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

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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

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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

developed but they have used specific antigens and their binding ligands, and are thus limited to measurement of only one specific type of antibody [12]. Accordingly, given their small size relative to other IgG binding proteins, high affinity, low cost and ability to bind to a range of IgG subtypes, recombinant variants of Streptococcal Protein G and Staphylococcal Protein A [13] were selected as potential candidate ligands for the FP binding assay. We hypothesized that according to Equation (1), Protein G would likely give a larger signal shift upon binding of IgG than protein A due to its smaller molecular volume (V) (as their molecular weights are 22 KDa and 45 KDa respectively) and reduced rotational correlation time (ϕ). To assess this, Protein G and A were initially labeled with fluorescein isothiocyanate (FITC), which is routinely utilized for FP spectroscopy [5] and FP was measured in the presence of varying concentrations of rIgG1 diluted in CD-CHO culture media at a fixed concentration (0.5 μM) of labeled Protein A or Protein G [Fig. 1A]. As predicted, it was clear that the FITC-Protein G ligand gave a much larger signal shift (3x) upon binding to rIgG1 than did FITC-Protein A over a bioprocess-relevant MAb analyte concentration range up to 160 mg L^{-1}. Thus, Protein G was utilized for further assay development.

Second, we compared alternative fluorophores. We hypothesized that a fluorophore with a long fluorescence lifetime would give the largest signal shift upon IgG binding (maximizing τ, see Equation (1)). However, an optimal fluorophore should also exhibit a high fluorescence intensity (as this allows accurate calculation of FP), minimal interference of ligand-analyte binding (e.g. via aggregation or steric hindrance), ease of conjugation, small molecular size and large Stokes shift. Fluorophores selected for comparative analysis were FITC (Ex. 485 nm, Em. 520 nm; brightness 80 $cm^{-1}mM^{-1}$; fluorescence lifetime, 4 ns), BODIPY FL (Ex. 502 nm, Em. 512 nm; brightness 88 $cm^{-1}mM^{-1}$; fluorescence lifetime, 5 ns) and Alexa647 (Ex. 650 nm, Em. 665 nm; brightness 79 $cm^{-1}mM^{-1}$; fluorescence lifetime, 1 ns). All fluorophores were covalently conjugated to recombinant Protein G at a 2:1 M ratio of dye to protein in bicarbonate buffer at pH 8.2. As shown in Fig. 1B, it is clear that FITC is the optimal fluorophore of those tested as it gives the largest polarization signal shift upon binding to rIgG. This is not entirely as predicted from Equation (1) as BODIPY FL has a slightly longer fluorescence lifetime than FITC, thus it is likely that other factors such as steric hindrance are influencing the results. Unexpectedly, use of the Alexa647 conjugated Protein G actually decreased the FP signal upon addition of IgG. We speculate that this may be a consequence of ligand aggregation caused by the fluorophore which is mitigated upon addition of rIgG. We conclude that FITC labeled Protein G was the optimal ligand, of those tested, for further assay development.

