Detergent Micelle Conjugates Containing Amino Acid Monomers Allow Purification of Human IgG near Neutral pH

Guy Patchornik[1], Gunasekaran Dhandapani[2], and Ellen Wachtel[3]

[1]Ariel University Faculty of Natural Sciences
[2]Affiliation not available
[3]Weizmann Institute of Science Faculty of Chemistry

May 12, 2022

Abstract

Industrial scale production of therapeutic monoclonal antibodies (mAbs) is commonly achieved with Protein A chromatography, a process that requires exposure of the antibody to strongly acidic conditions during the eluting step. Exposure to acid inactivates virus contaminants but may, in parallel, lead to antibody aggregation that must be eliminated or kept at acceptably low levels. This report seeks to provide a practical method for overcoming a long-standing problem. We show how Brij-O20 detergent micelles, conjugated by the amphiphilic [(bathophenanthroline)3:Fe2+] complex in the presence of amino acid monomers: phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), isoleucine (Ile) or valine (Val), efficiently capture polyclonal human IgG (hIgG) at neutral pH and allow its recovery by extraction either at pH 4 (85-97% yield) or at pH 6.3 (72-84% yield). Of the five amino acid monomers surveyed, Phe or Tyr produced the highest overall process yield at both pH 4 and 6.3. The monomeric state of the purified hIgG's was confirmed by dynamic light scattering (DLS). Potential advantages of the purification method are discussed.

Biotech Method

Detergent Micelle Conjugates Containing Amino Acid Monomers Allow Purification of Human IgG near Neutral pH

Gunasekaran Dhandapani,[1] Ellen Wachtel[2] and Guy Patchornik[1*]

[1] Department of Chemical Sciences, Ariel University, 70400, Ariel, Israel.

[2] Faculty of Chemistry, Weizmann Institute of Science, 76100, Rehovot, Israel.

.

*Corresponding author:

Email: guyp@ariel.ac.il

Abstract

Industrial scale production of therapeutic monoclonal antibodies (mAbs) is commonly achieved with Protein A chromatography, a process that requires exposure of the antibody to strongly acidic conditions during the eluting step. Exposure to acid inactivates virus contaminants but may, in parallel, lead to antibody aggregation that must be eliminated or kept at acceptably low levels. This report seeks to provide a practical method for overcoming a long-standing problem. We show how Brij-O20 detergent micelles, conjugated by the amphiphilic [$(bathophenanthroline)_3$:Fe^{2+}] complex in the presence of amino acid monomers: phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), isoleucine (Ile) or valine (Val), efficiently capture polyclonal

human IgG (hIgG) at neutral pH and allow its recovery by extraction either at pH 4 (85-97% yield) or at pH 6.3 (72-84% yield). Of the five amino acid monomers surveyed, Phe or Tyr produced the highest overall process yield at both pH 4 and 6.3. The monomeric state of the purified hIgG's was confirmed by dynamic light scattering (DLS). Potential advantages of the purification method are discussed.

Keywords

Antibody purification, Protein A, non-ionic surfactant micelles.

Abbreviations

DDW - Double distilled water, DLS - Dynamic light scattering, DMSO - Dimethyl sulfoxide, *E. coli* - Escherichia coli, hIgG - human IgG, IgG - Immunoglobulin G, Ile - isoleucine, Leu - leucine, Phe - phenylalanine, SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis, Tyr - tyrosine, Val - valine.

Introduction

Monoclonal antibodies (mAbs) are powerful detection tools in science [1, 2] and valuable therapeutic agents in medicine [3, 4]. Their significant contribution to human health derives from their strong binding affinity and high specificity towards various antigens, including proteins, carbohydrates, lipids and nucleotides, and , as such, allow their use in personalized medicine [5] and cancer therapy [6, 7]. In 2021 alone, 16 new mAbs were expected to be approved by the American Food and Drug Administration [8]. Clearly, as the global demand for diverse mAbs grows, increase in large scale production and expression levels of mAbs (*i.e.* upstream processing) combined with their purification (*i.e.* downstream processing), is becoming ever more challenging [9].

