Real-Time Detection of Monoclonal Antibodies in Protein A Breakthrough and in Process Samples

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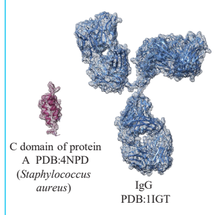
Introduction

Measurement of antibody concentrations is ubiquitous in biopharmaceutical process development and manufacturing. Purification of therapeutic monoclonal antibodies (mAbs) usually involves a protein A affinity capture step. Because column breakthrough of antibody in complex, UV-absorbing culture fluid cannot be readily detected in real time, processes are conservatively designed and column capacity often is underutilized, wasting adsorbent and reducing productivity.



We have developed a fluorescence-based monitoring technology which allows mix-and-read mAb detection in cell culture fluid (*Ref* 9), which may be useful in at-line assays and in clone and culture development, and here report the use of reporters immobilized on agarose monolith supports for continuous detection of IgG in column breakthrough. The agarose monolith structure was optimized using residence time distribution measurement across a range of cooling rates. Column effluent was continuously contacted with immobilized fluorescein-labeled Fc-binding ligands to produce an immediately-detectable shift in fluorescence intensity. The technology allows rapid and reliable monitoring of IgG in a flowing stream, without prior sample preparation. We observed significant shifts in fluorescence intensity at 0.05 g/L human IgG, sufficient to detect 5% breakthrough of a 1 g/L load within 4 minutes or 8 CV of the monolith at a flow rate of 0.5 mL/min. The fluorescence intensity response at different load concentrations was used to calibrate fluorescence intensity with IgG concentration.

Fluorescence Reporting

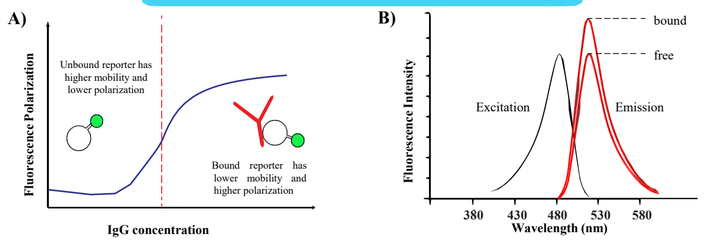


Figure 1. (A) **Fluorescence polarization**. Fluors are attached to Fc-binding ligands. When unbound, these reporters rotate faster and emit depolarized light. Larger molecules, such as antibodies, rotate slowly. The binding of the low molecular weight ligands to antibodies results in high fluorescence polarization. (B) **Fluorescence intensity**. Excitation (black) and emission (red) spectra of fluorescein. The typical fluorescence intensity of unbound affinity reporter is indicated by free and enhanced fluorescence after affinity reporter binds to IgG is indicated by bound.

Fc-binding Reporters

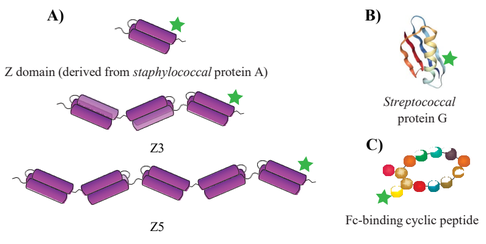


Figure 2. Affinity ligands used in this study. (A) Z domain derived from *staphylococcal* protein A *(Ref 7)*, trimer (Z3) and pentamer (Z5) are polymerized forms of the Z domain. (B) IgG-binding domain from *streptococcal* protein G (PDB: 2IGG). (C) 13-mer Fc-binding cyclic peptide *(Fc-III, Ref 8)*.