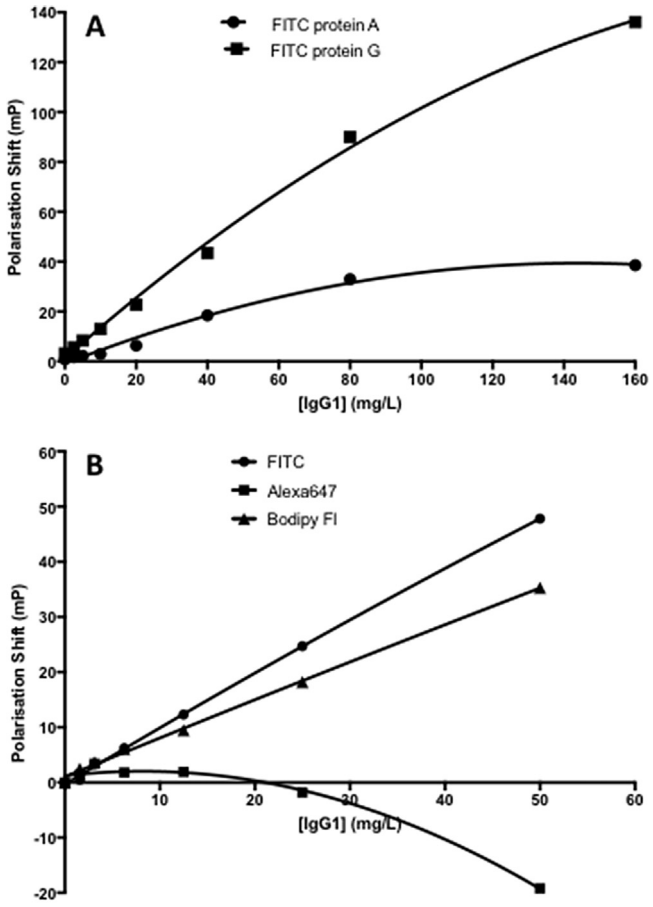

Fig. 1. Selection of IgG binding protein ligand and fluorophore conjugate for FP assay. (A) Comparison of FITC conjugated Protein A (●) and Protein G (■). Both ligands were incubated in CD-CHO at 6 μg per well with varying concentrations of rIgG1 for 30 min prior to measurement of FP (mP) shift relative to control medium lacking rIgG1. (B) Protein G was covalently labeled with different fluorophores, either FITC (●), Alexa647 (■) or BODIPY FL (▲) and FP shift at varying rIgG1 concentrations was determined as described.

Lastly, we sought to maximize FP signal shift upon IgG binding by optimization of Protein G labeling with FITC, both with respect to degree (mol/mol) and site of labeling. FITC covalently bonds to proteins via free amine groups, generally those on lysine side chains, but also on the N-terminus of the peptide. As labeling of lysine residues requires their deprotonation in an alkaline environment, then alteration of the labeling pH not only influences general lysine reactivity, but can also influence where the fluorophore attaches (i.e. at neutral pHs lysine amines are relatively unreactive thus the N terminal amine group is preferred). We hypothesized that site of attachment could influence the maximal FP shift as this could potentially influence Protein G binding to the analyte. To optimize labeling conditions, Protein G was labeled with FITC using labeling reaction solutions of varying pH and varying dye to protein ratios (Fig. 2A). Subsequently, Protein G-FITC conjugates were purified and their FP assay performance compared with respect to two metrics: (i) Protein G ligand fluorescence after background normalization, and (ii) maximal polarization shift determined by incubating a fixed Protein G ligand concentration (0.5 μM) with a saturating concentration of rIgG1 (100 mg L^{-1}).

As shown in Fig. 2A, at both FITC to Protein G ratios (2:1, 4:1) significantly increased Protein G-FITC conjugate fluorescence was achieved with increased labeling solution pH. However, this was associated with a concomitant reduction in maximum polarization shift on rIgG1 binding at pH > 7. We infer that this is a consequence of either (i) excess FITC label interfering with the Protein G binding [14] or that (ii) adjacent FITC molecules covalently bound to Protein G may influence their respective depolarization signals through quenching or energy transfer, however given that the dye:protein molar ratios were typically <1, this is unlikely to be the case. Based on these data, we sought to both maximize polarization signal and Protein G ligand fluorescence by increasing FITC to Protein G molar ratio in the labeling solution at pH 7. These data (Fig. 2B) show clearly that optimal ligand labeling conditions were obtained at FITC to Protein G labeling ratios >8:1. Under these conditions, maximal polarization shift on rIgG1 binding was maintained. We infer that these labeling conditions primarily favored N-terminal labeling, with relatively minimal binding elsewhere on the Protein G ligand. Spectroscopic analysis of the molar ratio of FITC to Protein G in the conjugate synthesized at an FITC to Protein G molar ratio of 8:1 at pH 7 yielded a fluorophore to protein ratio of 0.31: 1. This ligand was used for subsequent analyses.