The majority of mAbs are purified from cell culture *via* Protein A chromatography due to the high binding affinity [10] and specificity [11] of the Protein A ligand to a wide range of antibody (Ab) isotypes [12]. The unique properties of Protein A translate into excellent recovery yields (generally >90%) and high purity (>98%), both accomplished within a single chromatographic step [13], making Protein A chromatography the gold standard technology in Ab purification [14]. In general, mAbs are eluted very efficiently from Protein A resins under acidic conditions (*e.g.* 0.1 M sodium citrate, pH 3.3 [15]). Low pH is required for (a) weakening the interactions between the immobilized Protein A and the Fc domain of the target antibody; and for (b) inactivating any viruses which may be present as impurities in the system [16, 17]. However, low pH may also promote disruption of antibody secondary structure which can lead to aggregate formation [16-20]. Therefore, suppression or elimination of antibody aggregation , during both upstream and downstream processing, has become a subject of on-going investigation in bioprocess development [20]. The high antibody concentration present in the cell culture medium increases the frequency of protein self-adsorption events [16, 21, 22], which, of course, can also lead to aggregation. Several strategies and/or small molecule additives, aimed at minimizing antibody aggregation during column elution, have been tested. They have included for example: (a) addition of 0.5-1M urea [16] or (b) of arginine monomers [21] to the running buffer. Suppressing the formation of antibody oligomers is vital: studies have shown that such aggregates may reduce (i) therapeutic antibody potency; (ii) batch to batch reproducibility; as well as (iii) promoting undesirable immunogenic response [18, 23-25].

We have studied an alternative purification method that would avoid exposing polyclonal human IgG's to acidic conditions. This approach relies on a recently described non-chromatographic, ligand-free strategy that uses nonionic detergent micelles conjugated *via* the amphiphilic complex [$(\text{bathophenanthroline})_3$:$Fe^{2+}$] [26-28] as the purification platform. Such conjugated detergent micelles were found to quantitatively capture human and mouse IgG's at neutral pH, exclude hydrophilic protein background impurities and allow extraction of relatively pure IgG's without parallel co-extraction of background impurities or dissolution of the conjugated micelles [26-28]. The approach was found to function efficiently with three commercially available surfactant families characterized by polyethylene oxide (PEO) headgroups: Tween; Brij and Triton X-100. We note that PEO-based surfactants are approved for use in pharmaceutical formulations. However, it was found to be necessary to lower the pH to 3.8 during IgG extraction in order to achieve overall satisfac-

tory yield of pure antibodies (generally >80%, [28]). Here, we investigated the possibility of weakening the interactions between captured antibodies and the detergent matrix to such an extent that antibody capture would not be affected while efficient extraction could be performed at close to neutral pH.

Materials and methods

Sodium chloride (Sigma, S7653), Brij O-20 (polyoxyethylene (20) oleyl ether, Sigma, 436240,), Ex-CELL 610-HSF medium (Sigma, 14610C), glycine (Bio-lab 07132391), bathophenanthroline (GFS chemicals, C038446), ferrous chloride tetrahydrate (Sigma, 44939), human IgG (Lee-Biosciences, 340-21), phenylalanine (Sigma, P2126), tyrosine (Sigma, T3754), tryptophan (Sigma, T3300), isoleucine (Sigma, I2752), valine (Sigma, 94640), Polyethylene glycol-6000 (PEG-6000) (Sigma, 81260).

Preparation of 200 mM bathophenanthroline:DMSO:HCl solution

Into 90 µL of dimethyl sulfoxide (DMSO) and 10 µL of 25% HCl, 6.64 mg of bathophenanthroline are added and vortexed for 5 minutes until total dissolution is observed.

Preparation of 200 mM amino acid monomer:DMSO:HCl solution

Into 92.5 µL of dimethyl sulfoxide (DMSO) and 7.5 µL of 25% HCl, 3.3 mg of amino acid monomer (Phe, Tyr, Trp,Ile ,or Val) are added and vortexed for 5 minutes until total dissolution is observed.

Purification of human IgG (hIgG) *via* conjugated Brij-O20 micelles

Step I: Preparation of conjugated micelles

Conjugated detergent micelles were obtained by mixing equal volumes of**medium A** and **B** as follows: **medium A** was prepared by the addition of 6 µL of the amphiphilic chelator bathophenanthroline (200 mM in DMSO\HCl) and 4.5 µL of amino acid monomer (200 mM in DMSO\HCl) with 77 µL of: 0.7 mM Brij-O20 in DDW, with vigorous vortexing (10 seconds). 165 µL**of medium B** , containing 1 mM $FeCl_2$ in 20 mM NaCl, was then added to **medium A** with vigorous vortexing and further incubated for 5 minutes at 25°C. This was followed by the addition of 13 µL of 1M NaCl and incubation for 5 minutes at 25°C. Centrifugation (21,000 x g , 5 minutes at 19 °C) was applied, and the supernatant was removed from the resulting red pellet. The red pellet was washed once with 30 µL of cold 20 mM NaCl and centrifugation was repeated (21,000 x g , 5 minutes at 19 °C).