Batch IgG Detection

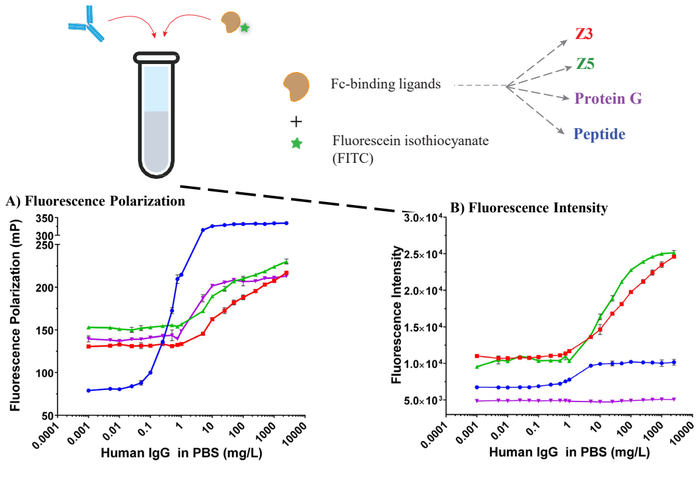


Figure 3. The FITC-labeled Fc-binding ligands (50 nM) were tested against increasing IgG concentrations. (A) Shift in fluorescence polarization was observed for all the Fc-binding ligands. (B) Interestingly, a significant increase in fluorescence intensity was observed for Z3 (■), Z5 (▲) and peptide (●).

Binding Site on IgG-Fc

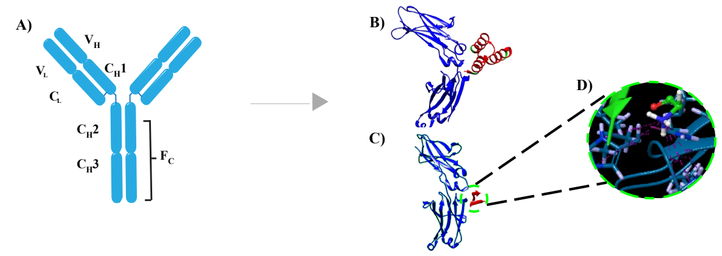


Figure 4. IgG complex with Fc-binding ligands. (A) IgG structure with protein A binding site, Fc (C_H2-C_H3). (B) The B-domain from protein A is a three-helix bundle (PDB: 5U4Y; 59 AAs); Z-domain is variant of the B-domain with Ala/Val at the N-terminus and a Gly/Ala substitution in Helix 2. (C) Fc-III peptide in complex with the Fc-region at the C_H2-C_H3 domain interface (PDB: 1DN2; 13 AAs). X-ray structure of the peptide bound to Fc suggests that the FITC attached to the N-terminus of the peptide by an aminocaproic acid linker is in proximity to His 433, which we speculate could affect its emission intensity in the bound state.

IgG Detection in Cell Culture Fluid

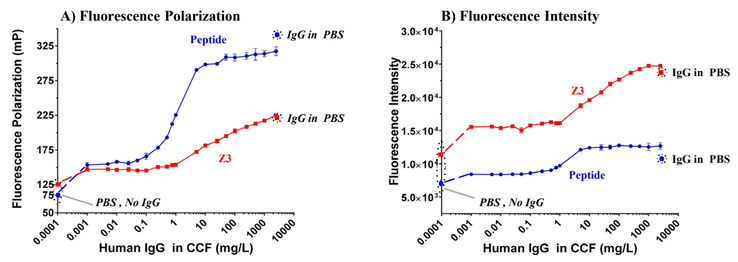
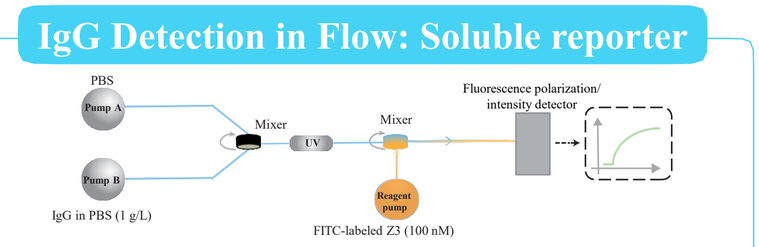


Figure 5. Human IgGs were spiked in Murine-Ab-depleted hybridoma culture fluid (IMDM with 10% FBS). The concentration of each Fc-reporter was 50 nM. Phenol red in the culture medium increases the level of background fluorescence. Presence of cell culture fluid does not significantly affect the human IgG-induced shift in fluorescence polarization (A) and fluorescence intensity (B).

