

# DEVELOPMENT OF AN ASSAY TO DETERMINE PROTEIN SENSITIVITY TO TUNGSTEN AND TO INVESTIGATE THE EFFECTS OF pH AND IONIC STRENGTH ON AGGREGATION

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## ABSTRACT

**Purpose:** Tungstate polyoxyanions have been implicated in the aggregation of proteins in prefilled syringes. Assays based on SEC-HPLC and turbidity were used to determine protein sensitivity to tungstate and to investigate the effects of pH and ionic strength on the aggregation of proteins by tungstate polyoxyanions.

**Methods:** Sensitivity to aggregation by tungstate was evaluated by incubating proteins at concentrations of 0.1- 1 mg/mL in microfuge tubes in 20 mM buffers pH 4-7 with tungstate at 1-100ppm. Stock solutions of tungstate and NaCl were prepared in the same buffers and readjusted to the nominal pH. After incubation overnight at 4°C, turbidity was measured spectrophotometrically at 350 nm. Protein precipitates were removed by centrifugation and the soluble protein was analyzed by SEC-HPLC.

**Results:** Protein aggregation in the presence of tungstate was observed from pH 4-5.5, but not at 6 or 7. Aggregation was blocked by increasing the ionic strength with NaCl. The concentration of salt required to prevent precipitation was protein-dependent, but also varied with both pH and tungstate concentration. Once formed, precipitates could be resolubilized by raising the pH to 7. Not all proteins precipitated in the presence of tungstate including those with low isoelectric points. Unlike glass prefilled syringes, which contained measurable amounts of tungsten and silicone, syringes made of plastic were both tungsten- and silicone-free.

**Conclusions:** Assays based on turbidity and SEC-HPLC can be used to evaluate protein sensitivity to tungsten in order to optimize formulations for the packaging of biologics in glass prefilled syringes. Ionic strength and pH are important elements in the aggregation of proteins by tungstate polyoxyanions. An acidic pH is necessary for the generation of polyoxyanions and aggregation by tungstate polyoxyanions may be blocked by increasing the ionic strength of the formulation. This suggests that the interaction of polyoxometalates with proteins is primarily electrostatic. Some proteins precipitate with tungstate more readily than others, but a net positive charge is essential. Although protein size does not appear to be important, other factors related to a protein's structure may play a role in determining binding and aggregation by tungstate polyoxyanions. Syringes made of plastic that are free of both silicone oil and tungsten, such as Daikyo Crystal Zenith® resin, should be considered for use with sensitive biomolecules.

## INTRODUCTION

The aggregation of protein therapeutic products has the potential to cause severe immune responses in patients. Silicone oil, which is used to lubricate glass prefillable syringes, has been reported to induce protein aggregation (Jones et al, 2005), and in several cases the aggregation of therapeutic proteins has been attributed to the presence of tungsten particles in prefilled syringes (Rosenberg, 2006). The source of tungsten in glass prefilled syringes appears to be due to tungsten oxide vapor deposits in the syringe funnel area and shed from the heated tungsten pins used to produce the channel through which the needle is mounted (Liu et al, 2010). Two recent *in vitro* studies have identified conditions required to induce protein aggregation by tungsten (Jiang et al, 2009; Bee et al, 2009).

The picture that has emerged from those investigations is that soluble tungsten polyanions generated at acidic pH (Pope, 1983) are responsible for tungsten-induced protein aggregation. In addition to being primarily electrostatic in nature and dependent on the concentration of both protein and tungsten, the interaction is partially reversible, and Bee et al (2009) have calculated that the protein co-precipitates with polytungstate as a charge-neutral complex. Both reports used soluble tungsten species from commercial sources and concluded that acidified Na<sub>2</sub>WO<sub>4</sub> was the most potent. In addition, Jiang et al (2009) prepared extracts from used tungsten pins and showed that these were also effective at inducing formation of protein aggregates.