Step II: IgG capture: Freshly prepared conjugated micelles were resuspended in 100 µL of serum-free medium (Ex-CELL 610-HSF) containing 4% PEG-6000 and the target hIgG at concentration 5 mg/ml. The suspension was vigorously vortexed for 45 sec and after 10 minutes of incubation at room temperature, centrifugation (21,000 x g , 5 minutes, 19°C) was applied. The supernatant was discarded and pellets were briefly washed with 30 µL of cold 20mM NaCl. An additional centrifugation step followed (21,000 x g , 5 minutes, 19°C) and the supernatant was removed.

Step III: IgG extraction: Conjugated detergent micellescontaining the target IgG were resuspended and incubated with 200 µL of 50 mM Gly, 30 mM NaCl at pH 4 or 200 mM Tris at pH 6.3 in 50 mM NaCl for 10 minutes at 25°C. Centrifugation followed (21,000 x g , 5 minutes, at 19 °C); the supernatant was removed and analyzed by SDS-PAGE.

Dynamic light scattering (DLS)

DLS measurements were performed by comparing commercial, pure (>95%) hIgG (1-2 mg/mL) that had not encountered detergent, with hIgG following capture and extraction *via*conjugated Brij-O20 micelles ± amino acid monomers. All hIgG samples were solubilized in 200 mM Tris pH 6.3 and 50 mM NaCl. Samples were centrifuged at 21,000 x g for 20 minutes at at 19 °C prior to measurement at 25°C. The intensity-weighted size distributions of hIgG were determined using the auto-correlation spectroscopy protocol of the Nanophox instrument (Sympatec GmbH, Clausthal-Zellerfeld, Germany).

Densitometry

Bands present in Coomassie-stained gels were quantified using the EZQuant program. http://www.ezquant.com/en/. Process yield was calculated by comparing the intensity of bands representing a known amount of commercial calibrated, pure IgG to the extracted target antibody at the end of the purification process.

Results and Discussion

Weakening the binding affinity between captured antibodies and the Brij O20 detergent micelle conjugates was investigated at pH 4 and at pH 6.3 by including amino acid monomers during conjugate formation. (**Figure 1)** Five amino acid monomers, three with aromatic side chains and two hydrophobic, were studied: phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), isoleucine (Ile), valine (Val) (**Figure 2, A**). Their relative contribution to process efficiency was evident*via* a spiking experiment. Polyclonal, commercial hIgG (>95% purity) (**Figure 2 A, lane 1)** was mixed with*E. coli* lysate, the latter serving as an artificial contaminating background (**Figure 2, A - lane 3**). The mixture was then added to the conjugated detergent micelles +/- amino acid monomers. Exclusion of hydrophilic impurities and extraction of hIgG from the conjugated micelles at pH 4 **(Figure 2, A - lanes 4-9**) followed. Relatively pure antibody was obtained with all amino acid monomers tested**(Figure 2, B).** These findings demonstrated that addition of amino acid monomers during micelle conjugation leads to improved process yields when extraction is carried out at pH 4 **(Figure 2, B**). Highest overall yield was obtained when the detergent matrix had been supplemented with Phe (97%) whereas Ile was the least efficient (85%)**(Figure 2, B**).

Increasing the pH to 6.3 during IgG extraction **(Figure 2, C**), produced an average recovery yield of only 54%. As anticipated, we must conclude that the less acidic conditions are unable to sufficiently weaken the binding affinity between the bound IgG and the surrounding detergent matrix. However, when either Phe or Tyr were added during the conjugation step, overall yields increased dramatically, reaching 82-84%. Trp was found to be the least efficient by ˜10% when compared to Phe or Tyr; Ile and Val showed significant contribution to process yield (76-78%) as compared to yield in their absence (54%) **(Figure 2, C**). Finally, DLS analysis was also utilized to assess whether recovered hIgG's extracted at pH 6.3 are monomeric (**Figure 3**). We found that extracted hIgG's are indeed monomeric and this was readily observed regardless of the particular amino acid added to the Brij-O20:[(bathophenanthroline)$_3$:Fe^{2+}] conjugates.