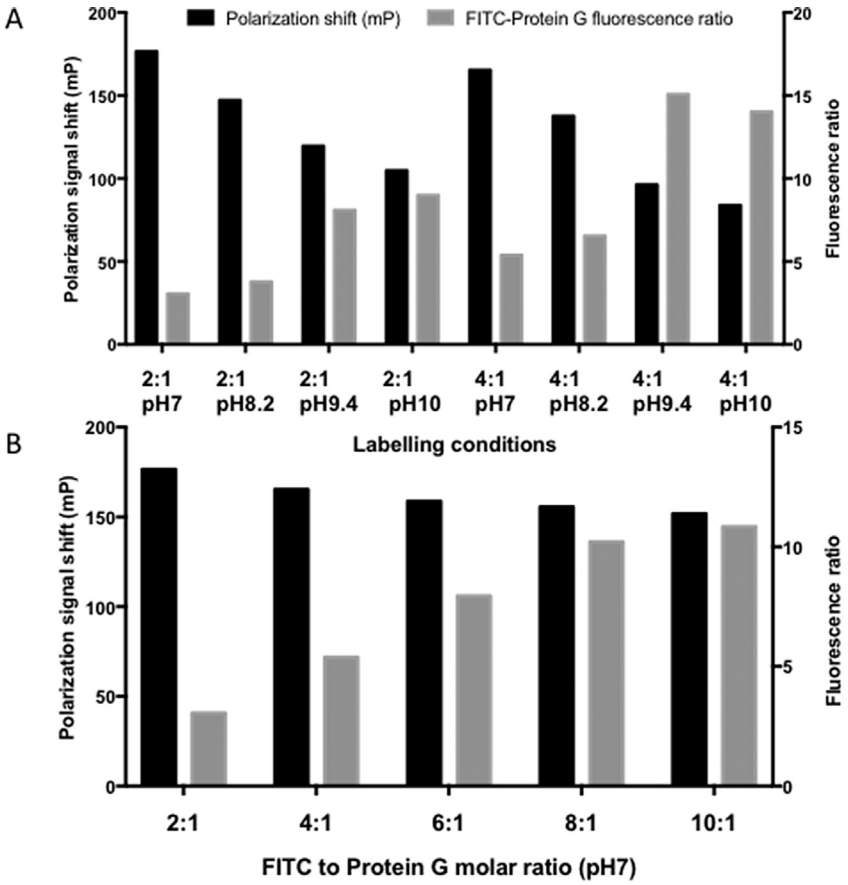

Fig. 2. Optimization of Protein G labeling with FITC. (A) Protein G was labeled with FITC at either at a molar ratio (fluorophore: protein) of either 2:1 or 4:1 at varying pH. In each case a measurement of FP shift at a fixed concentration (100 mg/L) of rIgG1 analyte and 6 μg labeled Protein G per well (black bars) was taken. Additionally Protein G ligand fluorescence was measured relative to background fluorescence of CD-CHO medium (grey bars). (B) Protein G was labeled with FITC at constant pH of 7 at varying molar ratios of fluorophore: protein prior to measurement of FP shift at a fixed concentration (100 mg/L) of rIgG1 analyte (black bars) and Protein G ligand fluorescence as above (grey bars). For each condition, dye to protein molar ratios were as follows: pH7/2:1 = 0.1:1, pH8.2/2:1 = 0.14:1, pH9.4/2:1 = 0.43:1, pH10/2:1 = 0.6:1, pH7/4:1 = 0.19:1, pH8.2/4:1 = 0.26:1, pH9.4/4:1 = 0.85:1, pH10/4:1 = 1.09:1, pH7/6:1 = 0.28:1, pH7/8:1 = 0.31:1, pH7/10:1 = 0.36:1.

