Analytical Biochemistry 534 (2017) 49–55

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

High-throughput quantitation of Fc-containing recombinant proteins in cell culture supernatant by fluorescence polarization spectroscopy

Ben Thompson [a], Jerry Clifford [b], Mike Jenns [c], Andrew Smith [c], Ray Field [c], Kalpana Nayyar [d], David C. James [a, *]

[a] Department of Chemical and Biological Engineering, The University of Sheffield, Sheffield, UK
[b] Valitacell Ltd, Pembroke House, 28-32 Upper Pembroke Street, Dublin 2, Ireland
[c] MedImmune, Granta Park, Cambridge, CB21 6GH, UK
[d] Abzena, Babraham Research Campus, Babraham, CB22 3AT, UK

ARTICLE INFO

Article history:
Received 6 June 2017
Received in revised form
7 July 2017
Accepted 11 July 2017
Available online 14 July 2017

Keywords:
Fluorescence polarization
Immunoglobulin
Assay

ABSTRACT

Measurement of recombinant protein product titer critically underpins all biopharmaceutical manufacturing process development, as well as diverse research and discovery activity. Here, we describe a simple rapid (<2 min per 96 samples) 96-well microplate-based assay that enables high-throughput quantitation of recombinant immunoglobulin G and Fc-containing IgG derivatives in mammalian cell culture supernatant over a wide dynamic range of 2.5–80 mg/L, using microplate fluorescence polarization (FP) spectroscopy. The solution-phase FP assay is based on the detection of immunoglobulin Fc domain containing analyte binding to FITC-conjugated recombinant Protein G ligand to measure analyte concentration dependent changes in emitted FP. For ease of use and maximal shelf life, we showed that air-dried assay microplates containing pre-formulated ligand that is re-solubilized on addition of analyte containing solution did not affect assay performance, typically yielding an across plate coefficient of variation of <1%, and a between-plate standard deviation below 1%. Comparative assays of the same samples by FP and other commonly used IgG assay formats operating over a similar dynamic range (Protein A HPLC and bio-interferometry) yielded a coefficient of determination >0.99 in each case.

Introduction

Recombinant biopharmaceuticals have become a dominant therapeutic platform - 8% annual growth and 7 of the top 8 best selling drugs in 2014 were biologics with the top 10 best selling biologics accounting for $70bn in sales in 2013. Recombinant monoclonal antibodies (MAbs) and MAb variants represent the largest and fastest growing group of drugs [1]. Engineered MAb derived therapeutics such as bispecifics and other immunoglobulin-derived fusion proteins currently occupy an increasing proportion of industrial development pipelines.

Measurement of recombinant product titer underpins all aspects of bioprocess development, from the selection of engineered clonal cell lines to monitoring of production process performance. Ideal assay technologies should enable: (i) accurate and selective measurement of product titer across a wide dynamic range, (ii) automated, high-throughput operation and (iii) be technically simple, robust and cost effective. Standard current methods for the quantitation of IgG are Protein A HPLC [2], ELISAs and Biolayer interferometry [3]. However, these methods suffer from drawbacks for the application of high throughput quantitation. Protein A HPLC, while regarded by many as “industry standard” is relatively low-throughput and requires specialist technical operation. ELISAs, while simple, are time consuming and require impractical levels of sample dilution for standard use. Biolayer interferometry, while high-throughput, generally incurs a large capital and consumable expenditure.

Most generic methods (ELISAs, HPLC and biolayer interferometry) for measurement of IgG in solution rely on binding of the analyte to a specific IgG binding protein, (typically recombinant staphylococcal Protein A) adsorbed or covalently attached to a solid phase. In contrast, the assay methodology presented here adopts a different strategy based on the direct measurement of binding of a

* Corresponding author.
E-mail address: d.c.james@sheffield.ac.uk (D.C. James).

http://dx.doi.org/10.1016/j.ab.2017.07.013
0003-2697/

small, highly specific IgG Fc binding protein, Protein G [4] covalently labeled with a fluorophore, to large Fc domain containing analytes (such as MAbs) in solution using fluorescence polarization (FP) spectroscopy. This entirely solution phase approach yields a microwell-based assay that eliminates the need for surface binding, washing or elution steps, which yields a much simpler and faster assay approach.

FP spectroscopy indirectly quantifies the relative size of molecular complexes by measuring their rotational speed (or diffusion) in solution [5]. When fluorophores are excited by polarized light, they preferentially emit light in the same plane of polarization when they are immobile. However, rotation of fluorophores in the time between absorption and emission of the photon (the fluorescence lifetime) has the effect of rotating the plane of the polarization of the emitted light. The more the molecules move in this time, the more the light is depolarized. As a result of small molecules rotationally diffusing faster in solution than larger molecules, the size of a molecule, with an associated fluorophore, can be measured using the degree of light depolarization according to Equation (1).

$$\left(\frac{1}{P}-\frac{1}{3}\right)=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1-\frac{\tau}{\phi}\right) \quad (1)$$

where

$$\phi=\frac{\eta V}{RT}$$

Equation (1): P is polarization, P_0 is fundamental polarization of the fluorophore, τ is the fluorescence lifetime of the fluorophore, ϕ is the rotational coefficient, η is the solution viscosity, V is the molecular volume, R is the universal gas constant and T is temperature.

