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Antibody mix-and-read assays based on fluorescence intensity probes

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ABSTRACT

Antibodies and Fc fusion proteins are a rapidly growing class of pharmaceuticals. Cell culture and purification process development and operation require frequent measurement of product concentrations, commonly by complex enzyme-linked immunosorbent assay and high-performance liquid chromatography methods. Here we report a fast (<30 s), and simple antibody Fc assay based on mix-and-read reporting by fluorescence emission. A soluble fluorescein-labeled Fc-affinity reporter produced by standard peptide synthesis is mixed with an Fc-containing sample to produce an immediate shift in both fluorescence polarization and intensity, compatible with on- and at-line measurements and microbio-reactor monitoring. We observed significant shifts in fluorescence intensity in Chinese hamster ovary cell culture fluid spiked with IgG and detected an adalimumab biosimilar down to 100 ng/mL (10^{-4} g/L), despite the interferents in the complex sample matrix. Neither the fluorescence polarization nor the fluorescence intensity assay is significantly affected by the addition of clarified lysate of 2 million CHO-k1 cells/mL, suggesting applicability even to cultures of low viability. Biochemical and molecular docking approaches suggest that the fluorescence intensity enhancement is caused by changes in the fluorophore's local microenvironment upon binding to IgG Fc, especially by interactions with Fc His433.

Abbreviations: CCF: Cell Culture Fluid; CHO: Chinese Hamster Ovary cells; ELISA: Enzyme Linked Immunosorbent Assay; Fc: Fragment Crystallizable of antibody; HPLC: High-Performance Liquid Chromatography; HP β CD: hydroxypropyl- β -cyclodextrin; IgG: ImmunoglobulinG; mAb: Monoclonal Antibody; PBS: Phosphate-Buffered Saline; PDB: Protein Data Bank; SpA: Staphylococcal protein A; SpG: Staphylococcal protein G.

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Introduction

Currently, 67 US FDA-approved monoclonal antibody (mAb) therapeutics represent *ca.* 60% of the total biopharmaceutical market.^{1,2} These complex biomolecules are challenging to produce at large scale, requiring elaborate process development. Antibody concentrations in culture fluid and process intermediates are most commonly measured by methods based on surface capture, such as enzyme linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), surface plasmon resonance and biolayer interferometry.³ These methods often require substantial method development and elaborate equipment, and have limitations of turnaround time, throughput, and/or ease of use. High-throughput approaches are increasingly used in mAb clone selection, cell culture optimization, and purification process development to reduce time to clinic, and rapid analytical methods are required to keep pace with the dynamic requirements of the bioprocessing environment. A simple, fast, mix-and-read assay based on simple instrumentation and reagents could therefore simplify and speed antibody process development.

Here we introduce a simple, rapid antibody assay based on homogenous, separation-free reporting of antibody crystallizable fragment (Fc) concentrations by fluorescence emission. Fluorescence-based homogenous detection methods report a target binding-induced change in the size of a protein-ligand complex (fluorescence polarization) or a change in fluorescence emission lifetime or intensity.^{4–8} Fluorescence-based methods are extensively used to characterize protein-protein and protein-ligand interactions.^{9–14} Recently, we showed the applicability of fluorescence-based methods in near real-time detection of antibodies in chromatographic separations.¹⁵

Here, we use a novel antibody-specific fluorescent affinity ligand to rapidly (<30 seconds) and quantitatively detect antibodies without separation. All aspects of the approach are parallelizable and automatable, and compatible with typical bioprocess development workflows. We also demonstrate its resistance to interference by complex sample matrices such as cell culture fluid. Finally, we use biochemical and molecular modeling approaches to investigate the structural basis of the observed response to Fc.

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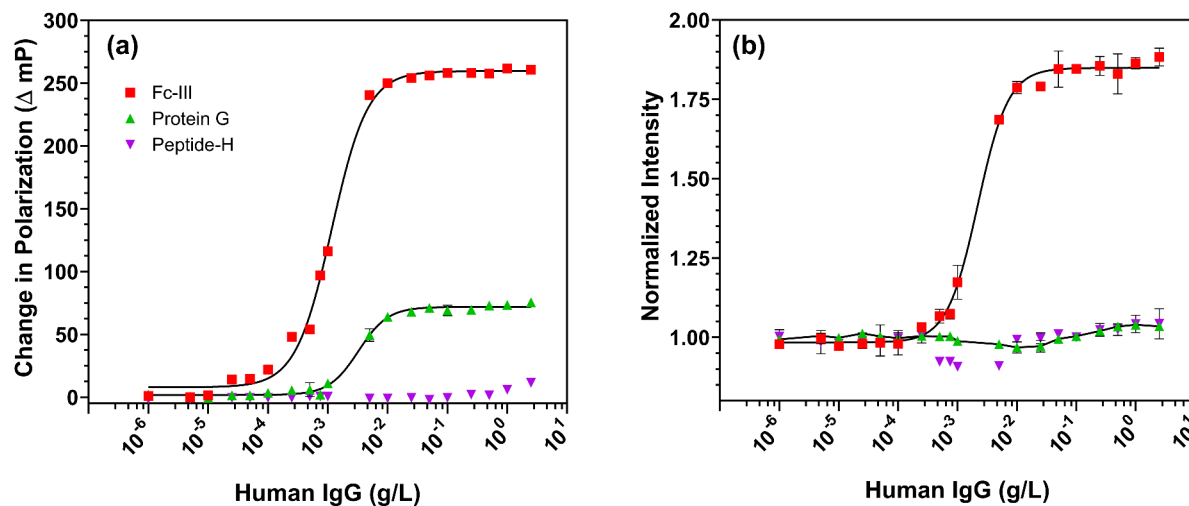


Figure 1. Change in fluorescence polarization (a) and intensity (b) of affinity probes Fc-III (red square), protein G (green triangle) and peptide-H (magenta inverted triangle) as a function of increasing IgG concentration. Error bars indicate standard deviation around the mean and are often smaller than the symbol. The solid black lines for Fc-III and protein G are a 4-parameter logistic fit. The normalized intensity is the fold change calculated by dividing the intensity by intensity of the probe alone (without IgG).

