples (9 mAbs and 2 ADCs) are shown in Figure 2-4. Changes in the electropherograms were observed where migration times and peak profiles were apparently different. Therefore, it was confirmed that the method is able to differentiate each sample specifically. The analyzed samples have a wide range of the pI value (from 7 to 9) and variation of drug distribution (DAR 0 to 8), which are common characteristics in biopharmaceuticals. Therefore, in regards to the specificity, the results indicate that the CZE method can fully cover almost all common variations of biopharmaceutical candidates and be applicable as a universal method for the use with identity tests.

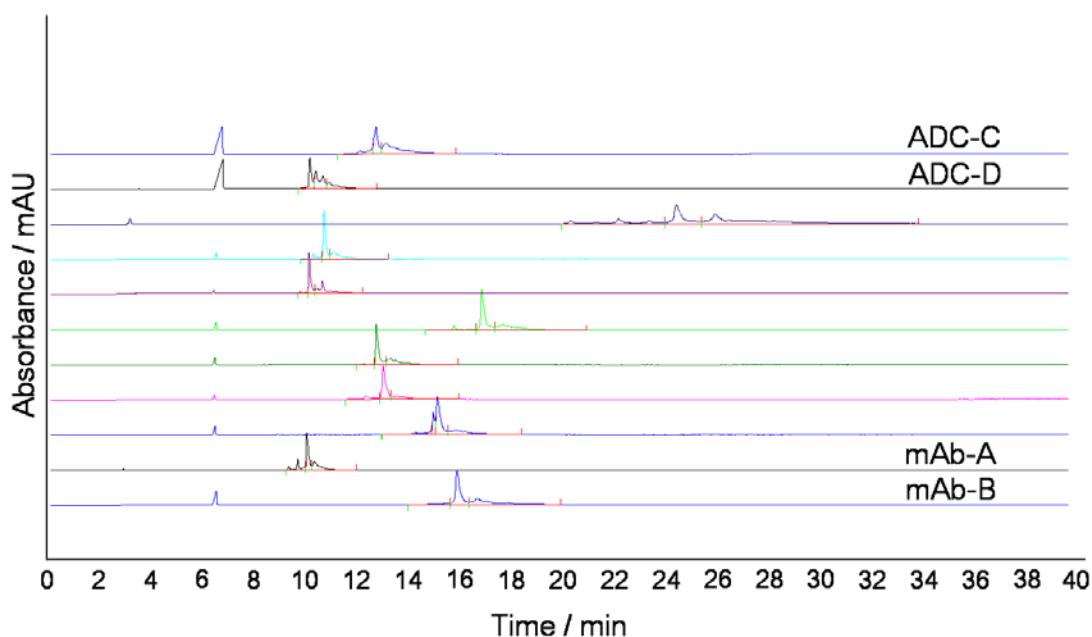


Figure 2-4. Electropherograms of the mAb-A, mAb-B, ADC-C, ADC-D and other mAb samples. In total, 11 samples (2 ADCs, 9 mAbs including mAb-A, mAb-B, ADC-C, and ADC-D) were analyzed with the same experimental conditions as in Figure 2-2.

2-3-2-2 Linearity

The correlation coefficients of all samples were more than 0.999. Area/Conc. (%) at each concentration of all samples was in the range of 82% to 123%. Therefore, the linearity in the range of 1% to 200% of the target concentration (2 mg mL⁻¹) was confirmed with good recovery.

2-3-2-3 Quantitation limit

The S/Ns of mAb-A, mAb-B, ADC-C and ADC-D obtained from the sample solutions having 1% of the target concentration were 13.0, 11.6, 10.1 and 13.9, respectively. The result of the repeatability using the concentration of the sample solutions showed that the RSD of the main peak area was less than 8.7%. Considering the content of the main peak of mAb-A, mAb-B, ADC-C and ADC-D, quantitation limits of the peak areas were determined to be 0.5%, 0.5%, 0.3% and 0.4%, respectively. These are sensitive enough to assess the purity of charge variants.

2-3-2-4 Precision (Repeatability and Intermediate precision)

Figure 2-5 shows the results of mAb-A and ADC-C as examples of a repeatability evaluation. The %RSD of the peak area of the main peak was less than 2.5% and the RSD of the migration time of the main peak was less than 0.4%. The 95% confidence interval of the SD of the migration time indicated that the 0.1 min difference in the migration time is sufficient to differentiate samples. Considering the results of the specificity (measurement of other samples), CZE specifically identified mAbs/ADCs from their peak profiles and their migration times. Therefore, the method shows a high precision and can be used as an identity test for biopharmaceuticals.

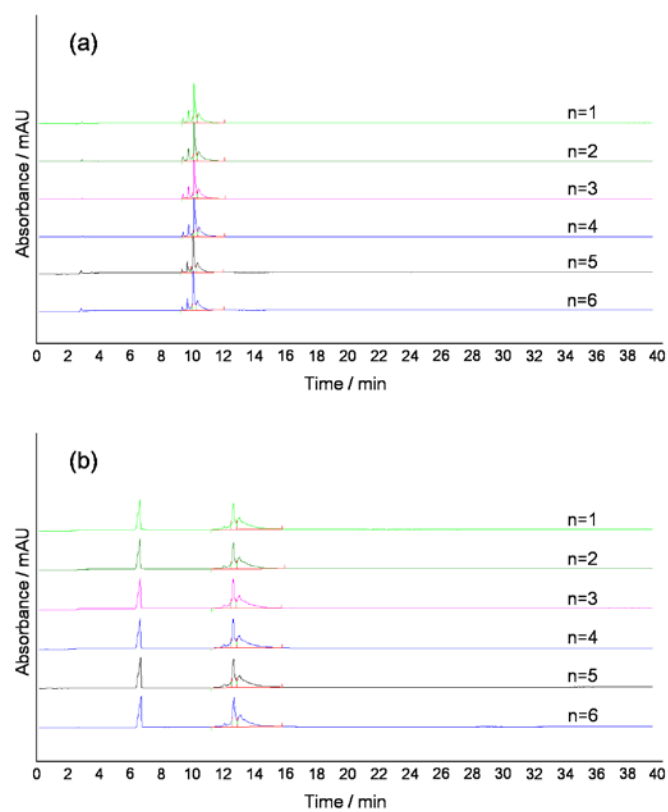


Figure 2-5. Repeatability of the (a) mAb-A and (b) ADC-C analysis. Experimental conditions were the same as those in Figure 2-2.

2-3-2-5 Accuracy

Accuracy was evaluated based on the results of the specificity, linearity and precision studies. They showed sufficient analytical performance, therefore, accuracy was confirmed.

2-3-2-6 Range

The results of the specificity, precision and accuracy studies satisfied required analytical performance. Therefore, the range was determined to be a concentration ranging between 1% to 200%.

2-3-2-7 Robustness (*Running buffer components*)

Figure 2-6 shows the effect of running buffer pH and HPMC concentrations on the electropherograms of mAb-A and ADC-C. The difference (%) of the main peak area was less than 9.0%. Peak area did not show any notable change in the range from 0.45% to 0.55% HPMC concentration at pH 5.7, or in the range from pH 5.6 to pH 5.8 with 0.50% HPMC. Therefore, the method was robust against the changes in pHs and HPMC concentrations of the running buffer.

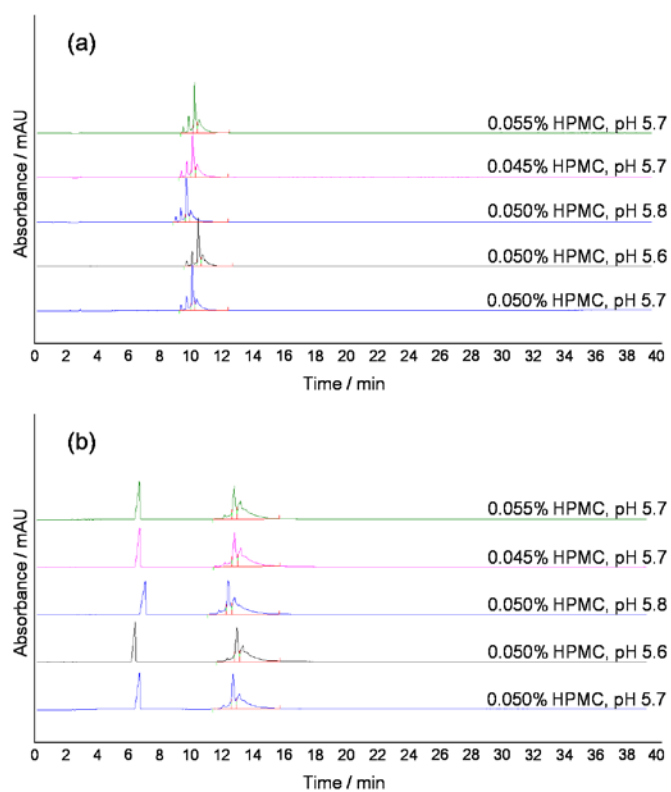


Figure 2-6. Effect of pH and HPMC concentration on the analysis of (a) mAb-A and (b) ADC-C. Experimental conditions were the same as those in Figure 2-2.

2-4 Conclusions

We developed and validated the CZE method to evaluate charge variants of mAbs and ADCs (ranging in *pI* from 7 to 9 and having DARs of up to 8). The method validation of CZE was conducted using two different mAbs and two different ADCs. The method was validated for use with identity and purity tests, and thus, can be a promising alternative to the IEF and CEX methods. The method showed quantitative results with high specificity, separation efficiency and precision. It should be noted that CZE is applicable for ADCs without any modification of the method. Therefore, the proposed CZE method shows the potential for the use in manufacturing process development, formulation development, and product characterization of biopharmaceuticals, including ADCs.

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Chapter 3

Identification and Characterization of a Thermally Cleaved Fragment of Monoclonal Antibody-A Detected by Sodium Dodecyl Sulfate-Capillary Gel Electrophoresis

3-1 Introduction

Monoclonal antibodies (mAbs) represent a promising class of therapeutics in the biopharmaceutical industry because of their advantage of having highly specific targeting towards a wider range of indications. More than 40 mAbs have been approved for the use of indications such as cancer and inflammatory diseases.¹⁻⁵

The fragmentation of mAbs is an important degradation pathway that impacts the quality of a final drug product.⁶⁻⁸ Fragments can be generated during the production and storage of the products depending on the temperature, pH, and formulation components.⁹⁻¹⁵ It is well known that peptide bonds in the hinge region of mAbs are susceptible to hydrolysis, generating fragments corresponding to the Fab region, Fc region, and antibody lacking one Fab arm.¹⁶⁻¹⁷ This fragmentation means a loss of purity (monomeric antibody) and directly affects the efficacy of mAbs. Not only that, the variations of fragmentation patterns are observed depending on mAbs' sequences because certain peptide linkages are prone to cleavage. When resulting fragments

contain complementarity determining regions (CDRs), they lose the efficacy of the mAbs. Therefore, fragmentation is recognized as a critical quality attribute that needs to be monitored to evaluate the purity of mAbs.

Analysis of fragments is conducted mainly by size-based separation methods, such as size exclusion chromatography (SEC), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and SDS-capillary gel electrophoresis (SDS-CGE).¹⁸⁻²⁰ SEC is a useful method for monitoring aggregates and hinge cleaved fragments such as Fab and lacking one Fab arm, but its separation efficiency is insufficient for detecting minor fragments.²¹ On the contrary, SDS-PAGE and SDS-CGE show a superior separation efficiency compared to SEC, and are able to monitor smaller fragments than Fab and lacking one Fab arm. SDS-CGE is a capillary format of SDS-PAGE and in comparison shows several advantages over SDS-PAGE, including automated operation, shorter separation time, and capability of accurate protein quantification.²²⁻³⁰ Therefore, SDS-CGE is now widely used for the precise evaluation of mAb fragmentation. However, the identification of fragments observed in SDS-CGE is challenging to carry out due to the difficulty of collecting analytical amounts of fractionations from capillaries. Consequently, few reports have been published regarding approaches to identifying observed fragments in SDS-CGE.³¹ Especially, an internal standard (10 kDa marker) is routinely used in SDS-CGE to normalize the migration time of separated peaks, and some degradation peaks of mAbs can co-migrate with the marker, preventing quantification of the purity. Thus, further development of methodologies that will allow the identification of SDS-CGE peaks is needed.

In this study, a fragment of mAb-A was detected close to the internal standard during the thermal degradation study. To identify the fragment confirmed in SDS-CGE, three

analytical procedures were employed: (1) in-gel digestion peptide mapping, (2) reversed phase liquid chromatography (RPLC) coupled with mass spectrometry (MS), RPLC–MS, and (3) the application of a Gelfree 8100 fractionation system following RPLC–MS. MS is a powerful technique able to confirm the primary structure of a molecule by intact-MS (top-down approach) and peptide mapping (bottom-up approach). In-gel digestion peptide mapping and RPLC–MS are authentic and straightforward ways to obtain molecular mass and/or the primary sequence of a fragment.³²⁻³⁷ The Gelfree 8100 fractionation system is a relatively new instrument used to collect samples separated by gel electrophoresis. After Gelfree fractionation, samples can be collected in a solution and applied to MS after the removal of detergents.³⁸⁻³⁹ To the best of our knowledge, however, the Gelfree 8100's applicability to intact-MS analysis for the characterization of mAbs has not yet been reported. Finally, in this study, SDS-CGE without an internal standard was demonstrated to assess the increased amount of the fragment, and the impact of the fragmentation on the efficacy was evaluated.

3-2 Experimental Section

3-2-1 Materials and reagents

The recombinant humanized mAb-A (subclass; IgG1, concentration; 20 mg mL⁻¹, formulation; 10 mM histidine, 10 w/v% sucrose, 0.01 w/v% polysorbate 80, pH5.6) was manufactured at Daiichi-Sankyo Co., Ltd.(Tokyo, Japan). The degradation samples were prepared by incubation in stability testing chambers (5 °C ± 3 °C and 25 °C ± 2 °C/60%RH ± 5%RH). The bare fused-silica capillary, SDS gel buffer, SDS sample buffer, acidic wash solution (0.1 N HCl), basic wash solution (0.1 N NaOH), and 10 kDa internal standard were purchased from AB Sciex (Brea, CA, USA), iodoacetamide (IAM), iodoacetic acid (IAA), dithiothreitol (DTT), 0.2 M sodium hydroxide (NaOH), tris (2-carboxyethyl)phosphine hydrochloride (TCEP), tris(hydroxymethyl)aminomethane (Tris), polysorbate 80 (PS80), ammonium hydrogen carbonate, methanol, acetonitrile (LC-MS grade), 2-propanol (IPA), formic acid (FA) and trifluoroacetic acid (TFA) from Wako Pure Chemical Industries (Osaka, Japan), 2-mercaptoethanol, 10 x Tris/Glycine/SDS buffer and bio-safe Coomassie stain from Bio-Rad Laboratories (Richmond, CA, USA), 12% Tris-Glycine gel and protein molecular weight marker from TEFCO (Tokyo, Japan), modified trypsin (sequencing grade) from Promega (Madison, WI, USA), a Tris acetate sample buffer (5x), 8% tris acetate cartridge and HEPES running buffer from Expedeon, Inc.(San Diego, CA, USA), and 2-morpholinoethanesulfonic acid and monohydrate (MES) from Dojindo Laboratories (Kumamoto, Japan).

3-2-2 SDS-CGE (Reduced/Non-reduced)

A PA800 plus system with a UV detector and 32 Karat software (AB Sciex, Brea, CA, USA) were used. The SDS-CGE separation was performed in a bare fused-silica capillary (50 μm i.d., 360 μm o.d., total length 30.2 cm, effective length 20.0 cm) at 25°C. The SDS gel buffer was used as running buffer. The mAb sample was diluted with the SDS sample buffer to make 95 μl of 1.0 mg mL^{-1} sample solution, then 2-mercaptoethanol (in reduced condition) or 250 mM IAM solution (in non-reduced condition) (5 μL) and 5 mg mL^{-1} 10 kDa internal standard (2 μl) were added to the sample solution. The sample solution was spun-down, heated at 75°C for 5 min, cooled at room temperature for 3 min, and SDS-CGE analysis was applied. Before the experiment, the capillary was pre-conditioned by flushing with a basic wash solution (0.1 N NaOH) for 3 min, an acidic wash solution (0.1 N HCl) for 1 min, water for 1 min, and SDS gel buffer for 10 min at 70 psi. The sample was electrokinetically injected for 20 s at 5 kV and separated for 35 min at 15 kV in a reverse polarity with detection at 220 nm. The data acquisition rate was set as 2 Hz. The sample storage temperature was set at 25 °C.

3-2-3 In-gel digestion peptide mapping: SDS-PAGE, in-gel digestion and peptide mapping

The sample solution of SDS-PAGE was prepared in the same way as described for SDS-CGE (Reduced/Non-reduced). The 10x Tris/Glycine/SDS buffer was diluted 10 times with water to use as running buffer. The sample solution and the molecular weight marker solution were applied to the 12% Tris-Glycine gel. A constant current of 20 mA was applied to the gel. After the gel electrophoresis analysis, the gel was stained with bio-safe Coomassie stain solution for 1 h, and washed with 40% methanol

for 3 h and lastly washed with water overnight.

For in-gel digestion, the fragment band was cut into cubes (*ca.* 1 mm³), and then washed with 50 mM ammonium hydrogen carbonate solution (400 µl) for 10 min and 50 mM ammonium hydrogen carbonate/50% acetonitrile solution (400 µl) for 10 min. After drying completely, the DTT solution was added and incubated at 56 °C for 60 min, subsequently, and the IAA solution was added and incubated in the dark at room temperature for 45 min. The gel was washed with 50 mM ammonium hydrogen carbonate solution and 50 mM ammonium hydrogen carbonate/50% acetonitrile solution in the same manner, and then dried. The enzyme solution (12.5 ng/µl trypsin) (20 µl) was placed on ice and incubated for 65 min. The incubated sample was added to 50 mM ammonium hydrogen carbonate solution (80 µl) and left overnight at 37 °C. The supernatant was moved to a new Eppendorf tube with 1% FA solution (100 µl) and sonicated for 15 min. A 5% FA/50% acetonitrile solution (100 µl) was added twice and sonicated. The supernatant was dried and dissolved with 0.1% FA solution.

The prepared sample was applied to RPLC–MS to obtain information of the primary sequence. The digested samples were separated by RPLC using an LC1200 (Agilent Technologies, Santa Clara, CA, USA) employing a Poroshell 120SB-C18 2.1 × 150 mm, 2.7 µm column (Agilent Technologies, Santa Clara, CA, USA). The mobile phase A was water-TFA (100:0.1, v/v) and mobile phase B was water-acetonitrile-TFA (10:90:0.1, v/v/v). A linear gradient program was set as (Time/B%) = (0/0), (5/0), (120/43), (120.01/100), (135/100), (135.01/0), (155/0) at the column temperature of 50°C. The flow rate was 0.2 mL min⁻¹ and UV detection was carried out at 220 nm. The separated peaks were detected by a mass spectrometer, LTQ/XL Orbitrap (Thermo Fisher Scientific, San Jose, CA, USA), equipped with an electrospray ion (ESI) source

set in the positive ion mode for the m/z range of from 300 to 2000.

3-2-4 RPLC–MS: intact-MS analysis and peptide mapping

For an intact-MS analysis, the samples were separated with RPLC using an LC-20 Prominence XR (Shimadzu Corporation, Kyoto, Japan) employing an Aeris Widepore XB-C8 300Å 2.1 × 100 mm, 3.6 µm column (Phenomenex, Torrance, CA, USA). The mobile phase A was water-TFA (1000:1, v/v) and mobile phase B was water-acetonitrile-IPA-TFA (100:200:700:1, v/v/v/v). A linear gradient program was set as (Time/B%) = (0/21), (3/21), (21/36), (21.01/100), (25/100), (25.01/21), (35/21) at the column temperature of 85 °C. The flow rate was 0.2 mL min⁻¹ and UV detection was carried out at 214 nm. The separated peaks were detected with a mass spectrometer, Q-Tof premier (Waters, Manchester, UK), equipped with an electrospray ion source set in the positive ion mode for the m/z range of from 1000 to 4000.

The sample preparation for peptide mapping was conducted as follows: The sample (20 µg) was dried completely and dissolved with an enzyme digestion buffer (100 mM Tris-HCl, 0.02 v/v% PS80, pH 8.0) (20 µl). The enzymatic digestion was carried out by then adding 0.2 mg mL⁻¹ trypsin solution (4 µl) and incubating at 37 °C overnight. The sample was reduced by adding 200 mM DTT solution (0.6 µl) and incubating at 37 °C for 30 min. The sample was alkylated by adding 200 mM IAA to 0.2 M NaOH solution (1.4 µl) and incubating in a dark condition at room temperature for 15 min. To quench the IAA, 200 mM DTT solution (0.8 µl) was added to the sample. The enzymatic reaction was stopped by adding 100 mM TCEP/1v/v% TFA solution (2 µl). The prepared sample was applied to RPLC–MS to obtain information of its primary sequence. The digested samples were separated by RPLC using an LC1200 employing

an AdvanceBio peptide map 2.1 × 150 mm, 2.7 μm column (Agilent Technologies, Santa Clara, CA, USA). The mobile phase A was water-TFA (1000:1, v/v) and mobile phase B was water-acetonitrile-TFA (400:3600:3, v/v/v). A linear gradient program was set as (Time/B%) = (0/0), (10/0), (100/45), (100.01/100), (115/100), (115.01/0), (117/100), (117.01/0), (135/0) at the column temperature of 50 °C. The flow rate was 0.2 mL min⁻¹ and UV detection was carried out at 220 nm. The separated peaks were detected by an LTQ/XL Orbitrap with ESI (positive ion mode) for the *m/z* range of from 300 to 2000.

3-2-5 Gelfree 8100 fractionation

A Gelfree 8100 fractionation system (Expedeon, Inc., San Diego, CA, USA) was used to fractionate samples by following the manufacturer's instructions. The sample was diluted with the tris acetate sample buffer (×5) and incubated at 75 °C for 5 min. The denatured sample was applied to the 8% tris acetate cartridge and fractionated following the procedure. The fractionated samples were concentrated by using centrifugal filters (Amicon Ultracel MWCO 3K) with a 10 mM MES buffer (pH 6.0). To confirm the fractionation of samples, samples were directly applied to SDS-CGE. For further MS analysis, the fractionated samples were applied to the detergent removal spin columns (Thermo Fisher Scientific, Inc., Waltham, MA, USA) to remove SDS. These samples were then applied to the intact-MS described in the previous section.

