

Hydration Phenomena in Concentrated Protein Solutions

by

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Declaration of Originality

I hereby declare that this thesis and the work presented herein were entirely my own work. Information presented and derived from the published and unpublished work of others has been acknowledged in the text and references are given.

A page from a journal of modern experimental physics will be as mysterious to the uninitiated as a Tibetan mandala. Both are records of enquiries into the nature of the universe.

Fritjof Capra, *The Tao of Physics* (1975), Ch. 2

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I understand:

‘It is a little star-dust caught, a segment of the rainbow which I have’

Henry David Thoreau, Walden (1854)

Abstract

The transport of water in glassy polymers is important in many fields but an underappreciated topic in protein science; though proteins are of rising importance in many industries. Similar to water transport in synthetic polymers, glassy proteins suffer from non-equilibrium effects during water sorption. However, the presence (or absence) of these phenomena is unclear. Here and for the first time, experimental evidence is reported for the presence and consequences of non-equilibrium relaxations during water sorption in proteins over long time scales based on gravimetric and calorimetric experiments. Non-Fickian water sorption kinetics were found in all experiments. It is demonstrated how water solubility in a glassy protein is highly dependent on experimental design and time scales. The apparent sorption kinetics were attributed to the convolution of two processes: one that is controlled by the concentration gradient; one that is driven by a slow relaxation of the protein. A relaxation–diffusion model was employed to model sorption kinetics and the respective time constants were estimated. Exothermic heats of mixing, that were both temperature and concentration dependent, were measured. Enthalpy of mixing time evolution data was in excellent agreement with gravimetric sorption kinetics. The plasticising effect of water onto the protein was further shown by differential scanning calorimetry of concentrated protein solutions. All this demonstrates the unsuitability of long established surface and lattice approaches to model or predict water sorption in proteins. Protein pressure–volume isotherms were obtained and used to model a protein’s water sorption isotherms by the non-equilibrium lattice fluid theory for the first time. It is anticipated that this work stimulates a revaluation of the design of water sorption experiments in glassy proteins and the interpretation of such data. This in turn is expected to profoundly affect formulation engineering and Quality-by-Design approaches for a wide range of protein-based products.

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Preamble

The role of water in biology is part of the canon of the some of the oldest and most extensively discussed problems in natural sciences. A chronological account could begin with Thales of Miletus (c. 624 – c. 546 BC), a pre-Socratic philosopher, who considered water to be the basis of all matter, including living matter, in his cosmological thesis. All the way through the ancient times, a fascination for water remained. By the onset of the Renaissance, a time when modern science was born, the fascination for water did only become greater. Leonardo da Vinci (1452 – 1519) formulated one of the seismic philosophical ideas of the Occident with his theory of analogy; a theory that connects the macrocosm of the world, and indeed universe, with its microcosm. And within his written accounts, I find one of the most beautiful accounts on the topic of ‘water’:

‘The water which rises in the mountain is the blood which keeps the mountain alive.’¹

As experimental data consequently became available, water was called upon to an even greater extent to explain incomprehensible biological and physico-chemical phenomena. It was only in the last century with the advances in natural and physical sciences that water was indubitably and to a substantial degree found responsible for many of the special properties of aqueous systems including those containing macromolecules. Water is the solvent of life. All living organisms interact with it. It is vital in maintaining protein structure and, thus, life.

¹ Leonardo da Vinci, from: *The Notebooks of Leonardo da Vinci: Philosophy I*. E MacCurdy (etd), 1938

Chapter 1. Introduction

The sorption and diffusion of water in glassy polymers is important in many fields of industrial and academic interest. A sub-class of polymers of rising interest are proteins. Proteins are large macromolecules that are made up of a sequence of amino acids and characterised by a unique three-dimensional structure that corresponds to their biologically active state. The native structure of a protein is a result of a fine balance among various molecular interactions including covalent linkages, hydrophobic interactions, electrostatic interactions, hydrogen bonding and van der Waals forces. [1]

Particular academic and industrial interest has been paid to enzymes, which are proteins with a capacity to catalyse specific chemical reactions, over the past few decades. From a life science perspective, enzymes are important as they are essential for many complex reactions governing life in both simple as well as more advanced organisms. In this case, ubiquitously interacting with water. On the other hand stands the industrial use of enzymes. Throughout the history of mankind, enzymes have been used in a wide range of processes including leather tanning, leavening of bread, or cheese making. [2] The commercial use of enzymes began around the end of the nineteenth century. Since 1959, bacterial alkaline proteases have been used as a formulation additive in industrial and household laundry detergents. Indeed, most (in terms of volume market share) of the enzymes produced on the industrial scale are used in the detergent industry. [3]

Be it in the context of life sciences or technical uses, amorphous enzymes/proteins are exposed to water vapour during processing, storage and use. At ambient conditions, most proteins are glassy. The accurate measurement and appropriate interpretation of the transport mechanism of water in glassy proteins is complex and, up-to-date, an underappreciated topic. The problem itself is not just posed by the uniqueness of each protein in terms of its physical and chemical properties

and its structure, but also by the non-equilibrium state of a glassy bio-polymer/protein and the complex chemical behaviour of water itself (e.g. hydrogen bonding). The following section provides a brief review of previous work that relates to the work contained in this thesis. The transport of water in glassy (bio-)polymers is classified here into two categories: (1) water sorption (i.e. solubility or mass uptake), and (2) water sorption kinetics (i.e. diffusion). Before this topic is discussed, a brief review on the polymer–protein analogy that is used throughout this thesis is presented. The interested reader can find more detailed reviews and accounts on theoretical backgrounds in the appropriate Chapters of this thesis.

1.1 PROTEIN–POLYMER ANALOGY

Most formulations of biological macromolecules are thought to be stabilised by converting the product (often a dilute aqueous solution) into the solid state. [4] This process is delicate as the removal of up to ninety nine percent of the water present in solution may cause severe losses of bioactivity. Therefore, there are only two established processes by which water is commonly removed; freezing or evaporation. As most proteins are thermally denatured at elevated temperatures and low temperatures reduce the kinetics of chemical or physical degradation processes generally (by inspection of the Arrhenius equation), freeze-drying has become the method of choice. Freeze-drying is the removal of water at very low temperatures by the

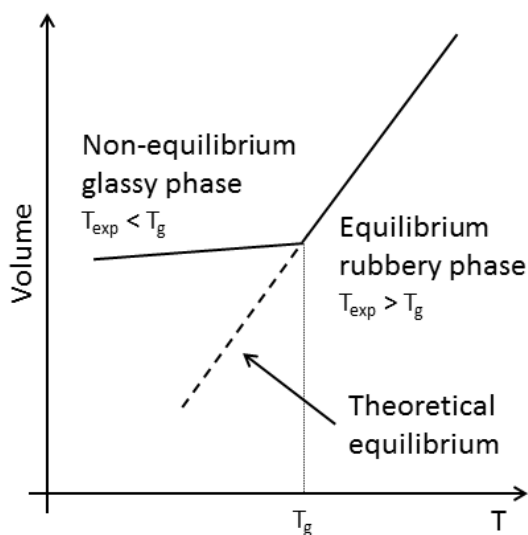


Figure 1 Schematic diagram of the specific volume of a glass-forming polymer as a function of temperature at isobaric conditions. T_g and T_{exp} denote the glass transition temperature and experimental temperature, respectively. The T_g may be defined as the temperature where polymer chains begin to exhibit large-scale co-operative movement. Adapted from reference [5].

sublimation of the frozen water; generally, the final solid product is amorphous for high-purity protein formulations.

Amorphous materials can exist in a rubber- or glass-like state depending on the temperature relative to their T_g . The case of semi-crystalline systems shall be excluded here. The glass transition is a reversible transition in amorphous materials from a hard relatively brittle state to a molten or rubber-like state. In other words, the glass transition temperature is the temperature at which the relaxation time for segmental motion of polymer chains approaches that of the experimental time scale. Figure 1 shows a schematic volume–temperature diagram of such amorphous system (where semi-crystallinity is excluded for the sake of simplicity) at isobaric conditions.

Initial work [6, 7] that aimed to measure the glass transition temperature for common proteins such as collagen and keratin did not show a specific heat capacity step increase that is so easily identifiable in simple polymers or glass-forming liquids as a sign of a glassy solid. As scanning calorimetry techniques improved, Tseratli and Smirnova [8] reported on the development of a pronounced heat capacity step increase/discontinuity on rescan after denaturation of collagen over a specific temperature window. Other workers [9-11] have also reported heat capacity jumps at near ambient temperature in food proteins and interpreted these as glass transitions despite the ‘smeared-out’ shape of the heat capacity increase. Further work by Sochava, Smirnova and co-workers [12, 13] showed a distinct heat capacity step increase that was enhanced upon denaturation for a selected group of proteins. All this work points towards the glassy, and indeed dynamic, nature of a protein materials.

Proteins are dynamic, not static systems. [14] They move intra-molecularly; their side chains can fold and rotate. [15, 16] Frauenfelder and co-workers [17] showed that intramolecular protein motions can be characterised and interpreted by analogy from other complex systems such as

glass-forming synthetic liquids. For simple cases, such as the binding of carbon monoxide to myoglobin, a relationship between intramolecular protein molecule motion and function could be drawn. [17]

Doster and co-workers studied the thermal transition of hydration water in myoglobin crystals [18] and glasses [19] at very low temperatures. They found a broad glass transition between 180 and 270 K. This transition temperature arises from the presence of amorphous water at super-cooled conditions.

Rasmussen and co-workers [20] studied dynamic properties of ribonuclease A down to very low temperatures. They found that ribonuclease A loses its catalytic function below the 220 K glass transition, but would bind a substrate or inhibitor rapidly at 228 K. Enzyme flexibility and function were found to be linked.

It is striking how well some of the properties in proteins can be described by polymer theory. On the basis of the work by Iben and co-workers [21] and Ansari and co-workers [22], it was suggested that hydrated proteins, here as individual molecules, exhibit high enough complexity to exhibit co-operative dynamics comparable to those of polymers and simpler glass-forming systems. [23]

As the field of polymer science advances, developing the analogy between the behaviour of proteins and simpler glass-forming liquids and polymers gives rise to one of the most exciting opportunities in the field of physical chemistry and chemical engineering of such systems.

1.2 WATER SOLUBILITY IN GLASSY POLYMERS/PROTEINS

The early studies on the sorption of water in glassy polymers, and following the previous section, I would like to include biological macromolecules such as proteins, were focused on the experimental measurement of the water solubility and understanding the energetics of the water sorption process (e.g. heats of sorption). [17, 24, 25] These early studies specifically investigated effects of polymer polar groups [25], polymer crystallinity [26], solvent-to-polymer surface area ratios, and temperature [27] on water solubility in numerous glassy systems. Prevailing from these studies are two ideas: (1) the need for models to adequately describe the water sorption isotherms in glassy polymers; and (2) the presence of associated water molecules within the polymer matrix (known as ‘clustering’).

The early work published on moisture sorption in proteins at near-ambient temperatures completely neglected the glassy character of such systems. There is a long line of tradition of modelling the solubility (and water sorption isotherms) using surface-based models. In 1944, first modelling studies of the water vapour sorption isotherm in proteins were published [28, 29] and employed the Brunauer–Emmett–Teller theory [30] of adsorption of gases in multi-molecular layers. Only a year later, Linus Pauling analysed the water sorption of water by proteins [31] and suggested that the initial process of water sorption (i.e. at low hydration level) could be thought of as the attachment of one water molecule to each polar amino side chain. This idea was widely explored during the second half of the twentieth century and among the most notable contributions stand those by Kuntz and Kauzmann [32] and D’Arcy and Watt [33]. Models of bulk-stoichiometric binding of water by proteins have been suggested by several authors [34, 35]. However, it was found that these models do not have general applicability [36, 37]. The heritage of these early studies still remains as, even up-to-date, surface approaches and bulk stoichiometric models are still used to describe protein hydration (e.g. [38]). Though, some more recent studies

have considered the solution character of protein–water systems. Tables 1 and 2 list commonly used surface adsorption theories and polymer theories, respectively, to describe the water sorption isotherm in proteins and/or polymers. Out of all the theories listed in Table 2, it is only the Flory–Huggins and Vrentas–Vrentas theories that have been employed to model water vapour sorption in lyophilised proteins.

A commonly employed model to describe water vapour sorption in a glassy polymer is the dual mode sorption theory [39]. This theory has received little application in the field of water vapour sorption by proteins. According to the dual mode sorption theory, there are two penetrant populations within the polymer. Based on the early work by Barrer and co-workers [40] and Vieth and Sladek [41] it was suggested that one of the populations is an immobilised Langmuir portion whilst the other portion dissolves directly into the polymer and is the Henry’s Law population. The dual mode sorption theory has been widely applied to fit penetrant solubility data but has no predictive capacity *per se*. [42-44]

Another commonly used model by various investigators [45-48] to describe water sorption in polymers is the Flory–Huggins [49] theory. This solution theory is based on the idea that the great dissimilarity in molecular sizes between polymer and penetrant need to be taken into account when computing the entropy of mixing. Its single model parameter is the binary interaction parameter χ that can be related to the van-Laar heat of mixing [50, 51] or the Hildebrand solubility [52] parameters. The Flory–Huggins theory was initially developed for rubbery polymers (under thermodynamic equilibrium constraints) and often underestimates the solubility of water in glassy polymers (e.g. reference [53]).

Hernandez and co-workers [54] established a modification of the dual mode sorption theory. They replaced the Henry’s Law term by the Flory–Huggins equation. The Langmuir portion was

Table 1 Non-extensive list of common surface adsorption theories used to describe the water sorption isotherm in biological products.

Isotherm	Year of report	Number of model parameters	Original Paper
Freundlich	1906	2	Freundlich, H.M.F. J. Phys. Chem. 1906, 57, 385-471
Langmuir	1918	1	Langmuir, I. J Am. Chem. Soc. 1918, 40, 1361-1402
BET	1938	2	Brunauer, S.; Emmett, P.H.; Teller, E. J. Am. Chem. Soc. 1938, 60, 309-319
Oswin	1946	2	Oswin, C.R. J. Soc. Chem. Ind. 1946, 65, 419-42
Hailwood–Horrobin	1946	3	Hailwood, A.J.; Horrobin, S. Trans. Faraday Soc. 1946, 42B, 84-92
Smith	1947	2	Smith, S.E. J. Am. Chem. Soc. 1947, 69, 646-651
Henderson	1952	2	Henderson, S.M. Agric. Eng. 1952, 33, 29–3
GAB	1966	3	Guggenheim, E.A. Application of Statistical Mechanics. Clarendon Press: Oxford, 1966, Vol.1, Ch. 11, pp 186-207
Caurie	1970	3	Caurie, M. J. Food. Techn. 1970, 5, 301-307
D’Arcy–Watt	1976	≥ 3	Watt, I.C.; D’Arcy, R.L. J. Polym. Sci. Symp. 1976, 55, 144-166
Peleg	1993	4	Peleg, M. J. Food Proc. Eng. 1993, 16, 21-37

Table 2 Non-extensive list of common equations of state for polymer systems and some solubility/mixing models (*).

Model	Year of report	Type	Original Paper
Bragg–Williams	1934	Lattice*	Bragg, W.L.; Williams, E.J. Proc. Roy. Soc. 1934, A145, 699
Flory–Huggins	1942	Lattice*	Flory, P.J. J. Chem. Phys. 1942, 10, 51-61
FOV	1964	Cell	Flory, P.J.; Orwoll, R.A.; Vrij, A. J. Am. Chem. Soc. 1964, 86, 3507-3514
Sanchez–Lacombe	1978	Lattice-fluid	Sanchez, I.C.; Lacombe, R.H. Macromolecules 1978, 11, 1145-1156
Simha–Somcynsky	1980	Hole model	Jain, R.K.; Simha, R. Macromolecules 1980, 13, 1501-1508
SAFT	1990	Tangent-sphere	Chapman, W.G.; Gubbins, K.E.; Jackson, G.; Radosz, M. Ind. & Eng. Chem. Res. 1990, 29, 1709-1721
Vrentas–Vrentas	1991	Glassy sorption, based on lattice*	Vrentas, J.S.; Vrentas, C.M. Macromolecules 1991, 24(9), 2404-2412
PHSC	1994	Tangent-sphere	Song, Y.; Lambert, S.M.; Prausnitz, J.M. Chem. Eng. Sci. 1994, 49, 2765.
NE-LF	1996	Lattice-fluid	Doghieri, F.; Sarti, G.C. Macromolecules 1996, 29, 7885-7896

still retained as in the original theory. This theory was developed specifically for water/glassy polymer mixtures and there have been numerous other modifications [52] of the dual mode sorption theory. [55-59] Though, none of these theories have predictive power.

More recently, Doghieri and Sarti [60-68] developed a theoretical framework to describe the sorption of gases and liquids in glassy polymers based on a non-equilibrium treatment of the lattice fluid theory by Sanchez and Lacombe [69]. Special examples of their original treatment are the non-equilibrium lattice fluid (NELF) model [70, 71] and the non-equilibrium statistical associating fluid theory (NE-SAFT) [71, 72]. Sarti and De Angelis [73] have employed the NELF model to predict the sorption of water in polycarbonate, whilst Davis and Elabd [74, 75] have used the NELF and NE-SAFT approach to predict the water solubility in various polymers. The SAFT and NE-SAFT theories can provide a realistic representation of polar penetrants (such as water) as they can account for molecular association. This phenomenon is not accounted for in the NELF approach.

Unlike non-polar penetrants, water diffusing in a glassy polymer interacts in the system via (1) interactions with the polymer and (2) self-association with adjacent water molecules (due to hydrogen bonding). Water is known to reside within one of three states (populations) within a glassy polymer matrix: (1) monomeric, or weakly associated water molecules; (2) larger, strongly hydrogen-bond water clusters (e.g. hexamers); and (3) water molecules that interaction with the polymer backbone. [76, 77] Zimm [78] and Lundberg [79] derived an equation from statistical mechanics (under similar assumptions as the Flory–Huggins theory) that quantifies the extent of penetrant (e.g. water) clustering in a binary mixture. Their clustering function can be calculated directly from water solubility measurements such as a water sorption isotherm and has been used extensively to classify water clustering indirectly in numerous water/polymer mixtures [80-82]. A major drawback in the light of glassy polymers is the equilibrium thermodynamic framework that

was employed in the derivation of the Zimm–Lundberg theory. The non-equilibrium nature of a glassy polymer can cause significant deviations. Davis and co-workers [75] studied the non-equilibrium effects observed during water vapour sorption in a variety of glassy polymers both from direct molecular-level measurements and mathematical analysis. In all of their systems studied, they observed non-Fickian diffusion kinetics that were manifested in a two-stage water sorption process. An initial stage of water uptake is followed by a second stage of continuous, gradual uptake during long experimental times. This phenomenon was rationalised by a diffusion–relaxation transport model and complemented by an *in situ* spectroscopic analysis of the states of water in the polymer. It was shown that clustering levels may exceed those estimated by theoretical analyses.

Whilst theories to describe water sorption in synthetic/simpler glassy polymers have seen a significant improvement in the last twenty years, water sorption phenomena in proteins are poorly understood relative to our understanding of synthetic polymers or natural homo-polymers. Amongst the earliest publications on the water solubility in proteins is the seminal review by McLaren and Rowen [83], though, Bull’s paper as presented at the Cleveland, Ohio, meeting of the American Chemical Society in 1944 [84] stands out as the first significant study in this field. Between then and the early 1990’s, a lot of progress in the understanding of water sorption and hydration phenomena in proteins was made. Gregory reviewed the protein hydration and glass transition behaviour [85] in 1995 and summarised four important conclusions:

- (a) Dry proteins are glassy and rigid but are plasticised by water. This plasticising water seems to enter the protein interior early in the hydration process and provides alternative mobile hydrogen-bond donors and acceptors for the peptide groups of the protein. Therefore, it facilitates segmental motion and rearrangement.

Table 3 The chemistry of protein hydration process. Reproduced with some omissions from Table II of [87]. License was obtained prior to reproduction; see Appendix 1.7

Hydration level (h) in g/g	Thermodynamics	Structure	Dynamics
0 – 0.07	Knee in sorption isotherm Larger differences in partial molar quantities for transfer into hydration layer Folded state stable; $T_m \gg$ At 0.05h: normalisation of pK At 0.07: phase transition (2D condensation)	Water interacts primarily with charged groups At 0.07h: transition in surface water from disordered to ordered and/or clustered state	Water mobility low Protein motions are frozen Enzymatic activity is negligible
0.07 – 0.25	Plateau in sorption isotherm Small differences in partial molar quantities for transfer of water into hydration layer T_m decreases strongly with increased hydration	Water interacts primarily with polar protein surface groups Water clusters centred on charged polar sites At 0.15h: long-range connectivity of surface water is established	Internal protein motions increase At 0.1 – 0.15h: common enzymes develop activity At 0.15h: long-range proton movements along percolative networks
0.25 – 0.38	Region of strong upswing in sorption isotherm, corresponding to thermodynamic quantities being close to bulk solvent values Phase transition (condensation)	At 0.25h: start of condensation of water onto weakly interacting unfilled patches of protein surfaces	Water motion increases strongly with increased hydration
0.38	Full hydration, defined as point at which major changes in thermodynamic properties are complete $T_m \approx$ solution value	Monolayer of water covers surface Interaction with charged and polar surface groups selects locally ordered arrangement of hydration water	Most of hydration water has mobility close to bulk water Full internal motions of proteins Dynamic thermodynamic coupling between hydration water and protein
> 0.38	Hydration forces reflect cooperative interactions of water in several layers about macromolecule		Motion of large groups at protein surface may require participation of adjacent bulk solvent (e.g. cooperative H-bond rearrangements).

(b) At low hydration levels, the glass transition temperature of the water/protein mixture is much higher than common water sorption measurement temperatures. Once sufficient water has been added to the protein, the glass transition temperature drops down below the measurement temperature. At 298 K, this typically occurs within a hydration range of $0.07 - 0.2 \text{ g/g}$. With further addition of water (i.e. increase in hydration), the glass transition temperature continues to decrease and reaches a value of $180 - 220 \text{ K}$ upon full hydration.

(c) Proteins undergo conformational changes (varying in extent from protein to protein) upon dehydration. These conformational changes typically occur at low hydration levels and depending on the dehydration rate may cause a broad distribution of conformational states in the dry protein (static disorder). The continuous conformational changes and the fact that the glassy–rubbery transition is both temperature and hydration dependent explains a wide spectrum of hysteresis effects in proteins.

(d) Many enzymes are found to be constructed from two functional domains. Each of these domains is thought to contain a glassy, rigid ‘knot’ which is embedded in a more mobile matrix. It is thought that the knots are the most important structural element in a protein and they would dictate the structure/conformation of the functional domains in which they are located. Due to their construction, the knots remain glassy through the 200 K glass transition which is solely undergone by the flexible matrix.

Table 3 summarises a widely accepted view the on chemistry of the protein at various hydration levels and is based on a picture of the hydration process of lysozyme developed by Careri and co-workers [86] and Rupley and co-workers [87]. It also points towards the present lack of detailed thermodynamic understanding of concentrated aqueous protein solutions. To a large extent, the non-equilibrium nature of glassy proteins and its impact on the water vapour sorption behaviour has been neglected. For a long time now, the prevailingly applied theories are based on surface

approaches. [88] Some interesting work, including that of Wolf and co-workers [89-92], is slowly establishing the solution character as well as the non-equilibrium nature of concentrated protein solutions when modelling their water sorption isotherms or, indeed, their phase behaviour.

1.3 WATER SORPTION KINETICS

The kinetics of liquid water sorption in a glassy polymer, commonly and confusingly referred to as water vapour sorption, are complex due to the time-dependent relaxation processes of the glassy polymer (as it is in a non-equilibrium state). [93] A glassy polymer is hard and brittle; its chains are in a pseudo-frozen (i.e. non-elastic) state allowing for only very limited rotational mobility. They contain free volume (i.e. void space) in the range of 0.1 to 10 %. All these effects result in a transport mechanism of water molecules during sorption that is more complex than that of rubbery polymers. [94]

When the polymer is in its rubbery equilibrium state (i.e. above the glass transition temperature), the relaxation of the polymer volume is instantaneous upon a change in conditions (e.g. mechanical or chemical stress, water ingress, temperature/pressure change) and, therefore, is not a time-dependent process. The elastic response to water is usually observed as Fickian diffusion behaviour. Fick's first law [95], which relates the flux (J_i) of i to its concentration gradient (∇C_i), can be used to describe Fickian diffusion:

$$J_i = -D_{ij}\nabla C_i \quad (1.1)$$

where D_{ij} is the diffusion coefficient of component i in component j . By conservation of mass and the use of Equation 1.1, Fick's second law of diffusion can be obtained. It is given by the following expression:

$$\frac{\partial C_i}{\partial t} = D_{ij}\nabla^2 C_i \quad (1.2)$$

where D_{ij} is typically assumed to be constant. Specific solutions to Equation 1.2 depend on the initial and boundary conditions as well as the sample geometry.

As the rubber is cooled down below its glass transition temperature (cf. Figure 1.1), the specific volume of the polymer (and indeed its enthalpy or entropy) deviates from that of the theoretical

equilibrium state. This volume is manifested as a trapped in void space between the pseudo-frozen polymer chains. The difference between the non-equilibrium glassy state specific volume and that of the theoretical rubbery equilibrium state is known as the fractional free volume of the glassy polymer. [96] Over long time scales, the polymer volume contracts (in most cases) and it is these time-dependent relaxation processes that often give rise to deviations from Fickian behaviour during water sorption. Equation 1.2 and its basic set of assumptions do not hold good in this case anymore.

Early water vapour sorption studies of ethylcellulose [97] and of polystyrene [98] showed non-Fickian behaviour that was manifested via (a) continuously increasing water solubilities at very long experimental times and (b) sorption-induced crazing of the glassy polymer due to water clustering. Water vapour sorption experiments of poly(methyl methacrylate) [99] revealed non-Fickian behaviour. Here, this non-Fickian behaviour was manifested not just in the sorption kinetics but also a pronounced sorption–desorption cycle hysteresis.

Stannet and co-workers carried out studies of water sorption in poly(acrylonitrile) [99, 100]. From their work, it was concluded that non-Fickian sorption kinetics of water arise from a slow polymer relaxation that occurred to accommodate the clustering of water molecules.

More recently, Potreck and co-workers [101] reported on the water vapour sorption kinetics in sulfonated poly(ether ether ketone) (S-PEEK). They observed Fickian behaviour accompanied by polymer relaxation at both low and high water vapour activities. It was suggested that the relaxation-controlled transport mechanism was the controlling and rate-determining process at higher water vapour activities.

In an attempt to understand the relationship between concentration-gradient controlled diffusion and that controlled by polymer relaxation, Vrentas and Duda [102, 103] introduced the concept of the diffusional Deborah number (De). The Deborah number is a dimensionless quantity; it is the

ratio of the times scales for concentration gradient-controlled diffusion over that of polymer relaxation. When $De > 1$, the observed diffusion behaviour is Fickian but obviously depends on sample geometry and scale (e.g. film thickness). When $De < 1$, the characteristic time of penetrant diffusion (as controlled by the concentration gradient) is longer than polymer relaxation. When $De \sim 1$, polymer relaxation and penetrant diffusion occur at similar time scales and the resulting diffusion behaviour is correspondingly complex.

Similar to the case of water solubility, our knowledge of water vapour sorption kinetics in glassy proteins is rudimentary compared to the behaviour of amorphous polymer systems. To my best knowledge, there is no significant contribution to this field that presents the water vapour sorption kinetics of a glassy model protein (such as lysozyme or bovine serum albumin) over a relevant temperature range. In fact, most work in the field of protein water sorption does not consider kinetic effects. In this fashion, whether an experiment was carried out in an interval or integral sorption mode is often unclear and the actual path-dependency of the solubility or sorption isotherm have not been explored for proteins as much as they were for simpler synthetic polymers. Good examples of investigations into this phenomena for synthetic polymers are references [104] and [105].

1.4 UNRESOLVED QUESTIONS IN PROTEIN HYDRATION

In this work, liquid water sorption in a number of lyophilised, amorphous model proteins was explored both experimentally and theoretically to examine the following questions:

Question 1: Can the wide differences in reported water vapour sorption isotherms in amorphous proteins be reconciled based on some underlying physical or chemical mechanism?

Question 2: Can the non-equilibrium nature of a glassy protein during water vapour sorption and, subsequently, the connection between concentration gradient controlled diffusion and relaxations on the basis of gravimetrically obtained sorption kinetics be established and through the use of a diffusion–relaxation model?

Question 3: Can polymer solution theories and non-equilibrium thermodynamic approaches be applied to model or predict the water solubility in glassy proteins; and can knowledge of the thermodynamic mixing properties such as the heats of sorption or the concentration dependence of the glass transition temperature be employed in order to develop effective isotherm/solubility models?

In order to develop a deeper understanding of water vapour sorption in amorphous proteins (Question 1) both in terms of the actual solute solubility as well as the kinetics of the process, a number of water transport experiments have to be carried out. The ability to accurately measure the mass uptake in proteins is of great importance. Figure 2 shows water vapour sorption isotherms for bovine serum albumin (BSA) obtained from five different research groups. Here and throughout the entire thesis, water activity is defined as

$$a_w = \gamma_w x_w = f_w / f_w^0 \quad (1.3)$$

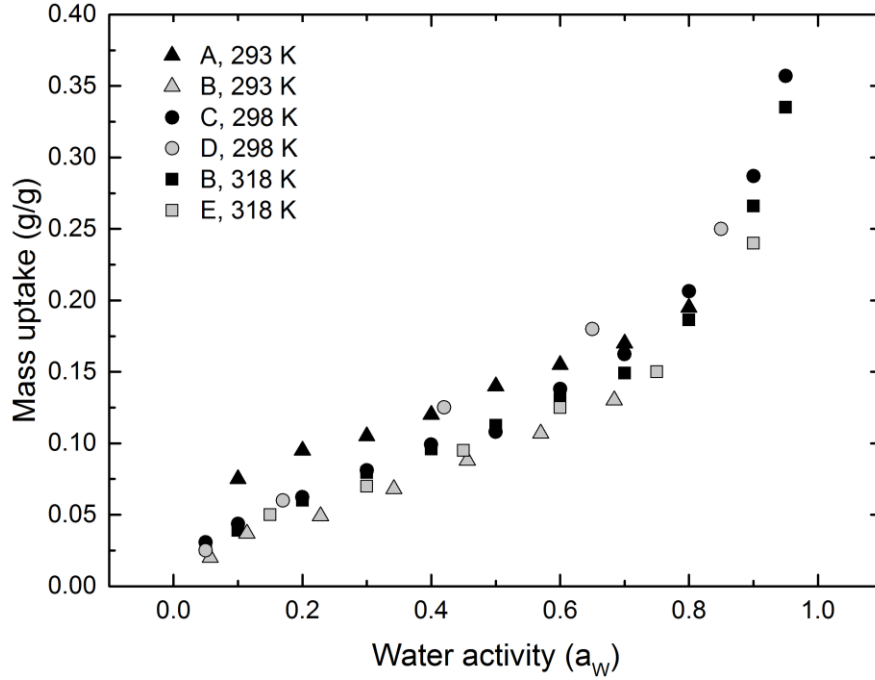


Figure 2 Comparison water vapour sorption isotherms of BSA as reported in literature at atmospheric pressure. (A) reproduced from [106]; (B) from [84]; (C) from [107]; (D) from [89]; and (E) from [108]. The diagram depicts the great variability in the published isotherms.

where a_w is the water activity, x_w is the mole fraction of water, γ_w is the activity coefficient of water, and f_w , f_w^0 are the fugacity of water in the system and under reference conditions, respectively. It is usually assumed that the gas phases behave ideally under conditions of ambient temperature and atmospheric pressure. Therefore, the ratio of fugacities can be taken as the ratio of pressures:

$$f_w/f_w^0 = p_w/p_w^0 \quad (1.4)$$

where p_w and p_w^0 are the vapour pressure of water in the system and of pure liquid water at the same temperature respectively. Following from this, Equation (1.1) can be rewritten in terms of relative humidity (R.H.) as

$$a_w = p_w/p_w^0 = \frac{RH}{100} \quad (1.5)$$

where RH is the percentage relative humidity of the air layer in equilibrium with the sample.

It is the great variability in the obtained results combined with the lack of serious attempts to rationalise these differences that is rather alarming. An investigation into the experimental mode (i.e. interval, integral, differential sorption) of the sorption experiments as well as the temperature dependence of the process is believed to shed light into the reported variability of water vapour sorption isotherms of proteins. Only a precise and reliable measurement of the isotherms can form a sound basis of any modelling attempts or predictions for such complex materials.

A gravimetric technique to capture and measure the sorption process is believed to provide a direct measurement of water sorption/diffusion and protein relaxation (Question 2). Previous diffusion–relaxation models for simple glass-forming systems and polymers have shown good agreement with experimental data. Up-to-date, there has been no important contribution in this field that showed the use of such concepts for protein–water systems. Capturing both sorption and relaxation phenomena in a single experiment, a more thorough understanding of these phenomena can be obtained. A diffusion–relaxation model is a means to quantitatively describe the time scales on which water sorption and glassy protein relaxation occur. It is expected that these investigations will also aid the experimental design of water sorption studies of proteins by elucidating the relationship between the time scales of the experiment and the present (or absent) non-equilibrium, glassy protein relaxation processes (as manifested in the water sorption kinetic data).

With a deeper understanding of diffusion–relaxation phenomena which occur during water transport in glassy proteins, more accurate and reliable water sorption isotherms can be reported. It will then be interesting to see how the solubility of water in glassy proteins can be modelled, and whether insight into the heats of sorption or the concentration-dependence of the glass transition temperature can aid in these modelling attempts (Question 3). There is an exciting opportunity here to investigate how these experimental ‘mixing’ properties underpin or eliminate the

fundamental bases of many of the commonly used treatments to model the isotherm. Lattice-based models have been used successfully in describing water sorption in glassy proteins [88], but experimental data must be regressed to the model. A true prediction of the sorption isotherms is difficult. On the other hand, equations of state, and particularly those developed within a non-equilibrium framework, have been used to predict both gas and liquid sorption in synthetic, glassy polymers but their application to simple globular proteins is yet awaited. Independent measurements of water and protein pressure–volume–temperature data, and the water–protein interaction parameter, are required for these theories and, in turn, could make these models truly predictive. To my best knowledge, there is no reported pure-component pressure–volume–temperature data published for the amorphous proteins studied here, thus, prohibiting the use of equation-of-state based approaches.

This work is expected to provide unique information on water sorption phenomena in glassy proteins. It is hoped to close the knowledge gap on a number of unresolved key questions in this field whilst the conclusion obtained will impact different fields of chemical engineering sciences as well as industrial formulation engineering.

1.5 THESIS OUTLINE

Chapter 2 examines the dependence of the water solubility/mass uptake studied by Dynamic Vapour Sorption of four model proteins (*bovine* serum albumin (BSA), *Bacillus sp.* protease (BSP), *Bacillus licheniformis* α -amylase (BLA), *porcine* pancreas lipase (PPL)) on the experimental temperature and mode (i.e. integral, interval sorption and desorption; cyclic and non-cyclic). Experimental data is regressed by common isotherm models and insufficiencies of these models are presented. For the work presented in Chapter 3-5, only bovine serum albumin was used due to the safety profile of the other model proteins; both the protease and amylase are classified as respiratory and contact sensitising chemicals (R 42/43). Further to this reason, the BSP and BLA studied in Chapter 2, which were not studied in greater depth, are known to suffer from chemical instabilities.

In Chapter 3, the kinetic water sorption data that make up the quasi-equilibrium isotherms of Chapter 2 are discussed for bovine serum albumin. A diffusion—relaxation model is applied to gain further insight into the observed anomalous kinetics.

Chapter 4 presents an analysis of the enthalpy and entropy effects during water sorption via both theoretical considerations as well as experimentally determined heats of sorption (in the interval sorption mode) for bovine serum albumin.

In Chapter 5, the water concentration dependence of the glass transition temperature for native bovine serum albumin is presented based on scanning calorimetric measurements and several models are employed to describe this process. The plasticising nature of water is discussed and the influence on the water solubility is explored.

Chapter 6 uses the NELF model to predict an interval water vapour sorption isotherm of bovine serum albumin. This prediction is based on measurements of a pressure—volume isotherm of bovine serum albumin (and amorphous porcine lipase and chicken egg white lysozyme).

Chapter 7 provides a summary of this work with key conclusions along with suggestions for future work.

1.6 REFERENCES

- [1] Rathore, N.; Rajan, R.S. *Biotechnol. Prog.* **2008**, 24, 504-514
- [2] Priest, F.G. *Extracellular Enzymes*. Wokingham, Van Nostrand Reinold UK Co., **1984**
- [3] Barfoed, H.C. in *Industrial Enzymology*, (Eds.: Godfrey, T.; Reichelt, J.), London, Macmillan Publishers, **1983**, p. 284
- [4] Franks, F. *Nature Biotechnology* **1994**, 12, 253-255
- [5] Sperling, L.H. *Introduction to Physical Polymer Science*. USA: Wiley Interscience, 2006
- [6] Haly, A. R.; Snaith, J. W. *Biopolymers* **1971**, 10, 1681-1699
- [7] Haly, A. R.; Snaith, J. W. *Biopolymers* **1968**, 6, 1355-1377
- [8] Tsereteli, G. I.; Smimova, O.I. *Biophysics* **1989**, 34 (5), 984-984
- [9] Singh, G. P.; Parak, F.; Hunklinger, S.; Dransfield, K. *Phys. Rev. Lett.* 1981, 47, 685-688
- [10] Parak, F.; Nienhaus, G. U. J. *Non-Cryst. Solids* **1991**, 131, 362-368
- [11] Nienhaus, G. U.; Frauenfelder, H.; Parak, F. *Phys. Rev. B* **1991**, 43, 3345-3350
- [12] Sochava, I. V.; Tseretoli, G. I.; Smirnova, O. I. *Biofizika* **1991**, 432-436
- [13] Sochava, I. V.; Smirnova, O. I. *Food Hydrocolloids* **1993**, 6 (6), 513-524
- [14] Linderstrom-Lang, K.U.; Scheliman, J.A. *Enzyme* **1959**, 1, 443-510
- [15] Gurd, F.R.N.; Rothgeb, T.M. *Adv. Prot. Chem.* **1979**, 33, 74-165
- [16] Careri, G.; Fasella, P.; Gratton, E. *Annu. Rev. Biophys. Bioeng.* **1979**, 8, 69-97
- [17] Frauenfelder, H.; Sligar, S.G.; Wolynes, P.G. *Science* **1991**, 254, 1598-1603
- [18] Doster, W.; Bachleitner, A.; Dunau, R.; Hiebl, M.; Luescher, E. *Biophys. J.* **1986**, 50(2), 213-219
- [19] Doster, W.; Cusack, S.; Petry, W. *Nature* **1989**, 337, 754-756
- [20] Rasmussen, B.F.; Stock, A.M.; Ringe, D.; Petsko, G.A. *Nature* **1992**, 357, 423-424

- [21] Iben, I.E.T.; Braunstein, D.; Doster, W.; Frauenfelder, H.; Hong, M.K.; Johnson, J.B.; Luck, S.; Ormos, P.; Schulte, A.; Steinbach, P.J.; Xie, A.H.; Young, R.D. *Phys. Rev. Lett.* **1989**, 62, 1916-1919
- [22] Ansari, A.; Jones, C. M.; Henry, E. R.; Hofrichter, J.; Eaton, W. A. *Science* **1992**, 256, 1796-1798
- [23] Green, J.L.; Fan, J.; Angell, C.A. *J. Phys. Chem.* **1994**, 98, 13780-13790
- [24] Doty, P.M.; Aiken, W.H.; Mark, H. *Ind. Eng. Chem.* **1946**, 38, 788-791
- [25] Katchman, B.; McLaren, A.D. *J. Am. Chem. Soc.* **1959**, 73, 2124-2127
- [26] Klute, C.H. *J. Appl. Polym. Sci.* **1959**, 1, 340-350
- [27] Doty, P.M.; Aiken, W.H.; Mark, H. *Ind. Eng. Chem.* **1946**, 38, 788-791
- [28] Bull, H.B. *J. Am. Chem. Soc.* **1944**, 66, 1499-1507
- [29] Shaw, T.M. *J. Chem. Phys.* **1944**, 12, 391-392
- [30] Brunauer, S.; Emmett, P.H.; Teller, E. *J. Chem. Phys.* **1938**, 60, 309-319
- [31] Pauling, L. *J. Am. Chem. Soc.* **1945**, 67(4), 555-557
- [32] Kuntz, I.D.; Kauzmann, W. *Adv. Prot. Chem.* **1974**, 28, 239-345
- [33] Watt, I.C.; D'Arcy, R.L. *J. Polym. Sci.* **1976**, 55, 155-166
- [34] Boki, K.; Kawasaki, N.; Takahashi, H. *J. Colloid Interface Sci.* **1993**, 161, 148-154
- [35] Leeder, J.D.; Watt, I.C. *J. Colloid Interface Sci.* **1974**, 48, 339-344
- [36] Hageman, M.J. *J. Agric. Food Chem.* **1992**, 40, 342-347
- [37] Chuang, J.C.; Morawetz, H. *Macromolecules* **1973**, 6, 43-47
- [38] Kocherbitov, V.; Arnebrant, T. *Langmuir* **2010**, 26(6), 3918-3922
- [39] Vieth, W.R.; Howell, J.M.; Hsieh, J.H. *J. Membr. Sci.* **1976**, 1, 177-220
- [40] Barrer, R. M.; Barrie, J. A.; Slater, J. *J. Polym. Sci.* **1958**, 27, 177-197
- [41] Vieth, W.R.; Sladek, K.J. *J. Colloid Sci.* **1965**, 20, 1014-1033

- [42] Kirchheim, R. *Macromolecules* **1992**, 25, 6952-6960.
- [43] Barrie, J.A.; Williams, M.J.L.; Munday, K. *Polym. Eng. Sci.* **1980**, 20, 20-29
- [44] Koros, W.J.; Chan, A.H.; Paul, D.R. *J. Membr. Sci.* **1977**, 2, 165-190
- [45] Cornejo-Bravo, J.M.; Siegel, R.A. *Biomaterials* **1996**, 17, 1187-1193
- [46] Arce, A.; Fornasiero, F.; Rodríguez, O.; Radke, C.J.; Prausnitz, J.M. *Phys. Chem. Chem. Phys.* **2004**, 6, 103-108
- [47] Rodríguez, O.; Fornasiero, F.; Arce, A.; Radke, C.J.; Prausnitz, J.M. *Polymer* **2003**, 44, 6323-6333.
- [48] Sharp, J.S.; Forrest, J.A.; Jones, R.A.L. *Macromolecules* **2001**, 34, 8752-8760
- [49] Flory, P.J. *Principles of Polymer Chemistry*; London: Cornell University Press, **1953**
- [50] Van Laar, J.J., *Sechs Vortrage uber das thermodynamische Potential*, Vieweg, Braunschweig, **1906**
- [51] Van Laar, J.J. *Die Thermodynamik einheitlicher Stoffe und binarer Gemische*, Noordhoff, Groningen, **1935**
- [52] Hildebrand, J. H. *The Solubility of Non-Electrolytes*; New York: Reinhold, **1936**
- [53] Hancock, B.C.; Zografi, G. *Pharmaceutical Research* **1993**, 10, 1262-1267
- [54] Hernandez, R.J.; Giacin, J.R.; Grulke, E.A. *J. Membr. Sci.* **1992**, 65, 187-199
- [55] Wang, J.-S.; Naito, Y.; Kamiya, Y. *J. Polym. Sci., Part B: Polymer Physics* **1996**, 34, 2027-2032
- [56] Nadakatti, S.M.; Kim, J.H.; Stern, S.A. *J. Membr. Sci.* **1995**, 108, 279-291
- [57] Kumazawa, H.; Bae, S.-Y. *J. Appl. Polym. Sci.* **1996**, 60, 115-121
- [58] Barbari, T.A. *J. Polym. Sci., Part B: Polymer Physics* **1997**, 35, 1737-1746
- [59] Feng, H. *Polymer* **2007**, 48, 2988-3002
- [60] Doghieri, F.; Sarti, G.C. *Macromolecules* **1996**, 29, 7885-7896

