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Birgit Mueller-Chorus
West Pharmaceutical Services, Germany, birgit.mueller-chorus@westpharma.com

Erica Tullo
West Pharmaceutical Services, Germany

Vinod Vilivalam
West Pharmaceutical Services, Germany

Lloyd Waxman
West Pharmaceutical Services, Germany

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Effect of Agitation on Protein Aggregation in Vials Made from Glass or Plastic

Authors: Dennis Liu, Lloyd Waxman, Vinod Vilivalam, Birgit Mueller-Chorus, and Erica J. Tullo

For several types of proteins, it has been demonstrated that potential for aggregation resulting from mechanical agitation is much lower when packaged in vials comprising Crystal Zenith® (CZ), a cyclic olefin polymer, as compared to vials comprising glass.

Background:

The increasing use of proteins as therapeutics has focused attention on the need to maintain the stability of these labile molecules during both storage and shipment. The physical degradation of therapeutic proteins can arise from aggregation and adsorption in primary containers, from chemical damage due to exposure to light, and from leachables including oxidants, free radicals, and metal ions. In addition to loss of potency and valuable drug product, there is growing evidence that protein aggregates are capable of inducing an immune response that could neutralize the effect of the drug (Rosenberg). In instances where the drug product is similar or identical to an endogenous protein, the development of cross-reacting antibodies could lead to potentially life-threatening consequences for the patient.

Although the trend in the pharmaceutical industry has been to package therapeutic proteins, particularly monoclonal antibodies at high concentration, in prefilled syringes, vials continue to be used as primary containers for multiple-use applications including vaccines as well as for the reconstitution of lyophilized drug products. For this reason, we have compared the effect of mechanical stress on protein aggregation in vials made of glass, the material most widely used in their manufacture, with vials made from the plastic cyclic olefin polymer.

Objectives

1. To develop and characterize a simple stress model to compare the stability of biologics in vials made of different materials.

2. To investigate the aggregation of various classes of proteins including antibodies, enzymes, and peptide hormones in vials made of glass and plastic.

Materials and Methods

Vials:
Presterilized 2 mL vials made of glass or the plastic Daikyo Crystal Zenith cyclic olefin polymer. Stoppers were laminated with Flurotec® film (a fluoropolymer), to minimize the effect of the elastomeric component on protein stability.

Proteins:
Rabbit IgG was obtained from Rockland Immunochemicals. Therapeutic proteins were purchased from a local pharmacy.

Buffers:
Proteins were dissolved in or diluted into one of two buffers –

   PBS – 20 mM sodium phosphate/ 150 mM NaCl (pH 6.8)

   EPO – 20 mM sodium phosphate/ 2.7 mM sodium citrate/100 mM NaCl (pH 6.9)

Methods:
• Aggregation: Visual inspection of particulate formation and quantification of turbidity changes were made by measuring changes in absorbance at 350 nm before and after storage and/or agitation of samples in vials. Loss of protein due to aggregation was estimated by the decrease in absorbance of the solution at 280 nm or by SE-HPLC at 214 nm and 280 nm after centrifugation to remove insoluble material.

• SE-HPLC was carried out on a Waters Model 2696 liquid chromatography system using a GE Life Sciences Superdex 200 column (1x30 cm). Protein elution was monitored at 214 nm and 280 nm and the area under the protein peak was compared to controls that were stored in vials that had not been agitated. The elution buffer consisted of 20 mM sodium phosphate/ 150 mM NaCl (pH 6.8).

• Agitation: The vials were stoppered and sealed with an aluminum crimp and placed horizontally on an orbital shaker at 200 rpm at room temperature (RT) up to 120 hours. The concentration of each protein was typically 1 mg/mL unless otherwise noted. Vials were filled with 1.0 mL of protein solution and all samples were run in triplicate.

• Measurements: For each protein, the absorbance at 350 nm and 280 nm were measured at the start of the experiment to determine protein concentration and to establish a baseline for turbidity measurements. After filling the vials, the remaining solution was stored in a glass screw cap vial at 4°C ("Stock") until the experiment was completed. Controls consisted of storing filled vials at both 4°C and at RT without agitation. However, the absorbance of solutions stored at RT did not differ measurably from those stored at 4°C or from the Stock.

Results

Preliminary experiments indicated that MAb1 aggregated when shaken in glass vials. To develop a set of standardized test conditions, the speed of the orbital shaker which produced measurable aggregation and the optimal sample volume for aggregation was determined. Figure 1 shows that when glass vials were mounted horizontally on the shaker aggregation was negligible until the speed of rotation was increased to at least 200 rpm. None of the molecules in this study aggregated below 200 rpm (data not shown). At this speed the optimum sample volume was 1.0 mL (Figure 2). These parameters are comparable to those used in similar studies (Hawe, et al.). When the vials were mounted upright, aggregation did not occur (Figure 3). These results are consistent with the notion that the dimension of the liquid-air interface and parameters such as the velocity of agitation can affect the rate and extent of aggregation (Hawe, et al.). During horizontal rotation, there is clearly more intense mixing, bubble break up, and bubble entrainment, compared to vertical rotation. This enhances exposure of the protein molecules to the air-liquid interface where unfolding followed by irreversible aggregation and/or particle formation can occur. Under these conditions, by comparison with glass vials, aggregation of MAb1 in vials made of CZ was much less. Aggregation was also time-dependent (data not shown) and most of the studies were carried out for 96 hours.