Results

Affinity probes for IgG

The candidate fluorescent IgG reporters Fc-III (2 kDa), protein G (SpG, 21.6 kDa) and peptide-H (1.3 kDa) were evaluated by measuring the change in fluorescence polarization or intensity of the probe (50 nM) as human IgG concentration was varied from 0 to 2.5 g/L (Figure 1). At low IgG concentrations, unbound probes rotate rapidly in solution and randomize orientation while in the excited state, emitting with low polarization. At higher IgG concentrations the probes bind to IgG, and the probe-IgG complex (>150 kDa) rotates slowly in solution, leading to polarized emission. As expected, the fluorescence polarization values of all probes increased with IgG concentration (Figure 1a). The Fc-III probe showed the highest polarization dynamic range, with a 260 mP difference between bound and free probe (Δ mP). The dynamic range for SpG was 75 mP; peptide-H did not reach saturation at the highest IgG

concentration offered (2.5 g/L), though an increase of 11 mP was seen. The lowest IgG concentration required to produce half-maximal response for Fc-III, SpG and peptide-H was 0.001 g/L, 0.005 g/L and 1 g/L, respectively. Although Fc-III and SpG have been reported to have comparable binding affinity for IgG, Fc-III gave a 3.5-fold larger polarization dynamic range when compared to SpG, presumably because of its low molecular weight. Notably, substantial changes in polarization were observed down to 1–10 mg/L IgG, suggesting excellent limit of detection.

Unexpectedly, labeled Fc-III fluorescence intensity increased strongly with increasing IgG concentration. As shown in Figure 1b, Fc-III fluorescence intensity increased *ca.* 1.9-fold between 0 and 2.5 g/L IgG, with most of the increase occurring below 10 mg/L IgG. The fluorescence intensities of SpG and peptide-H, by contrast, increased by less than 5% even at 2.5 g/L IgG. IgG binding-induced fluorescence enhancement for fluorescein-labeled protein A (SpA) has

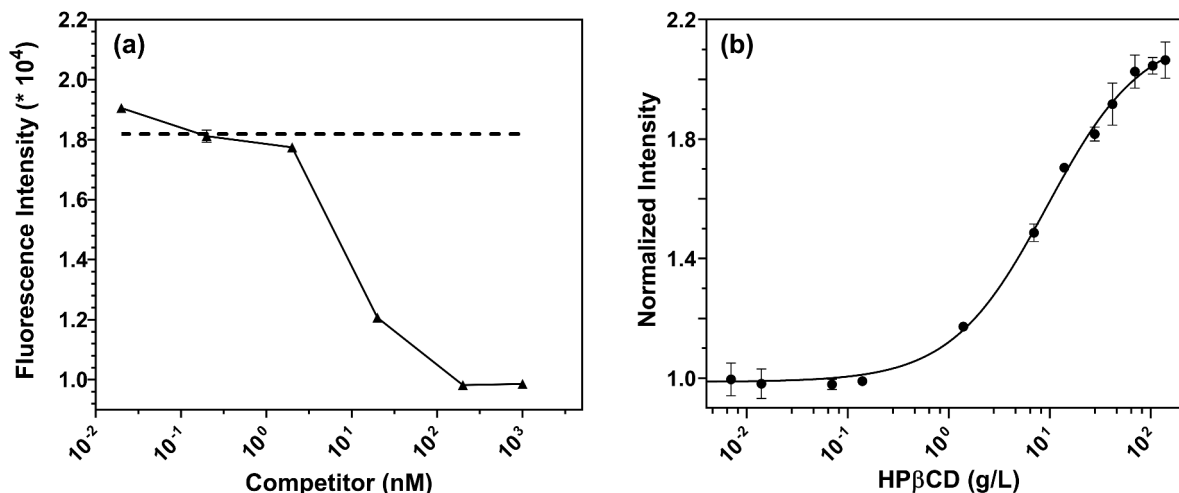


Figure 2. (a) Competitor SpA reduces fluorescence intensity of IgG-Fc reporter mixtures. Serial dilutions of unlabeled SpA were prepared in PBS buffer. A mixture of human-IgG (33 nM, 0.005 g/L) and Fc-III probe (50 nM, 0.00001 g/L) was mixed with competitor SpA at final concentrations from 0.02 nM to 1 μ M followed by measurement of fluorescence intensity. The average fluorescence intensity in absence of competitors is indicated by dashed line. The solid line indicates intensity measurements with the unlabeled competitor, SpA. (b) Increased fluorescence intensity of Fc-III probe with increasing concentrations of HP- β -cyclodextrin.

