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# Investigation of the mechanism of protein adsorption on ordered mesoporous silica using flow microcalorimetry

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ABSTRACT

The adsorption of bovine serum albumin (BSA) and lysozyme (LYS) on siliceous SBA-15 with 24 nm pores was studied using flow microcalorimetry; this is the first attempt to understand the thermodynamics of protein adsorption on SBA-15 using flow microcalorimetry. The adsorption mechanism is a strong function of protein structure. Exothermic events were observed when protein–surface interactions were attractive. Entropy-driven endothermic events were also observed in some cases, resulting from lateral protein–protein interactions and conformational changes in the adsorbed protein. The magnitudes of the enthalpies of adsorption for primary protein–surface interactions decrease with increased surface coverage, indicating the possibility of increased repulsion between adsorbed protein molecules. Secondary exothermic events were observed for BSA adsorption, presumably due to secondary adsorption made possible by conformational changes in the soft BSA protein. These secondary adsorption events were not observed for lysozyme, which is structurally robust. The results of this study emphasize the influence of solution conditions and protein structure on conformational changes of the adsorbed protein and the value of calorimetry in understanding protein–surface interactions.



## 1. Introduction

Protein adsorption on ordered mesoporous materials (OMMs) has been shown to have great potential in a wide range of applications, such as bioseparations, biosensors, and enzyme catalysis. High and rapid protein loading within the mesopores of OMMs (such as SBA-15) is a characteristic feature of these materials [1]. A number of studies have been performed to understand the effects of pore size [2], adsorbent surface chemistry [3,4], protein structure [5], and solution conditions [1] on protein adsorption. The similarity between the pore size of the OMM and the dimensions of the protein complicates mechanistic interpretations using data from conventional measurements (isotherms and rate studies) due not only to possible involvement of external surface area but also to non-idealities originating from intermolecular interactions between adsorbed molecules. Consequently, the data obtained by isotherm and rate studies are not always sufficient to pinpoint the underlying mechanism of protein interaction with SBA-15 materials. Direct visualization of protein adsorption using confocal scanning laser microscopy (CSLM) has significantly improved the understanding of protein adsorption in OMMs [6–12]. However, CSLM can only provide insight into mass transfer during protein adsorption on these materials; it cannot provide information on the energetics of the system. Previous studies confirmed that SBA-15 materials with pore size of 24 nm can be effectively used to adsorb proteins in the pores utilizing the maximum available surface area [1,2]. Various siliceous and ligand terminated OMMs have been used for protein adsorption; however the mechanism of protein adsorption under different solution conditions has not yet been established.

In the past, researchers have turned to calorimetry to explore the underlying mechanism of protein adsorption [13–17]. The equilibrium capacity for a protein on a surface is determined by the Gibbs free energy change of adsorption, $\Delta G_{ads}$. This free energy change, in turn, depends upon the enthalpy change of adsorption, $\Delta H_{ads}$, which can be measured using calorimetry. Protein adsorption can be exothermic or endothermic [13–17]. Exothermic adsorption is associated with attractive forces between the surface and the adsorbed proteins, between the adsorbed proteins, or both [13–17]. Endothermic adsorption is often attributed to changes of protein conformation or orientation upon adsorption or to the associated release of solvent from the surface; in this case the adsorption process is entropy-driven [13–17]. When protein adsorption involves multiple phenomena (such as folding or water release) exotherms and endotherms have been observed at different times during the adsorption process [13–17].

Calorimetry is a valuable tool to understand protein–adsorbent surface interactions, due to its capability to detect minute ther-

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mal changes ($10^{-6}$ K). These temperature changes can be converted to heats of adsorption using an experimentally determined calibration factor. Exothermic events result in temperature increases, while endothermic events produce temperature decreases. Based on calorimetric measurements, Thrash and Pinto [15] suggested that in protein ion-exchange chromatography (IEC) the repulsive interaction between the adsorbed protein molecules is a more significant contributor to endothermic adsorption than are solution non-idealities and surface dehydration. These interactions can be modulated by the cations present in the mobile phase. In the case of hydrophobic supports, exothermic events were observed. In a different study, it was postulated that endothermic adsorption might be the result of protein restructuring, protein reorientation, and/or water release from the surface [18,19]. The conformational changes for adsorbed proteins appeared to be greater in the absence of salt [16].