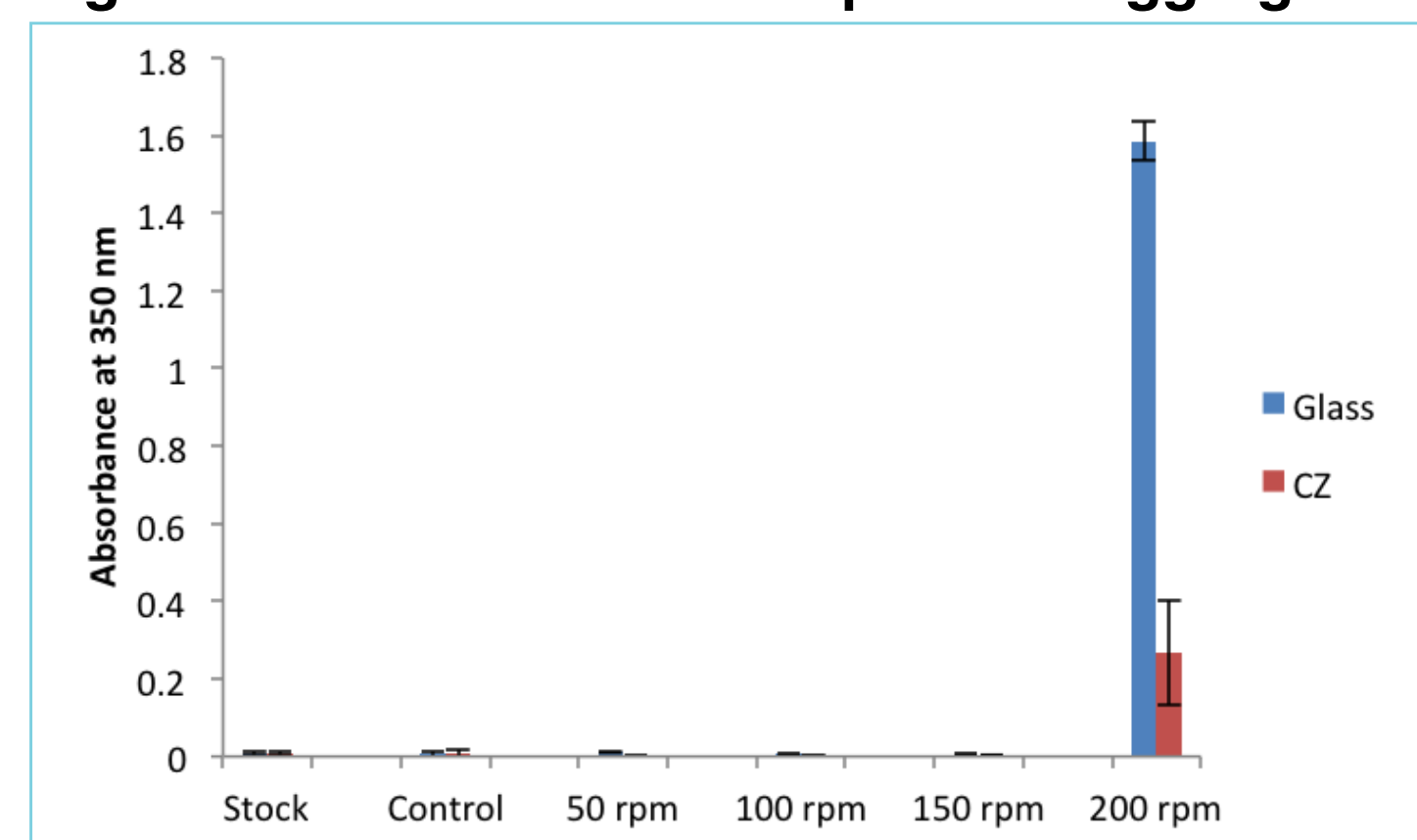### Figure 1: Effect of rotation speed on aggregation of MAb1

Figure 1: 2 mL vials were filled with 1mL of a solution of MAb1 which were agitated for 96 hr. Turbidity was measured spectrophotometrically by the absorbance at 350 nm.

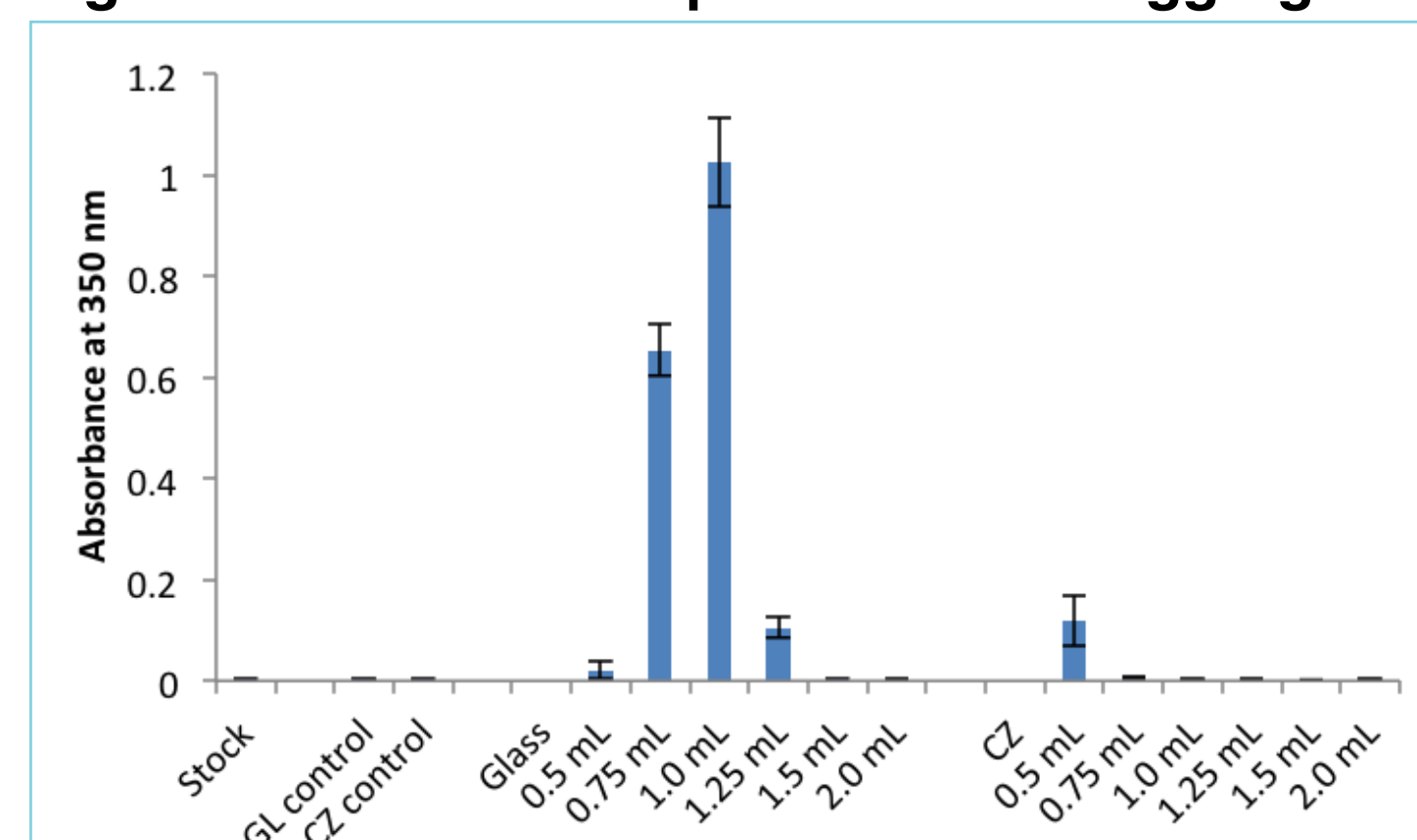### Figure 2: Effect of sample volume on aggregation of MAb1