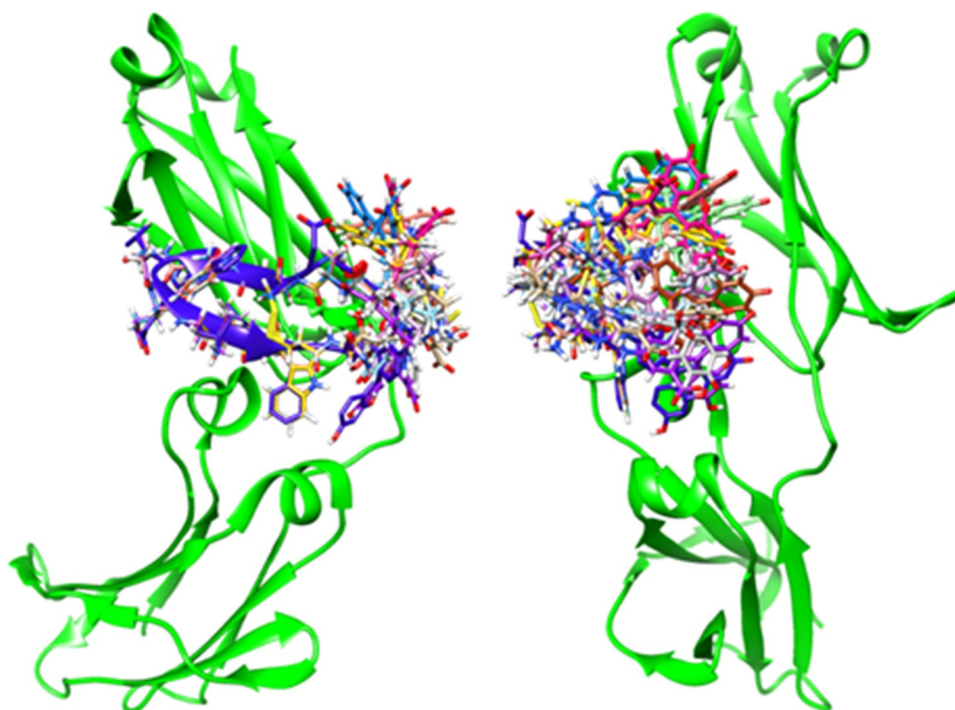


Figure 3. Ensemble of AutoDock Vina-predicted positions of fluorescein-labeled Fc-III bound to IgG-Fc (Left: front view; Right: side view).

previously been reported.^{16,17} Each SpA molecule contains 60 lysine residues, making site-specific labeling of SpA using amine-reactive fluorescent dyes challenging. Small, synthetic peptides like Fc-III offer chemical homogeneity, site-specific labeling, scalability and reliability of supply, and potentially cost advantages.

To confirm the specificity of the Fc-III-IgG interaction, we evaluated the effect of competing SpA (which binds to the same location on Fc as Fc-III) on the change in fluorescence intensity of Fc-III/IgG mixtures. **Figure 2a** shows that the emission intensity of a mixture of IgG (0.005 g/L; 30 nM) and Fc-III probe (0.0001 g/L; 50 nM) is markedly diminished by the presence of unlabeled SpA. With 500 nM SpA, the fluorescence intensity of the Fc-III/IgG mixture fell to that of free Fc-III probe (50 nM). These results indicate that the observed intensity enhancement results from specific interactions between IgG and Fc-III.

The sensitivity of fluorescein emission to perturbations in the local environment is well documented.^{18–21} It is possible that the fluorescence intensity enhancement we observed could result from relocation of the fluorophore into a relatively hydrophobic environment upon binding. To investigate this, we evaluated the effect of an inclusion agent, hydroxypropyl- β -cyclodextrin (HP β CD),^{22–26} which presents a hydrophobic cavity surrounded by hydrophilic surface.²⁶ For the Fc-III probe (50 nM), the addition of HP β CD resulted in a 2.1-fold increase in fluorescence intensity (**Figure 2b**), a value comparable to the 1.8-fold increase observed during the Fc-III/IgG interaction. Such cyclodextrin-induced fluorescence enhancement of fluorescein has been reported previously²⁷ and suggests the involvement of the fluorescein label in the binding interactions.

Molecular docking analysis

AutoDock Vina and Schrödinger Glide were used together for more reliable prediction of the mode of binding of the Fc-III probe to IgG-Fc. The docking score and the binding interactions of the docked poses were used to select biologically reasonable binding poses. The AutoDock Vina results predict that the Fc-III peptide moiety of the fluorescein-labeled Fc-III binds to the same portion of the IgG as seen in the peptide-Fc complex crystal structure, with a root-mean-square deviation of about 1 Å. Due to the flexibility of the linker, there are multiple predicted binding orientations of the fluorescein (**Figure 3**).

The specific interactions in the two top-ranked Schrödinger Glide docking predictions of the labeled Fc-III/IgG-Fc complex are shown in **Figures 4 and 5**. The interactions between the Fc-III peptide and the IgG-Fc are similar to those seen in the crystal structure.²⁷ Several hydrogen bonds are formed between the Fc-III peptide and IgG-Fc residues Ile253, Ser254, Arg255, Glu380, His433, Asn434 and Tyr436, while Arg255, Glu380 and Glu382 form three salt bridges with the ligand. The interaction of the fluorescein with amino acid residues on IgG is primarily driven by the affinity between Fc-III and IgG-Fc. Residues Glu430 and Leu432 form two hydrogen bonds with the fluorescein structure. Pi-Pi stacking was also found for residue His433 with the xanthene moiety of fluorescein. The surrounding hydrophobic residues form hydrophobic interactions with the ligand. The specific interactions of the fluorescein with IgG-Fc residues may affect the fluorescence polarization, and especially intensity. In addition, in the free fluorescein-labeled Fc-III probe, the proximity of the adjacent tryptophan residue to the fluorescein could reportedly induce quenching.^{28,29} Given the flexible

