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A rapid method for simultaneous evaluation of free light chain content and aggregate content in culture media of Chinese hamster ovary cells expressing monoclonal antibodies for cell line screening

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The goal of developing a monoclonal antibody (mAb) production process is high productivity and high quality. Because the productivity and quality of mAbs depend on cell line properties, the selection of cell lines suitable for large-scale production is an important stage in process development for mAb production. The light chain (LC) is important for antibody folding and assembly in the endoplasmic reticulum; cell lines that secrete a large amount of LCs in the medium secrete high-quality antibodies with high productivity. LC contents in culture media have been estimated by western blotting, reverse-phase high-performance liquid chromatography, and enzyme-linked immunosorbent assay. However, these analyses require fine tuning of experimental conditions for each antibody analyzed. Here we report a rapid and simple high-sensitivity size-exclusion chromatography (HS-SEC) method to evaluate the contents of low-molecular weight species (LMWS, mainly consisting of LC monomers and dimers) and high-molecular weight species (HMWS, aggregates) in the media for cell line screening. Because LMWS and HMWS are important indicators of productivity and quality, respectively, for cell line screening, HS-SEC will be useful in the first step of cell line selection needed for large-scale production.

[**Key words:** Therapeutic antibody; Size-exclusion chromatography; Low-molecular weight species; High-molecular weight species]

Therapeutic monoclonal antibodies (mAbs) have been widely used for their high antigenic specificity, long serum half-life, and low incidence of undesirable side effects; the global market for therapeutic mAbs is growing annually (1). Because large doses of therapeutic mAbs are usually required to achieve clinical success, mAb production processes with high productivity are needed to reduce the expense to patients. The quality of the therapeutic mAbs expressed is also critical, given that mAbs produced from Chinese hamster ovary (CHO) cells are heterogeneous owing to aggregation (2,3) and various post-translational modifications (4,5). This aggregation and modification of antibodies may lead to a reduction in biological activity and/or an increase in undesirable side effects under certain circumstances (6,7). The content of aggregated species is the most critical source of heterogeneity; this is because aggregates may induce immunogenic responses and cause adverse events following administration (6,7). Thus, the aggregate content of antibody should be precisely controlled.

Because the productivity and quality of mAbs depend on cell line properties, the selection of cell lines suitable for large-scale production is an important stage in process development for monoclonal antibody production. This selection process is time consuming as well as labor intensive (8), and various properties of cell lines and mAbs produced by cell lines should be evaluated during this screening (9). In particular, the light chain (LC) is important for antibody folding and assembly in the endoplasmic reticulum, and cell lines that secrete a large amount of LC show high productivity (10–13) and high quality (9,10,14). To date, LC contents in culture media have been estimated by western blotting (14), reverse-phase high-performance liquid chromatography (RP-HPLC) (10), and enzyme-linked immunosorbent assay (ELISA) (15); however, these analyses require fine tuning of experimental conditions for each antibody analyzed: optimization of the amount of both the mAb of interest and primary antibodies against it for western blotting and ELISA and optimization of gradient conditions for RP-HPLC. High-molecular weight species (HMWS) has been determined in a separate analysis by size-exclusion chromatography (SEC) of protein A-purified samples (16) aside from estimate of LMWS. In addition, when the culture media are purified/concentrated by protein A, LC not associated with the heavy chain (HC) is removed, and not all antibody aggregates are retained; LC and HMWS cannot be accurately evaluated after protein A purification/concentration. For this reason, a rapid, simple, and exhaustive method for evaluating both LC and HMWS at the same time in the culture media at the cell screening stage is desirable.

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We aimed to develop a rapid and simple high-sensitivity size-exclusion chromatography (HS-SEC) method that enables the simultaneous evaluation of both LC and HMWS in culture media and can be applied without fine tuning of experimental conditions for each antibody analyzed. Although termed as SEC in our previous report (9), analyses of HMWS and low-molecular weight species (LMWS) in culture media have already been performed by using the present HS-SEC. We found negative relationships between HMWS(%) and titers and positive relationships between LMWS(%) and the titers for days 5 and 7 using 28 cell lines (9). In addition, we found a negative association between the aggregate contents and LMWS(%) in culture media by stepwise multiple linear regression analyses (9). Because we did not describe the details of the HS-SEC in order to focus the results obtained by using the method in our previous article (9), we report the qualification of the HS-SEC method and its advantages over conventional SEC method in the present article.

MATERIALS AND METHODS

Cell culture and antibody production Cell culturing to produce trastuzumab (trade names: Herclon and Herceptin) using 125-ml Erlenmeyer flasks was performed according to our previous report (9), and cell cultures using 2-L glass bioreactors were performed according to our previous report (17). Culturing was performed for 14 days, and culture supernatants were stored at below −20°C. In addition to trastuzumab, two monoclonal IgG1 antibodies having a kappa LC (designated as mAb-A and mAb-B) were expressed in CHO cells under optimized culture conditions.

Purification of antibodies Trastuzumab was purified, where indicated, with a Protein A HP SpinTrap (GE Healthcare, Buckinghamshire, UK). Procedures were modified slightly from the manufacturer's instructions; binding buffer, elution buffer, and neutralizing buffer employed were Dulbecco's phosphate-buffered saline (DPBS, Thermo Fisher Scientific, Waltham, MA, USA), 20 mM sodium citrate (pH 3.2), and 1.5 M Tris, respectively. The volume of neutralizing buffer added was 4.6 μL, and all centrifugation steps were performed at 100 ×*g* for 30 s during the entire procedure.