3-3 Results and Discussion

3-3-1 mAb-A degradation sample fragment peak detection in SDS-CGE

To evaluate the stability of mAb-A, SDS-CGE was employed for analysis of the mAb-A initial sample and mAb-A degradation sample (25 °C for 6 months). SDS-CGE detected new peaks as shown in Figure 3-1. In both non-reduced and reduced conditions, the fragment peak gradually increased close to the internal standard (10 kDa marker). In the reduced condition (Figure 3-1a), a new fragment appeared before the heavy chain peak. In the non-reduced condition (Figure 3-1b), a shoulder peak appeared in the monomer peak. These results suggested that the fragment peak was considered to be a substance related to the heavy chain. The fragment peak co-migrated with the internal standard, thus preventing accurate quantification of the purity. The identification and accurate quantification of the new fragment peak were carried out in following experiments. We conducted three analytical procedures (gel-based, RPLC-based and gelfree-based approaches) to identify the fragment peak confirmed by SDS-CGE. Table 3-1 summarizes pros and cons of these methodologies.

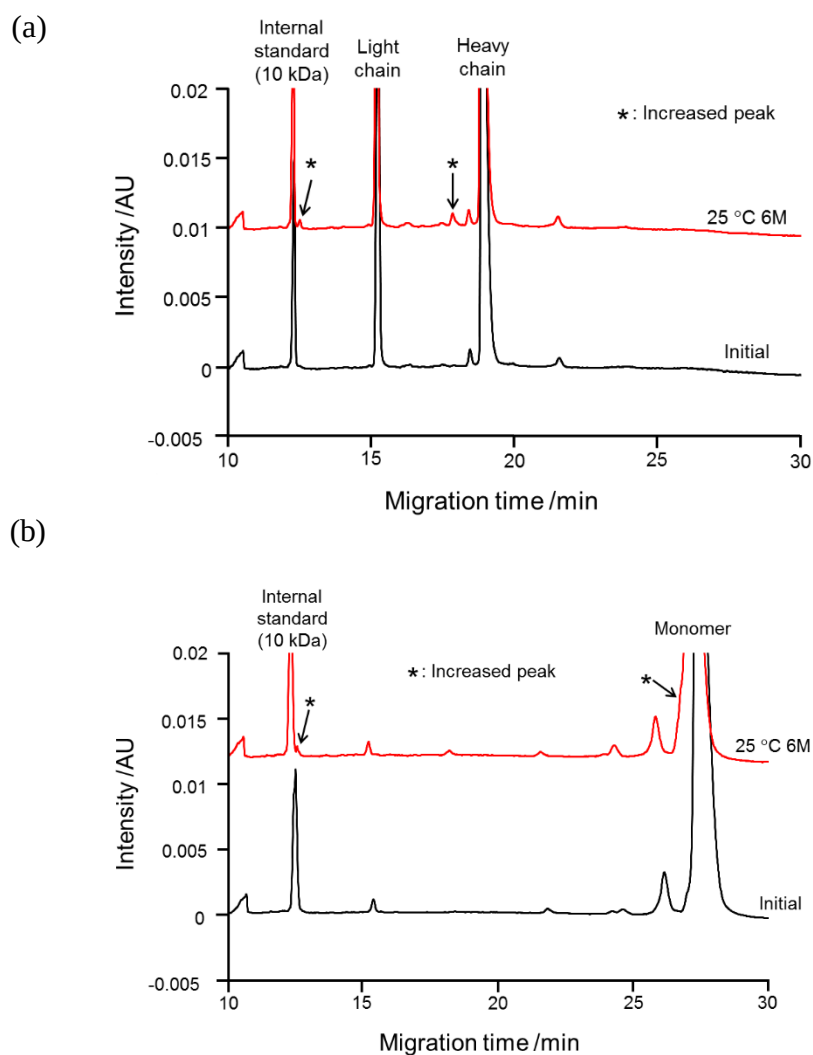


Figure 3-1. Electropherograms of the mAb-A initial sample (lower trace) and degradation sample (upper trace) obtained by SDS-CGE (a) reduced and (b) non-reduced conditions. Analytical conditions: bare capillary, 50 μm i.d. (360 μm o.d.) $\times$ 30.2 cm, 20 cm effective; separation voltage, -15 kV; detection, 220 nm; capillary temperature, 25 $^{\circ}\text{C}$; sample storage temperature, 25 $^{\circ}\text{C}$; injection, - 5 kV for 20 s; running buffer, SDS gel buffer. The internal standard peak, light chain peak, heavy chain peak, monomer peak, and increased peaks are indicated. Especially, the increased peak close to the internal standard is the focus of this study.

Table 3-1. Pros and Cons of Gel-based, RPLC-based, and Gelfree-based approaches to characterizing detected SDS-CGE peaks

Methodology	Pros	Cons
Gel-based approach - SDS-PAGE - in-gel digestion - peptide mapping	• Similar separation methodology to SDS-CGE	• Labor-intensive (ex. SDS-PAGE for 1 day, collection of separated bands manually and preparation for peptide mapping samples for 1 day, RPLC-MS analysis for 1 day) • Intact-MS analysis is impossible due to the requirement of enzymatic digestion for sample preparation
RPLC-based approach - intact-MS - fractionation - peptide mapping - SDS-CGE spike test	• Intact-MS analysis is available directly • Peptide mapping is available after fractionation of peaks	• Different separation methodology to SDS-CGE (the spike test is necessary to confirm the identity of the separated peaks) • Limited separation efficiency compared to SDS-PAGE, SDS-CGE (in some cases, target molecules cannot be separated)
Gelfree-based approach - fractionation - SDS-CGE - intact-MS	• Similar separation methodology to SDS-CGE • MS analysis is available after removal of SDS	• Labor-intensive (ex. optimization of fractionation for 1 day)

3-3-2 Gel-based approach: SDS-PAGE and in-gel digestion peptide mapping

To identify the fragment peak from SDS-CGE, we conducted SDS-PAGE and in-gel digestion peptide mapping of the fragment bands, because, while collecting desired bands demands labor-intensive work, in-gel digestion peptide mapping is a well-established analytical method. The 10 kDa band and HC Unknown band (shown in Figure 3-2) were collected and reduced, alkylated, and digested with trypsin. The digested samples were analyzed by RPLC–MS to elucidate the structure of the fragments. The identification of the 10 kDa band and HC Unknown band are summarized in Table 3-2. The results indicated that the 10 kDa band was HC1-101 or HC1-104. Although the fragment was confirmed as a heavy chain related substance, the identification of the cleavage site was somewhat difficult because HC102-H104 was cleaved by trypsin as a short peptide, which was not retained by the RPLC column. Also, obtaining accurate mass information of the whole molecule was impossible due to the requirement of enzymatic digestion for sample preparation. Therefore, other methodologies to confirm the integrity of the fragment, meaning the molecular mass of the intact fragment, were conducted by an RPLC-based approach.

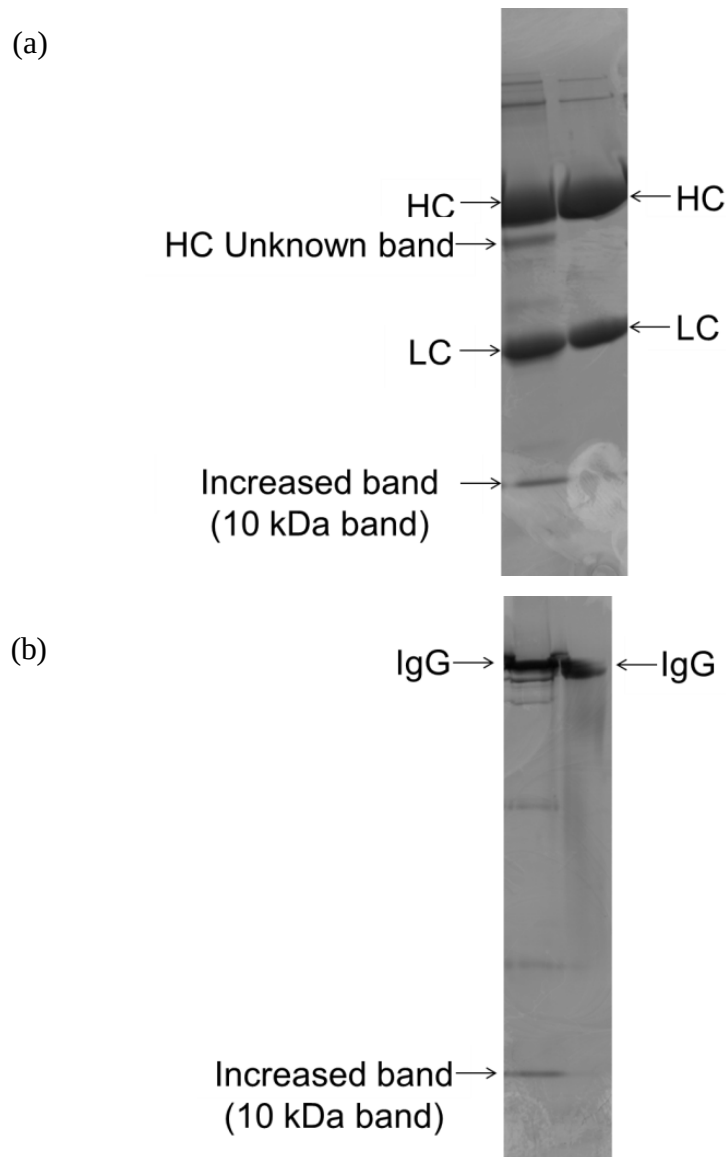


Figure 3-2. Gel image of SDS-PAGE applying mAb-A initial sample (right lane) and degradation sample (left lane) in (a) reduced condition and (b) non-reduced condition. Analytical conditions: gel, 12% Tris-Glycine; migration, 20 mA constant current; detection, bio-safe Coomassie stain. The 10 kDa band, LC band, HC band, HC unknown band and IgG band are indicated. The 10 kDa band corresponds to the increased peak in SDS-CGE.

Table 3-2. Identified peptides of the in-gel digestion samples (a) 10 kDa band and (b) heavy chain unknown band with peptide mapping

(a) 10 kDa band

Sequence	Identified	RT (min)	Delta (ppm)
HC1-12	MS/MS	64.30	-0.08
HC13-19	ND	ND	ND
HC20-23	ND	ND	ND
HC24-38	ND	ND	ND
HC39-65	ND	ND	ND
HC66-67	ND	ND	ND
HC68-87	MS/MS	86.22	-0.15
HC88-101	MS/MS	67.02	-0.43
HC102-104	ND	ND	ND

ND: Not detected

(b) Heavy chain unknown band

Sequence	Identified	RT (min)	Delta (ppm)
HC105-123	MS/MS	83.10	-0.63
HC124-135	MS/MS	71.42	-0.67
HC136-149	MS/MS	67.57	-0.98
HC150-212	ND	ND	ND
HC213-215	ND	ND	ND
HC216	ND	ND	ND
HC217-220	ND	ND	ND
HC221-224	ND	ND	ND
HC225-250	ND	ND	ND
HC251-257	MS/MS	53.26	-0.51
HC258-276	MS/MS	77.24	-0.46
HC277-290	MS/MS	74.73	-0.43
HC291-294	ND	ND	ND
HC295-303	ND	ND	ND
HC304-319	MS/MS	99.45	-1.04
HC320-322	ND	ND	ND
HC323-324	ND	ND	ND
HC325-328	ND	ND	ND
HC329-336	MS/MS	54.42	-0.84
HC337-340	ND	ND	ND
HC341-342	ND	ND	ND
HC343-346	ND	ND	ND
HC347-357	ND	ND	ND
HC358-362	ND	ND	ND
HC363-372	MS/MS	72.45	-0.45
HC373-394	MS/MS	84.07	-0.12
HC395-411	MS/MS	88.06	-0.11
HC412-416	MS/MS	32.41	-0.68
HC417-418	ND	ND	ND
HC419-441	MS/MS	77.87	-0.59
HC442-448	ND	ND	ND

ND: Not detected

3-3-3 RPLC-based approach: intact-MS, peptide mapping and SDS-CGE

RPLC–MS is a straightforward method for obtaining substance molecular mass, therefore, intact-MS was conducted to confirm the molecular mass of the fragment peak. As shown in Figure 3-3 (a), the new fragment peak was eluted around 7 min. Figure 3-3 (b) shows the deconvolution mass spectra of the peak of 11700 Da. Therefore, from the results of in-gel digestion and RPLC–MS, the fragment was considered to be HC1-104 (theoretical mass: 11701 Da). And thus, from the results of in-gel digestion and RPLC–MS, the fragment was identified as HC1-104.

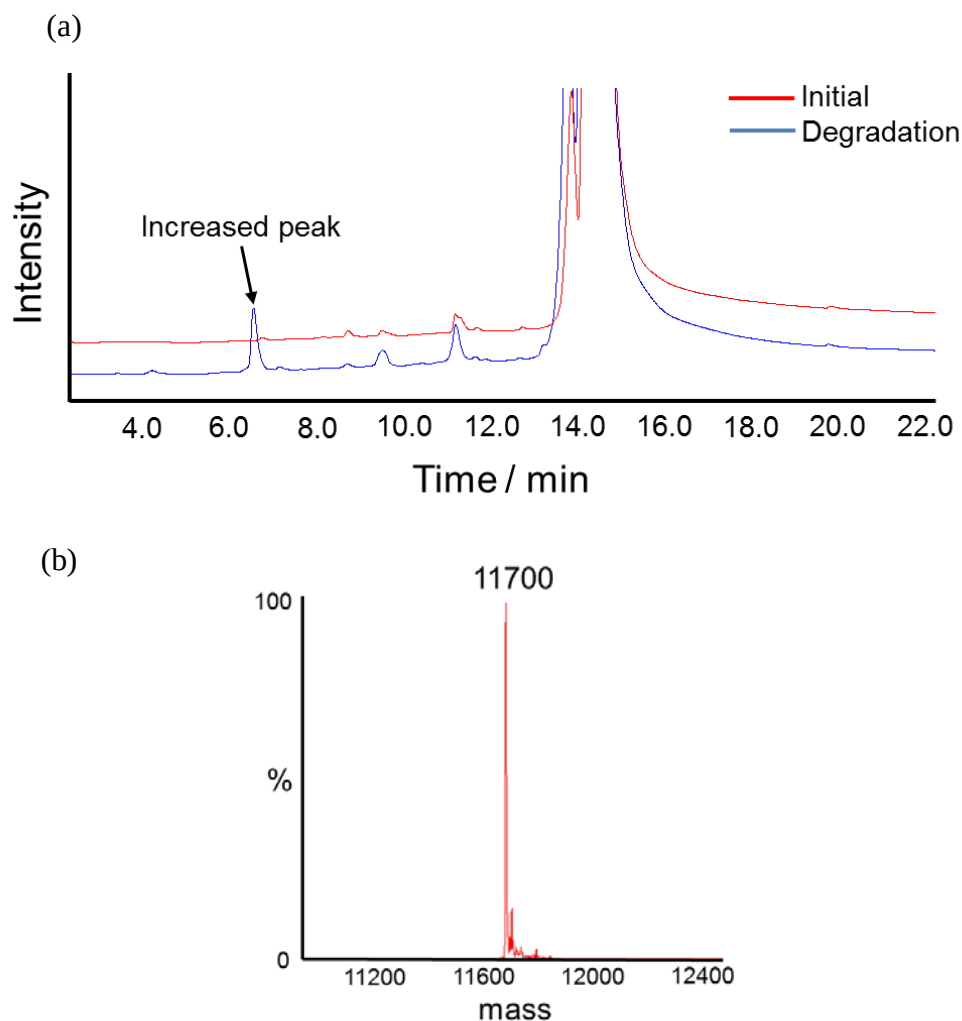


Figure 3-3. RPLC–MS chromatogram of mAb-A initial sample (upper trace) and degradation sample (lower trace) in non-reduced condition. The increased peak is indicated in the UV chromatogram (a) and its deconvoluted mass spectra (11700 Da) is shown in (b). Analytical conditions: analytical column, Aeris Widepore XB-C8 column (100 ×2.1 mm i.d., 300Å, 3.6 µm); mobile phase A, water-TFA (1000:1, v/v), mobile phase B, water-acetonitrile-IPA-TFA (100:200:700:1, v/v/v/v); flow rate, 0.2 ml min⁻¹; gradient program, 21% to 36% mobile phase B for 18 min; column temperature, 85 °C; UV detection, 214 nm; MS condition, ESI positive for the *m/z* of 1000 to 4000.

To confirm the amino acid sequences of the HC1-104 fragment peak, the fragment peak was fractionated and applied to tryptic peptide mapping. The identification via MS/MS analysis is summarized in Table 3-3 and shows good agreement with the results of the in-gel digestion peptide mapping. Taken together with the results of the intact-MS analysis, the fragment was successfully identified as HC1-104.

Table 3-3. Identified peptides of the RPLC fractionated sample with peptide mapping

Sequence	Identified	RT (min)	Delta (ppm)
HC1-12	MS/MS	57.83	-0.53
HC13-19	MS/MS	31.16	-0.76
HC20-23	MS/MS	24.08	-1.04
HC24-38	MS/MS	79.80	-0.24
HC39-65	MS/MS	85.65	0.38
HC66-67	ND	ND	ND
HC68-87	MS/MS	74.51	-0.19
HC88-101	MS/MS	60.08	0.06
HC102-104	ND	ND	ND

By using this RPLC method, we could fractionate the desired peak and identify it. However, the separation methodologies of RPLC and SDS-CGE are completely different; briefly, RPLC is a separation method based on differences of hydrophobicity of molecules, whereas the SDS-CGE method is based on their size differences. Therefore, a spiking test of the fractionated sample from SDS-CGE was important for confirming its identity. The fragment peak separated by RPLC was fractionated and applied to SDS-CGE. As shown in Figure 3-4, we confirmed that the RPLC fractionated peak migrated close to the internal standard of SDS-CGE. In addition, the fragment peak was increased by spiking the RPLC fractionated sample to the initial and degradation samples (Figure 3-5). Thus, the fractionated sample was confirmed as a species identical to the fragment. And therefore, the fragment migrating close to the internal standard peak was confirmed as HC1-104. To achieve characterization of fragments more efficiently, the Gelfree 8100 fractionation system was then newly introduced and fragments were evaluated further.

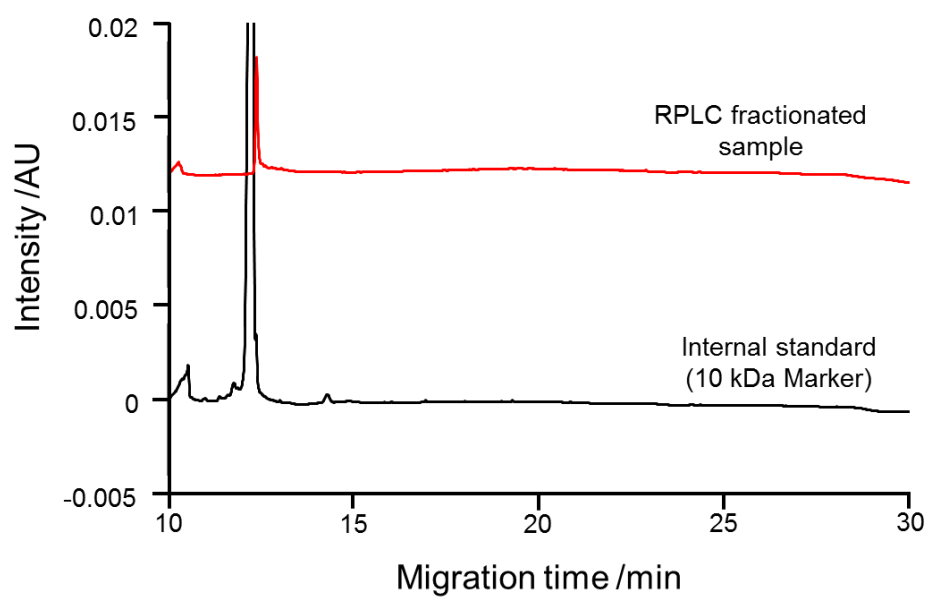


Figure 3-4. Electropherogram of RPLC fractionated sample (upper trace) and the internal standard (lower trace) in SDS-CGE. The fractionated sample migrated close to the internal standard. Experimental conditions were the same as in Figure 3-1.

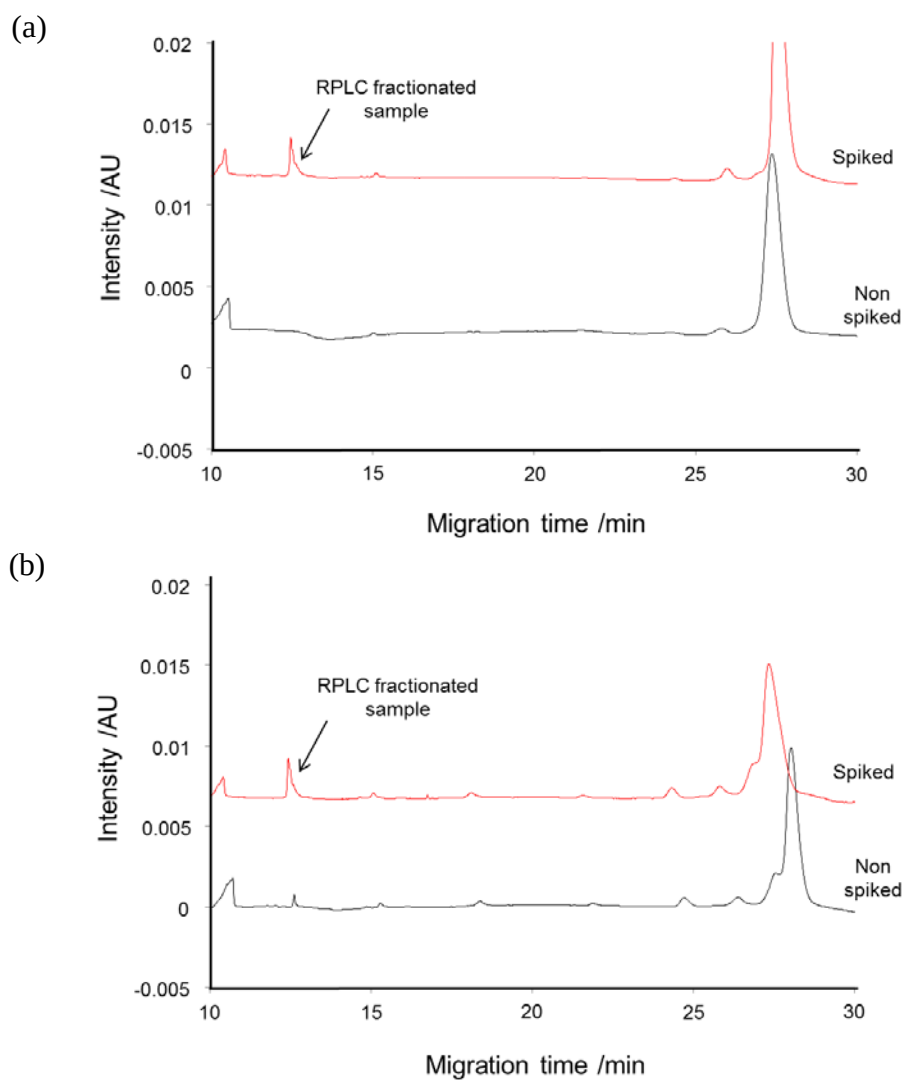


Figure 3-5. Spiking tests of RPLC fractionated sample to the mAb-A initial sample (a) and to the degraded sample (b). The increased peak is indicated in the spiked test. Experimental conditions were the same as in Figure 3-1.