IgG Detection in Flow: Soluble reporter



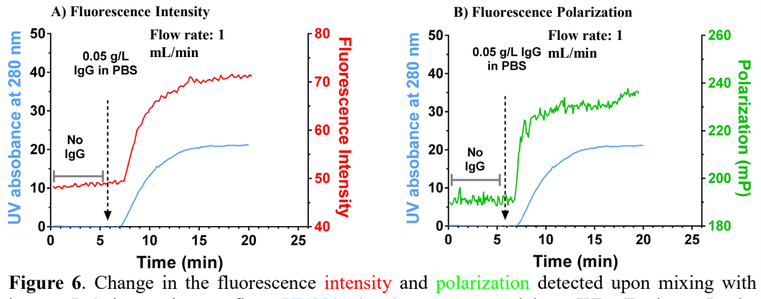
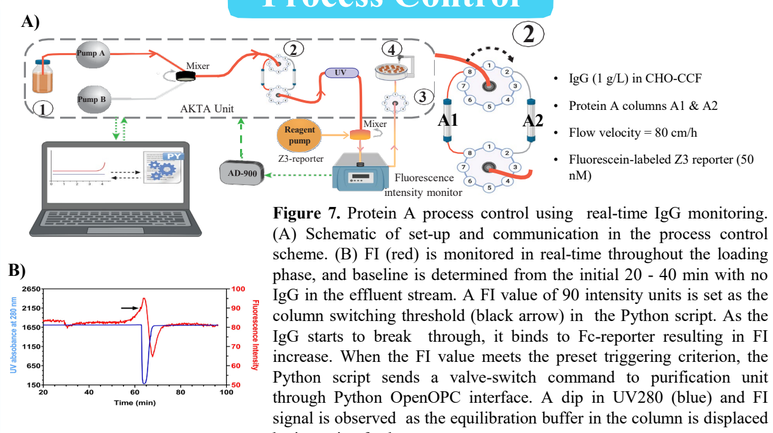


Figure 6. Change in the fluorescence intensity and polarization detected upon mixing with human IgG in continuous flow. UV280 absorbance measured by AKTA Explorer. In the absence of IgG in the flow (region before the dashed arrow), the fluorescence intensity (red) and polarization (green) remain low. Fluorescence intensity and polarization increase as the IgG-rich stream flows through the system. The polarization detector is situated downstream of the intensity detector. The system can detect 50 mg/L IgG in a flow of 1 mL/min. The dashed arrow indicates the start of IgG-rich stream.

Process Control



- IgG (1 g/L) in CHO-CCF
- Protein A columns A1 & A2
- Flow velocity = 80 cm/h
- Fluorescein-labeled Z3 reporter (50 nM)

Figure 7. Protein A process control using real-time IgG monitoring. (A) Schematic of set-up and communication in the process control scheme. (B) FI (red) is monitored in real-time throughout the loading phase, and baseline is determined from the initial 20 - 40 min with no IgG in the effluent stream. A FI value of 90 intensity units is set as the column switching threshold (black arrow) in the Python script. As the IgG starts to break through, it binds to Fc-reporter resulting in FI increase. When the FI value meets the preset triggering criterion, the Python script sends a valve-switch command to purification unit through Python OpenOPC interface. A dip in UV280 (blue) and FI signal is observed as the equilibration buffer in the column is displaced by incoming feed.