We have used both turbidity measurements and SEC-HPLC to monitor aggregation by Na<sub>2</sub>WO<sub>4</sub>. In addition to confirming the results of recent studies, we show that under the extreme conditions of low pH and ionic strength used to investigate this phenomenon *in vitro* that most proteins are susceptible to precipitation by soluble tungsten. Our data suggest that the net charge on a protein is a predictor of precipitation by tungsten. We also show that prefilled syringes made of plastic do not contain silicone oil or tungsten, which are not used in their manufacture. Therefore, for proteins sensitive to aggregation by tungsten or silicone oil, plastic syringes are an attractive alternative to syringes made of glass.

## METHODS

### Preparation of Reagents

Buffers used in these studies had a concentration of 20 mM and were prepared at 23-25°C. Sodium acetate was used at pH 4.0, 4.5, 5.0 and 5.5. Sodium citrate was used at pH 6.0 and sodium phosphate at pH 7.0. Stock solutions of tungstate were prepared at a concentration of 10,000ppm by dissolving sodium tungstate powder in each buffer and readjusting the pH. The UV spectrum (350 nm – 260 nm) of each solution of tungstate was unchanged after centrifugation (15,000 x g/15 min) indicating total solubilization of the tungstate salt and the absence of particulate material. Other concentrations were prepared by serial dilution of the 10,000ppm solutions. Stock solutions of 1 M NaCl were prepared at each pH to study the effect of ionic strength on protein precipitation by tungstate.

Protein solutions were prepared by dissolving the powder in deionized water or in buffer to a concentration of 1 or 5 mg/mL. Proteins which were obtained as concentrated solutions were diluted in either water or buffer. The protein concentrations were verified spectrophotometrically using published extinction coefficients. The properties of some of the proteins used in this study are listed in Table I.

**Table I: Biophysical properties of proteins used in this study**

| Protein   | M <sub>r</sub> x 10 <sup>-3</sup> | # Basic Groups | Experimental pI | Calculated pI | Calculated Net Charge at pH 4 | Calculated Net Charge at pH 5 |
|-----------|-----------------------------------|----------------|-----------------|---------------|-------------------------------|-------------------------------|
| BSA       | 66.4                              | 104            | 4.7-5.2         | 5.6           | + 64                          | + 17                          |
| ChyA      | 25.7                              | 21             | 8.8-9.6         | 8.5           | + 15                          | + 7                           |
| Ovalbumin | 42.9                              | 43             | 4.5-4.7         | 5.2           | + 22                          | + 1                           |
| Lysozyme  | 14.3                              | 19             | 10.5-11.0       | 9.3           | + 12                          | + 9                           |
| Pepsin    | 34.5                              | 5              | 1, 2.2-2.8      | 3.2           | - 12                          | -32                           |

### Protein Aggregation Assays

Assays were carried out in siliconized microfuge tubes in a volume of 1200  $\mu\text{L}$ . The reaction mixture consisted of 120  $\mu\text{L}$  protein solution, 960  $\mu\text{L}$  buffer and 120  $\mu\text{L}$  tungstate stock solution at the same pH. The final concentration of protein was typically 0.1 – 0.5 mg/mL. The mixtures were stored overnight at 4°C prior to analysis. Controls which contained buffer in place of tungstate were run to determine whether pH alone had an effect on protein aggregation. In experiments to examine the effect of ionic strength, aliquots of stock solutions of NaCl prepared at various pHs were added to the incubation mixtures instead of buffer.

Two methods were used to measure protein aggregation:

Protein turbidity was determined spectrophotometrically by measuring the change in absorbance at 350 nm after storage compared to a control which lacked tungstate.

Aggregation was quantitated by SEC-HPLC analysis of the same samples following centrifugation (15,000  $\times$  g/10 min) to remove insoluble aggregates. Soluble protein was injected onto a GE Amersham Superdex 200 column (1  $\times$  30 cm) controlled by a Waters 2695 liquid chromatography system. Proteins were detected by their absorbance at 280 and 220 nm. Recovery was determined by comparing the area under the peak relative to the control. The elution buffer was 20 mM sodium phosphate pH 7.0 containing either 150 or 500 mM NaCl.