- [61] De Angelis, M.G.; Sarti, G.C.; Doghieri, F. *Ind. & Eng. Chem. Res.* **2007**, 46, 7645-7656
- [62] Carlà, V.; Hussain, Y.; Grant, C.; Sarti, G.C.; Carbonell, R.G.; Doghieri, F. *Ind. & Eng. Chem. Res.* **2009**, 48, 3844-3854
- [63] De Angelis, M.G.; Sarti, G.C.; Doghieri, F. *J. Membr. Sci.* **2007**, 289, 106-122
- [64] Doghieri, F.; Ghedini, M.; Quinzi, M.; Rethwisch, D.G.; Sarti, G.C. *Desalination* **2002**, 144(1-3), 73-78
- [65] Pinnau, I.; Freeman, B.D., Eds.; *ACS Symp. Ser.* **2004**, 876, 55-73
- [66] Carlà, V.; Wang, K.; Hussain, Y.; Efimenko, K.; Genzer, J.; Grant, C.; Sarti, G.C.; Carbonell, R.G.; Bonavoglia, B.; Storti, G.; Morbidelli, M. *Ind. & Eng. Chem. Res.* **2006**, 45, 1183-1200
- [67] Doghieri, F. *Macromolecules* **2005**, 38, 10299-10313
- [68] Giacinti Baschetti, M.; Doghieri, F.; Sarti, G.C. *Ind. & Eng. Chem. Res.* **2001**, 40, 3027-3037
- [69] Sanchez, I.C.; Lacombe, R.H. *Macromolecules* **1978**, 11(6), 1145-1156
- [70] Doghieri, F.; Sarti, G.C. *J. Membr. Sci.* **1998**, 147, 73-86
- [71] Chapman, W.G.; Gubbins, K.E.; Jackson, G.; Radosz, M. *Fluid Phase Equilibria* **1989**, 52, 31-38
- [72] De Angelis, M.G.; Doghieri, F.; Sarti, G.C.; Freeman, B.D. *Desalination* **2006**, 193, 82-89
- [73] Sarti, G.C.; De Angelis, M.G. *AIChE Journal* **2012**, 58, 292-301
- [74] Davis, E.M.; Minelli, M.; Giacinti Baschetti, M.; Sarti, G.C.; Elabd, Y.A. *Macromolecules* **2012**, 45, 7486-7494
- [75] Davis, E.M. Water sorption and diffusion in glassy polymers. PhD thesis, Drexel University, **2013**
- [76] Du, A.; Koo, D.; Theryo, G.; Hillmyer, M.A.; Cairncross, R.A. *J. Membr. Sci.* **2012**, 396, 50-56
- [77] Modesti, M.; Dall'Acqua, C.; Lorenzetti, A.; Florian, E. *J. Membr. Sci.* **2004**, 229, 211-223

- [78] Zimm, B.H. *J. Chem. Phys.* **1953**, 21, 934-935
- [79] Zimm, B.H.; Lundberg, J.L. *J. Phys. Chem.* **1956**, 60, 425-428
- [80] Ranade, G.; Stannett, V.; Koros, W.J. *J. Appl. Polym. Sci.* **1980**, 25, 2179-2186
- [81] Kilburn, D.; Claude, J.; Mezzenga, R.; Dlubek, G.; Alam, A.; Ubbink, J. *J. Chem. Phys. B* **2004**, 108, 12436-12441
- [82] Weinmüller, C.; Langel, C.; Fornasiero, F.; Radke, C.J.; Prausnitz, J.M. *J. Biomed. Mat. Res. Part A* **2006**, 77A, 230-241
- [83] McLaren, A.D.; Rowen, J.W. *J. Polym. Sci.* **1951**, 8, 289-324
- [84] Bull, H.B. *J. Am. Chem. Soc.* **1944**, 66, 9, 1499-1507
- [85] Gregory, R.B. (ed.) Protein–solvent interactions. Marcel Dekker: New York. **1995**, Ch. 3
- [86] Careri, g.; Gratton, E.; Yang, H.P.; Rupley, J.A. *Nature* **1980**, 284, 572-573
- [87] Rupley, J.A.; Gratton, E; Careri, G. *Trends Biochem. Sci.* **1983**, 8, 18-22
- [88] Shlambin, S.L.; Hancock, B.C.; Zografí, G. *Eur. J. Pharma. Biopharma.* **1998**, 45, 239-247
- [89] Antonov, Y.; Eckelt, J.; Sugaya, R.; Wolf, B.A. *J. Phys. Chem. B* **2013**, 117, 5497-5502
- [90] Antonov, Y.; Wolf, B.A. *Langmuir* **2014**, 30(22), 6508-6515. Wolf, B.A. *Ind. Eng. Chem. Res.* **2013**, 52, 3530-3536
- [91] Antonov, Y.; Wolf, B.A. *Biomacromolecules* **2005**, 6, 2980-2989
- [92] Antonov, Y.A.; Wolf, B.A. *Biomacromolecules* **2006**, 7, 1562-1567
- [93] Frisch, H.L. *Polymer Eng. Sci.* **1980**, 20, 2-13
- [94] George, S.C.; Thomas, S. *Progr. Polym. Sci.* **2001**, 26, 985-1017
- [95] Fick, A. *Annalen de Physik* **1855**, 170, 59-86
- [96] Victor, J.G.; Torkelson , J.M. *Macromolecules* **1987**, 20, 2241-2250
- [97] Barrer, R.M.; Barrie, J.A. *J. Polym. Sci.* **1958**, 28, 377-386
- [98] Pogany, G.A. *Polymer* **1976**, 17, 690-694

- [99] Stannet, V.; Haider, M.; Koros, W.J.; Hopfenberg, H.B. *Polymer Eng. Sci.* **1980**, 20, 300-304
- [100] Stannet, V.T.; Ranade, G.R.; Koros, W.J. *J. Membr. Sci.* **1982**, 10, 219-233
- [101] Potreck, J.; Uyar, F.; Sijbesma, H.; Nijmeijer, K.; Stamatialis, D.; Wessling, M. *Phys. Chem. Chem. Phys.* **2009**, 11, 298-308
- [102] Vrentas, J.S.; Jarzebski, C.M.; Duda, J.L. *AIChE Journal* **1975**, 21, 894-901
- [103] Vrentas, J.S.; Duda, J.L. *J. Polym. Sci.: Polymer Physics Edition* **1977**, 15, 441-45
- [104] Kruger, K.M.; Sadowksi, G. *Macromolecules* **2005**, 38(20), 8408-8417
- [105] Vrentas, J.S.; Duda, J.L.; Ni, Y.C. *J. Polym. Sci. Phys. Ed.* **1977**, 15(11), 2039-2045
- [106] Luthra, S.; Lechuga-Ballesteros, D.; Kalonia, D.S.; Pikal, J.M. *J. Pharm. Sci.* **2007**, 96, 1974-1982
- [107] Amberg, C.H. *J. Am. Chem. Soc.* **1957**, 79 (15), 3980-3984
- [108] Kachel, S.; Scharfer, P.; Schabel, W. *Chem. Eng. Process Process Intens.* **2013**, 68, 45-54

1.7 APPENDIX

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Chapter 2. Liquid water sorption in amorphous proteins

2.1 INTRODUCTION

Proteins are increasingly used as therapeutic agents and as active ingredients in various biopharmaceutical, household and personal care formulations. However, their formulation strategies differ significantly from conventional low-molecular weight actives such as pharmaceuticals which are usually in a crystalline dosage form. In most applications, proteins are produced as dry, amorphous powders using spray- or freeze-drying which offer increased relative storage stability over liquid state formulations.

The sorption and diffusion of water in hydrophilic glassy polymers is important in many fields and has been studied extensively both experimentally and theoretically [1]. However, the water sorption behaviour of proteins is less well understood. As the hydration level affects the structure and function of proteins, the knowledge of the hydration behaviour of these amorphous protein formulations is crucially important for virtually all applications. For example in freeze-dried protein formulations the residual water content of the final amorphous product cake is used as a Quality-by-Design property.

Biological systems do not function without the presence of some water. For instance, fully dehydrated enzymes are not biologically active, though as little water as a single water ‘mono-layer’ restores their biological function [2]. A comprehensive knowledge of hydration properties and mechanisms is important both during processing (e.g. freeze drying, spray drying, foam drying) and final product storage for amorphous protein solids. Extensive reviews on the hydration of nominally ‘dry’ proteins were published by Kuntz and Kauzmann [3] and subsequently by Rupley and Careri [4].

Generally, the amount of water that is sorbed by amorphous protein formulations is greater than that which can be accounted for by a surface adsorption mechanism. Water is taken up into the bulk of hydrophilic materials with typical water contents of up to twenty five percent by mass for high water activities ($a_w > 0.9$). Characteristic protein–water sorption isotherms exhibit two deviations from isotherm linearity as shown in Figure 3: one at low water activities and the other at high activities. The deviation at low activities ($0 < a_w \leq 0.25$) appears as a “shoulder” and is a typical feature observed of a “Type II” isotherm for gas adsorption processes. [5] This phenomenon is different to a “Type III” isotherm where no initial shoulder occurs. The deviation at high activities (typically $a_w > 0.7$) appears as an exponential upswing in sorbed moisture content.

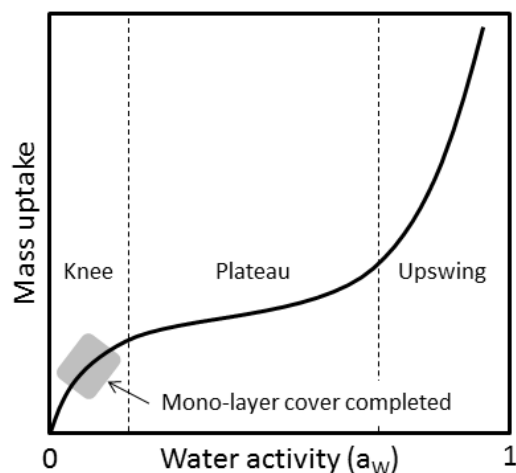


Figure 3 Schematic isotherm featuring the typical deviations from isotherm linearity as observed in amorphous high-purity protein formulations. This typical water sorption isotherm may be divided into three regions: knee, plateau, and upswing.

In view of the apparent similarity of water vapour absorption isotherm shapes to those obtained from gas adsorption experiments, it has been common practice to fit water vapour sorption data to classical BET isotherms derived for gas adsorption processes. [6] Since the original work by

Pauling [7], the generally accepted view was that surface-based or stoichiometric models provide an appropriate framework to describe hydration phenomena in such ‘dry’ proteins. By far the most popular approach has been to use the well-known BET equation [8]:

$$W = \frac{W_m C_B (p/p_0)}{[1 - (p/p_0)][1 - (p/p_0) + (C_B (p/p_0))]} \quad (2.1)$$

where W is the mass of water absorbed per unit mass of solid, p/p_0 is the relative vapour pressure of water, and W_m and C_B are constants. For the gas adsorption experiment, W_m denotes the mass of gas forming a complete monolayer on the solid surface, and it is usually taken to represent the point or region where the initial shoulder occurs. The use of the BET equation and its derivatives (e.g. GAB equation) for predicting water vapour sorption in amorphous pharmaceutical and proteins has been reported and reviewed by Zografi [9]. In this review, Zografi points out that water uptake into amorphous solids decreases the glass transition temperature of the solid whilst advocating the use of surface-based approaches. He suggested that water in amorphous solids can exist in both a ‘bound’ and ‘solvent-like’ state with probably two types of ‘bound’ states. [9]

This picture is in line with early attempts in understanding the binding of sorbed water. A generally accepted view (cf. Table 3), based on both theoretical considerations and experimental evidence, was that the sorption isotherms for proteins could be classed into three distinct regions [10, 11]. Here, the first region is binding of water to highly active sites such as charged and highly polar groups. The second region is ascribed to the transition from an absorbed monolayer to multilayer coverage. This process occurs via the binding of water to weaker sorption sites such as the peptide backbone and polar surface groups. Any additional water binding in the second region occurs via local clustering near charged and highly polar groups and through the filling of voids created by swelling of the protein. The third and final region, the multilayer region, occurs with condensation of water at very weak binding sites and layering of loosely held water. [12] So, the

general belief has been that the behaviour of water sorbed by a protein system can be predicted using adsorption isotherms and existing mathematical models to identify several areas of the isotherm that represent distinctly different characteristics of the water transport mechanism. For water activities giving rise to mass uptake below the Brunauer-Emmet-Teller (BET) monolayer coverage region, generally 5 – 9 % water content depending on the particle size, the mobility of the absorbed water and conformational flexibility of the protein are limited. [12] At or near this level of hydration, there is some indication that secondary relaxations of the amorphous system may occur and many decomposition pathways become observable over experimentally detectable time frames. [4] The dynamic mobility of the system then slowly increases with increasing hydration level. Degradation reactions dependent upon the mobility of the water and/or the protein are, therefore, greatly enhanced at temperatures and/or moisture contents above T_g . [13] At high residual moisture contents, the possibility of chemical reactions occurring (cleavage, oxidation, deamidation, denaturation, aggregation, etc.) is increased. [14]

Water is a complex solvent due to its polarity and ability to form hydrogen bonds, both with itself and with polymers, as well as its tendency to cluster or cause plasticisation of hydrophilic polymers. [15] This greatly complicates the analysis of water sorption in macromolecules. Whilst, water vapour sorption isotherms for amorphous proteins have been reported for more than half a century now [16], the understanding of the protein hydration mechanism is incomplete.

This prevailing picture of water surface adsorption by proteins does not take into account the structural and dynamical changes of the protein associated with hydration. More recent analyses have considered the water–protein system to be analogous to a simple aqueous solution. [17] Early work showed disagreement with the simplest solution models (e.g. Raoult’s law, Henry’s law) and concluded that protein–water systems did not behave as ideal or regular solutions. [17] Following this study, several investigators attempted to apply solution theories that had been developed for

synthetic amorphous polymeric systems to describe their data for bio-polymers. Perhaps the most simple, famous and most widely used of these solution models is the Flory–Huggins model [18].

Despite these earlier studies, the nature of the water sorption process, including the glassy characteristics of amorphous protein materials, has been largely neglected. As a result, the vast majority of reported studies of the water vapour sorption of amorphous proteins neglects non-equilibrium effects (e.g. structural, volumetric relaxation). The experimental data reported are largely affected and complicated by ‘sample history’, both prior to the start of the experiment as well as during the actual measurement of a water sorption isotherm. Whilst some studies of water sorption in glassy synthetic polymers have considered these non-equilibrium effects (cf. Chapter 1), the influence of sample history on the solubility of water and sorption kinetics has not been explored sufficiently for protein systems.

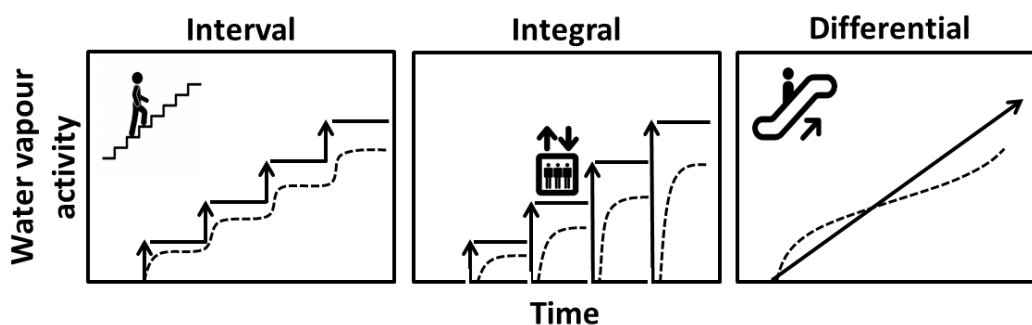


Figure 4 Schematic representation of different modes of vapour sorption experiments. The schematic shows the water activity — time plane for the different modes.

A gravimetric sorption experiment can typically be carried out in three modes: (a) *interval* sorption, where the solvent activity is subsequently raised in a stepwise fashion through one continuous measurement; (b) *integral* sorption, where the solvent activity is raised from zero up to one specific value and the mass uptake is recorded (typically with a new specimen for each measurement); or (c) *differential* sorption, where an activity ramp is applied in one continuous

measurement which can be understood as a special case of interval sorption at infinitely small activity intervals. It should be understood by the reader that each one of these three sorption modes creates a different experiment-induced sample history.

From the countless studies of equilibrium water vapour sorption and sorption isotherms for amorphous, water-compatible polymers (synthetic and natural) reported, two general characteristics have become widely acknowledged. One is, such experiments do not usually represent a true thermodynamic equilibrium measurement since the polymer substrate is undergoing viscoelastic relaxation due to plasticisation by sorbed water. [19] Secondly, such experiments are not conducted at a constant fictive temperature with respect to the ΔT -governed viscoelastic properties of the polymer, as the glass transition temperature decreases during the experiment as the moisture content increases. [20] Therefore, any rational molecular interpretation of the water vapour isotherm for glassy biopolymers must consider the time dependent and kinetic behaviour which is key to understanding the characteristic sigmoidal shape of these isotherms. In this context, it is crucial to understand the influence of the experimental sorption mode employed (i.e. integral, interval, or differential; and cyclic or non-cyclic).

While much of Section 1.2 ('Water solubility in glassy polymer/proteins) gave a detailed insight into the background and chronology of previous work on water sorption in glassy polymers and the molecular chemistry of protein hydration, it is the aim of this Chapter to bridge the gap between these two topics. In this study, water sorption and desorption experiments were carried out in integral and interval mode for four model protein systems over a wide range of temperatures. A detailed analysis of the quasi-equilibrium isotherms obtained was carried out. (The corresponding sorption kinetics are analysed and discussed in Chapter 3.) Different theoretical sorption isotherm models are then tested against the experimental data.

2.2 EXPERIMENTAL SECTION

2.2.1 Materials

Bovine serum albumin (BSA) (lyophilised powder, catalogue number 05470), protease from *Bacillus* *sp.* (BPS) (lyophilised powder, catalogue number P4789), α -amylase from *Bacillus* *licheniformis* (BLA) (lyophilised powder, catalogue number A4551), and *porcine* pancreas lipase (PPL) (lyophilised powder, catalogue number L0382) were purchased at the highest commercially available grade from Sigma Aldrich (Gillingham, United Kingdom). Ultrapure deionised water (resistivity $\sim 16\text{ M}\Omega \cdot \text{cm}$) was used for all sorption experiments.

2.2.2 Methods

A Dynamic Vapour Sorption (DVS) analyser Advantage 1 (Surface Measurement Systems, London, United Kingdom) was used for all sorption experiments. Figure 5 shows a schematic diagram of the instrument layout. DVS is a well-established method to measure the vapour sorption isotherms, where the vapour phase may be water or an organic solvent. The ultra-microbalance records mass uptake/loss of a sample with $\pm 0.1\text{ }\mu\text{g}$ mass resolution within 1 second data fidelity.

For each run, as-received protein samples ($\sim 6 - 10\text{ mg}$) were loaded into the sample pan. After closing the apparatus, the system was purged with dry air at the relevant experimental temperature ($283.15 - 333.15\text{ K}$) until a constant sample weight was achieved. By ‘constant’, a change of weight of no more than 0.05 weight percent during a 30 *min* time interval was considered. This performance was typically achieved within ten hours. Water vapour is injected in a flow mode at mass-flow-controlled rates (total flowrate 200 *sccm*) to achieve the specified mixture of dry and ‘wet’ air. Experiments were carried over a range of vapour activities, a_w , from

0.03 to 0.60, sometimes up to $0.90a_w$. Assuming an ideal gas phase of pure water vapour, i.e. neglecting the vapour pressure of BSA, the water activity is then approximated according to $a_w = p/p_w^0$, where p is the pressure and p_w^0 is the water saturation pressure at the experimental temperature. The experiments were computer-automated and carried out in both cyclic and non-cyclic interval sorption and desorption mode, as well as integral sorption mode.

All graphs presented were prepared with OriginLab Pro 9.1 (OriginLab, Northampton, MA), and Maple 15 (MapleSoft Europe Ltd., Cambridge, UK) was used for data analysis.

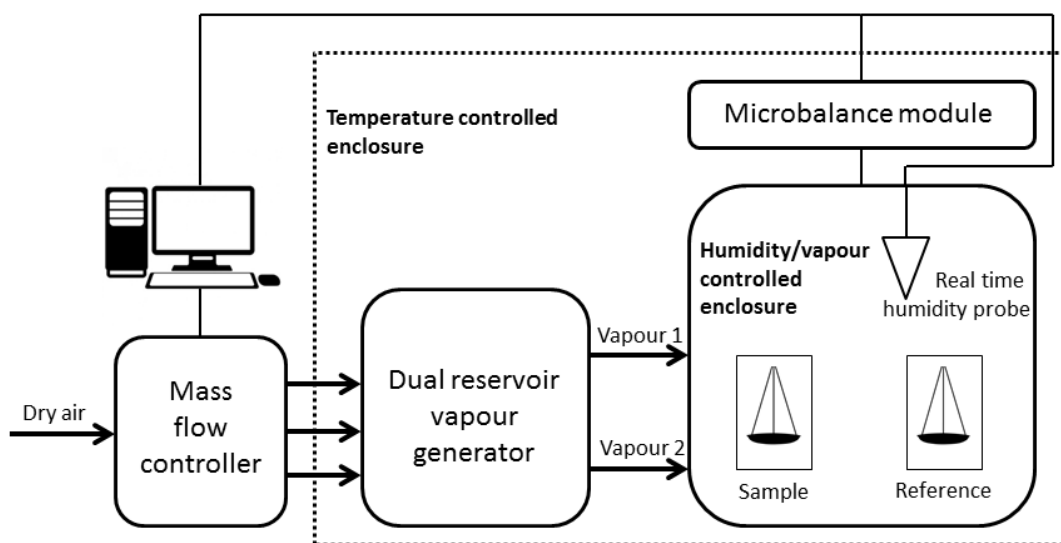


Figure 5 Schematic diagram of Dynamic Vapour Sorption analyser. The schematic depicts the set-up of a DVS system, where a microbalance module is placed within a temperature-controlled enclosure.

2.3 RESULTS AND DISCUSSION

2.3.1 Bovine Serum Albumin: Interval Sorption

Figure 6 shows a typical set of raw data from a gravimetric interval sorption–desorption experiment for water/BSA at 293.15 K. The quasi-equilibrium water uptake at each activity interval is defined as the mass ω_t at time t where:

$$\left(\frac{d\omega_t}{dt}\right)_{T,P} \rightarrow 0 \quad (2.2)$$

From this expression, a quasi-equilibrium water sorption and desorption isotherm can be calculated. An example of this isotherm is shown in Figure 7 which displays a sorption/desorption isotherm typical for water/protein systems. The desorption isotherm does not return to zero mass uptake origin at zero water activity as the 800 *min* time interval is not sufficient to dry out the material completely. Hence, mass transfer equilibrium was not achieved at that point.

It is a well-known fact that penetrant–polymer systems exhibit different sorption isotherms above and below the glass transition temperature of the pure polymer. It was observed [21] that the isotherms curve away in a convex manner from the penetrant pressure axis as the penetrant pressure is increased for sorption in rubbery polymers in the sense of:

$$\left(\frac{\partial^2 p_1}{\partial \omega_1^2}\right)_T < 0 \quad (2.3)$$

where p_1 is the penetrant pressure in the gas/vapour phase and ω_1 is the mass fraction of the penetrant in the liquid polymer-rich phase (in g/g). For penetrant sorption in a glassy polymer, it was observed [22, 23] that the sorption isotherms generally curve toward the penetrant pressure axis with increasing penetrant pressure in the sense of:

$$\left(\frac{\partial^2 p_1}{\partial \omega_1^2}\right)_T > 0 \quad (2.4)$$

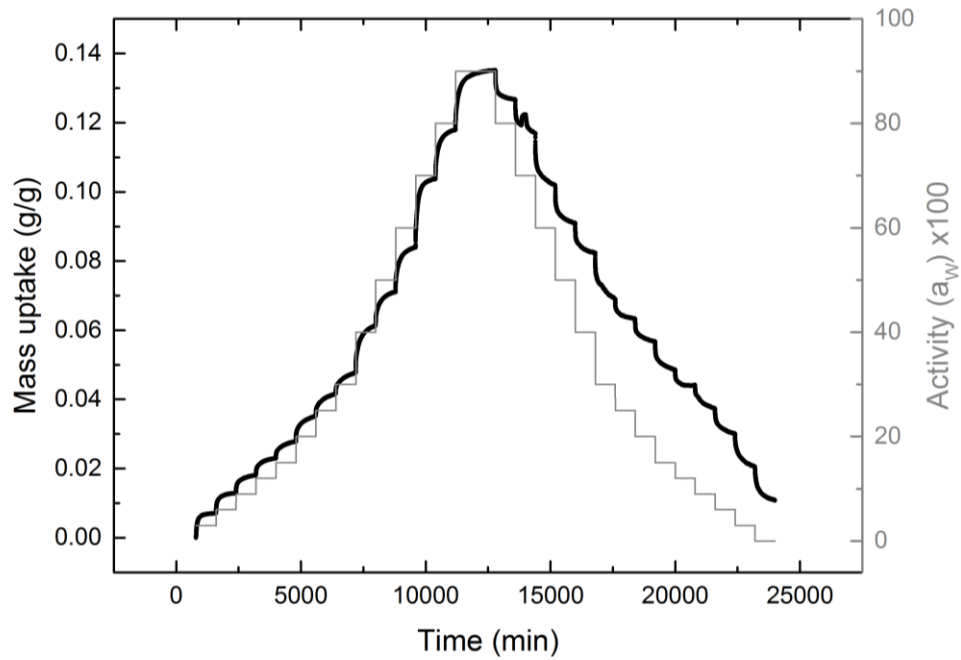


Figure 6 Gravimetric cyclic water sorption and desorption time curves (primary axis) and water activity x100 (secondary axis) for water/BSA at 293.15 K.

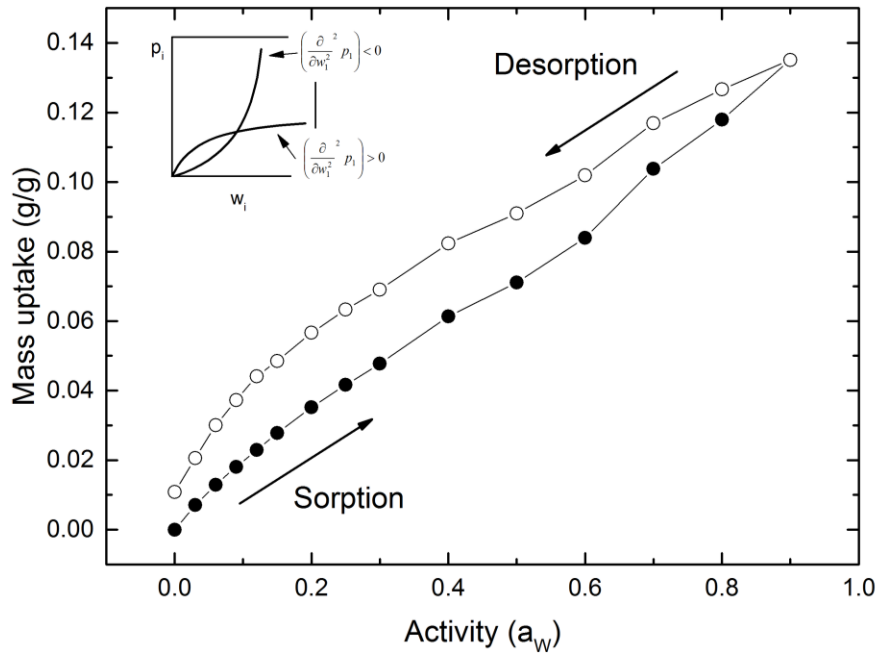


Figure 7 Typical cyclic interval; sorption and desorption isotherm for water/BSA at 293.15 K. Experimental error smaller than symbol size. (n=2) The inset relates back to Equations 2.3 and

2.4

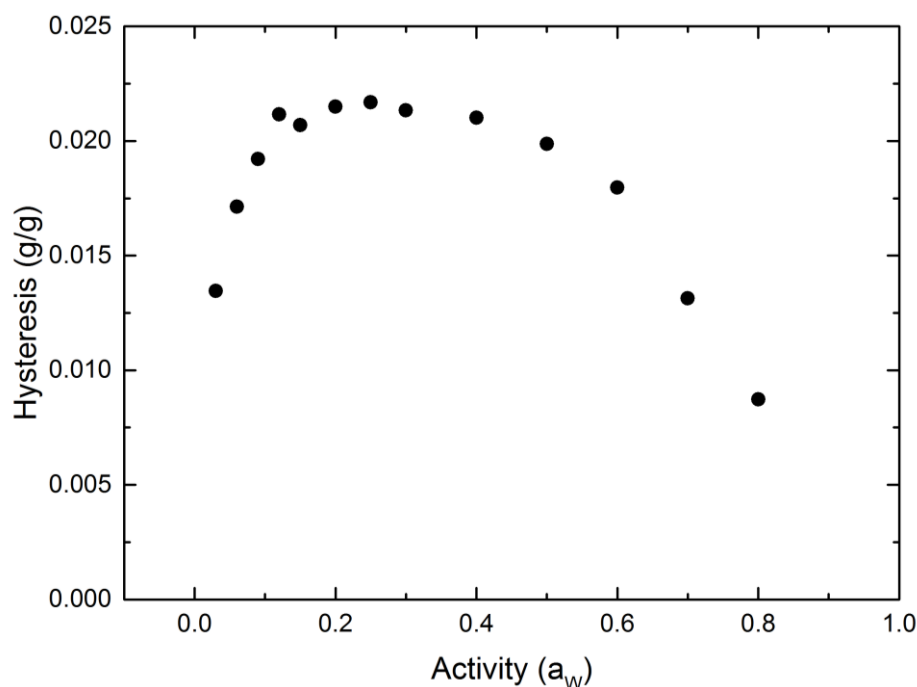


Figure 8 Typical sorption–desorption hysteresis for water/BSA at 293.15 K as calculated from the isotherms shown in Figure 7. The sorption hysteresis is highest at intermediate water activities. Experimental error smaller than symbol size. (n=2)

However, it was noticed from early on that glassy water–polymer systems often exhibit an isotherm of the shape described by Equation 2.3 rather than Equation 2.4. [24] Therefore, the shape of the isotherms here cannot be explained with such a simplistic view. [24] Equations (2.3) and (2.4) can be related to the experimental data shown in Figure 7 by the definition of activity (cf. Equations 1.3 to 1.5). The inset in Figure 7 depicts the relation between Equations 2.3 and 2.4. with an experimentally measured isotherm.

The hysteresis of the isotherm, where the sorption segment lies below the desorption segment and desorption ‘appears’ to take place at a hypothetical effective lower temperature despite the constant experimental temperature, is commonly observed in water–protein systems as well as water–polymer systems. [4] Repeated cycling of a single sample can affect the level of hysteresis

further which makes the situation even more complicated to interpret. [25] Figure 8 shows the sorption–desorption hysteresis for the system water/BSA at 293.15 K. The extent of hysteresis is greatest at moderate activities, below and above which hysteresis levels decrease.

Formally, a system showing hysteresis is not expected to exhibit one unique relationship between an external independent variable x and an internal dependent variable y . [26]

Strictly speaking, the presence of sorption–desorption hysteresis does undermine the theoretical basis of virtually all sorption isotherm models. All of the theories summarised in Table 1 (cf. Chapter 1), which are surface based approaches, were derived under the assumption of thermodynamic equilibrium. Although the same applies the majority of the theories summarised in Table 2 (cf. Chapter 1) as polymer theories/equations of state, the density of the equation of state theories can be used as an internal order parameter; it is used as a time-dependent parameter. Therefore, non-equilibrium features could be modelled.

Whilst only shown here for water/BSA at 293.15 K, hysteresis is highly reproducible under most experimental conditions. Typically, the isotherm information is converted into free energies of transfer of water from a bulk vapour or solvent phase to the protein, and from the temperature dependence of the isotherm, enthalpies of transfer are typically estimated. These calculations are completed under the assumption of equilibrium. Therefore, if hysteresis is present, the equilibrium state may not be sufficiently defined for a thermodynamic analysis.

Several explanations for hysteresis of water-sorption in proteins have been offered: (1) conformational changes within the protein that occur during sorption at low levels of hydration and reverse slowly during desorption compared to the rates during sorption [27]; (2) metastable states associated with phase transitions within the protein (i.e. adsorbate) that correlate to ‘S-shaped’ thermodynamic functions (the so called van der Waals loops) [28]; (3) capillary condensation within voids of the solid material [29]; (4) true equilibrium is only achieved during

desorption where the process is not limited by nucleation of water on charged sites and numbers of clusters can exceed the number of charged sites hence giving rise to higher desorption isotherm [25, 30]. Bryan [31] has discussed further possible models based on a thermodynamic analysis.

Much of the evidence to suggest that conformational changes of the protein in the region of low hydration cause sorption–desorption hysteresis support the hypothesis of hysteresis as a molecular phenomenon [25, 31, 32]. In fact, investigations into the sorption hysteresis loop of lysozyme by Bryan [31] support the analysis that continuous changes in protein conformation occur with changing hydration levels. In this sense and by the formal definition of hysteresis, which is the system's dependence of its outputs on current and past inputs, it is clear that water vapour sorption in BSA may not be treated by models based on an equilibrium thermodynamic framework and neither may BSA be treated as a static solid matrix of sorption sites but as a dynamically changing solid substrate.

2.3.2 Bovine serum albumin: Integral sorption

The integral sorption experiment is fundamentally different to the interval sorption experiment – though it is an experiment rarely reported. During integral sorption, a dry sample is taken and equilibrated against one specific water activity, and in this study the weight change is measured for 800 *min*. The experiment is then repeated with a new sample at a different activity so there are no sample history effects. During interval sorption, it is one sample that is continuously, in subsequent steps equilibrated against an increasing water activity.

Figure 9 shows the isotherms for the two modes of operation for the system water/BSA at 293.15 K. Up to an activity of $0.15a_w$, there is no statistical difference between the mass uptake during interval and integral sorption. Above this activity, the moisture uptake during integral

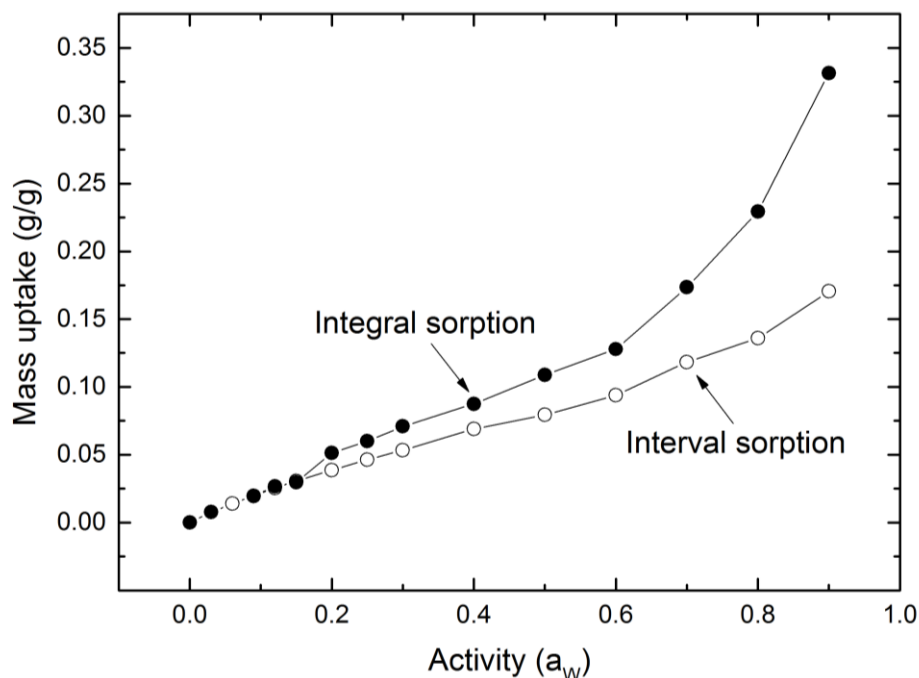


Figure 9 Comparison between interval and integral water sorption. Integral (solid circle) and interval (open circle) water sorption isotherms of BSA at 293.15 K. Experimental errors are smaller than symbol size. $n = 3$ for interval sorption data, $n = 3$ for integral sorption data at 0.03, 0.15, 0.20 and 0.90 a_w ; otherwise $n = 1$.

sorption is significantly higher. In fact, the discontinuity at $0.15a_w$ is observed as a sharp step-increase. This phenomenon cannot be explained with a simple equilibrium theory. So, if the presence of sorption–desorption hysteresis is a manifestation of the continuous conformational changes of a protein that occur with changing levels of hydration, then the comparison of the interval and integral sorption experiments might suggest that Δa_w might be the driving force for this change during a water sorption experiment.

These results suggest that the water mass uptake/solubility may not be a state but a path variable, that is highly dependent on the experimental mode. This analysis is a surprising result. As of yet, an alternate explanation of this phenomenon cannot be offered. The kinetics of the mass transfer

process have to be considered as non-equilibrium effects are expected to give rise to this unusual sorption behaviour. This topic shall be further considered in Chapter 3.

2.3.3 Bovine serum albumin: Non-cyclic interval desorption

In Bryan's study from 1987 [31], the following was stated: "*The idea that a protein molecule might have a [water (de-)sorption] 'memory' of its past history and that this 'memory' might be lost when the protein surface is covered by water is certainly speculative. It is not hard to think of interesting biological implications.*" It was based on his study into the thermodynamics of water sorption–desorption in proteins. Here, both hysteresis effects during interval sorption–desorption cycle experiments of the system water/BSA as well as pronounced differences between interval and integral sorption isotherms have been shown. Both phenomena corroborate Bryan's statement.

Figure 10 shows the case of a non-cyclic interval desorption experiment. Starting with a fully hydrated sample at an activity of $0.90a_w$, the activity was subsequently decreased in the fashion of the typical interval experiments. The starting point of this experiment corresponds to the integral sorption experiment from dry up to an activity of $0.90a_w$. There is no 'memory' caused by previous interval sorption steps, unlike posed during the sorption–desorption cycle where the desorption isotherm's starting point coincides with the last measurement point of interval sorption isotherm.

As the activity decreases, the water concentration decreases but still lies significantly above the isotherm of the integral water sorption experiments. For $0 < a_w \leq 0.15$, both the cyclic and non-cyclic interval desorption isotherms overlay. At low hydration, heats of mixing in water/protein systems are highly exothermic [33], so that the binary thermodynamic interaction is favourable and strong. Similarly, there is no statistically relevant difference between mass uptake for $0 <$

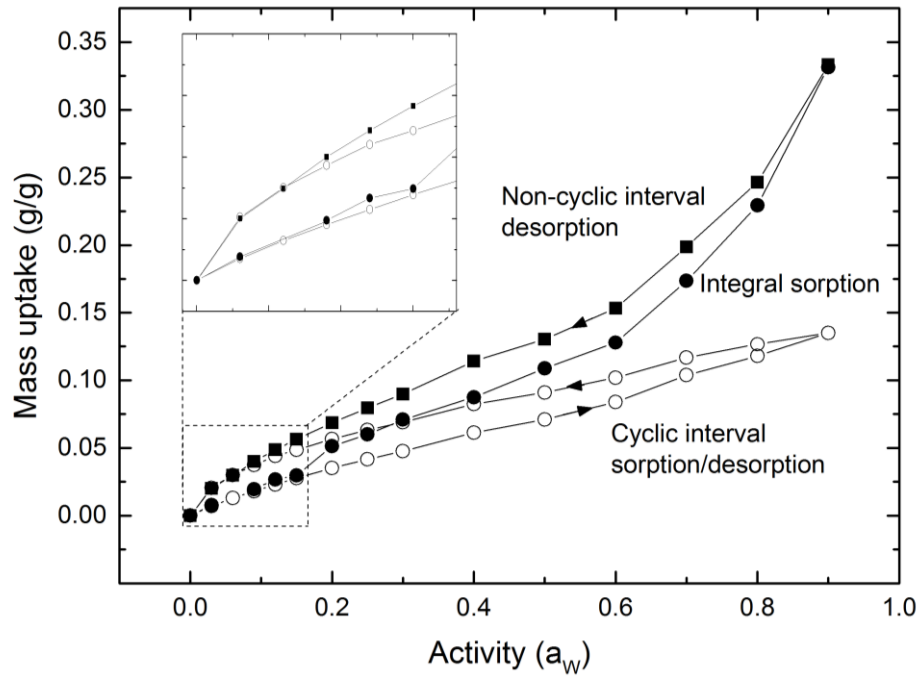


Figure 10 Comparison between integral, cyclic interval sorption, and non-cyclic interval desorption isotherms. Integral sorption (solid circle), interval sorption–desorption cycle (open circle), and non-cyclic interval desorption (solid box) water sorption isotherms of BSA at 293.15 K. Experimental errors are smaller than symbol size. $n = 3$ for non-cyclic interval desorption.

$a_w \leq 0.15$ interval and integral sorption. On the basis of this data, it is suggested that the experimental mode is less important than the direction (i.e. sorption or desorption) of the experiment at low hydration.

Lüscher-Mattli and Ruegg [25] performed water sorption experiments of α -chymotrypsin and tropocollagen and suggested that only a desorption isotherm (and not the absorption branch of a sorption cycle) may represent thermodynamic equilibrium regardless of the prior treatment of the sample. To this point, it has to be added that only the non-cyclic interval desorption isotherm may therefore represent thermodynamic equilibrium.

While the interpretation of the observed phenomena is difficult and does require an analysis of the kinetics (see Chapter 3), there is one statement that can be made with certainty now regarding the system water/BSA: full hydration of the BSA molecule is not achieved during the interval sorption–desorption cycle.

The results shown here corroborate the findings by Wolf and co-workers [63] who showed that the vapour pressures (for a given composition of the mixture) are considerably larger in the case of water loading than in the case of water removal (as predominantly practiced with the measurements of p_{vap}) for water/BSA mixtures.

2.3.4 Temperature-dependent interval sorption of different proteins

Whilst anomalies have been established for the system water/BSA at 293.15 K, the general applicability of these anomalies to water–protein systems were tested by measuring sorption isotherms for four globular model proteins over a wide and processing-/application- relevant temperature range. Alongside *bovine* serum albumin (BSA), water sorption isotherms were measured for an amorphous protease from *Bacillus sp.* (BPS), an α -amylase from *Bacillus licheniformis* (BLA), and a *porcine* pancreas lipase (PPL). These three proteins are technical enzymes relevant to the personal care and household industry (i.e. biological laundry detergents, automated dish-washing tablets). Figures 11 to 14 show the interval water sorption isotherms for these systems.

For water/BSA, the mass uptake at a given activity decreases with increasing temperature except for the isotherm at 293.15 K at which mass uptake is lowest. For water/BPS and water/PPL, there is little temperature-dependence of the mass uptake for low and intermediate activities. At high

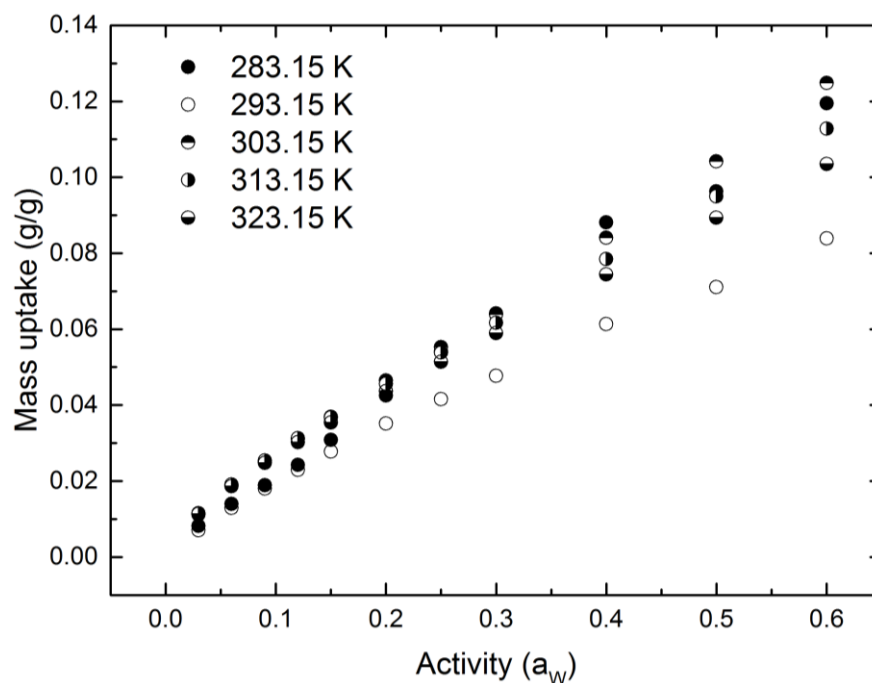


Figure 11 Typical interval sorption isotherms for the system water/BSA between 283.15 and 323.15 K.