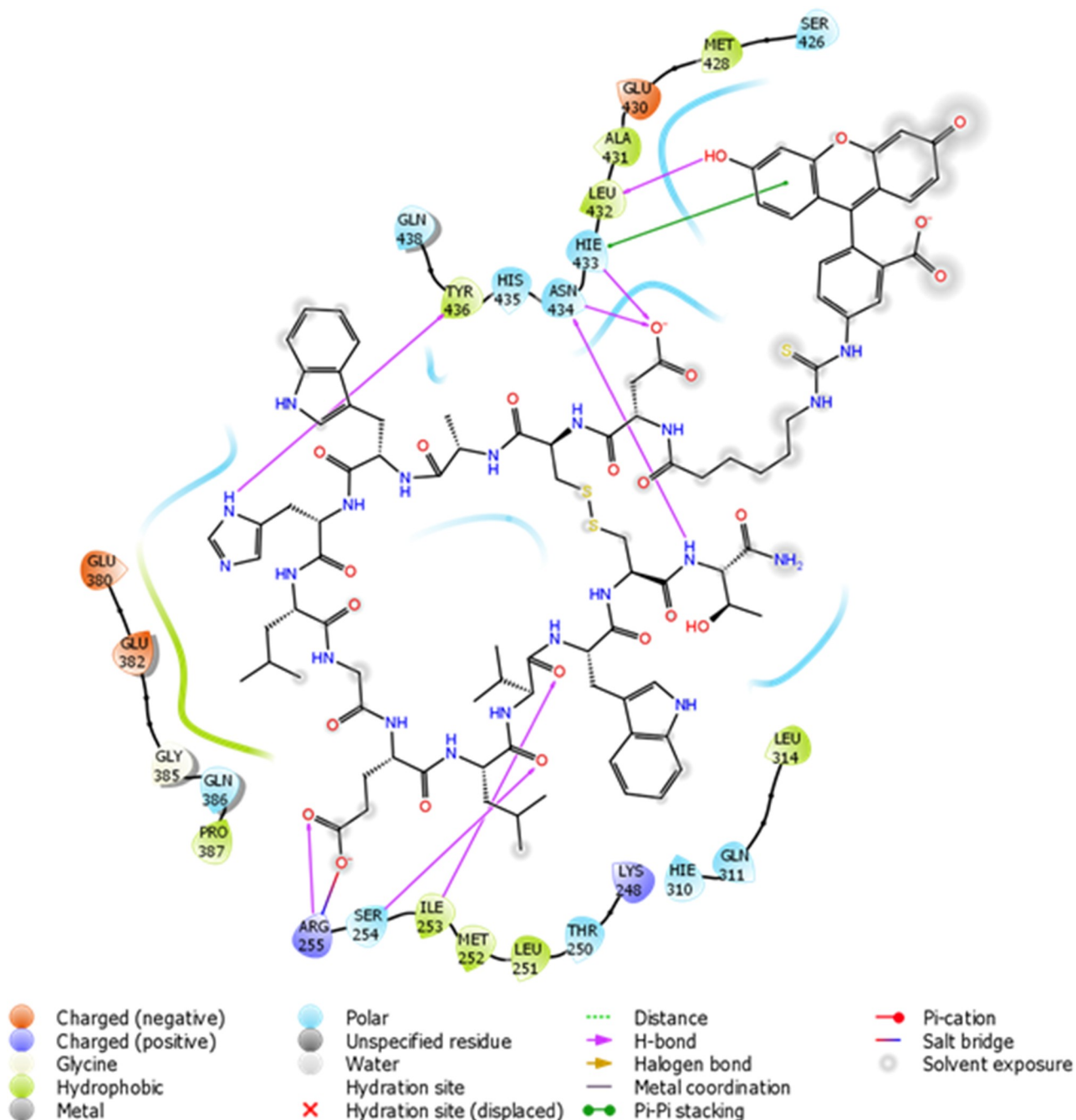


Figure 4. Intermolecular interactions in the highest-ranked Schrödinger Glide prediction of binding of fluorescein-labeled Fc-III to IgG-Fc.

(aminohexanoic acid) linker between fluorescein and the N-terminus of Fc-III, Fc-III/IgG binding may involve reorientation of the fluorophore away from tryptophan. Interestingly, the IgG-binding domains of SpA are devoid of tryptophan,³⁰ yet the fluorescein labeled SpA shows similar changes in fluorescence intensity as reported here upon binding to IgG,^{16,17,31} suggesting a limited role of tryptophan-induced quenching. The competition and molecular docking studies substantiate the involvement of the fluorophore in the binding interactions. In addition, the Fc-III and HP β CD, experiments provide evidence of the hydrophobicity-dependent change in fluorescence intensity.

Assay performance

The effect of Fc-III probe concentration on IgG detection limit was evaluated (Figure 6). As shown in Figure 6a, Fc-III probe concentrations of 25 nM – 500 nM gave similar fluorescence polarization changes from 0 to 2.5 g/L IgG, with the half-response IgG concentrations varying from 0.0005 to 0.01 g/L depending on probe concentration (Fig. S1). Figure 6b shows fluorescence intensity responses for peptide concentrations of 25 nM to 500 nM. At 25 nM Fc-III, the lowest concentration of IgG (0.00025 g/L) showed a 1.16-fold increase in fluorescence intensity, while 0.005 g/L IgG was required to show an