3-3-4 Gelfree-based approach: SDS-CGE of the Gelfree 8100 fractionation samples, and intact-MS analysis

Gelfree 8100 fractionation was introduced to collect the desired fraction based on the same separation methodology of SDS-CGE. The recovery and reproducibility of the Gelfree 8100 fractionation method were evaluated. The recovery was more than 86% and the reproducibility (RSD) of the recovery was less than 8% (n=4). The fractionated sample was directly applied to SDS-CGE and we achieved an efficient separation of the sample based on the size differences. Figure 3-6 shows the SDS-CGE electropherograms of Gelfree 8100 fractionation samples and suggests that fractionated samples were separated corresponding to their molecular sizes. Fr.2, 3, and 4 show the fragment peaks that migrated close to 10 kDa. To confirm the intact molecular weight of the samples, these fractions were used for further structural analysis.

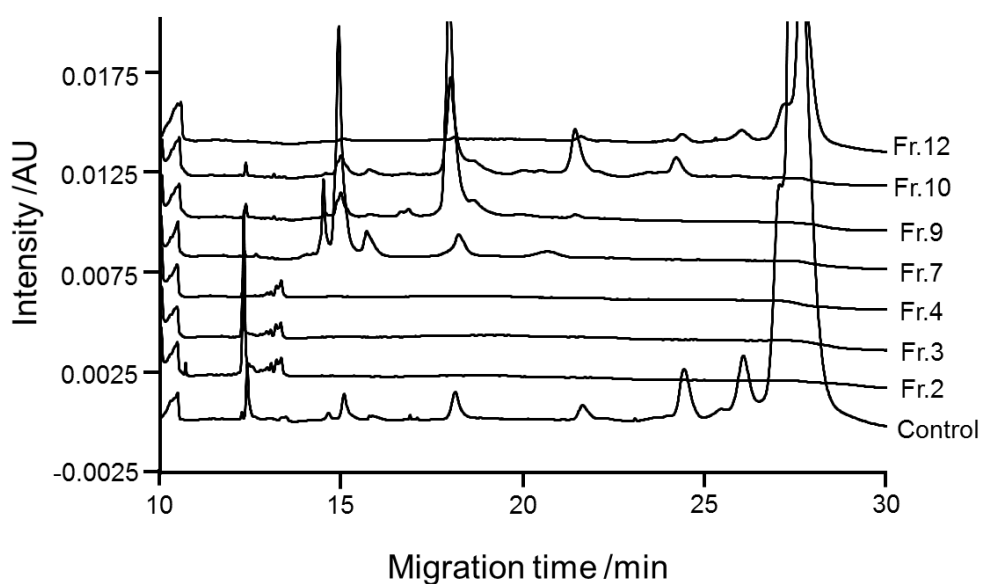


Figure 3-6. SDS-CGE electropherogram of Gelfree fractionated samples. The Gelfree fractionated samples were concentrated by using centrifugal filters and applied to SDS-CGE directly. Control means unfractionated degradation sample. The large peaks of Fr.7, Fr.9, Fr.10 were identified as light chain peak, heavy chain peak, and heavy-light chain peak, respectively. The large peaks at 24 min and 26 min were heavy-heavy chain and heavy-heavy-light chain, respectively. These peaks are common fragments during thermal degradation. Experimental conditions were the same as in Figure 3-1.

Prior to the intact-MS analysis, however, removal of the surfactant (which was bound to the sample or was contained in the sample solution) with a detergent removal column was necessary due to the sample treatment of Gelfree 8100 fractionation requiring SDS to denature proteins. Removing SDS is crucial to obtaining an MS spectrum because it causes sample ion suppression and deteriorates the quality of the MS spectrum. Therefore, the samples were treated by a detergent removal column, and then applied to the intact-MS analysis. The deconvolution mass was assigned as 11701 Da (Figure 3-7), which matched well to the theoretical mass of HC1-104. Other fractionated samples were also applied to intact-MS analysis in the same manner and their intact molecular weights were elucidated. In this way, Gelfree 8100 fractionation samples were successfully applied to intact-MS by removing SDS. The Gelfree 8100 data corresponded well to the data obtained by the in-gel digestion peptide mapping and RPLC-MS. To our knowledge, the application of a Gelfree 8100 fractionation system for mAb structural analysis and its comparison with other approaches has not yet been well documented. Our investigation showed that the Gelfree 8100 fractionation was well suited to confirming the peaks detected in SDS-CGE in terms of both the separation methodology, and applicability to mass analysis.

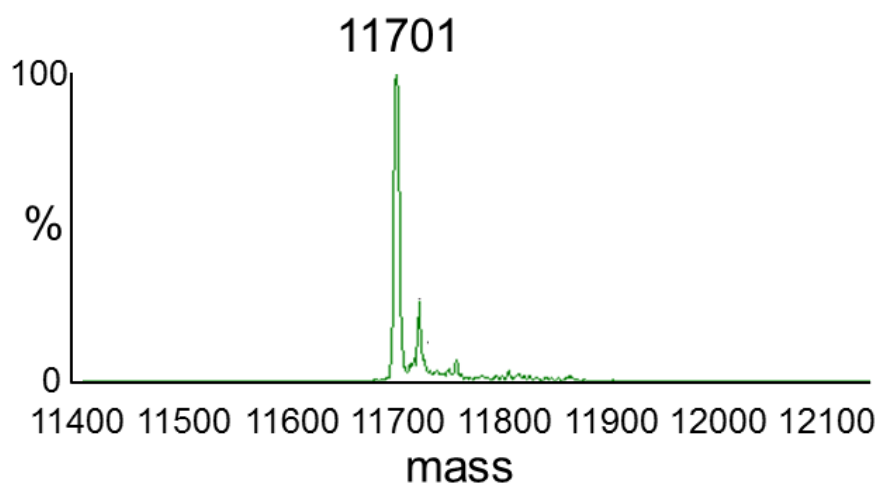


Figure 3-7. Intact-MS spectra of Gelfree fractionated sample. Experimental conditions were the same as in Figure 3-3.

In conclusion, these structural analyses revealed that the fragment was HC1-104, and the cleavage site was Arg-Asp. The primary sequence of Xaa-Asp has been reported as the cleavable site, and the schematic mechanism is considered to be as depicted in the literature¹⁵, however, the cleavage of the Arg-Asp sequence in mAbs has not been reported so far. The HC1-104 fragment of the mAb contained CDRs, and the cleaved site of Arg-Asp was found to be in the middle of CDR3. Therefore, the region could be exposed to solvents and become susceptible to fragmentation. The counterpart to HC1-104, the larger fragment of HC, H105-449, was also identified by in-gel digestion peptide mapping and RPLC-MS. The H105-449 corresponded to the increased peak appeared at 18 min in Figure 3-1 (a).

3-3-5 SDS-CGE (without using the internal standard) for mAb-A thermal degradation samples

We conducted SDS-CGE without using the internal standard to quantify the amount of the fragment peak. Figure 3-8 shows electropherograms of mAb-A thermal degradation samples. The 10 kDa fragment peak of the degradation samples was clearly detected and quantitated in the modified method. As shown in Figure 3-9, the fragment increased linearly along with the temperature. This result suggested that the fragment is generated by hydrolytic cleavage. Thermal fragmentation is the most frequent degradation for mAbs, therefore, the dependence on temperature should be carefully monitored to assure the stability of the biotherapeutics. In addition, the HC1-104 fragment contained CDRs, and thus could affect the antigen binding activity. The loss of CDR regions directly results in the loss of efficacy for mAb-A. Thus, this peak must be monitored as an impurity peak. However, the increased amount of the fragment was 0.2% per year at 5 °C storage, and this stability is considered to be acceptable. Therefore, the impact on the efficacy was limited during the storage at 5 °C. The rate of the thermal degradation and resultant cleaved sites would be varied depending upon a mAb's sequences, therefore its careful evaluation should be conducted. The information, including cleavable sites of the mAb and the rate of its degradation, is useful for choosing and/or designing stable antibodies in research areas.

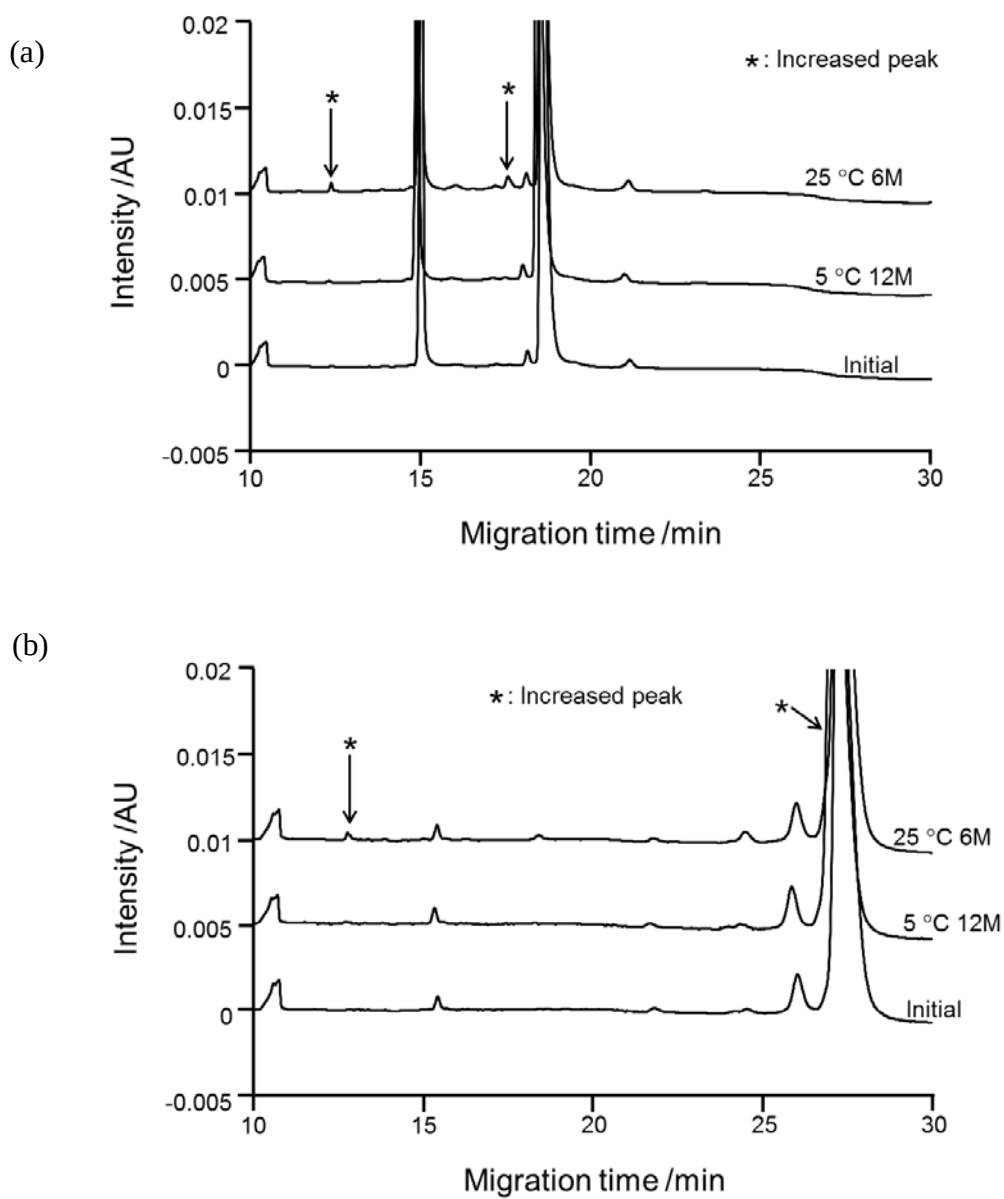


Figure 3-8. The electropherograms of SDS-CGE without using internal standard in (a) reduced condition and (b) non-reduced condition. Experimental conditions were the same as in Figure 3-1.

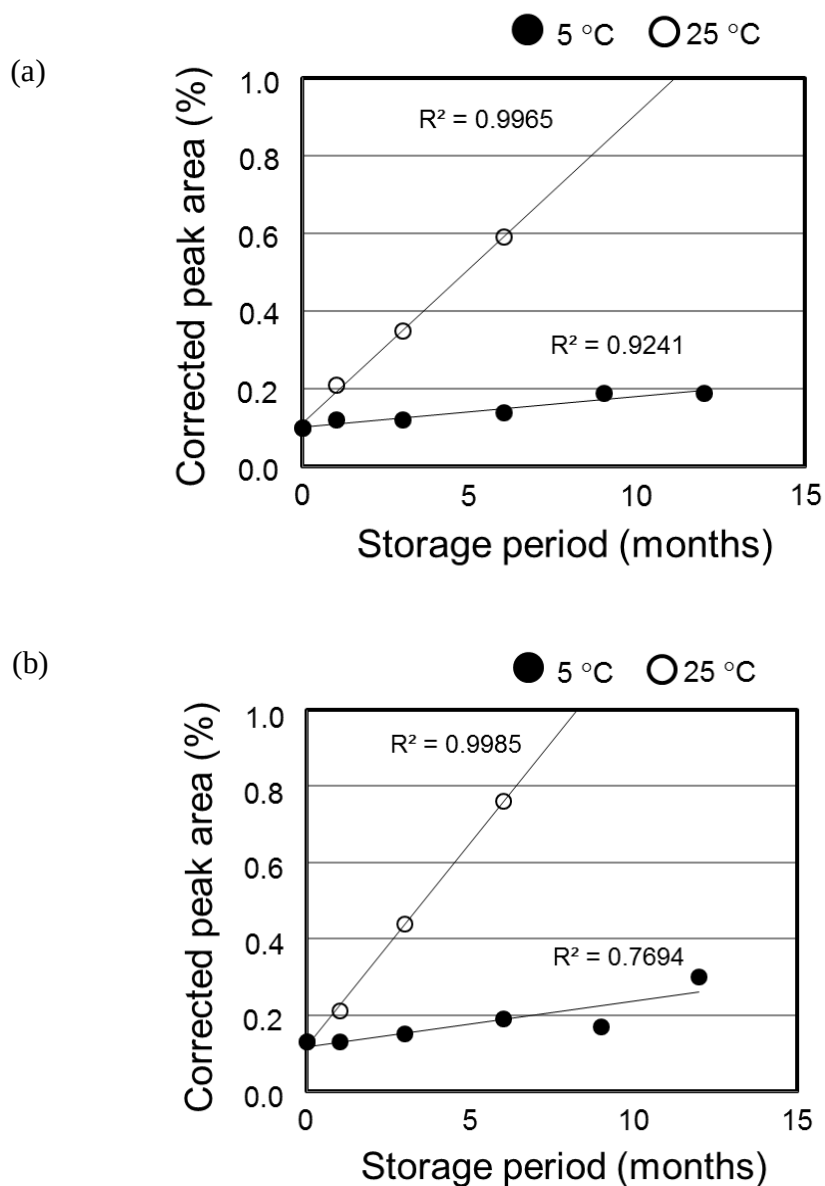


Figure 3-9. The plot of increased amount of the fragment in SDS-CGE without using the internal standard in (a) reduced condition and (b) non-reduced condition. The closed circle describes the increased amount of 10 kDa fragment at 5 °C and the open circle describes that at 25 °C.

3-4 Conclusions

We confirmed a fragment of mAb-A that migrated close to the internal standard (10 kDa marker) of SDS-CGE and increased by thermal degradation. The fragment was identified by in-gel digestion peptide mapping, RPLC-MS, and Gelfree 8100 fractionation. The fragment was HC1-104, which is involved in CDRs, and thus affects the antigen binding activity, meaning the efficacy of mAb-A. Finally, the increased amount of HC1-104 was reassessed by SDS-CGE without using the internal standard and was evaluated as increasing 0.2% per year at 5 °C. The identification of a fragment confirmed in SDS-CGE often becomes challenging, therefore, combining available fractionation methods and structural identification methods (including top-down and bottom-up approaches) is important to identifying the fragment. In this case, the internal standard used in SDS-CGE made it difficult to monitor the identified fragment peak, supporting the argument that an internal standard should be carefully chosen depending on its antibody degradation profile. We believe that the proposed approach in this study will be useful and applicable to the quality evaluation of biotherapeutics, including complex protein pharmaceuticals such as vaccines, and antibody-drug conjugates.

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Chapter 4

New platform for simple and rapid protein-based affinity reactions

4-1 Introduction

A variety of antibody-based medicines have been approved in recent years.¹⁻² These products have high annual returns due to their high selectivity toward their target antigens, relatively low levels of side effects, and stability in vivo; in addition, 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 rapid optimization using high-throughput chromatography.⁸⁻⁹ Indeed, for certain antibodies, more than 100 kg is required at the clinical investigation stage.¹⁰⁻¹² Usually, separation media in which protein A is immobilized onto a crosslinked-agarose adsorbent are used for the analysis and purification of an antibody¹³⁻¹⁵ as a suitable separation antibodies.¹⁶⁻¹⁹ 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.²⁰⁻²² 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.²³⁻²⁵ For purification of biomolecules, the monolithic structure is suitable for rapid reactions because the flow-through pores are integrated with the skeleton.²⁶⁻³¹ 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 ($>10\ \mu\text{m}$), 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 or spongy monolith as a novel separation medium.³²⁻³³ The spongy monolith is prepared simply by blending a thermoplastic resin above its melting point with water-soluble pore templates. After removal of the pore template by washing with water, the resultant spongy monolith contains large flow-through pores of $>10\ \mu\text{m}$ in diameter, and a column made of the spongy monolith facilitates separation mediated by hydrophobic interactions and/or ion exchange at a high flow rate. Additionally, the spongy monolith can be prepared in any shapes and easily packed into a column. Therefore, we expected that the spongy monolith containing specific functional groups, such as epoxy groups, would be useful for affinity chromatography and overcome the limitations of current media.

4-2 Experimental Section

4-2-1 Materials and reagents

Poly(ethylene-co-glycidyl methacrylate) (Sigma Aldrich), pentaerythritol (Toso), recombinant human IgG1 antibody (Daiichi-Sankyo), bovine serum albumin (BSA) (Sigma Aldrich), IgG1, kappa from human myeloma plasma (Sigma Aldrich), IgG2, kappa from human myeloma plasma (Sigma Aldrich), pierce recombinant Protein A (Life Technologies), PBS Tablets (TaKaRa), acetonitrile, HPLC grade (Wako Pure Chemical Industries), acetonitrile, LC-MS grade (Wako Pure Chemical Industries), sodium dihydrogenphosphate dihydrate (Wako Pure Chemical Industries), sodium chloride (Wako Pure Chemical Industries), 2 M sodium hydroxide (Wako Pure Chemical Industries), 2 M hydrochloric acid (Wako Pure Chemical Industries), Pepsin from porcine gastric mucosa (Sigma Aldrich), acetic acid (Wako Pure Chemical Industries), tris(hydroxymethyl)aminomethane (Wako Pure Chemical Industries), formic acid (Wako Pure Chemical Industries), trifluoroacetic acid (TFA) (Wako Pure Chemical Industries). Pure water was obtained from Milli-Q Gradient A10 (Merck Millipore).

4-2-2 Instruments

Scanning electron microscope, TM-1000 (Hitachi High-Technologies). LC analyses for IgG with PDA were operated by a LC system, LC-20 Prominence (Shimadzu Co.). LC-MS analyses for IgG were operated by a LC system, LC-20 Prominence XR (Shimadzu Co.), a mass spectrometer, Q-TOF premier (Waters Co.), and a protein analysis soft, MassLynx V4.1 (Waters Co.). LC-MS analyses for the digestion with

Pepsin were operated by a LC system, LC1100 or LC 1200 (Agilent Technologies), a mass spectrometer, LTQ/XL Orbitrap (Thermo Fisher Scientific), and a peptide mapping soft, PepFinder 2.0 (Thermo Fisher Scientific). MS conditions with a Q-TOF premier were follows: capillary, 3.5 kV; sampling cone, 80.0 V; source temperature, 100 °C; cone gas flow, 50.0 (L/Hr), and with a LTQ/XL Orbitrap were follows: spray voltage, 4 kV; capillary temperature, 325 °C; capillary voltage, 10 V.

4-2-3 Preparation of a spongy monolith

35 w% of poly(ethylene-co-glycidyl methacrylate) (PEGM), in which glycidyl methacrylate of 8% is contained, 52 w% of pore templates (pentaerythritol), whose particle size in diameter was classified around 10 µm, and 7 w% 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. The porosity of the spongy monolith calculated by a void volume on LC was 65% and the diameter of its cross section across its entire length was 4.8 mm. (PEGM-SpM).