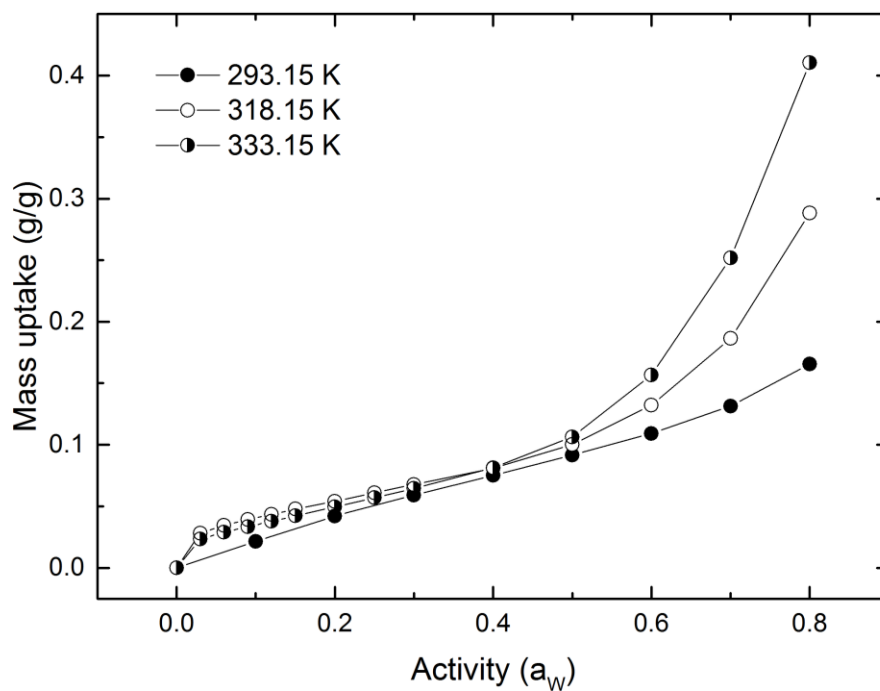


Figure 12 Typical interval sorption isotherms for the system water/BSP between 293.15 and 333.15 K.

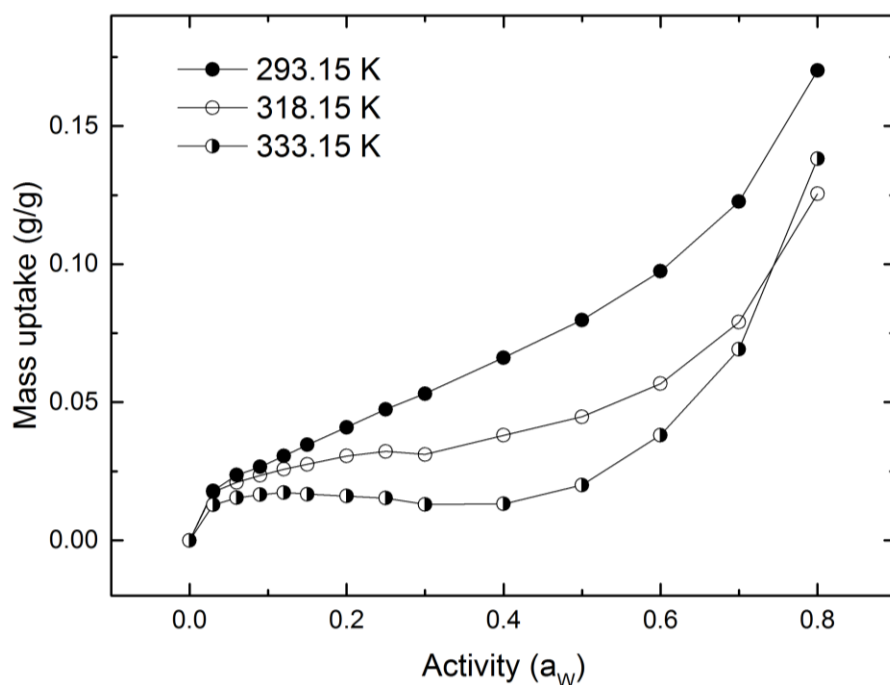


Figure 13 Typical interval sorption isotherms for the system water/BLA between 293.15 and 333.15 K.

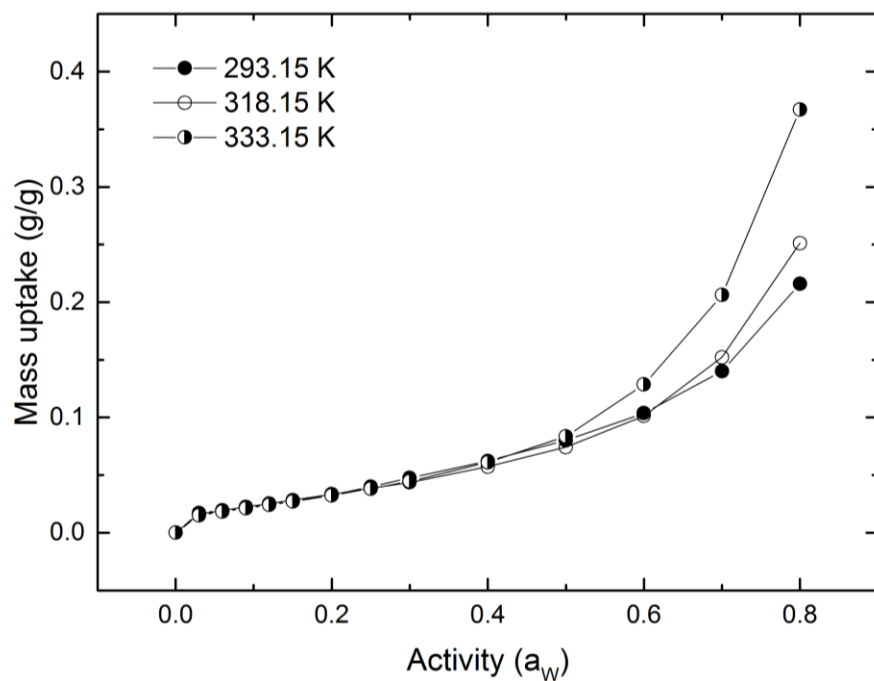


Figure 14 Typical interval sorption isotherms for the system water/PPL between 293.15 and 333.15 K.

activities, the sorption capacity increases with increasing temperature. This behaviour could be seen as a rubbery characteristic. For the system water/BLA, the mass uptake at constant activity decreases with an increase in temperature. Further, the knee of the isotherm (present at low activities) seems to develop into a pronounced local maximum at elevated temperatures. This maximum cannot be interpreted by classical equilibrium theories and must be interpreted as a direct manifestation of a conformational or phase change of the protein molecule. To my best knowledge, there has been no report of the development of a local maximum in the water mass uptake by an amorphous protein as reported here.

Traditionally, heats of sorption/mixing were estimated from the temperature dependence of the water sorption isotherms. (Some of this work is reviewed in Chapter 4.1.) From the analysis of the temperature dependence of the sorption isotherms reported here, it appears that such analysis could almost be ‘pot luck’ in the sense that the choice of temperatures used might give rise to a misleading picture. For instance, if one was to take the water sorption isotherms of BSA at 303.15, 313.15, and 323.15 K to estimate heats of mixing, a different picture would be drawn if one used the isotherms at 293.15 and 303.15 K. Therefore, there is significant level of arbitrariness in estimating heats of sorption from the temperature dependence of water sorption isotherms for proteins.

Furthermore, it is obvious now that no routine experimental analysis can yield mechanistic insight into water sorption phenomena in glassy–rubbery proteins, and the analysis of such data is greatly complicated by the uniqueness of each protein, both in terms of structure and chemistry.

2.3.5 Isotherm modelling: D’Arcy–Watt model

Classical sorption theories draw a formal and clear distinction between *adsorption* and *absorption*. Adsorption is confined to the physical interface between a substrate and its

environment. Absorption is the sorption process occurring within a substrate. Various theoretical models to treat surface adsorption have been proposed [34-37]. Further attempts [8, 16, 38-42] were made later to treat multi-layer sorption. Some of these models are listed in Table 1 (cf. Chapter 1). Absorption was thought to be essentially internal *adsorption* where a sorbing gas or vapour would diffuse from the surface of the sorbent into its interior. [43]

The sigmoidal isotherms of many synthetic and natural macromolecular substrates with water cannot be accurately described by these simple models. A study on a broad range of isotherm models for polymer–water systems was carried out by Barrie [44]. While the isotherm model of Brunauer, Emmett, and Teller [8] fits the experiment water uptake of polymers and proteins up to activities of about $0.50a_w$ [10], serious discrepancies between the experimental isotherm and that predicted by the theory occur at higher activities. This limited success is despite some of the fundamental unsuitability as posed by the key assumptions of the theories. Solutions theories such as proposed by Flory [45] and Huggins [46] can give reasonable agreement with experimental data at high solvent activities for both proteins [9] and synthetic polymers [47]. The use of the Flory–Huggins theory is discussed in latter sections.

From a stoichiometric analysis of water sorption in keratin fibres [48] and proteins [49], D’Arcy and Watt [11] suggested an isotherm model for sorption in non-homogeneous sorbents. They suggested that absorbed water is associated with polar side chains, with peptide and amide groups, and forms a hydrogen bond network at high water activities. Their isotherm model is a composite curve comprised of three contributions: (1) monolayer sorption by strong binding sites in the form of a Langmuir-type isotherm; (2) monolayer adsorption by weakly binding sites linearly related to the vapour activity; and (3) multilayer/hydrogen bond network formation at high activities that is described by a modified Langmuir-type contribution. The equation is:

$$W = \sum_{i=0}^l \frac{K'_i K_i a_w}{1 + K_i a_w} + C a_w + \frac{k' k a_w}{1 - k a_w} \quad (2.5)$$

where W is the weight of the sorbate (e.g. water) adsorbed by the sorbent (e.g. polymer), $K'_i = mn_i/N$ is the number of primary sites n_i of type i , multiplied by the molecular weight of the sorbate (m) and divided by Avogadro's number (N); K_i is a constant and measure of the attraction between the sorbate and its sorption sites. K'_0 and K_0 are both equal to zero. l is the number of different types of sorption sites for primary adsorption that are described by a Langmuir isotherm. C is a constant for the linear approximation on specific sites. $k' = mD/N$ is the number of secondary types, multiplied by the molecular weight of the sorbate and divided by Avogadro's number. Equation 2.5 is a general expression; for particular systems one might find one or more of the terms inoperative; then, the appropriate constants in Equation 2.5 will assume zero values.

Figure 15 shows the isotherms for water sorption into porcine lipase at 333.15 K fitted by the D'Arcy–Watt model. A good fit (coefficient of determination (R^2) = 0.99899) between the regressed and experimental isotherm was achieved. However, with five fitting parameters a reasonable fit should be expected. The regressed isotherm parameters were found to be: $l = 1$, $K'_1 = 0.0158$, $K_1 = 5$, $C = 0$, $k' = 0.0667$, and $k = 1.051$. The middle term of Equation 2.5 seems to be inactive as $C = 0$ and corresponds to the situation where some sites have weaker affinities for the sorbate such that the monolayer is only partly completed even at activities near the saturation vapour pressure.

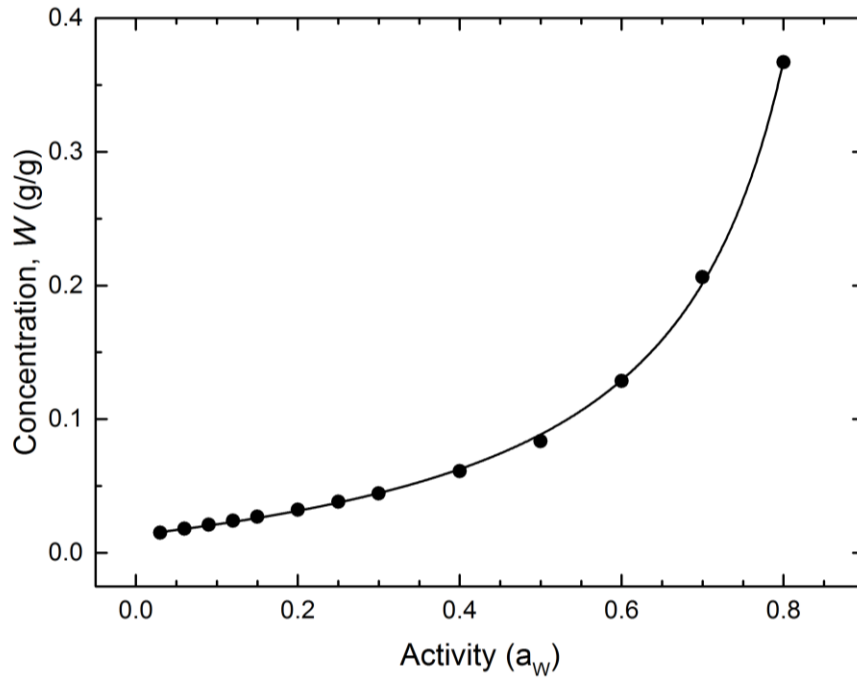


Figure 15 Comparison between experimentally obtained water sorption isotherm for water/PPL and D'Arcy-Watt model. Water sorption isotherm for PPL at 333.15 K fitted by the D'Arcy–Watt model (Equation 2.5). The regressed isotherm parameters were found to be: $l = 1$, $K'_1 = 0.0158$, $K_1 = 5$, $C = 0$, $k' = 0.0667$, and $k = 1.051$.

The D'Arcy–Watt model does not provide any insight into the underlying molecular phenomena that either (a) give rise to the observed sorption behaviour or (b) are caused by the sorbing water. Whilst this sorption model was developed in the context of water sorption into amorphous proteins it is thought that its underlying framework is neither theoretically sound nor would it provide the basis for a molecularly realistic and insightful improvement of the model. It neglects the glassiness of the protein and the solution character of the process, especially at intermediate to high water activities.

2.3.6 Isotherm modelling: Dual mode sorption

Meares [50] was one of the first researchers to suggest that inhomogeneities present in a polymer (that are due to the inability of the sub- T_g polymer matrix to relax to thermal equilibrium) have a pronounced impact on penetrant solubility and transport. He proposed a ‘dual-mode’ mechanism in which the first mode is that of an ordinary solution and the second mode is that of adsorption of penetrant molecules into voids or holes that were frozen into the glass at T_g . Vieth, Michaels and Barrie [51, 52] suggested that the nonlinear sorption isotherm often observed in glassy polymers could be represented by:

$$C = C_D + C_H = k_D p + C_H' b p / (1 + b p) \quad (2.6)$$

where C is the total concentration of penetrant molecules, C_D is the concentration of ‘dissolved’ molecules, C_H is the concentration of molecules adsorbed in holes or microvoids, p is the partial pressure of the penetrant in the reservoir, k_D is the Henry’s Law constant, and b is the hole affinity constant. The Langmuir population, C_H , is at least partially immobilised. Whilst this equation is empirical in nature, its ability to represent experimental data has been shown for a wide range of polymers. [53]

Figures 16 to 19 show the dual mode sorption model regressions for water sorption in BSA, BSP, BLA, and PPL over a wide range of temperatures. A good fit was achieved for all proteins and temperatures up to activities of $0.60a_w$, except for the α -amylase (BLA) where the sorption isotherms feature local maxima in sorption uptake at intermediate activities. Furthermore, it was not possible to fit the high-activity end of the isotherms with the dual mode sorption model as the model cannot account for the strong upswing in concentration in this region. This issue was expected from both a mathematical analysis of the dual mode sorption theory and phenomenological considerations. Henry’s Law cannot capture the solution character of this type

of solvent–polymer systems. Neither can Equation 2.6 produce the upswing mathematically nor do the assumptions hold good for when the system goes into ‘solution mode’ at such high activities. It is a question as to whether the material is no longer glassy at high water activities, thus, causing the failure of the dual mode sorption theory.

Chiou and co-workers [54] have argued that it is common for sorption isotherms of glassy polymers to show a shape characteristic of glassy polymers at low solvent activities and a shape characteristic of sorption in rubbery polymers at high solvent activities.

They found that the penetrant concentration at which the transition from glassy-like to rubbery-like sorption behaviour takes place decreases with increasing temperature. The effect has been attributed to solvent induced plasticisation of the polymer matrix.

The departure from glassy sorption behaviour can be analysed by an inspection of the first- and

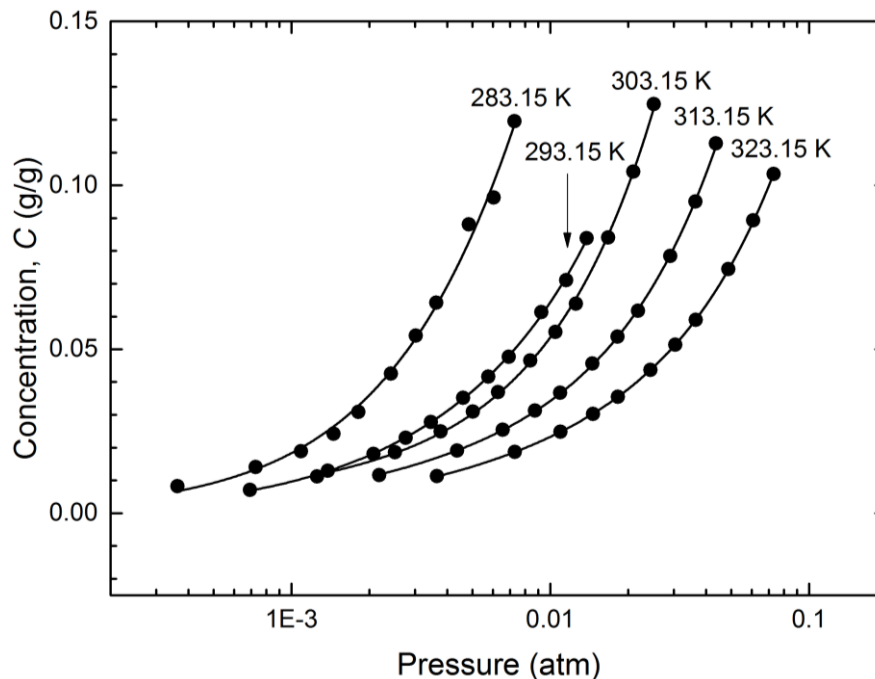


Figure 16 Dual mode sorption (Equation 2.6) regression of interval water vapour sorption in BSA at different temperatures. The sorption isotherms were plotted in the typical way when using dual mode sorption theory.

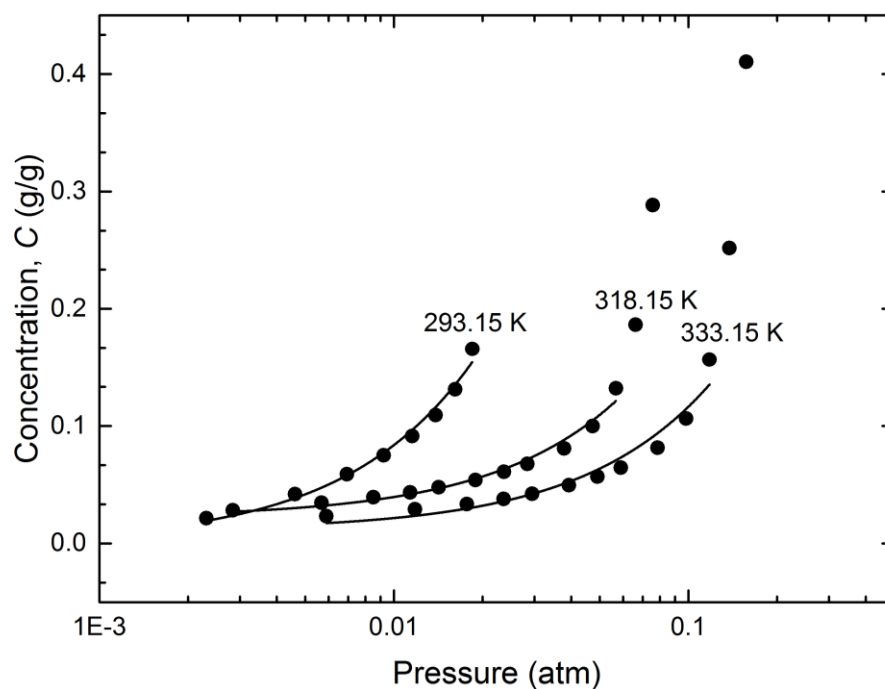


Figure 17 Dual mode sorption (Equation 2.6) regression of interval water vapour sorption in BSP at different temperatures.

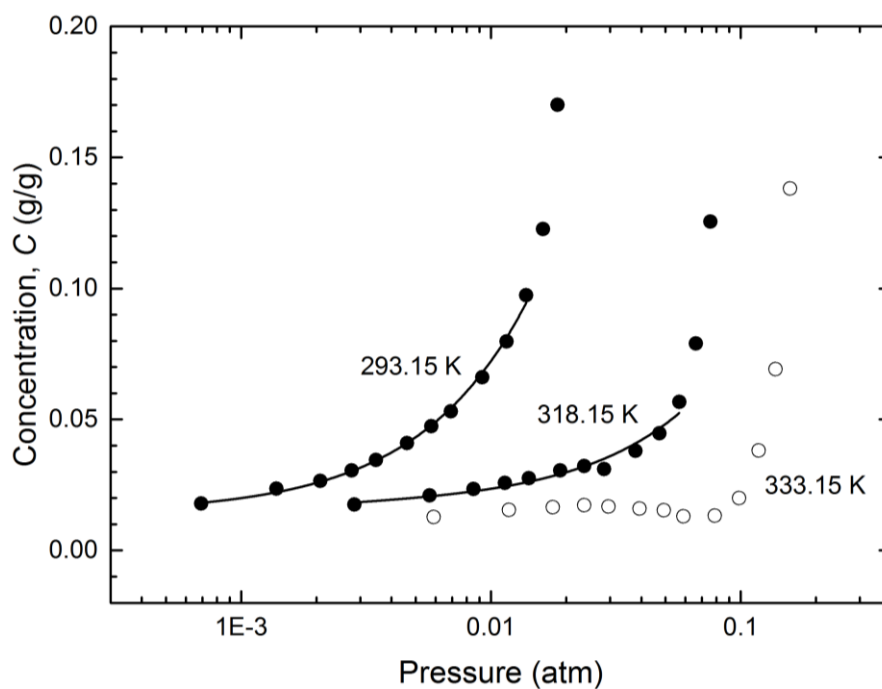


Figure 18 Dual mode sorption (Equation 2.6) regression of interval water vapour sorption in BLA at different temperatures.

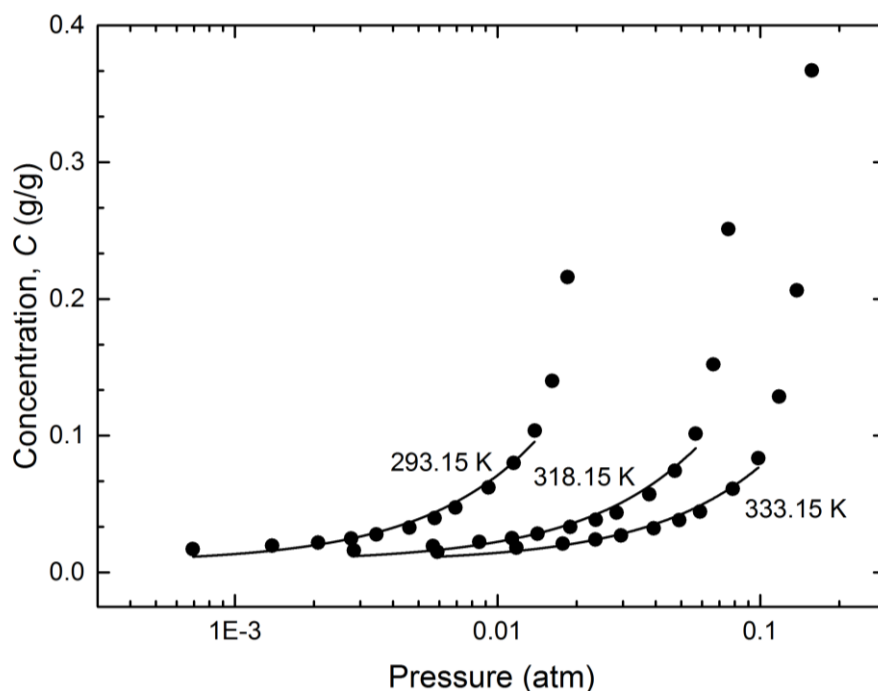


Figure 19 Dual mode sorption (Equation 2.6) regression of interval water vapour sorption in PPL at different temperatures.

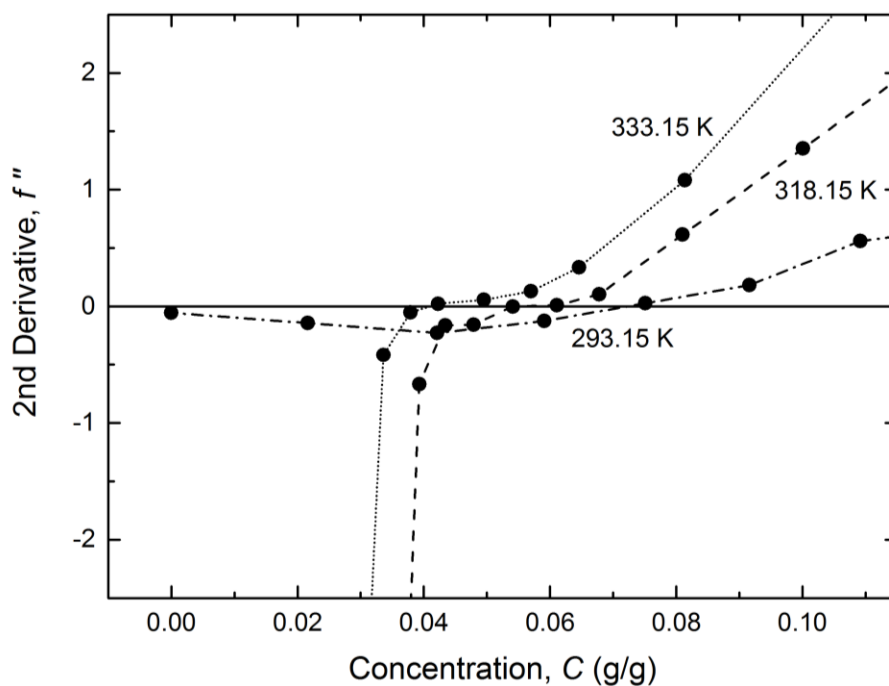


Figure 20 Second-order derivative of the water sorption isotherm with respect to water activity, here plotted against concentration for the system water/BPS.

second-order derivatives of the sorption isotherm. The isotherm's inflection point may signify the departure from glassy sorption behaviour. Mathematically, the necessary condition for an inflection point is $f''(x) = 0$. In this sense, an analysis of the isotherms is possible. Figure 20 shows how the characteristic concentration, where glassy sorption behaviour is lost, varies with temperature for water sorption in BPS. However, the analysis is not self-consistent as it does not represent the same trend for the four model proteins of interest investigated here. What is more is that the predicted transition concentration is thought to be unrealistically low and not in line with the chemistry of protein hydration as presented in Table 3 (cf. Chapter 1).

The classic dual mode sorption theory does not provide a suitable framework to describe the water sorption process into glassy bio-polymers over the whole concentration range. As water is a plasticiser, theories that allow for glassy-rubbery transition events during sorption need to be tested.

2.3.7 Isotherm modelling: Flory–Huggins solution theory

A highly useful approximate equation describing the free energies of mixing in (rubbery) polymer systems was derived independently by Staverman and Van Santen [55, 56], Huggins [46, 57], and by Flory [45, 58] on the type of lattice models qualitatively described by Meyer [59]. In their lattice model, the mixture is represented by a number of regularly arranged lattice sites, each of size V_ℓ expressed as molar volume. Each polymer molecule is thought to be composed of a number of segments of size V_ℓ occupying m_i lattice sites so that:

$$m_i = \frac{V_{m,i}}{V_\ell} \quad (2.7)$$

where $V_{m,i}$ is the molar volume of species i . If there are n_i moles of species i , the total volume of the system is:

$$V = V_\ell \sum n_i m_i \quad (2.8)$$

where the sum runs over all species (i.e. full occupancy of lattice sites is assumed). The handiest composition variables are volume fractions of the species, defined in the model by:

$$\phi_i = \frac{n_i V_i}{V} \quad (2.9)$$

Considering a strictly binary mixture of species 1 (solvent) and 2 (polymer). Then, the Flory–Huggins free energy of mixing is given by:

$$\frac{\Delta G}{NRT} = \left(\frac{\phi_1}{m_1}\right) \ln \phi_1 + \left(\frac{\phi_2}{m_2}\right) \ln \phi_2 + \chi \phi_1 \phi_2 \quad (2.10)$$

From this, an isotherm equation can be formulated as

$$\ln a_w = \ln(\phi_1) + \left(1 - \frac{1}{\chi}\right) \ln(\phi_2) + \chi \phi_2^2 \quad (2.11)$$

It has become widely accepted [60] in the literature on thermodynamic properties of polymer solutions that the binary interaction parameter (χ) is concentration dependent. Various molecular grounds can be advanced to support this functionality. This parameter denoted here by χ here has been approximated in a power series in concentration with empirical coefficients [60]. Leaving a detailed discussion of molecular reasons aside, χ is approximated in a phenomenological fashion and according to an equation of the form:

$$\chi = a + b\phi_1 + c\phi_1^{-1} \quad (2.12)$$

By fitting Equation 2.11 to each data point along a gravimetrically determined water sorption isotherm (such as presented in Figure 7 for instance), the concentration dependence of the binary interaction parameter χ can be evaluated. For a given water activity, the corresponding mass uptake is measured in an isothermal DVS experiment (a resulting sorption isotherm is shown in

Figure 9 for instance). This mass uptake can then be turned into a volume fraction, which then turns Equation 2.11 into a one-parameter model. So, substituting experimental data into Equation 2.11 and equating for χ at each activity yields a series of χ values as a function of volume fraction or water activity (as they are interrelated in the Flory–Huggins theory).

Figures 21 to 24 show this evaluation for the four model proteins and shows a good fit of such data by Equation 2.12. From this type of analysis it could be inferred that the binary interaction is both concentration- and temperature-dependent. It was not possible to reproduce the abnormalities (i.e. the local sorption maximum) of BLA at 318.15 and 333.15 K. Furthermore, the regression for water/BSA at 283.15 K indicated complex behaviour that cannot be approximated by a low-order polynomial. Apart from these cases, the regressed interaction parameters were represented accurately by Equation 2.12. The quality of this regression will then determine the quality of the representation of the experimental data by the Flory–Huggins isotherm.

Traditionally, the interaction parameter χ was defined as a dimensionless heat of mixing according to the van Laar equation:

$$\Delta H_m = kTN_1\phi_2\chi_{1,2} \quad (2.13)$$

where these symbols have the usual meaning. The only parameter on the right hand side of Equation 2.13 that can have a negative sign is χ itself. So, this result implies that the heat of mixing is exothermic $\chi < 0$ and endothermic for $\chi > 0$. This means that the heat of mixing would change from exothermic at low activities to endothermic at intermediate and high activities for the four systems studied here.

The point where $\chi = 0$ corresponds to the state of athermal mixing (i.e. purely combinatorial), ϕ_c . For water sorption in lipase or α -amylase, values for ϕ_c decrease with increasing temperature. For protease, values for ϕ_c increase with increasing temperature. For BSA, values for ϕ_c increase up to a temperature of 313.15 K and decrease at higher temperatures.

As a good representation of the regressed binary interaction parameters was achieved by Equation 2.12, it could be argued that the hydration process of the proteins studied here falls into the same category as commonly studied solvent–polymer mixtures. The observed shapes of the concentration dependence of χ are similar to those of solvent–polymer mixtures that it is almost tempting to neglect some of the assumptions (i.e. rubber system, no volume changes, no directional interactions) of the Flory–Huggins theory, and interpret the results with this simplistic solution theory. Extrapolating out of the studied concentration range into the dilute range raises the question of the validity of the estimated $\chi(\phi_1)$ functions. In Equation 2.12, the constant a gives the value for χ as $\phi_1 \rightarrow 1$. Typical values for $\chi(\phi_1 \rightarrow 1)$, that is under infinite dilution, are around 0.50 but the value shown here are one order of magnitude larger. In Chapter 4, a direct experimental investigation into the enthalpies and entropies of mixing is reported. In particular, it is questionable whether the predicted change of sign from exothermic mixing to endothermic mixing (at higher water activities) can be directly measured. As of yet, a complete and rigorous molecular interpretation of water sorption in proteins is not possible from a pure Flory–Huggins treatment. More advanced thermodynamic parameters and functions need to be analysed.

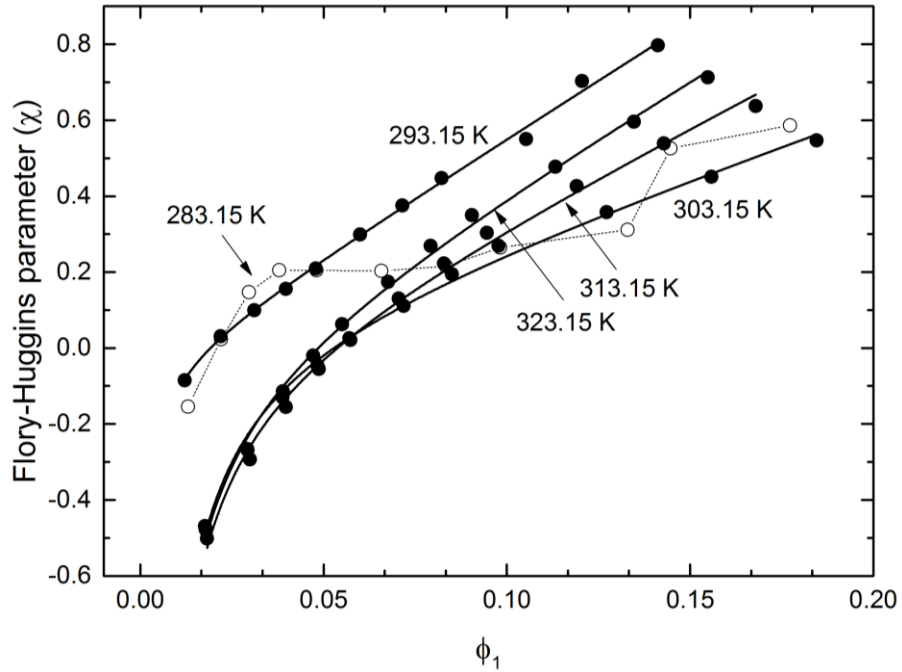


Figure 21 Flory–Huggins interaction parameters for the system water/BSA at different temperatures. Fit by Equation 2.12 with parameters: for $T = 293.15$ K parameter are $a = -0.0469$, $b = 6.089$, $c = -0.0013$; for $T = 303.15$ K parameters are $a = 0.0165$, $b = 3.325$, $c = -0.0100$; for $T = 313.15$ K parameters are $a = -0.0735$, $b = 4.762$, $c = -0.0099$; for $T = 323.15$ K parameters are $a = -0.0817$, $b = 5.618$, $c = -0.0093$.

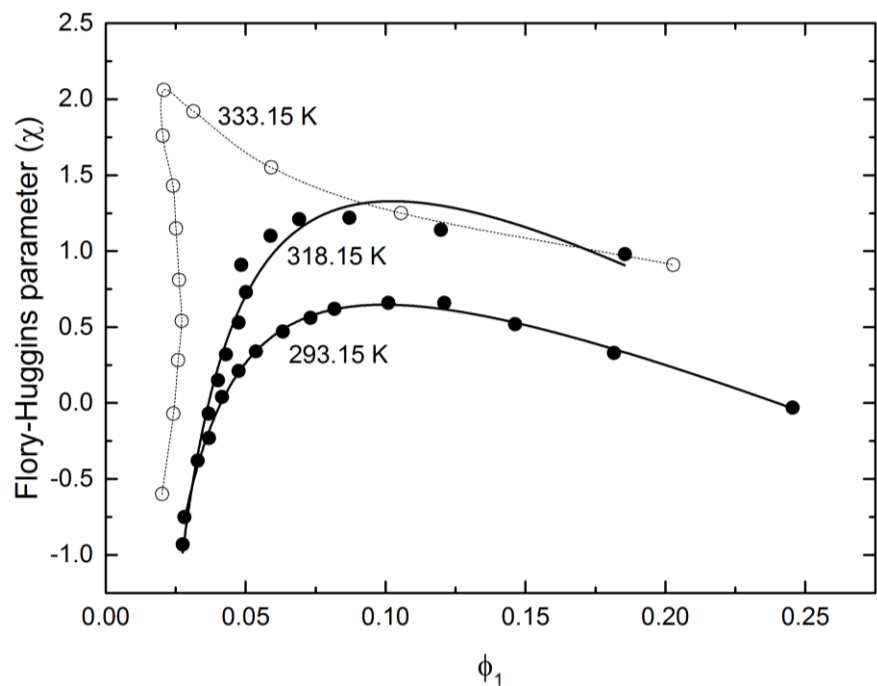


Figure 22 Flory–Huggins interaction parameters for the system water/BLA at different temperatures. Fit by Equation 2.12 with parameters: for $T = 293.15$ K parameters are $a = 2.21$, $b = -7.87$, $c = -0.08$; for $T = 318.15$ K parameters are $a = 3.65$, $b = -11.34$, $c = -0.12$.

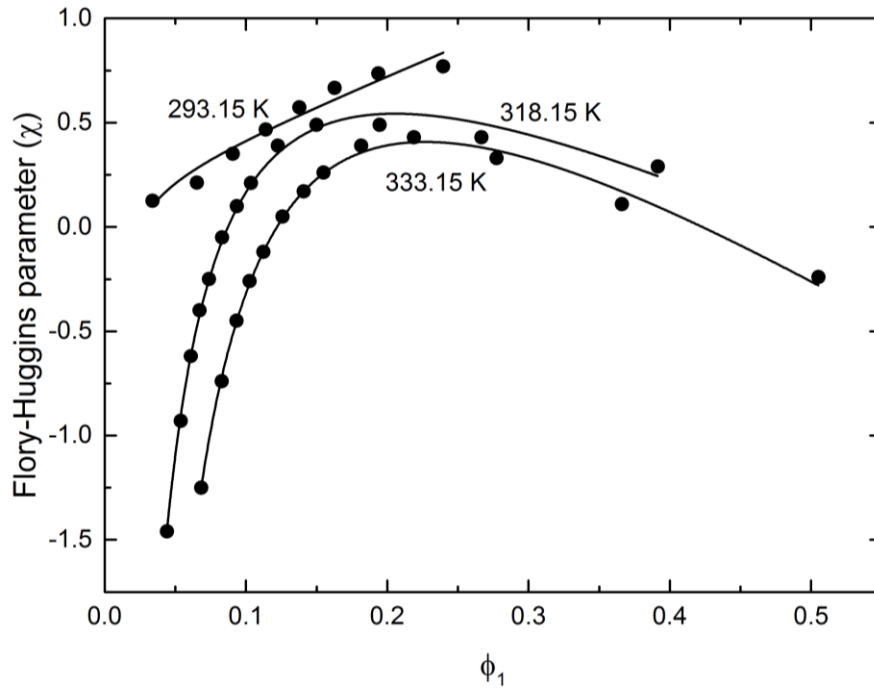


Figure 23 Flory–Huggins interaction parameters for the system water/BSP at different temperatures. Fit by Equation 2.12 with parameters: for $T = 293.15$ K parameters are $a = 0.2020$, $b = 2.757$, $c = 0.0066$; for $T = 318.15$ K parameters are $a = 1.935$, $b = -3.387$, $c = -0.1431$; for $T = 333.15$ K parameters are $a = 2.4481$, $b = -4.493$, $c = -0.2317$.

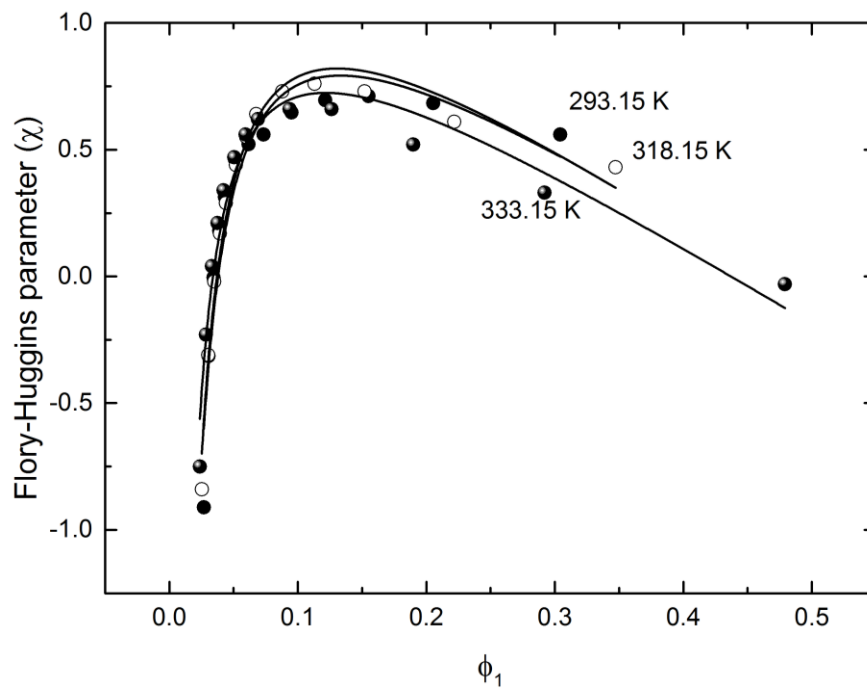


Figure 24 Flory–Huggins interaction parameters for the system water/PPL at different temperatures. Fit by Equation 2.12 with parameters: for $T = 293.15$ K parameters are $a = 1.676$, $b = -3.318$, $c = -0.0588$; for $T = 318.15$ K parameters are $a = 1.728$, $b = -3.476$, $c = -0.0592$; for $T = 333.15$ K parameters are $a = 1.499$, $b = -3.183$, $c = -0.0472$.

2.3.7 Water clustering analysis: Zimm–Lundberg theory

It would be highly desirable now to understand the states of water within the water/protein mixture. The Zimm and Lundberg cluster integral [61], which assumes negligible compressibility of the system, provides a theory to evaluate the extent of clustering of the solvent molecules inside the polymer matrix theoretically. These clusters are imagined as chains of water molecules that extend from the H-bonding sites within the polymer.

In their initial study, Zimm and Lundberg applied the theory to water/collagen, toluene/polystyrene, and benzene/rubber. The cluster integral can be directly obtained from quasi-equilibrium sorption data using the relation:

$$\frac{G_{11}}{v_1} = -(1 - \phi_1) \left[\frac{\partial(a_w/\phi_1)}{\partial a_w} \right]_{P,T} - 1 \quad (2.14)$$

where G_{11} is the cluster integral, v_1 is the partial molar volume, ϕ_1 the volume fraction for water molecules. The quantity G_{11}/v_1 gauges the tendency of sorbed molecules to cluster. If $G_{11}/v_1 = -1$, the solution is ideal: water molecules exclude their own volumes to the other water molecules but have no other effect on their distribution. $G_{11}/v_1 = 0$ means that clustering is just sufficient to overcome the excluding effect of the central water molecule. If $G_{11}/v_1 < -1$, the water molecules prefer to remain isolated.