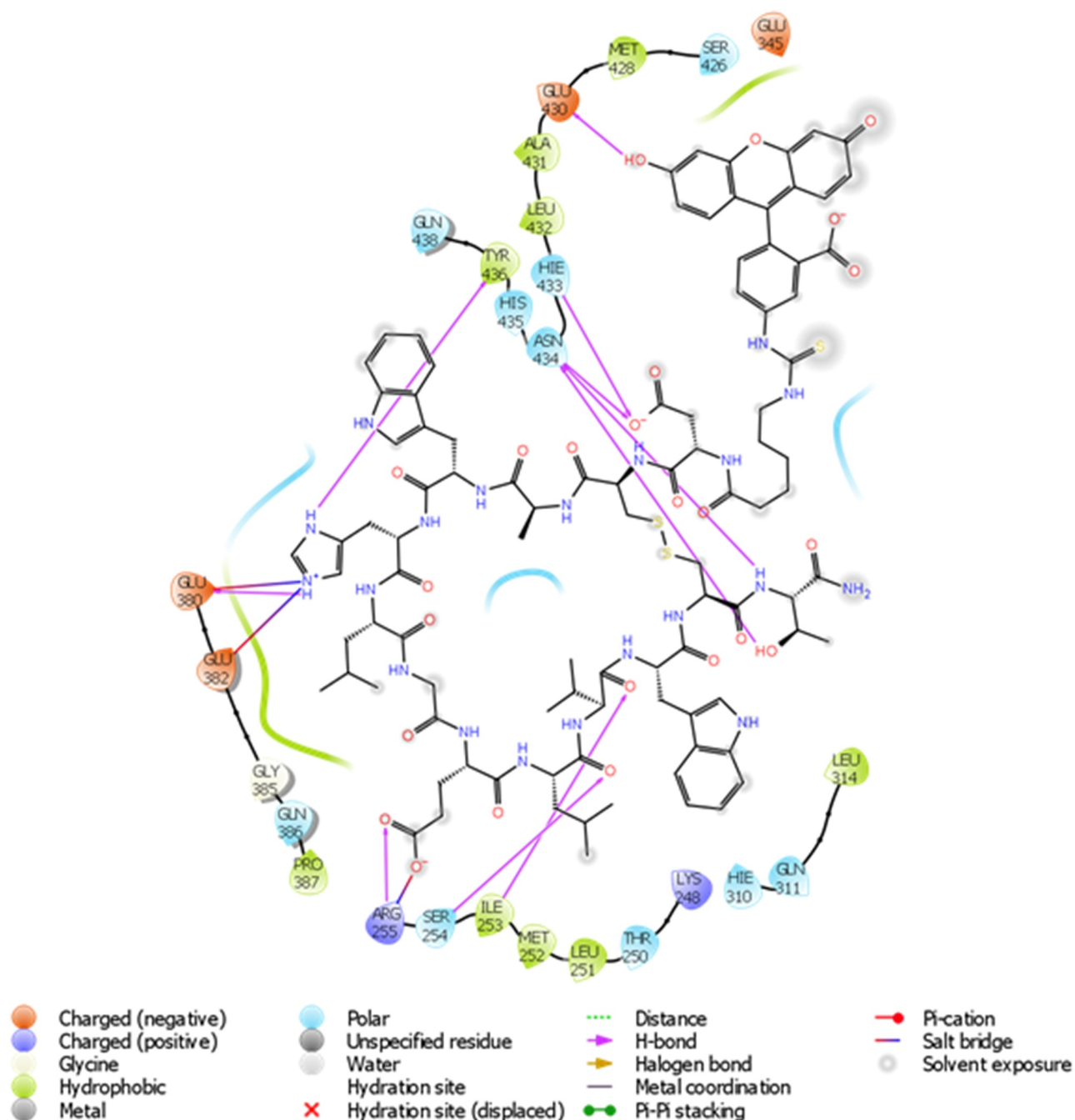


Figure 5. Intermolecular interactions in the second-highest-ranked Schrödinger Glide prediction of binding of fluorescein-labeled Fc-III to IgG-Fc.

equivalent fold increase for 500 nM Fc-III probe. Notably it can be observed that the dynamic range for the IgG-assay is 50 times wider, 0.0001 to 2.5 g/L, at 25 nM of probe.

Such reporter concentration optimization is commonly used during immunoassay development and calibration.^{32–34} The linear quantitation range of the assay can be adjusted by changing probe concentration, suggesting it could be adapted to a range of applications ranging from clone selection to process development and process analytical technology. As shown in [Figures 6C](#), 50 nM Fc-III gave a linear response to IgG concentrations of 0.0001 g/L to 0.001 g/L, while at 2000 nM Fc-III the linear range of the assay shifted to 0.01 g/L – 0.1 g/L.

IgG detection in cell culture fluid

The Fc-III IgG assay was tested in realistic bioprocess samples to identify any sample matrix effects. Cell culture fluid (CCF) from the null Chinese hamster ovary (CHO)-S cell line served as a complex, realistic sample matrix.^{35–37} CHO-CCF contains over 6,000 impurities, including host cell proteins, nucleic acids, and lipids, originating from the media and from cell secretion and lysis during normal cell death and shear stress during harvest.^{38,39} The background fluorescence of the CHO-CCF used in this study was comparable to 25 nM of Fc-III probe. The use of a higher Fc-III probe concentration (100 nM) was sufficient to overcome background fluorescence from the CHO-CCF. The resultant