This paper reports the results of a study of the adsorption of bovine serum albumin (BSA) and lysozyme (LYS) on siliceous SBA-15 with 24 nm pores. This is the first attempt to understand the thermodynamic aspect of protein adsorption on SBA-15 using flow microcalorimetry. The effects of solution conditions (pH and temperature) and protein structure on the enthalpy of adsorption are reported.

## 2. Experimental

### 2.1. Materials

BSA (A 7030) and lysozyme (L 6876) were purchased from Sigma (St. Louis, MO, USA) and used without further purification. SBA-15 with a pore size of 24 nm (SBA-15-240 Å) was used as a support material. The SBA-15-240 Å was synthesized using the procedures described by Katiyar et al. [2]. The carrier fluids for the experiments were 50 mM acetate buffer (pH 4.8) and 50 mM phosphate buffer (pH 7.6). Lysozyme sample concentrations were 10–60 mg/mL; BSA sample concentrations were 20–80 mg/mL.

### 2.2. Flow microcalorimetry

A Miscroscal FMC 3Vi flow microcalorimeter (FMC; Gilson Instruments, Westerville, OH, USA) was used for calorimetric measurements; the FMC system is described in detail elsewhere [13]. The FMC contains a column (sample cell) with a volume of 0.171 mL. The flow through the cell is regulated by two precision syringe micropumps; the mobile phase flow rate was 1.65 mL/h and the sample loop was 0.770 mL. Two highly sensitive thermistors are interfaced with the sample cell to detect the small temperature changes associated with adsorption on the sample. The cell is thermally insulated to minimize heat loss during the experiments and the impact of changes in laboratory conditions on the calorimetric results. Experiments can be performed at various cell temperatures using a block heater; the experiments reported here were performed at 25 and 35 °C.

The FMC cell was initially packed with approximately 25 mg of solid and evacuated to remove air trapped inside the porous adsorbent. Once evacuated, the sample was wetted with the buffer and allowed to reach thermal equilibrium, indicated by a constant calorimeter output. A known protein sample (concentration and volume) was then introduced into the cell by switching a multiport valve. Protein adsorption caused a change in cell temperature, which was converted to a heat signal using a calibration factor. The effluent, with un-retained protein, was collected until equilibrium was attained. The protein concentration in the effluent was determined from the absorbance at 280 nm using a UV spectrophotometer (Spectronic 1001, Milton Roy). A mass balance was used to calculate the amount of protein adsorbed. Peak deconvolution was performed using the PEAKFIT software package. These data were used to calculate the specific heats of protein adsorption.

**Table 1**
Enthalpy of lysozyme (LYS) adsorption onto SBA-15-240 Å at pH 8 and 25 °C. Sample size: 0.770 mL; mobile phase rate: 1.65 mL/h.

| LYS initial concentration (mg/mL) | Loading (mg/g) | $\Delta H_{ads}$ (kJ/mol) |
|---|---|---|
| 10 | 253 | −381 |
| 30 | 675 | −98 |
| 60 | 855 | −91 |

$\Delta H_{ads}$: Enthalpy of adsorption.

## 3. Results and discussion

This study reports on the adsorption of two proteins, lysozyme (LYS) and bovine serum albumin (BSA) on SBA-15-240 Å. Physical characterization of the SBA-15-240 Å used in the study reported here has been presented elsewhere (sample SBA-15-3 in Ref. [2]). SBA-15 has surface chemical properties similar to other silica materials. The surface silanol groups are of particular importance, as these groups provide acidity and, at low pH, the possibility of hydrogen bonding. The isoelectric point of SBA-15 is $3.7 \pm 0.3$, higher than that of more conventional silicas (~2) [20]. These materials also have some hydrophobic character, as evidenced by significant protein adsorption under conditions where electrostatic interactions are not favorable for adsorption. At the conditions of the work reported here, pH 4.8 and 7.8, the SBA-15-240 Å will have a net negative surface charge.

The proteins used in this study have different isoelectric points (11.1 for lysozyme [21] and 4.6 for BSA) and different degrees of structural stability. Lysozyme is classified as a structurally robust or "hard" protein [22], while BSA is a "soft" protein. A similar concept of hard and soft proteins is presented by Brandes et al. [21]. Relatively simple adsorption behavior can be expected for hard proteins, while more complex adsorption behavior can be expected for soft proteins.