Figure 25 shows the results of the Zimm-Lundberg analysis for water/BSA during both interval and integral sorption at 293.15 K. For the interval sorption experiment, values for G_{11}/v_1 are initially negative. As more water is sorbed, the clustering function rises, finally reaching a value around one which is typical for a polymer. [61] This result suggests that there is no appreciable water clustering in BSA during this experiment. During integral sorption, the trend follows that for the interval sorption experiments for water concentrations greater than 0.05 g/g.

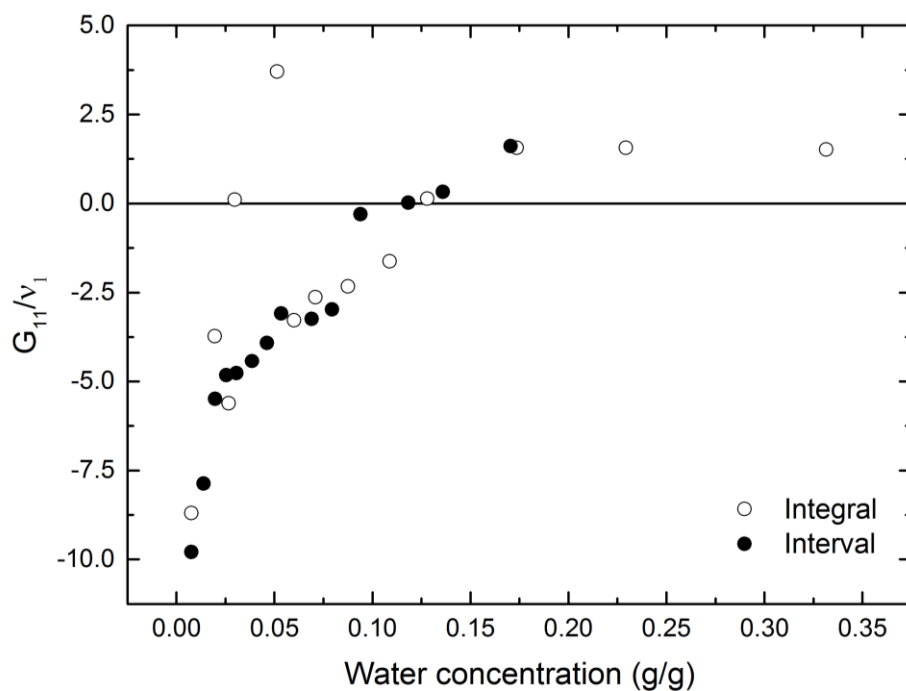


Figure 25 Zimm-Lundberg water clustering analysis for sorption in BSA during interval and integral sorption at 293.15 K.

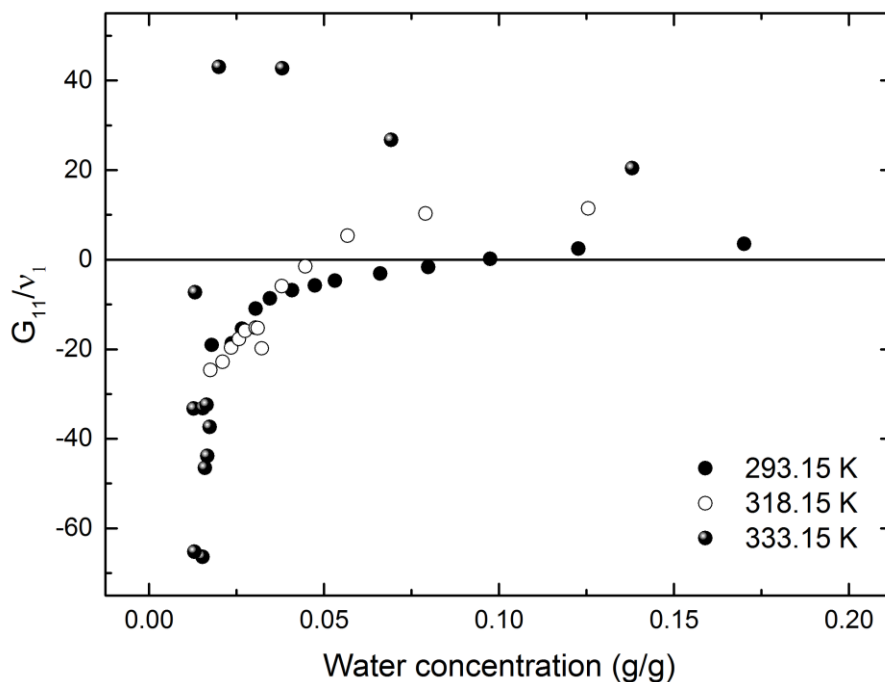


Figure 26 Zimm-Lundberg water clustering analysis for interval sorption in BLA at different temperatures.

Below this, there is a sharp initial rise from negative values at very low hydration up to a positive value at about 0.04 g/g . This behaviour arises from the discontinuity of the integral sorption isotherm around those water concentrations (cf. Figure 9). So indeed this result suggests that great care has to be taken when using this theoretical framework to make judgements on water clustering.

Further problems arise when analysing the water sorption isotherms at different temperatures of the α -amylase. Figure 26 shows the corresponding clustering analysis. Whilst the G_{11}/v_1 analysis of the water sorption isotherm at 293.15 K might appear to be insightful to the reader, inconsistencies arise at higher temperatures where no clear trend can be seen. Indeed, this result was expected due to the complex shape of the water sorption isotherms of the α -amylase (cf. Figure 13).

Only the systematic analysis of water sorption behaviour in proteins suggests that the Zimm–Lundberg analysis might be unsuitable to treat systems as complex as proteins. Davis and Elabd [62] stressed that great caution must be taken when using the Zimm–Lundberg analysis as the sole analysis to describe the extent of clustering in glassy polymers. They complement their theoretical analysis with spectroscopic measurements of water bands. Furthermore, the Zimm–Lundberg model was derived under the assumption of thermodynamic equilibrium. It is obvious that an equilibrium framework may not describe these studied systems adequately. Therefore, the Zimm–Lundberg cluster analysis does not provide a suitable theory to explain the pronounced difference in sorption capacity between different experimental modes and, indeed, the temperature dependence of the sorption isotherms.

2.4 CONCLUSION

An extensive set of experimental water sorption in four amorphous model proteins was collected using a gravimetric vapour diffusion technique. Sorption mass uptake and observed anomalies suggest a mechanistic picture beyond that of simple surface adsorption. The experiments were carried out in integral and interval mode, under sorption, desorption, and cyclic sorption–desorption modes.

During cyclic sorption–desorption, pronounced hysteresis was found. Furthermore, the mass uptake during integral sorption is significantly higher than during interval sorption. During the non-cyclic interval desorption experiment, where the sample was fully hydrated at $0.90a_w$ before the water activity was decreased in intervals, the water concentration was even higher. All this data suggests a strong history dependence of the mass uptake/loss of the water (de-)sorption experiment and points towards nonequilibrium effects in the glassy protein upon water sorption.

Following this notion, there was no general temperature dependence of the experimental isotherms within the studied temperature and activity ranges. This hugely complicates any isotherm modelling analysis. The suitability of a surface model (D’Arcy–Watt), a surface–solution model (Dual mode sorption), and a solution theory (Flory–Huggins) in representing experimental isotherms were tested. The D’Arcy–Watt theory represented experimental data well but did not provide a molecular insight into the sorption process. The dual mode sorption theory did not represent experimental data for activities higher than $0.60a_w$. The Flory–Huggins theory with a concentration- and temperature-dependent interaction parameter allowed for good representation of the experimental isotherms, and is unique in the sense that models of this class allow insight into the entropy and enthalpy landscape of the binary systems. It is yet to be shown whether the implicated $\Delta H - \Delta S$ plane of the Flory–Huggins theory can be experimentally accessed and be thought of as a good representation of experimentally determined enthalpies and entropies of

mixing. The Zimm–Lundberg cluster integral was used to evaluate the states of sorbed water though uncertainty over the overall applicability of the Flory–Huggins theory, under which the cluster integral was derived, prohibited any reliable mechanistic insight.

2.5 REFERENCES

- [1] Frisch, H.L. *Polym. Eng. Sci.* **1980**, 20(1), 2-13
- [2] Rupley, J.A.; Yang, PH; Tollin, G, **1980** In: Water in Polymers, ed. SP Rowland, pp. 110-20. Washington, DC: Am. Chem. Soc.
- [3] Kuntz, I.D.; Kauzmann, W, *Adv. Protein Chem.* **1971**, 93, 514-516
- [4] Rupley, J.A.; Careri, G, *Adv. Protein Chem.* **1991**, 41, 38-172
- [5] Hiemenz P.C. **1986** Principles of Colloid and Surface Chemistry. Marcel Dekker, New York
- [6] McLaren, A.D.; Rowen, J.W. *J. Polym. Sci.* **1951** 7, 289-324
- [7] Pauling, L. *J. Am. Chem. Soc.* **1945**, 67(4), 555-557
- [8] Brunauer, S.; Emmett, P.H.; Teller, E. *J. Am. Chem. Soc.* **1938**, 60, 309-319
- [9] Zografi, G. *Drug. Dev. Indust. Pharm.* **1988**, 14, 1905-1926
- [10] Leeder. J.D.; Watt I.C. *J. Colloid Interface Sci.* **1974**, 48, 339-344
- [11] Watt, I.C.; D'Arcy, R.L. *J Polym. Sci. Symposium* **1976**, 55, 144-166
- [12] Hageman MJ in Ahern TJ and Manning MC (Ed.). **1992**. Part A: Chemical and Physical Pathways of Protein Degradation. Plenum Press, New York, pp. 273-309
- [13] Franks, F. *Cry. Lett.* **1990**, 11, 93-110
- [14] Towns, J.K. *J. Chromatogr. A* **1995**, 705, 115-127
- [15] Schult, K.A.; Paul, D.R. *J Appl. Polym. Sci.* **1996**, 61(11), 1865-76

- [16] Bull, H.B. *J. Am Chem. Soc.* **1944**, 66, 1499-1507
- [17] Kuntz, I.D., Kauzmann, W. *Adv. Protein Chem.* **1974**, 28, 239-245
- [18] Flory PJ. **1953**. Principles of Polymer Chemistry. Cornell University Press, New York
- [19] Apicella, A.; Hopfenberg, H.B. *J. Appl. Polym. Sci.* **1980**, 25, 2179-86
- [20] Watt, I.C. *J. Macromol. Sci.-Chem.* **1980**, A14, 245-255
- [21] Vrentas, J.S.; Vrentas, C.M. *Macromolecules* **1991**, 24, 2404-2412
- [22] Fleming, G. K.; Koros, W. J. *Macromolecules* **1986**, 19, 2285-2291
- [23] Toi, K.; Paul, D. R. *Macromolecules* **1982**, 15, 1104-1107
- [24] Toi, K.; Paul, D. R. *Macromolecules* **1982**, 15, 1104-1107
- [25] Lüscher-Mattli, M.; Ruegg, M. *Biopolymers* **1982**, 21, 403-418
- [26] Knittel, D.R.; Pack, S.P.; Lin, S.H.; Eyring, L. *J. Chem. Phys.* **1977**, 67 (1), 134-142
- [27] Bryan, W.P. *J. Theor. Bio.* **1980**, 87, 639-661
- [28] Hill, T.L. *J. Chem. Phys.* **1947**, 15, 767-777
- [29] McLaren, A.D.; Kirwan, J.P. *J. Gen. Virol. Pt. I* **1976**, 33, 155-157
- [30] Rupley, J.A.; Gratten, E.; Careri, G. *Trends Biochem. Sci.* **1983**, 8, 18-22
- [31] Bryan, W.P. *Biopolymers* **1987**, 26, 1705-1716
- [32] Cerofolini, G.F.; Cerofolini, M. *J. Colloid Interface Sci.* **1980**, 78, 65-73

- [33] Luthra, S.; Lechuga-Ballesteros, D.; Kalonia, D.S.; Pikal, M.J. *J. Pharm. Sci.* **2007**, 96(8), 1974-82
- [34] Langmuir, I. *J. Am. Chem. Soc.* **1918**, 40, 1361-1403
- [35] Freundlich, H. *Colloid and Capillary Chemistry*, London, Methuen, **1926**
- [36] Frumkin, A.; Slygin, A. *Acta Physicochim.* **1935**, 3, 791-801
- [37] Brunauer, S.; Love, K.D, Keenan, R.G. *J. Am. Chem. Soc.* **1942**, 64, 751-758
- [38] Cassie, A.B. *Trans. Faraday Soc.* **1945**, 41, 458-464
- [39] Cassie, A.B. Symp. Fibrous Proteins (Society of Dyers & Colourists) London, **1946**, p. 87.
- [40] Hailwood, A.J. Horrobin, S. *Trans. Faraday Soc.* **1946**, 42B, 84-102
- [41] De Boer, J.H.; Zwicker, C. *Z. Phys. Chem. B*, **1929**, 3, 407
- [42] Bradley, R.S.; *J. Chem. Soc.* **1936**, 1467-1474
- [43] Hayward, D.O.; Trapnell, B.M.W. *Chemisorption*, London, Butterworths, **1964**
- [44] Barrie, J.A. *Diffusion in Polymers*, Crank, J.; Park, G.S., eds, Academic Press London, **1968**, Ch. 8
- [45] Flory, P.J. *J. Chem. Phys.* **1942**, 10, 51-61
- [46] Huggins, M.L. *Ann. N.Y. Acad. Sci.* **1943**, 44, 431-443
- [47] Rowen, J.W.; Simha, R. *J. Phys. Chem.* **1949**, 53, 921-930
- [48] Watt, I.C.; Leeder, J.D. *J. Text. Instr.* **1968**, 59, 353-364

- [49] Watt, I.C.; Leeder, J.D. *J. Text. Instr.* **1968**, 59, 353-364
- [50] Meares, P. *J. Am. Chem. Soc.* **1954**, 76, 3415-3422
- [51] Michaels, A.S.; Vieth, W.R.; Barrie, J.A. *J. Appl. Phys.* **1963**, 34, 1-12
- [52] Michaels, A.S.; Vieth, W.R.; Barrie, J.A. *J. Appl. Phys.* **1963**, 34, 13-20
- [53] Vieth, W.R.; Howell, J.M.; Hsieh, J.H. *J. Membr. Sci.* **1976**, 1, 177-230
- [54] Chiou, J.S.; Maeda, Y.; Paul, D.R. *J. Appl. Polym. Sci.* **1985**, 30, 4019-4029
- [55] Staverman, A.J.; Van Santen, J.H. *Recl. Trav. Chim.* **1941**, 60, 76-84
- [56] Staverman, A.J. *Recl. Trav. Chim.* **1941**, 60, 640-649
- [57] Huggins, M.L. *J. Chem. Phys.* **1941**, 9, 440-449
- [58] Flory, P.J. *J. Chem. Phys.* **1941**, 9, 660-661
- [59] Meyer, K.H. *Z. Physik. Chem. (Leipzig)* **1939**, B44, 383
- [60] Tompa, H. *Polymer Solutions*, Butterworth Scientific, **1956**
- [61] Zimm, B.H.; Lundberg, J.L. *J. Phys. Chem.* **1956**, 60(4), 425-428
- [62] Davis, E.M.; Elabd, Y.A. *J. Phys. Chem. B* **2013**, 117, 10629-10640
- [63] Antonov, Y.; Eckelt, J.; Sugaya, R.; Wolf, B.A. *J. Phys. Chem. B* **2013**, 117, 5497-5502

Chapter 3. Non-equilibrium sorption of water in glassy bovine serum albumin

3.1 INTRODUCTION

Water sorption and diffusion in glassy biopolymers is important in many fields, including foods, pharmaceuticals and personal health care. The accurate measurement and appropriate interpretation of the underlying mass transfer mechanisms of water sorption in glassy biopolymers are further complicated by the nonequilibrium nature of the glassy biopolymer and the complex behaviour of water (e.g. hydrogen bonding). Studies on synthetic polymers and simple chain-like bio-polymers [1-4] point towards the great importance of this topic. However, there has been no detailed study of the water sorption kinetics in glassy protein powders. Experimental investigations are further complicated here by the inability to cast thin films or produce spherical particles of homogenous size and shape.

Polymers above their glass transition temperature are known to respond quickly to a change in their environmental conditions [5]. For instance, changing the temperature would almost immediately lead to the attainment of a new equilibrium volume. A rubbery polymer film that is exposed to a constant solute activity of an appreciably smaller solute molecule capable of diffusing in the film (i.e. the penetrant) achieves a constant penetrant concentration rapidly at the film interface. The time scales of this sorption equilibrium are much shorter than the characteristic times of penetrant diffusion. Moreover, there appears to be a unique mode of sorptive dispersal of the penetrant.

In a glassy polymer, significant contributions to the overall mass transfer processes are expected to arise from long relaxation time-events. During penetrant sorption, the molecular motions of portions or whole polymer chains are not sufficiently rapid to completely homogenise the

penetrant's concentration field. [6] Therefore, local concentration heterogeneities might form. Penetrant molecules can be expected to reside in holes or irregularly shaped voids with very different intrinsic diffusional mobility compared to the situation in the corresponding equilibrium, rubbery state.

The mathematical description [5] of the transport process of penetrant sorption in rubbery films, that is systems without free volume or holes, is typically assumed to satisfy the following assumptions: (1) mass transfer occurs by molecular diffusion according to Fick's first and second laws; (2) the boundary conditions that are usually applied at the film surfaces $x = 0, l$ are constant values of the penetrant concentration c by means of the rapid establishment of the sorption equilibrium. In a homogenous film of sufficient area, Fick's laws yield the diffusion equation for one dimensional isothermal, isopiestic diffusion:

$$\frac{\partial c}{\partial t} = \frac{\partial}{\partial x} \left\{ D(c) \frac{\partial c}{\partial x} \right\} \quad 0 < x < l \quad (3.1)$$

The binary diffusion coefficient $D(c)$ can be thought of as the product of a thermodynamic factor, that is the partial derivative of the chemical potential difference of the penetrant with respect to the logarithm of its concentration, and a molecular mobility factor. Both of these factors are functions of the local thermodynamic variables. That is, c , the absolute temperature T and the total impressed hydrostatic pressure difference Δp ; but *not* time t .

The boundary conditions are usually given as:

$$c(0, t) = c_0 \quad (3.2)$$

and

$$c(l, t) = c_1 \quad (3.3)$$

where c_0 and c_1 are constants. Further, the boundary problem requires an initial condition:

$$c(x, 0) = c_i \quad (3.4)$$

with c_i typically chosen based on experimental data. Depending on the system, c_i could be a constant but also a function of x . Further, $D(c)$ has to be continuously differentiable with respect to x at least once satisfying the inequality:

$$0 < D(c) \leq D_0 < \infty \quad (3.5)$$

in $0 \leq x \leq l$ in order for the boundary conditions to be well posed. In Equation 3.1, the lower bound prevents diffusion against the concentration gradient (i.e. ‘up-hill’ diffusion) and the upper bound prevents states of infinite dissipation.

The specification of the boundary value problem as per Equations 3.2 to 3.4 together with Equation 3.5 specify what is known as *Fickian diffusion*. Crank and Fujita [5] have extensively reported on the experimental characteristics of Fickian sorption and permeation curves. The great

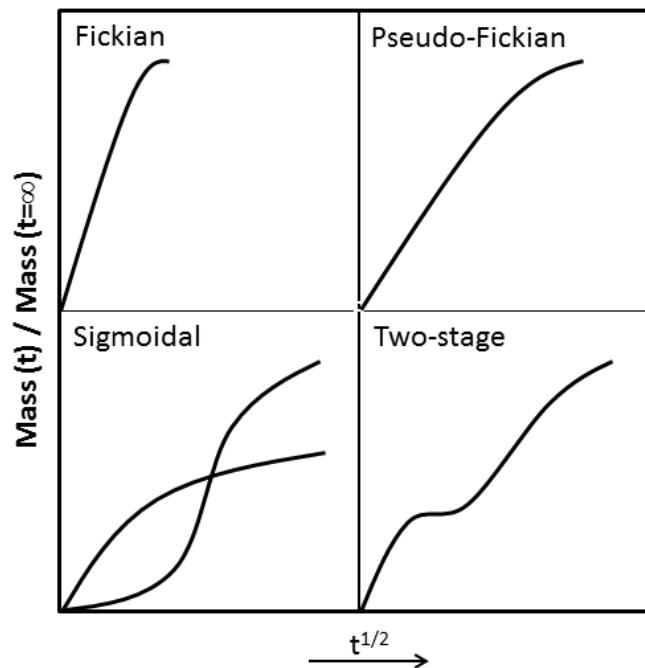


Figure 27 Schematic of non-Fickian or anomalous sorption curves compared with Fickian-type curves. Adapted from Rodgers [11].

importance of Fickian diffusion is that it *is a reference state* which is experimentally recognised and corresponds to the typical response in penetrant transport of rubbery polymers.

Departures from Fickian diffusion can occur for many reasons. For instance, a failure to establish sorption equilibrium at a film surface (even in a rubbery film), say because of an appreciable evaporation rate, would result in sorption curves exhibiting an inflection point making them sigmoidally non-Fickian. Further instances of this kind of behaviour have been collectively termed as ‘non-Fickian’ or ‘anomalous’ diffusion by King [7], Crank and Park [8] and Long and co-workers [9, 10]. It is often found that the features of non-Fickian diffusion are observed in glassy polymers and vanish at (or in few instances somewhere above) the glass transition temperature of the system.

A commonly used classification scheme for non-Fickian diffusion and sorption in glassy polymers was originally proposed by Alfrey, Gurnee and Lloyd [12], also used by Crank [13] and extended by Frisch [14]. Three basic classes of behaviours are distinguished: (1) Case I or Fickian in which the rate of diffusion is significantly less than that of relaxation due to structural, mechanical etc. modes of the polymer–penetrant system. Sorption equilibrium is established instantaneously which in turn results in time independent boundary conditions. Therefore, there is no dependence on swelling kinetics. (2) Case II or Super Case II transport is characterised by rapid diffusion compared with the relaxation processes. However, the sorption process is affected by a pronounced dependency on the swelling kinetics. (3) Anomalous or non-Fickian diffusion occurs when the rates of the diffusion and relaxation processes are at comparable time scales.

Further to this analysis, it is known that that the sorption curves of simple gas and vapour penetrants for glassy polymers contain a contribution from a Henry’s Law solubility-type term and a Langmuir adsorption contribution. This phenomenon has been described as dual mode sorption. The Langmuir contribution is interpreted as the manifestation of partially immobilised penetrant

molecules in fixed holes or irregular cavities in the polymer matrix. It has been argued that Case II/Super Case II, anomalous diffusion and dual mode sorption features are interrelated and a direct consequence of the glass transition of the mixture.

The different classes of sorption curves can be distinguished by the shape of the initial portion of the sorption curves, M_t/M_∞ , as a function of time:

$$M_t/M_\infty = Kt^n \quad (3.6)$$

with the constants K and n . Here, n shows how the mass transfer scales with time. For $n = \frac{1}{2}$, diffusion is Fickian (Case I), while for Case II, $n = 1$, and for Super Case II, $n > 1$. The diffusion for Case I is controlled by the diffusion parameter. In Case II, the diffusion process is controlled by the velocity of an advancing diffusion front. This front marks the boundary between the swollen polymer gel and the glassy core. It signifies the innermost limit of dispersion or penetration. For non-Fickian or anomalous diffusion, $0.5 < n < 1$ which occurs when diffusion and relaxation rates are comparable. The special diffusion cases of Super Case II ($n > 1$) and Pseudo-Fickian ($n < 0.5$) diffusion were created much later.

Above the glass transition temperature, T_g , diffusion of a penetrant into a polymer is, under appropriate experimental conditions, found to be Case I. This behaviour is not the case for polymer–penetrant systems below their glass transition temperature. In studies with penetrant vapours at near atmospheric partial pressures or less, it is commonly argued that the behaviour can be divided into two categories depending on the experimental temperature, T , relative to the critical temperature of the penetrant vapour, T_c . Here, T_c is not to be confused and denotes the temperature at the critical point. For $T > T_c$ and $T > T_g$, not only is Henry's Law valid due to the sufficiently small solubility of the penetrant but also the concentration dependence of the diffusional mobility is negligible effectively giving rise to the approximation of a constant

diffusion coefficient. For $T_c \geq T$ and $T > T_g$, neither a concentration independent diffusion coefficient (due to sufficiently large sorption) nor its independence of pressure can be assumed. (Note, this is the situation for the experiments to be presented in this Chapter.) While water and some other strongly hydrogen bonding vapour penetrants may exhibit a special behaviour due to the complex nature of their interactions with the polymer, most penetrants (i.e. organic molecules) plasticise the polymer and, therefore, act in an anti-cluster manner. The equilibrium sorption results in a material that can be treated as a homogenous molecular solution and approximated by theories such as the Flory–Huggins solution theory.

At or below the T_g , diffusion and sorption of vapour penetrants are greatly complicated. The following common sorption anomalies have been reviewed [14] : (1) pseudo-Fickian behaviour in which the sorption curve when plotted against the square root of time shows an unusually small initial linear region or resembles Fickian curves but departs from film thickness scaling. (2) Sigmoid shaped sorption curves when M_t/M_∞ is plotted against the square root of time. Commonly, an inflection point is observed at $M_t/M_\infty \approx 0.5$. Here, rates of desorption initially exceed rates of sorption, but then become slower and the curves cross. (3) Two-stage sorption: after an initial rapid uptake, the sorption curve as a function of square root of time approaches a quasi-equilibrium followed by a slow approach to a true equilibrium. The true equilibrium might lie above the quasi-equilibrium uptake or below it for systems that feature a sorption overshoot. (4) Time-dependent surface concentrations that give rise to an initial time lag.

In this Chapter, the sorption and diffusion of water vapour in amorphous bovine serum albumin was investigated with a gravimetric sorption technique run in three different modes (interval, integral, ‘forced’ interval sorption) at different experimental temperatures (283.15, 293.15, 303.15, 313.15, 323.15 K; all below the glass transition temperature of ‘dry’ BSA). Non-Fickian behaviour was observed under all three experimental modes and over the entire temperature and

activity range studied. Two distinct phenomena were observed to occur over distinct time periods (two-stage sorption behaviour) within the same experimental time scale: (1) diffusion driven by the water concentration gradient, and (2) diffusion driven by a slower (in relation to the first process) polymer matrix relaxation. The kinetic sorption curves for the interval sorption experiment are regressed by a diffusion–relaxation model to determine the Deborah (De) number.

3.2. EXPERIMENTAL SECTION

3.2.1 Materials

Bovine serum albumin (BSA) (lyophilised powder, catalogue number 05470) was purchased at the highest commercially available grade from Sigma Aldrich (Gillingham, United Kingdom). Ultrapure deionized water (resistivity $\sim 16\text{ M}\Omega \cdot \text{cm}$) was used for all sorption experiments.

3.2.2 Methods

See Section 2.2.2 for a detailed methodology.

As experimental errors were found to be significantly smaller than the symbol size used here; the experimental kinetic data shown in this Chapter is not average across multiple experiments at the same conditions. The diagrams depict what is defined as a typical set of data for the given experiment unless otherwise stated.

3.3. RESULTS AND DISCUSSION

3.3.1 Interval sorption curves and diffusion behaviour

Figures 28 to 31 show the sorption curves for selected water activity steps between 283.15 and 323.15 K. (For a more complete data set see Appendix section 3.6.1.) The total mass absorbed of water per dry mass of bovine serum albumin is plotted against the square root of time. There has been no previous report of this type of data for BSA or indeed other model proteins using a gravimetric technique with the accuracy and wide breadth of experimental conditions presented here. Plotting against the square root of time enhances the visibility of the data for small t and the trend for large t while it ‘lumps’ the data at intermediate t .

An initial time lag ($< 5 \text{ min}$) in the kinetic data was observed in all experiments. It is most likely an experimental artefact: it is not possible to raise solvent activity and equilibrate surface concentrations immediately. As a consequence, a sigmoidal shape of the sorption curve is expected at the very early stage of the sorption time-curve. [13]

Throughout the entire activity range studied here, the kinetic data for the experiments conducted between 303.15 and 323.15 K group together and follow a similar trend. Similarly, the data at 283.15 and 293.15 K group together but follow a different trend with significantly slower sorption kinetics. Overall, it is important to point out that the temperature trend of the kinetic sorption curves is directly manifested in the (quasi-)equilibrium sorption isotherms (cf. Figure 11).

True sorption equilibrium is not achieved during the 800 *min* activity intervals; be it manifested in the shape of a slow, continuous and gradual mass uptake sorption curve or a sorption overshoot for long experimental times. The sorption curves for temperatures between 303.15 and 323.15 K change from the slow continuous, gradual uptake at long experimental times to the overshooting

curves at the activity step change $0.30 \rightarrow 0.40 a_w$. All this points towards the non-equilibrium nature of the water transport in a glassy protein.

As a polymer glass undergoes the transition to its super-cooled liquid, there is a decrease in molecular enthalpy and volume that is accompanied by a molecular rearrangement and an increase in internal hydrogen bonding within the amorphous polymer matrix itself. Therefore, there will be less sites for water to sorb. (An alternative statement could be made via the chemical potential difference avoiding the idea of ‘sites’.) This phenomenon can be seen as a sorption overshoot under certain experimental conditions, where diffusion and relaxation rates operate on comparable time scales. Below 303.15 K, the slow continuous, gradual uptake behaviour mode is dominating within the studied activity range. Interestingly, both phenomena are manifestations of relaxation-controlled diffusion.

Other investigators have observed similar behaviour for the sorption of small solvent molecules in glassy polymers [14] but never for amorphous proteins. Generally, the behaviour is attributed to the non-equilibrium state of the glassy polymer. Here, diffusion is initially driven by the concentration gradient of the penetrating solvent. At longer times, this mass transfer process is controlled by the relaxation or swelling of the polymer that results from the stress imposed by the penetrant. [14, 15] If the experiment was carried out above the T_g of the polymer, the polymer would be in an equilibrium state instantaneously (i.e. elastically) responding to the stress imposed by the penetrating water. Therefore, these rapid relaxation phenomena can commonly not be observed at typical experimental time scales (due to the time resolution of gravimetric techniques).

Generally, the reason for non-Fickian diffusion behaviour is assumed to be twofold [16]: (1) The characteristic relaxation time of the viscoelastic swelling or deswelling of a polymer is close to the characteristic diffusion time. Therefore, the kinetics of swelling directly influence the kinetics of the mass transfer process (e.g. by changing the diffusion coefficient or solubility with

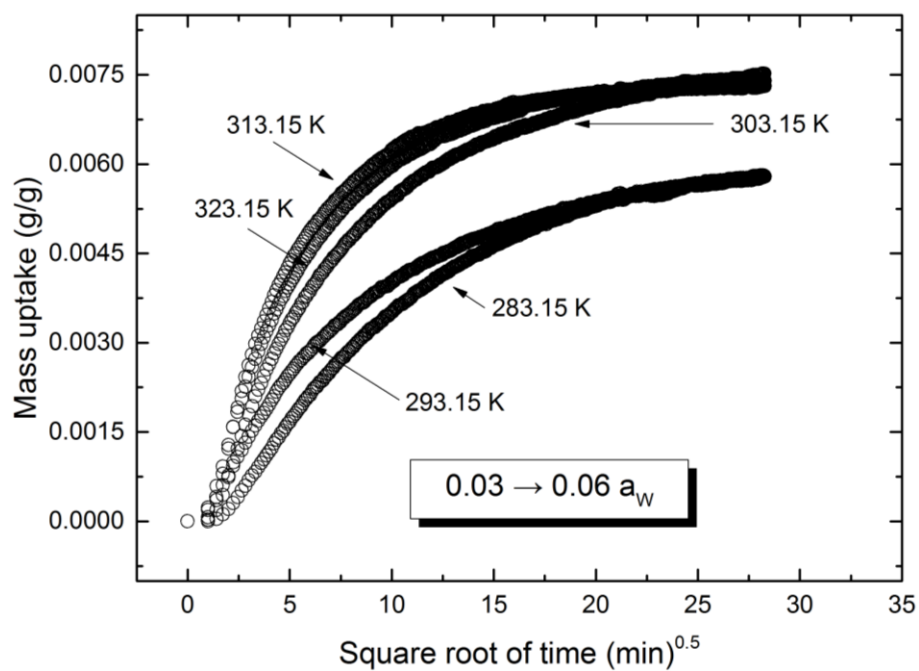


Figure 28 Experimental interval sorption curves for water/BSA for an activity change from 0.03 to 0.06 a_w as a function of temperature.

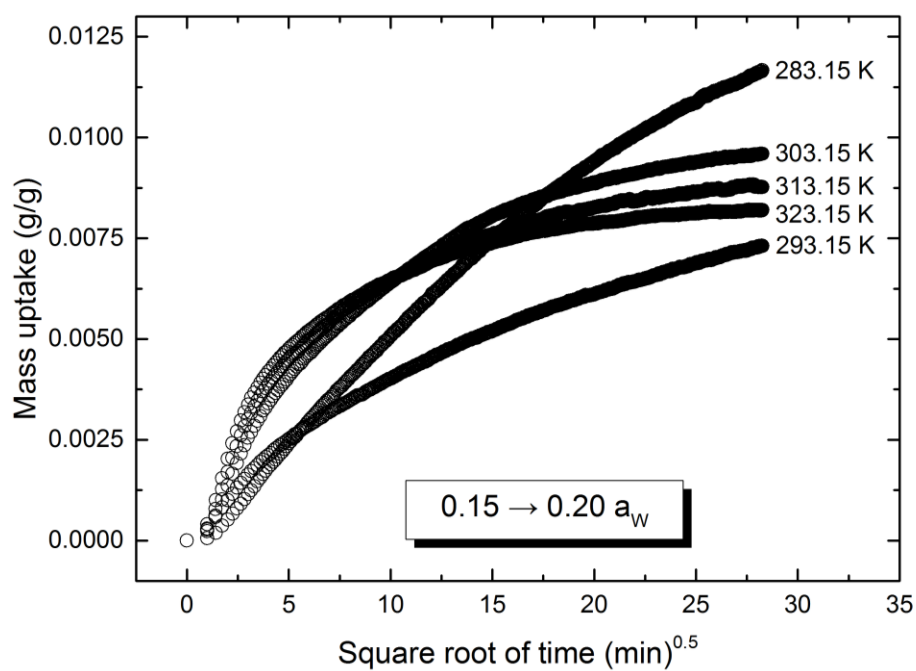


Figure 29 Experimental interval sorption curves for water/BSA for an activity change from 0.20 to 0.25 a_w as a function of temperature.

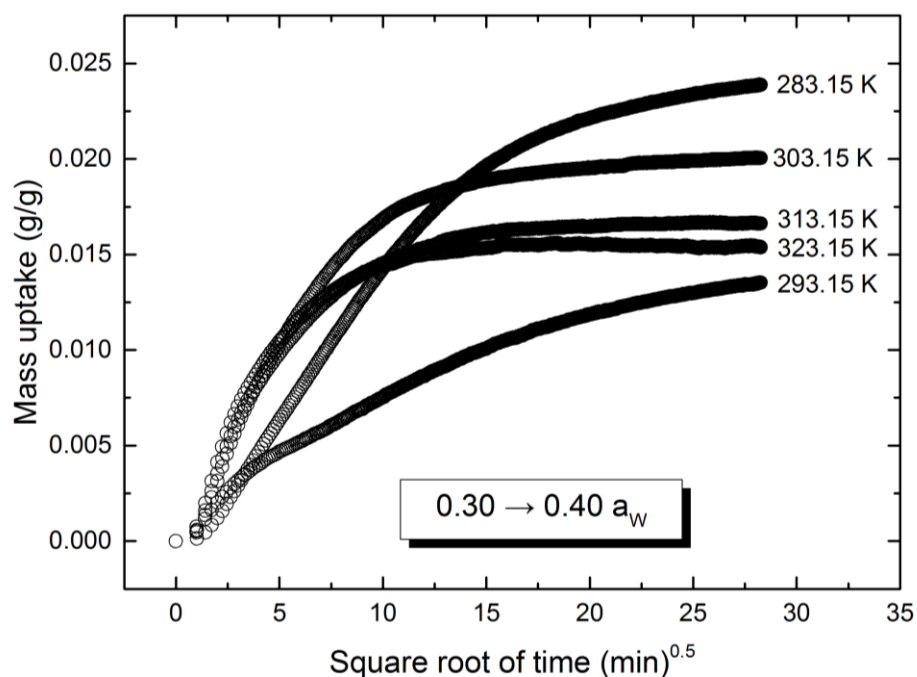


Figure 30 Experimental interval sorption curves for water/BSA for an activity change from 0.30 to 0.40 a_w as a function of temperature.

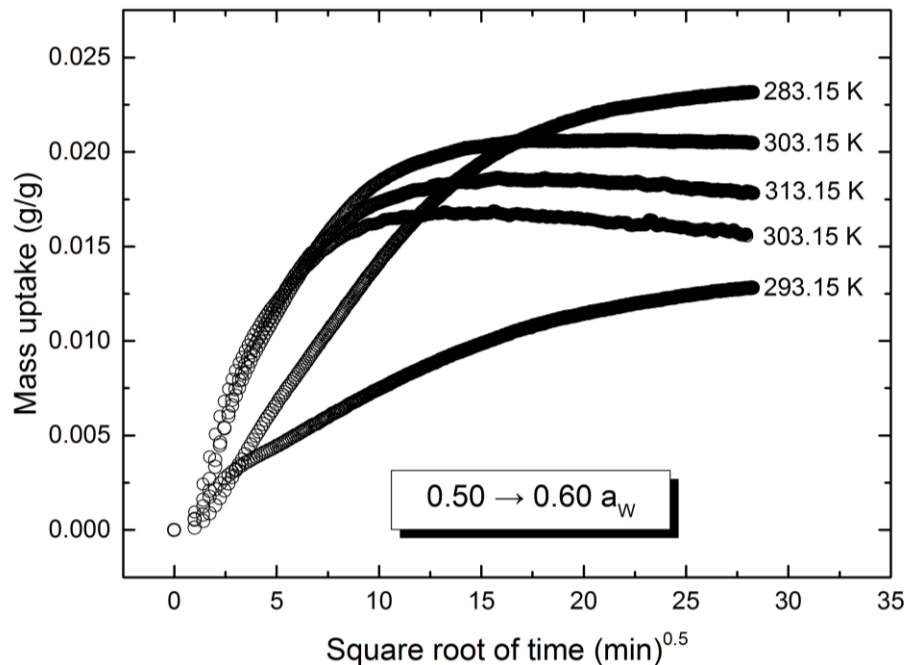


Figure 31 Experimental interval sorption curves for water/BSA for an activity change from 0.50 to 0.60 a_w as a function of temperature.

time). (2) The concentration profiles lead to different swelling potentials throughout the polymer matrix. To compensate for this, e.g. surface chains could be compressed and inner elements extended. Crank described these processes ‘volume viscous relaxation’ and ‘differential swelling stress’ models, respectively.

To judge whether an individual interval sorption experiment can be described with a single and constant relaxation time, the analysis of Petropoulos and Roussis [17] is applied. For their analysis to hold good, the concentration interval has to be sufficiently small. Plotting the sorption curves as $\ln(1 - M_t/M_\infty)$ against time t should give a straight line for systems that can be described by a single and constant relaxation time. Any deviations from this linearity are attributable to a strong concentration dependence of the corresponding relaxation time(s). For systems that feature a sorption overshoot, a modified expression is used, $\ln(1 - M_t/M_{max})$ is plotted against time t .

Special sorption curves for the activity changes $0.03 \rightarrow 0.06a_w$, $0.12 \rightarrow 0.15a_w$, $0.25 \rightarrow 0.30a_w$, and $0.50 \rightarrow 0.60a_w$ are plotted in Figures 32 and 33 at different temperatures. (For a more complete picture, see additional data in Appendix 3.6.2) As this analysis is only fundamentally sound for long-term diffusion ($M_t/M_\infty > 0.5$), the initial curvature of all sorption curves for short times can be neglected. Towards longer times, the sorption curves show a strong curvature; whilst at intermediate times, sorption curves feature a quasi-linear regime.

The systems featuring overshoot behaviour exhibit even more complex kinetics. There is a distinct local minimum after which values of $\ln(1 - M_t/M_{max})$ decrease again. The minimum signifies the onset of the overshoot in the sorption curve. The time-to-onset at a specific interval step decreases with increasing temperature. For instance, for the interval step from $0.50 \rightarrow 0.60a_w$ the onset time decrease from about *520 min at 303.15 K*, to *330 min at 313.15 K*, down to *250 min at 323.15 K*. Neither the experiments with the smallest concentration interval nor the one with the largest show a significantly different shape of the curve and, therefore, it was

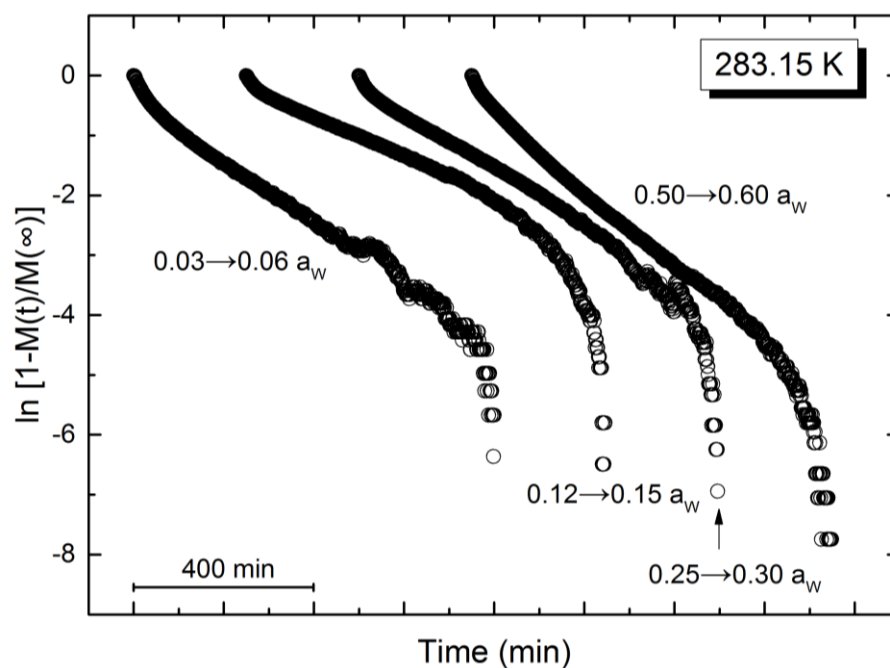


Figure 32 First-order kinetic analyses of the interval sorption curves for water/BSA at 283.15 K for selected activity intervals.

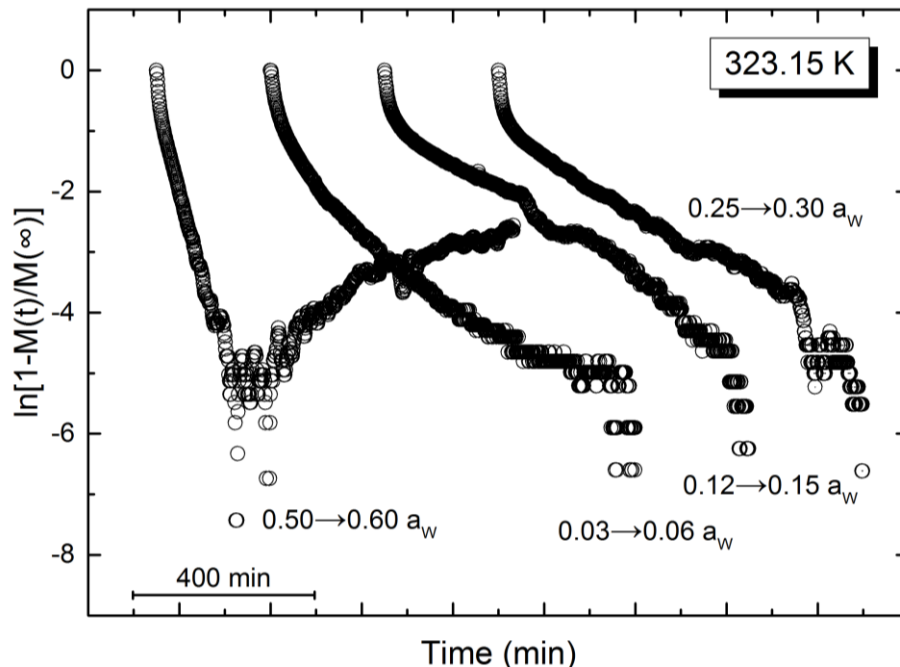


Figure 33 First-order kinetic analyses of the sorption curves for water/BSA at 323.15 K for selected activity intervals.