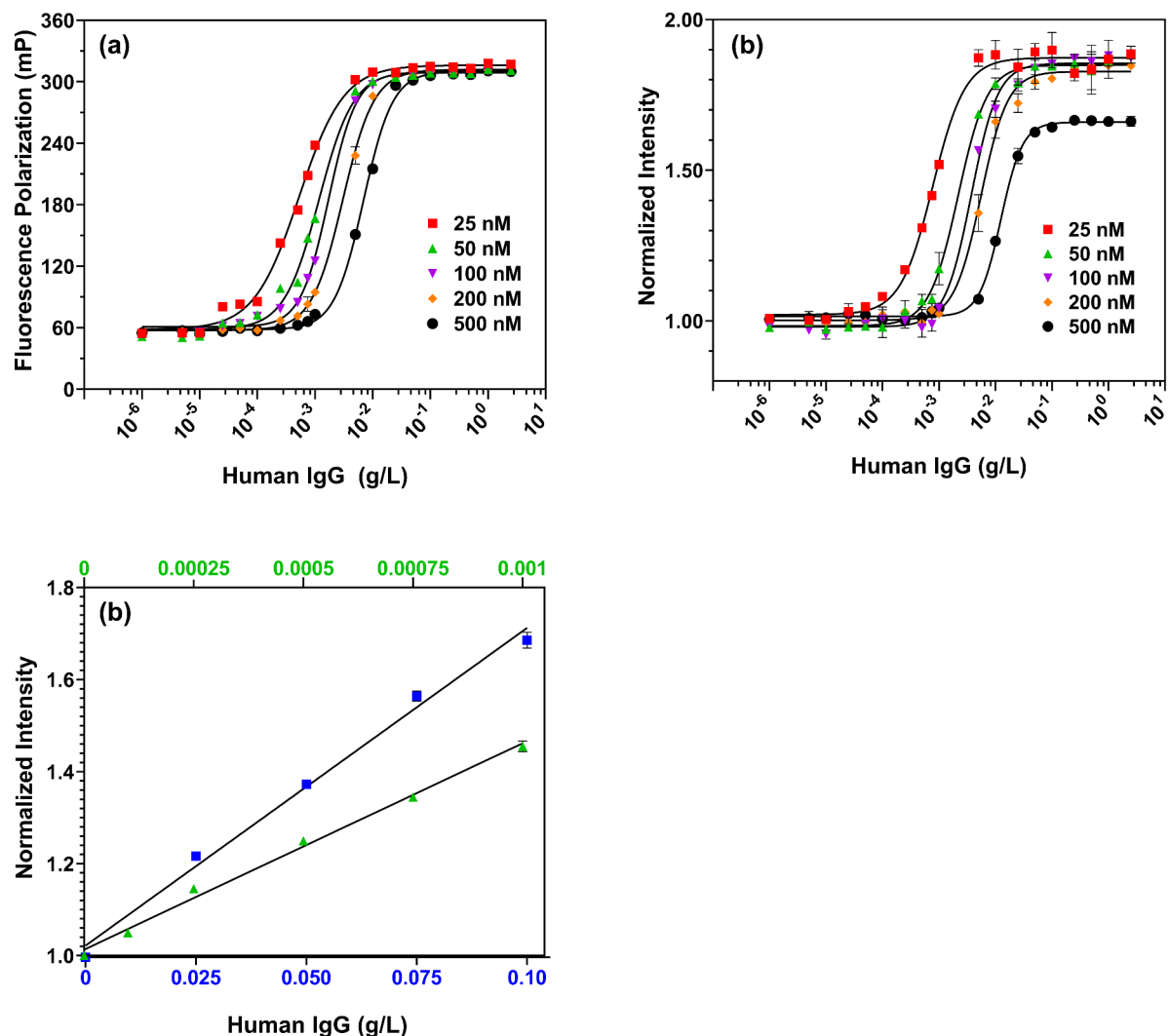


Figure 6. Fluorescence polarization (a) and intensity responses (b) for different concentrations of Fc-III probe (25–500 nM) to IgG of varying concentrations (0–2.5 g/L). (c) Quantitation of low (10^{-4} – 10^{-3} g/L; top x-axis) and high (0.025–0.1 g/L; bottom x-axis) IgG concentrations with 50 nM (triangle; top axis), and 2000 nM (square; bottom axis) fluorescein labeled Fc-III.

fluorescence intensity of the free Fc-III probe at 100 nM in CHO-CCF was about three times the background fluorescence signal. As shown in Figures 7, 100 nM Fc-III probe showed nearly identical fluorescence polarization changes for polyclonal human IgG and adalimumab biosimilar in CCF. In this case, an IgG concentration of 0.00075 to 0.001 g/L was required for half-maximal polarization response, and the fluorescence intensity of Fc-III probe increased by ~1.8-fold with 2.5 g/L adalimumab (IgG1 mAb) and human IgG. The observed limits of detection (signal > blank intensity + 3StdDev of blank) by fluorescence intensity were 0.0001 g/L for adalimumab biosimilar and 0.00025 g/L for human IgG (a heterogeneous mixture of IgG subtypes 1–4 that binds Fc-III less well than pure IgG1).^{40,41} As a control for possible interference with the assay by soluble components liberated by cell lysis during culture or harvest, centrifuged CHO cell lysate prepared as described below was added to cell culture fluid containing 0.01–1 g/L human IgG in an amount equivalent to 2 million lysed cells per mL, and produced no significant change in either polarization or intensity assay results (Fig. S2). The Fc-III probe IgG limit of detection is comparable to those of HPLC or ELISA, albeit with a faster, simpler workflow.

The detection limit for murine IgG1, to which Fc-III binds relatively poorly,⁴¹ was 0.1 g/L, 1000 times that for human IgG. Even this higher detection limit could be useful in applications involving higher murine IgG concentrations (0.1 g/L to 2.5 g/L), including many murine hybridoma cultures.

The Fc-III peptide was selected to bind to a region of IgG-Fc that interacts with four structurally different proteins, including proteins A and G. While murine and human IgG antibodies differ in this region,⁴² histidines H433 and H435 of IgG are conserved, and molecular docking suggested His433 Pi-Pi stacking interactions with the fluorescein. This possibility is further supported by the observation of fluorescence intensity enhancement with both human and mouse IgG.

Discussion

Antibody concentrations are routinely measured in culture fluid and process streams during clone and process development, and in manufacturing. Common UV absorbance methods suffer in this application from interference by medium components, and by proteins and nucleic acids liberated from cells lysed during culture or harvest. Antibody concentrations, therefore, are most