In order to begin to rationalize the experimental results described above, one should distinguish between (i) the downstream effect of acidic pH on the conformational state of IgG molecules; and (ii) the nature of the amino acid monomer /nonionic detergent micelle interaction. The latter may be the basis of our ability to extract hIgG's at close to neutral pH and at relatively high purity and yield. Low pH, during antibody elution from the Protein A column, as well as during extraction from our conjugated micelle matrix, is liable to produce a denaturation/renaturation equilibrium, thereby weakening IgG binding affinity and facilitating extraction on the one hand, but leading to aggregate formation on the other. Sjogren *et al.,*[29] have increased our insight into preferential interaction between peptides and nonionic surfactant micelles. Their observation of broad NMR signals from tyrosine and phenylalanine, as compared to lysine, implied that there is a higher degree of solvent exposure for lysine residues than for the aromatic side chains, suggesting that peptide and detergent primarily interact through the aromatic rings of the peptide. Furthermore, the NOESY spectra displayed NOE cross-peaks between the aromatic ring protons of the phenylalanine residues and the protons in the surfactant hydrocarbon chains. This finding confirmed that the aromatic rings and the alkyl chains of the surfactant are, on average, near each other. All the NMR results support the conclusion that interactions taking place between aromatic rings of the peptides and the surfactant alkyl chains are dominant. We therefore tentatively suggest that the presence of aromatic rings, *e.g.* , of phenylalanine or tyrosine, in the hydrophobic core of the Brij micelles is responsible for weakening the binding of polyclonal hIgG molecules even at pH 6.3, while not giving rise to protein denaturation nor to aggregate formation.

In conclusion,

Addition of Phe or Tyr amino acid monomers during conjugation of Brij-O20 micelles *via* the [(bathophenanthroline)$_3$:Fe^{2+}] amphiphilic complex, appears to weaken the interaction of captured IgG's

with the nonionic detergent matrix at pH 6.3. This is best demonstrated by the ability to efficiently extract hIgG (82-85%yield) at pH 6.3 whereas in the absence of amino acid monomers, overall process yield is only 54%. Using Phe or Tyr in antibody purification protocols is attractive: the amino acid monomers are (i) non-toxic; (ii) relatively inexpensive; and importantly, (iii) do not lead to antibody denaturation. Antibody aggregation which can occur at low pH is thereby avoided.

Acknowledgments

G. P. thanks Ariel University for their support.

Data availability statement: The data that support the findings of this study are openly available in [repository name e.g "figshare"] at http://doi.org/[doi], reference number [reference number].

All authors wish to emphasize that:

The manuscript does/does not contain experiments using animals. The permission of the national or local authorities (giving the permission or the accreditation no. of the laboratory and of the investigator) should be stated if animal experiments are included. If no such rules or permissions have been implicated in the particular country, this must be stated. www.biotechnology-journal.com Manuscript submission: http://mc.manuscriptcentral.com/btj Contact the Editorial Office at: btj@wiley.com Page 7 of 18 vi) The manuscript does/does not contain human studies. If such studies are included, it should be stated that local Ethical Committee approval was received for the studies and that the informed consent of all participating subjects was obtained. Permission statements relating to points 4 and 5 should also be included in the text of the manuscript.

References

1. Goldman, R.D., *Antibodies: indispensable tools for biomedical research.* Trends Biochem Sci, 2000.**25** (12): p. 593-5.

2. Herzenberg, L.A., S.C. De Rosa, and L.A. Herzenberg, *Monoclonal antibodies and the FACS: complementary tools for immunobiology and medicine.* Immunology Today, 2000.**21** (8): p. 383-390.

3. Alkan, S.S., *Monoclonal antibodies: the story of a discovery that revolutionized science and medicine.* Nat Rev Immunol, 2004. **4** (2): p. 153-6.

4. Weiner, G.J., *Building better monoclonal antibody-based therapeutics.* Nat Rev Cancer, 2015.**15** (6): p. 361-70.

5. Liu, J.K., *The history of monoclonal antibody development - Progress, remaining challenges and future innovations.* Ann Med Surg (Lond), 2014. **3** (4): p. 113-6.

6. Mullard, A., *Maturing antibody-drug conjugate pipeline hits 30.* Nat Rev Drug Discov, 2013.**12** (5): p. 329-32.

7. Sievers, E.L. and P.D. Senter,*Antibody-drug conjugates in cancer therapy.* Annu Rev Med, 2013.**64** : p. 15-29.

8. Kaplon, H. and J.M. Reichert,*Antibodies to watch in 2021.* MAbs, 2021. **13** (1): p. 1860476.

9. Ecker, D.M., S.D. Jones, and H.L. Levine, *The therapeutic monoclonal antibody market.* mAbs, 2015.**7** (1): p. 9-14.

