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Note

Calorimetric study of bovine serum albumin dilution and adsorption onto polystyrene particles

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Abstract

Titration calorimetry was used to investigate the interaction between a model antigen, bovine serum albumin (BSA), and a model particulate carrier, polystyrene (PS). The binding enthalpy was much higher than reported in the literature for a similar system and did not display a sigmoidal binding curve. These experiments may have accessed low coverage surface sites due to the irreversible nature of protein binding and stepwise titration. An important correction is the heat of dilution of the protein solution. Two regimes were observed: at low concentrations of BSA (below ca. 0.3% (w/v)) an exothermic dilution enthalpy of ca. $-100\,mJ\,mg^{-1}$ was determined, whereas at higher concentrations of BSA values of ca. $-20\,mJ\,mg^{-1}$ were obtained. Solution rheological data also showed a change at 0.3% (w/v) BSA, so we hypothesise that the fraction of the BSA as monomers, dimers and polymers in solution changes at approximately 0.3% (w/v).

Keywords: Adsorption; Dilution; Titration calorimetry; Bovine serum albumin; Protein; Binding

Some vaccines are under development in which the antigen is adsorbed onto a particle, which can be delivered mucosally rather than parenterally and have the potential to stimulate a stronger immune response when compared to the antigen in solution. For such a vaccine to work, the antigen must be presented in a form that stimulates the immune system: that is, in a conformation that functionally matches the natural antigen (O'Hagan and Valiante, 2003). This work aims to characterise the adsorption process for future particulate vaccine carrier optimisation. The adsorption of a model antigen, bovine serum albumin (BSA) onto a model particulate carrier, polystyrene (PS) was studied using isothermal titration calorimetry.

A Thermometric (Järfälla, Sweden) 2277 Thermal Activity Monitor (TAM) was used with a titration unit for all experiments. The calorimeter reaction cell (4 ml volume) was filled with 2.8 ml of 0.9% (w/v) NaCl and 233 μl of PS particle suspension (ca. 2.7% solids) (Polysciences, Warrington, PA 18976, USA)

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and stirred using an acrylic turbine style stirrer. For dilution control experiments the PS suspension was substituted with deionised water. For all experiments, the system was allowed to stabilise for approximately 24 h during which an electrical calibration (for full scale deflection) was performed. Twenty injections of freshly dissolved BSA solution (in 0.9% (w/v) NaCl) were made, once every 2 h. The energy output from a given injection was found by integration of the power output over the 2 h following the injection (area under the peak) using Digitam version 4.1 software (Thermometric).

The calorimeter measures the heat change of the whole sample (Q). This includes all processes as well as the binding reaction of interest, as summarised by Eq. (1). Thus, control experiments are required to find the enthalpy change of all processes so the binding enthalpy can be found by subtraction.

$$Q = Q_{\text{friction}} + \Delta H_{\text{particle dilution}} + \Delta H_{\text{BSA dilution}} + \Delta H_{\text{binding}} \quad (1)$$

The heat of friction (Q_{friction}) was negligible: (-0.09 ± 0.26) mJ per injection and the heat of particle dilution ($\Delta H_{\text{particle dilution}}$) small: (0.18 ± 0.25) mJ per injection for the 1.05 μm latex particles and (0.37 ± 0.21) mJ for the 0.11 μm particles.

The importance of the BSA dilution control is shown by Fig. 1, which shows a sample power output against time graph. This is for experiments with and without particles present: binding and dilution experiments respectively. Fig. 1 shows that the magnitude of the power output was similar in both dilution and binding experiments. Thus in order to find the binding enthalpy (the difference between the binding and dilution experiments), knowledge of the dilution process is required. Fig. 1 also shows that the reaction is slow: the heat output lasts for the duration of the two hour injection period.

Results for the enthalpy change of dilution of various concentrations and volumes of BSA are shown in Fig. 2. Twenty injections of BSA solution were made into the cell, initially containing 0.9% (w/v) NaCl only. Thus, for the second and subsequent injections, the enthalpy change is that of comparatively concentrated BSA injections mixing with dilute BSA in the cell. The dilution ratio is fairly large: 200:1, for 15 μl injections. The results are plotted as cumulative enthalpy, which is the sum of all the injection enthalpies from the first injection to the injection for the particular result point. The curves on Fig. 2 lie in one of two regions depending on the concentration of BSA in the injections: low concentrations have a more exothermic dilution enthalpy: ca. $-100\,\text{mJ mg}^{-1}$, compared to ca. $-20\,\text{mJ mg}^{-1}$ for concentrations above around 0.3% (w/v) BSA. This trend of dilution enthalpy is that less heat is released from diluting the more concentrated protein sample, which is the opposite of diluting simple salts. This trend and the existence of a critical concentration suggest a change in the state of the BSA in solution at ca. 0.3% w/v).