### Tungsten and Silicone Content of Prefilled Syringes

Tungsten was analyzed using ICP-MS on a Perkin-Elmer/SCIEX Elan DRC II. Tungsten was extracted from glass or plastic syringes by drawing up 1 mL 2%  $\text{HNO}_3$  or 1 mL 5%  $\text{NH}_4\text{OH}$  into each and sonicating the filled syringes for 1 hour at 50°C. The extracts were dispensed and the syringes were flushed with an additional 1 mL of solvent. The extract and the rinsate were pooled and brought to 3 mL with water. The lower limit of quantification is  $\sim$ 2.5 ng/mL, corresponding to  $\sim$ 1  $\mu\text{g}$ /syringe. Syringes were also incubated with each solvent for 7 days at room temperature prior to sonication.

Silicone was determined by atomic absorption spectroscopy on a Perkin Elmer AAnalyst 100. The needles were cut off four Daikyo Crystal Zenith® (CZ) 1 mL long syringes or four glass syringes ( $\sim$ 100  $\text{cm}^2$  barrel surface area) as close to the barrel as possible. The syringes were extracted with 50 mL methyl isobutyl ketone to solubilize the silicone. Blanks were prepared similarly. The lower limit of quantification is  $\sim$ 8  $\mu\text{g}$ /mL, corresponding to 100  $\mu\text{g}$ /syringe.

## RESULTS AND DISCUSSION

### Aggregation by Tungstate Requires Acidic pH

Assays were carried out with bovine IgG (blgG) over the pH range 4.0 - 7.0 at 1, 10 and 100ppm tungstate. Figure 1 shows that the blgG monomer is quantitatively precipitated by 10 and 100ppm tungstate at pH 4.0 to 5.5, but is not affected at pH 6.0 or 7.0. The data also show that lower tungstate (1ppm) is not as effective as higher concentrations at precipitating blgG.

Figure 1: Effect of pH on the precipitation of blgG by tungstate

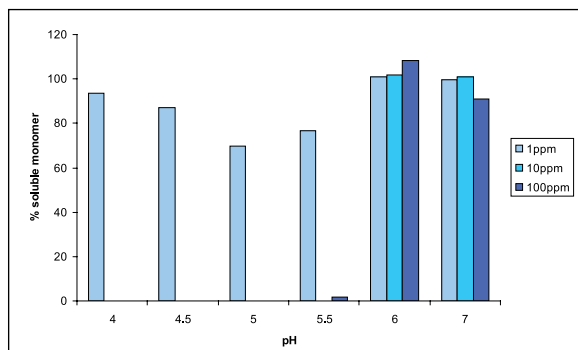


Figure 1: blgG at 50  $\mu\text{g}$ /mL was incubated over the pH range 4.0 – 7.0 with 1, 10 or 100ppm tungstate prepared at the same pHs. The amount of soluble monomer was quantitated by SEC-HPLC after centrifugation to remove precipitated protein.

### The Effect of Ionic Strength on Protein Precipitation by Tungstate

The effect of ionic strength on the precipitation of proteins by tungstate was investigated since many drug formulations contain varying amounts of salt. Figure 2 shows the effect of ionic strength on the precipitation of solutions of ChyA and blgG by tungstate at pH 5.0. The data indicate that 100 mM NaCl was sufficient to completely block the precipitation of ChyA by tungstate. In contrast, greater than 500 mM NaCl would be required to completely prevent precipitation of blgG under the same conditions. In addition, higher concentrations of salt were necessary to block aggregation in the presence of 100ppm tungstate than 10ppm tungstate, and higher ionic strength was required to block aggregation by tungstate at pH 5.0 than at pH 5.5 (data not shown). These experiments show that the interaction of tungstate with proteins is primarily electrostatic and that the interaction is stronger at lower pH.