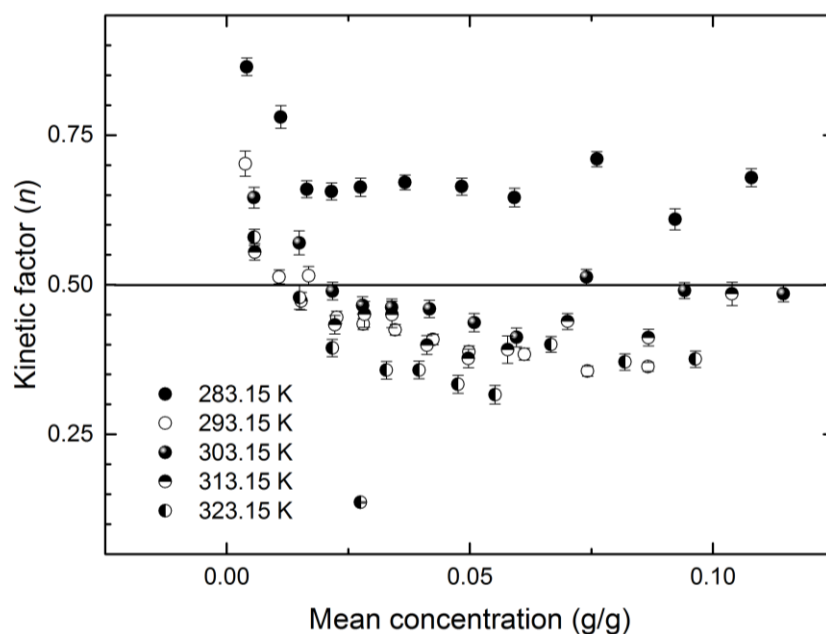


Figure 34 The kinetic factor n as regressed from Equation 3.6 for the system water/BSA between 283.15 and 323.15 K during interval sorption. Error bars denote confidence interval.

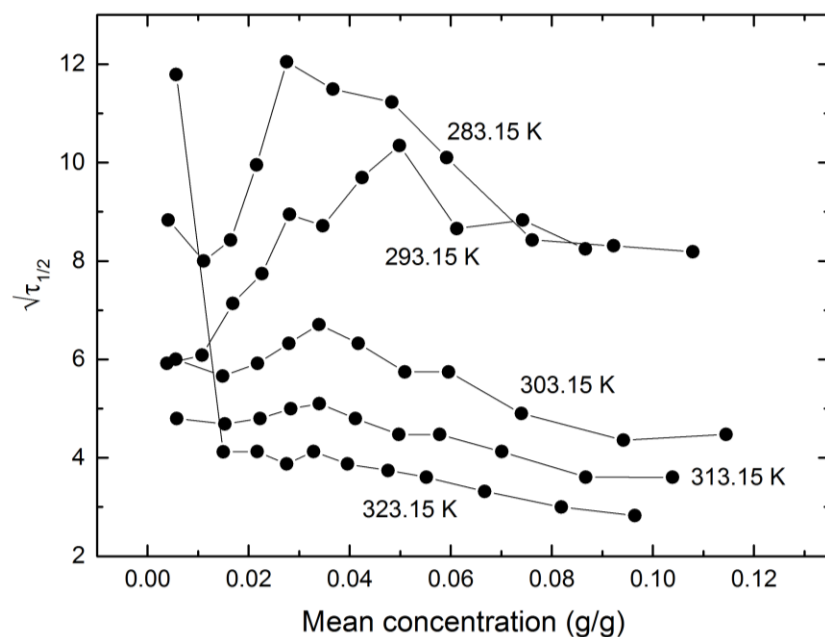


Figure 35 Interval sorption half times for the system water/BSA between 283.15 and 323.15 K.

assumed that the concentration intervals were sufficiently small for this analysis to hold good. Overall, the analysis suggests that there are at least two concentration independent relaxation constants required to describe the sorption process of water into BSA over the studied temperature and activity range, or alternatively one constant that is concentration-dependent.

The most commonly used method to characterise the nature of the transport process is to fit the initial portion ($M_t/M_\infty < 0.5$) of the sorption curve with Equation 3.6. Unfortunately, the lyophilised BSA powder is heterogeneous in particle size and shape; and many other factors other than glassy relaxations may produce anomalous behaviour (e.g. non-planar geometry, time-dependent surface concentrations), even if the rate-controlling process is Fickian at the molecular level.

The results for the system water/BSA between 283.15 and 323.15 K are shown in Figure 34. For the initial stages of sorption and the entire activity range studied for the sorption experiment at 283.15 K, anomalous ($0.5 < n < 1$) diffusion kinetics were found. After the initial stages of sorption, the sorption process becomes pseudo-Fickian ($n < 0.5$) for the experiments at $T \geq 293.15$ K. This implies that the initial portion of the sorption curve persists for shorter times than in the Fickian case.

The half-time of a mass transfer process is another important kinetic indicator. It is fundamentally related to the diffusion coefficient and is the value of t/d^2 for which $M_t/M_\infty = 0.50$. Figure 35 shows values for $t_{1/2}^{0.5}$ for the water/BSA sorption experiment at different temperatures. The values for $t_{1/2}^{0.5}$ are plotted against the mean concentration for each activity step change. As the non-equilibrium nature of the sorption data directly affects the estimated value for the diffusion half-time, no error bars are plotted here. However, the greater the differential mass uptake at an activity step, the smaller the error. This implies that the experimental error is

relatively smaller towards the higher mean concentrations (as $\Delta a_w = 0.1$ here). So, qualitative confidence can be assigned here.

By and large, the diffusion half times decrease with increasing temperature. At high hydration levels and high temperatures, sorption is expected to be fast. This behaviour is clearly manifested in the grouping and trend of the data between 303.15 and 323.15 K.

The attainment of ‘equilibrium’ is slower at low hydration levels. Pikal and co-workers [18] report a similar behaviour for the water vapour desorption of lysozyme, bovine serum albumin, and immunoglobulin. This conclusion is in line with Kocherbitov and co-workers [19] who observed slow desorption kinetics for water/lysozyme at low hydration levels. They suggested that the structure of a protein changes upon removal of water in a way to compensate for the absence of water by increasing protein–protein interactions. [20, 21] Therefore, sorption at low hydration levels is a slow process.

3.3.2 Integral sorption curves and diffusion behaviour

The integral sorption experiment is fundamentally different to the interval sorption experiment in the sense that there is no sample ‘history’ at the beginning of each sorption experiment. A specific activity is experimentally achieved in one step as opposed to the numerous intervals leading up to a specific activity in interval sorption. Therefore, the thermodynamic driving force (via the chemical potential) and/or the concentration gradient are significantly larger here which is expected to affect the rates of concentration-gradient controlled diffusion and relaxation-controlled diffusion. Recall (cf. Figure 9), the sorption capacity during integral sorption is significantly

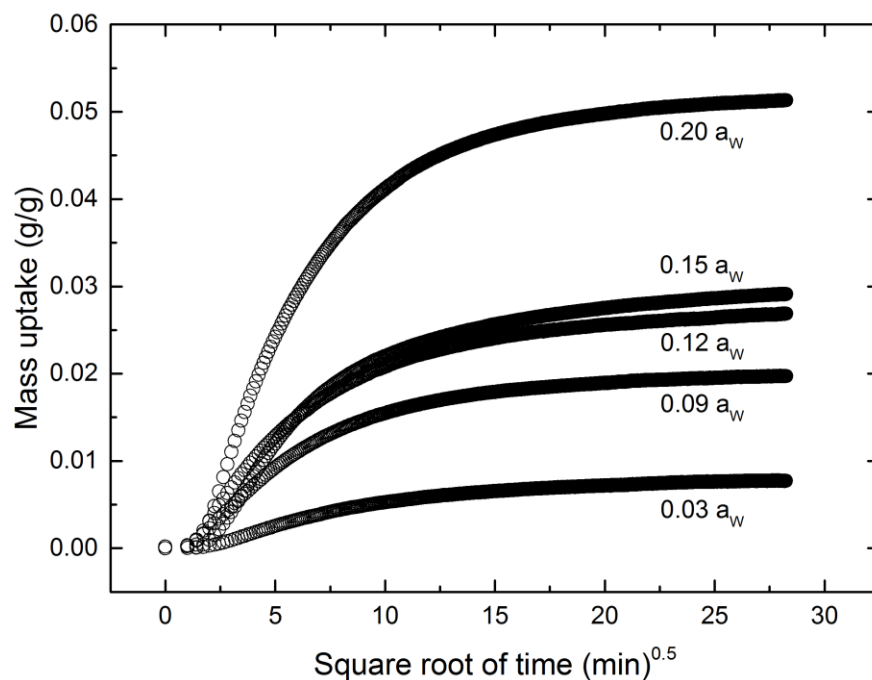


Figure 36 Experimental integral sorption curves for water/BSA at 293.15 K for activity changes from 0.00 up to 0.20 a_w .

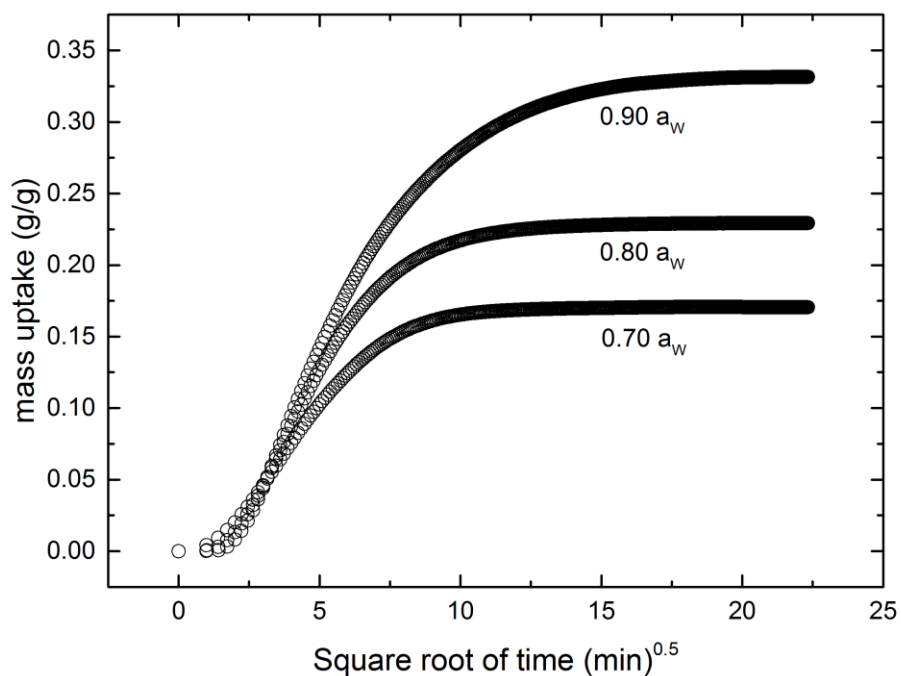


Figure 37 Experimental integral sorption curves for water/BSA at 293.15 K for activity changes from 0.00 up to 0.90 a_w .

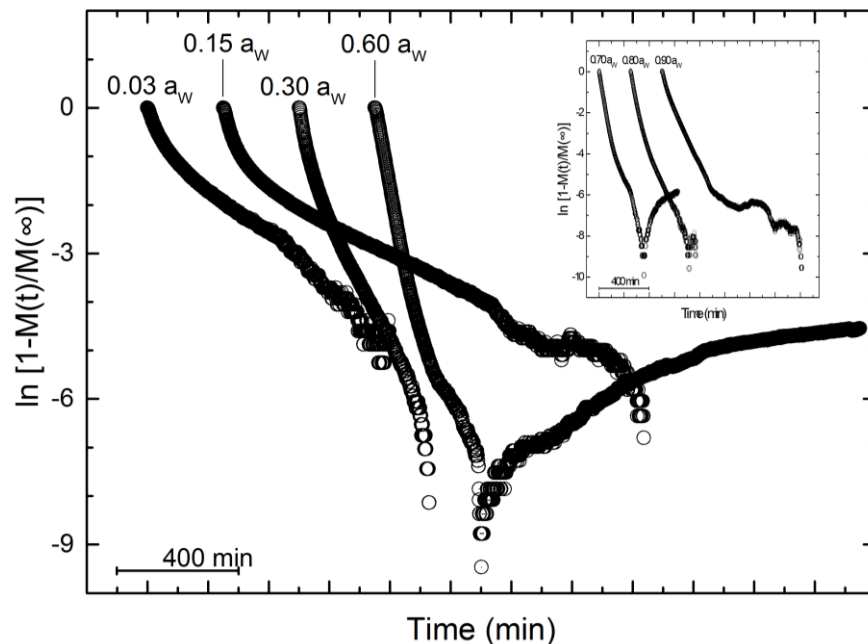


Figure 38 First-order kinetic analyses of the integral sorption curves for water/BSA at 293.15 K for selected activity intervals. Inset shows how trend is retained at high water activities. (cf. Figure 59)

higher in the system water/BSA for activities higher than $0.15 a_w$. Figures 36 and 37 show some of the integral sorption curves for the system water/BSA at 293.15 K over the activity range from 0.03 up to $0.90 a_w$. (For a more complete picture, see additional data in Appendix section 3.6.3)

An initial time lag can be seen for all experiments in the form of the sigmoidal shape of the sorption curve at short times – an experimental artefact. The sorption curves up to an activity of $0.30 a_w$ display a slow, continuous and gradual uptake for the long-time range ($M_t/M_\infty > 0.50$) of the sorption curve. For $a_w \geq 0.60$, small and kinetically slow sorption overshoots are observed. Compared to the interval sorption experiment, the integral sorption curves seem to suffer not as strongly from diffusion anomalousness.

Similarly to the analysis in the previous section, the analysis of Petropoulos and Roussis [17] is applied. Figure 38 shows the results. (For a more complete picture, see additional data in

Appendix 3.6.4) Strong deviations from linearity were found in all experiments indicating that the relaxation process is either concentration dependent or there are at least two relaxation rate constants acting that are independent of concentration.

Similarly to previous sections, the kinetic sorption factor n was estimated from the initial portion of the sorption curves according to Equation 3.6. Figure 39 shows the results. Values for n as a function of concentration lie within the anomalous regime ($0.5 < n < 1$). The comparison to the interval sorption experiment at 293.15 K, where mostly pseudo-Fickian behaviour ($n < 0.5$) was observed, reveals that the mass transport process during integral sorption is mechanistically different.

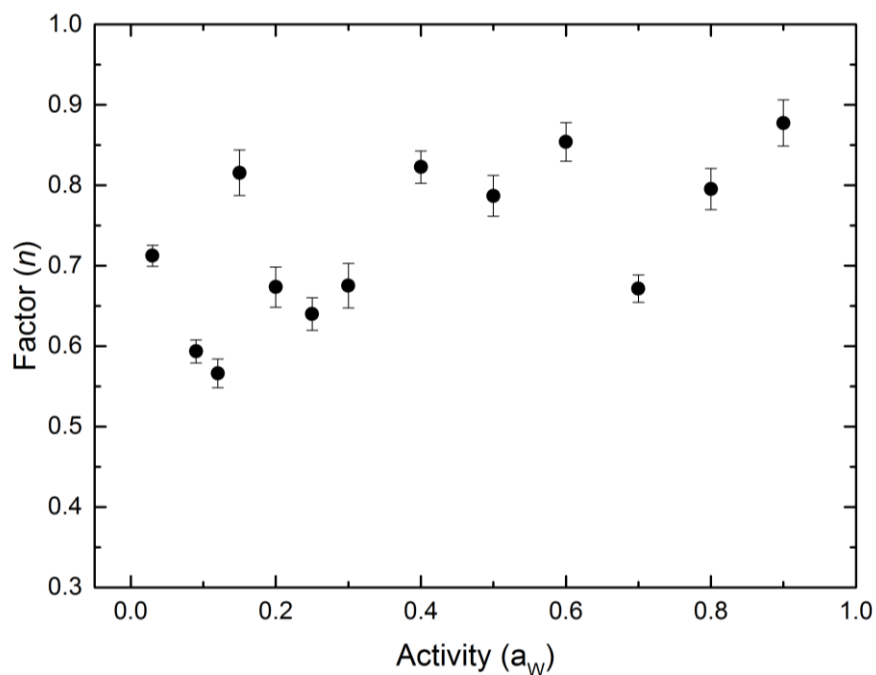


Figure 39 The kinetic factor n as regressed from Equation 3.6 for the system water/BSA at 293.15 K during integral sorption. Error bars represent confidence interval.

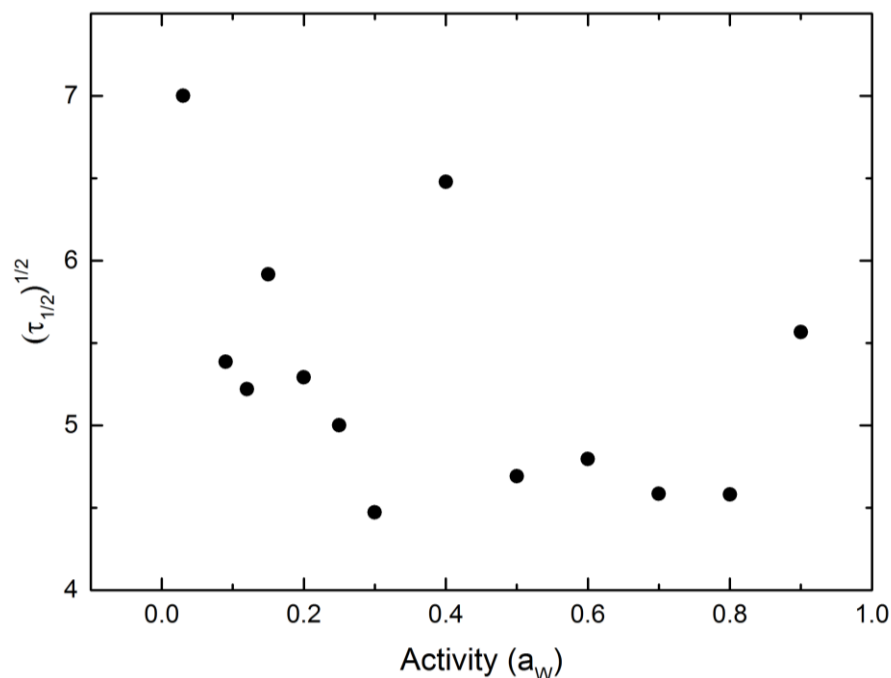


Figure 40 Estimated integral sorption half times for water/BSA at 293.15 K. (n=3)

This phenomenon is also reflected in the diffusion half-times, as plotted against the mean concentration in Figure 40 for the integral sorption experiments. The values are smaller, i.e. sorption is faster, compared to the interval sorption experiment at the same temperature (cf. Figure 35).

3.3.3 Forced interval sorption and diffusion behaviour

In order to test the influence of the interval time length on the sorption kinetics and the subsequent isotherm, sorption experiments of the system water/BSA were carried out at 293.15 K at different interval time lengths (200, 400, 800 min). This type of experiment is described as ‘forced’ interval sorption; ‘forced’ in the sense that there is not sufficient time to reach equilibrium before a subsequent activity change is imposed onto the system.

Figure 41 shows the resulting isotherms which by no means should be thought of as equilibrium isotherms. True mass transfer equilibrium was not reached during the respective measurements; though, it can be expected that the longer the system is equilibrated at a certain water activity the closer the system should be to the attainment of equilibrium. Whilst less pronounced at low

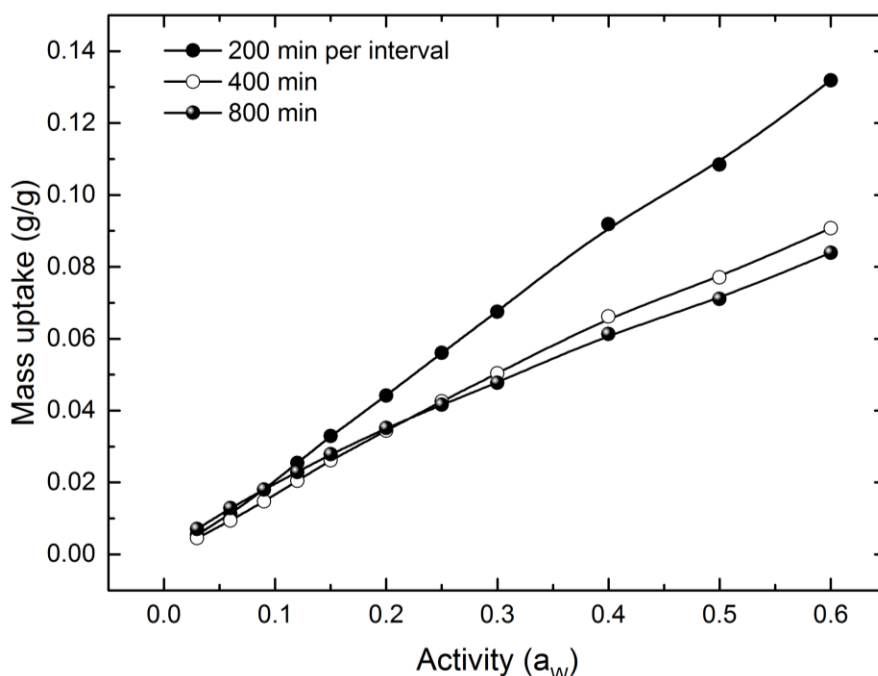


Figure 41 Interval sorption isotherms for the system water/BSA at 293.15 K for different time-for-sorption lengths (200, 400, 800 min). (n=3)

hydration/water activities ($a_w \leq 0.2$), a significant increase in water uptake with decreasing interval time lengths was observed for higher activities.

This observation is a truly surprising finding that challenges common views on water transport in glassy proteins. It can only be explained by the presence of glassy-state protein relaxations during sorption that gain increasing influence on the mass uptake/solubility of water as the time scale of the experiment increases. The long-time scale protein relaxations lead to a decrease in glassy free volume; therefore, the phenomenon of increasing water uptake with decreasing

experimental time scales might be explained by the presence of higher levels of free volume (during experiments at decreased time scales), hence, giving rise to a higher water uptake.

Figures 42 and 43 show interval sorption curves for the forced sorption experiment with an interval time length of 200 min per activity step. Up to an activity of $0.30a_w$, sorption curves do not feature the typical non-linear regime (near M_t/M_∞), which mathematically signifies the

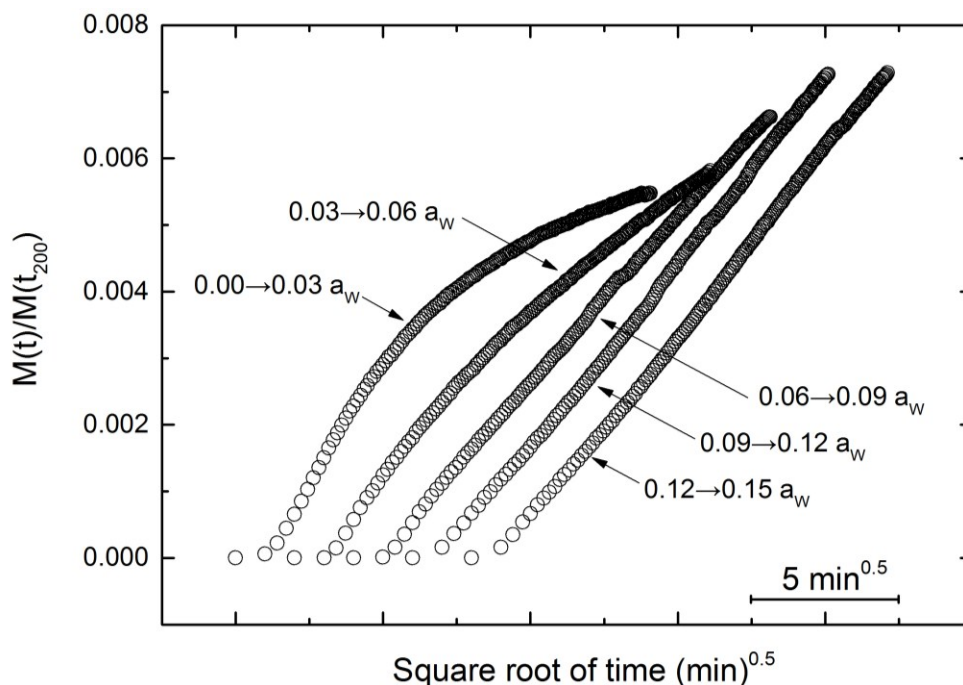


Figure 42 ‘Forced’ interval sorption curves for water/BSA for activity changes up to $0.15 a_w$ at 293.15 K.

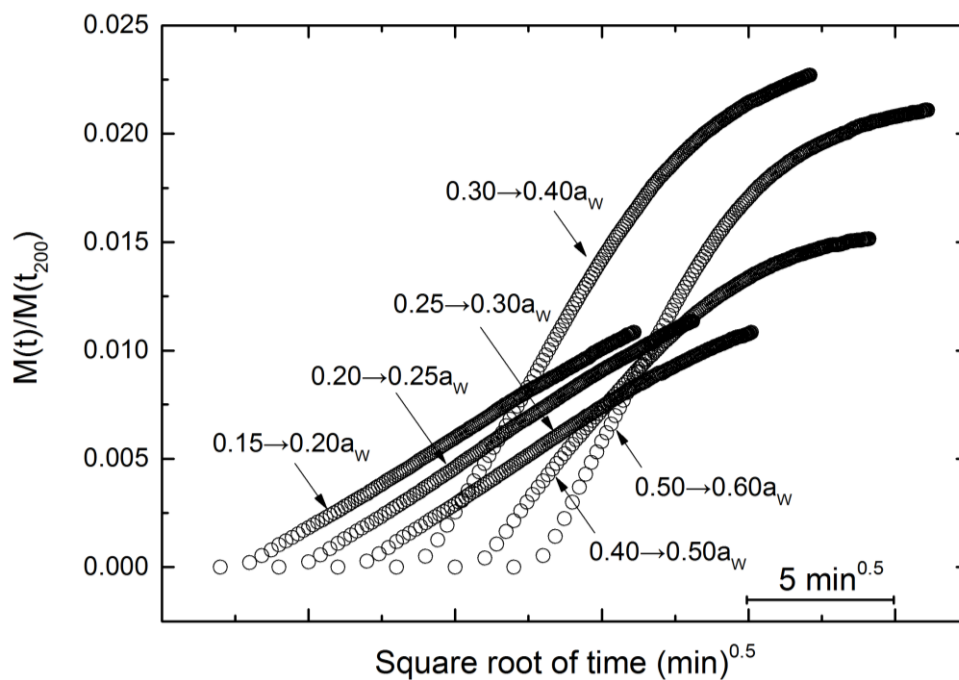


Figure 43 ‘Forced’ interval sorption curves for water/BSA for activity changes up to $0.60 a_w$ at 293.15 K.

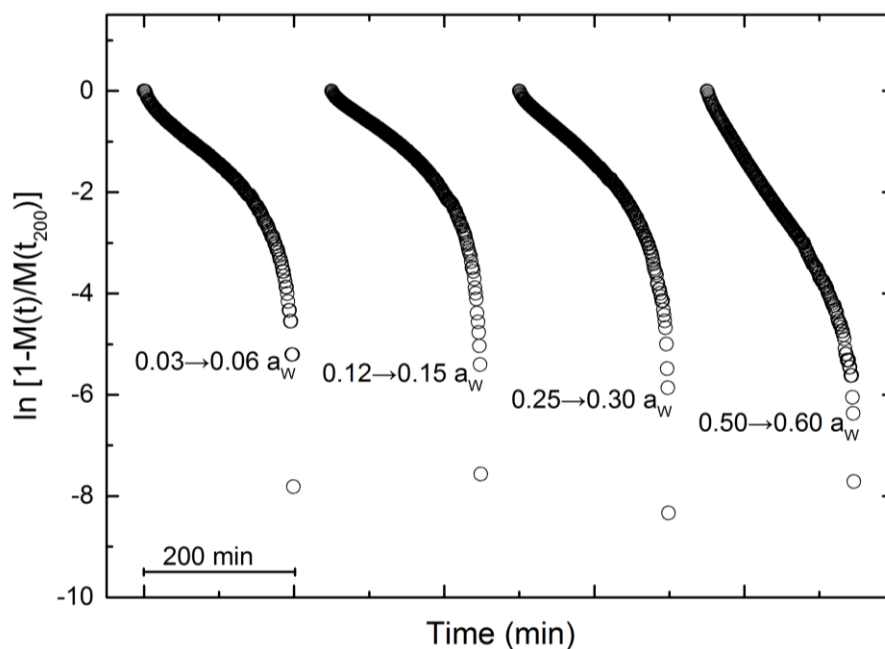


Figure 44 First-order kinetic analyses of the ‘forced’ interval sorption curves for water/BSA at 293.15 K for selected activity intervals.

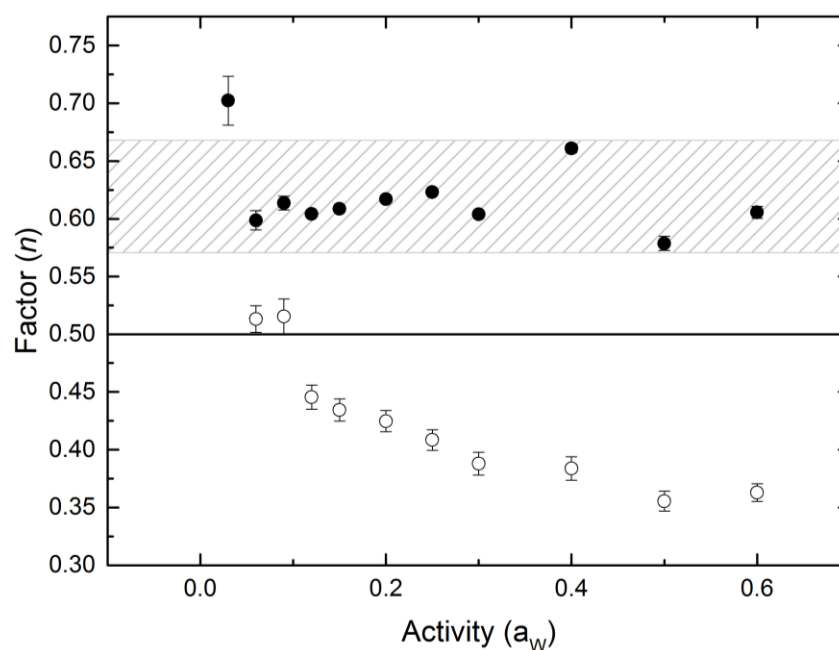


Figure 45 The kinetic factor n as regressed from Equation 3.6 for the system water/BSA at 293.15 K during ‘forced’ interval sorption (solid circle) compared to the 800min interval sorption experiment (open circle).

approach of equilibrium, but remain almost linear with the square root of time. This implies that the overall and apparent sorption kinetics are not relaxation controlled to the extent observed for longer time-for-sorption experiments (cf. section 3.3.1). This supports the notion of a ‘forced’ sorption experiment.

Similarly to the analysis in the previous sections, the analysis of Petropoulos and Roussin [17] is applied. Figure 44 shows the result. Deviations from linearity were found indicating that relaxations are still occurring within the experimental time scales but overall not as pronounced as during the 800 min time-for-sorption experiment.

In order to test this, a fit of the initial portion of the sorption curves to Equation 3.6 was performed. Figure 45 shows the results. Except for the first activity step ($0.03a_w$), values for n lie in the non-Fickian regime, between 0.575 and 0.675. It can be recalled that for the 800 min

interval time length experiment, values for n were almost entirely smaller than 0.50 lying in the pseudo-Fickian regime.

1.3.4 Modelling diffusion–relaxation phenomena

The interval water sorption kinetic data can be analysed by means of a two-stage diffusion model. During integral sorption, the concentration gradients are significantly larger which makes it even less likely to obtain a constant diffusion coefficient for a sorption experiment. Therefore, this analysis is constrained to the interval sorption experiments as present in Section 3.3.1.

The most prominent two-stage theory is that by Berens and Hopfenberg [15]; other have also proposed diffusion–relaxation models for glassy polymers. [22, 23] The simplest geometry, as adapted in the kinetic analysis of Berens and Hopfenberg [15] is a collection of spherical particles of uniform size. The total amount M_t of penetrant sorbed at time t per unit weight of polymer may be expressed as:

$$M_t = M_{t,F} + M_{t,R} \quad (3.7)$$

where $M_{t,F}$ and $M_{t,R}$ are the contribution of Fickian diffusion and relaxation processes, respectively. Solution of Fick's law for sorption by a sphere according to Crank [13] gives:

$$M_t = M_{\infty,F} \left(1 - \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp(-n^2 k_F t) \right) \quad (3.8)$$

where

$$k_F = 4\pi^2 D/a^2 \quad (3.9)$$

and $M_{\infty,F}$ is the equilibrium amount of sorption, D is the diffusion coefficient, n is the series of integers, and a is the sphere diameter. Berens and Hopfenberg suggest first order kinetics for the

relaxation process with a rate proportional to the difference between $M_{t,R}$ and $M_{\infty,R}$, the equilibrium amount of sorption due to relaxation; thus:

$$\frac{dM_{t,R}}{dt} = k(M_{\infty,R} - M_{t,R}) \quad (3.10)$$

Upon integration and allowance for the possibility of several relaxation processes with different rates k_i and individual equilibrium contributions $M_{\infty,i}$, the result is:

$$M_t = M_{\infty,F} \left(1 - \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp(-n^2 k_F t) \right) + \sum_i M_{\infty,i} [1 - \exp(-k_i t)] \quad (3.11)$$

To interpret observed experimental sorption data, a diffusion model is required that is able to capture the variety of behaviours observed: Fickian diffusion with time lag (at $\ll t$) and anomalous diffusion (at $\gg t$). It is important that these two features operate at different times during the sorption curve. However, in order to reduce the number of fitting parameters, it was not attempted to capture the initial time lag or to include more than one relaxation time constant.

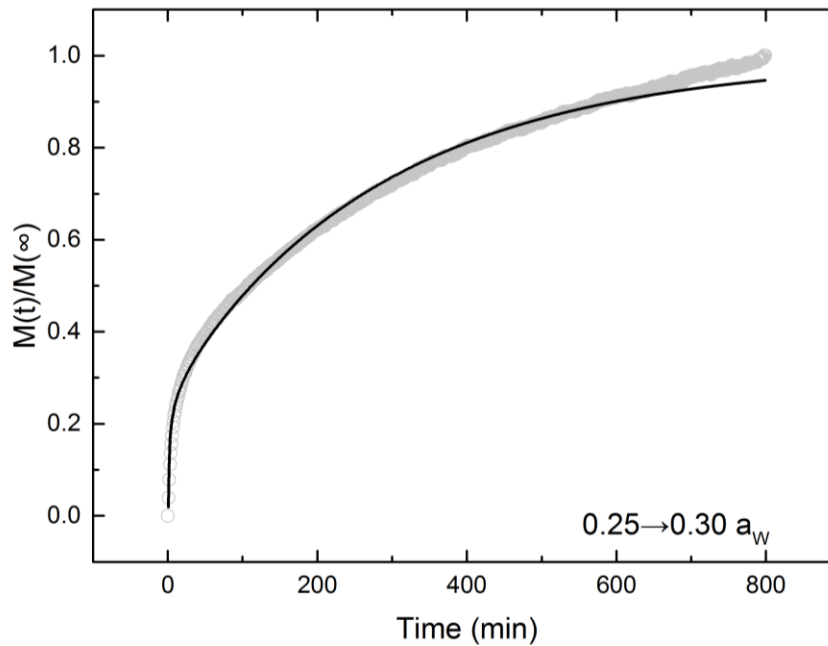


Figure 46 Interval sorption curve for an activity change from 0.25 to 0.30 a_w for the system water/BSA at 293.15 K. (—) Fit by Equation 3.11.

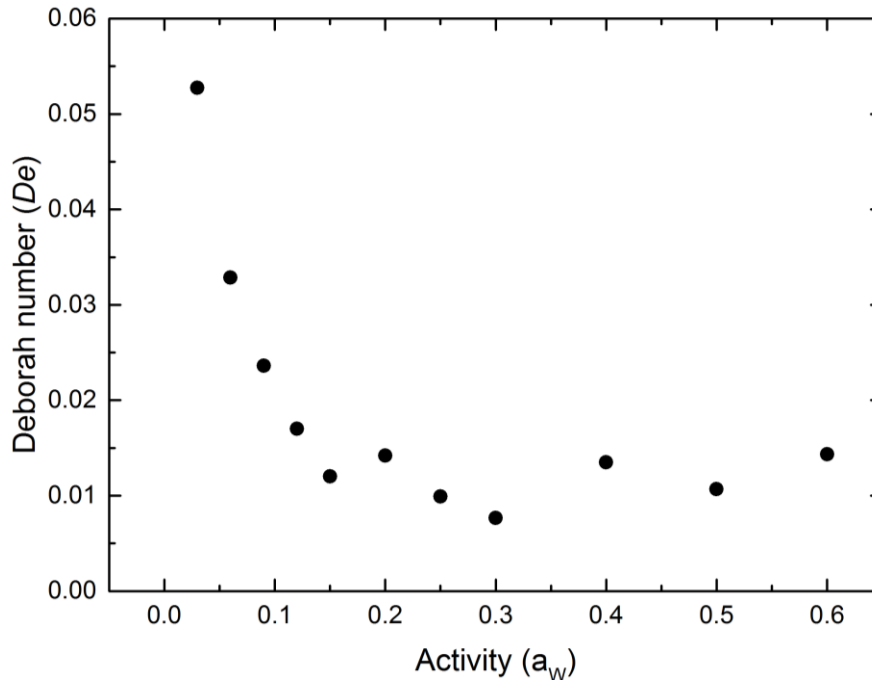


Figure 47 Estimated Deborah number (De) from the interval sorption curve fits by Equation 3.11 for the system water/BSA at 293.15 K.

Figure 46 shows a regressed sorption curves using this approach. The regression results of the diffusion–relaxation model are show in Table 4. A good agreement between the experimental results and the model regressions were found. Comparing the diffusion time constant, k_F , and relaxation time constant, k_R , in Table 4 gives mechanistic insight into the two-stage diffusion–relaxation phenomena.

In their original work, Berens and Hopfenberg propose that when $k_F \gg k_R$, rapid Fickian kinetics should be observed follow by a slower and distinct separate, relaxation-controlled sorption stage. For $k_F \approx k_R$, no clear distinction between the two stages is possible. The parameter f_F denotes the ratio of $M_{\infty,F}/M_{\infty,i}$.

The ratio of the diffusion time ($\tau_F \approx L^2/D$) and relaxation time ($\tau_R \approx 1/k_R$) is referred to as the diffusion Deborah (De) number [24]. A high De corresponds to the case of $k_F \gg k_R$ and a low

De (approaching the limit value of one) corresponds to $k_F \approx k_R$. For $De \ll 1$, the diffusion process is relaxation controlled. Figure 47 shows the estimated De numbers. Strong relaxation was found for the interval sorption experiments at 293.15 K. Up to $0.30 a_W$, De decreases by about one order of magnitude after which it stays constant around $De \approx 0.015$.

An inspection of the relaxation contribution to the overall water transport process yields more insight. If the value of k_F is substituted back into the second term of the RHS of Equation 3.11 in the sense of $t = 1/k_F$, then $M_{t,R}/M_{\infty,R} = 1 - e^{-1} \approx 0.63$. That means, that more than half of the relaxation-sorbed water is taken up. Values for $1/k_F$ lie in the between 120 to 290 *min*. Relating back to Section 3.3.3, where interval sorption isotherms of different experimental time scales were presented, it can now be understood that the present relaxations are not pronounced at shorter experimental time scales. In this sense, relaxations (i.e. volume contractions) cause the decreased water uptake during the 800 *min* interval time length sorption experiment in BSA.

Table 4 Regression results for the diffusion–relaxation model for interval water sorption in BSA at 293.15 K. Experimental water sorption kinetic data was regressed by Equation 3.11.

Activity	k_F	$\pm$	k_R	$\pm$	f_F	$\pm$
a_w	1/min		1/min		1/min	
0.03	0.152	0.00614	0.00802	6.24E-05	0.34914	0.00539
0.06	0.2058	0.00602	0.00676	4.75E-05	0.47428	0.004
0.09	0.19525	0.00702	0.00461	2.95E-05	0.44764	0.00397
0.12	0.26261	0.01035	0.00447	2.07E-05	0.3955	0.00315
0.15	0.31791	0.0203	0.00382	1.95E-05	0.34741	0.00373
0.20	0.28068	0.01378	0.00399	1.88E-05	0.3686	0.00334
0.25	0.36956	0.02593	0.00366	1.64E-05	0.31943	0.00339
0.30	0.44893	0.03932	0.00344	1.53E-05	0.29985	0.00344
0.40	0.35364	0.01366	0.00477	1.38E-05	0.32573	0.00218
0.50	0.43009	0.02338	0.0046	1.58E-05	0.31944	0.00262
0.60	0.36209	0.01298	0.00519	1.48E-05	0.33506	0.00212

3.4 CONCLUSION

Experimental sorption data obtained via a gravimetric method run in three different modes (interval, integral, ‘forced’ interval sorption) at different experimental temperatures (283.15, 293.15, 303.15, 313.15, 323.15 K; all below the glass transition temperature of ‘dry’ BSA) showed non-Fickian behaviour under all three experimental modes and over the entire temperature and activity range studied. The nature of the non-Fickian behaviour was analysed and found to differ between the three different experimental modes. It is suggested that the non-Fickian behaviour resulted of the nonequilibrium state of the glassy biopolymer (BSA). Two-stage sorption kinetics were reported, where diffusion driven by the water concentration gradient is followed by a slower (in relation to the first process) polymer matrix relaxation controlled diffusion process. A diffusion–relaxation model confirms the observed two-stage behaviour and allowed the estimation of Deborah numbers. These numbers were found to be smaller than one indicating strong relaxation control of the overall diffusion process under experimental time scales. Further, the time constants of the diffusion and relaxation process, respectively, revealed that the relaxation is kinetically ‘overridden’ during the 200 *min* interval time length sorption experiment as there is not sufficient time for the relaxation to manifest itself significantly in the water uptake/solubility.