4-2-4 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 (Figure 4-1). The diameter of the spongy monolithic column was greater than the internal diameter of the empty column (4.6

mm). 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 shrunken 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 LC 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 packing³⁴⁻³⁵ condition.

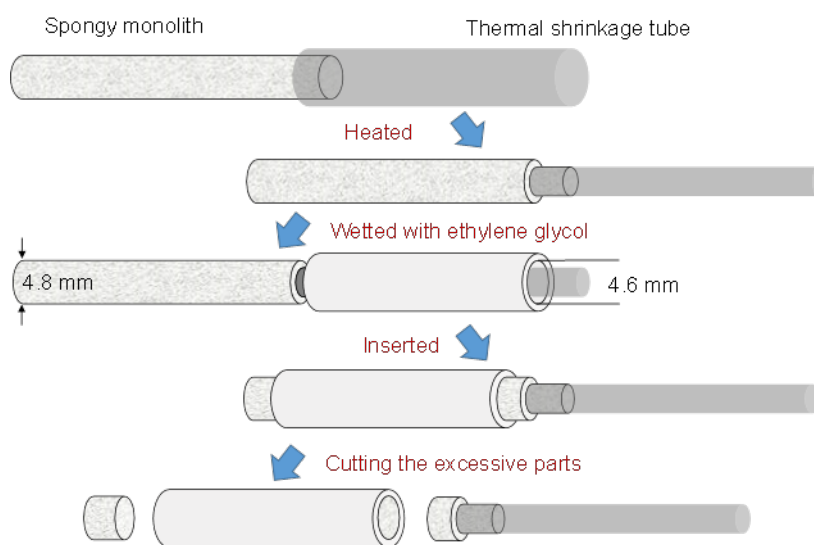


Figure 4-1. Schematics of the packing procedures of spongy monoliths.

4-2-5 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 (MeCN) and pure water were passed through the PEGM-SpM at room temperature for 5 mL in each solvent. The protein A solution (1 mg mL⁻¹) was fulfilled into the PEGM-SpM completely, and then the column was incubated at 37 °C for 16 h. The completed column was washed with pure water for 1 h at 1 mL min⁻¹. (ProA-SpM).

4-2-6 Preparation of a pepsin immobilized column

Pepsin of 15 mg was dissolved into 5 vol% formic acid aqueous solution of 5 mL. For conditioning the column, MeCN and pure water were passed through the PEGM-SpM at room temperature for 5 mL in each solvent. The pepsin solution (3 mg mL⁻¹) was fulfilled into the PEGM-SpM completely, and then the column was incubated at room temperature for a week. The completed column was washed with 5 vol % formic acid aqueous solution for 1 h at 1 mL min⁻¹. (Pep-SpM).

4-2-7 Conditions for RPLC

For RPLC evaluations, a linear gradient was employed using 0.1 vol% trifluoroacetic acid (TFA) aqueous solution (A) and 0.1 vol% TFA in MeCN at 1.0 mL min⁻¹ under 40 °C. The gradient condition was utilized at 100% A to 100%B for 20 min, and 100%B for 20 to 30 min. For an affinity separation of the ProA-SpM, a 50 mM phosphate buffer with 150 mM NaCl pH 7.5 (A) and pH 2.5 (B) was employed at 25 °C.

Regarding Figure. 4-5 (b), a linear gradient was utilized at 100% A (0 to 5 min), 100% A to 100% B (5 to 15 min), and 100% B (15 to 25 min). For the other figures, the stepwise gradient was employed. The condition at 1.0 mL min^{-1} was 100% A (0–2.4 min) and 100% B (2.41 to 9.6 min). The gradient conditions were optimized in response to the flow rate.

4-2-8 Fractionation and determination of IgG from cell culture

To know the possibility for the affinity separation with the ProA-SpM, the real sample was utilized for the separation. Protein A load sample, which was obtained just by simple filtration with membrane filter ($0.2 \text{ }\mu\text{m}$) to remove the cells, was directly injected into the ProA-SpM with the same conditions as above. The peak of the seemed to IgG was manually collected (Fraction 2) and the flow through fraction was also collected (Fraction 1). Both fractions, the original supernatant, and a standard IgG were analyzed by LC with a TOF-mass spectrometer. For the intact-MS analysis, the samples were separated with RPLC using an LC-20 Prominence XR (Shimadzu) employing an Aeris Widepore XB-C8 $300 \text{ }\text{\AA}$ $2.1 \times 100 \text{ mm}$, $3.6 \text{ }\mu\text{m}$ column (Phenomenex). The mobile phase A was water/TFA (1000/1) and mobile phase B was water/MeCN/IPA/TFA (100/200/700/1). A linear gradient was set as (Time/B%) = (0/21), (3/21), (21/36), (21.01/100), (25/100), (25.01/21), (35/21) at the column temperature of $85 \text{ }^{\circ}\text{C}$. The flow rate was 0.2 mL min^{-1} and UV detection was carried out at 214 nm . The separated peaks were detected with a Q-Tof premier (Waters), equipped with an electrospray ion source set in the positive ion mode for the m/z of 1000 to 4000. The parent molecular weights were estimated by a deconvolution of multi ions with MaxEnt1³⁶⁻³⁷ (Waters).

4-2-9 Online digestion of an antibody by the Pep-SpM and LC–MS analysis for peptide mapping

A reduced antibody sample was prepared with 0.05 M acetic acid and 0.05 M tris(2-carboxyethyl) phosphine hydrochloride in aqueous solution (the concentration of the antibody, 10 mg mL⁻¹), and then the solution was stirred at 75 °C for 15 min. The antibody solution was passed through the Pep-SpM at 10 or 100 mL h⁻¹, and the eluted solution was collected during every 1 min. On the other hand, the reduction antibody solution was also reacted with a pepsin solution as the comparison for 1 or 150 min. Both the collected fraction by online digestion in the Pep-SpM and by treating in solution were analyzed typical LC. LC conditions are follows; column, AdvanceBio PeptideMap 2.1 × 150 mm; mobile phase, 0.1 vol% TFA in water as mobile phase A and 0.1 vol% TFA in MeCN, 0 to 55% B for 0 to 30 min, 100% B for 30.1 to 40 min; temperature, 50 °C; flow rate, 0.2 mL min⁻¹, which is corresponding to Figure 4-15 using UV detection. For LC–MS analyses, instead mobile phase condition was follows; 0.1 vol% TFA in water as mobile phase A and 0.1 vol% TFA in 90% MeCN aqueous, 0 to 43% B for 5 to 120 min, 100% B for 120.1 to 135 min, which is corresponding to Figure 4-17, Figure 4-18, and Table 4-2 obtained by PepFinder 2.0. The separated peaks were detected by a mass spectrometer, LTQ/XL Orbitrap (Thermo Fisher Scientific), equipped with an electrospray ion source set in the positive ion mode for the m/z of 300 to 2000. MS/MS fragmentation analysis was conducted by using following conditions: the parent ions were fragmented with HCD at the isolation width of 6.0 Da and the collision energy of 35 V. Table 4-2 indicates all the peptides detected by LC–MS containing the origin (light or heavy chain), the number of amino

acid of each terminal, and the length. Here, the number of amino acid was assigned that the number 1 is first amino acid from the N-terminal, and the total number of amino acids are 213 and 449 in light chain and heavy chain³⁸⁻³⁹, respectively.

4-3 Results and Discussion

4-3-1 Preparation of a protein A immobilized column

In this study, we prepared a novel spongy monolith consisting of poly(ethylene-co-glycidyl methacrylate) (PEGM). After the monolith was packed into a column, protein A was immobilized onto the media in situ, and the affinity reaction was quantitatively examined and validated under high-throughput conditions. In an additional application of the new platforms, we immobilized the digestive enzyme pepsin onto the spongy monolith and performed online flow digestion of an antibody, and then determined the primary structure of the antibody from the peptide fragments.

As shown in Figure 4-2, a PEGM spongy monolith (PEGM-SpM) was successfully prepared with the expected morphology. In brief, the average pore size of the prepared PEGM-SpM was $\sim 10\ \mu\text{m}$, as determined by mercury porosimeter (Figure 4-3), whereas no meso-pores were detected by nitrogen-gas adsorption analysis. The PEGM-SpM was packed into a stainless-steel column by a simple method (Figure 4-1) similar to that used in our previous study. After the column was conditioned with methanol and water, a protein A solution ($1.0\ \text{mg mL}^{-1}$ in PBS) was passed through the column, and then incubated at $37\ ^\circ\text{C}$ for 16 h after both ends of the column were sealed. No morphological alteration was observed following the protein A modification (Figure 4-4).



Figure 4-2. Protein A immobilized spongy monolith (ProA-SpM). Physical appearances of Pro-SpM including primary materials, a molded item, and a packed column.

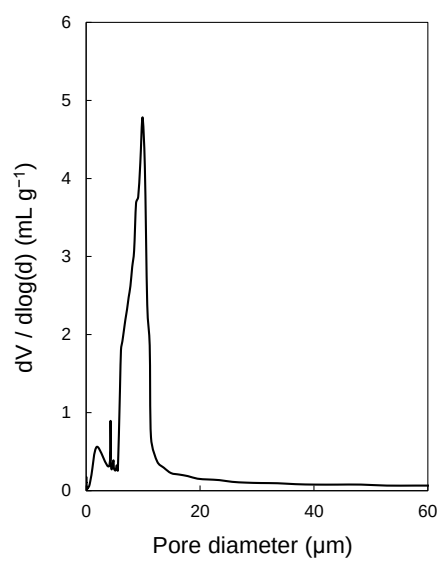


Figure 4-3. Pore distribution of a PEGM-based spongy monolith by mercury porosimetry.

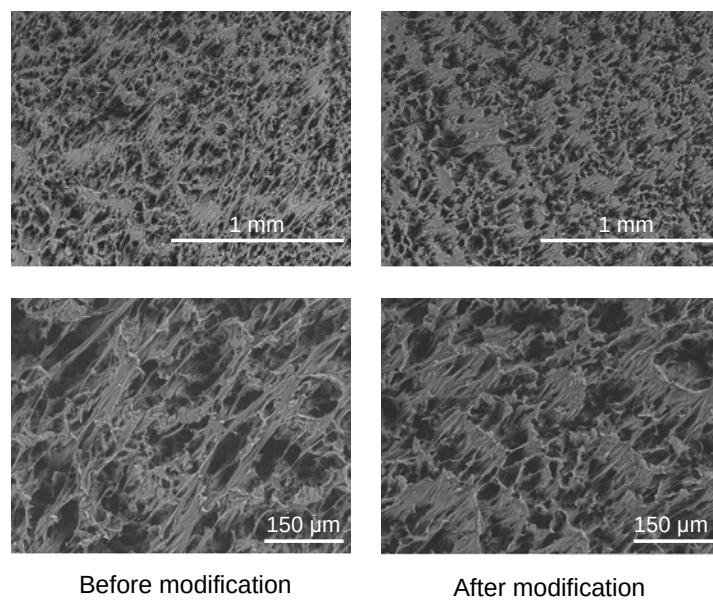


Figure 4-4. SEM images of the spongy monolith before and after immobilization with Protein A.

To confirm the effect of the modification, we then analyzed the column (ProA-SpM) by liquid chromatography (LC). As shown in Figure 4-5 (a), IgG1 was strongly retained on the original PEGM-SpM via hydrophobic interaction in a typical reversed-phase LC (RPLC) mode. On the other hand, the ProA-SpM exhibited significantly less hydrophobic interaction, resulting in faster elution of IgG1. A similar phenomenon was observed when BSA was used as a solute (Figure 4-6). These results indicated that protein A effectively covered the skeleton surface of the monolith, dramatically suppressing non-selective hydrophobicity. Next, we confirmed the affinity of the ProA-SpM via simple pH-gradient LC, which is commonly employed to evaluate protein A-immobilized columns because the interaction between protein A and IgG occurs only at pH >7. The resultant chromatograms are summarized in Figure 4-5 (b). As expected, IgG1 was selectively retained on the ProA-SpM and released by a one-step pH gradient. By contrast, BSA was quickly eluted without any retention, and PEGM-SpM adsorbed IgG1 due to its high hydrophobicity. Additionally, another IgG family member, IgG2, was also effectively separated on the ProA-SpM (Figure 4-7). In a simplified validation, we injected various amounts of IgG1 into the ProA-SpM, and found that the linear range of peak area was at least 1.0–250 µg (Figure 4-5 (c)). In addition, we evaluated the accuracy by continuous analyses (n = 6), and estimated the relative standard deviation (RSD) as 0.7%. According to these results, this simply prepared ProA-SpM column had affinity similar to that of commercially available protein A-immobilized columns.

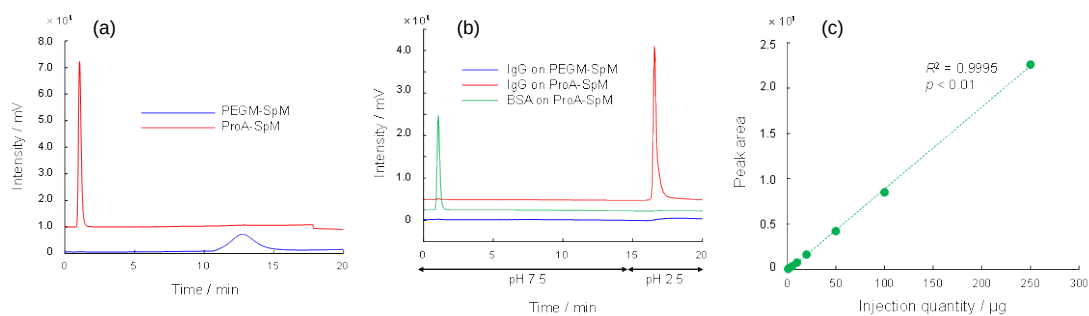


Figure 4-5. UV Chromatograms of IgG and BSA on the PEGM-SpM and ProA-SpM. (a) Reversed-phase chromatograms of IgG 1 with the PEGM-SpM and ProA-SpM. (b) Affinity separation of IgG1 by a stepwise pH gradient. (c) A relation between the injected amount of IgG1 and the peak area with the ProA-SpM.

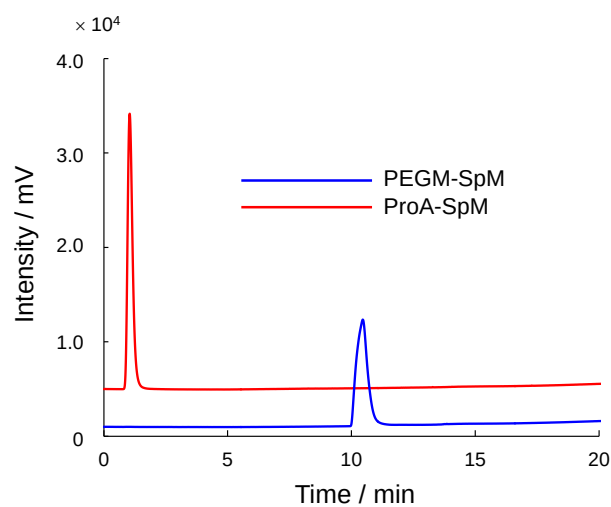


Figure 4-6. Chromatograms of BSA with PEGM-SpM or ProA-SpM.

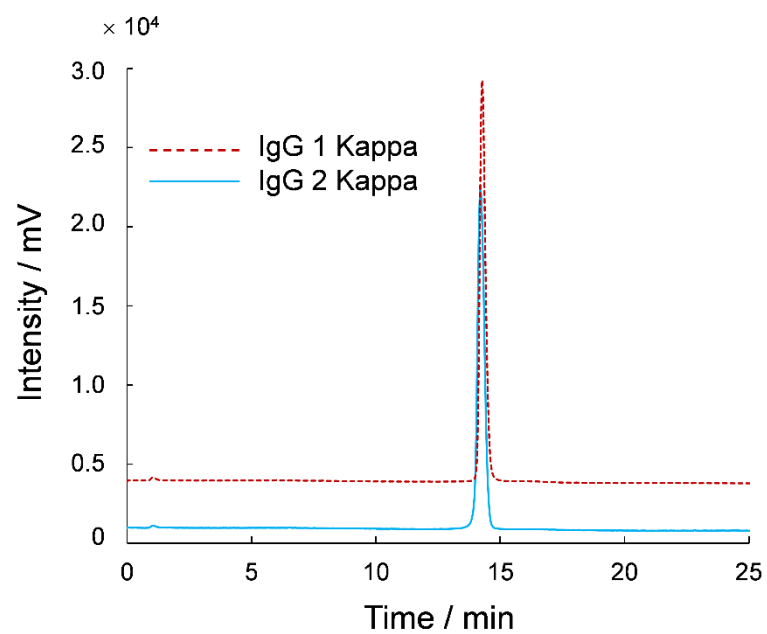


Figure 4-7. Chromatograms of IgG standards with ProA-SpM by pH gradient.

4-3-2 Adsorption capacity of Protein A

To be most useful, an affinity column must have abundant adsorption capacity for the ligand(s) of interest. To evaluate the maximum adsorption capacity due to immobilized protein A, we performed a frontal analysis, a method commonly utilized to evaluate capacity by LC⁴⁰⁻⁴¹, of both our column and a commercially available protein A-immobilized column (ProA-Column). This analysis revealed that the densities of immobilized protein A in the ProA-SpM and ProA-Column were 1.0–4.2 nmol g⁻¹ and 5–21 nmol g⁻¹, respectively (Figure 4-8). Although the density of immobilized protein A was slightly lower in the ProA-SpM, the adsorption capacity for IgG1 was comparable between the two columns (0.31 mg for the ProA-SpM and 0.32 mg for the ProA-Column). Therefore, our ProA-SpM had sufficient capacity to serve as an affinity column for the effective separation of IgG.

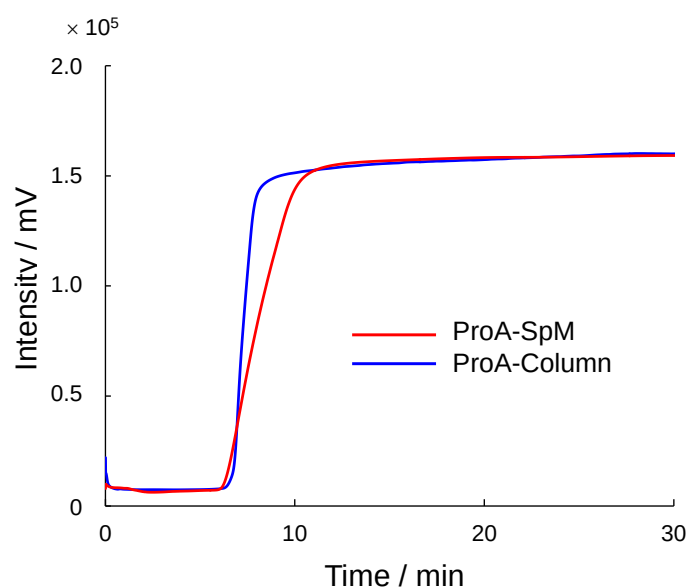


Figure 4-8. Frontal analyses using IgG standard with ProA-Column and ProA-SpM.

4-3-3 Protein A affinity chromatography at high flow rate

The most important advantage of the spongy monolith is its potential for high-throughput elution. To evaluate this feature, we carried out a similar affinity separation using IgG1 as the solute under various flow rate conditions; the results are summarized in Figure 4-9. When the ProA-Column was utilized at a higher flow rate, the flow-through fraction was presented in front of the solvent peak, as shown in Figure 4-9 (a). By contrast, the ProA-SpM allowed higher recovery, even at a high flow rate. Figure 4-9 (c) shows the chromatograms for both the ProA-Column and ProA-SpM at a flow rate of 9.0 mL min^{-1} . Obviously, the collected and flow-through peaks were completely different from each other. The backpressure and recovery of IgG on the columns at each flow rate are summarized in Figure 4-10 (a) and (b), respectively. For the ProA-SpM, both backpressure and recovery were superior to those of the ProA-Column.

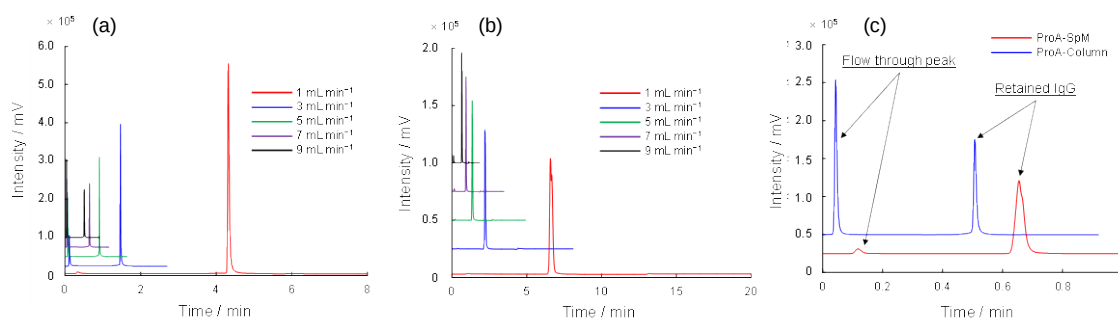


Figure 4-9. The UV chromatograms for IgG on protein A immobilized column under various flow rates. Affinity separations with a various flow rates with the ProA-Column (a) and ProA-SpM (b). (c) Rapid separation of IgG from a protein A load sample with the ProA-Column or ProA-SpM at 9.0 mL min⁻¹.