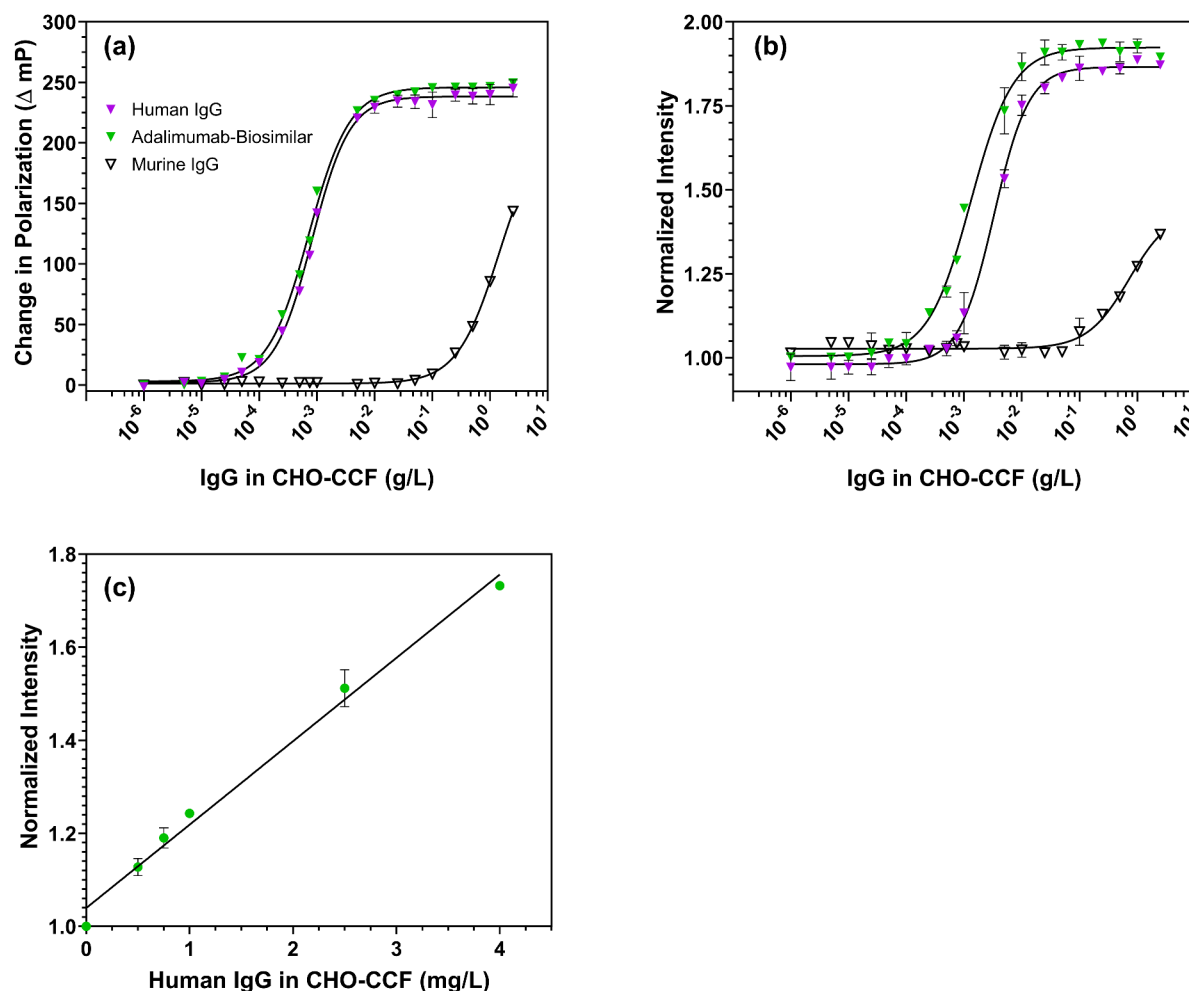


Figure 7. Detection of different IgG analytes in CHO cell culture fluid. (a) Fluorescence polarization and (b) fluorescence intensity of 100 nM Fc-III probe with polyclonal human IgG (magenta), Adalimumab biosimilar IgG1 (green) and murine-IgG1 (black) in CHO-CCF. (c) Linear variation of fluorescence intensity of 100 nM Fc-III probe with human IgG in CHO-CCF.

commonly measured by ELISA, chromatography, or surface plasmon resonance, each of which requires substantial instrumentation and has a low throughput, incompatible with modern high-throughput approaches to clone selection, cell culture optimization, and purification process development and operation.

Here we introduce a simple, rapid, mix-and-read antibody assay based on homogenous, separation-free reporting of antibody Fc concentrations by fluorescence intensity, readable by simple instrumentation including standard microtiter plate readers. The ready production of the reporter by standard peptide synthesis, and its tolerance of complex culture fluid even when spiked with clarified cell lysate, suggests that the IgG assay could be very practically useful in bioprocess environments. The use of simple fluorescence intensity instrumentation facilitates ease of implementation and use in a typical bioprocess environment. Its great sensitivity (below 1 mg/L), and potentially the judicious use of unlabeled competitor or lower-affinity Fc-reporter would allow the assay to quantify even the lowest and highest IgG titers practically encountered in bioprocessing.

The unexpected enhancement of fluorescence intensity upon IgG/Fc-III probe binding is intriguing and potentially very useful for quantitative IgG detection in homogenous assays. The biochemical and molecular docking approaches used here help elucidate the origins of this fluorescence intensity enhancement

in changes in the local microenvironment of the fluorophore. This fast (< 30 s), quantitative and tunable IgG-assay can be used for IgG quantitation in cell line development, automated clone screening, purification and other bioprocess operations.

Materials and methods

Affinity binders and fluorescent labeling

The mAb reporter probes were prepared by modifying candidate affinity ligands with fluorescein. The Fc-binding peptide Fc-III (DCAWHLGELVWCT-NH₂)²⁷ was modified with an N-terminal-fluorescein coupled to an aminohexanoic acid (Ahx) spacer (Fluorescein-Ahx-DCAWHLGELVWCT-NH₂). Peptide-H (HWRGWV)⁴³ was modified with fluorescein in the same way. Fluorescein-labeled peptides were synthesized by GenScript and were used as supplied. Fluorescein-labeled protein G (product code PGF) was purchased from ProteinMods.