Evidence in the literature shows that the BSA undergoes conformational changes upon adsorption. Brandes et al. [21]. studied the adsorption of BSA on hydroxyapatite (HP) particles, and showed that BSA is partially folded during adsorption, and the surface-bound state is distinctly different from both the native state and the thermally denatured state in solution. Larsericsdotter et al. [23] concluded that adsorption onto silica reduces the structural stability of BSA, but not so significantly that internal residues are exposed to an aqueous solvent phase. BSA is often used in the literature as an example of a globular "soft" protein with a high amount of α-helical structure [21]. It was observed that during adsorption BSA primarily lost its α-helix structure [24] with a significant increase in unordered domains [25]. BSA adsorption on a sepharose-based hydrophobic surface was observed to be driven by entropic forces under linear conditions, however under non-linear conditions the adsorption was driven by enthalpy change [26].

FMC studies of LYS-SBA-15-240 Å interactions as a function of sample loading were performed at pH 8 and room temperature (25 °C); the thermograms obtained in these experiments are shown in Fig. 1, and the corresponding calculated heats of adsorption are summarized in Table 1. Feed concentrations were 10, 30, and 60 mg/mL; these feed concentrations resulted in LYS loadings of 253, 675, and 855 mg/g, respectively. pH 8 is the pH condition closest to the isoelectric point of lysozyme that could be used because the silica adsorbent is not stable at pH greater than 8.5 [27]. At pH 8, LYS is positively charged and the silica surface is negatively charged. A single exothermic peak is observed, which indicates strong attractive protein–surface interactions and negligible struc-

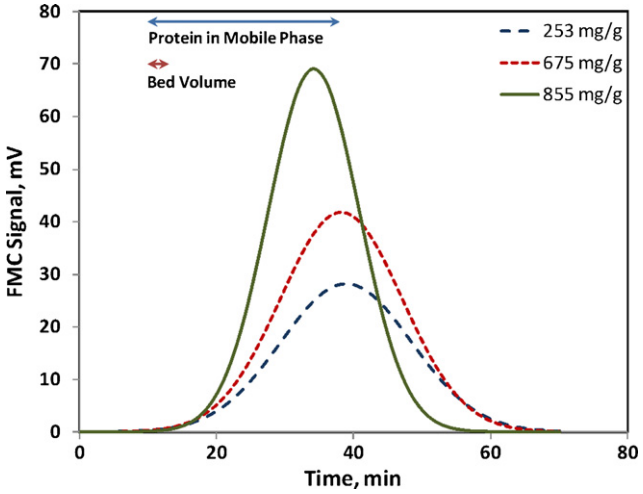


**Fig. 1.** Thermograms for lysozyme (LYS) adsorption on SBA-15-240 Å at pH 8 and 25 °C at three LYS loadings.

tural rearrangement during adsorption. In each experiment, the exotherm began when the protein sample reached the adsorbent. The magnitude of $\Delta H_{ads}$ decreased as the lysozyme loading is increased. This decrease in $\Delta H_{ads}$ can be attributed to repulsive lateral interactions between adsorbed lysozyme molecules; these repulsive interactions increased in magnitude as the loading increased [13,15,19]. The shape of the adsorption isotherm for LYS on SBA-15-240 Å [2] indicates that multilayer adsorption of LYS occurs at higher loadings. For the overloaded conditions studied here, the distribution coefficient for LYS decreases as the mobile phase LYS concentration decreases. This results in faster movement of LYS through the bed, explaining the shift of the timing of exotherm peak to lower times at higher loadings. The peak of the exotherm at the lowest protein loading corresponds to the end of the protein sample, so it is also possible that the shift in the timing of the peak is due to rapid saturation of the outer regions of the adsorbent particles.