Figure 2: Effect of ionic strength on the precipitation of proteins by tungstate

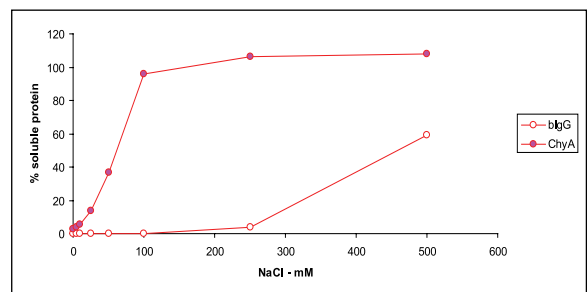


Figure 2: ChyA and blgG at 0.5 mg/mL were incubated at pH 5.0 with 100ppm tungstate and increasing concentrations of NaCl at the same pH. After centrifugation to remove precipitated protein, the soluble protein was quantitated by SEC-HPLC.

### Precipitation by Tungstate Is Dependent on the Relative Concentration of Tungstate and Protein

In addition to being dependent on the concentration of tungstate, precipitation is also a function of protein concentration. Figure 3 shows that the concentration of tungstate required to precipitate 50% of the ChyA (IC50) shifted to the right with increasing protein concentration, and there was a linear relationship between the IC50 and protein concentration (data not shown). At the IC50 the molar ratio between tungsten and protein was  $\sim$ 19:1. These results support a model that would require binding of multiple tungstate molecules to initiate protein precipitation.

Figure 3: Effect of tungstate concentration on the precipitation of ChyA at different concentrations

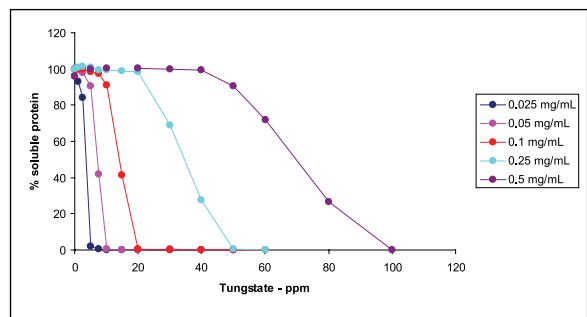


Figure 3: ChyA at 0.025, 0.05, 0.1, 0.25, and 0.5 mg/mL was incubated overnight at pH 4.0 with tungstate from 1-100ppm. After centrifugation to remove precipitated protein, the protein in the soluble fraction was quantitated by SEC-HPLC.

### Turbidity Can Also Be Used to Evaluate Protein Aggregation by Tungstate

Measurement of protein turbidity caused by tungstate aggregation provides a rapid alternative to SEC-HPLC. Turbidity was monitored spectrophotometrically by measuring the change in absorbance at 350 nm, a wavelength where the absorbance of most proteins is negligible. Figure 4 shows a comparison of data obtained for four proteins using both methods. The IC50s for both methods are 10-20ppm tungstate. An assay based on turbidity could also be performed in a microplate format which would increase throughput and reduce sample handling.

**Figure 4: Comparison of protein aggregation by SEC-HPLC with turbidity**

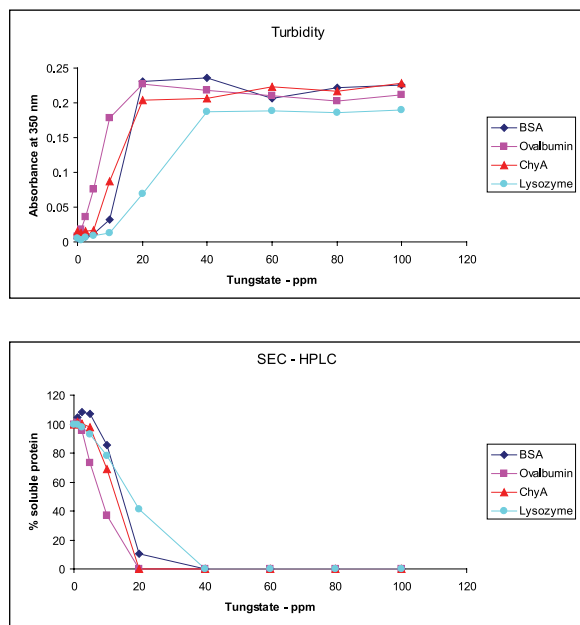


Figure 4: Proteins at 100  $\mu\text{g}/\text{mL}$  were incubated overnight at pH 4.0 with tungstate from 1-100ppm. After measuring the absorbance at 350 nm, the samples were centrifuged to remove insoluble aggregates and the soluble protein was quantitated by SEC-HPLC.