Figure 2: 2 mL vials were filled with different volumes of MAb1 and shaken for 96 hr at 200 rpm. Turbidity was measured spectrophotometrically by absorbance at 350 nm.

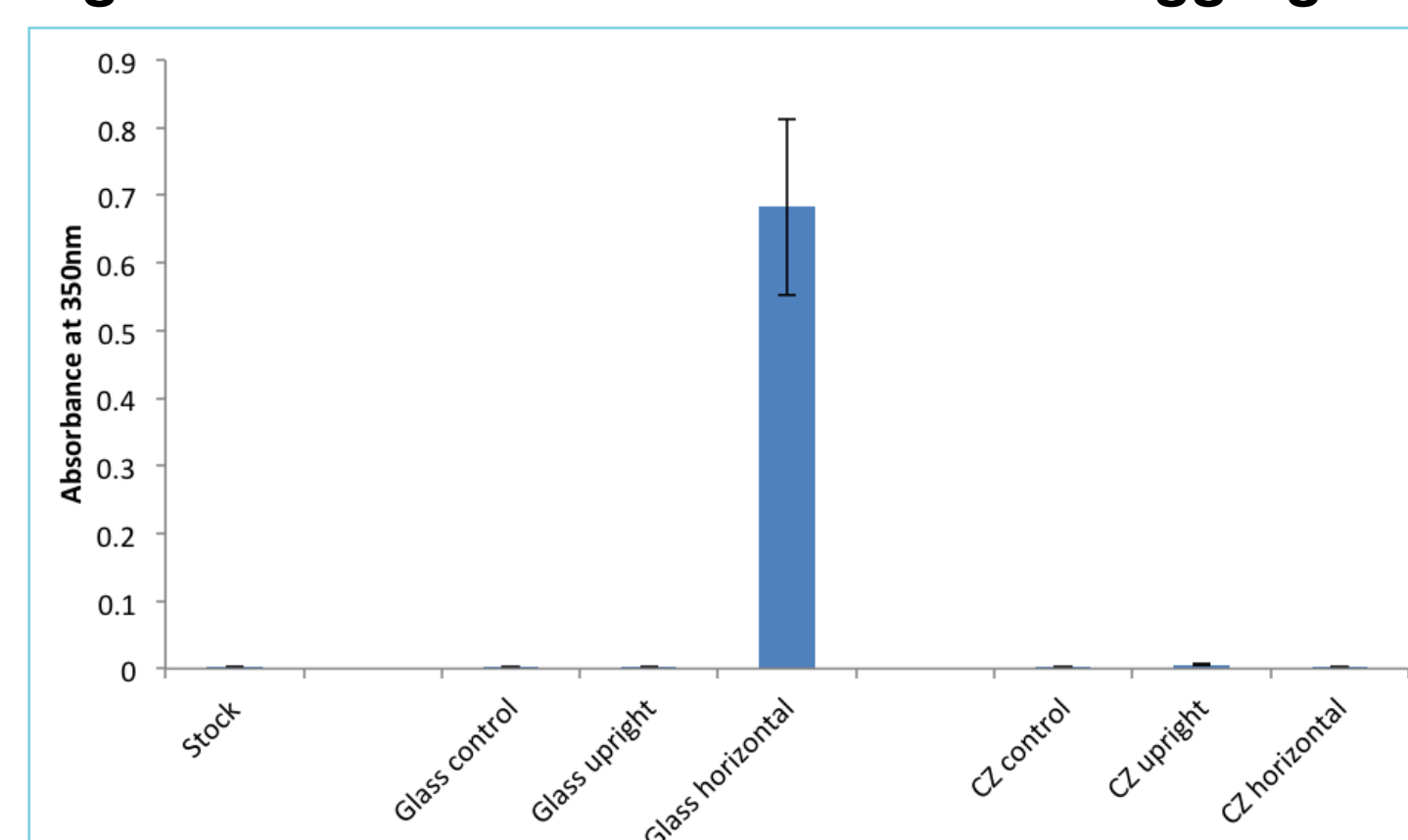### Figure 3: Effect of vial orientation on aggregation of MAb1

Figure 3: 2 mL vials were filled with 1 mL of MAb1 and shaken at 200 rpm for 96 hr. Vials were mounted either horizontally or upright on the shaking platform. Aggregation was measured by turbidity at 350 nm.

Under the conditions established above, we examined the aggregation of several other therapeutic MAbs, a therapeutic fusion protein and rabbit IgG. Figure 4 shows that under the conditions of this study rabbit IgG aggregates less in vials made of CZ than of glass. Quantitation of the amount of soluble protein by absorbance of the solutions after centrifugation to remove insoluble aggregates shows that 94% was soluble in CZ vials after shaking while 60% remained soluble in glass vials. Similarly, when MAb2 was shaken under the same conditions only 13% of the protein remained soluble in glass vials while 58% was soluble in vials made of CZ (Figure 5). These results indicate that CZ is superior to glass in preventing aggregation in vials under mechanical stress. One model of surface-induced aggregation proposes that adsorption of protein monomer onto the container surface promotes partial unfolding. These conformationally-altered monomers may aggregate either on the surface or perhaps after release back into solution due to mechanical forces. We suggest that this mechanism or one similar which occurs more efficiently on glass than on CZ may be applicable here.