3.5 REFERENCES

- [1] Davis, E.M.; Elabd, Y.A. *J. Phys. Chem. B* **2013**, 117, 10629-10640
- [2] Davis, E.M.; Elabd Y.A. *Ind. Eng. Chem. Res.* **2013**, 52, 12865-12875
- [3] Davis, E.M.; Minelli, M.; Baschetti, M.G.; Jr.; Elabd, Y.A. *Ind. Eng. Chem. Res.* **2013**, 52, 8664-8673
- [4] Davis, E.M.; Minelli, M.; Baschetti, M.G.; Jr.; Sarti, G.C.; Elabd, Y.A. *Macromolecules*, **2012**, 45, 7486-7494
- [5] Crank, J.; Park, G.S. (eds.) "Diffusion in Polymers", Academic Press, London, **1968**
- [6] George, S.C.; Thomas, S. *Prog. Polym. Sci.* **2001**, 26, 985-1017
- [7] King, G. *Trans. Faraday Soc.* **1945**, 41, 325-332
- [8] Crank, J.; Park, G.S. *Trans. Faraday Soc.* **1951**, 47, 1072-1084
- [9] Long, F.A.; Kokes, R.J. *J. Am. Chem. Soc.* **1953**, 75, 2232-2237
- [10] Long, F.A.; Thompson, L.J. *J. Polym. Sci.* **1955**, 15, 413-426
- [11] Rodgers, C.E. **1965**. Physics and chemistry of the organic solid state. (Eds. Fox, D.; Labes, M.M.; Weissberger, A.) Vol. II, Chapter 6. Interscience, New York
- [12] Alfrey, T.; Gurnee, E.F.; Lloyd, W.G. *J. Polym. Sci.* **1966**, C12, 249-261
- [13] Crank, J. "The Mathematics of Diffusion", 2nd Ed., Clarendon Press, Oxford, **1975**
- [14] Frisch, H.L. *J. Polym. Eng. Sci.* **1980**, 20(1), 2-13
- [15] Berens, A.R.; Hopfenberg, H.B. *Polymer* **1978**, 19, 489-496
- [16] Sanopoulou, M.; Petropoulos, J. *Macromolecules* **2001**, 34, 1400-1410
- [17] Petropoulos, J.H.; Roussis, P.P. In: Permeability of Plastic Films and Coatings to Gases, Vapor and Liquids. Hopfenberg, H.B., Ed.; Plenum: New York, **1974**, p. 219

- [18] Luthra, S.; Lechuga-Ballesteros, D.; Kalonia, D.S.; Pikal, J.M. *J Pharm. Sci.* **2007**, 96, 8, 1974-1982
- [19] Kocherbitov, V.; Arnebrant, T.; Soderman, O. *J. Phys. Chem. B* **2004**, 108, 19036-19042
- [20] Prestrelski, S.J.; Tedeschi, N.; Arakawa, T.; Carpenter, J.F. *Biophys. J.* **1993**, 65, 661-671
- [21] Gregory, Adv. *Protein Chem.* **1995**, 41, 37-112
- [22] Carla, V.; Hussain, Y.; Grant, C.; Sarti, G.C.; Carbonell, R.G. Doghieri, F. *Ind. Eng. Chem. Res.* **2009**, 48, 3844-3854
- [23] Dimos, V.; Sanopoulou, M. *J. Appl. Polym. Sci.* **2006**, 100, 2278-2288
- [24] Vrentas, J.S.; Jarzebski, C.M.; Duda, J.L. *AIChE J.* **1975**, 21, 894-901

3.6 APPENDIX

3.6.1 Interval Sorption Curves

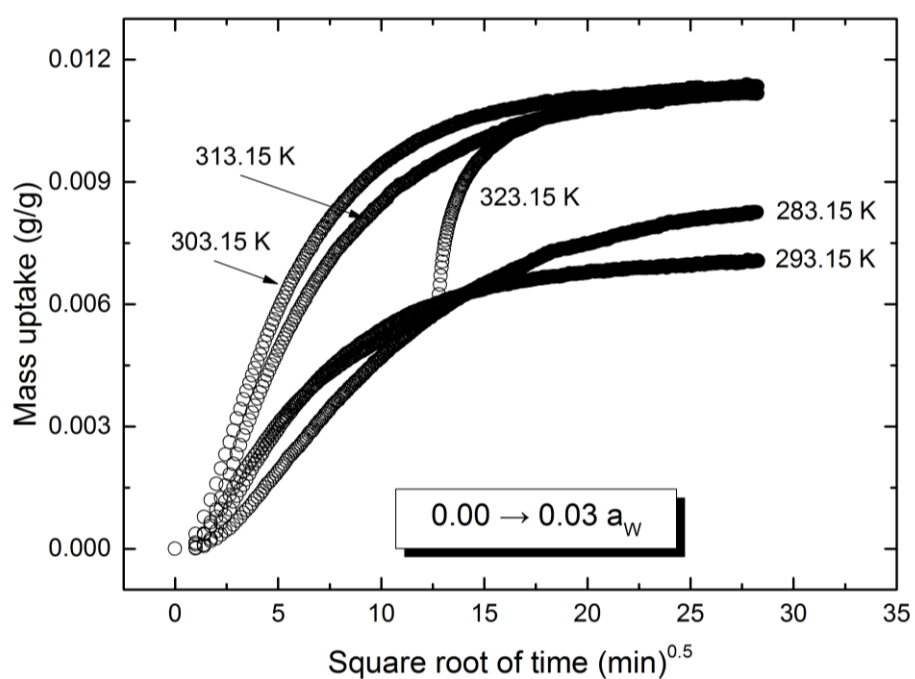


Figure 48 Experimental interval sorption curves for water/BSA for an activity change from 0.00 to 0.03 a_w as a function of temperature.

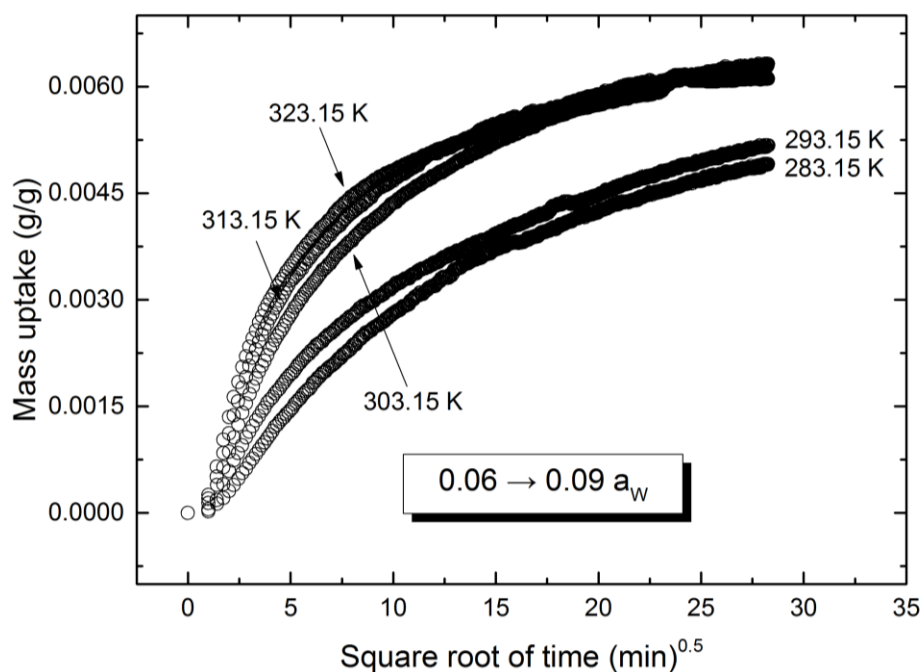


Figure 49 Experimental interval sorption curves for water/BSA for an activity change from 0.06 to 0.09 a_w as a function of temperature.

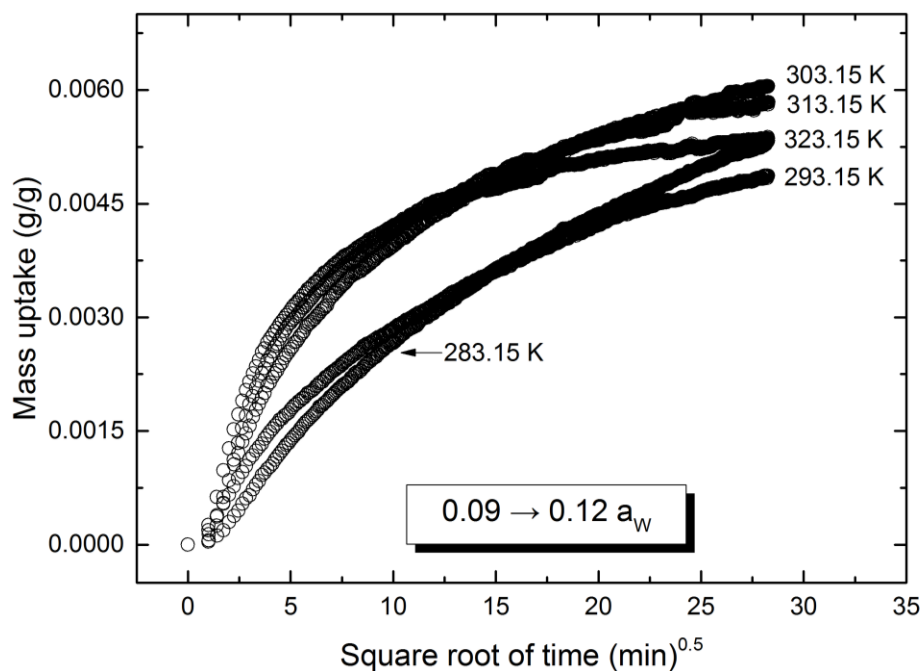


Figure 50 Experimental interval sorption curves for water/BSA for an activity change from 0.09 to 0.12 a_w as a function of temperature.

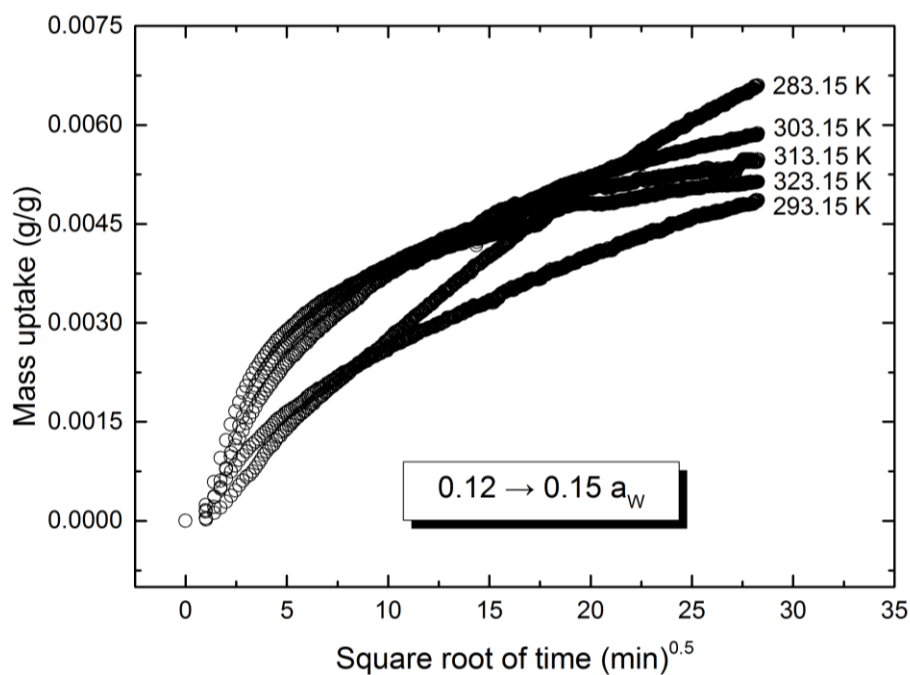


Figure 51 Experimental interval sorption curves for water/BSA for an activity change from 0.12 to 0.15 a_w as a function of temperature.

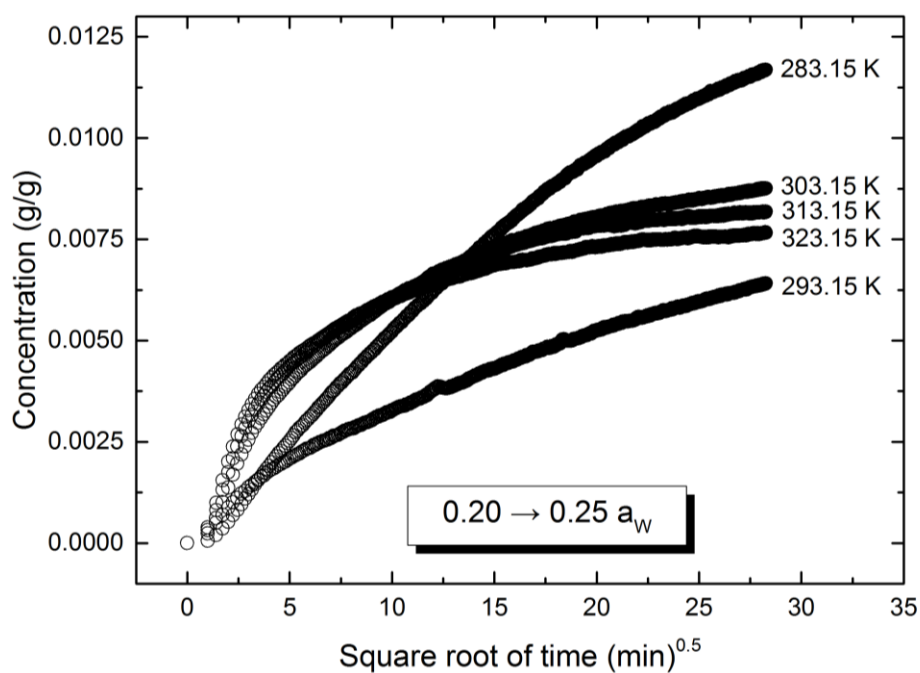


Figure 52 Experimental interval sorption curves for water/BSA for an activity change from 0.20 to 0.25 a_w as a function of temperature.

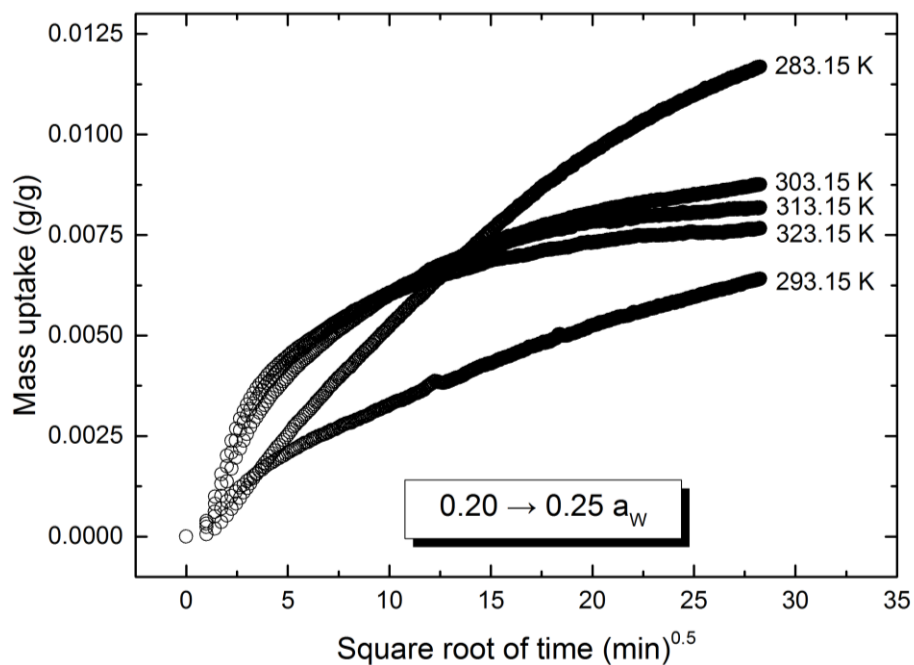


Figure 53 Experimental interval sorption curves for water/BSA for an activity change from 0.25 to $0.30 a_w$ as a function of temperature.

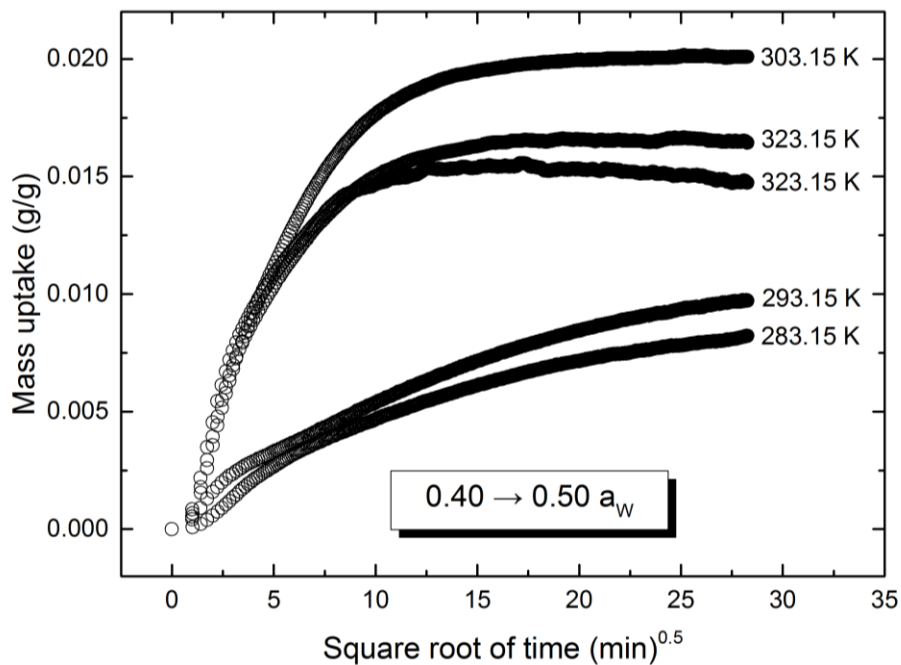


Figure 54 Experimental interval sorption curves for water/BSA for an activity change from 0.40 to $0.50 a_w$ as a function of temperature.

3.6.2 Special interval sorption curves

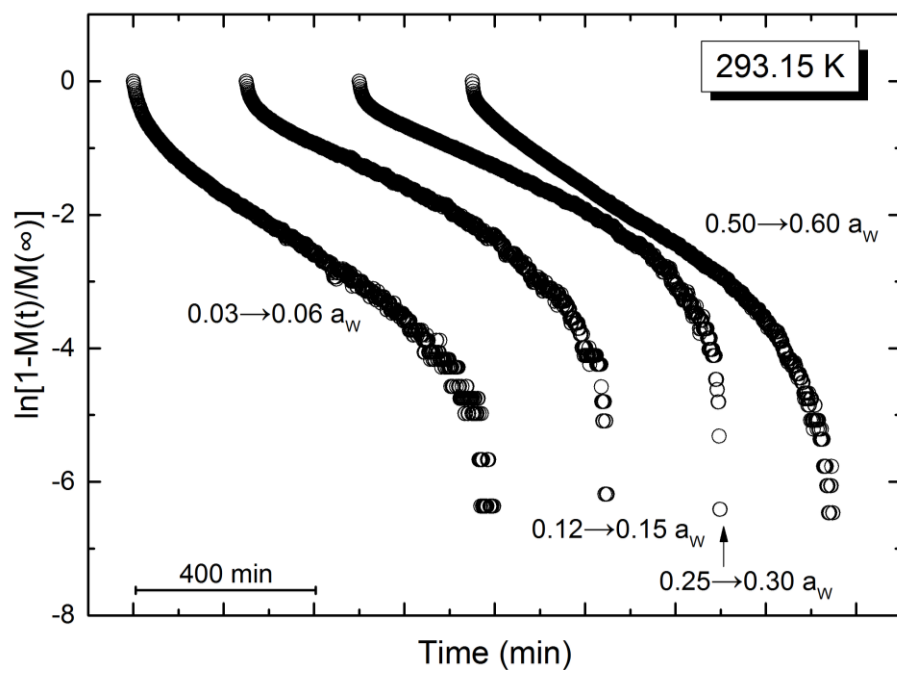


Figure 55 First-order kinetic analyses of the sorption curves for water/BSA at 293.15 K.

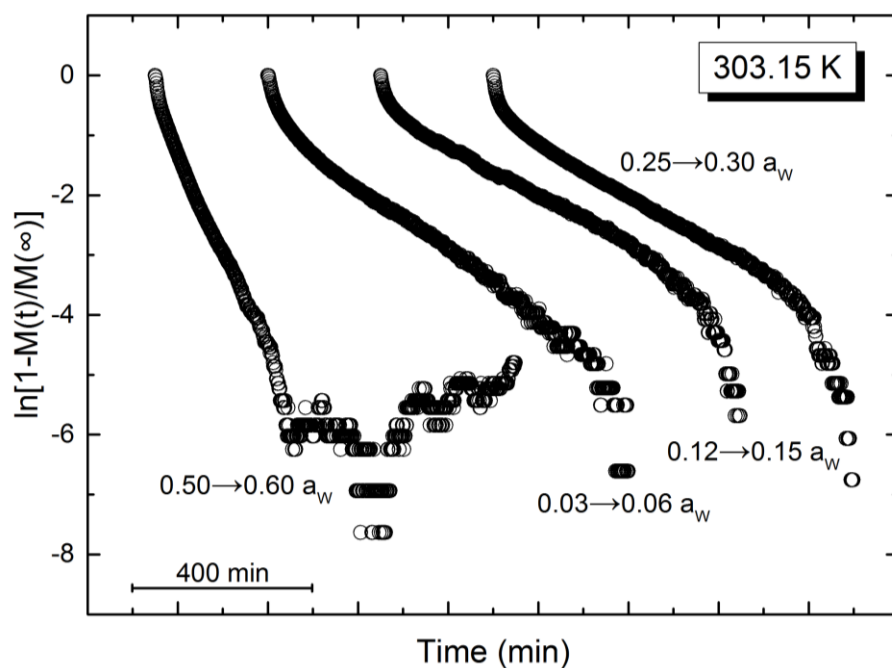


Figure 56 First-order kinetic analyses of the sorption curves for water/BSA at 303.15 K.

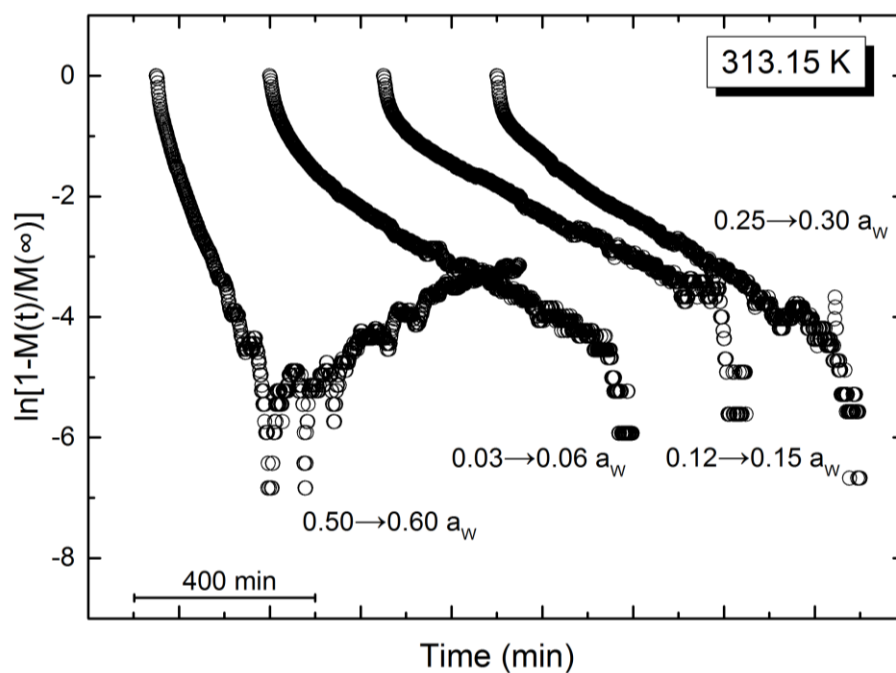


Figure 57 First-order kinetic analyses of the sorption curves for water/BSA at 313.15 K.

3.6.3 Integral sorption curves

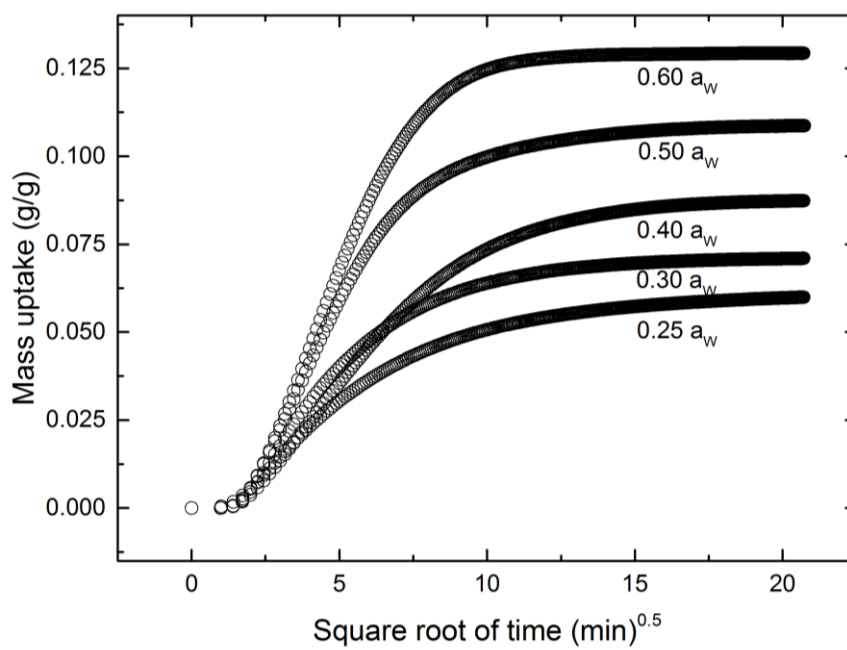


Figure 58 Experimental integral sorption curves for water/BSA at 293.15 K for activity changes from 0.00 up to 0.60 a_w .

3.6.4 Special integral sorption curves

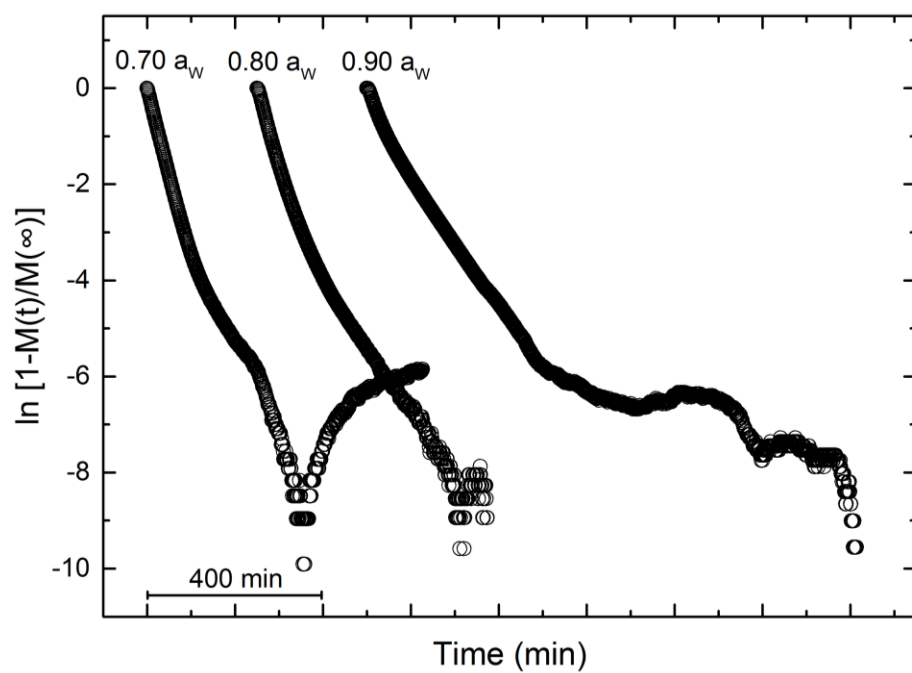


Figure 59 First-order kinetic analyses of the integral sorption curves for water/BSA at 293.15 K.

Chapter 4. Enthalpy–entropy effects during water sorption in bovine serum albumin

4.1 INTRODUCTION

The hydration of biological macromolecules plays a crucial role in determining their structure, stability, dynamics, and function. [1] There is a significant difference in thermodynamic properties between hydrated and bulk water in protein systems [2-5]. It is obvious that understanding the nature of the hydration process of proteins requires the explanation of the effects of water on the protein itself and vice versa. For this reason, a quantitative study of the contributions of the water and protein to the thermodynamic functions that govern the hydration process is of profound importance.

There are typically two different approaches by which protein–water interactions are studied. In one approach, protein–water interactions are studied in the dilute aqueous solution region. In the other approach, changes in the protein's thermodynamic, structural or dynamic properties are examined at relatively low hydration levels (i.e. concentrated solution with a solid). The latter approach is particular useful when aiming to access thermodynamic information on the hydration process.

From the temperature dependence of the water sorption isotherms, differential heats of water sorption can be calculated. Lüscher-Mattli and Ruegg [6, 7] reported this data for lysozyme and α -chymotrypsin from water sorption data between 298.15 and 313.15 K. Bone [8] studied the water vapour sorption by lysozyme in the temperature range of 279.15 – 319.15 K. A strong dependence of the hydration enthalpy with water concentration was found and with increasing water content, the hydration enthalpy approaches the enthalpy of condensation of pure water as might be expected. The basic assumption of this analysis is: the enthalpy does not depend on

temperature. However, this assumption is not correct as the heat capacities of both water and proteins depend on temperature. [9]

Isothermal calorimetry is a direct experimental approach for measuring the thermodynamic of proteins during hydration. Amberg [10] was the first to do so and measured the heats of water vapour sorption on bovine serum albumin at 293.15 K within a water uptake range of 0 to 0.12 *g/g*. Almong and Schrier [11] calorimetrically measured the enthalpies of solution of dried and hydrated ribonuclease A at 298.15 K within a water uptake range of 0 to 0.26 *g/g*. Here, the heat of solution is the enthalpy change associated with the dissolution of a dried or hydrated ribonuclease A in water at constant pressure resulting in infinite dilution. They found that the enthalpy of solution was negative (exothermic) for low water activities, and as the water content increased, the enthalpy of solution approached a value of zero. More recently, Smith and co-workers [12] measured the heat of water sorption by lysozyme with an isothermal calorimeter in the range of water activities between 0 and 0.895 a_w at 298.15 K. Kocherbitov and co-workers [13] studied the hydration enthalpy of lysozyme over the complete water activity range. Pikal and co-workers [14] simultaneously measured water desorption isotherms and enthalpies for lysozyme, bovine serum albumin, and monoclonal immunoglobulin at 293.15 K. Sirotkin and co-workers [15-18] studied the heat effects of hydration–dehydration of numerous proteins over the entire water activity range. The interaction enthalpy was found not just to depend on temperature and concentration but also on the initial water content and hydration history. This observation is an important finding. In many way it challenges the validity of published interaction enthalpies of protein/water systems.

The calorimetrically measured enthalpies contain information on the binary water–protein interaction (and a history aspect). This enthalpy includes information about corresponding conformational changes related to protein structure and glass transition. It is now generally

recognised that proteins undergo a glass-like transition with increasing hydration with water acting as a plasticising solvent. [2] However, the exact mechanism of the glass transition in proteins is not fully understood. [19] For instance, the connection between the low temperature second-order transition (180 – 220 K) and that above 273.15 K in hydrated proteins is still unclear.

Structural changes strongly affect the water sorption behaviour of proteins. There have been reports indicating that the α -helix and β -sheet content of a solid lyophilised protein is different to the content when in solution [20, 21]. It can be expected that the transitions between different protein conformations will change the numbers of interaction sites for water and the thermodynamics of those interactions. None of these facts are appropriately considered in the current water sorption models for water–protein systems.

A further anomaly of the water sorption process in glassy proteins is the observed enthalpy–entropy compensation event. In essence, enthalpy–entropy compensation refers to the experimental observation of a linear scaling between the changes in entropy and enthalpy for a set of related reactions/processes. The subject is surrounded by considerable controversy and no general, truly statistical mechanical explanation has been offered as of yet. Enthalpy–entropy compensation effects during protein hydration were first reported by Lüscher-Mattli and Ruegg [7] and more recently by Kocherbitov and Arnebrant [19]. While Lüscher-Mattli and Ruegg interpreted the compensation effect solely in terms of phase transitions of the water molecules, Kocherbitov and Arnebrant explain the compensation effect from the point of view of hydration of the protein–protein interface. However, it should be noted that such compensation is ubiquitous when comparing enthalpies with residual entropies.

In this work, experimentally determined interval heats of sorption of water vapour into glassy bovine serum albumin at 293.15, 313.15 and 323.15 K within the activity range of 0.06 – 0.60 a_w are reported. The measurements were carried out in an experimental mode to mimic and

complement the gravimetric interval sorption isotherm measurements reported (cf. Section 2.3.4). In fact, the time evolution of the heat flow event is compared with the sorption kinetics. The calorimetric data is then analysed in terms of its thermodynamic excess functions. Enthalpy–entropy compensation effects are also discussed. The Chapter’s focus is then directed towards the Flory–Huggins analysis (cf. section 2.3.7), in particular the binary interaction parameter (χ), and its extension by Vrentas and Vrentas in order to establish a fundamental discrepancy between experimentally determined interaction enthalpies and those predicted by the van-Laar/Flory-Huggins theory.

4.2 EXPERIMENTAL SECTION

4.2.1. Materials

Bovine serum albumin (BSA) (lyophilised powder, catalogue number 05470) was purchased at the highest commercially available grade from Sigma Aldrich (Gillingham, United Kingdom). Ultrapure deionised water (resistivity $\sim 16 \text{ M}\Omega \cdot \text{cm}$) was used for all sorption experiments.

4.2.2 Methods

See Section 2.2.2. for detailed methodology on Dynamic Vapour Sorption experiments used in this Chapter.

A Thermal Activity Monitor III(*) (TA Instruments Inc., Utah, United States) was used in conjunction with a 4 mL relative humidity perfusion unit. Experiments were performed isothermally at 293.1500, 313.1500 and 323.1500 K. Dry nitrogen was used as a carrier gas. The total gas flow was controlled by two *EL-Flow* mass flow controllers (Bronkhorst, Ruurlo, Holland). The total flow rate was set to 100 mL/h. The total flow is divided into two lines, a wet line and a dry line. Control over the water activity in the sample chamber was achieved by manipulating the flow-mix ratio of the wet and dry lines. The criterion for thermal stability was defined as a maximum slope of the heat flow of 50 nW/h with a standard deviation of no more than 12.5 nW for a linear fit over a 20 min interval.

Experiments with a typical sample weight of 5 mg were conducted in the interval sorption mode. The sample was first equilibrated under dry gas ($0.00a_w$) until the heat flow signal was stable at zero. The activity was subsequently increased in $0.03a_w$ steps to $0.15a_w$, followed by measurements at 0.20, 0.30, 0.40 and $0.60a_w$. The duration of each step was dictated by the

respective heat flow and had to meet the stability criterion for at least 180 *min* before the next step.

(*) The Thermal Activity Monitor III used in this study is a commercial state-of-the-art flow calorimeter set-up. Experiments were carried out at Procter & Gamble Co. Newcastle Innovation Centre (Newcastle, United Kingdom) between August and November 2014. I would like to acknowledge the support by Michelle Jackson, Tara Aziz and Simon Greener in facilitating these experiments (all Procter & Gamble Co.).

4.3. RESULTS AND DISCUSSION

4.3.1 Isothermal flow calorimetry

Before starting the actual sorption experiment, a stable calorimeter baseline has to be established after insertion of the sample ampoule. Figure 60 shows the heat flow (normalised by sample weight) as a function of time at 293.15 K under ‘dry’ conditions. From this type of data, a linear baseline was constructed for each individual calorimetric experiment. As typical baseline fluctuations are about $3 - 6 \times$ orders of magnitude smaller than the heat event associated to an activity step change, a linear fit was assumed to be suitable.

Figures 61 to 63 show the raw data of typical interval heat of sorption experiments at 293.15, 313.15 and 323.15 K, respectively. Here, the heat flow from the sample ampoule to the heat sink (reservoir) is plotted for each activity increment. The area under the curve (i.e. integral) is the heat of sorption in units of energy/heat per gram of bovine serum albumin to go from an initial activity to a final one in a step-wise fashion. The observed shoulders of the heat flow data in Figures 61 to 63 occur reproducibly but there is no clear temperature or concentration dependence. If any physical meaning might be attributed than it is a support of the notion of ‘long times to equilibrium’, i.e. dynamical changes within the protein-water mixture occur over experimentally detectable timescales.

Figure 64 shows a typical integral curve of this type of data. After some algebra and employing the sorption isotherms for the appropriate temperatures (cf. section 2.3.1), the heat of sorption in units of energy per moles of water at a given mixture concentration can be calculated. These are the usual units employed for this type of data. The calculation is based on the assumption that the calorimeter mimics the gravimetric experiment perfectly; that is, (a) water vapour sorption kinetics and well as quasi-equilibrium solubility are identical in both experiments, and (b) the

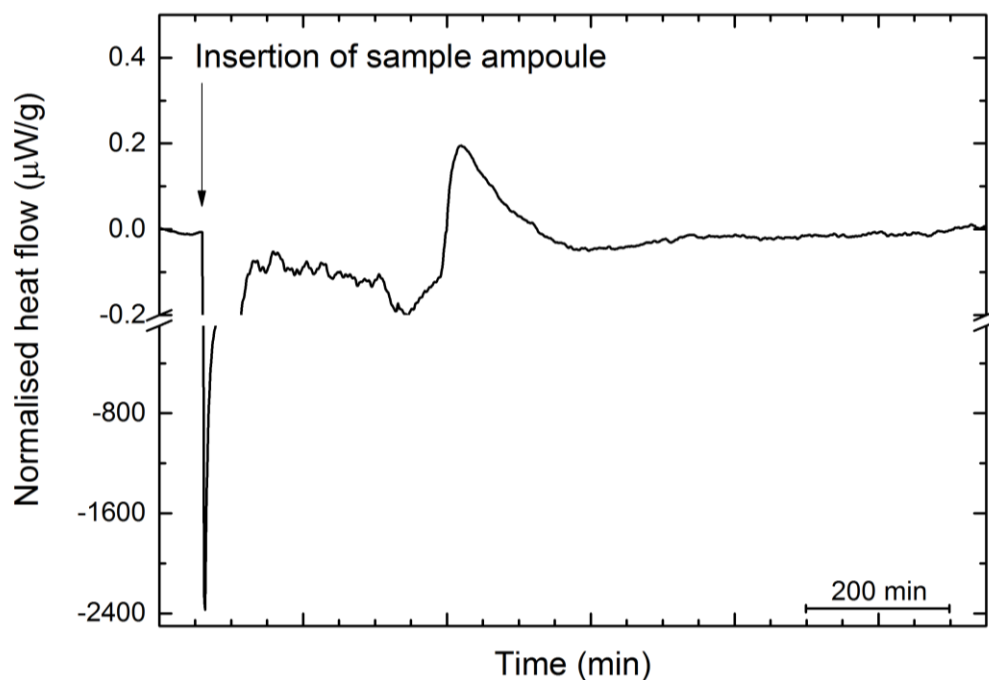


Figure 60 A typical heat flow curve during sample insertion and baseline stabilisation of the calorimeter; here at 293.15 K.

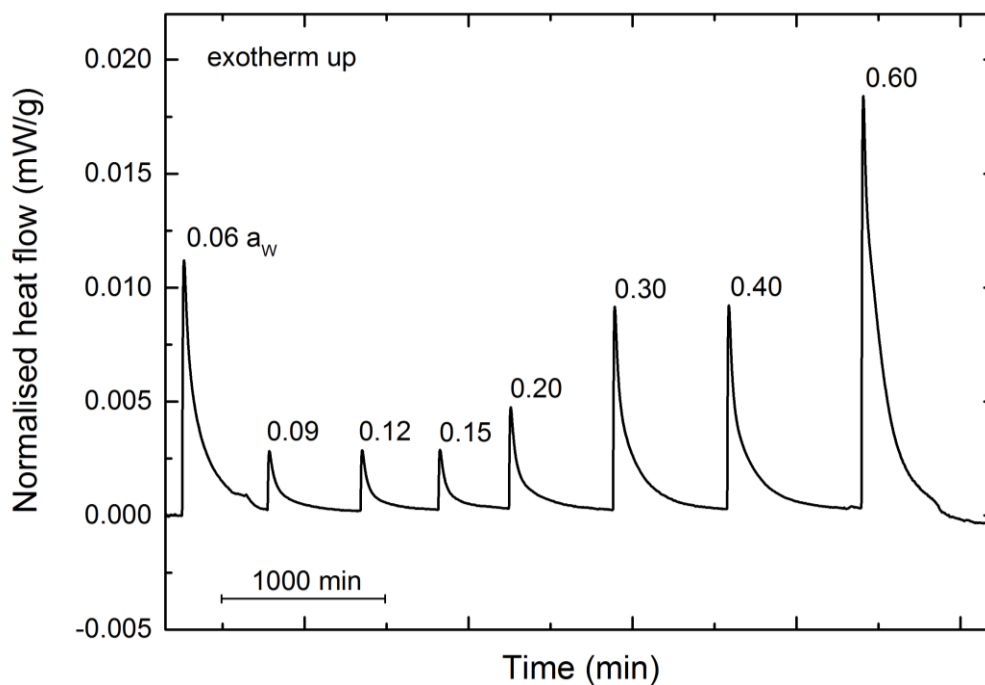


Figure 61 A typical heat flow curve during interval water vapour sorption of BSA at 293.15 K.

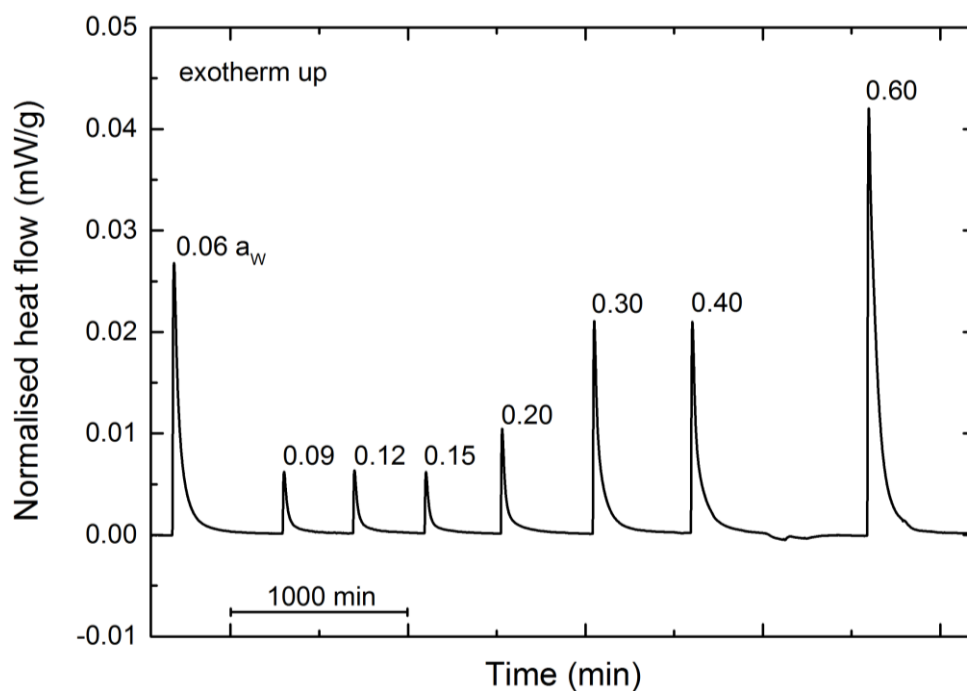


Figure 62 A typical heat flow curve during interval water vapour sorption of BSA at 313.15 K.

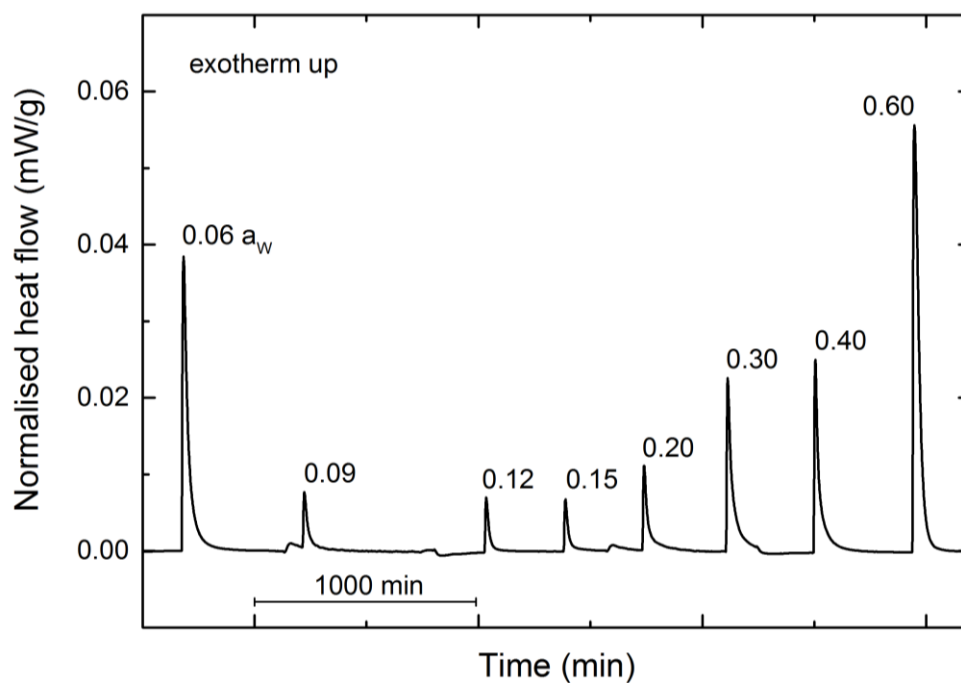


Figure 63 A typical heat flow curve during interval water vapour sorption of BSA at 323.15 K.