The interaction of BSA with SBA-15-240 Å at pH 4.8 and 25 °C shows more interesting behavior. Thermograms obtained at different BSA feed concentrations (20, 50, and 80 mg/mL) are shown in Fig. 2; these different initial concentrations correspond to the BSA loadings of 224, 358, and 564 mg/g, respectively. Two exothermic events were observed in each of these tests; the enthalpies of

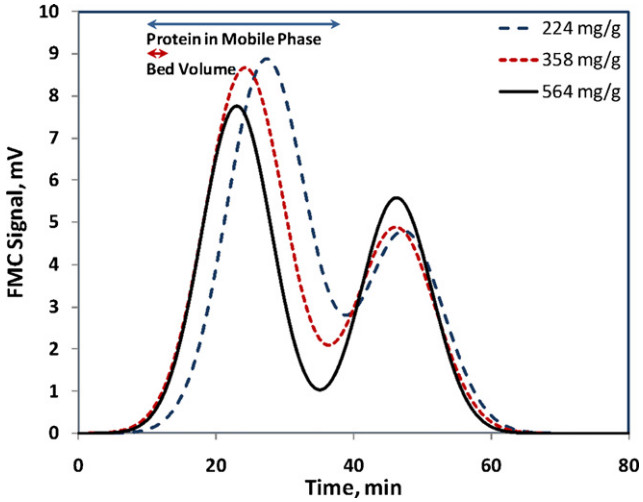


**Fig. 2.** Thermograms for bovine serum albumin (BSA) adsorption on SBA-15-240 Å at pH 4.8 and 25 °C at three BSA loadings.

**Table 2**
Enthalpy of bovine serum albumin (BSA) adsorption onto SBA-15-240 Å at pH 4.8 and 25 °C. Sample size: 0.770 mL; mobile phase rate: 1.65 mL/h.

| BSA initial concentration (mg/mL) | Loading (mg/g) | $\Delta H^{I}_{ads}$ (kJ/mol) | $\Delta H^{II}_{ads}$ (kJ/mol) |
|---|---|---|---|
| 20 | 224 | −360 | −182 |
| 50 | 358 | −221 | −124 |
| 80 | 564 | −112 | −81 |

$\Delta H^{I}_{ads}$: Enthalpy of adsorption during first event.
$\Delta H^{II}_{ads}$: Enthalpy of adsorption during second event.

adsorption are summarized in Table 2. The first exotherm, $\Delta H^{I}_{ads}$, results from attractive protein–surface interactions. As with LYS, the first exotherm for BSA adsorption begins when the protein sample reaches the adsorbent. However, the first exotherm peaks well before the end of the protein sample. Reduction of $\Delta H^{I}_{ads}$ with increase in loading may be due to increased repulsive interactions between the protein molecules. At higher loading, repulsive lateral interactions between the adsorbed protein molecules, which are endothermic, might increase, reducing the net heat of adsorption at higher loadings ($\Delta H^{I}_{ads}$) [18,28]. The first peak occurs later at the lowest BSA loading than at higher BSA loadings; the difference in peak timing can be explained as a consequence of the concentration dependence of the distribution coefficient of the protein.

The second exothermic peaks, $\Delta H^{II}_{ads}$, are aligned at approximately 47 min for the concentrations studied here, well after the end of the protein sample. This timing indicates that the second exotherm is not the result of the formation of secondary adsorbed layers, consistent with the adsorption isotherm [2]. This second peak can be attributed to the secondary adsorption of BSA molecules. After the primary BSA adsorption on the SBA-15 surface, the BSA molecules undergo conformational changes that create new sites on the protein surface which are available for favorable interactions with the adsorption surface. This conclusion is supported by the work of Kondo et al. [19,29], which shows that hemoglobin binding with CdS particles may be the result of a primary binding interaction followed by conformational changes favorable for secondary adsorption. Soderquist and Walton [30] reported that the protein adsorption process is characterized by three stages: initial adsorption, slow conformational change with irreversible adsorption, and at longer times, desorption of small amounts of denatured protein. Conformational changes during protein adsorption are reported to be primarily endothermic [15]. The simultaneous occurrence of conformational changes (endothermic) and secondary adsorption (exothermic) during the second peak results in the lower magnitude $\Delta H^{II}_{ads}$; an endothermic event is buried in the second exothermic peak. The magnitude of $\Delta H^{II}_{ads}$ decreases as BSA loading increases. This trend can be explained by the fact that the protein–surface interactions are stronger at lower protein loadings [19]. These strong interactions are responsible for greater conformational changes, which drive the secondary adsorption process.

A separate study of the adsorption kinetics of BSA and LYS on large pore SBA-15 materials using confocal microscopy showed that equilibrium is achieved within about 20 min. A similar observation is reported by Soderquist and Walton [30] who found that the majority of protein was adsorbed within 10 min followed by slow uptake to final equilibrium. These observations show that the effects of mass transfer resistance are negligible in this study, and thus show that the secondary event is not the result of mass transfer resistance effects.