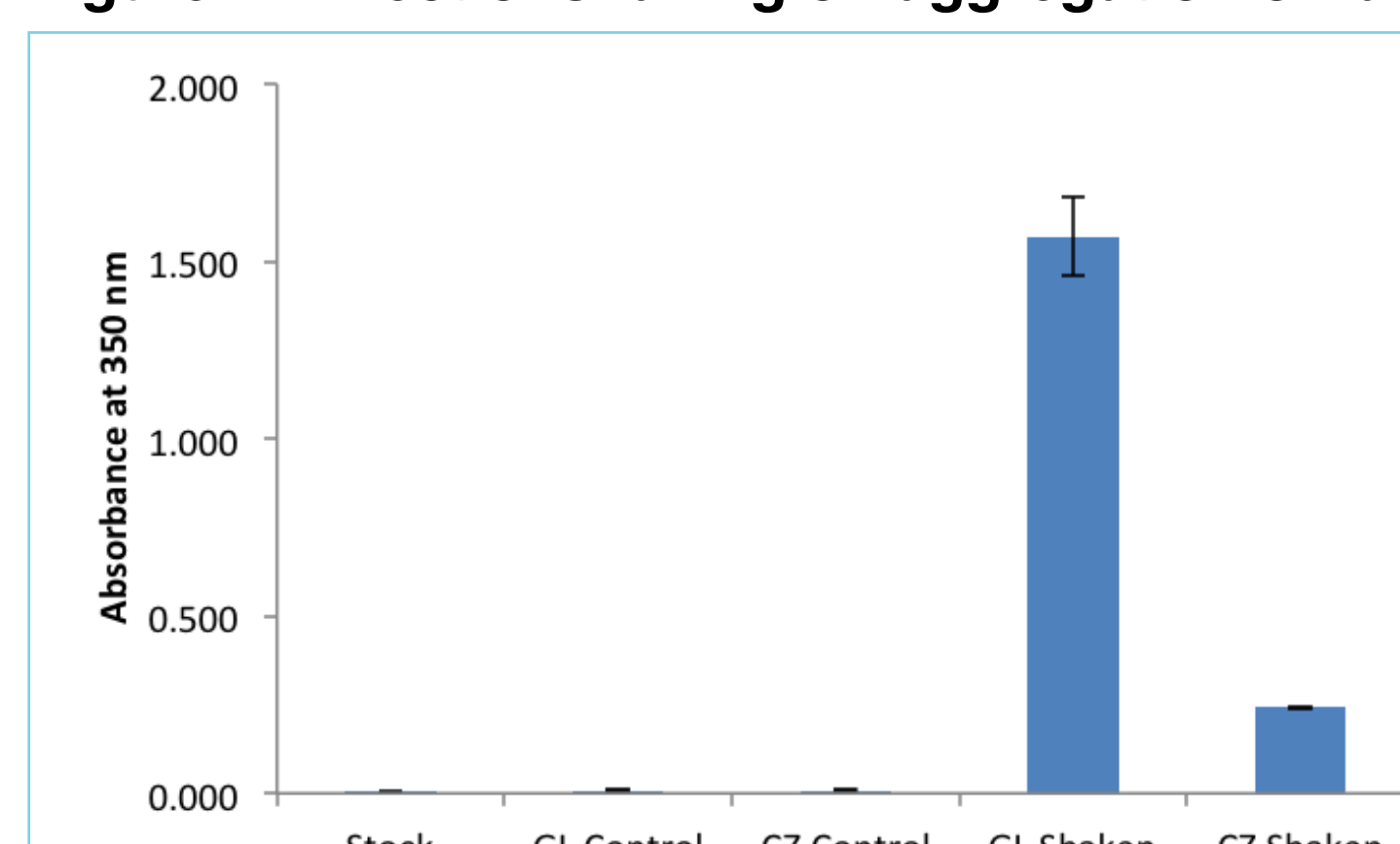### Figure 4: Effect of shaking on aggregation of rabbit IgG in vials

Figure 4: 2 mL vials were filled with rabbit IgG and shaken for 96 hr at 200 rpm. Aggregation was measured by turbidity at 350 nm (top) and at 280 nm after centrifugation to remove insoluble protein (bottom).

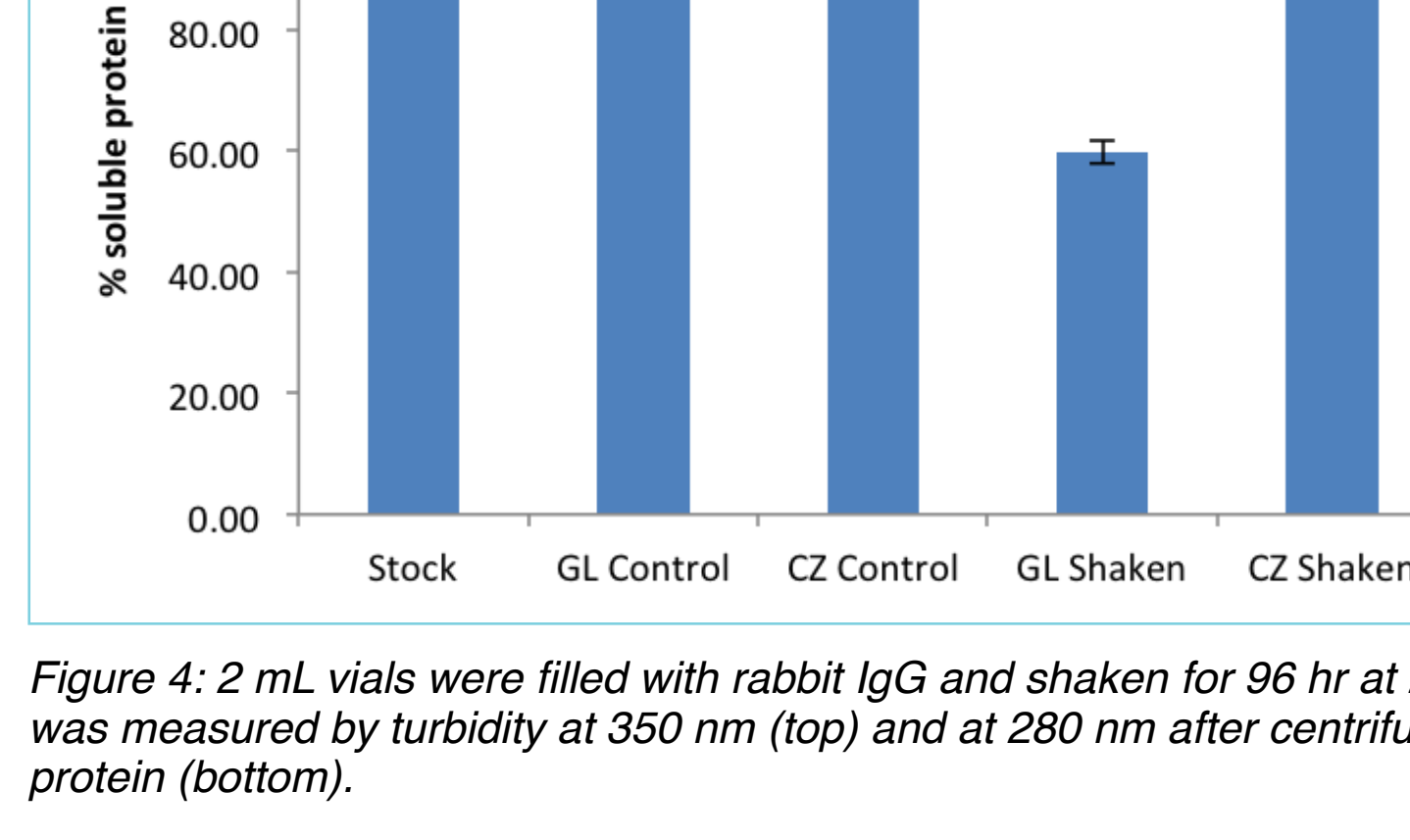### Figure 5: Effect of agitation on aggregation of MAb2 in vials