10. Hober, S., K. Nord, and M. Linhult, *Protein A chromatography for antibody purification.*Journal of Chromatography B, 2007. **848** (1): p. 40-47.

11. DeLano, W.L., et al.,*Convergent solutions to binding at a protein-protein interface.*Science, 2000. **287** (5456): p. 1279-83.

12. Page, M. and R. Thorpe,*Purification of IgG Using Protein A or Protein G* , in *The Protein Protocols Handbook* , J.M. Walker, Editor. 2002, Humana Press: Totowa, NJ. p. 993-994.

13. Azevedo, A.M., et al.,*Partitioning of human antibodies in polyethylene glycol–sodium citrate aqueous two-phase systems.* Separation and Purification Technology, 2009. **65** (1): p. 14-21.

14. Low, D., R. O'Leary, and N.S. Pujar, *Future of antibody purification.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **848** (1): p. 48-63.

15. Mazzer, A.R., et al.,*Protein A chromatography increases monoclonal antibody aggregation rate during subsequent low pH virus inactivation hold.* Journal of chromatography. A, 2015. **1415** : p. 83-90.

16. Shukla, A.A., P. Gupta, and X. Han, *Protein aggregation kinetics during Protein A chromatography. Case study for an Fc fusion protein.* J Chromatogr A, 2007.**1171** (1-2): p. 22-8.

17. Ejima, D., et al., *Effects of acid exposure on the conformation, stability, and aggregation of monoclonal antibodies.* Proteins, 2007. **66** (4): p. 954-62.

18. Cromwell, M.E., E. Hilario, and F. Jacobson, *Protein aggregation and bioprocessing.* Aaps j, 2006.**8** (3): p. E572-9.

19. Hari, S.B., et al.,*Acid-induced aggregation of human monoclonal IgG1 and IgG2: molecular mechanism and the effect of solution composition.*Biochemistry, 2010. **49** (43): p. 9328-38.

20. Paul, A.J., K. Schwab, and F. Hesse, *Direct analysis of mAb aggregates in mammalian cell culture supernatant.* BMC Biotechnol, 2014. **14** : p. 99.

21. Shukla, A.A., et al.,*Downstream processing of monoclonal antibodies–application of platform approaches.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **848** (1): p. 28-39.

22. Gagnon, P., et al.,*Transient conformational modification of immunoglobulin G during purification by protein A affinity chromatography.* J Chromatogr A, 2015. **1395** : p. 136-42.

23. Chi, E.Y., et al., *Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation.* Pharm Res, 2003. **20** (9): p. 1325-36.

24. Joubert, M.K., et al.,*Classification and characterization of therapeutic antibody aggregates.* J Biol Chem, 2011. **286** (28): p. 25118-33.

25. Fekete, S., et al., *Theory and practice of size exclusion chromatography for the analysis of protein aggregates.* J Pharm Biomed Anal, 2014. **101** : p. 161-73.

26. Dhandapani, G., et al., *A general platform for antibody purification utilizing engineered-micelles.* MAbs, 2019. **11** (3): p. 583-592.

27. Dhandapani, G., et al.,*Role of amphiphilic [metal:chelator] complexes in a non-chromatographic antibody purification platform.* J Chromatogr B Analyt Technol Biomed Life Sci, 2019. **1133** : p. 121830.

28. Dhandapani, G., et al.,*Nonionic detergent micelle aggregates: An economical alternative to protein A chromatography.* N Biotechnol, 2021. **61** : p. 90-98.

29. Sjögren, H., et al.,*Interactions between charged polypeptides and nonionic surfactants.* Biophys J, 2005. **89** (6): p. 4219-33.

Figures

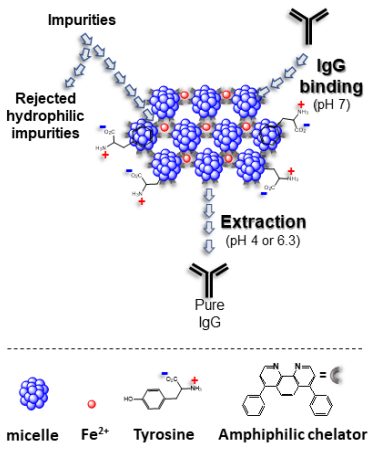

Figure 1: Cartoon of IgG purification protocol *via* the conjugated micellar aggregate matrix comprising Brij-O20, the [(bathophenanthroline)$_3$:Fe^{2+}] amphiphilic complex and tyrosine amino acid monomers (Tyr).

