Analytica Chimica Acta 821 (2014) 54–61

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

A fluorescence anisotropy method for measuring protein concentration in complex cell culture media

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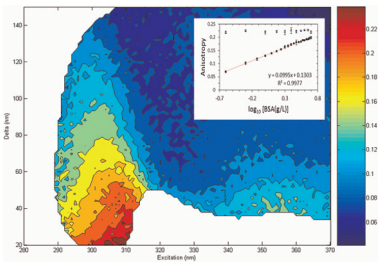
HIGHLIGHTS

- Quantitative method for monitoring the BSA content of complex cell culture media.
- Anisotropic emission of the large molecule protein is clearly separable from the small fluorophore emission.
- Has a limit of detection for BSA of 13.8 $\mu g\,mL^{-1}$ and a useable range of 0.1 to ~4 $mg\,mL^{-1}$.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 January 2014
Received in revised form 6 March 2014
Accepted 7 March 2014
Available online 12 March 2014

Keywords:
Fluorescence
Anisotropy
Total synchronous fluorescence spectroscopy (TSFS)
Bioprocess
Protein

ABSTRACT

The rapid, quantitative analysis of the complex cell culture media used in biopharmaceutical manufacturing is of critical importance. Requirements for cell culture media composition profiling, or changes in specific analyte concentrations (*e.g.* amino acids in the media or product protein in the bioprocess broth) often necessitate the use of complicated analytical methods and extensive sample handling. Rapid spectroscopic methods like multi-dimensional fluorescence (MDF) spectroscopy have been successfully applied for the routine determination of compositional changes in cell culture media and bioprocess broths. Quantifying macromolecules in cell culture media is a specific challenge as there is a need to implement measurements rapidly on the prepared media. However, the use of standard fluorescence spectroscopy is complicated by the emission overlap from many media components. Here, we demonstrate how combining anisotropy measurements with standard total synchronous fluorescence spectroscopy (TSFS) provides a rapid, accurate quantitation method for cell culture media. Anisotropy provides emission resolution between large and small fluorophores while TSFS provides a robust measurement space. Model cell culture media was prepared using yeastolate (2.5 $mg\,mL^{-1}$) spiked with bovine serum albumin (0 to 5 $mg\,mL^{-1}$). Using this method, protein emission is clearly discriminated from background yeastolate emission, allowing for accurate bovine serum albumin (BSA) quantification over a 0.1 to 4.0 $mg\,mL^{-1}$ range with a limit of detection (LOD) of 13.8 $\mu g\,mL^{-1}$.

1. Introduction

Macromolecule production using cell culture requires the use of complex nutrient media to maintain optimal physicochemical conditions for productive growth. Cell culture media have very diverse and complex chemical compositions. Despite the recent trend towards the use of chemically defined media, there are many systems where components like complex biogenic hydrolysates [1] or proteins [2] are key constituents added to improve cell growth.

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http://dx.doi.org/10.1016/j.aca.2014.03.007
0003-2670/

Most media for mammalian cell culture are complex mixtures of components with component concentrations ranging from ppm to 5–10% w/w, making comprehensive analysis challenging. However, the analysis and control of cell culture media variance is critical in industrial biotechnology. Conventional methods for media monitoring and specific component analysis consist of different hyphenated chromatographic techniques that make routine analysis time consuming and expensive [3,4]. For measuring protein concentration in media solutions (without purification), the only viable option is to utilise one of the specific colorimetric based assays (*e.g.* Lowry, Bradford, and BCA assays) [5]. Unfortunately, these methods require various degrees of sample manipulation and reagent addition, neither of which are desirable.

Vibrational spectroscopy has been used for monitoring compositional changes in cell culture media, but problems can arise with spectral resolution and weak analytical signals in solution [6–10]. This is particularly the case for the macromolecular constituents of media like insulin and serum albumin where concentrations are low, typically 1–10 $\mu g\,mL^{-1}$and 1–5 $mg\,mL^{-1}$ [11], respectively. Fluorescence spectroscopy is an attractive alternative for near real time on-line/at-line and non-destructive monitoring of biogenic process samples and raw materials quality control [12]. This is because some significant constituents of culture media are intrinsically fluorescent, like riboflavin, pyridoxine, and the amino acids phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr), and furthermore they can be detected at low concentrations (mg–$\mu g\,mL^{-1}$) [12–15]. However, the compositional complexity of cell culture media causes problems with fluorophore emission overlap. For example, the intrinsic fluorescence, of a protein like bovine serum albumin (BSA), is dominated by emission from Trp with lesser contributions from Tyr and Phe [16].