Static fluorescence polarization and intensity measurements

Static fluorescence experiments were performed in Corning 96-well flat-bottom black microtiter plates (CLS3650, Sigma). Purified human IgG (IHUIGGAP) was purchased from

Innovative Research, Inc. Human IgG1 adalimumab biosimilar Exemptia was from Zydus Cadila, India. The antibodies were diluted to the desired working concentration in phosphate-buffered saline (PBS) or in null CHO-S CCF (a generous gift of Prof. Ruben Carbonell and Golden LEAF Biomanufacturing Training and Education Center, North Carolina State University). Briefly, CHO-S cells were grown in CD CHO medium (Cat# 10743002, Thermo Fisher Scientific) supplemented with 4 mM L-glutamine to a cell density of 3.31×10^6 cells/mL, with 96.1% viability.⁴⁴ Upon harvest, the CCF was clarified by centrifugation at 8000×g for 30 min followed by filtration through a 0.2 μm polyethersulfone membrane filter (Cat# 10040, VWR). To test for possible interference with the assay by cell components liberated by cell lysis into culture fluid, CHO-k7 cells grown in Gibco/F12 (1:1) (1x) Dulbecco's Modified Eagle Medium (the generous gift of Rohan Kulkarni and Navin Varadarajan, University of Houston) were harvested by centrifugation (500×g, 10min), lysed by sonication (20 kHz, 20s, 3 cycles), and centrifuged (10000 ×g, 20 min). This clarified lysate was added to the clarified CHO culture fluid derived from a culture with 96.1% viability described above, and these samples spiked with human IgG1 and tested as described below. In a typical batch experiment, 125 μL of mAb-spiked PBS or CHO-CCF was transferred to a microtiter plate well, followed by the addition of 125 μL of the fluorescent affinity ligand diluted in PBS to the required concentration. The microplate was immediately placed in a filter-based fluorescence polarization/intensity microplate reader (Tecan Infinite F200 PRO; Ex 485 nm/Em 535 nm). For experiments involving the use of competitors, 5 μL (2% of the overall volume) of PBS (negative control) or competitor in PBS was added to a microwell containing 0.001 g/L human IgG and 50 nM fluorescein-labeled affinity ligand (250 μL) and incubated for 120 min (a time found sufficient for equilibration) before fluorescence polarization and fluorescence intensity measurements.

Fluorescence polarization is defined by the following equation:

$$FP(mP) = \left(\frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \right) \times 1000$$

Where I_{\parallel} is the intensity with Ex/Em polarizers set parallel, and I_{\perp} is the intensity with polarizers set perpendicular. Fluorescence polarization values are usually expressed in units of millipolarization (mP).

Molecular docking of labeled FC-III

The X-ray crystallographic structure by Delano *et al.* of the 13-residue cyclic peptide Fc-III DCAWHLGELVWCT-NH₂ in complex with IgG-Fc (PDB ID: 1DN2) was used for docking studies.²⁷ The X-ray crystal structure of IgG-Fc (PDB ID: 1DN2) was retrieved from the RCSB protein data bank.²⁷ The structure of the fluorescein-labeled Fc-III was generated based on the structures of Fc-III and fluorescein and energy-minimized for 1000 cycles using the OPLS_2005 force field in Schrödinger Maestro (Schrödinger, LLC).

Combining different docking methods often can achieve more reliable cross-validated predictions.⁴⁵ Therefore, two different molecular docking methods were used in this study to obtain binding modes of fluorescein-labeled Fc-III and IgG-Fc. AutoDock Vina is a widely used open-source docking program.⁴⁶ The IgG-Fc and fluorescein-labeled Fc-III structures were prepared with AutoDock Tools.⁴⁷ All hydrogens were added and Gasteiger charges were assigned.⁴⁸ The center of the docking grid box was placed at the center of geometry of the engineered peptide in complex with IgG-Fc in the crystal structure (PDB ID: 1DN2). The grid box extended beyond the binding site, with 35 points along each edge of the box, 1.0 Å apart. The IgG-Fc was enclosed in a grid box of 35 Å in each direction to include the potential ligand-binding site. In order to find the best conformations, the level of search exhaustiveness and the maximum number of binding modes were set at 16 and 20, respectively. The AutoDock Vina docking score was used to rank the different docking poses by binding affinity. Docking calculations were repeated 3 times with different random seeds.^{49,50,51}

The Schrödinger Glide program was separately used to further investigate the interactions between IgG-Fc and fluorescein-labeled Fc-III.⁵⁰ The protein IgG-Fc structure retrieved from PDB ID: 1DN2²⁷ was prepared by the Protein Preparation Wizard in the Schrödinger Maestro visualizer. Hydrogens were added and bond orders were assigned. Ionization states were assigned at pH 7.0. Using ProtAssign, the hydrogen positions in the structure of the protein IgG-Fc were optimized for hydrogen bonding and energy minimized to eliminate potential steric clashes before docking. The fluorescein-labeled Fc-III was prepared with the LigPrep platform in the Schrödinger Maestro visualizer. Possible ionization states were generated at pH 7 with the Epik module in Schrödinger. Hydrogen atoms were added, tautomers generated, specified chiralities were retained, and the geometries of the fluorescein-labeled Fc-III were optimized. Finally, after preparation, six output structures of the fluorescein-labeled Fc-III were generated for further docking calculations. The ligand was treated as fully flexible. The docking grid was generated with the Receptor Grid Generation tool in the Schrödinger Maestro visualizer. The default van der Waals radius scaling factors were used. The center and the size of the grid box (35 Å) were defined according to the position of the engineered peptide in complex with IgG-Fc in the crystal structure (PDB ID: 1DN2). The standard-precision docking score was used to rank the docking poses. For each fluorescein-labeled Fc-III structure after preparation, a maximum of 50 poses were saved after docking calculations. Fc-III is a disulfide-bonded cyclic peptide and no modification was made to the peptide Fc-III moiety. Therefore, the peptide portion of the ligand was internally constrained (rigid), while the fluorescein label was free to change conformation. All the other settings remained at their defaults in Schrödinger.⁴⁹⁻⁵¹

Competing interests

Authors UP, BV, KK, and RCW on this manuscript are listed as inventors on a pending patent application on this work, all other authors declare they have no competing interests.

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Disclosure statement

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