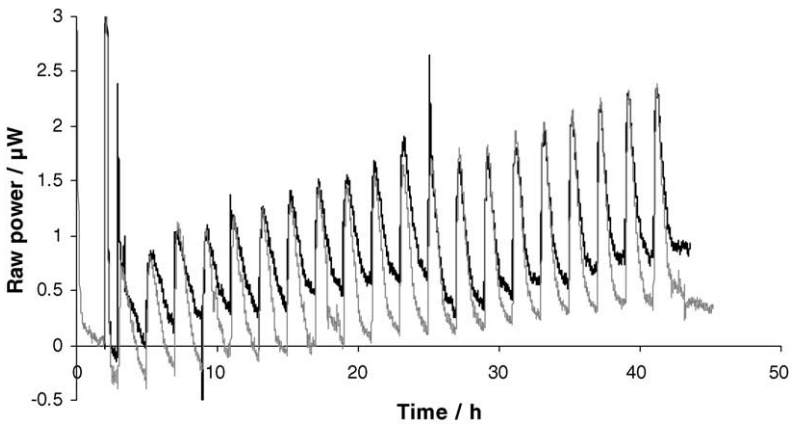

Fig. 1. Titration calorimetry raw power output graph. Black curve: binding to 1.05 μm PS suspension, grey curve: dilution control. Both experiments had twenty 15 μl injections of 0.2% (w/v) BSA in 0.9% (w/v) NaCl into 3.0 ml in the cell. The first peak is from calibration.

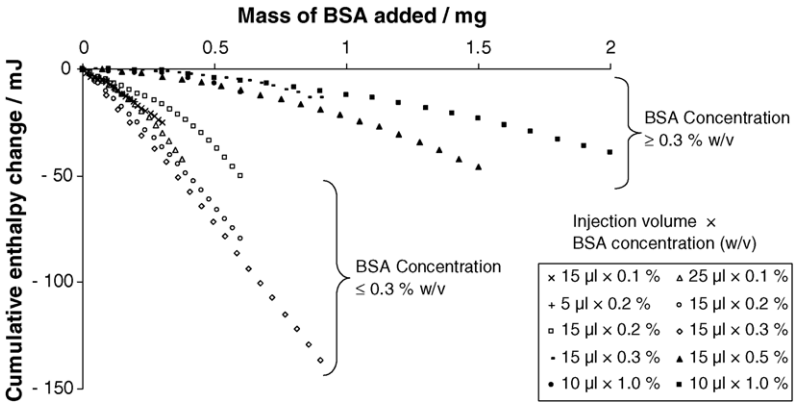

Fig. 2. Dilution enthalpy of BSA injected into 0.9% (w/v) NaCl showing the variation with BSA concentration and injection volume. The results are plotted as cumulative enthalpy, which is the sum of the enthalpies of all the injections up to the point.

Fig. 2 also shows some difference in dilution enthalpy between solutions of the same concentration, with both different and with the same injection volume. Changes in injection volume should not affect the dilution enthalpy, when plotted as a function of mass of protein. For example, three 5 μl injections of 0.2% (w/v) BSA should have the same enthalpy as one 15 μl injection. Differences may be due to sample handling or random error in the calorimetric measurements.

There has been some work published on dilution of BSA. Filisko et al. (1986), using a mix calorimeter and 2% BSA (in sodium chloride/hydrochloric acid buffer, pH 6.1) considered the heat of dilution of BSA negligible and did not report it. Phuong-Nguyen et al. (1982) used a mix calorimeter to dilute BSA (0.4 mM = 2.6% (w/v)) in phosphate buffer (0.1 M, pH 7.4) with more phosphate buffer (1:1.3 ratio). The heat output was 24.1 mJ for the 91 mg undialysed sample, which is a dilution enthalpy of $-0.26\,\mathrm{mJ\,mg^{-1}}$ (of BSA). Polyakov et al. (1986) used a flow calorimeter to dilute BSA (2.0–7.5% (w/v)) in water (pH 6.6–6.8, adjusted with strong acid or base) at a 1:1 ratio. They concluded that the dilution of BSA was practically athermal (less than $\pm 5\,\mu\mathrm{J\,mol^{-1}}$ of water, which corresponds to less than $\pm 14\,\mathrm{nJ\,mg^{-1}}$ of BSA for 2% (w/v) solution) and no trends in dilution enthalpy with concentration were observed.