Evaluation of assay performance

Selection of Protein G as an assay ligand was based on its known selectivity for the Fc region of mammalian IgGs [15], including human and mouse (subclasses 1–4, [16]). Therefore, we hypothesized that the FP assay would be generally applicable to both common IgG variants as well as engineered IgG derivatives containing an Fc domain. Whilst the magnitude of the FP shift observed may be affected by analyte molecular mass according to Equation (1), other unpredictable variables such as molecular conformation and differences in Protein G binding affinity may also affect assay performance. Using standard assay conditions (0.5 μM FITC-Protein G in 120 μL CD-CHO culture medium), we tested both kappa and lambda (light chain) variants of recombinant human IgG1, a bispecific MAb and an Fc-containing fusion protein with BSA used as a control. These data are shown in Fig. 3A and B. Both IgG1 variants and the bispecific MAb exhibited similar calibration curves up to 100 mg L^{-1} analyte concentration, whereas the fusion protein exhibited an increased polarization shift at lower concentrations. We conclude that the FP assay is a suitable method to measure all IgG-derived Fc-containing analytes with a molecule specific standard curve necessary for accurate quantitation.

It is acknowledged another factor which could lead to different shaped binding curves is aggregation of the analyte. The effect of this would be 2-fold in that a larger rotating molecule formed by aggregation would lead to a slightly larger polarization shift upon binding and additionally aggregation could obscure Fc binding sites and lead to reduced protein G binding. However it should be highlighted that most IgG assays, would be sensitive to reductions in accuracy caused by large degrees of analyte aggregation.

Subsequently, we compared quantitation of recombinant IgG1 (rIgG1) titer in conditioned cell culture media samples by FP to Protein A HPLC and biolayer interferometry methods. Both of these methods rely on binding of IgG analyte to Protein A adsorbed on a solid phase and, in our hands, have a high correlation to yield of purifiable rIgG and hence represent an 'industrial standard' comparator. A range of in-process rIgG1-containing samples from clarified cell culture supernatants were quantified using both methods in parallel to the FP method (Fig. 4). Highly comparable (coefficient of determination > 0.99) analyte quantitation across a 10-fold concentration range was observed in both cases.

Assay dynamic range is a fundamental component of assay performance. As described above, linear dynamic range for IgG1 quantitation using the standard FP assay in cell culture media was typically between 2.5 and 80 mg L^{-1}. Whilst polarization shift limits the maximum analyte concentration measurement, assay CV increases relatively at low analyte concentrations as there was a typically constant assay standard deviation of ±1 mP (±0.6 mg L^{-1}) across all analyte concentrations. However, other elements of upstream bioprocess development such as "transient production",