Figure 5: 2 mL vials were filled with 1.0 mL MAb2 or MAb3 and agitated for 96 hr at 200 rpm. Soluble protein was determined by the absorbance of the solution at 280 nm after centrifugation to remove insoluble material.

Some proteins were more resistant to aggregation than others under the conditions used in this study. MAb3 aggregated less in glass vials than either MAb1 or MAb2 and it was not possible do detect aggregation in vials made of CZ using turbidity measurements. The fusion protein FP1 showed no reproducible aggregation in either kind of vial in several experiments (Figure 6).

Figure 6: Effect of agitation on aggregation of MAb3 and FP1 in vials
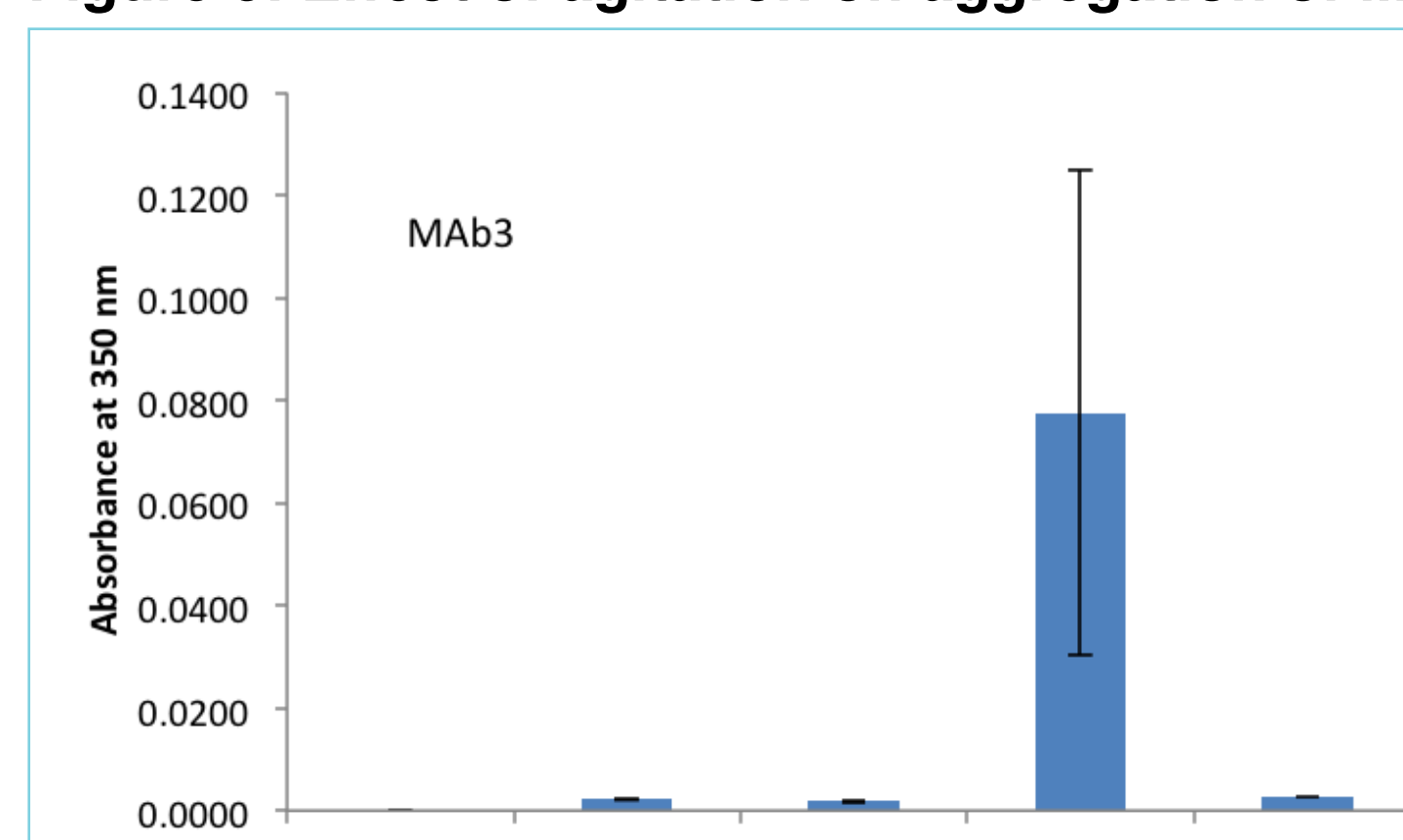

Figure 6: 2 mL vials were filled with 1.0 mL MAb3 or FP1 and shaken for 96 hr at 200 rpm. Aggregation was measured by turbidity at 350 nm.