FP is measured by excitation of a ligand-bound fluorophore in solution with plane polarized light with measurement of the intensity of light emitted in the plane parallel to the incident light (polarized proportion) and perpendicular to the incident light (depolarized portion). The FP is expressed as a normalized difference of these two intensities [6] which is typically in milli polarization units (mP) according to Equation (2). The mP values for polarization can theoretically range from −333.33 to 500 units [7] however for fluorophore molecules freely rotating in solution, values range from 0 to 500 units [8,9].

$$mP=\frac{(\text{Parallel intensity}-\text{Perpendicular intensity})\times 1000.}{(\text{Parallel intensity}+\text{Perpendicular intensity})} \quad (2)$$

Equation (2): Consequently, when fluorescently labeled Protein G ligand is unbound, it tumbles rapidly and depolarizes the light more than when it is bound to a significantly larger IgG (or Fc-containing) analyte. We demonstrate that under the assay conditions described the change in polarization observed upon fluoro-labeled Protein G ligand binding can be used to accurately measure the mass of recombinant IgG, or any IgG Fc-containing variant in clarified cell culture media across a wide dynamic range. Relative to other methods, the FP-based microplate-based assay is simple, rapid, cost effective and readily automatable.

Materials and methods

Biological materials

Assay development and optimization was performed on cell-free culture supernatant samples generated by a range of recombinant IgG producing and non-producing Chinese hamster ovary (CHO) cell lines cultivated in suspension in commercially available (e.g. CD-CHO, Life Technologies, Paisley, U.K.) and proprietary chemically defined growth media using standard laboratory procedures as described previously [10]. Prior to analysis, cell culture samples were centrifuged at 5000×*g* for 5 min. Recombinant human IgG1 standards, recombinant Protein A, truncated recombinant Protein G [11] were purchased from Sigma (Poole, U.K).

Analytical methods

Volumetric titre was calculated using either protein A affinity chromatography or BioLayer Interferometry. Protein A affinity chromatography used a protein A ImmunoDetection® sensor cartridge (Applied Biosystems, Warrington, UK), coupled to an Agilent Series 1260 HPLC (Agilent, Berkshire, UK), according to the manufacturer's instructions. BioLayer Interferometry used an Octet 384QK with Protein A biosensors (Pall ForteBio Europe, Portsmouth) that were used according to the manufacturer's instructions. In both methods, a reference standard of purified product was run alongside the samples for calibration.

FP spectroscopy

Fluorescently labeled Protein G ligand and recombinant IgG diluted in chemically defined CHO medium in a total volume of 120 μL were incubated for 30 min at room temperature (RT) in 96-well, half-area, black-walled, non-binding surface microplates (Corning, New York, U.S.A.) prior to measurement of FP (as mP, Eqn. (2)) using a PheraStar Plus FP microplate reader (BMG Labtech, Germany).

Fluorophore conjugation

Recombinant Protein G and Protein A at a concentration of 5 mg mL^{-1} were covalently labeled at primary amines with varying concentrations of fluorophores in sodium carbonate-bicarbonate buffer at RT at varying pH (see results section) Labeled proteins were subsequently purified using G20 Sephadex desalting columns (GE, Uppsala, Sweden) equilibrated in PBS prior to concentration using 10 KDa cutoff Amicon spin concentrator tubes (Merck Millipore, Nottingham, UK). Fluorescein isothiocyanate (FITC) was obtained from Sigma. BODIPY-FL and Alexa647 were both obtained from Life Technologies. Dye to protein ratios were determined using A280 and A495 with e0.1% extinction coefficients of 0.95 for protein G' (Mw 20 KDa) and 195 for FITC (Mw 389Da). A correction factor of 0.35*A495 was applied to the A280 reading prior to calculation.

Results and discussion

Assay design and optimization

For a high precision FP assay, ideally a large polarization signal shift would occur upon ligand binding to the analyte. This maximizes both assay resolution and dynamic range, where we define resolution as ability to significantly differentiate between two concentrations of analyte. First, based on Equation (1) we hypothesized that a relatively small immunoglobulin binding ligand would give the largest signal shift upon binding to a typical IgG1 (±150 KDa). We also considered other factors such as amenability to fluorescent labeling, cost, commercial availability, binding affinity, stability and specificity. Moreover, we desired generic application to a wide variety of IgG or IgG derived molecules regardless of their target antigen. Similar FP assays have been