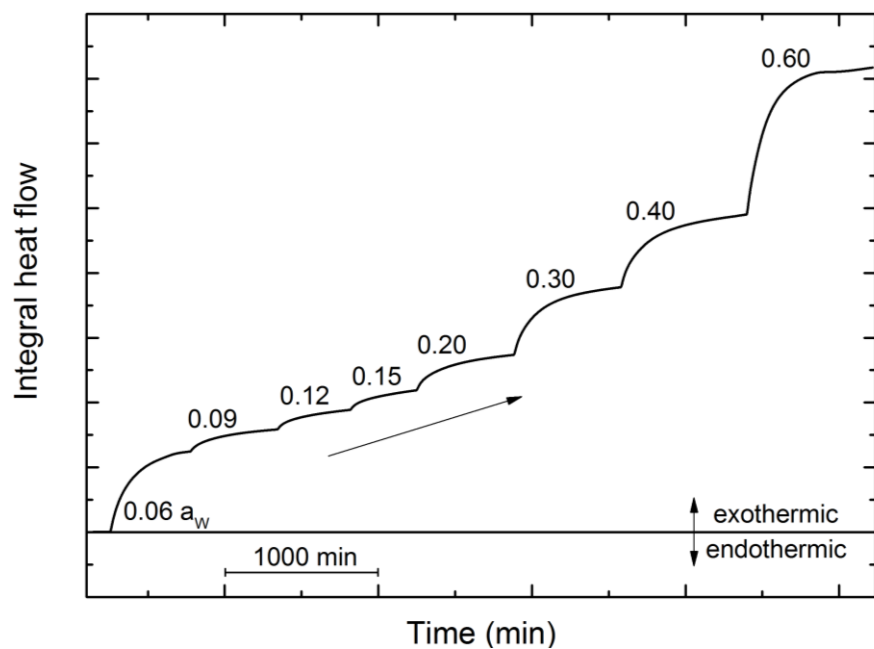


Figure 64 A typical integral heat flow curve (in arbitrary units) of the interval water vapour sorption experiment of BSA at 293.15 K.

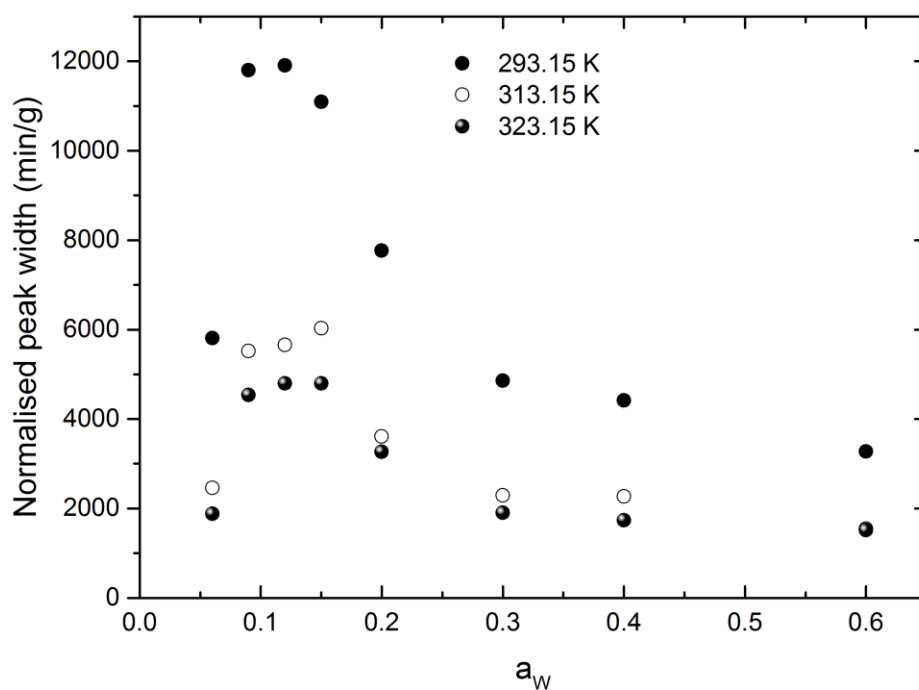


Figure 65 Normalised peak width of the heat flow curve as a function of water activity and temperature for the system water/BSA. $n = 3$; Error bars smaller than symbol size.

‘thermodynamic path’ of the gravimetric sorption experiment is the same as in the calorimetric measurement. This implies, both experiments are isothermal with regards to the sorption temperature (that requires heat reservoirs to be sufficiently large), and the time scales of sorption at each activity interval have to be similar.

Similarly to the kinetic analysis of the gravimetrically studied water vapour sorption process, basic kinetic indicators of the calorimetrically studied water vapour sorption process can be computed. As the two different experiments were carried out in experimental modes to complement one another and were carried out on similar time scales (i.e. time spent per activity interval), a comparison between the calorimetric and gravimetric measurements is thought to be reasonable.

Figure 65 shows the peak width of the heat flow of each activity increment. The data points are normalised by the initial concentration gradient at each interval, which is the driving force for the mass transfer process. The peak width decreases with increasing temperature making the heat event a faster process at elevated temperatures. For low hydration (up to $\approx 0.20 a_w$), the peaks are broad. For higher levels of hydration, the peaks become noticeably narrower indicating a faster thermal event. A similar trend was suggested on the basis of gravimetric sorption data (cf. Figure 35).

Figure 66 shows the concentration dependence of the heat of sorption subtracted by the heat of water condensation. It is common practise (e.g. [13]) to assume

$$\Delta H_{sorption}(P, T, \phi_1) = \Delta H_{mix}(P, T, \phi_1) + \Delta H_{condensation}(P, T, \phi_1) \quad (4.1)$$

The enthalpies presented now are molar enthalpies of mixing (ΔH_{mix}) of liquid water with BSA rather than molar enthalpies of sorption ($\Delta H_{sorption}$) of water from vapour. The heat of mixing (ΔH_{mix}) is defined as the heat that is taken up or released during the mixing process of two or more non-reacting chemical substances. The molar heat of sorption ($\Delta H_{sorption}$) is the

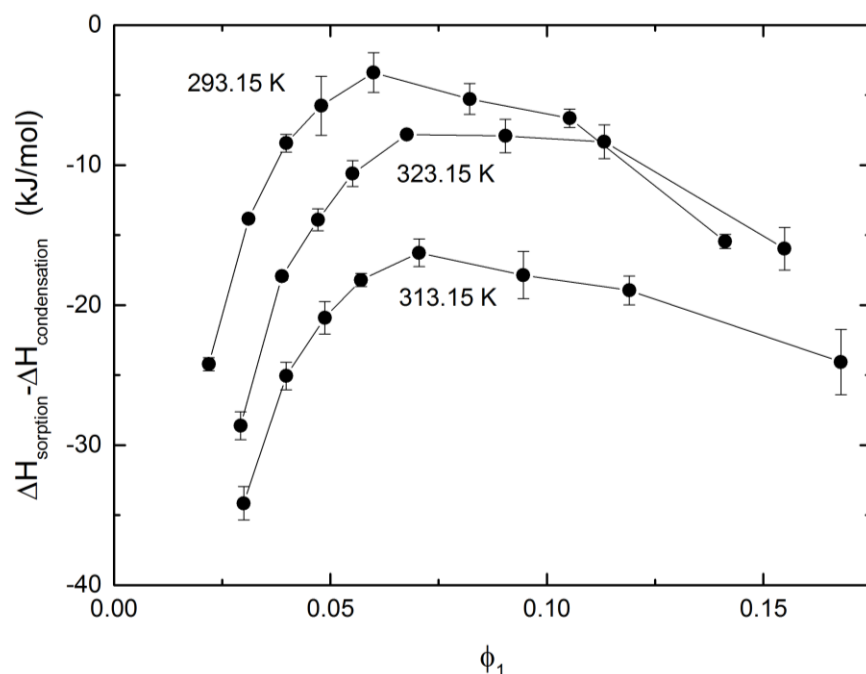


Figure 66 Heat of mixing of water/BSA as a function of water concentration and temperature. $n = 3$.

experimentally determined heat change upon an water activity step increase during the calorimetric experiment.

There is no comparable data published for the system water/BSA in a similar experimental mode. Though, water sorption enthalpies are within the same order of magnitude for common water/protein systems. As established in Chapters 2 and 3, water sorption in BSA is highly dependent on the experimental path. This dependency also applies to the thermal events caused by water sorption.

The enthalpy curves can be divided into two parts corresponding to different regimes of sorption. This division of the hydration process into several regimes has been proposed before [8, 12, 22, 23] but it was only the work of Kocherbitov and co-workers [13, 24, 25] during their work on lysozyme hydration that pointed away from site heterogeneity as the basis for the division and towards aggregation properties and the glass transition event instead.

Regime 1: $0.00 < \phi_1 \leq 0.06$. The main features of regime 1 are highly exothermic ΔH_{mix} and slow sorption of water. The slow sorption is counterintuitive and to explain this phenomenon not just protein–water but also protein–protein interactions need to be considered. Protein–protein interactions could be manifested in the form of hydrophobic association, hydrogen bonds between polar elements and backbone atoms, or salt bridges (ionic interactions) between oppositely charged amino acid side chains. As BSA is glassy at low water contents and within the temperature range studied, it can be expected that molecular voids are present between the individual protein molecules. In regime 1, the water contents are not high enough to fill these voids forcing the protein molecule to undergo slow conformational transitions in order to reduce the volume of the system. The slow sorption kinetics in this regime have already been discussed (cf. section 3.3.1) in the light of gravimetric results and are typical for glassy systems. Values for ΔH_{mix} decrease up to a local minimum in the magnitude of ΔH_{mix} which signifies the boundary between regime 1 and 2.

Regime 2: $\phi_1 > 0.06$. The main features of regime 2 are exothermic and increasing ΔH_{mix} , high water solubility, and a decreasing heat flow peak width. After regime 1, very hydrophilic and polar sites of the protein molecules are hydrated (both on the surface as well as within the protein interior). This makes the protein–protein interactions weaker while the protein–water interactions do not change much (as hydration is low). In regime 2, it is where activities are higher and significant amount of water is sorbed causing the system to gradually loose its glassy characteristics. As sorption is faster in this regime (i.e. peak width is decreasing), it can be expected that conformational response of the protein to water is occurring either faster or to a lesser degree relative to regime 1. The conformational changes/volume contractions were expected to occur at an expense of the enthalpic driving forces of the sorption process. Therefore, ΔH_{mix} is tending towards more negative values again.

4.3.2 Kinetic analysis of heat of sorption time evolution data

Figures 67 to 69 offer a comparison between the integrated heat flow time evolution data and the mass uptake (during interval sorption) corresponding to an activity change $0.06 \rightarrow 0.09 a_w$, as a function of time at the three temperatures. (For a more complete picture see additional data in Appendix 4.6.1.) To our best knowledge, this type of comparison for water sorption in glassy polymers has not been reported previously.

For a sufficiently small activity interval change and a rubbery polymer, the two curves are expected to superimpose as the heat event would be controlled by the Fickian sorption process. However, it has been established in previous chapters that the water/BSA system is (a) glassy at low hydration levels, (b) water sorption is non-Fickian, and (c) the BSA matrix undergoes structural changes during sorption with long times-to-equilibrium. Therefore, the fact that the two curves do not superimpose ordinarily can be interpreted as a manifestation of competing exothermic and endothermic events during sorption.

In general, many features of the sorption curves are reflected in the integral heat curves. For small t , the difference between the dimensionless mass uptake and integral heat curve decreases with increasing temperature and hydration level. This means that the endothermic contribution to the process is less dominating at small t . So, if protein relaxations were an endothermic process, than this observed trend might point towards a process that is dominated by an entropic contribution. Usually, the higher the temperature, the more favoured are endothermic processes (if the entropy contribution was only small or independent of temperature). Moreover, it was observed, only for large t , the two curves tend towards superimposition.

Overshooting integral heat curves are a manifestation of endothermic processes for large t . It is interesting that these curves are not accompanied by overshooting sorption curves during experimental time scales and conditions. The overshooting integral heat feature appears in systems

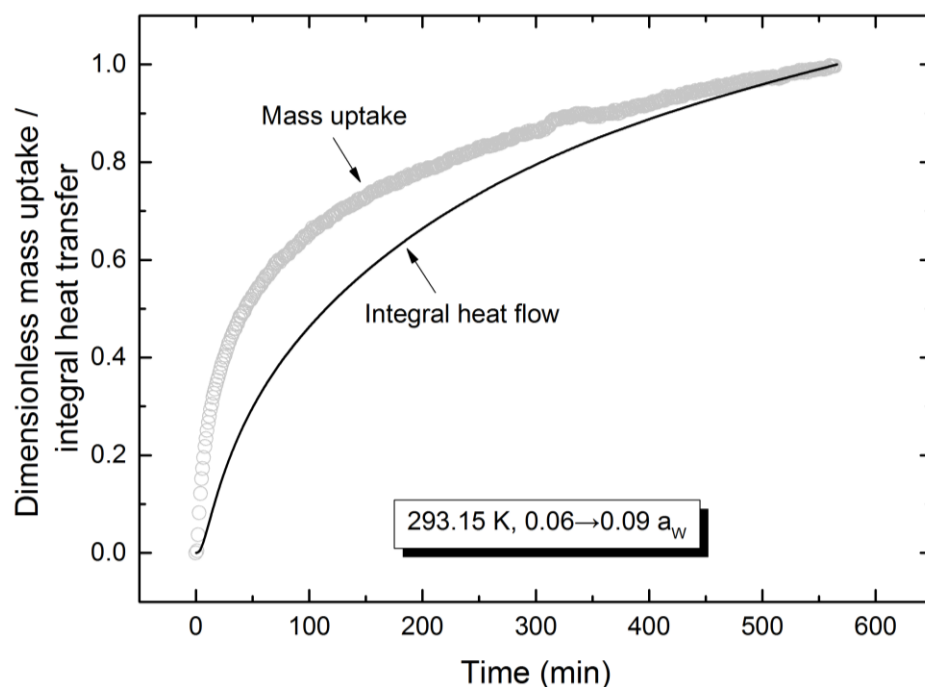


Figure 67 Normalised integral heat flow and mass uptake during interval water sorption of BSA at 293.15 K for an activity change of 0.06 → 0.09 a_w .

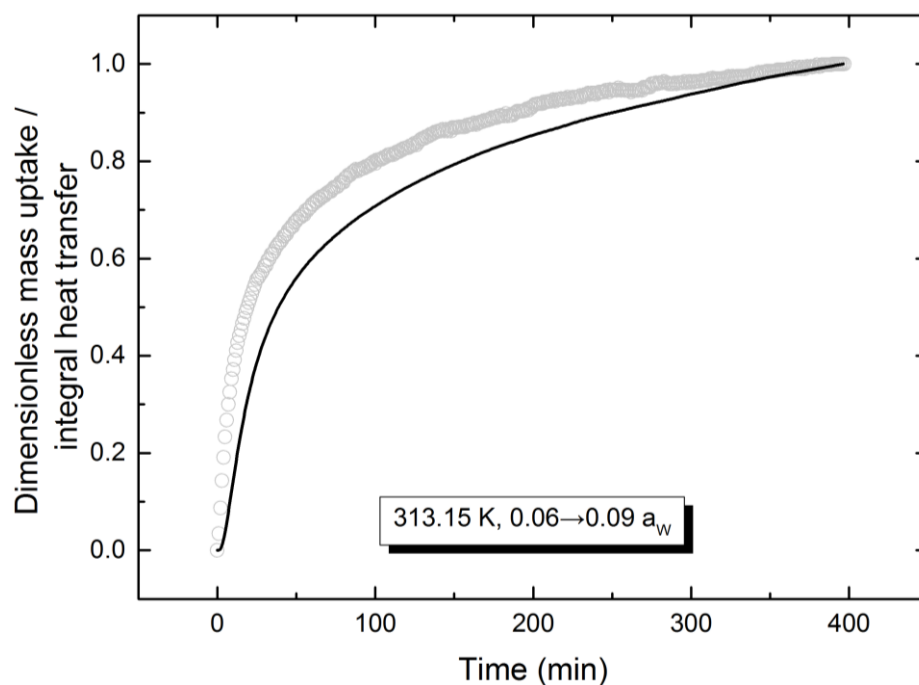


Figure 68 Normalised integral heat flow and mass uptake during interval water sorption of BSA at 313.15 K for an activity change of 0.06 → 0.09 a_w .

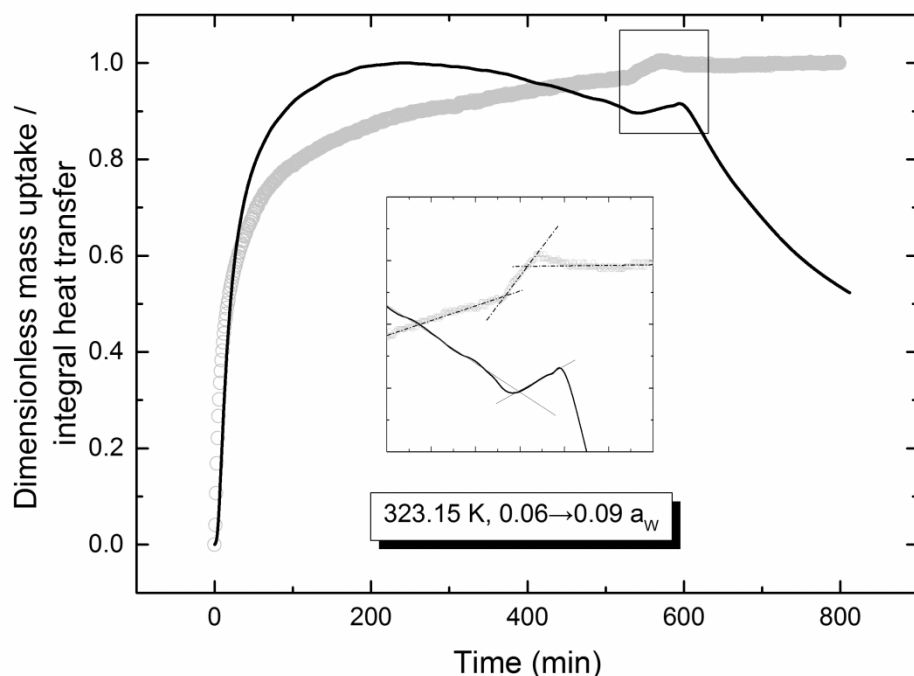


Figure 69 Normalised integral heat flow and mass uptake during interval water sorption of BSA at 323.15K for an activity change of $0.06 \rightarrow 0.09 a_w$.

at elevated temperatures and/or intermediate sorption levels but not within the activity range presented here at 293.15 K.

Another interesting feature is found in Figure 69 for water vapour sorption into BSA at an activity increment from 0.06 to $0.09a_w$ at 323.15 K. The mass uptake curve from the gravimetric measurement shows a smooth step-wise increment with an onset about 530 *min* after the activity increment (inset of Figure 69). Unlike in Figure 67, where an insignificant ‘bump’ was observed around 300 *min*, there are two important findings from this feature.

Firstly, the calorimetric experiment mimics the gravimetric measurement excellently. Otherwise, the feature would not be manifested in both signals. Whilst there is uncertainty as to how to evaluate the onset time of this feature for both experiments, a simple tangent construction suggests similar onset times (529 *min* for gravimetric experiment, 533 *min* for calorimetric experiment).

Secondly, even at very low hydration, sorbing water has a pronounced impact on protein characteristics and structure. It can be expected that the water at this hydration level (0.01864 g/g at $0.06a_w$) is strongly bound to the protein at high energy sites and voids and, therefore, it seems unlikely that this feature arises from some higher-order phase transition in the ‘water phase’. It is interpreted as a conformational change/response of the BSA molecules to water sorption.

4.3.3 Thermodynamic excess functions of hydration

In the thermodynamics applied to solutions of nonelectrolytes or macromolecules it is often preferred to describe their deviation from ideal solution behaviour (or indeed any reference state) by excess thermodynamic functions. The term originated from the work of Scatchard and Hamer [26]. This type of analysis has been carried out for water–protein systems by Sirotkin and co-workers [15-18].

A thermodynamic function of a real binary system can be described by an excess function Z^E as the difference between the observed thermodynamic function Z^M and that for an ideal binary mixture (or reference state) Z_{id}^M according to:

$$Z(P, T, w_i)^E = Z(P, T, w_i)^M - Z(P, T, w_i)_{id}^M \quad (4.2)$$

Any deviation from zero quantifies the extent to which the real binary system is non-ideal. This variation may arise from specific interactions between its components (e.g. hydrogen bonding, charge–charge interactions). For a binary system, the values of Z^E are composed of two contributions:

$$Z^E(P, T, w_1) = w_1 \bar{Z}_1^E(P, T) + w_2 \bar{Z}_2^E(P, T) \quad (4.3)$$

where w_i denotes the weight fractions of components 1 (water) and component 2 (protein) and $w_1 + w_2 = 1$.

Excess partial enthalpies. The measured interaction enthalpy during water sorption into bovine serum albumin can be treated as the excess enthalpy ΔH^E . The value for H^E is made up by the enthalpies of the solution of dried and hydrated protein in pure liquid water (here at atmospheric pressure):

$$H^E = w_1[\bar{H}_{w,protein}^h - \bar{H}_{w,liquid}] + w_2[H_P^h - H_P^\ominus] \quad (4.4)$$

where $\bar{H}_{w,protein}^h$ is the partial enthalpy of water bound to the protein, $\bar{H}_{w,liquid}$ is the partial enthalpy of pure liquid water, H_P^h is the partial enthalpy of the hydrated protein, and $H_P^\ominus$ is the partial enthalpy of the dried protein, all at the reference temperature and pressure.

The excess partial molar enthalpies of the water and protein may be calculated from experimental data according to

$$\bar{Z}_1 = Z^E - w_2 \left(\frac{\partial Z^E}{\partial w_2} \right)_{T,P} = \bar{H}_1^E \quad (4.5)$$

$$\bar{Z}_2 = Z^E - w_1 \left(\frac{\partial Z^E}{\partial w_1} \right)_{T,P} = \bar{H}_2^E \quad (4.6)$$

at a given P, T . Here, Z^E is the excess property of interest, e.g. excess enthalpy as H^E . The excess enthalpy is defined as the difference between the actual interaction enthalpy H and the interaction enthalpy of an ideal solution, $\sum_i H_i x_i$, where H_i is the pure-component molar enthalpy. Therefore, it is clear that $H^E = H - \sum_i H_i x_i$ denotes the heat of mixing. The partial molar excess enthalpies at any composition x_i can then be determined.

Excess partial Gibbs energies. The Gibbs-Duhem equation can be applied to calculate the excess Gibbs free energies. For a binary mixture, the Gibbs-Duhem relation yields:

$$n_1 d\mu_1 + n_2 d\mu_2 = 0 \quad (4.7)$$

where μ_1 and μ_2 are the partial molar chemical potential of the water and protein, respectively.

At constant temperature and pressure:

$$d\mu_i = RTd(\ln a_i) \quad (4.8)$$

where a_i is the activity of water or protein in the binary mixture. So, from Equation 4.7 and 4.8 one can write:

$$d(\ln a_2) = -\frac{1-x_2}{x_2}d(\ln a_1) \quad (4.9)$$

where x_i denotes the mole fraction of water or protein.

The activity coefficients, γ_i , are given by:

$$a_i = x_i\gamma_i \quad (4.10)$$

For an ideal system ($\gamma_i \equiv 1$), Equation 4.9 can be written as:

$$d(\ln x_2) = -\frac{1-x_2}{x_2}d(\ln(1-x_2)) \quad (4.11)$$

For a real (non-ideal) system, Equation 4.9 can be written as:

$$d(\ln \gamma_2) + d(\ln x_2) = -\frac{1-x_2}{x_2}d(\ln \gamma_1) - \frac{1-x_2}{x_2}d(\ln(1-x_2)) \quad (4.12)$$

Subtracting Equation 4.11 from Equation 4.12 yields:

$$d(\ln \gamma_2) = -\frac{1-x_2}{x_2}d(\ln \gamma_1) \quad (4.13)$$

Integrating Equation 4.13 from $\ln \gamma_1^0$ at $x_2 = 1$ to $\ln \gamma_0$ at x_2^* gives:

$$\ln \gamma_2 = - \int_{\ln \gamma_1^0}^{\ln \gamma_1} \frac{1-x_2}{x_2} d(\ln \gamma_1) = - \int_{\ln \gamma_1^0}^{\ln \gamma_1} \frac{n_1}{n_2} d(\ln \gamma_1) \quad (4.14)$$

Now, the excess partial Gibbs free energy of water and protein, respectively, can be calculated according to Equations 4.15 and 4.16:

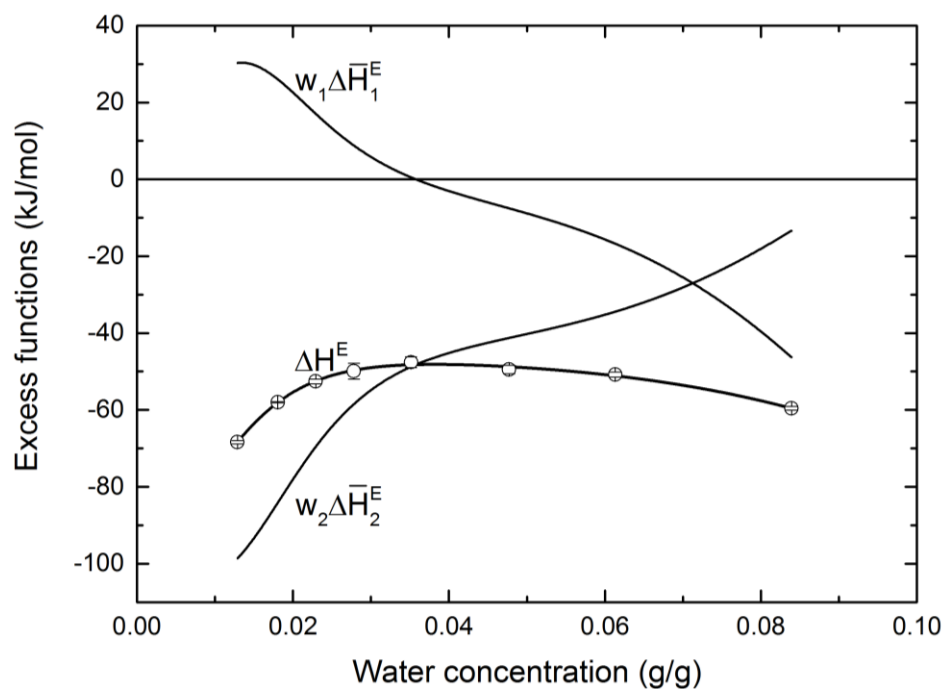


Figure 70 Thermodynamic excess enthalpies for the system water/BSA at 293.15 K. (○)
Experimental heat of sorption.

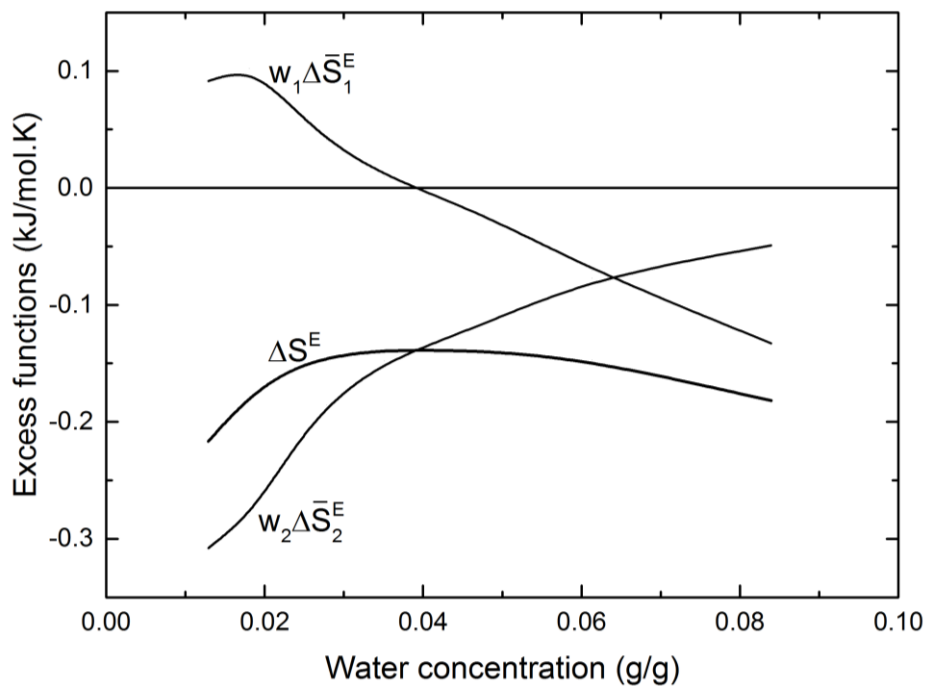


Figure 71 Thermodynamic excess entropies for the system water/BSA at 293.15 K.

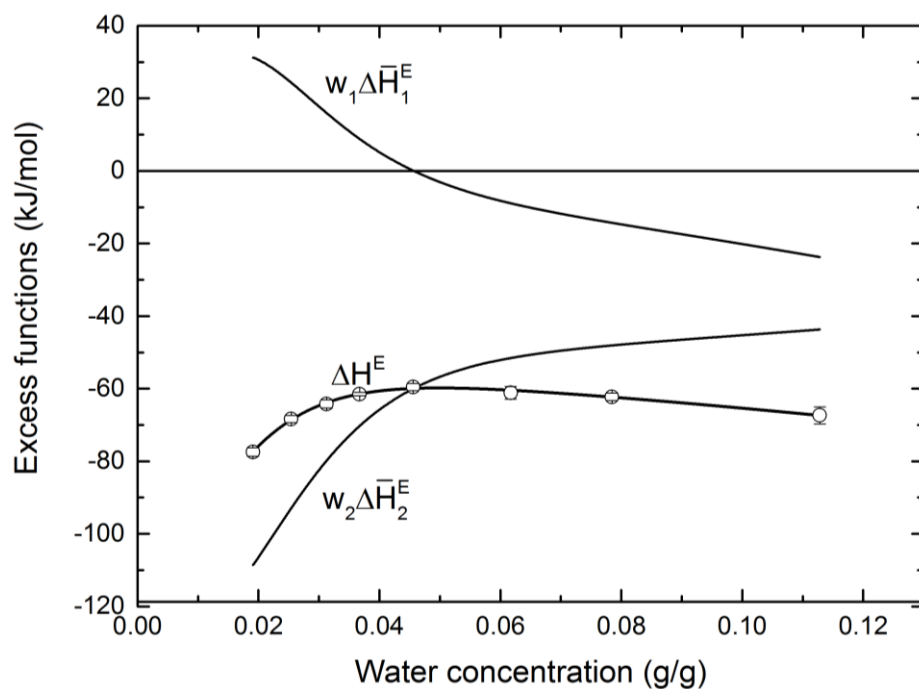


Figure 72 Thermodynamic excess enthalpies for the system water/BSA at 313.15 K. (○)

Experimental heat of sorption

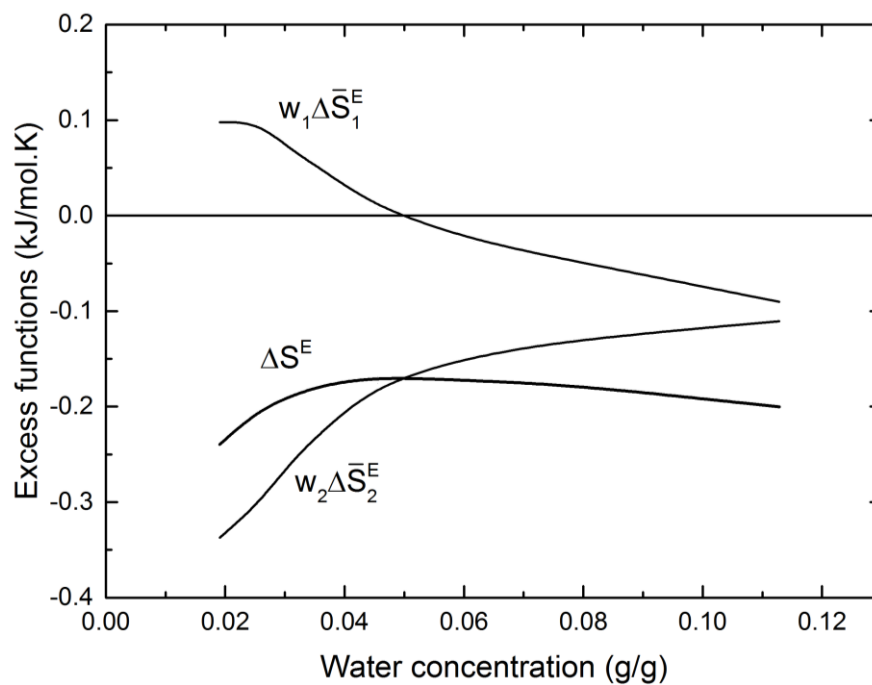


Figure 73 Thermodynamic excess entropies for the system water/BSA at 313.15 K.

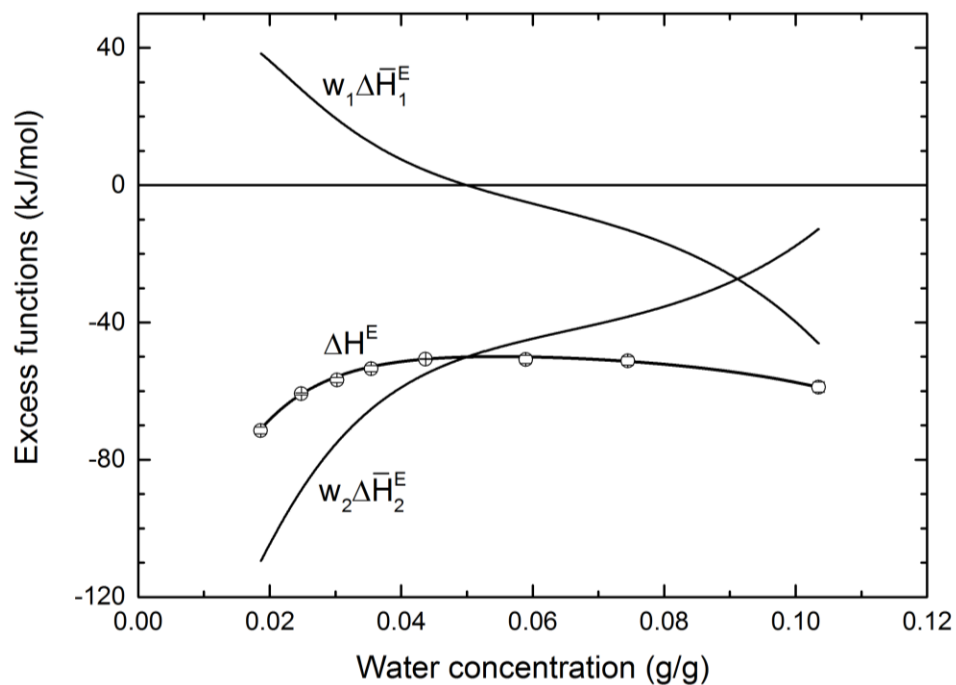


Figure 74 Thermodynamic excess enthalpies for the system water/BSA at 323.15 K. (○)

Experimental heat of sorption

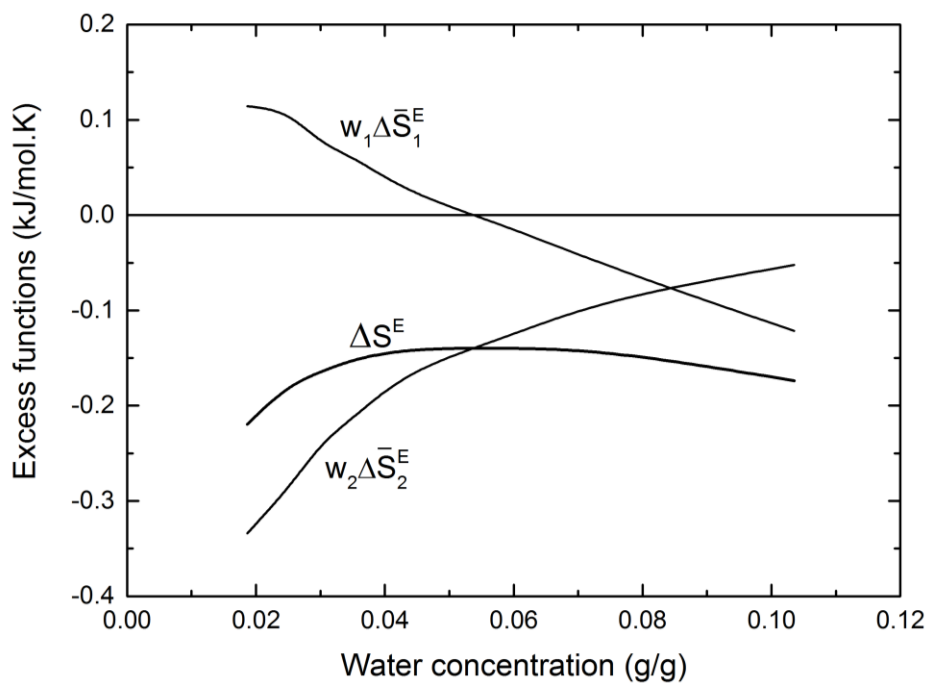


Figure 75 Thermodynamic excess entropies for the system water/BSA at 323.15 K.

$$\mu_2^E = - \int_{-\infty}^{\mu_1^E} \frac{n_1 M_1}{n_2 M_2} d(\mu_1^E) \quad (4.15)$$

$$\mu_1^E = \frac{RT}{M_1} \ln \gamma_1 \quad (4.16)$$

where μ_1^E and μ_2^E denote the partial excess Gibbs energy of water and protein, respectively; M_1 and M_2 denote the molecular weight of water and protein, respectively.

The excess Gibbs energy of mixing can be calculated from Equation 4.17:

$$G^E = w_1 \mu_1^E + w_2 \mu_2^E \quad (4.17)$$

Excess partial entropies. The partial excess entropies can be calculated according to Equations 4.18 to 4.20:

$$S^E = \frac{H^E - G^E}{T} \quad (4.18)$$

$$\bar{S}_1^E = S^E - w_2 \left(\frac{\partial S^E}{\partial w_2} \right)_{T,P} \quad (4.19)$$

$$\bar{S}_2^E = S^E - w_1 \left(\frac{\partial S^E}{\partial w_1} \right)_{T,P} \quad (4.20)$$

where $\bar{S}_1^E$ and $\bar{S}_2^E$ are the excess partial entropies of water and protein.

Figures 70, 72, and 74 show the dependency of the ΔH^E values on the hydration level of bovine serum albumin BSA at 293.15, 313.15 and 323.15 K. In order to carry out the excess function analysis, the experimental heats have to be smoothened or fitted by some phenomenological function. Without any physical basis, an asymmetric double sigmoidal peak function was employed and found to represent the data within the experimental errors. The ΔH^E values were estimated using Equation 4.3 and as outlined above. The $\Delta \bar{H}_1^E$ and $\Delta \bar{H}_2^E$ were calculated using Equations 4.5 and 4.6, respectively. Figures 71, 73, and 75 show the corresponding hydration level dependence of the ΔS^E values. The $\Delta \bar{S}_1^E$ and $\Delta \bar{S}_2^E$ were calculated using Equations 4.18 to 4.20.

In an ideal binary mixture of components 1 and 2, the average 1–2 interactions are the same as the average 1–1 and 2–2 interactions. For non-ideal mixtures, the three types of interactions have different strengths. As shown, the ΔH^E values differ significantly from zero pointing towards the non-ideality of the 1–2 (water–protein) interaction. Overall, the process was found enthalpically favourable ($\Delta H < 0$) and entropically unfavourable ($\Delta S < 0$). This behaviour is typical for a hydrophilic hydration process ($\equiv \Delta \bar{H}^E, \Delta \bar{S}^E < 0$). As the process is occurring spontaneously ($\Delta G < 0$), it can be suggested that the process is in enthalpy-control.

Both the partial enthalpy and entropy excess functions depend most strongly upon water content especially at low water contents. This analysis could be verified by an inspection of the first-order derivative of these functions with respect to concentration at constant temperature and pressure. (Not shown here.)

Due to limitations imposed by the experimental set-up, it was not possible to determine the pure-component limits (or measure very close to them) of the heat of mixing, that is in particular as $\phi_1 \rightarrow 0$. It has to be pointed out that the curves shown in Figures 70 to 75 should all meet the origin at $\phi_1 = 0$ by definition.

A division of the sorption process into distinct regions similar to that employed in the previous section can now be made on the basis of partial molar excess functions. The observed maximum in

Table 5 Thermophysical properties of pure saturated water from reference [27].

Temperature	Enthalpy of condensation	Entropy of condensation
<i>K</i>	<i>kJ/mol</i>	<i>kJ/mol·K</i>
293.15	-44.1953	-0.15078
313.15	-43.3438	-0.14439
323.15	-42.9117	-0.13279

ΔH^E , that is present at the three temperatures at between 0.04 to 0.06 g/g, is interpreted as the point where the way water interacts with the amorphous protein changes fundamentally. Pikal and co-workers [14], who measured the enthalpy of desorption for BSA at 293.15 K, detected a minimum in their heat of desorption data of BSA at a water concentration of about 0.17 g/g. As it is understood from the investigation into the different modes of water sorption (see Chapters 2 and 3) it has to be pointed out that the significant difference between Pikal and co-workers' desorption minimum and the here presented sorption maximum of the heats of sorption is in line with our reasoning.

At low hydration levels ($w_1 \approx 0 - 0.05$), the values for $\Delta \bar{H}_1^E$ are positive (thermodynamically unfavourable) while values for $\Delta \bar{H}_2^E$ are negative (thermodynamically favourable). Similarly, values for $\Delta \bar{S}_1^E$ are positive (thermodynamically favourable) while values for $\Delta \bar{S}_2^E$ are negative (thermodynamically unfavourable).

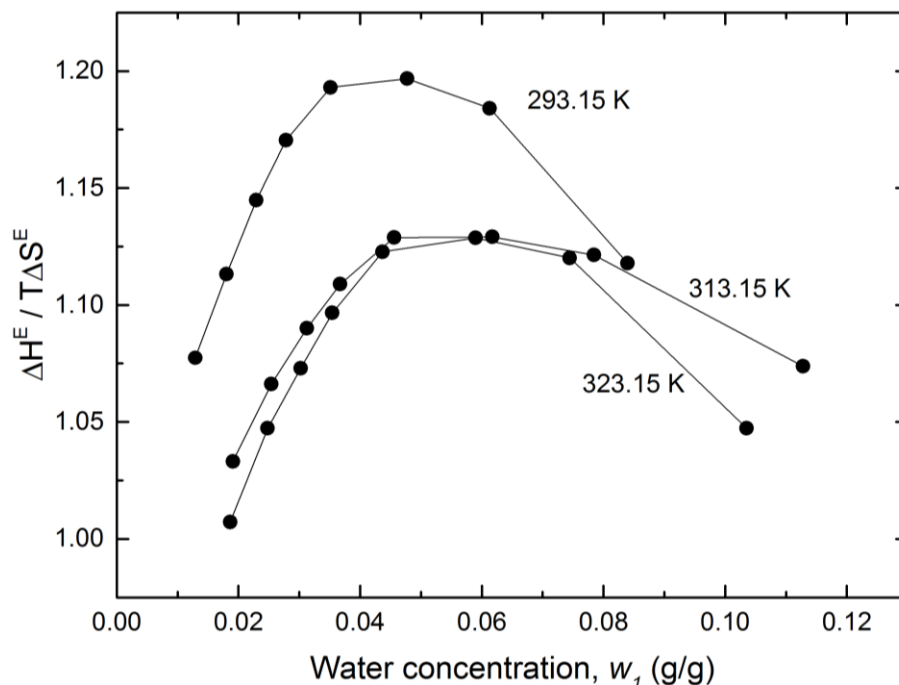


Figure 76 Enthalpy/Entropy ratio for interval water vapour sorption of BSA at different temperatures.