Surfactants are frequently added to biotherapeutic formulations in order to prevent surface denaturation (Wang, et al.). Therefore, Polysorbate 80 (PS80) (0.03-01%, w/v) was added to determine if it could prevent the aggregation of MAb2 when shaken in glass vials. At 0.03%, the lowest concentration tested, PS80 eliminated the aggregation of MAb1 (Figure 7).

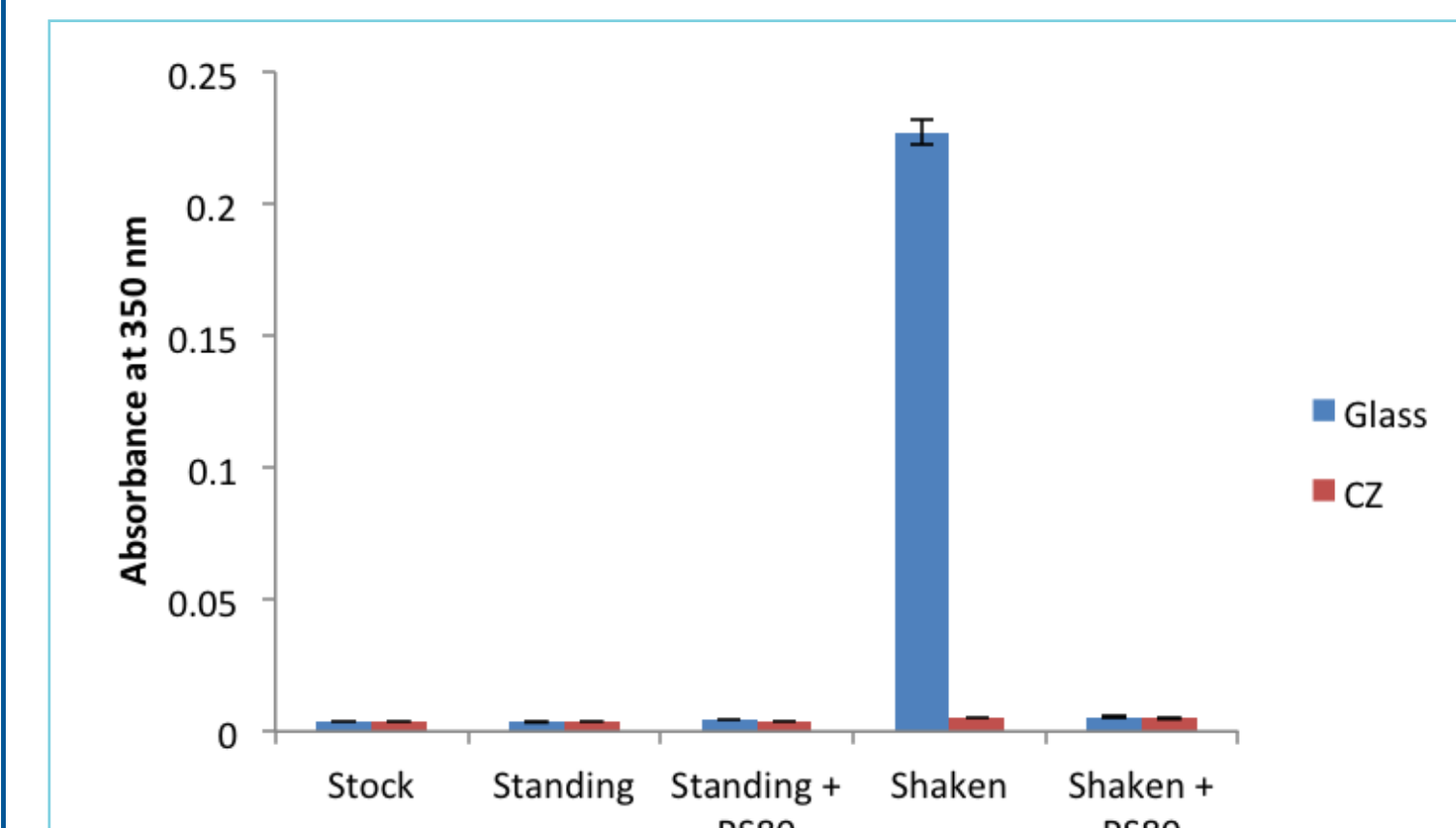### Figure 7: Effect of Polysorbate 80 on the aggregation of MAb1 in shaken vials

Figure 7: 2mL vials were filled with 1.0 mL MAb1 and a small volume of an aqueous solution of PS80 was added to a final concentration of 0.03%. The vials were shaken for 96 hr at 200 rpm and the turbidity was measured at 350 nm.

The properties of aggregates formed by agitation in glass vials was also examined. Since PS80 was able to prevent aggregation in shaken vials, whether or not the surfactant could reverse preformed aggregates was tested. Figure 8 shows that when PS80 was added to a solution of aggregates of MAb1 and incubated for one week, there was essentially no change in the turbidity of the solution, indicating that surfactant was unable to reverse aggregates of MAb2. Dilution of the aggregates into fresh buffer also failed to dissociate the aggregates (data not shown).