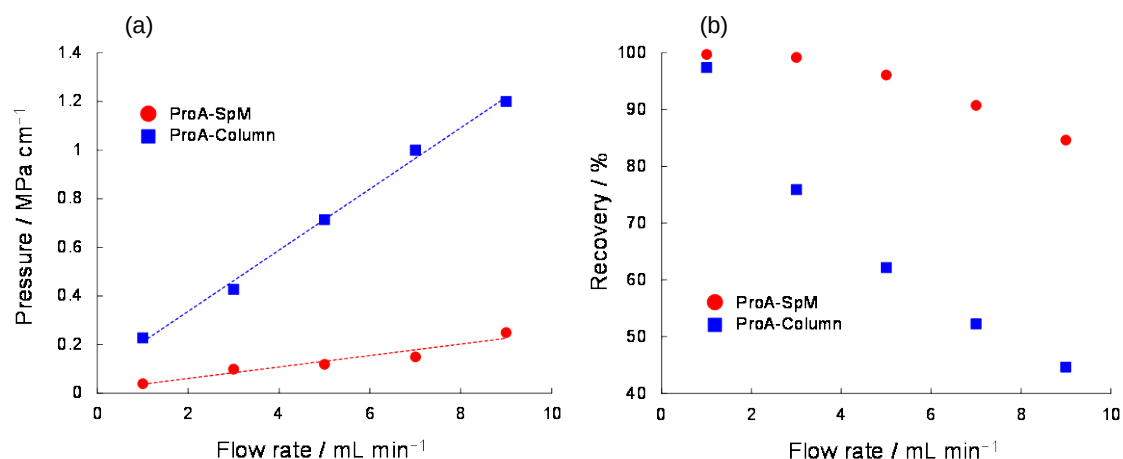


Figure 4-10. Back pressure and recovery of IgG on the ProA-Column and ProA-SpM. (a) Comparison of back pressure on LC eluted by 50 mM phosphate buffer with 150 mM NaCl as a mobile phase using the ProA-Column or ProA-SpM. (b) Comparison of the recovery for IgG with the ProA-Column or ProA-SpM, total amount was estimated by sum of the peak area among the flow though peak and IgG.

A potential reason for these significant differences, especially in recovery at a high flow rate, is that the affinity interaction between a protein-based ligand and an antibody under a higher flow rate is generally not effective in a column in which spherical and porous beads are packed, due to the lower accessibility caused by slower mass transfer. Usually, in an LC analysis, the van Deemter equation (1)⁴² is employed to determine the plate height, H , which is defined as diffusion per column length and directly contributes to the separation efficiency:

$$H = A d_p + (B D_m)/u + (C d_p^2 u)/D_m \quad (1)$$

Here, d_p , D_m , and u are the diameter of the packed particle, diffusion coefficient of the solute, and linear velocity, respectively, and A , B , and C are constants corresponding to eddy diffusion, longitudinal diffusion, and mass transfer, respectively. Under higher linear velocity, mass transfer is usually predominant, resulting in low separation efficiency. For interactions among macromolecules (e.g., protein–protein interactions), slow mass transfer may provide fewer chances for encounter; thus, most of the IgG was eluted without interaction at high flow rates on the ProA-Column. On the other hand, the spongy monolith contains only macro-size flow-through pores, and protein A should be immobilized only on the surface of the monolithic skeleton. Therefore, we anticipated that the ProA-SpM would allow effective interaction between protein A and IgG 1. Additionally, the linearity of recovery at a higher flow rate (9.0 mL min⁻¹) is similar to that at a lower flow rate (Figure 4-11). Furthermore, we investigated the ruggedness of the ProA-SpM. As a result of 100 times repeated analyses with IgG under 9.0 mL min⁻¹, the RSDs of the retention time and recovery of IgG were estimated

as 0.45% and 0.41%, respectively. Also, the recovery of IgG was kept over 99% even after washing with 0.1 M aqueous NaOH (5 times), which is commonly used as an evaluation for the ruggedness of affinity columns. These results suggested that the ProA-SpM had enough ruggedness as an affinity column. According to these results, we anticipate that the ProA-SpM could be used as a novel affinity separation medium for high-throughput purifications.

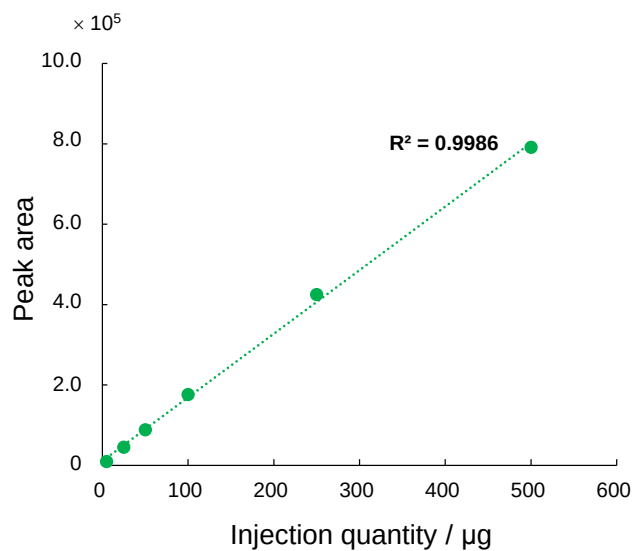


Figure 4-11. Linearity of injected IgG under rapid elution, 9.0 mL min^{-1} .

4-3-4 Fractionation and determination of IgG from cell culture

To demonstrate purification of IgG from cell culture samples, we used the ProA-SpM. Cell culture supernatant treated using typical procedures was separated with a simple pH gradient using a variety of flow rates. Then, the peak likely to contain IgG was manually fractionated, and the chromatograms of free supernatant and a standard IgG are shown in Figure 4-12 (a). To confirm the presence of IgG in the fraction, the collected sample was analyzed by authentic RPLC with time-of-flight (TOF) mass spectrometry (MS) (TOF-MS), which is usually employed for the proteins separation using a reversed-phase column with MS detection. As shown in the obtained chromatograms (Figure 4-13) generated by UV detection, both the supernatant and the first fraction contained a major peak and a few minor peaks, whereas the collected fraction clearly contained only one peak, which corresponded to the standard IgG peak. A comparison of total ion chromatograms is provided in Figure 4-12 (b). Similar to the UV chromatograms, the collected sample exhibited a clear peak without any other minor peaks. After deconvolution of the original MS results, all the peaks from the original supernatant could be assigned, and the observed MS numbers are summarized in Table 4-1. In addition, the MS spectra of each peak are described in Figure 4-14. These results indicate that the collected fraction contained the selectively separated IgG moiety from the natural cell culture sample. Finally, the concentration of IgG in the supernatant of the cell culture was estimated as 0.42 mg mL⁻¹. Thus, we successfully demonstrated affinity separation based on the protein A-IgG interaction at high throughput using the newly developed spongy monolith. The method exhibited a good reproducibility and efficient recovery over a wide range of concentrations.

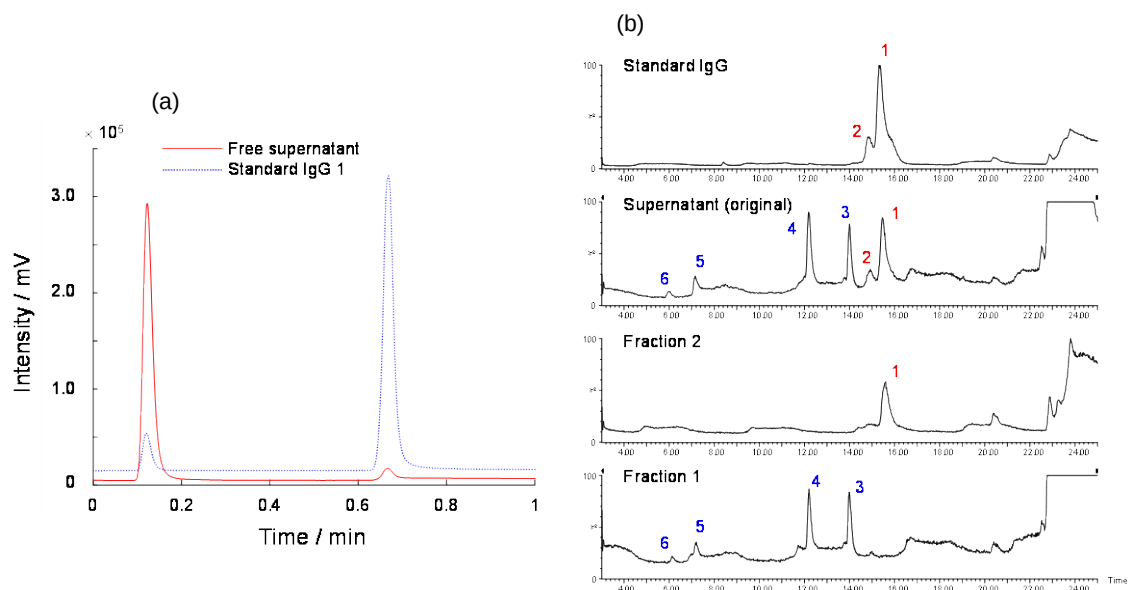


Figure 4-12. Affinity separation of cell supernatant for isolation of IgG. (a) UV chromatograms of a supernatant in a protein A load sample and a standard IgG1 with the ProA-SpM. (b) Total ion chromatograms in RPLC.

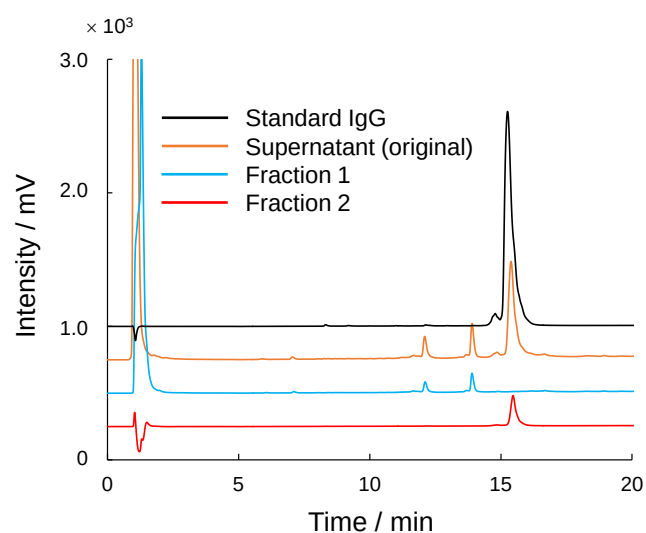
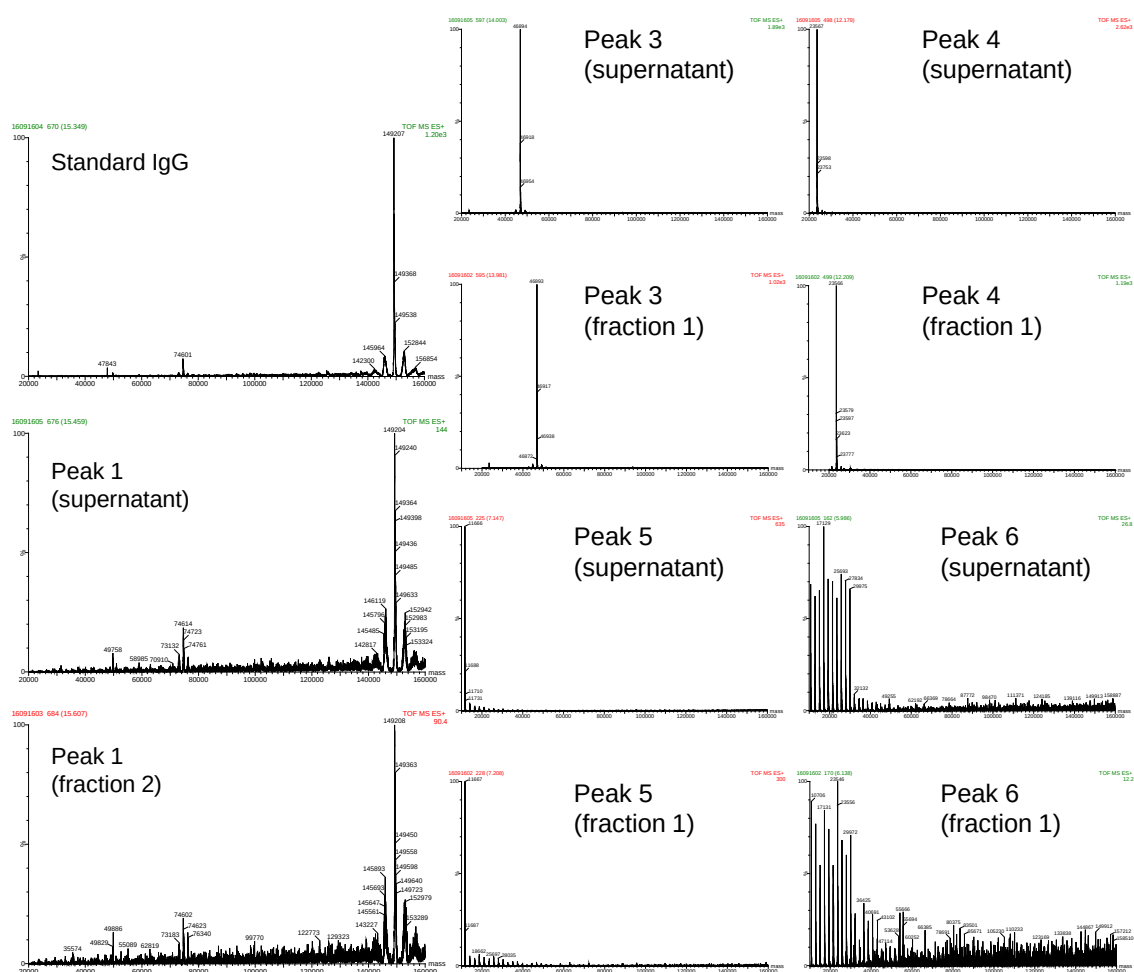


Figure 4-13. The UV chromatograms of a Protein A load sample.

Table 4-1. Peak identifications from a protein A load sample. Fractions 1 and 2 were collected from the separation (Figure. 4-12 (a)) from the front and back peaks, respectively. The peaks are corresponding to Figure. 4-12 (b). The observed mass was estimated by a deconvolution of multi ions with MaxEnt1.

Sample	Peak No.	Retention Time (min)	Observed mass (Da)
Standard IgG	1	15.35	149207
	2	14.81	23382
			101380 125840
Supernatant (original)	1	15.46	149204
	2	14.93	23384
	3	14.00	46894
	4	12.18	23567
	5	7.15	11666
	6	5.99	-
Fraction 2 (IgG fraction from ProA-SpM)	1	15.61	149208
Fraction 1 (flow through fraction from ProA-SpM)	3	13.99	46893
	4	12.21	23566
	5	7.21	11677
	6	6.14	-



4-3-5 Online digestion of an antibody by the Pep-SpM and LC-MS analysis for peptide mapping

As mentioned above, the newly developed spongy monolith was suitable for a high-throughput affinity reaction. Because the immobilization of the proteins was based on a simple reaction with epoxy groups in the monolith, we believe that this material could be used for a variety of protein-based reactions. To explore this idea further, we carried out high-throughput online digestion using a digestive enzyme, pepsin. The immobilization of pepsin was performed successfully by a method similar to the one used for protein A. Pepsin is an aspartic protease that cleaves peptide bonds between hydrophobic and preferably aromatic amino acids, such as phenylalanine, tryptophan, and tyrosine. In this evaluation, an antibody solution was introduced into the pepsin-immobilized spongy monolith (Pep-SpM), and the eluted fraction was analyzed by LC-MS. For comparison, samples in a simple solution containing pepsin and the antibody were also analyzed to confirm the cleaved peptides. The UV chromatograms are summarized in Figure 4-15. As expected, longer reaction in solution yielded larger peptide fragments. On the contrary, in online digestion with the Pep-SpM, the peptide fragments were much larger, even though a faster flow rate (100 mL h⁻¹) was employed. When a slower flow rate (10 mL h⁻¹) was used, the detected peaks were almost the same as those in solution samples treated for 150 min. The numbers of peptides detected in LC-MS, as a function of the number of amino acids and elution time, are summarized in Figure 4-16. Both figures demonstrate that a slower flow allowed for more extensive digestion. These results clearly showed that effective cleavage occurred in the Pep-SpM. To confirm the sequence of each peptide, a quantitative analysis was also carried out. The theoretical alignment of amino acids

in the antibody and the theoretical pepsin digestion fragments (digestion sites: N terminal, F, I, M, Y, W, V; C terminal, C, D, E, F, L, M, T, W, Y) were compared against results generated by the PepFinder 2.0 based on the LC–MS data. All assignment data are summarized in Table 4-2. Coverage for the primary amino acid alignment, as determined from those results, is shown in Table 4-3. In both elution flows, all 213 residues in the light chain were detected. Additionally, coverage of the heavy chain corresponded to the flow speed; i.e., a slower flow provided higher coverage. These results also supported the idea that efficient online digestion occurred in the Pep-SpM. Finally, we showed that the reproducibility of the online digestion was satisfactory at both flow rates, as shown in Figure 4-17 and Figure 4-18. All these results indicate that the spongy monolith can be used for the effective online digestion.

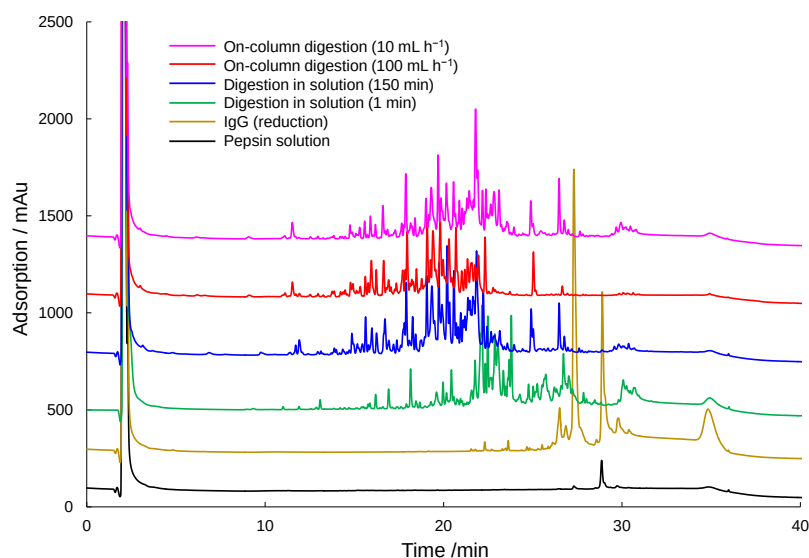


Figure 4-15. Online digestion of a recombinant human IgG1 antibody with the Pep-SpM. UV chromatograms of the digested antibody in solution or online with the Pep-SpM.

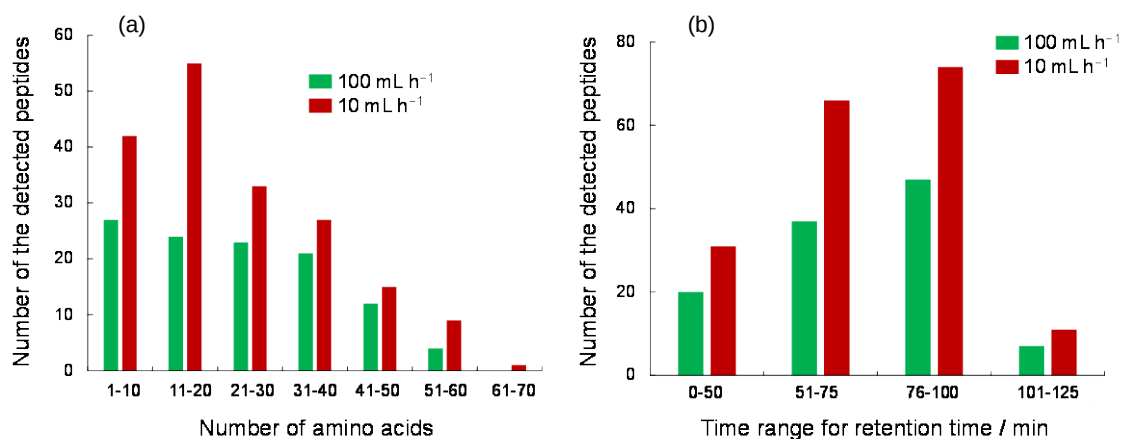


Figure 4-16. Detected peptides by the online digestion of an antibody by the Pep-SpM. (a) The number of the assigned peptides against the number of the composed amino acids. (b) The number of the assigned peptides against the elution time in LC separation.

Table 4-2. Assigned peptides by online digestion of a recombinant human IgG1 antibody with Pep-SpM. These peptides were assigned by PepFinder 2.0. based on all the peptides detected by LC-MS containing the origin (light or heavy chain). The number of amino acids of each terminal and the length are summarized. Here, the number of amino acid was assigned that the number 1 is first amino acid from N-terminal.