This overlap of media amino acid and protein emission makes protein quantification difficult using standard emission spectroscopy. The situation can sometimes be resolved by the use of multi-dimensional fluorescence (MDF) spectroscopy techniques like excitation emission matrix (EEM) or total synchronous fluorescence spectroscopy (TSFS) where one interrogates a much wider fluorescence emission space. In EEM measurements, a series of emission spectra are collected, each at a different excitation wavelength. The excitation wavelength changes incrementally so that a multi-dimensional plot of emission spectra *versus* excitation wavelength is obtained, the EEM ($\lambda_{ex} \times \lambda_{em} \times I_{\lambda ex,\ \lambda em}$) [17]. In TSFS (for example constant-wavelength mode), both excitation and emission monochromators are scanned simultaneously at the same rate with a fixed wavelength separation ($\Delta\lambda$). By sequentially collecting spectra over a range of $\Delta\lambda$ increments $\gtrsim$10 nm, one can generate a multi-dimensional TSFS spectrum ($\lambda_{ex} \times \Delta\lambda \times I_{\lambda em,\ \Delta\lambda}$) [18]. The key advantage of TSFS over EEM is the ability to easily avoid Rayleigh scatter artefacts for example when characterising very complex multi-fluorophore mixtures such as crude petroleum oils where light scatter can be a big problem [19]. One disadvantage with MDF-based methods is that data analysis of the complex data generally requires the use chemometric techniques such as multi-way PCA [20] or factor based methods like PARAFAC [21] or MCR [22].

Combining TSFS/EEM data with chemometrics provides a rapid method for the characterization of individual fluorescent components in complex mixtures such as cell culture media and bioprocess broths [12,15,23,24]. In the context of rapid quality control/assessment of protein containing media formulation, one needs to measure protein concentration rapidly and accurately during media preparation while simultaneously verifying correct gross composition. Gross composition can easily be assessed using EEM [12,23], and sometimes it is feasible to quantify specific fluorophores using a modified standard addition method [15]. However, this approach only works for a narrow analyte concentration range because of sensitivity to varying inner-filter/matrix effects as composition changes. Therefore, for the protein concentration measurements, we use another approach and exploit the intrinsic size (strictly speaking, the rotational correlation time) related information in the fluorescence emission.

Fluorescence anisotropy characterizes the polarization state of the emitted light, and this in turn can be related to the physical size and/or restricted movement of a fluorophore [25,26]. Polarization measurements can introduce a new dimension to regular MDF spectroscopy of complex materials that offers a new insight on the origin of the emitting fluorophore [27,28]. Components in complex mixtures can be differentiated by their hydrodynamic volume and rotational speed [29]. Fluorescence anisotropy as a technique has been applied to the study of protein interactions, protein structure, reaction kinetics that occur with changes in molecule rotational time, changes of local viscosities and protein distribution in cellular membranes [16,25,30–34]. However, the method has only rarely been applied to complex biogenic mixture analysis [35–38], and the use of MDF with anisotropy is not common. Polarized EEM fluorescence spectroscopy has been used to study multi-component fluorescent dyes mixtures and spin-coated polymeric thin films [39–41], but we are unaware of any application to biogenic systems.

This study showed how anisotropy incorporated into MDF measurements can accurately characterise and quantify protein in cell culture media. Fluorescence anisotropy discriminates between fluorescence emissions of small molecule media components and the large molecule protein component. Yeastolate (a complex hydrolysate) spiked with BSA was used as a realistic model cell culture media akin photophysically to those used in industrial biotechnology. BSA emission [42–44] overlaps completely with the complex emission of yeastolate which contains appreciable amounts of both Trp and Tyr [12,24].

2. Materials and methods

2.1. Materials

Bacto™ TC and Difco™ Yeastolate (the water soluble portion of autolyzed yeast) was purchased from BD. Bovine serum albumin (>99%), Rhodamine B (Rh-B), glycerol (99.5%), phosphate buffer saline (PBS) tablets, and Bradford reagent were purchased from Sigma–Aldrich. All materials were used without further purification. Stock yeastolate solution (5 $mg\,mL^{-1}$) was prepared by first dissolving 1.25 g yeastolate in sterilized high purity water (250 mL) and then membrane filtering (0.22 μm) before aliquotting under sterile conditions. A sterile, filtered BSA stock solution (10 $mg\,mL^{1}$) was also prepared. 80 different mixture samples were prepared by spiking 2 mL of the yeastolate solution with BSA aliquots varying from 0 to 2 mL. Samples were brought to a final volume of 4 mL by PBS buffer addition. The sample set had a BSA concentration range of 0–5 $mg\,mL^{-1}$ with a constant yeastolate concentration of 2.5 $mg\,mL^{-1}$. All samples were prepared in triplicate, and reproducibility was determined at 0.5 and 4 $mg\,mL^{-1}$ BSA by preparing each sample 10 times. A second sample set was prepared in the same manner using PBS buffer in place of the yeastolate solution. All samples were stored at −70 °C to prevent compositional changes in yeastolate and left to defrost overnight prior to analysis. These were then pipetted into 1 cm pathlength quartz cuvettes (Lightpath Optical, UK) and sealed under sterile conditions. The same samples were also analysed using the Bradford assay with an excess of 30 parts reagent to one part sample, according to the product technical bulletin (Sigma, Bradford Reagent Technical Bulletin, Product Number B 6916). Samples were analysed almost immediately after addition of Bradford reagent directly into the quartz cuvettes.