Purification of antibodies with a protein A affinity column in other experiments followed that in our previous report (16).

Antibody concentration determination The antibody titer in culture media was determined with a protein A column according to our previous report (9). The antibody concentration of each protein A-purified sample was determined by absorbance at 280 nm according to our previous report (16).

HS-SEC for analyses of culture media To estimate the proportions of low-molecular weight species (LMWS), high-molecular weight species (HMWS), and main species in culture media, we developed the following HS-SEC method. HS-SEC was performed with a TSKgel SuperSW3000 column (4.6 mm i.d. × 30 cm; Tosoh Corporation, Tokyo, Japan) preceded by a guard column. The TSKgel SuperSW3000 column exhibits higher sensitivity than the TSKgel G3000SWXL (Tosoh Corporation) commonly used in SEC because of its smaller inner diameter (4.6 vs. 7.8 mm) and smaller diameter of resin particles (4 vs. 5 μm).

The mobile phase consisted of 50 mM sodium phosphate, 500 mM NaCl, and 5% (v/v) ethanol (pH 7.0). The isolation conditions were as follows: flow rate, 0.175 mL/min; column temperature, 25°C; injected protein, 5 μg (in 20 μL); and detection wavelength, 215 nm. The sample was diluted with DPBS to 250 μg mAb/mL. The relative molecular mass (M_r) of each peak was estimated against a gel filtration standard (product 151-1901, Bio-Rad Laboratories, Hercules, CA, USA). The HPLC analysis was performed on a Dionex UltiMate 3000 (Thermo Fisher Scientific) or a Waters 2695 Alliance HPLC system (Waters, Milford, MA, USA). Trastuzumab, which has kappa LCs, was used in the experiment unless otherwise specified.

The content of free LC (monomer and dimer of LC) was estimated using anti-kappa LC antibody as follows. The decrease in the peak height of the main LMWS in HS-SEC upon the addition of anti-kappa LC antibody (product ab19977, Abcam, Cambridge, UK; anti-LC Ab) corresponds to the free LC. In the first step, the amount of anti-LC Ab needed to eliminate the peak of LMWS in HS-SEC was determined. After the addition of various amounts of anti-LC Ab, the samples were incubated overnight at 2°C–8°C and centrifuged at 15,000 ×*g* for 15 min, and the supernatants were analyzed by HS-HPLC. To evaluate the contribution of impurities in the anti-LC Ab reagent, the experiments were also performed without mAb. In the second step, the ratio of anti-LC Ab to mAb in the sample was set at 70:1 (w/w), and 15 culture medium samples from different cell lines containing various proportions of LMWS were analyzed. To eliminate the contribution of impurities of anti-LC Ab, the

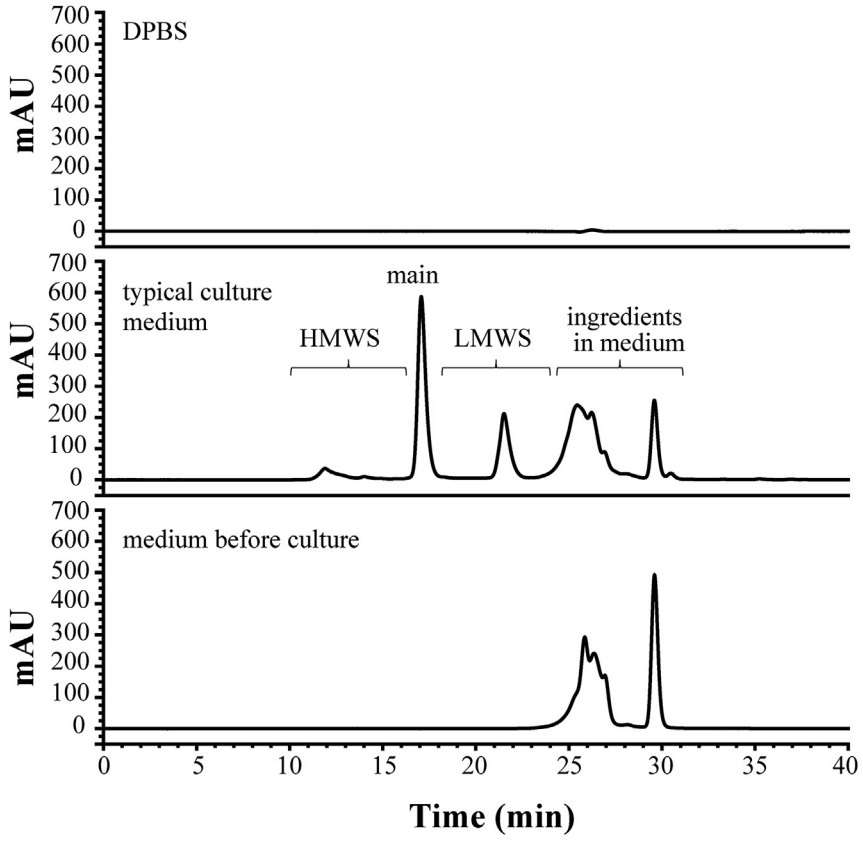

FIG. 1. Chromatograms of high-sensitivity size-exclusion chromatography of Dulbecco's phosphate-buffered saline (top), typical culture medium, which was taken from batch 3 of our previous report (16) on day 14 (middle), and medium before culture (bottom). mAU, milli-absorbance unit; LMWS, low-molecular weight species; HMWS, high-molecular weight species.