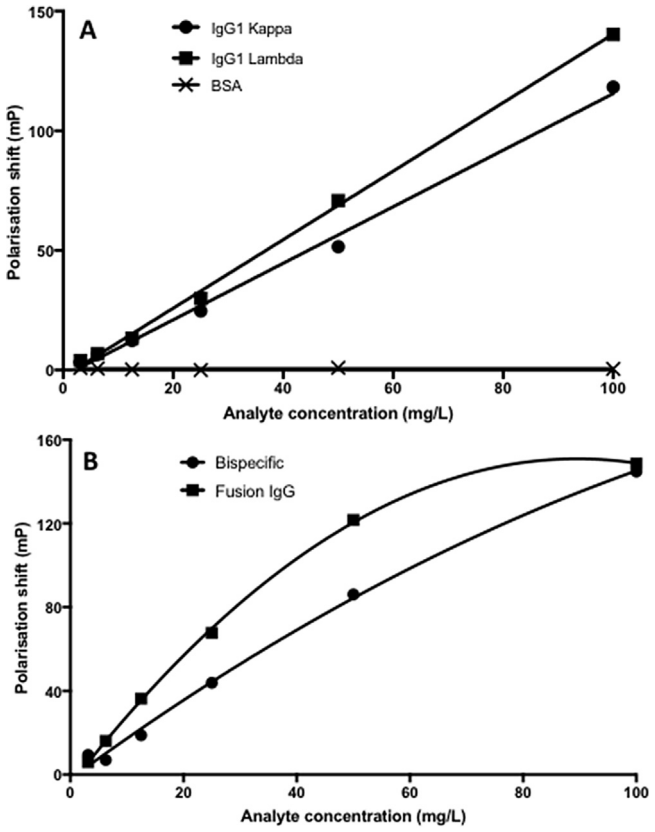

Fig. 3. Evaluation of different Fc-containing recombinant protein analytes. (A). Comparison of FITC conjugated Protein G ligand binding to rIgG1 kappa (●) and lambda (■) light chain variants with BSA as control (×) and (B) a recombinant bispecific MAb (●), a recombinant Fc-fusion protein (■). FITC-Protein G ligand was incubated in CD-CHO medium at 6 μg per well with varying analyte concentrations for 30 min prior to measurement of FP (mP) shift.

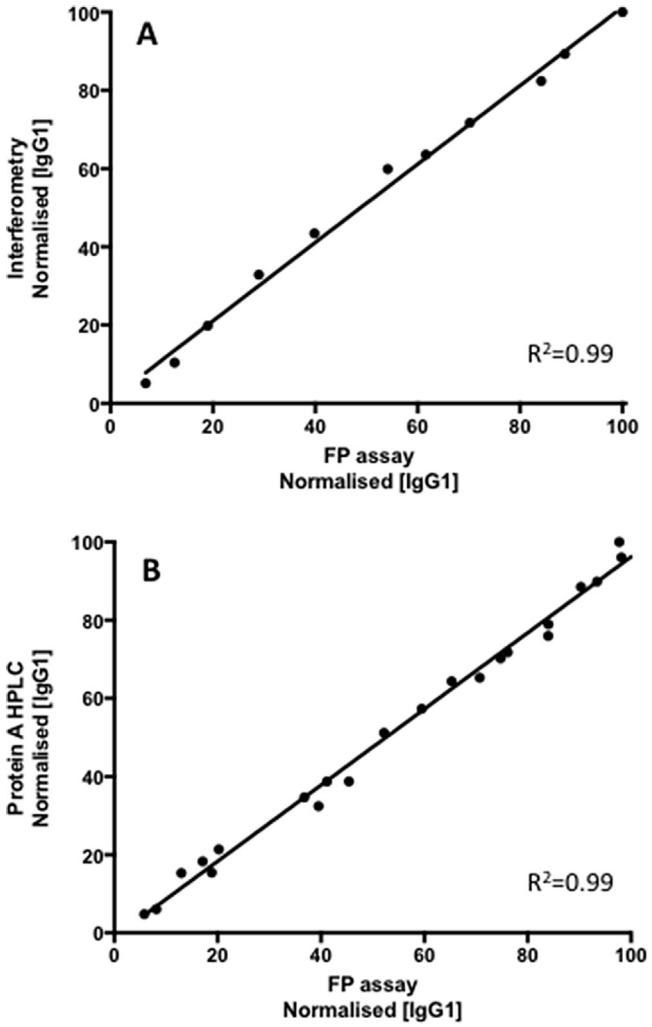

Fig. 4. Comparative analysis of rIgG1 quantitation by fluorescence polarization spectroscopy, biolayer interferometry and Protein A HPLC assays. rIgG1 titer in a range of conditioned media samples from fed-batch bioreactors was quantified by fluorescence polarization assay using FITC-Protein G ligand at 6 μg per well. The same samples were then analyzed by either biolayer interferometry (A) or Protein A HPLC (B). Data is shown as normalized units in both cases.