Flow Rate 100 mL h ⁻¹							Flow Rate 10 mL h ⁻¹						
Retention Time (min)	Identified peptide						Retention Time (min)	Identified peptide					
	Chain	N-terminal		C-terminal		Length		Chain	N-terminal		C-terminal		Length
16.2563	L	Y	87	Q	90	4	16.2486	L	Y	87	Q	90	4
25.186	L	K	125	V	131	7	25.1773	L	K	125	V	131	7
25.4079	H	Q	177	L	181	5	25.3485	H	Q	177	L	181	5
30.0255	L	Y	91	Y	94	4	26.2261	L	S	161	T	171	11
30.2895	L	F	83	T	85	3	30.024	L	Y	91	Y	94	4
30.6824	H	D	401	F	406	6	30.2692	L	F	83	T	85	3

31.4681	L	Y	87	S	93	7	30.625	H	D	401	F	406	6
31.884	L	Y	86	Q	90	5	31.4988	L	Y	87	S	93	7
35.2608	H	I	379	E	382	4	31.8938	L	Y	86	Q	90	5
37.6997	L	T	5	L	11	7	35.2807	H	I	379	E	382	4
37.7475	L	K	148	E	160	13	37.7173	L	T	5	L	11	7
38.1499	L	A	84	Q	90	7	37.9938	L	A	84	Y	87	4
42.2943	L	Y	86	S	93	8	38.3718	H	Y	182	S	186	5
43.1654	L	D	1	M	4	4	39.3537	L	Q	123	V	131	9
44.0371	L	F	83	Y	86	4	41.7937	H	I	255	T	262	8
44.7465	L	Y	87	Y	94	8	42.3163	L	Y	86	S	93	8
45.4826	L	V	132	L	134	3	43.8953	L	I	75	D	82	8
45.5874	H	V	264	D	272	9	44.0758	L	F	83	Y	86	4
48.0523	L	Y	36	L	46	11	44.4621	H	V	5	S	17	13
48.7965	H	I	255	C	263	9	44.487	H	T	69	L	79	11
50.6965	L	L	47	Y	49	3	44.7377	H	Y	94	M	103	10

51.5222	H	V	371	D	378	8	44.7598	L	Y	87	Y	94	8
52.798	L	T	179	E	194	16	45.4787	L	V	132	L	134	3
53.3235	L	F	83	Q	90	8	45.6127	H	V	264	D	272	9
54.9483	L	F	83	Y	87	5	46.1097	H	E	1	L	4	4
55.1311	L	L	135	E	142	8	46.2323	H	N	84	V	93	10
58.0703	H	I	255	V	264	10	46.2734	L	S	181	E	194	14
59.2466	H	L	370	D	378	9	48.0088	L	Y	36	L	46	11
59.4285	L	D	1	L	11	11	48.2478	L	T	74	D	82	9
60.5378	L	V	195	C	213	19	48.6997	H	I	255	C	263	9
62.1063	L	A	143	E	160	18	48.8763	L	S	161	T	177	17
62.1547	L	K	24	W	35	12	50.722	L	L	47	Y	49	3
62.5981	L	D	1	D	17	17	51.4312	H	V	371	D	378	8
63.453	L	K	125	L	134	10	51.8283	L	S	12	T	22	11
63.6346	H	V	429	G	448	20	51.8554	L	A	143	W	147	5
64.09	H	T	413	F	425	13	52.3511	H	E	359	L	367	9

64.5711	L	S	161	L	178	18	52.6215	H	E	1	L	4	4
65.308	L	R	95	V	114	20	52.769	L	T	179	E	194	16
67.1361	L	T	72	D	82	11	52.9656	H	V	188	T	199	12
67.854	L	V	33	L	46	14	54.1503	L	F	71	T	74	4
68.1168	L	Y	94	V	114	21	55.0588	L	W	50	D	70	21
68.4145	H	W	383	L	400	18	55.0588	H	M	254	C	263	10
68.6245	L	S	181	C	213	33	55.2225	H	Y	182	V	187	6
69.0118	H	F	407	L	412	6	55.496	L	V	131	L	134	4
70.125	L	Y	91	V	114	24	56.5512	H	V	187	T	199	13
70.7921	L	T	179	C	213	35	58.0617	H	I	379	N	392	14
71.1183	H	S	426	G	448	23	58.0617	H	I	255	V	264	10
71.6	H	V	371	E	382	12	58.8873	L	S	12	C	23	12
72.026	L	L	47	D	70	24	59.2177	H	L	370	D	378	9
72.2769	L	K	24	L	46	23	59.3608	H	H	431	G	448	18
72.6228	L	Y	87	V	114	28	59.3608	L	D	1	L	11	11

73.8955	H	W	383	F	406	24	60.5088	L	V	195	C	213	19
73.9915	L	C	23	L	46	24	60.7397	L	L	178	E	194	17
74.2131	L	L	178	C	213	36	61.4625	L	E	194	C	213	20
74.6945	L	R	95	F	115	21	61.6085	H	V	429	K	449	21
74.8552	L	D	1	T	22	22	62.0635	L	A	143	E	160	18
74.8943	L	Y	86	V	114	29	63.689	H	V	429	G	448	20
75.0746	L	L	135	E	160	26	63.689	L	C	23	W	35	13
75.848	L	A	84	V	114	31	64.0969	H	T	413	F	425	13
75.989	L	S	161	E	194	34	64.2954	H	L	237	F	243	7
77.028	L	Y	94	F	115	22	64.5605	L	S	161	L	178	18
77.2946	H	Y	182	T	199	18	64.9275	H	T	413	S	428	16
78.4786	L	Y	91	F	115	25	65.261	L	R	95	V	114	20
79.0127	L	D	1	C	23	23	65.5468	H	E	1	S	17	17
79.8161	H	I	379	L	400	22	65.5468	H	T	309	E	320	12
79.9533	L	L	47	F	71	25	66.9422	H	V	371	A	380	10

80.2163	L	Y	87	F	115	29	67.0433	L	T	72	D	82	11
81.6356	L	F	71	D	82	12	67.0433	H	V	148	T	157	10
81.7403	L	V	132	E	142	11	67.1793	H	S	428	G	448	21
82.128	L	Y	86	F	115	30	67.8288	L	V	33	L	46	14
82.358	H	I	379	F	406	28	68.0648	L	Y	94	V	114	21
82.8493	L	S	161	C	213	53	68.3037	H	W	383	L	400	18
83.4791	L	S	170	C	213	44	68.591	L	S	181	C	213	33
83.5848	L	T	72	F	83	12	68.591	L	A	32	L	46	15
83.5848	L	S	12	L	46	35	68.9706	H	F	407	L	412	6
83.813	H	F	407	F	425	19	69.0472	H	S	426	K	449	24
84.1388	L	Y	36	D	70	35	69.1245	H	T	368	D	378	11
84.656	L	L	47	T	74	28	69.2903	H	Y	200	L	236	37
85.5998	L	W	50	D	82	33	69.697	H	Y	393	F	406	14
85.8136	H	Y	409	K	449	41	70.0908	L	Y	91	V	114	24
86.1053	H	T	413	G	448	36	70.7877	L	T	179	C	213	35

86.6843	L	F	83	F	115	33	71.0471	H	S	426	G	448	23
87.4643	H	Y	409	G	448	40	71.4985	H	Y	409	F	425	17
88.6947	L	V	132	E	160	29	71.6321	H	V	371	E	382	12
89.3583	L	R	95	V	131	37	71.8381	H	L	147	T	157	11
90.4489	L	Y	94	V	131	38	71.9752	L	L	47	D	70	24
90.8482	L	Y	91	V	131	41	72.4119	H	E	359	L	370	12
91.0745	H	F	407	K	449	43	72.6163	L	Y	87	V	114	28
91.4385	L	D	1	L	46	46	72.6163	H	I	104	L	114	11
91.6291	L	Y	87	V	131	45	72.7808	H	V	381	L	400	20
92.645	L	F	83	L	124	42	73.914	H	W	383	F	406	24
92.645	H	V	371	F	406	36	73.9905	L	C	23	L	46	24
93.2428	L	L	47	D	82	36	74.2305	L	L	178	C	213	36
94.6313	L	W	50	F	83	34	74.6902	L	R	95	F	115	21
94.6313	L	F	71	F	83	13	74.8489	L	L	135	W	147	13
94.6313	H	V	158	L	176	19	74.8985	L	Y	86	V	114	29

95.4596	L	S	12	D	70	59	74.8985	L	D	1	T	22	22
96.3011	L	W	50	Y	86	37	75.8386	L	Y	172	E	194	23
96.7856	L	F	71	Y	86	16	75.8798	L	A	84	V	114	31
96.8625	L	Y	36	D	82	47	76.0312	L	S	161	E	194	34
98.8544	L	R	95	L	134	40	76.3668	H	L	370	E	382	13
99.4037	L	L	47	T	85	39	76.4683	H	E	1	L	18	18
99.5636	L	Y	94	L	134	41	77.0042	L	Y	94	F	115	22
99.7238	L	Y	91	L	134	44	77.0748	H	D	401	L	408	8
100.019	L	Y	87	L	134	48	77.1763	H	T	166	L	176	11
100.229	H	Y	200	M	254	55	77.279	H	Y	182	T	199	18
100.66	L	L	47	F	83	37	77.279	H	P	191	T	199	9
101.745	L	L	47	Y	86	40	77.6782	L	S	161	L	180	20
102.219	L	Y	36	F	83	48	77.7542	L	L	135	T	177	43
102.878	L	Y	36	Y	86	51	78.4331	L	Y	91	F	115	25
104.821	H	V	148	L	176	29	78.498	H	Y	200	V	242	43

							78.6612	L	L	47	F	62	16
							79.0015	L	D	1	C	23	23
							79.1568	H	T	309	E	335	27
							79.2615	H	V	115	L	144	30
							79.5169	H	V	115	C	146	32
							79.8138	H	I	379	L	400	22
							79.9655	L	L	47	F	71	25
							80.2291	L	Y	87	F	115	29
							80.8587	H	T	309	V	350	42
							81.2565	H	Y	182	Y	200	19
							81.4668	H	V	264	W	279	16
							81.549	H	Y	182	L	195	14
							81.7878	L	V	132	E	142	11
							82.1408	L	Y	86	F	115	30
							82.3517	H	I	379	F	406	28

							82.5123	L	S	167	C	213	47
							82.8535	L	S	161	C	213	53
							83.243	H	F	407	S	428	22
							83.5777	L	S	12	L	46	35
							83.5777	L	T	72	F	83	12
							83.8712	H	V	264	G	283	20
							83.8712	H	F	407	F	425	19
							84.0317	L	Y	36	D	70	35
							84.3237	H	T	413	K	449	37
							84.6498	L	L	47	T	74	28
							85.0453	H	T	309	C	369	61
							85.0453	H	T	309	L	367	59
							85.2045	L	L	135	E	194	60
							85.5982	L	W	50	D	82	33
							85.7765	H	Y	409	K	449	41

							86.0538	H	T	413	G	448	36
							87.0462	H	Y	200	F	243	44
							87.2253	H	Y	182	L	236	55
							87.4913	H	Y	409	G	448	40
							87.7194	L	L	47	L	73	27
							88.5248	L	T	5	L	46	42
							88.5248	L	Y	36	F	71	36
							88.687	L	V	132	E	160	29
							88.9361	H	V	115	L	147	33
							89.2455	L	R	95	V	131	37
							90.3494	L	Y	94	V	131	38
							90.8672	L	Y	91	V	131	41
							91.059	H	F	407	K	449	43
							91.374	L	D	1	L	46	46
							91.5013	L	Y	87	V	131	45

							92.2118	H	V	371	L	400	30
							92.2118	L	V	132	W	147	16
							92.637	H	V	371	F	406	36
							93.1772	L	L	47	D	82	36
							94.5832	L	W	50	F	83	34
							94.5832	H	V	158	L	176	19
							94.5832	L	F	71	F	83	13
							94.7828	H	L	370	F	406	37
							95.3685	L	S	12	D	70	59
							96.8033	L	Y	36	D	82	47
							97.4023	H	V	158	L	181	24
							98.197	H	Y	200	L	253	54
							98.7978	L	R	95	L	134	40
							99.2843	H	N	203	M	254	52
							99.479	L	Y	94	L	134	41

							100.058	H	Y	200	M	254	55
							100.639	L	L	47	F	83	37
							102.965	H	V	115	T	157	43
							104.781	H	V	148	L	176	29
							105.764	H	V	148	L	181	34
							106.385	H	L	147	L	176	30
							107.125	H	L	147	L	181	35
							108.022	H	V	158	T	199	42
							108.586	H	G	145	L	176	32
							109.074	H	G	145	L	181	37
							112.548	H	V	148	T	199	52

Table 4-3. Identified amino acids from a recombinant human IgG1 antibody using the Pep-SpM. The ratio of the detected amino acids was estimated from the all the results of peptides mapping by PepFinder 2.0.

	100 mL h⁻¹	10 mL h⁻¹
Light chain (213 residues)	100% (213/213)	100% (213/213)
Heavy chain (449 residues)	45.7% (205/449)	82.4% (370/449)

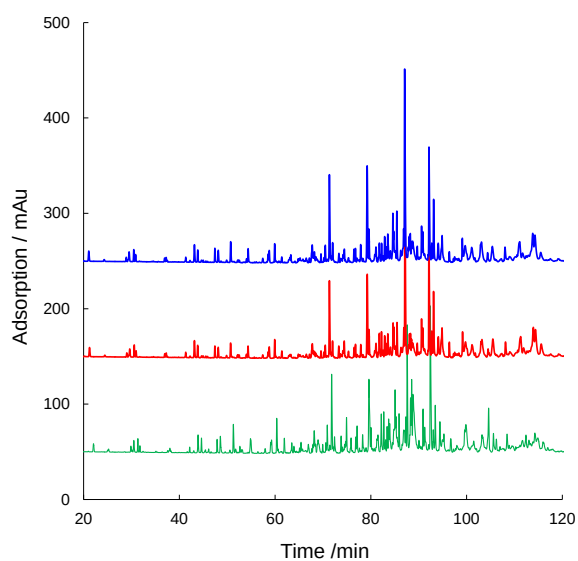


Figure 4-17. The UV chromatograms for repeatability of online digestion with the Pep-SpM under 10 mL h⁻¹ as flow rate.

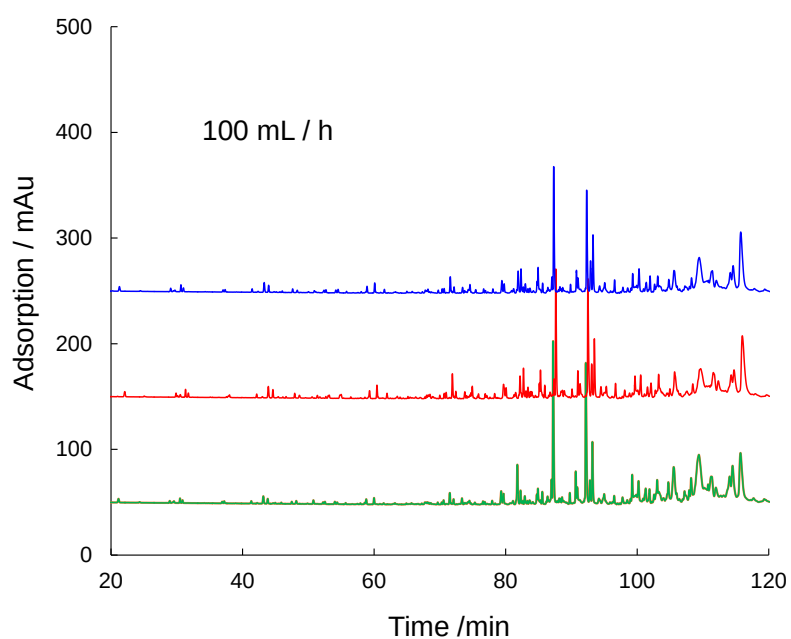


Figure 4-18. The UV chromatograms for repeatability of online digestion with Pep-SpM under 100 mL h⁻¹ as flow rate.

4-4 Conclusions

In summary, we proposed a new platform for protein-based affinity reaction. A spongy monolith containing epoxy groups was effectively used in affinity separation with protein A and digestion with pepsin. Both results demonstrated the utility of the new platform for rapid-flow affinity reactions. We believe that this new platform will be useful for variety of protein-based reactions with rapid flow rates and low costs. Additionally, the platform can be easily scaled up, and we anticipate that future efforts will contribute to purification of antibody-based medicines at the plant level.

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Chapter 5

Tunable Separations Based on a Molecular Size Effect for Biomolecules by Poly(ethylene glycol) Gel-based Capillary Electrophoresis

5-1 Introduction

Gel electrophoresis (GE) is one of powerful tools for the efficient separation of biomolecules, such as polysaccharides, nucleic acids, and proteins. Therefore, a variety of GE methods have been widely employed for the separations in the field of biochemistry, medical science, pharmacology, and food science.¹⁻¹² In most cases, a simple molecular sieving effect is employed for these separations, so that we have a number of possibilities for using separation media corresponding to the targeting compounds. In fact, a great number of applications using GE have been reported for the separations of polysaccharides¹³⁻¹⁵ and proteins¹⁶⁻²¹, especially the applications to DNAs separations have been widely examined.²²⁻²⁷ Furthermore, the formats of electrophoresis are not only slab gels and capillary gel electrophoresis (CGE) but microchips in recent researches.²⁸⁻³¹ In general, agarose gels and polyacrylamide (PAA) gels are usually utilized for GE.³²⁻³³ The agarose gel can be easily prepared and allows the effective separation of DNAs in the range of 0.1–60 k base pair (bp) by

controlling the concentration of agarose. However, the agarose gel is not suitable for the separation of small size differences because of its larger pores. On the other hand, the pore sizes of a PAA gel are controllable, so that the smaller DNAs can be separated. Meanwhile, the range of the suitable molecular size is limited in the PAA gels. Additionally, the toxicity of the acrylamide monomer and the non-specific interactions by amide groups toward biomolecules are also problematic in using PAA.³⁴ Instead of these gels, poly(ethylene glycol) (PEG) has attracted attention as another separation medium in GE. As well known, PEG shows several advantages, including higher biocompatibility, lower non-specific interaction, and low toxicity of the monomers. In addition, a variety of PEG derivatives having several ethylene oxide (EO) units are commercially available. Therefore, we expected that the PEG-based hydrogels can be useful for the separation medium in GE.³⁵⁻³⁶ As previous studies regarding the PEG-based separation in GE, X. Dou et al. reported the separation of RNA fragments ranged from 100 to 10,000 nt in PEG and polyethylene oxide (PEO) solutions with different molecular weight and different concentration in capillary electrophoresis.³⁷ Furthermore, T. Sakai et al. reported unique PEG-based separations by CGE using tetra-PEG, and reported the physical and chemical properties of the PEG gels related to the separation behavior based on the molecular sieving effect.³⁸⁻⁴² Similar to these interesting results, we also reported the PEG-based hydrogels using PEG dimethacrylate (PEGDMA) as a crosslinker, and applications to the responsible swelling/shrinking gel⁴³, the protein imprinting⁴⁴⁻⁴⁵, and the tunable molecular separations in GE.⁴⁶ Despite of these studies regarding PEG-based media in GE, the fundamental evaluations concerning the relations between the concentrations of the monomers contributing the

crude density of the polymer network and the molecular sieving effect had never been discussed. In this study, we aim to develop universal media for the efficient separation based on the molecular size in CGE: a variety of PEG-based hydrogels were prepared with PEGDMA by changing the concentration and EO unit in a capillary to control the polymer network. The separable ranges of the molecular weight for glucose and DNA ladders were evaluated with the prepared capillaries by CGE. Additionally, the separation of sugars carved out from monoclonal antibodies (mAbs) was demonstrated as a practical application in the CGE analysis.

5-2 Experimental Section

5-2-1 Materials and reagents

Methanol of the HPLC grade, acetic acid, sodiumcyanoborohydride (NaBH_3CN), tetrahydrofuran (THF), tris(hydroxymethyl)aminomethane (Tris), boric acid, sodiumhydroxide (NaOH), hydrochloric acid (HCl), acrylamide, 2,2'-azobis-[2-(2-imidazolin-2-yl)propane] (AIZP), N,N'-methylenebisacrylamide (MBAC), and ethylenediaminetetraaceticacid (EDTA) were purchased from Nacalai Tesque (Kyoto, Japan), 3-(trimethoxysilyl)propylmethacrylate (γ -MAPS) from Tokyo Chemical Industry (Tokyo, Japan), 2,2-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (AIYP), ammonium peroxodisulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), D(+)-glucose, maltose monohydrate, and maltoheptaose from Wako Pure Chemical Industries (Osaka, Japan), PEGDMA (9G, 14G, and 23G; M_w = 536, 736, and 1136, respectively) and glucose oligomer from Shin-Nakamura Chemical (Wakayama, Japan), YOYO-1 and DNA ladder from Thermo Fisher Scientific K. K. (Yokohama, Japan), 9-aminopyrene-1,4,6-trisulfonic acid (APTS) from Sigma-Aldrich Japan (Tokyo, Japan). Deionized water was obtained from a Milli-Q Direct-Q 3UV system (Merck Millipore, Tokyo, Japan). A fused-silica capillary was purchased from Polymicro Technologies (Phoenix, AZ, USA).

5-2-2 Instruments

CE analyses were carried out by a P/ACE MDQ (Beckman Coulter, Fullerton, CA, USA) with a laser-induced fluorescence (LIF) 488 nm Laser Module. Measurements

of pH of all the solutions were carried out by an F-52 pH meter (Horiba, Kyoto, Japan). IX71 (Olympus, Tokyo, Japan) and Eppendorf Thermomixer (Eppendorf AG, Hamburg, Germany) were used as a fluorescence microscope and a mixer, respectively.

5-2-3 Preparation of gel capillaries

A fused silica capillary (50 cm × 50 μm i.d.) was flushed with 1.0 M aqueous NaOH for 1 h, water for 5 min, 1.0 M aqueous HCl for 2 h, and methanol for 15 min by a syringe pump followed by N₂ gas. Then, the capillary was reacted with 50 vol% γ-MAPS in methanol at 40 °C for 16 h by flushing with a syringe pump. Finally, the reacted capillary was washed with methanol and dried, and then a vinyl-modified capillary was obtained. To prepare the gel capillaries with PEGDMA or PAA, solutions for diluting monomers were prepared. An 89 mM aqueous Tris-boric acid solution was prepared (1 × TB, pH 8.64). EDTA was diluted with 1 × TB to 2 mM (1 × TBE, pH 8.31). A PAA solution was prepared with acrylamide/MBAC at 40%T (total monomer concentration) and 5%C (weight percentage of crosslinker). Each pre-polymerization solution shown in Table 5-1 or Table 5-2 was filled into the vinyl-modified capillary and sealed tightly with Teflon tape in the end of the capillary. The capillaries were left for 16 h at 65 °C in water bath with AIZP or at room temperature with APS/TEMED. Then, both the end of the capillary were cut for 5 cm length to remove the void and fix to the CE instrument.

Table 5-1. Composition of PEGDMA-based hydrogels.

Crosslinker (9G, 14G, or 23G) (mg)	Initiator	Solvents ^a (1×TB or 1×TBE) (mL)	Ratio of crosslinker (volume%)
57		2.95	1.7
68		2.94	2.0
79		2.93	2.4
159	10% APS aq., 57 µL	2.86	4.7
238	TEMED, 11.4 µL	2.78	7.1
318	or	2.71	9.4
398	AIYP, 5.7 mg	2.64	11.8
477		2.57	14.2
557		2.49	16.5
637		2.42	18.9

^a TB buffer and TBE buffer were utilized for the capillaries to be analyzed glucose ladder and DNA, respectively.

Table 5-2. Composition of PAA-based hydrogels.

	PAA solution (5%C) (µL)	Initiator	Solvents ^a (1×TB or 1×TBE) (mL)
3%T	225		2.78
5%T	375		2.63
10%T	750	10% APS aq., 15 µL	2.25
15%T	1125	TEMED, 3 µL	1.88
20%T	1500		1.50

^a TB buffer and TBE buffer were utilized for the capillaries to be analyzed glucose ladder and DNA, respectively.