The absence of a second peak in the LYS adsorption thermograms and the presence of this second peak for BSA adsorption can be related to the concept of "hard" and "soft" proteins described by Norde and Anusiem [22], who studied the adsorption of BSA and LYS

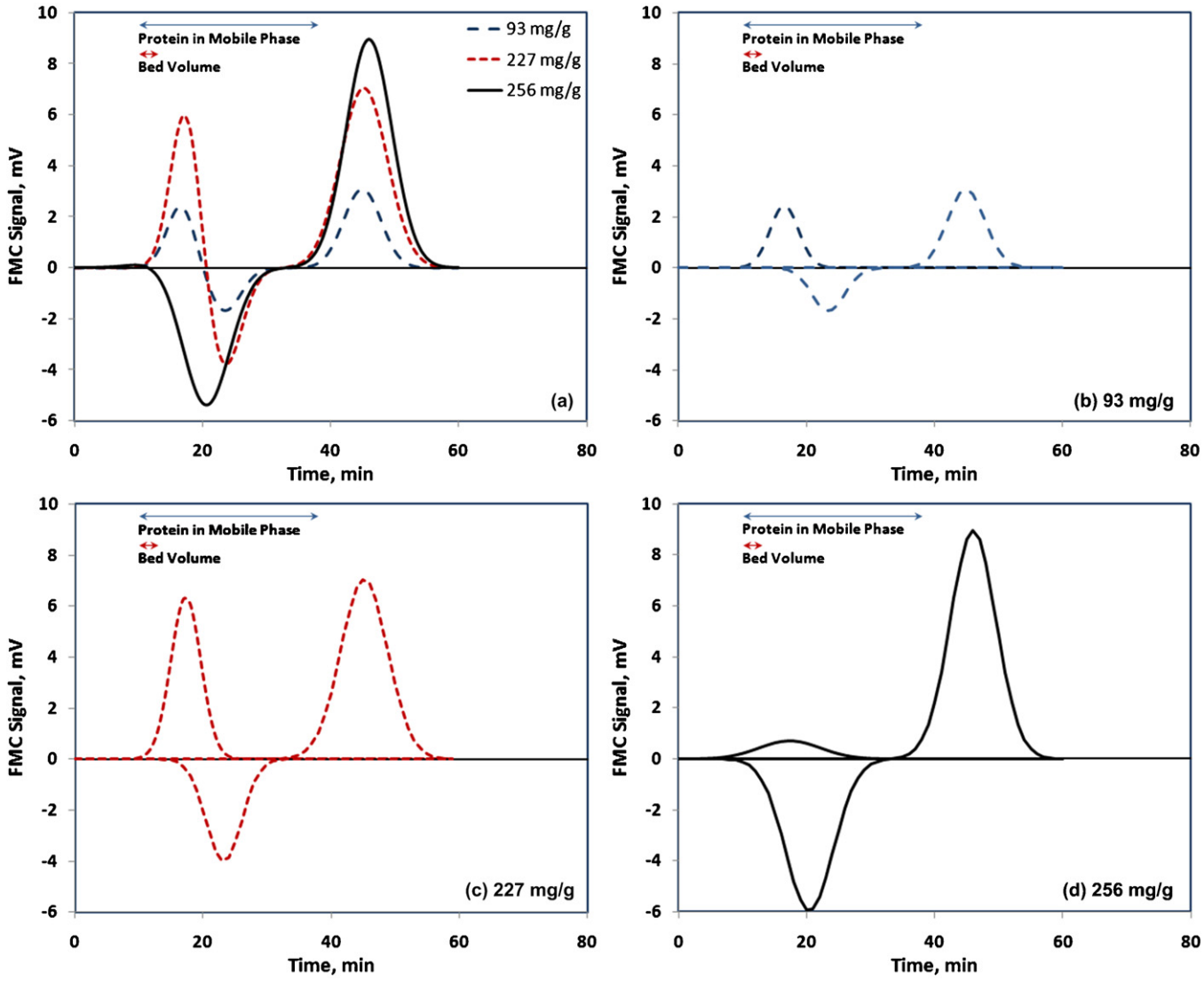


**Fig. 3.** Thermograms for bovine serum albumin (BSA) adsorption on SBA-15-240 Å at pH 7.6 and 25 °C at three BSA loadings. (a) Total heat flow; (b) deconvoluted peaks for 93 mg/g BSA loading; (c) deconvoluted peaks for 227 mg/g BSA loading; and (d) deconvoluted peaks for 256 mg/g BSA loading.

adsorbed onto fine silica particles. They found that BSA undergoes conformational changes upon adsorption; however, the structure of LYS was not affected by adsorption due to its robustness.