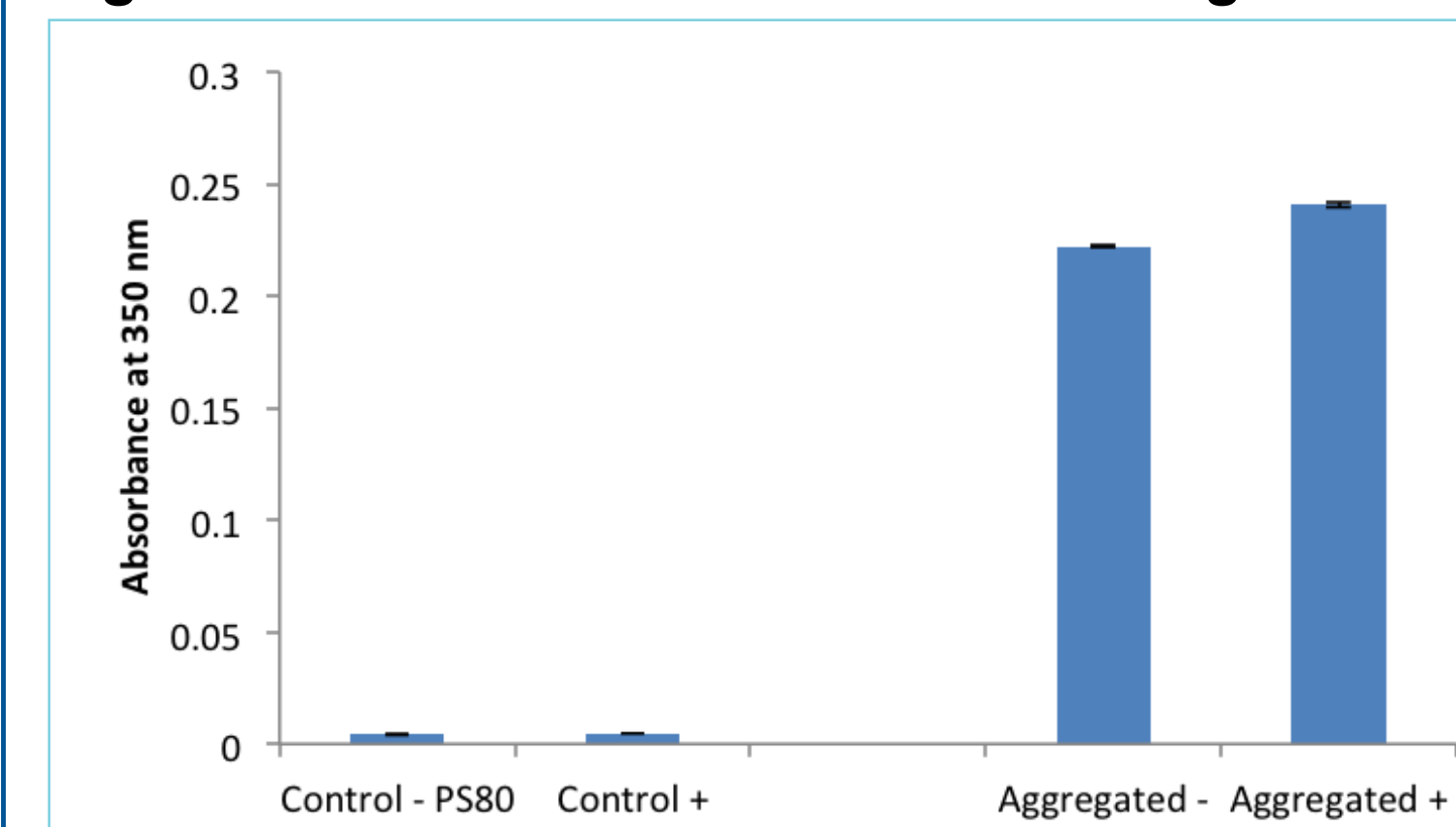### Figure 8: Effect of surfactant on reversing MAb1 aggregates

Figure 8: Aggregates of MAb1 were formed by shaking in glass vials for 48 hr. The aggregates were pooled and redistributed into fresh glass vials. A concentrated aqueous solution of PS80 was added to a final concentration of 0.1% to one set of vials while the second set received the same volume of water. The vials were then stored at 4°C for one week and the turbidity of the solutions was measured at 350 nm.

Since MAb1 did not aggregate significantly in CZ vials under the experimental conditions used in this study, a solution of aggregates formed by shaking MAb1 in glass vials was tested to see whether or not it could catalyze the aggregation of fresh MAb1 in vials made of CZ. However, as shown in Figure 9, aggregate formation ceased in CZ vials while it continued to increase in vials made of glass. In a related experiment, when aggregates formed in glass vials for 24 hr were transferred directly to fresh vials and shaken, aggregation continued to take place in glass vials but did not increase in vials made of CZ (data not shown).