early stage cell clone screening and vector development require the measurement of much lower concentrations of antibody in solution. To address this need for a higher sensitivity assay, we hypothesized that as the FP signal obtained depends on the proportion of Protein G ligand which is bound (i.e. the largest FP signal is obtained when all of the probe is bound), then use of a lower concentration of FITC-Protein G ligand in the assay would yield maximal FP signal at lower rIgG analyte concentrations. Therefore, we compared assay performance using a range of probe concentrations from 1.4 μM to 0.225 μM. As shown in Fig. 5A, these data reveal that lower concentrations of Protein G ligand shift the analyte detection range significantly lower, such that at a Protein G ligand concentration of 31.25 nM, an assay linear dynamic range between 0.5 and 6 mg L^{-1} (assay CV< 10% at 1 mg L^{-1}) could be obtained.

However, use of lower amounts of Protein G ligand substantially reduced the ligand to background fluorescence ratio such that increased fluorescence of conditioned media (e.g. via metabolite leakage from cells, changes in composition) systematically influenced assay performance, increasing the measured FP signal (Fig. 5B) yielding artifactually elevated analyte measurements. This issue, not evident with the standard assay, was resolved by performing a subtraction of parallel and perpendicular background fluorescence intensity prior to calculation of the polarization signal (mP) for both samples. As shown in Fig. 5C, this correction entirely removed the effect of conditioned media fluorescence enabling accurate measurement of rIgG1 analyte concentration.

Assay format development

To further simplify the use of the assay, it was hypothesized that FITC-labeled Protein G ligand could be stably pre-coated and dried onto the microwell assay plate to enable simple reconstitution, sample addition and rapid, high-throughput measurement using a FP microplate spectrometer. We considered it of paramount importance that the ligand coating process supported rapid and consistent re-solubilization of ligand from the well surface to give precise FP measurements. To investigate this, plates were coated with FITC-labeled Protein G at 6 μg/well in the presence of a variety of solvents prior to air drying at 30 °C in a dehumidified chamber, specifically (i) 2 mM potassium phosphate (KPO_4), (ii) 0.384 g/L bovine serum albumin, (iii) phosphate buffered saline (PBS), (iv) deionized water and (v) 0.1% sucrose. Whilst all solvents exhibited acceptable performance after reconstitution in binding buffer (20 mM acetate buffer pH 5, 1.28 g/L BSA), followed by addition of CD-CHO media containing rIgG standards, KPO_4 gave on average the lowest plate to plate standard deviation (Fig. 6A), 0.38 mP, and was selected for convenience. To assess the shelf-life of air-dried