BSA adsorption thermograms at pH 7.6 and 25 °C as a function of loading are presented in Fig. 3; the enthalpies of adsorption obtained from these experiments are presented in Table 3. Mobile phase BSA concentrations were 20, 50, and 60 mg/mL, corresponding to stationary phase loadings of 93, 227, and 256 mg/g. At pH 7.6, both SBA-15-240 Å and BSA have negative surface charges; thus, the primary interaction between the BSA and the SBA-15 is expected to be weaker than at pH 7.6 due to the presence of repulsive protein–surface and protein–protein interactions. These repulsive interactions increase as the BSA loading on the surface is increased [19]. The first event ($\Delta H^{I}_{ads}$), which begins when the protein sample reaches the adsorbent, is under these conditions exothermic for all cases; however the magnitude of this event is minimal at the highest loading (256 mg/g) due to increased lateral interactions between the similarly charged BSA molecules (Table 3). These lateral interactions, combined with primary interactions, cause conformational changes in the adsorbed BSA molecules. These changes are reflected by the second, endothermic events ($\Delta H^{II}_{ads}$) shown in Fig. 3 and reported in Table 3. The endothermic events occur while protein adsorption is still in progress, The third event ($\Delta H^{III}_{ads}$), which is exothermic, is again due to the secondary adsorption of BSA molecules. The increase in $\Delta H^{III}_{ads}$ with BSA loading is consistent with greater conformational change and increased lateral interaction between adsorbed BSA molecules. The absence of a second endothermic peak in Fig. 2 was explained by the strong primary protein–surface interactions at pH 4.8, which masked the endothermic events produced by structural rearrangement peak completely. Thus the presence of a strong exothermic event (due to strong protein–surface interactions) and a weak endothermic

**Table 3**
Enthalpy of bovine serum albumin (BSA) adsorption onto SBA-15-240 Å at pH 7.6 and 25 °C. Sample size: 0.770 mL; mobile phase rate: 1.65 mL/h.

| BSA initial concentration (mg/mL) | Loading (mg/g) | $\Delta H^{I}_{ads}$ (kJ/mol) | $\Delta H^{II}_{ads}$ (kJ/mol) | $\Delta H^{III}_{ads}$ (kJ/mol) |
|---|---|---|---|---|
| 20 | 93 | −73 | 72 | −152 |
| 50 | 227 | −84 | 57 | −180 |
| 60 | 256 | −17 | 138 | −204 |

$\Delta H^{I}_{ads}$: Enthalpy of adsorption during first event.
$\Delta H^{II}_{ads}$: Enthalpy of adsorption during second event.
$\Delta H^{III}_{ads}$: Enthalpy of adsorption during third event.

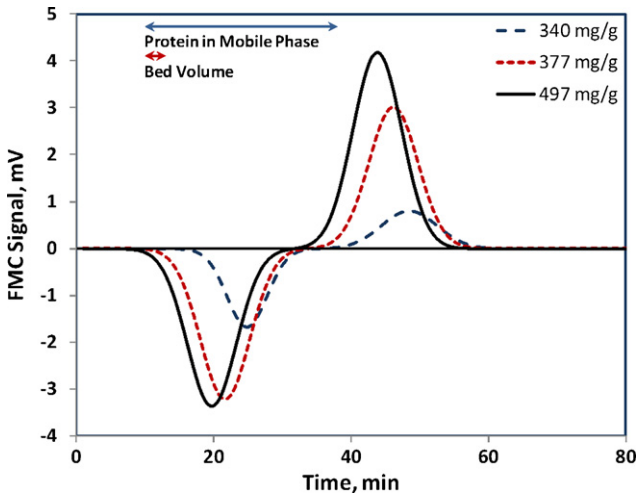


**Fig. 4.** Thermograms for bovine serum albumin (BSA) adsorption on SBA-15-240 Å at pH 4.8 and 35 °C at three BSA loadings.

event (due to minimal protein–protein interactions) at pH 4.8 result in the observation of a single exothermic event. The magnitudes of the exothermic events driven by primary protein–surface interactions are weaker at pH 7.6, allowing both events to be observed as shown in Fig. 3.