The dilution enthalpy reported in the literature is thus much lower than that measured in this study, for which there may be a number of reasons. Firstly, the BSA concentrations used by Phuong-Nguyen et al. (1982) and Polyakov et al. (1986) are higher than the experimental range used in this study. Given that the results of our study showed a lower dilution enthalpy for higher initial BSA concentrations, it is possible that the lower enthalpy reported is due to the higher BSA concentration. In the current study, small injections of BSA solution into a large volume of medium were studied (large dilution ratio of between 120:1 and 600:1). However, those in the literature have much smaller dilution ratios (around 1:1), so are further from infinite dilution, so a lower enthalpy would be expected. Furthermore, both literature studies used different buffer media, which can change the protein binding properties and convolute the results from the heat of buffer ionisation. The study by Polyakov et al. (1986) in the flow calorimeter did not specify the residence time but stated that it is intended for studies of rapid reaction. Therefore, we speculate that the residence time is shorter than the 2 h per injection used in our experiments, hence lower dilution enthalpy would be expected. All of the studies described in the literature that report the dilution enthalpy of BSA, used BSA concentrations above the critical concentration of ca. 0.3% (w/v). Hence, they did not observe an enthalpy—concentration dependence.

The behaviour of the BSA solution was further investigated by solution rheological measurements. A CSL2-500 controlled stress rheometer (TA Instruments, Leatherhead, UK) was used with a 4 cm diameter, 2° angle cone. All experiments were of the equilibrium flow type. The results, in Fig. 3, show a change of rheological behaviour at 0.3% (w/v) BSA,

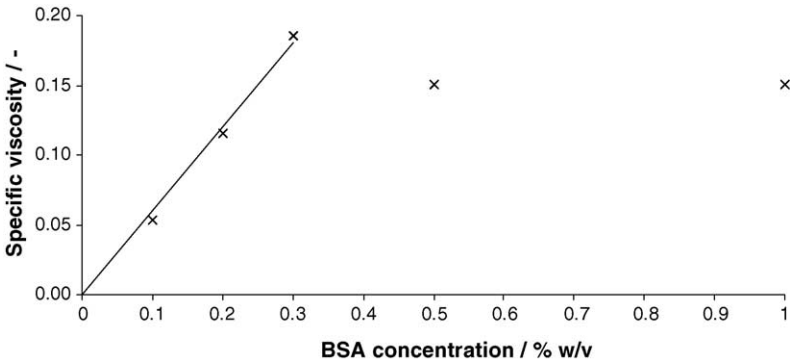

Fig. 3. Changes in specific viscosity $((\mu-\mu_{solvent})/\mu_{solvent})$ with BSA concentration. Freshly prepared solutions in 0.9% (w/v) NaCl.

consistent with the change in behaviour observed by titration calorimetry. Previous studies of the viscosity of BSA (Tanford and Buzzell, 1956; Monkos, 1996) have not observed this affect because they studied concentrations above 0.5% and 1.8% (w/v), respectively. For BSA concentrations below 0.3% (w/v), Fig. 3 shows a linear change in specific viscosity with BSA concentration, hence constant reduced viscosity (= specific viscosity/concentration) (Harding, 1997). For concentrations above 0.3% (w/v), the comparatively constant specific viscosity corresponds to a decrease in reduced viscosity. This could be caused by a decrease in the hydrodynamic diameter of the BSA molecules or a reduction in the attractive forces between the molecules (Harding, 1997). It is well known that BSA changes conformation with pH in solution (Tanford et al., 1955; Carter and Ho, 1994). However, all of our experiments were conducted at a pH of approximately 6.5, which corresponds to the normal ("N") conformation, so the hydrodynamic radius is expected to be constant. It has been reported that a proportion of BSA in solution is in the form of dimers and polymers (Yang et al., 1990; Giacomelli et al., 1997). Hence, we postulate that the fraction of BSA monomers is higher in solutions with less than 0.3% (w/v) BSA, a trend observed by Gabaldón (2002) for renaturing BSA. If monomers have more interaction with each other than dimers and polymers this explains the more exothermic dilution enthalpy and higher reduced viscosity in dilute (<0.3% (w/v)) solutions.

Models of protein adsorption to surfaces have a number of stages (Norde and Lyklema, 1979; Calonder and Van Tassel, 2001): protein molecules collide with the surface; initial interaction, for example, modulated through electrostatic forces and hydrophobic interaction; and bond strengthening, for example, by conformational change of the adsorbed material.