Figure 9: Effect on aggregation of MAb1 spiked with aggregated MAb1
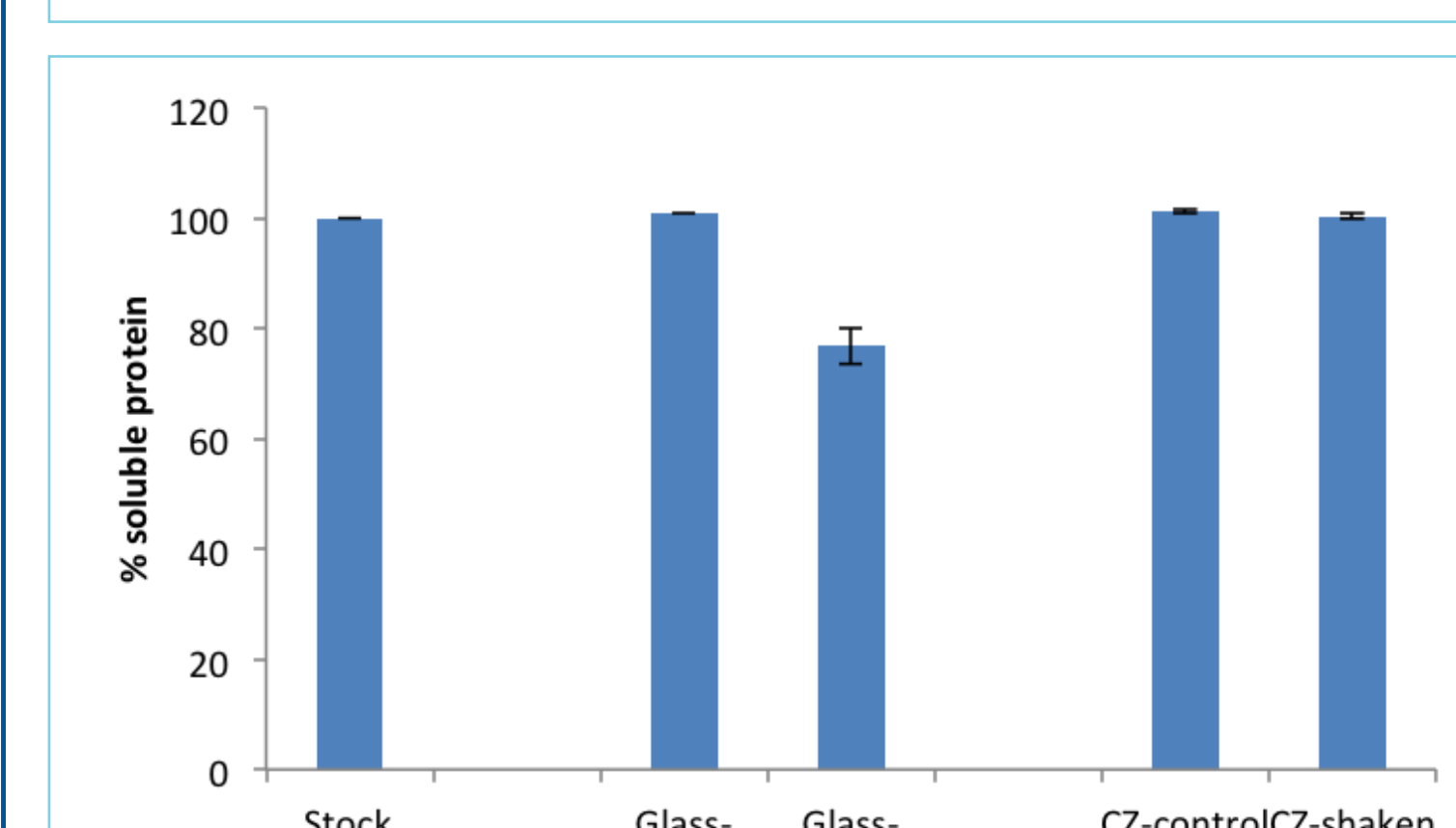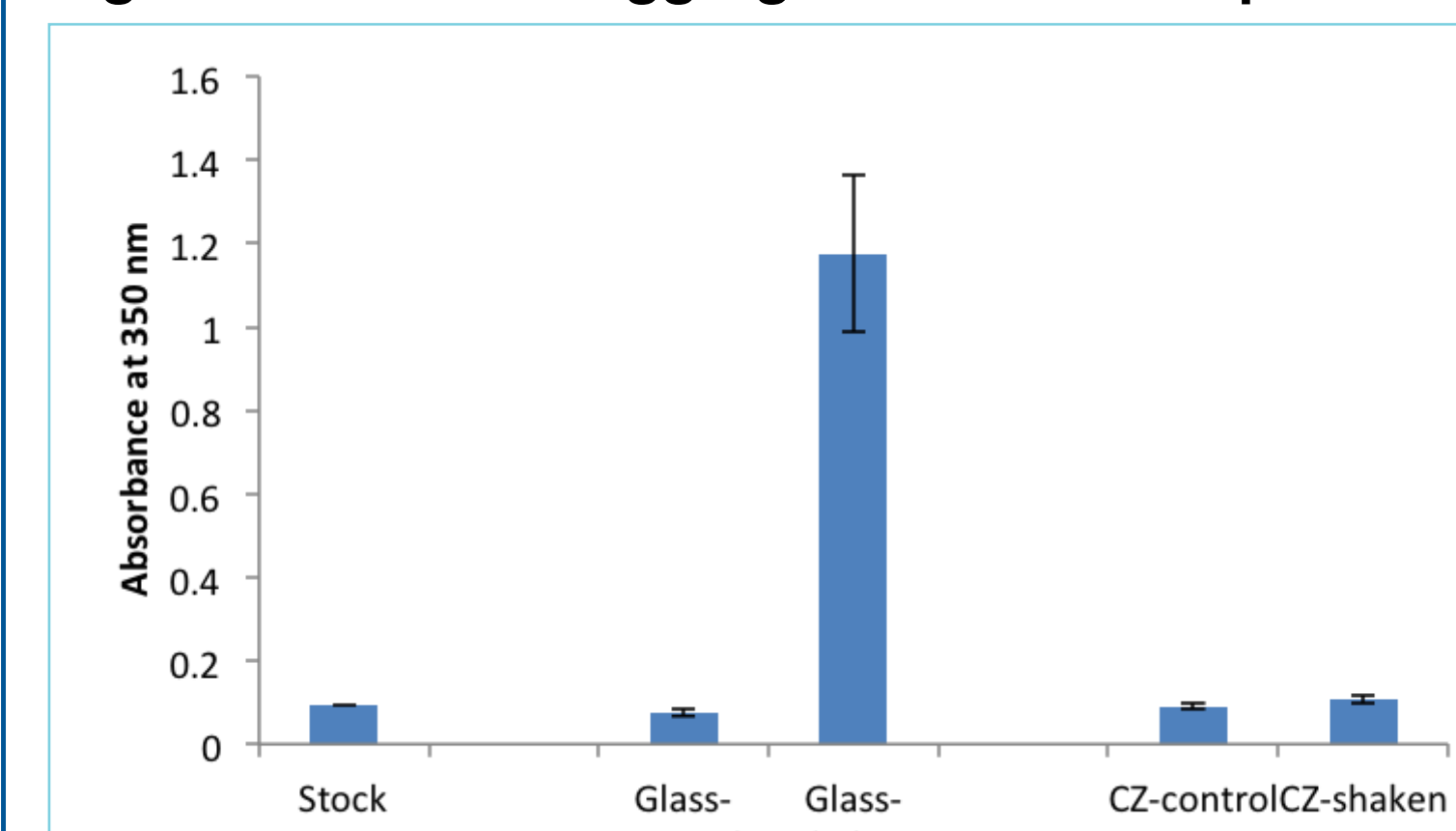

Figure 9: Aggregates of MAb1 were produced by shaking in glass vials for 96 hr, corresponding to 17% aggregated protein. One volume of this material was diluted with nine volumes of unaggregated MAb1 and redistributed into fresh vials made of glass or CZ. The spiked material was shaken for 96 hr and aggregation was measured by turbidity at 350 nm as well as at 280 nm after centrifugation to remove insoluble material. Aggregation in the glass vials after dilution and shaking corresponded to 20% of the total protein.

Conclusions

• In general, proteins showed a reduced extent of aggregation in vials made of CZ compared to glass vials when subjected to vigorous agitation.

• Although the air-liquid interface plays a major role in mechanically-induced aggregation, the surface properties of the primary container are also important to evaluate.

• Rotation at high speed on an orbital platform shaker is a simple model of mechanical stress to examine the effects of agitation on the aggregation of therapeutic proteins.

• The application of this method can be of use in evaluating vials for storage and administration of biologics.

References

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