BSA adsorption thermograms for SBA-15-240 Å at pH 4.8 and 35 °C are shown in Fig. 4 and the corresponding enthalpies of adsorption are summarized in Table 4. Mobile phase BSA concentrations were 20, 50, and 80 mg/mL, producing stationary phase concentrations of 340, 377, and 497 mg/g. BSA adsorption at 35 °C resulted in higher BSA loadings than were obtained at 25 °C. In Fig. 4, the first, endothermic event ($\Delta H^{I}_{ads}$) indicates that the BSA adsorption is driven by entropic effects. This initial endotherm begins as the protein sample reaches the adsorbent. Similar conclusions for linear isotherm loadings were obtained by Esquibel-King and Pinto [26]. The increased BSA loading with increase in temperature indicates that weakly hydrophobic forces and van der Waals forces may drive BSA adsorption. The endothermic event for BSA adsorption at higher temperature may be the result of a large release and rearrangement of the water molecules that surround the hydrophobic residues on the surface, and the structural changes due to protein–surface interactions are consistent with an entropy-driven process [26]. Recently, Ueberbacher et al. [31] showed that the adsorption of BSA on Butyl Sepharose 4 FF led to secondary structure changes and the α-helix content was reduced by approximately 24%. Ueberbacher et al. further stated that the increase in adsorption temperature can significantly increase the extent of structural change. The percentage of unfolded proteins increased exponentially with increasing temperature. While the initial BSA–surface interaction is driven by van der Waals forces, which are exothermic in nature, the higher temperature and weak structural stability induce changes in the secondary structure of BSA. This highly endothermic process is dominant during the initial part of the curve shown in Fig. 4. The second, exothermic peak ($\Delta H^{II}_{ads}$), which occurs after the protein sample has ended, is a result of secondary adsorption due to creation of new adsorption sites on adsorbed BSA molecules after conformational changes during the primary adsorption process.

**Table 4**
Enthalpy of bovine serum albumin (BSA) adsorption onto SBA-15-240 Å at pH 4.8 and 35 °C. Sample size: 0.770 mL; mobile phase rate: 1.65 mL/h.

| BSA initial concentration (mg/mL) | Loading (mg/g) | $\Delta H^{I}_{ads}$ (kJ/mol) | $\Delta H^{II}_{ads}$ (kJ/mol) |
|---|---|---|---|
| 20 | 340 | 29 | −16 |
| 50 | 377 | 47 | −45 |
| 80 | 497 | 44 | −58 |

$\Delta H^{I}_{ads}$: Enthalpy of adsorption during first event.
$\Delta H^{II}_{ads}$: Enthalpy of adsorption during second event.

This work reported here is a first attempt to understand the protein–surface interactions on mesoporous SBA-15 materials using flow microcalorimetry. The results of this study will be useful in understanding biomolecular adsorption on SBA-15 materials at different conditions, providing insight for designing better materials for biocatalysis and bioseparations.

## 4. Conclusions

Calorimetric measurements of heats of adsorption for BSA and lysozyme on SBA-15-240 Å materials demonstrate the importance of solution conditions and protein structure on protein adsorption. Adsorption tests using two proteins with different structural stability, BSA (soft) and lysozyme (hard), show that the adsorption mechanism is a strong function of protein structure. Exothermic events were observed for attractive protein–surface interactions. However, endothermic events were also observed in some cases; these resulted from lateral protein–protein interactions and conformational changes in protein molecules adsorbed on the SBA-15 surface. Adsorption is entropy-driven during endothermic events. The magnitudes of the enthalpies of adsorption for primary protein–surface interactions decrease with increased surface coverage by protein, indicating repulsive interactions between adsorbed protein molecules. The second exothermic events observed for BSA adsorption in the later part of the thermograms are due to secondary adsorption made possible by conformational changes in the soft BSA protein. The structural robustness of lysozyme minimizes the conformational changes upon adsorption, eliminating secondary adsorption. The results of this study emphasize the influence of solution conditions and protein structure on the degree of conformational changes on the adsorbent surface.

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