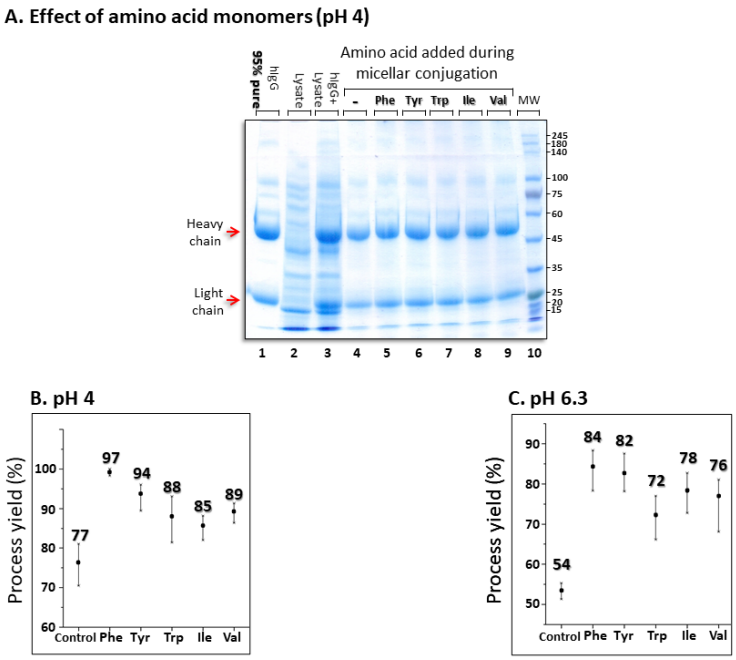

Figure 2: A. SDS-PAGE (Coomassie stained) analysis of process efficiency, extraction at pH 4: Lane 1: Total hIgG added to the system; lane 2: *E. coli.* lysate added as an artificial contamination background; lane 3: *E. coli* lysate and total hIgG; lanes 4-9: hIgG recovered via conjugated micellar aggregates comprising: Brij-O20, the [(bathophenanthroline)$_3$:Fe^{2+}] complex and amino acid monomers (see the Materials and Methods section); lane 10: Molecular weight markers (KDa). Red arrows indicate reduced heavy and light chains of hIgG, respectively. **B.** Process yield when human IgG (input concentration, 5 mg/ml), is extracted at pH 4, with addition of indicated amino acid monomers, while the Control lacks amino acid monomers; **C.** As in **B** , but with extraction at pH 6.3. Error bars are calculated as the standard deviation of 4 replicate measurements conducted on different days. Quantitation was accomplished as described in the Materials and Methods section.

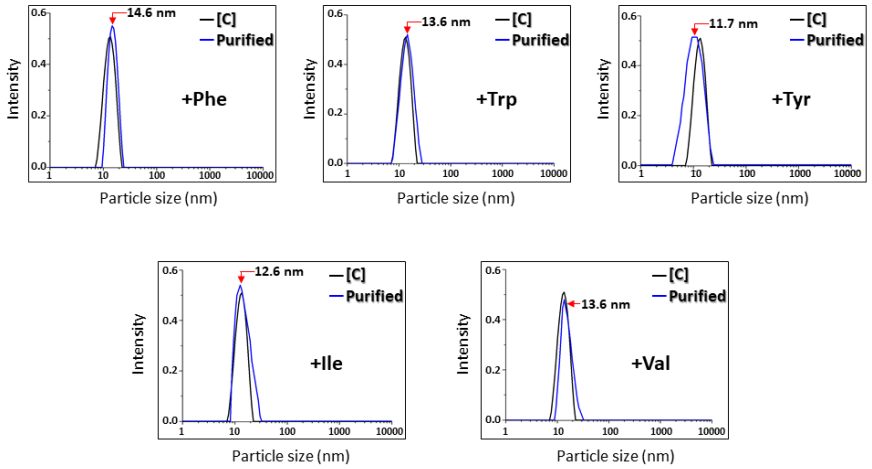

Figure 3: Dynamic light scattering (DLS) . The particle size distribution of commercial (95% purity) hIgG, [**C**] that never encountered conjugated Brij-O20 micelles (black lines), as determined by dynamic light scattering at 25°C, and the particle size distribution of hIgG extracted from detergent micelle conjugates at pH 6.3 in the presence of amino acid monomers (blue lines).