Immobilized Reporters for Continuous IgG Monitoring

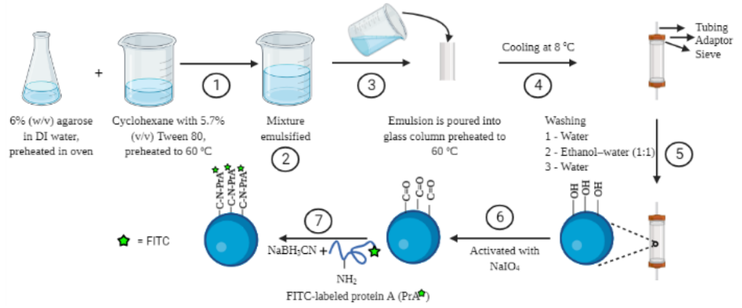


Figure 8. Monolith preparation and activation, and immobilization of FITC-labeled protein A ligand on agarose monolith. In step 1, equal volumes of preheated 6% (w/v) agarose in DI water and cyclohexane with 5.7% Tween 80 (v/v) were mixed. In step 2, the emulsion was created by vigorous mixing. In step 3, the emulsion was poured into the glass column. In step 4, agarose rods were solidified at 8 °C. After 5 minutes, the glass column was fitted with a flow distributor sieve, adaptor, and tubing. In step 5, the organic phase was removed by pumping water, ethanol-water (1:1, v/v), and finally degassed water through the column. In step 6, the monolith surface was activated with sodium periodate, followed by water and 1x PBS washes. In step 7, the activated surface was conjugated with FITC-labeled protein A and the Schiff base was reduced with sodium cyanoborohydride.

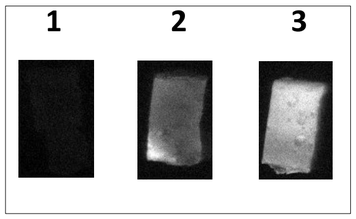


Figure 9. Visualization of reporter loading. Immobilization of FITC-labeled protein A on agarose monoliths was visualized by UV excitation and imaging under the ethidium bromide emission filter. 1. control, no loading of reporter, 2. batch mode, and 3. frontal loading. In the batch mode, the reporter is introduced in the monolith and Schiff base reaction was allowed to happen overnight. In the frontal loading, the reporter was continuously introduced in the monolith at a very slow flow rate till breakthrough was complete.

Cooling temperature	Residence time (sec)	Number of theoretical plates/m	Asymmetry factor
8 °C (faster cooling)	19.1	153.3	1.5
	13.4	164.4	1.8
	8.9	215.0	1.8
Room temperature	19.1	4.9	5.3
	13.4	0.4	4.8
	8.9	5.0	4.1

Table 1. Effect of agarose cooling rate (step 4, Figure 8) on NaCl residence time distribution and asymmetry. Agarose cooling was carried out at room temperature and at 8 °C.

IgG Detection in Flow: Immobilized reporter

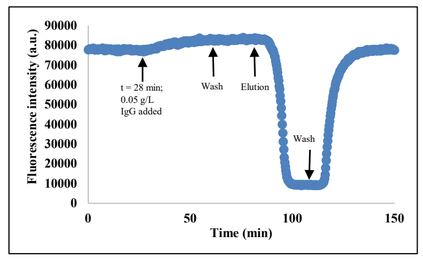


Figure 10. Detection of 5% breakthrough of 1 g/L IgG concentration using fluorescence intensity. Time course of the fluorescent intensity of the FITC-labeled protein A immobilized on the agarose monolith. IgG concentration of 0.05 g/L in 1x PBS introduced into the monolith starting at 28 min. Elution was done with glycine (100 mM, pH 3.5) and washing was done with 1x PBS.

Conclusions

- Fluorescence polarization and intensity approaches can be used to detect process-relevant levels of targets in column breakthrough and in batch samples.
- Fluorescence intensity is especially attractive due to the availability of inexpensive commercial detectors, and simpler automation.
- Fluorescence polarization is very generally applicable, including in the absence of intensity-reporting ligands, and may also be applicable to viruses, such as lentivirus - levels in cell culture fluid.
- Fluorescence intensity shows significant increase (7% from the baseline value) after loading 5% breakthrough of 1 g/L IgG concentration. Further work needs to be devoted to reducing the fluorescence baseline variability and to monolith regeneration.

Acknowledgements

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