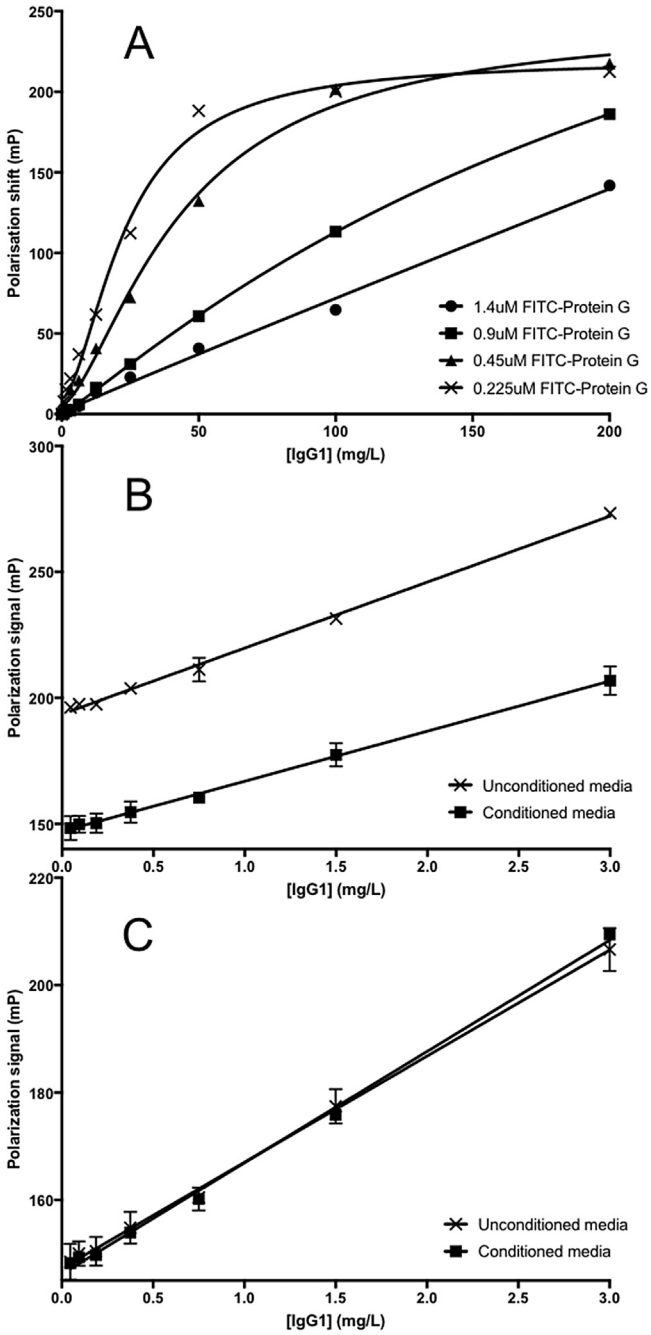

Fig. 5. Development of a high sensitivity FP assay. (A) FITC-Protein G ligand at varying final concentrations in a 60 μl volume were incubated for 30 min with a range of rIgG1 concentrations prior to measurement of polarization signal shift. (B) 60 μl FITC-Protein G ligand at a concentration of 31.25 nM was added to 60 μl of varying concentrations of IgG1 diluted in fresh (X) or 8-day conditioned CD-CHO media (■) prior to measurement of polarization signal shift. (C) As for (B) after subtraction of sample background fluorescence lacking Protein G ligand.