5-2-4 Sample preparations

APTS labeled glucose ladder (G1~G20) was prepared as follows: 2.5% aqueous APTS 3.0 μL , 100 mM aqueous glucose ladder 3.0 μL , acetic acid 2.25 μL , and water 6.75 μL were mixed in a polypropylene tube and stirred. After adding 1.0 M NaBH_3CN in THF 5.0 μL , the mixture was reacted at 55 $^{\circ}\text{C}$ in water bath for 2 h. Then, the mixture was diluted with a 1 $\times$ TB buffer to 100 μL . By the same procedures, glucose, maltose, maltopentaose, and maltoheptaose were labeled by APTS. YOYO-1 labeled DNA ladder (100~1500, 2072 bp, 1 $\mu\text{g}/\mu\text{L}$) was prepared as follows: DNA ladder 10 μL and 100 μM YOYO-1 20 μL were mixed in a polypropylene tube and left at room temperature for 1 h. Then, the mixture was diluted for 100 times with a 1 $\times$ TBE buffer. Each DNA containing 100, 500, 1000, and 1500 bp was labeled by the same procedures.

5-2-5 Preparation of the APTS labeled sugars carved out from mAbs

For real sample analyses, sugars carved out from mAbs were prepared. The APTS labeled sugars from mAbs-A and -B were separated by CGE with a PEGDMA-based gel capillary. Each monoclonal antibodies, mAbs-A and mAbs-B of 2.0 mg mL^{-1} were treated with the enzyme solution containing *N*-glycanase. After incubating at 50 $^{\circ}\text{C}$ for 15 min, the finishing reagent was added to the solutions, and then the supernatant was dried up after centrifugation. The dried samples were reacted with a 9-aminopyrene-1,4,6-trisulfonic acid (APTS) for fluorescent labeling.

5-2-6 Conditions of CGE

CGE analyses were carried out by a P/ACE MDQ with the capillaries (total length 40 cm, effective length 10 cm, 50 μ m i.d.), injection of 4 kV for 10 s, applied voltage of 4 kV, and detection of LIF (ex 488 nm, em 520 nm). The buffered solutions for glucose and DNA ladders were a 1 $\times$ TB buffer (pH 8.64) and 1 $\times$ TBE buffer (pH 8.31), respectively.

5-3 Results and Discussion

5-3-1 *Effect of polymerization*

In this study, we employed the binary polymerization procedure to prepare gel capillaries. Firstly, a thermal polymerization using AIYP as a radical initiator at 60 °C was examined. As a result, an electric current was not observed in most of the prepared capillaries by the thermal polymerization in typical electrophoresis even though a high voltage was applied (4.0 kV to 8.0 kV). On the other hand, an effective current (1.8 μ A to 2.2 μ A at 4.0 kV) was observed in the gel capillaries prepared by a redox polymerization at ambient temperature. According to these results, we expected that the shrinkage during the polymerization affected to the generation of the current. In fact, as a result of the observation by the optical microscope, a number of voids were found around the edge of the capillary (see Figure 5-1) although the continuous gel formation was confirmed in the center of the capillary. These discontinuous gel formations were assumed to be constructed by the shrinkage during the polymerization and prevented the generation of the current. To confirm the gel shrinkage, the bulk polymers were prepared by both the polymerization methods using a few PEGDMA having different EO chains. The photos of the gels are shown in Figure 5-2. These photos clearly show that the shrunk and white turbidity polymers were settled in the tube prepared by the thermal reaction, whereas the transpicious polymers observed without any settling in the redox reaction. In general, the polymerization rate was much higher in the thermal radical polymerization than that of the redox one at lower temperature, so that the polymers were precipitated easily in the thermal reaction.

Especially, when we used PEGDMA with a shorter EO chain or 9G, the obvious precipitation was observed by the hydrophobic interaction at higher temperature because the hydrophobicity of PEGDMAs depends on the length of the EO chain. Similar results were confirmed in our previous study.⁴⁷ According to these results, we employed the redox polymerization using APS and TEMED to prepare the PEGDMA-based gel capillaries.

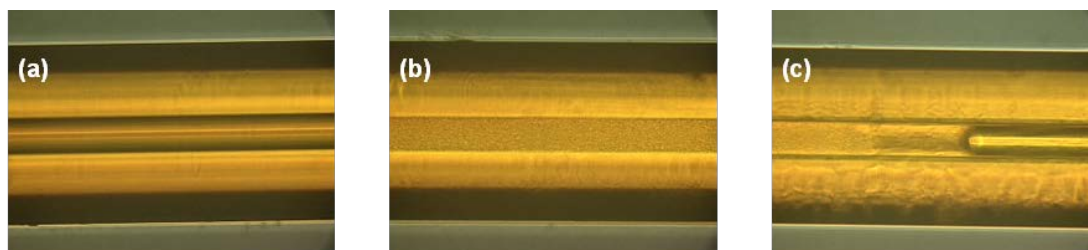


Figure 5-1. Observation of the gel capillary by a phase contrast microscope. (a) an original open tubular capillary, (b) center and (c) edge of the capillary filling with the PEGDMA gel prepared by thermal radical polymerization using AIYP.

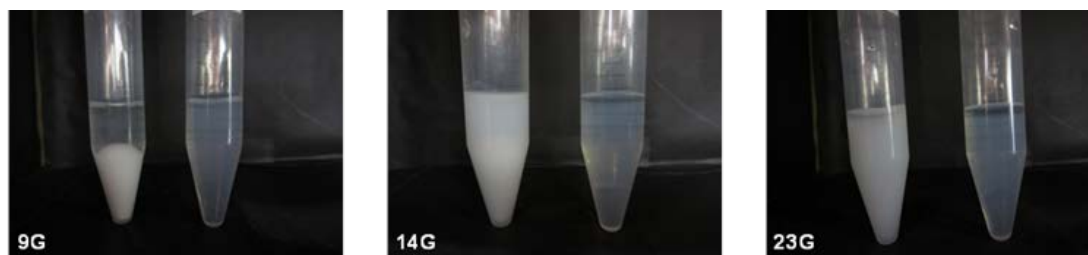


Figure 5-2. Photos of the prepared PEGDMA gels. Left, polymerization by thermal radical polymerization using AIYP; right, polymerization by redox polymerization using APS/TEMED.

5-3-2 Effect of the concentration and EO unit in PEGDMA gel

As well as PAA-based gels, the separations based on the molecular size are controllable by the alteration of the tuning gel network. To know the range of the separable molecular weight in the gel capillaries by CGE, a variety of the gel capillaries using PEGDMA with the multiple concentrations and EO units were prepared. At first, the limitation of the low and high concentrations of PEGDMAs were investigated. At a PEGDMA concentration lower than around 2 vol%, the gelation was not completed, so that the effect of the electroosmotic flow (EOF) was dominant and no separation of the glucose ladder was achieved. On the contrary, at a concentration higher than 17 vol%, the polymerization was too fast to fulfill the pre-polymerization solution into the capillary. Then, the gel capillaries, which could be confirmed the migration of glucose ladder, were evaluated to know the separation based on the molecular size by CGE.

The electrophoretic mobility (μ_e) of the glucose ladder against the molecular weight in each gel capillary is summarized in Figure 5-3. In every capillaries using different EO units, μ_e of all the glucose oligomers became smaller and the differences of μ_e among G1 to G20 were also decreased, as increasing the amount of PEGDMA. The reason of these results were dependent on the crosslinking density; briefly the lower crosslinking density provided the appropriate interference against the mobility of glucose oligomers with a higher molecular weight. On the other hand, the higher crosslinking density allowed the interference only for the lower molecular weight and decreasing the mobility of all the glucose ladders. Accordingly, the differences of μ_e among the lower molecular weight and higher molecular weight became small.

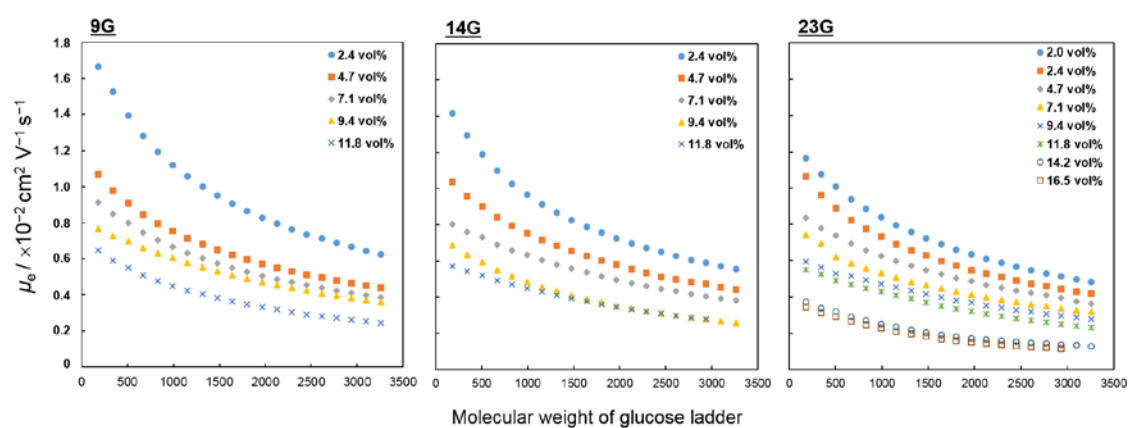


Figure 5-3. Electrophoretic mobility of glucose ladder in CGE using PEGDMA gel capillaries. Capillary, 50 μm i.d. $\times$ 40 cm (effective, 10 cm); applied voltage, 4 kV; injection, 4 kV–10 s; detection, LIF, ex 488 nm, em 520 nm; buffer, 1 $\times$ TB (pH 8.64).

As another investigation, the effect of the number of EO units in PEGDMA toward the separation based on the molecular size was evaluated. In Figure 5-4, μ_e of the glucose ladder in the gel capillary prepared with the same amount of PEGDMAs having different EO units are summarized. In comparison by each EO unit, μ_e was decreasing as using larger EO units. Additionally, the differences of μ_e were gradually smaller as using larger amount of PEGDMA and finally any alterations were not observed in the gel capillaries prepared with the highest amount. As mentioned above, the higher crosslinking density causes the interference of the mobility for the lower molecular weight. However, the further high crosslinking density provided almost the same effect and much lower μ_e for all the glucose ladders.

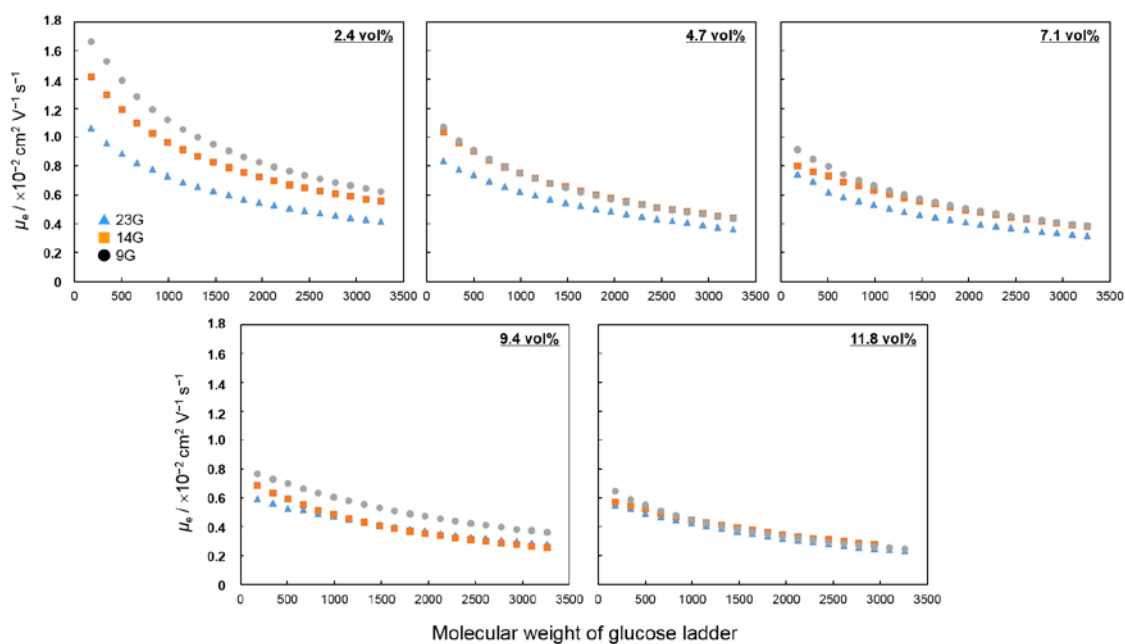


Figure 5-4. Comparison of the electrophoretic mobility of glucose ladder by alteration of the concentration of PEGDMAs in CGE. The conditions were the same as Figure 5-3.

Furthermore, the effect of the initiator amount was evaluated with the gel capillary prepared with 23G-PEGDMA (2.4 vol%) by changing only the amount of the initiator, APS (10%, 5.0%, 2.5%, and 1.0% aqueous solutions). As results of μ_e of the glucose ladder (Figure 5-5), similar values were obtained except for the capillary prepared with 10% APS; briefly the values were smaller than that of using 10% APS. During the radical polymerization, the termination reaction rate is proportional to the square of the concentration of radical species. Therefore, it is assumed that the lower concentration of APS provided the polymer network with the higher molecular weight, resulting in the smaller μ_e . These results suggested that the control of the initiator amount also affected the molecular size effect. Consequently, we can control the separation based on the molecular size by tuning the crosslinking density, EO units of PEGDMA, and the amount of an initiator, especially the tuning by the EO units of PEGDMA at lower concentration affected effectively. In fact, in the case of the separation of APTS-labeled glucose ladder, the separation is operated by the differences of the mobility based on the mass-to-charge ratio. As shown in Figure 5-6, APTS-labeled glucose ladder can be separated even by simple electrophoresis using an open-tubular capillary with much higher mobility. However, when using PEGDMA-gel capillaries, we can control the mobility based on the differences of the molecular size by the interference in the polymer network.

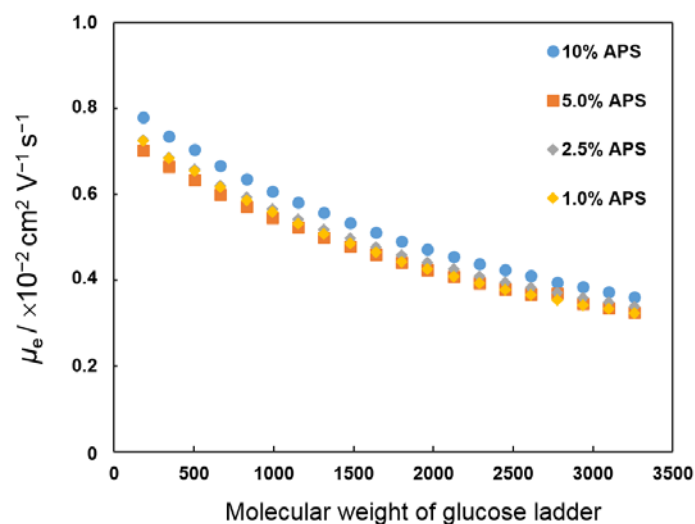


Figure 5-5. Comparison of the electrophoretic mobility of glucose ladder by alteration of the concentration of an initiator, APS in CGE. The conditions were the same as Figure 5-3.

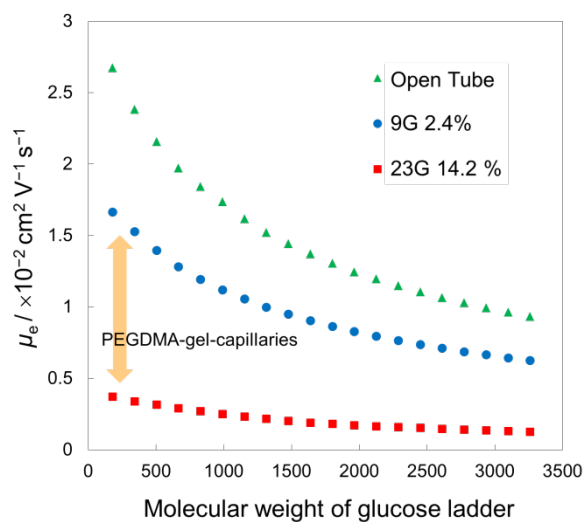


Figure 5-6. Comparison of the electrophoretic mobility of glucose ladder between an open tubular and PEGDMA-gel capillaries. The conditions were the same as Figure 5-3.

To reveal the potential separation efficiency of the PEGDMA-based gel capillaries, the typical gel capillary prepared with PAA was also evaluated. The value of μ_e of each glucose ladder in the PAA-based capillaries shown in Table 5-2 is described in Figure 5-7 (a). Additionally, the summary of the results by the PEGDMA-based capillaries, which was including all the results in Figures 5-3 and 5-4, are shown in Figure 5-7 (b). As well as the PEGDMA-based capillaries, μ_e of glucose ladder was smaller by increasing %T in the PAA-based capillaries, which shows the higher gel density provided the slower migration. In point of the range for the separation based on the molecular size, the PEGDMA-based capillaries showed much superior to the PAA-based capillaries.

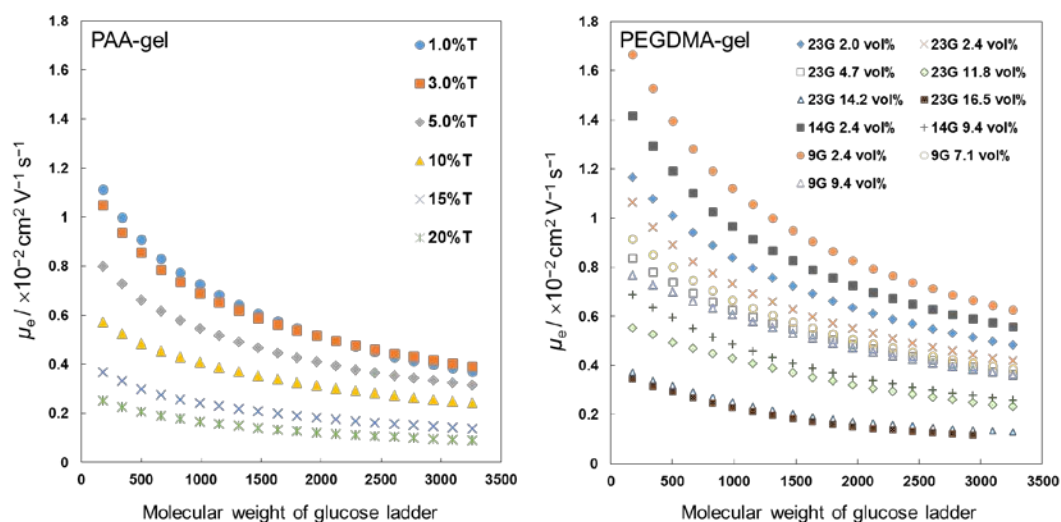


Figure 5-7. Comparison of the electrophoretic mobility of glucose ladder between PAA-gels and PEGDMA-gel capillaries. The conditions were the same as Figure 5-3.

Then, we demonstrated the separation of the APTS labeled glucose ladder using the optimized PEGDMA-based capillary. The electropherograms of the glucose ladder in the PAA and PEGDMA-based capillaries are shown in Figure 5-8. According to these results, the gel capillaries produced in this study provided the similar potential as separation media based on the molecular size as the typical PAA capillary. Furthermore, the suitable reproducibility was obtained for the capillary; in brief the gel capillaries prepared with the same conditions provided the almost similar separation efficiency for glucose ladders as shown in Figure 5-9.

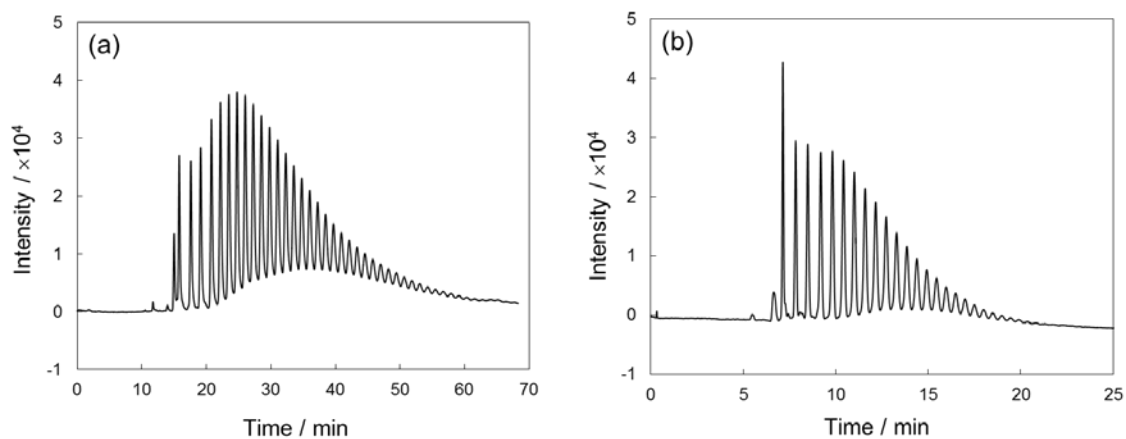


Figure 5-8. Electropherogram of the glucose ladder in CGE. (a) PAA-10%T, (b) PEGDMA-23G-9.4%. The conditions were the same as Figure 5-3.

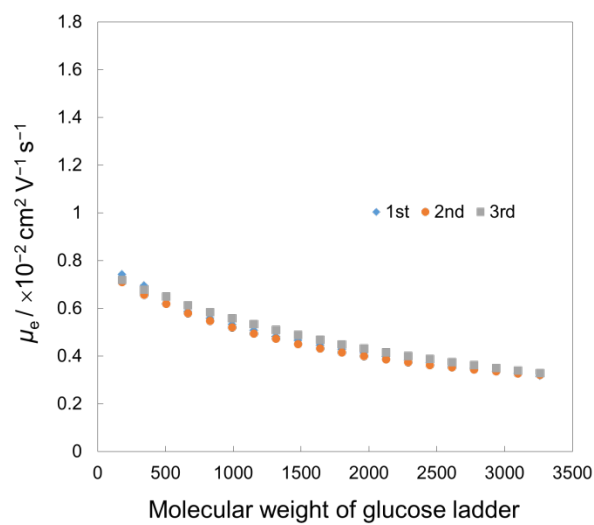


Figure 5-9. Reproducibility of the PEGDMA-gel capillaries (23G 7.1%). The conditions were the same as Figure 5-3.