The binding energy was calculated per mass of protein added and is shown in Fig. 4. Surface saturation is expected to occur at around 1–2 mg m^{-2} (experimentally measured by Van Oss and Singer (1966) and by Tamai et al. (1990)). Any aggregation of the particles will cause some error in the exposed surface area calculation but this will not affect the overall trend of the data on Fig. 4. No aggregation was observed for the 0.36 or 1.05 μm diameter particles or for any particle size on addition of the 0.9% (w/v) NaCl to the latex stock. However, addition of BSA to the 0.11 μm diameter latex in 0.9% (w/v) NaCl caused visual aggregation of particles. As the aggregation appears to be caused by binding of the BSA, the surface area per bound BSA molecule should be unaffected.

None of the curves on Fig. 4 have a sigmoidal shape as is expected from chemical binding (for example, the binding of Ba^{2+} to 18-crown-6 ether, as reported by Briggner and Wadsö (1991)). A calorimetric study of HSA adsorption onto PS in the literature showed a total adsorption enthalpy of less than ±5 mJ m^{-2}, depending on pH (Norde and Lyklema, 1978). In this study, the binding enthalpy is order of ±20 mJ m^{-2} per injection. Norde and Lyklema (1978) reported that the heat output from the binding reaction was complete within 15 min, in contrast to our experiments where heat output continued for around two hours (Fig. 1). The experiments in this paper are stepwise titration but those of Norde and Lyklema (1978) were a single step

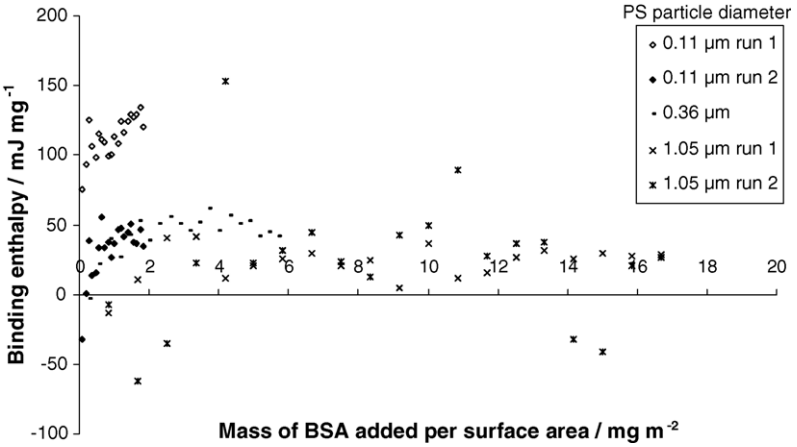

Fig. 4. Affect of PS particle size on BSA binding enthalpy. 15 μl injections of 0.2% (w/v) BSA in 0.9% (w/v) NaCl was used throughout and the same volume of particle suspension for all particle sizes. The heat output from binding experiments was dilution corrected and normalised by mass of BSA added.

and used a far higher concentration of PS suspension (ca. 8% (w/w) rather than ca. 0.2% (w/v)) and a BSA concentration of 9% (w/v), which is more than the critical concentration of 0.3% (w/v), discussed above. The low particle concentration was chosen for our experiments to mimic typical concentrations used in vaccine preparation, which has caused the binding signal to be dominated by the dilution enthalpy as shown in Fig. 1.

Other studies of macromolecular adsorption using stepwise titration calorimetry have been reported in the literature. For example, adsorption of immunoglobulin G to silica (Kamyshny et al., 2003); lysozyme and α-lactalbumin to PS (Haynes and Norde, 1995); and ethoxylated surfactants to PS (Wesemeyer et al., 1993) also did not show a sigmoidal change in enthalpy with the amount of macromolecule added. Hence, there is a difference in macromolecular adsorption and chemical binding, measured calorimetrically, presumably because of the more complicated molecular structure of proteins and multiple bonds formed on binding (Norde and Giacomelli, 1999).

The dilution and rheology experiments suggest that in BSA solutions of concentrations above approximately 0.3% (w/v) fewer of the BSA molecules exist as monomers. These dimers or polymers do not seem to rapidly dissociate when diluted. Consequently, the state of the BSA molecules depends on the processing steps taken, so not surprisingly will show different properties during processes such as adsorption. This lack of reversibility due to aggregation may have major significance for any experiment following dilution of BSA solutions of concentration greater than 0.3% (w/v). As protein adsorption is usually irreversible (Haynes and Norde, 1994) the history of the adsorbed sample will affect subsequent adsorption, which may explain why the binding curves do not show the expected sigmoidal shape.

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