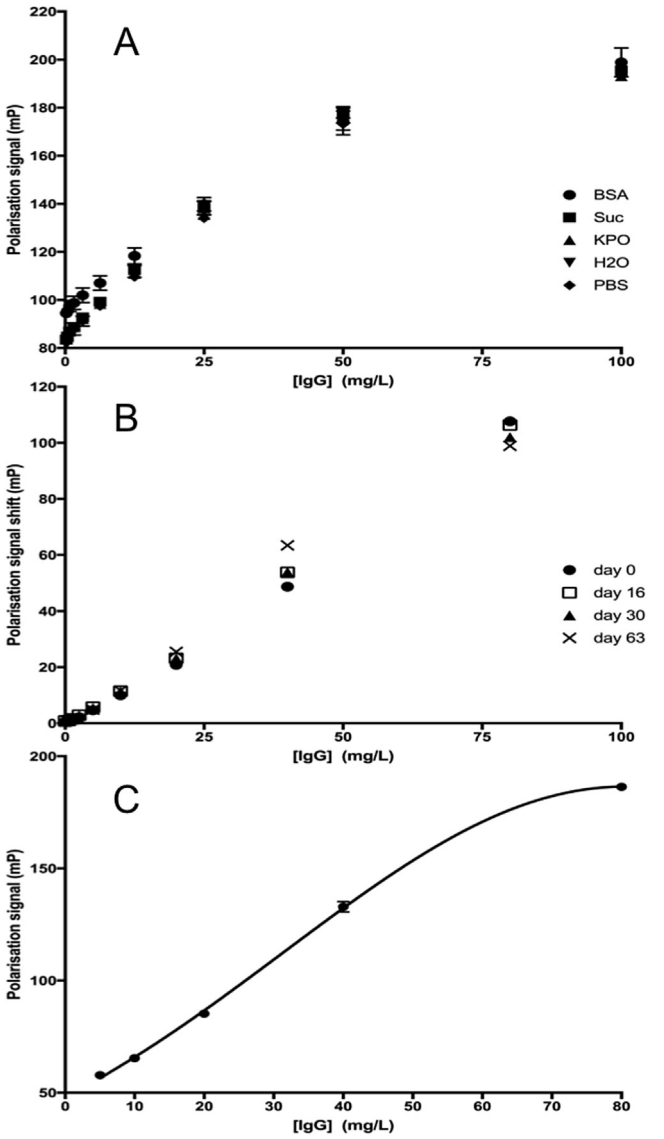

Fig. 6. Development and characterization of dried microplate assay format. (A) Comparative evaluation of FITC-Protein G ligand solvents. Microplates were coated with FITC-labeled Protein G at 6 μg per well in the presence of a variety of solvents (KPO_4 (▲), BSA (●), PBS (◆), deionized water (▼) and sucrose (■)) prior to air drying at 30 °C for 12 h in a dehumidified chamber prior to reconstitution with CD-CHO medium containing varying concentrations of rIgG1 and measurement of FP signal shift. (B) Accelerated stability study. Dried microplates containing FITC-labeled Protein G at 6 μg per well in KPO_4 buffer (as above) were stored at 37 °C for varying lengths of time prior to measurement of rIgG concentration by FP. (C) Microplate to microplate variance in measurement of rIgG1 concentration across triplicate microplates. Data are shown as the mean ± S.D.

microplates containing Protein G ligand in KPO_4 buffer, an accelerated stability study was performed. Microplates were stored at 37 °C for a period of 63 days and assay performance was evaluated throughout this period as described above (Fig. 6B). It was evident that microplate assay performance was not substantially altered. Calculation of chilled product shelf-life based on the Arrhenius law [17] predicted that assay plates would be stable for a period of at least 13 months at 4 °C. Finally, using the optimal coating process, an automated, robotic microplate preparation process was established to support manufacture of FP assay plates at scale and microplates were sampled to evaluate variance in assay performance. As shown in Fig. 6C, minimal variation between the assay microplates was observed. The standard deviation of signal within the plates was around 1 mP and the standard deviation between the plates was around 1.3 mP.

Conclusion

Through optimization of choice of protein ligand, fluorophore and peptide labeling conditions, we report the development of a

novel, rapid, simple and precise FP-based assay for titer measurements of IgG and Fc-containing derivatives. This assay has considerable advantages over alternative assays utilized to measure immunoglobulins, namely simplicity, economy, speed and high throughput. The FP assay is simple requiring no instrument set up and minimal sample preparation. The assay is considerably more economical than other high throughput assays and additionally as it takes <2 min to read 96 samples on the plate reader with 30 min sample preparation time, this is also an extremely rapid method for IgG quantitation. Moreover, given the simplicity of the assay, it is highly amenable to automation. Furthermore, we have adapted our assay to stable dried plate format for ease of use as a commercial assay.

'Quality by design' concepts are becoming increasingly important in biomanufacturing to gain control of critical quality attributes of the product [18]. This necessitates the ability to rapidly measure multiple bioprocess attributes simultaneously in a high-throughput manner. This is increasingly of importance in continuous biomanufacturing systems where the online monitoring of process attributes is vital to ensure consistent product quality. This study highlights the suitability of FP assays for use in bioprocess development and control due to the simplicity of assay development and the ease of use to enable measurement of molecular interactions (typically a specific ligand and a molecule of interest such as IgG, host cell protein, glycoform etc) in the liquid phase, to give instant quantitation of a molecule of interest with no need for washing, lengthy incubation periods or complex equipment. Additionally, the observed attributes of FP are mechanistically predictable which greatly facilitates planning and assessment of potential assay viability and subsequent assay optimization.

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