5-3-3 Separation of DNA ladder

Unlike the separation of glucose ladder, the simple molecular sieving effect can be evaluated with DNA ladder. Then, these separations of DNA ladder were investigated by using the PEGDMA-based capillaries prepared with several concentrations and EO units of PEGDMA. The electropherograms of DNA ladder are shown in Figure 5-10 and Figure 5-11. Comparing the results shown in Figure 5-10 (a) to (c), the separations of DNAs in the range of 100–300 bp, 100–900, and 100–1100 bp were accomplished by using 2.36 vol%, 4.73 vol%, and 9.39 vol% of PEGDMA-23G, respectively. Similar tendency was also observed when PEGDMA-14G or -9G was employed as the crosslinker as shown in Figure 5-11. However, the separation efficiency was much lower than that of PEGDMA-23G even though the higher concentration was applied. Additionally, when the PAA-based capillaries were employed for the separation of DNAs, the similar separation differences were observed as shown in Figure 5-10 (d, e). In brief, the DNAs in the range of 100–900 bp could be effectively separated in the PAA-5%T capillary. The capillaries prepared with further PAA-%T provided longer analysis time (> 200 min) and upset of the baseline (Figure 5-11 (g)), or no-detection because of the slower migration due to the high density of PAA. Consequently, as well as mentioned in the separation of glucose ladder, the PEGDMA-based capillaries are effectively utilized as the separation medium due to the molecular sieving in CGE.

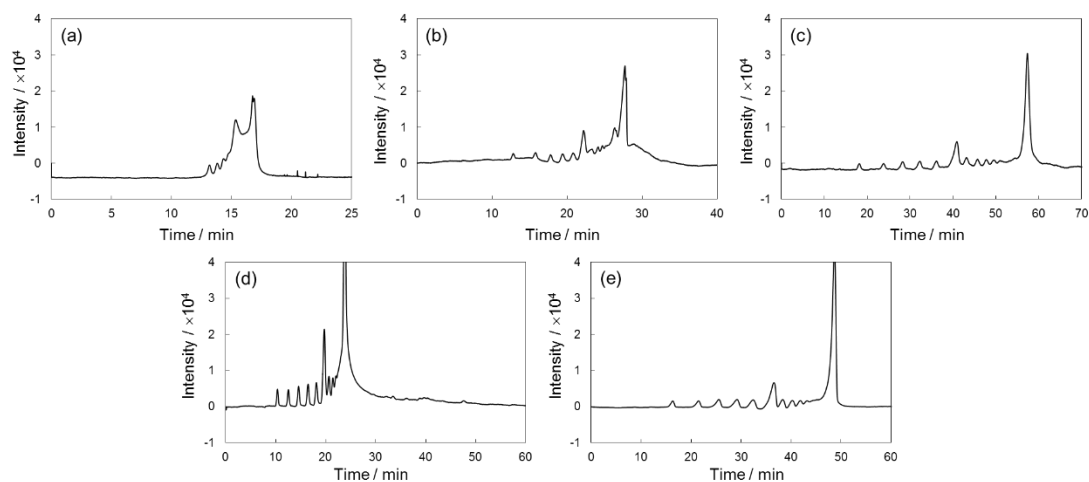


Figure 5-10. Electropherogram of the DNA ladder in CGE using PEGDMA and PAA. (a) PEGDMA-23G-2.4%, (b) PEGDMA-23G-4.7%, (c) PEGDMA-23G-9.4%, (d) PAA-3%T, (e) PAA-5%T. Capillary, 50 μm i.d. $\times$ 40 cm (effective, 10 cm); applied voltage, 4 kV; injection, 4 kV–10 s; detection, LIF, ex 488 nm, em 520 nm; buffer, 1 $\times$ TBE (pH 8.31).

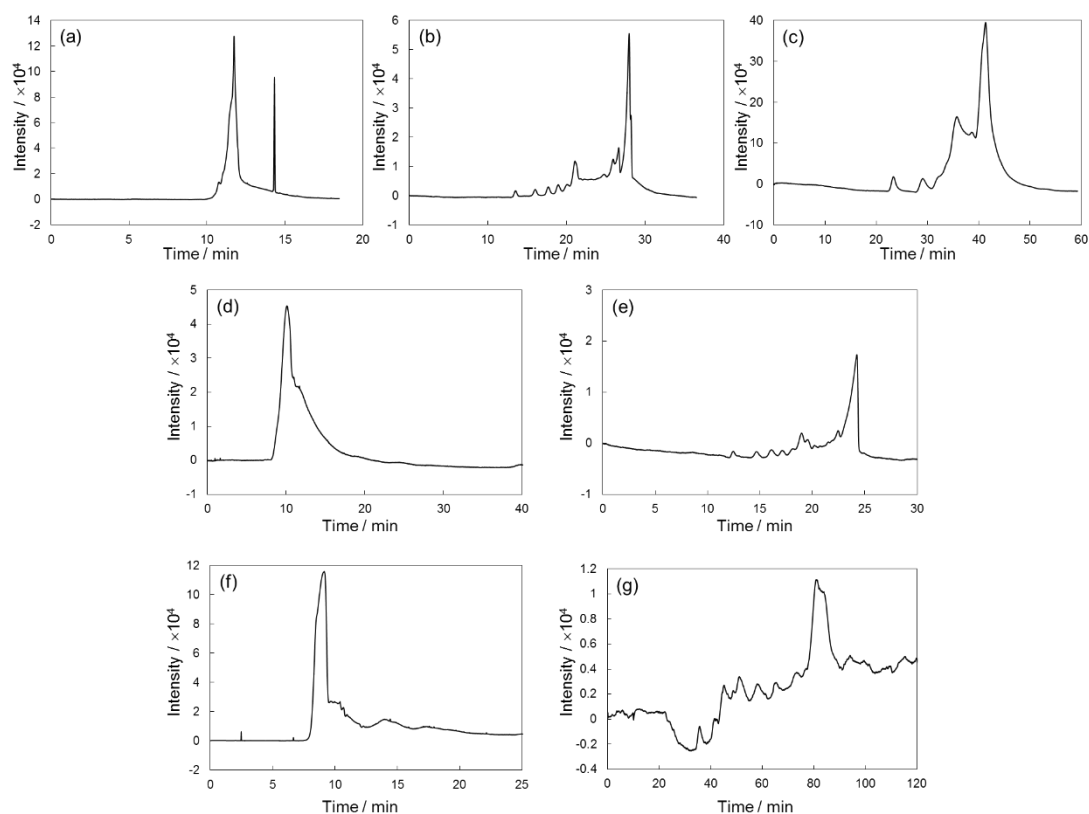


Figure 5-11. Electropherograms for DNA ladder in GCE. (a) PEGDMA-14G-2.4%, (b) PEGDMA-14G-4.7%, (c) PEGDMA-14G-9.4%, (d) PEGDMA-9G-2.4%, (e) PEGDMA-9G-4.7%, (f) PAA-1%T, (g) PAA-10%T. The conditions were the same as Figure 5-10.

5-3-4 Separation of the sugars carved out from mAbs

Finally, to demonstrate the separation of real samples, sugars carved out from mAbs were employed as the sample. The sugars were carved out with *N*-Glycanase from mAbs-A and -B, and then labeled with APTS. The electropherograms of the sugar samples in CGE with PEGDMA-14.2% are shown in Figure 5-12. The peak assignment was carried out by referring the results obtained from typical capillary electrophoresis (see Figure 5-13). A few sugars in each sample were effectively separated due to the molecular weight described in Figure 5-12. According to these results, the PEGDMA gel capillary could be used for the separation of biomolecules by the typical molecular size differences. We believe that the PEGDMA gel capillaries will be useful for the effective separation of biomolecules and the separation range based on the molecular size can be easily tuned by altering the concentration and/or EO units of PEGDMA.

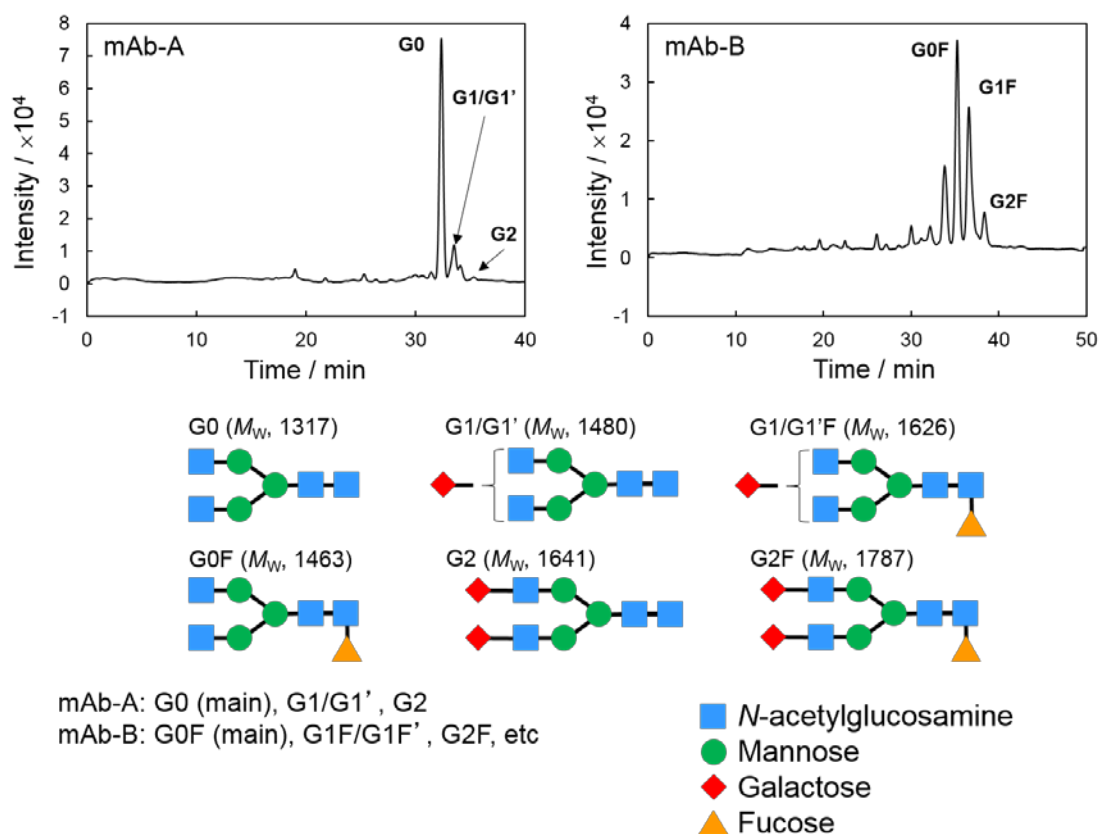


Figure 5-12. Separation of the sugars carved out from mAbs and the structure of the sugar chains. Capillary, PEGDMA-14.2%, 50 μ m i.d. $\times$ 40 cm (effective, 10 cm); applied voltage, 4 kV; injection, 4 kV –10 s; detection, LIF, ex 488 nm, em 520 nm; buffer, 1 $\times$ TB (pH 8.64).

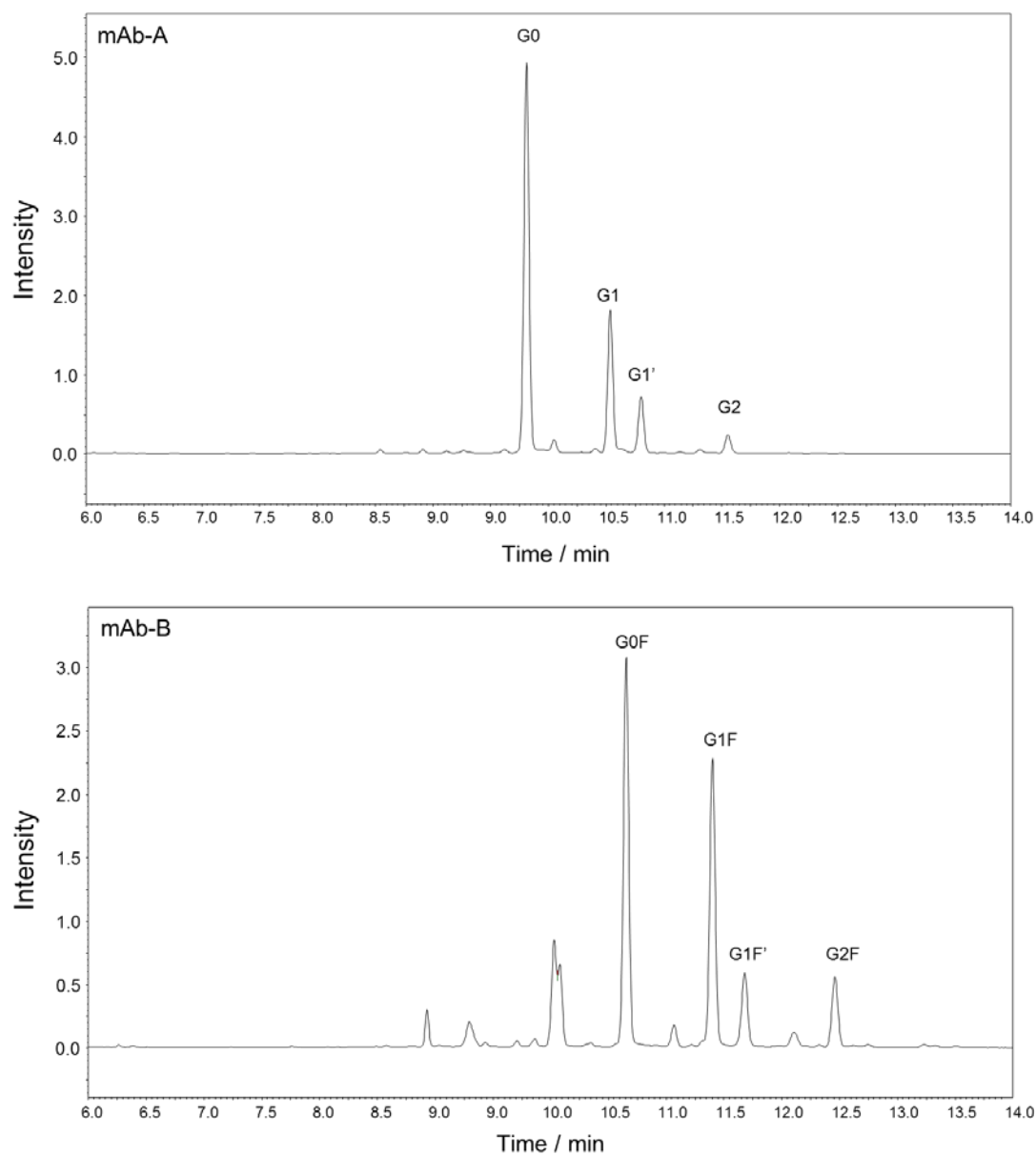


Figure 5-13. Separation of the sugars carved out from mAbs in capillary electrophoresis. Capillary, N-CHO Capillary, 50 μm i.d. $\times$ effective length 40 cm (total length 50 cm) (Beckmann), applied voltage, 30 kV; injection, 2.0 psi-12 s; detection, LIF, ex 488 nm, em 520 nm; buffer, carbohydrate separation gel buffer (Beckmann).

5-4 Conclusions

We successfully reported the possibility of the PEGDMA-based hydrogel capillary as a new separation medium enabling the separations based on the molecular size in CGE. To prepare the gel capillary, the redox polymerization using APS and TEMED was suitable. The CGE separations of both glucose and DNA ladders using the PEGDMA-based capillaries suggested that the concentration and EO units of PEGDMA affected the range of the separable molecular weight due to the interference molecular mobility or the simple molecular sieving effect. Additionally, as a practical application, the sugars carved out from mAbs were effectively separated due to the differences in the molecular size effect in PEGDMA-gel CGE. We expect that the PEGDMA-based gel capillaries can be used for these separation of biomolecules by the effective molecular size effect along with the typical gel capillaries such as a PAA-based capillary.

5-5 References

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General Conclusions

In this thesis, studies on the development of various separation methodologies based on chromatographic and electrophoretic techniques were described. Application studies of the developed methods were also carried out for the quality evaluation of actual biopharmaceuticals.

In the Chapter 2, the author validated a CZE method for confirming the identity and purity of the separated charge variants of mAbs or ADCs. The validation includes the evaluation of the specificity, linearity, quantitation limit, precision (repeatability and intermediate precision), accuracy, range, and robustness. The method was applicable to the majority of mAbs and ADCs (with *pI* from 7 to 9 and a drug to antibody ratio up to 8), requiring no modification of the method conditions. The proposed CZE method showed reproducible separation profiles, while CEX showed low reproducibility and deficient separation profiles due to an undesirable interaction between the separation column and the low molecular weight drugs combined with the ADCs. Since CZE is able to minimize this undesirable interaction during the separation, it proved to be a useful separation methodology for evaluating charge variants of ADCs. The validation of CZE for assessing ADCs was successfully demonstrated for the first time, and showed that CZE was suitable for the separation method for detecting the charge heterogeneity of ADCs.

The Chapter 3 describes a novel and comprehensive approach to identifying a

fragment peak of mAb-A detected by SDS-CGE. New impurity peak was detected close to the internal standard (10 kDa marker) of SDS-CGE. The peak increased about 0.5% under a 25 °C condition for 6 months. Generally, identification of fragments observed in SDS-CGE is challenging due to the difficulty of collecting analytical amounts of fractionations from the capillary. In-gel digestion peptide mapping and RPLC–MS were employed to elucidate the structure of the fragment. In addition, a Gelfree 8100 fractionation system was newly introduced to collect the fragment and the fraction was applied to the structural analysis of a mAb for the first time. These three analytical methods showed comparable results, proving that the fragment was a fraction of heavy chain HC1-104. The fragment contained CDRs, which are significant to antigen binding. Therefore, this fragmentation would affect the efficacy of mAb-A. In addition, SDS-CGE without the 10 kDa marker was demonstrated to clarify the increased amount of the fragment, and the experiment revealed that the impurity peak increases 0.2% per year in storage at 5 °C. The combination of the three analytical methodologies successfully identified the impurity peaks detected by SDS-CGE, providing information critical to assuring the quality and stability of the biotherapeutics.

In the Chapter 4, the author developed a spongy-like porous polymer (spongy monolith, SpM) consisting of poly(ethylene-co-glycidyl methacrylate) (PEGM) with continuous macropores that allowed efficient *in situ* reaction between the epoxy groups and proteins of interest. The average pore size of the prepared PEGM-SpM was 10 ~ μm , as determined by mercury porosimeter, whereas no meso-pores were detected by nitrogen-gas adsorption analysis. Immobilization of protein A on the SpM

(ProA-SpM) enabled high-yield collection of IgG from cell culture supernatant even at a high flow rate (9 mL min^{-1}). The ProA-SpM showed sufficient ruggedness as an affinity column, and the method exhibited a good reproducibility and efficient recovery over a wide range of concentrations. In addition, the immobilization of pepsin on the SpM enabled the efficient online digestion at a high flow rate. These results demonstrated the utility of the SpM as new platform for rapid-flow affinity reactions.

In the Chapter 5, a novel CGE technique with PEG-based hydrogels for the effective separations of biomolecules containing sugars and DNAs based on a molecular size effect was reported. The gel capillaries were prepared in a fused silica capillary modified with 3-(trimethoxysilyl)propylmethacrylate using a variety of the PEG-based hydrogels. After the fundamental evaluations in CGE regarding the separation by the molecular size effect depending on the crosslinking density, the optimized capillary provided the efficient separation of glucose ladder (G1 to G20). In addition, another capillary showed the successful separation of DNA ladder in the range of 10–1100 base pair, which is superior to an authentic acrylamide-based gel capillary. For both glucose and DNA ladders, the separation ranges against the molecular size were simply controllable by alteration of the concentration and/or units of ethylene oxide in the PEG-based crosslinker. Finally, the separations of real samples, which included sugars carved out from mAbs were demonstrated, and then the efficient separations based on the molecular size effect were achieved.

In summary, the author suggested several novel methodologies in this thesis: (1) CZE

for charge variants evaluation of ADCs, (2) identification of size variants by Gelfree 8100 fractionation, (3) spongy monolith column for affinity reactions between mAbs and Protein A or Pepsin, (4) PEG based tunable molecular sieving matrix for DNA and glycan analysis. These approaches showed superior points compared to conventional methods from the aspects of simplicity, throughput, and applicability.

Analysis of biopharmaceuticals faces difficulty of separation by chromatographic and electrophoretic methods due to their complexity; several variants are caused by size, charge, and, glycosylation differences. Especially, high separation efficiency is very important to identify and quantify impurities of biopharmaceuticals generated during production and/or storage. The author's findings, including the usefulness of CZE, identification approaches of SDS-CGE peaks using another fractionation system, and PEG-based separation media, will contribute to the analysis of biopharmaceuticals in detail. In addition, to produce biopharmaceuticals more cost-efficiently, new separation media will show higher binding capacity to the mAbs with lower costs needs to be developed. The SpM column showed some of desirable features, indicating the promising potential to replace the conventional separation media.

In conclusion, the obtained findings throughout the studies will contribute to the progress in quality evaluation of biopharmaceuticals, especially analysis of charge, size, affinity, and *N*-glycosylation differences in detail. The author believes that this thesis will become a milestone for further applications of CE and LC, and contribute to advance of industry in the near future.

List of Publications

Chapter 2. “Validation of Capillary Zone Electrophoretic Method for Evaluating Monoclonal Antibodies and Antibody-Drug Conjugates”,

Kei Kubota, Naoki Kobayashi, Masayuki Yabuta, Motomu Ohara, Toyohiro Naito, Takuya Kubo, Koji Otsuka;

Chromatography **2016**, 37, 117–124.

Chapter 3. “Identification and Characterization of a Thermally Cleaved Fragment of Monoclonal Antibody-A Detected by Sodium Dodecyl Sulfate-Capillary Gel Electrophoresis”,

Kei Kubota, Naoki Kobayashi, Masayuki Yabuta, Motomu Ohara, Toyohiro Naito, Takuya Kubo, Koji Otsuka;

Journal of Pharmaceutical and Biomedical Analysis **2017**, 140, 98–104.

Chapter 4. “New Platform for Simple and Rapid Protein-based Affinity Reactions”,

Kei Kubota, Takuya Kubo, Tetsuya Tanigawa, Toyohiro Naito, Koji Otsuka;

Scientific Reports **2017**, 7, 178.

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