### Aggregation by Tungstate Is Correlated with Protein Net Charge

Since the interaction of tungstate polyoxyanions appears to be primarily electrostatic, proteins with a net positive charge at a particular pH should be precipitable at that pH. Table I summarizes the physical properties of several of the proteins used in this study. When tested at pH 4, there was little difference between the IC50s for BSA (pI 4.2-4.9), ovalbumin (pI 4.5-4.7) and the basic proteins ChyA (pI 8.8-9.6) and lysozyme (pI 10.5-11) (Figure 4), four proteins which differ in mass, isoelectric point, net charge and total number of positive charges (Table I). However, the protein pepsin, which has a pI below 3 and a net negative charge at pH 4 (Table I), was not precipitated by tungstate even at 1000ppm (data not shown). This result is consistent with the proposal that the net charge on a protein is a key determinant of precipitability.

Based on these observations, we tested whether we could predict the pH range over which a protein would be precipitated by tungstate. We compared the pH dependence of ovalbumin (pI 4.5-4.7) with lysozyme (pI 10.5-11.0). Figure 5 shows that lysozyme could be precipitated from pH 4 to 5.5, a range over which it remains positively charged. In contrast, ovalbumin was only precipitable at pH 4 and 4.5, but at pH 5, slightly above its isoelectric point and close to where it is electroneutral, it was no longer precipitable.

**Figure 5: Effect of pH on the precipitation of ovalbumin and lysozyme**

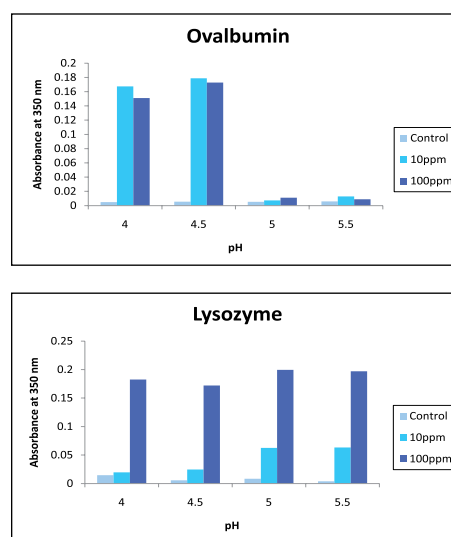


Figure 5: Ovalbumin and lysozyme at 100  $\mu\text{g}/\text{mL}$  were incubated overnight at pH 4.0-5.5 with tungstate at 10 and 100ppm. Turbidity was measured spectrophotometrically at 350 nm.

### The Interaction of Proteins with Tungstate Is Reversible

To determine whether protein/tungstate precipitates are reversible, a set of precipitates of BSA and blgG were prepared using pH 5.0 buffer and 100ppm tungstate, conditions shown to quantitatively precipitate each protein. The precipitates were resuspended in 1200  $\mu\text{l}$  (the standard assay volume) in pH 5.0 buffer with or without 0.5 M NaCl or in pH 7.0 buffer with or without 0.5 M NaCl. Table II shows that dilution into incubation buffer in the absence of tungstate did not solubilize the precipitates. The addition of 0.5 M NaCl at low pH solubilized about 11% of blgG. Resuspension in pH 7.0 buffer solubilized greater than 90% of BSA and ~60% of blgG. However, inclusion of 0.5 M NaCl in pH 7.0 buffer completely solubilized blgG. Analysis by SEC-HPLC also permitted an examination of the sizes of the resolubilized species. Both blgG and BSA contained slightly more dimer and higher molecular weight aggregates than were present in the control solutions.

**Table II: Reversibility of protein aggregates**

|                              | BSA Monomer | BSA Total | blgG Monomer | blgG Total |
|------------------------------|-------------|-----------|--------------|------------|
| Control                      | 100         | 100       | 100          | 100        |
| pH 5.0 buffer                | 0           | 0         | 0            | 0          |
| pH 5.0 buffer/<br>0.5 M NaCl | 0           | 0         | 10.6         | 10.7       |
| pH 7.0 buffer                | 94.0        | 98.1      | 55.0         | 58.7       |
| pH 7.0 buffer/<br>0.5 M NaCl | 93.7        | 100.3     | 88.5         | 99.7       |

Note: Protein precipitates were resuspended under each of the conditions listed in the table and stored overnight at 4°C. The suspensions were centrifuged to remove insoluble protein and the soluble protein was analyzed by SEC-HPLC.

### Tungsten-free, Silicone-free Prefilled Syringes for Sensitive Proteins

In contrast to staked needle syringes made of glass, the CZ syringe system does not utilize a tungsten pin to create an opening for inserting the needle. Consequently, this syringe system is tungsten-free and therefore a suitable alternative for the packaging of sensitive protein therapeutics. An analysis of tungsten was carried out on prefilled syringes made of CZ and glass. Table III shows that the amount of extractable tungsten in the CZ syringes is at the level of the solvent blank while the amount of tungsten in syringes made of glass is significant.

Silicone is also present in glass syringes, used as a lubricant. Silicone has been observed to cause aggregation, deformation and inactivation of native protein structure (Jones et al, 2005; Markovic, 2006). We therefore compared the silicone content of glass syringes with ones made of CZ, which do not contain silicone oil as a lubricant. Table IV shows a comparison of the amount of silicone in glass and plastic prefilled syringes. In contrast to syringes made of glass, plastic syringes are silicone-free.

**Table III: Tungsten content of prefilled syringes**

| Sample          | ng tungsten/syringe   |                     |
|-----------------|-----------------------|---------------------|
|                 | 5% NH <sub>4</sub> OH | 2% HNO <sub>3</sub> |
| CZ 1 mL IN Long | 0                     | 0                   |
| Glass 1 mL Long | 117                   | 46                  |

Note: Tungsten was extracted by drawing up 1 mL of solvent and sonicating for 1 hour at 50°C. The extract was expelled and the syringes were rinsed with an additional 1 mL of solvent and brought to 3 mL with water. The extracts were analyzed for tungsten by ICP-MS. Values shown are the mean of two (glass) or four (CZ) syringes extracted with either 5% NH<sub>4</sub>OH or 2% HNO<sub>3</sub>. The values obtained for the CZ syringes were identical to the solvent blank.

**Table IV: Silicone content of prefilled syringes**

| Sample          | µg silicone/syringe |
|-----------------|---------------------|
| CZ 1 mL IN Long | 0                   |
| Glass 1 mL Long | 203.5               |

Note: Values are the mean of two (glass) or four separate experiments (CZ) in which four syringes were extracted with methylisobutylketone to solubilize silicone. Prior to extraction, needles were first cut off as close to the barrel as possible. The extracts were analyzed by atomic absorption spectroscopy.

## SUMMARY

- Turbidity and SEC-HPLC were used to characterize protein aggregation by tungstate. Consistent results were obtained with both techniques, although SEC-HPLC can potentially provide information about the types of soluble aggregates which are formed.

- Aggregation by tungstate is a complex process and is influenced by a number of factors. These include pH, ionic strength, the relative concentration of both protein and tungstate, and the nature of the protein.
- Reversibility of aggregation by salt and raising the pH strongly suggests that protein aggregation is primarily a function of electrostatic interactions between the protein and polytungstate anions. The tendency of a protein to aggregate at a particular pH can be correlated with its net charge.
- To minimize the interaction of protein therapeutics with tungsten in prefilled syringes made of glass, formulations should be optimized at pH 6 or above preferably to include salt.
- The CZ 1 mL Long syringe system is not manufactured using a tungsten pin and is both tungsten- and silicone-free. It is ideally suited for biopharmaceutical drug delivery.

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