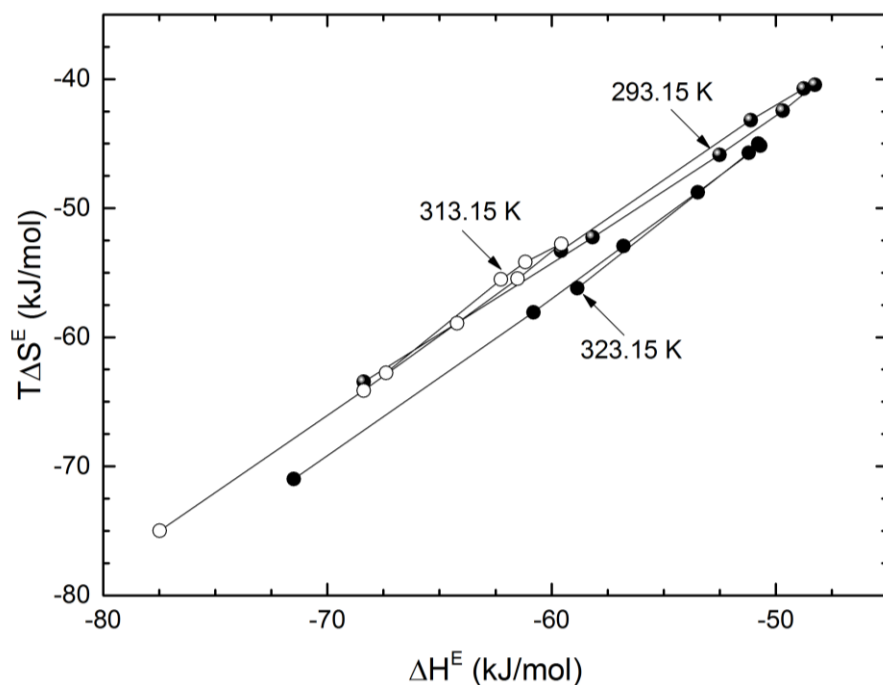


Figure 77 Enthalpy–entropy plane of the system water/BSA at different temperatures.

At these water contents, and within the experimental temperature range, the protein can be expected to be in the glassy (rigid) state. [2] In the glassy state, there are strong restrictions on conformational transitions as the protein is more or less frozen into a wide distribution of conformational states which correlates to the negative entropic (i.e. unfavourable) contribution of $\Delta\bar{S}_2^E$. The positive entropic contribution by the partial excess entropy function for water, $\Delta\bar{S}_1^E$, is common for systems that undergo intramolecular hydrogen bonding.

The dominating contribution of the partial molar excess entropy ($\Delta\bar{S}_2^E$) and enthalpy ($\Delta\bar{H}_2^E$) function for the protein over the contributions by the excess functions of water ($\Delta\bar{S}_1^E$ and $\Delta\bar{H}_1^E$) suggests that the total changes in the excess thermodynamic functions are governed by dynamic changes within the protein itself. It is not merely the addition of water reflecting these changes. In this concentration range, hydration of charged functional groups occurs. [3]

At moderate hydration levels ($w_1 > 0.05$), hydration of polar groups occurs typically up to a water hydration levels of 0.25. In this concentration range, the values for both $\Delta\bar{H}_1^E$ and $\Delta\bar{S}_1^E$ are negative and decrease with increasing concentration. This transition corresponds to the formation of a spanning, three-dimensional hydrogen bond network of water molecules. It is expected that this process is enthalpically favourable at the expense of entropy. Hence, unfavourable values for $\Delta\bar{S}_1^E$ were predicted. It is believed that the formation of this hydrogen bonded water network occurs as a quasi-two-dimensional percolation transition of the hydration water at the protein surface [28]. Interestingly, the values for both $w_1\Delta\bar{H}_1^E$ and $w_1\Delta\bar{S}_1^E$ approach those of the vapour-liquid condensation process (cf. Figures 70-75). Table 1 lists thermo-physical properties of pure water. Whilst outside the concentration range of the calorimetric study, it is expected that ‘structured’ water [29] with similar thermodynamic properties to that of bulk water is formed at higher water concentrations.

Figures 70 to 75 show that the excess enthalpy and entropy curves have distinctive slopes compensating for each other. This feature is an indicator for the presence of enthalpy–entropy compensation. Figure 76 shows a plot of $\Delta\bar{H}^E/T\Delta\bar{S}^E$ as a function of water concentration. Within the temperature and concentration range studied, $\Delta\bar{H}^E/T\Delta\bar{S}^E \geq 1.0$ indicating that the hydrophilic hydration process is enthalpy controlled.

Figure 77 shows a plot of $T\Delta S^E$ against ΔH^E as estimated from the calorimetric analysis. A linear fit of the individual data sets (when plotting $\Delta H^E(T)$ against $\Delta S^E(T)$) would yield the ‘compensation temperature’ ($\hat{\beta}$) as the slope and the Gibbs free energy of compensation as the intercept. $\hat{\beta}$ was found to lie within the range of 250 – 260 K. This result lies within the range of hydration of proteins and small molecules ($\hat{\beta} = 250 - 315$ K) as reported by Lumry and Rajender

[29]. They suggested that the observed enthalpy–entropy compensation process arises from a coupling of protein processes to water via the expansions or contractions of the protein.

Furthermore, it has to be questioned whether the suggested enthalpy–entropy compensation is purely a manifestation of the heterogeneity of the sorption sites or whether true thermodynamic compensation takes place. The interaction energies of water with different amino acid groups vary in strength. [19] When water is absorbed at a high energy site (such as an ionisable group), the hydration energy is strongly exothermic. However, the probability to find such site is lower, particularly with increasing concentration. That implies that the entropy effect at this site is strongly negative. When water is absorbed at a low energy site, the hydration energy is higher (less exothermic). The entropy of hydration that corresponds to such site is higher because the number of such sites is typically larger. Therefore, one could think that the observed enthalpy–entropy plane is a manifestation of site heterogeneity. Though, on the basis of Figure 77 and experimental evidence by Kocherbitov and Arnebrant [19], it can be suggested that the mechanism of enthalpy–entropy compensation dominates considerations over the heterogeneity of sorption sites.

4.3.4 Deviations from the van-Laar/Flory-Huggins theory

The calorimetrically measured heats of sorption contain information on the binary interactions, the general Flory–Huggins theory should reflect (or even quantitatively reproduce) the observed trends. The binary interaction parameter in the Flory–Huggins theory can be thought of as a dimensionless excess property as it describes the deviation from athermal mixing of the binary mixture. Whilst the van-Laar theory is more than one-hundred years old and approaching it might be seen as driven by a purely historic interest, it is widely used to date. In the Flory–Huggins theory, the heat of mixing (i.e. the departure from athermal mixing) is given by the van-Laar equation:

$$\Delta H_m = kTN_1\phi_2\chi_{1,2} \quad (4.21)$$

In this sense, a heat of mixing can be calculated according to the van-Laar/Flory–Huggins theory. Figure 78 shows the difference ($\Delta H^E - \Delta H_{van-Laar}$) as a function of water volume fraction where ΔH^E is the calorimetrically determined excess enthalpy of the sorption process and $\Delta H_{van-Laar}$ denotes the from regressed interaction parameters (cf. section 2.3.7) predicted heats of mixing. This difference describes the departure of the real water/BSA system from simple van-Laar mixing. A significant difference was found. This difference is concentration dependent and resembles the shape of the calorimetrically measured enthalpies (ΔH^E). Recalling the results on the temperature dependence of the sorption isotherms for water/BSA (cf. section 2.3.1), where the solubility of water in BSA increased according to $293.15 < 323.15 < 313.15$ K, it can be concluded: the higher the solubility of water at a given activity, the greater the difference ($\Delta H^E - \Delta H_{van-Laar}$). This suggests that the van-Laar/Flory–Huggins theory cannot account for the real interactions of a protein with a polar solvent such as water. Specific interactions, which are *per se* not considered in the deviation of the Flory–Huggins theory, lead to the disagreement between the measured and predicted enthalpies. To illustrate the implications of this finding, the enthalpy–entropy plane for the system water/BSA was plotted again in Figure 79. Here, the entropy is the combinatorial Flory–Huggins entropy of mixing and the enthalpies are either the calorimetrically determined excess enthalpies (ΔH^E) or the van-Laar heat of mixings ($\Delta H_{van-Laar}$) as predicted from the isotherm regressions (via χ). Both representations vary significantly with the calorimetrically observed enthalpy–entropy compensation (cf. Figure 77). Indeed, both analyses suggest a very different energetic landscape of the process. It can be proposed, that the

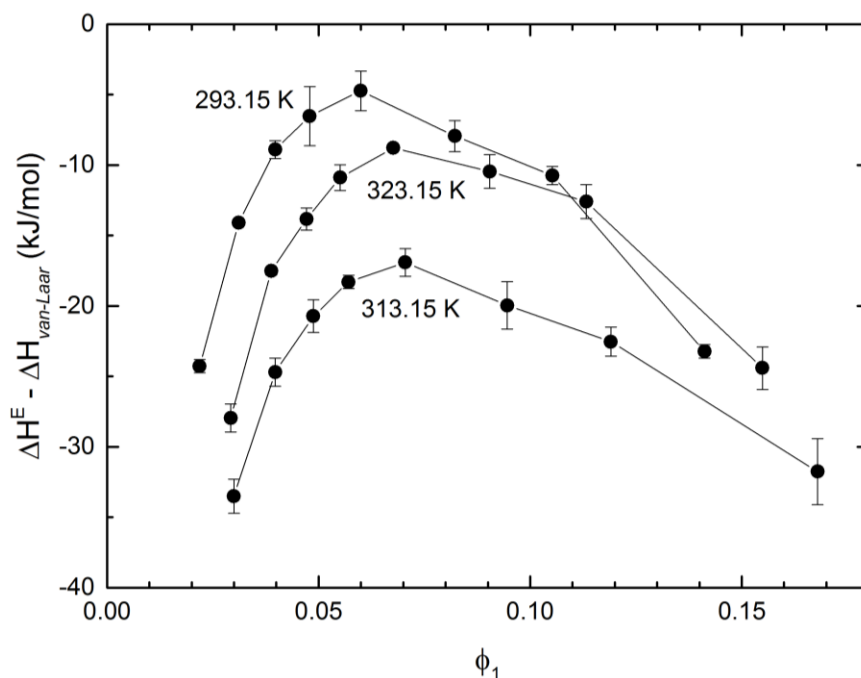


Figure 78 Difference between experimentally estimated heat of mixing and van-Laar enthalpy for water/BSA at different temperatures.

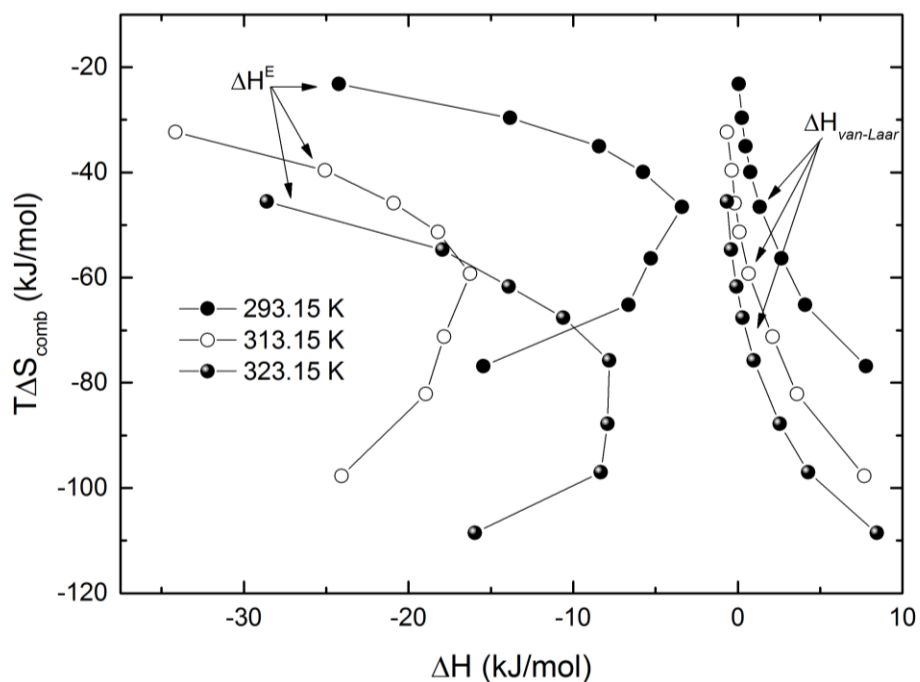


Figure 79 Enthalpy–entropy plane for combinatorial mixing with van-Laar heat and experimentally measured ones for the system water/BSA.

combinatorial Flory–Huggins mixing, which gives the formulation of the entropy in the Flory–Huggins theory, does not adequately represent the water/BSA system. There appears to be a fundamental problem with the way both the enthalpy as well as the entropy of mixing are defined in the Flory–Huggins theory. A problem already pointed out by Wolf and co-workers [30-34].

In the Flory–Huggins theory, the change in the enthalpy of mixing is that only arising from the formation of solvent–polymer contacts, which replace some of the old solvent–solvent or polymer–polymer contacts. Thus, the corresponding change in internal energy of the system upon mixing is given by:

$$\Delta U_{mix} = q_{12}\Delta\varepsilon_{12} \quad (4.22)$$

where

$$\Delta\varepsilon_{12} = \varepsilon_{12} - \frac{1}{2}(\varepsilon_{11} + \varepsilon_{22}) \quad (4.23)$$

Here, q_{12} denotes the number of newly formed contacts during mixing, ε_{ij} denotes the respective energy involved in the formation of a contact between the components i and j . Under the assumption of $\Delta V_{mix} = 0$ and making use of $H = U + PV$, the enthalpy of mixing can be expressed as:

$$\Delta H_{mix} = q_{12}\Delta\varepsilon_{12} \quad (4.24)$$

Assuming that the probability for a given lattice sites to be occupied by a solvent molecule is equal to its volume fraction, N_2 polymer molecules are surrounded by $N_2\phi_1mz$ solvent molecules. This represents the number of contacts q_{12} . Hence, the total change in enthalpy of mixing is given by:

$$\Delta H_{mix} = N_2\phi_1mz\Delta\varepsilon_{12} = N_1(\phi_2z)\Delta\varepsilon_{12} \quad (4.25)$$

In order to avoid using the co-ordination number z , which is difficult to measure in a real mixture, the binary interaction parameter was introduced:

$$\chi_{1,2} = \frac{z\Delta\varepsilon_{12}}{kT} \quad (4.26)$$

Recalling the derivation of $\chi_{1,2}$ (Equations 4.22 to 4.26) allows a deeper understanding of the problem between Flory–Huggins predictions and measured enthalpies. The assumption of no mixing volume change ($\Delta V_{mix} = 0$) is thought to be the predominant source of error here.

Without going into the derivation of the entropy of mixing it has to be pointed out that there are two major simplifications that are thought to contribute to the discrepancy in predicted and observed solution behaviour: (1) the size of each polymer segment is the same as that of a solvent molecule so that $m = v_2/v_1$ where v_i is the molar volume of component 1 and 2; (2) all lattice sites must be occupied making the lattice incompressible.

Having established this fundamental discrepancy, it is now interesting to understand the impact of the discrepancy between theory and experiment. Both traditional as well as more advanced treatments of the binary Flory–Huggins interaction parameter use the χ -parameter as what was thought to be a meaningful quantity in interpreting solvent–polymer phase behaviour. It is the purpose of the following paragraphs to explore the types of misleading molecular information that would be extracted from a purely theoretical analysis.

As differences between theoretical predictions and observed phase behaviour appeared, Flory postulated [35] that a contribution to the entropy of mixing (due to reorientation effects in the mixture) should be considered in the sense of Equations 4.27 to 4.29

$$\pi = \pi_H + \pi_S \quad (4.27)$$

$$\pi_H = -T \left(\frac{\partial \pi}{\partial T} \right) \quad (4.28)$$

$$\pi_S = \pi - \pi_H \quad (4.29)$$

where π is a dummy variable. By plotting χ as a function of the inverse temperature for different constant concentrations, one can obtain such information. The results of this analysis are presented in Figures 80. Despite quantitative discrepancies that are carried within the values of χ , it should

be interesting to see what kind of insight can be gained by carrying out this analysis, or whether false conclusions would arise.

The enthalpic contribution, displayed in the upper part of Figure 80, demonstrates the following features. Between 293.15 and 323.15 K, the enthalpic contribution decreases from positive (endothermic) values to negative (exothermic) values as temperature increases. For the experiment at 283.15 K, a different behaviour is observed not just suggesting an exothermic heat of mixing for most of the water activities but more importantly, that there might be an energetic/mechanistic change of the sorption process somewhere between 283.15 and 293.15 K. The enthalpic contribution seems to be unfavourably large for the system water/BSA at 293.15 K and 303.15 K over the entire concentration range studied. Only for the systems at $T \geq 313.15$ K, exothermic enthalpic contributions are reported, similarly to the experiment at 283.15 K.

The concentration dependence of the entropic contribution χ_S reproduces the dependence of χ_H as a mirror image, as suggested by phenomenological considerations. Enthalpy and entropy cannot be changed independently. For $293.15 \leq T \leq 313.15$ K, the initial noncombinatorial entropy of dilution is negative, that is in favour of the process. For concentrations above $\phi_1 = 0.04$ at 313.15 K and for the whole concentration range studied at 283.15 and 323.15 K, values of χ_S are positive making the process of dilution entropically unfavourable. It is suggested, sorption at 283.15 and 323.15 K is largely entropically unfavorable.

A comparison with the excess thermodynamic functions as determined from the calorimetric results shows that the analysis of χ , and its constituents χ_H and χ_S , leads to a picture of the water sorption process of bovine serum albumin that is not in line with the picture gained from calorimetric experiments. Per definition of χ_H , this result was expected as the temperature dependence of the water sorption isotherms of BSA do not follow a clear temperature trend.

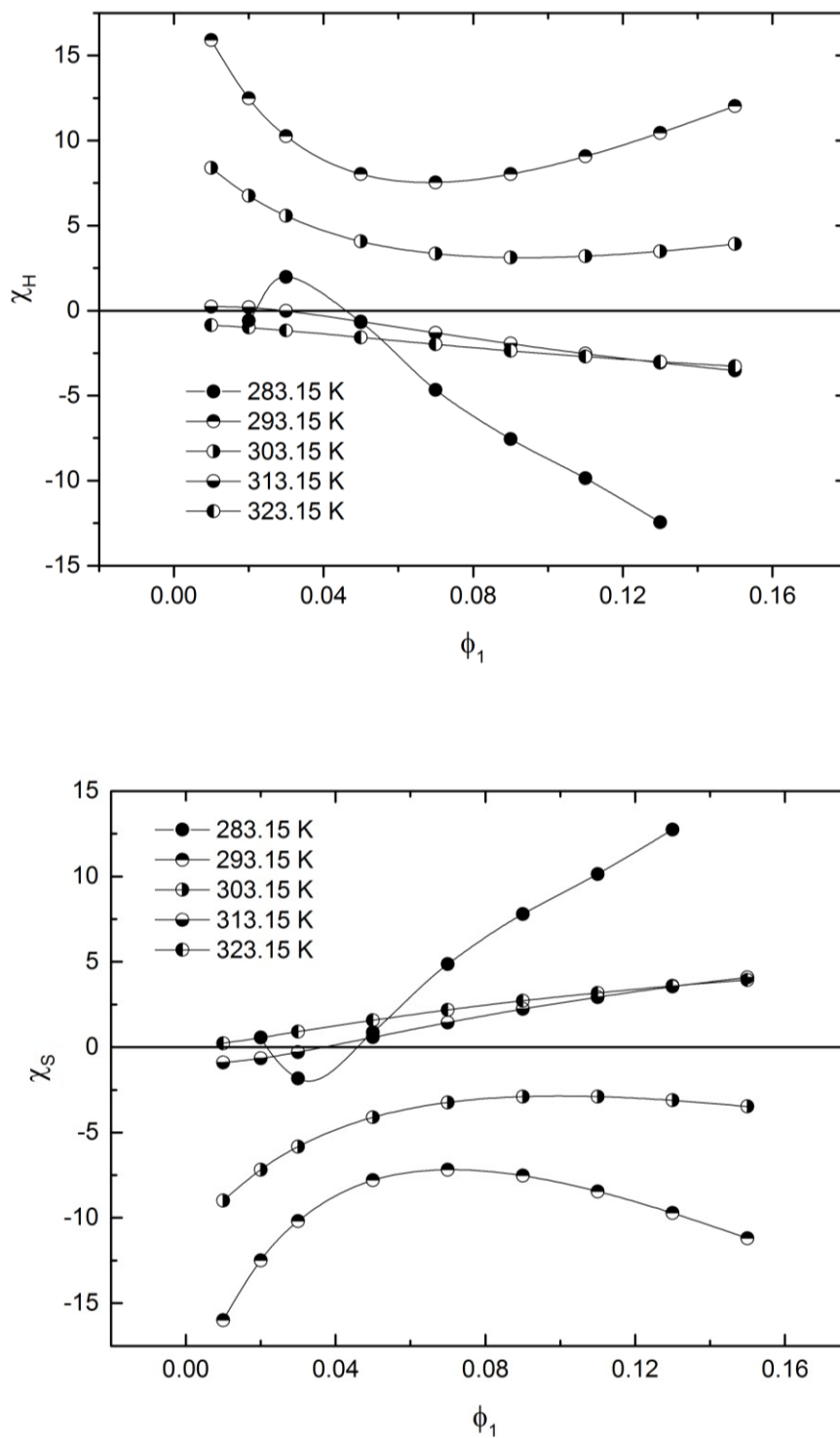


Figure 80 Concentration dependence of χ_H and, the enthalpic part of the Flory-Huggins interaction parameter (upper part), and χ_S , its entropic contribution (lower part), for the different temperatures studied for the system water/BSA.

Wolf [36] suggested a paradigmatic change in our view of the χ interaction parameter. Their theoretical analysis was performed by subdividing the dilutions process into two separable states. It was suggested that an effect of contact formation between solvent and polymer segments at fixed chain conformation is followed by a contribution of the conformational changes taking place in response to dilution. His theory has proven to give insight into simpler solvent/polymer systems but as discrepancies between the experimental energetic landscape and that proposed by the Flory–Huggins theory are substantial, further analyses of the binary interaction parameter χ were not carried out. However, as Wolf's theory itself points towards the multitude of physico-chemical process and interactions important to describe the sorption (or indeed phase) behaviour of more complex polymer solutions, his development should be regarded as an improvement over traditional treatments of the binary interaction parameter in such mixtures.

4.4 CONCLUSION

In this study, the water vapour sorption of bovine serum albumin was studied up to an activity of 0.60 a_w at 293.15, 313.15, and 323.15 K using an isothermal flow calorimeter. The calorimetric experiment was carried out in an interval sorption mode in order to complement the gravimetric water vapour sorption isotherms of bovine serum albumin. Strong evidence is reported here that there is excellent agreement as features of the gravimetric sorption curve are retained in the integral heat flow time evolution data of the calorimetric measurement. From the heats of sorption, heats of mixing as well as partial molar thermodynamic excess functions were computed. As traditional approaches to estimate the heats of mixing for water/protein systems assumed a temperature independent enthalpy, it has to be pointed out that this assumption can no longer be employed. On the basis of the heats of mixing, the sorption process can be divided into two regimes. At low levels of hydration ($\phi_1 < 0.06$), sorption is highly exothermic, the process is slow and conformational changes occur at experimental time scales. At increased hydration levels ($\phi_1 > 0.06$), sorption is faster, the solubility of water is much higher and it appears that the system is increasingly losing its glassy characteristic. The analysis of the thermodynamic partial excess functions supported the division of the sorption process into two regimes and revealed an energetic landscape typical for hydrophilic hydration. Water vapour sorption in bovine serum albumin is enthalpically favourable and entropically unfavourable within the studied concentration and temperature range when entropy refers to excess entropies here. The heats of sorption analysis revealed what appears to be an irreconcilable discrepancy between the van-Laar/Flory–Huggins theory and the experimentally determined heats of mixing.

4.5 REFERENCES

- [1] Sirotkon, V.A.; Khadiullina, A.V. *J. Therm. Anal. Calorim.* **2014**, 118, 951-959
- [2] Gregory, B.B. Protein–Solvent interaction. New York, Marcel Dekker, **1995**
- [3] Rupley, J.A.; Careri, G. *Adv. Protein Chem.* **1991**, 41, 37-122
- [4] Kuntz, I.D.; Kauzmann, W. *Adv. Prot. Chem.* **1974**, 28, 239-345
- [5] Bagchi, B. *Chem. Rev.* **2005**, 105, 3197-3219
- [6] Luscher-Mattli, M; Ruegg, M. *Biopolymers* **1982**, 21, 403-418
- [7] Luscher-Mattli, M.; Ruegg, M. *Biopolymers* **1982**, 21, 419-429
- [8] Bone, S. *Phy. Med. Biol.* **1996**, 41, 1265-1275
- [9] Yang, P.H.; Rupley, J.A. *Biochemistry* **1979**, 18, 2654-2661
- [10] Amberg, C.H. *J. Am. Chem. Soc.* **1957**, 79, 3980-3984
- [11] Almong, R.; Schrier, E.E. *J. Phys. Chem.* **1978**, 88, 1701-1702
- [12] Smith, A.L.; Shirazi, H.M.; Mulligan, S.R. *Biochim. Biophys. Acta* **2002**, 1594, 150-159
- [13] Kocherbitov, V.; Arnebrant, T.; Soderman, O. *J. Phys. Chem. B.* **2004**, 108, 19036-42
- [14] Luthra, S.; Lechuga-Ballesteros, D.; Kalonia, D.S.; Pikal, J.M. *J. Pharmac. Sci.* **2007**, 96 (8), 1974-1982
- [15] Sirotkin, V.A.; Korolev, D.V.; Silakova, A.E. *Russ. J. Chem. Phys. A.* **2007**, 81, 1341-1345
- [16] Sirotkin, V.A.; Korolev, D.V. *Thermochim. Acta* **2005**, 432, 246-253
- [17] Sirotkin, V.A.; Khadiullina, A.V. *J. Chem. Phys. B.* **2011**, 115, 15110-15118
- [18] Sirotkin, V.A.; Khadiullina, A.V. *J. Therm. Anal. Calorim.* **2014**, 118, 951-959
- [19] Kocherbitov, V.; Arnebrant, T. *Langmuir* **2010**, 26, 6, 3918-3922
- [20] Griebenow, K; Klibanov, A. *Proc. Nat. Acad. Sci. U.S.A.* **1995**, 92, 10969-10976

- [21] Constantino, H.R.; Griebenow, K.; Mishra, P.; Langer, R.; Klibanov, A. *Biochim. Biophys. Acta* **1995**, 1253, 69-74
- [22] Towns, J. K. *J. Chromatogr. A* **1995**, 705, 115-127
- [23] Yang, P. H.; Rupley, J. A. *Biochemistry* **1979**, 18, 2654-2661
- [24] Kocherbitov, V.; Arnebrant, T. *J. Phys. Chem. B* **2006**, 110, 20, 10144-10150
- [25] Kocherbitov, V.; Latynis, J.; Misiunas, A.; Barauskas, J.; Niaura, G. *J. Phys. Chem. B* **2013**, 117, 17, 4981-4922
- [26] Scatchard, G.; Hamer, W. J., *J. Am. Chem. Soc.* **1934**, 57, 1805-1809
- [27] Wagner, W.; Pruss, A. *J. Phys. Chem. Ref. Data* **2002**, 31, 2, 387-535
- [28] Olenikova, A.; Smolin, N.; Brovchenko, I.; Geiger, A.; Winter, R. *J. Phys. Chem. B* **2005**, 109, 1988-98
- [29] Lumry, R.; Rajender, S. *Biopolymers* **1970**, 9, 10, 1125-1227
- [30] Antonov, Y.; Eckelt, J.; Sugaya, R.; Wolf, B.A. *J. Phys. Chem. B* **2013**, 117, 5497-5502
- [31] Antonov, Y.; Wolf, B.A. *Langmuir* **2014**, 30(22), 6508-6515
- [32] Wolf, B.A. *Ind. Eng. Chem. Res.* **2013**, 52, 3530-3536
- [33] Antonov, Y.; Wolf, B.A. *Biomacromolecules* **2005**, 6, 2980-2989
- [34] Antonov, Y.A.; Wolf, B.A. *Biomacromolecules* **2006**, 7, 1562-1567
- [35] Flory, P.J. *Principles of Polymer Chemistry*; Cornell University Press: Ithaca, NY, London, **1953**
- [36] Wolf, B.A. *Macromol. Chem. Phys.* **2003**, 204, 1381-1390

4.6 APPENDIX

4.6.1 Kinetic analysis of heat sorption data

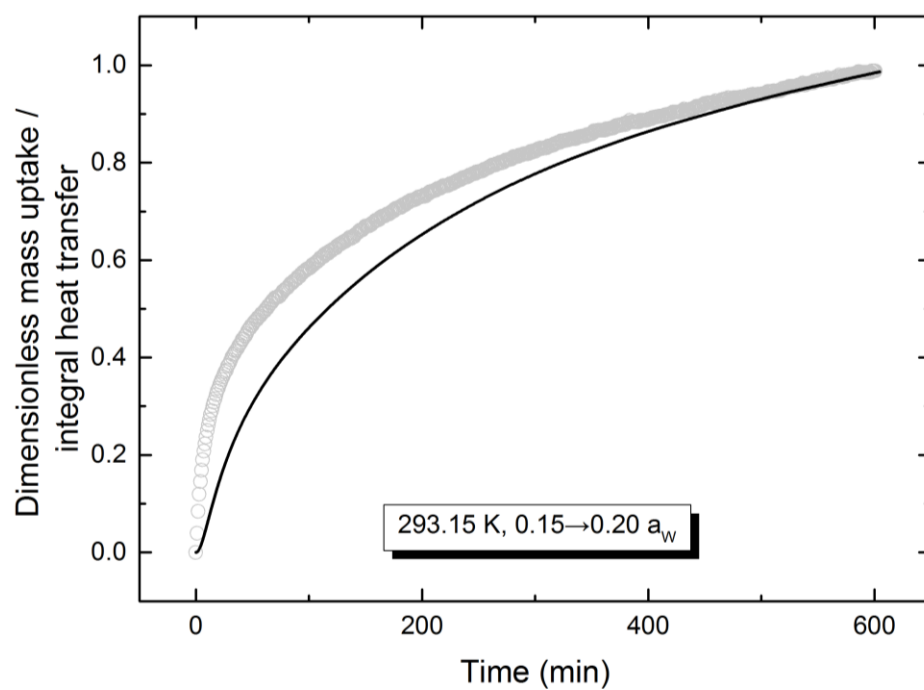


Figure 81 Normalised integral heat flow and mass uptake during interval water sorption of BSA at 293.15 K for an activity change of 0.15 $\rightarrow$ 0.20 a_w .

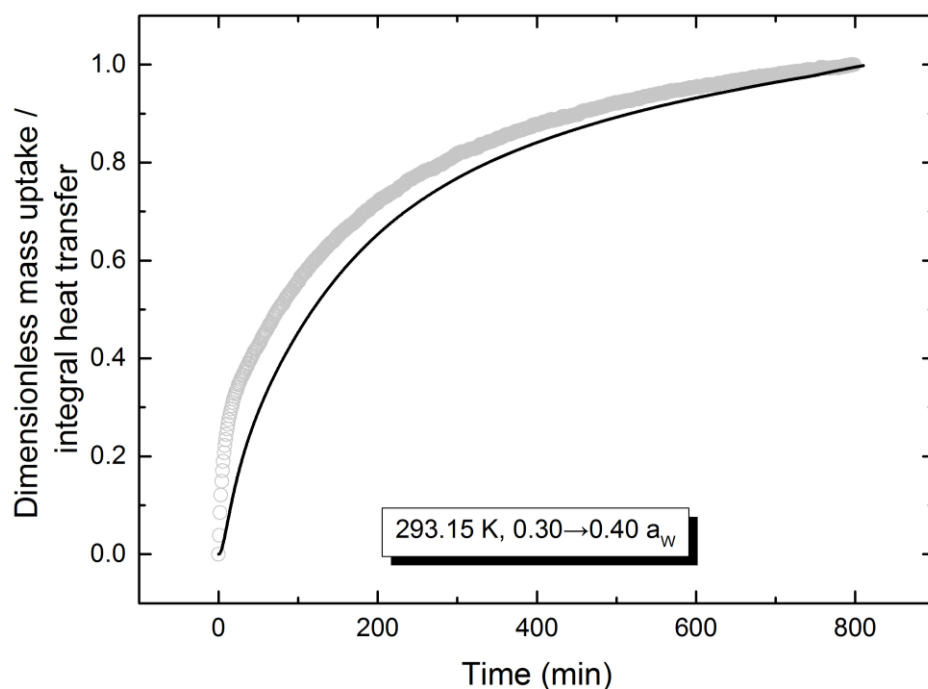


Figure 82 Normalised integral heat flow and mass uptake during interval water sorption of BSA at 293.15 K for an activity change of 0.30 $\rightarrow$ 0.40 a_w

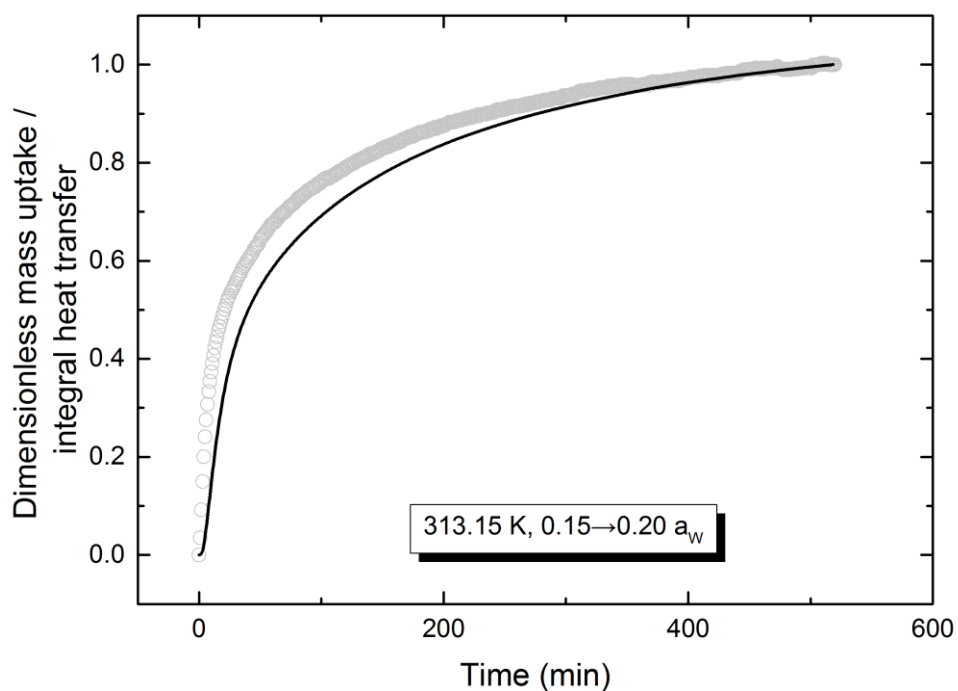


Figure 83 Normalised integral heat flow and mass uptake during interval water sorption of BSA at 313.15K for an activity change of 0.15 $\rightarrow$ 0.20 a_w .

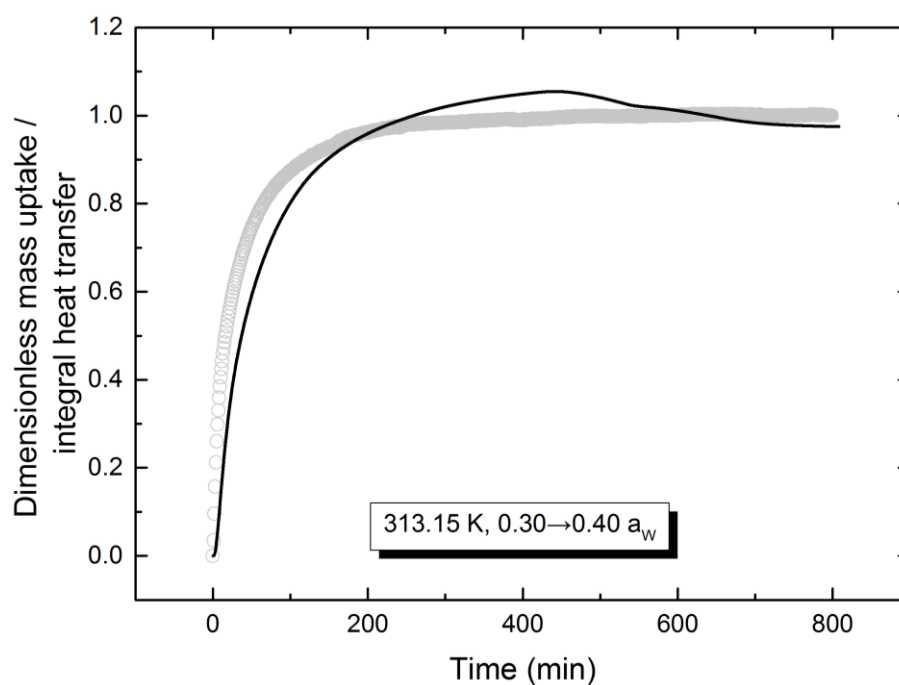


Figure 84 Normalised integral heat flow and mass uptake during interval water sorption of BSA at 313.15K for an activity change of 0.30 $\rightarrow$ 0.40 a_w .

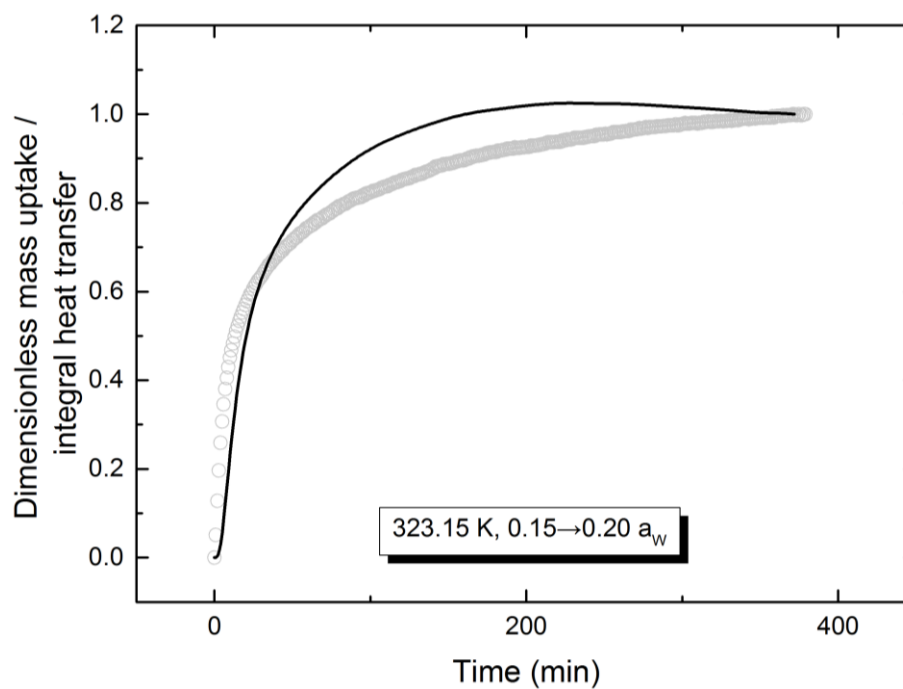


Figure 85 Normalised integral heat flow and mass uptake during interval water sorption of BSA at 323.15K for an activity change of 0.15 $\rightarrow$ 0.20 a_w .

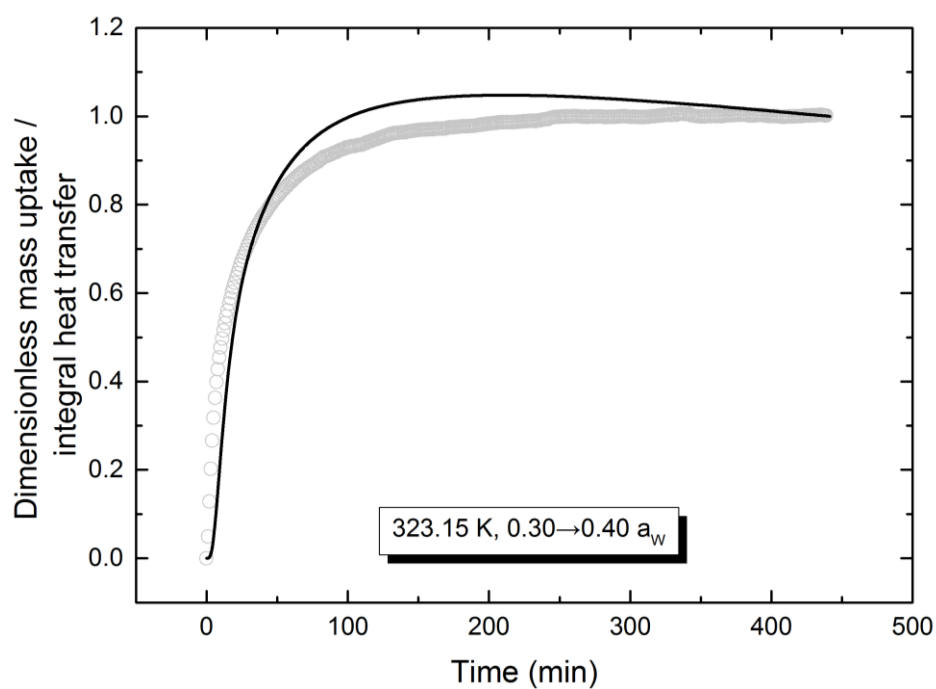


Figure 86 Normalised integral heat flow and mass uptake during interval water sorption of BSA at 323.15K for an activity change of 0.30 → 0.40 a_w .

Chapter 5. Influence of water on thermal transitions in bovine serum albumin

5.1 INTRODUCTION

Ever since the investigations by Tamman in the 1920s it has been known that liquids can be transformed into the glassy state upon cooling if crystallisation can be suppressed. [1] A glass may be defined as a material which is an ordinary liquid at high temperatures and whose thermodynamic extensive quantities (volume V , and entropy S) are moved out of equilibrium as the temperature is lowered past some temperature T_g which depends on the cooling rate. Above T_g , relaxation times associated with viscosity are very small and less than the time scale of the experiment. Below T_g , they are greater. However, this description applies to the formation of crystals too. To exclude crystal formation, a necessary requirement for the formation of a glass is continuous extensive thermodynamic quantities at T_g and no change of spatial symmetry through the glass transition; simplistically, the glass is a frozen in liquid.

Figure 87 is a schematic representation of the thermodynamics of glass-forming systems at constant pressure. A super-cooled liquid is classified as metastable; it is stable against small perturbations. This behaviour is shown in the schematic depiction of the free energy landscape in Figure 87(A). The super-cooled liquid is located in a local free energy minimum; its configuration is retained over infinitely large time scales. Classical equilibrium thermodynamics are typically applied to this state. Upon further cooling, a change in slope in some second-order thermophysical properties (i.e. specific heat, compressibility, coefficient of thermal expansion) can be detected. The temperature at which this change in slope occurs is known as the glass transition temperature. The glass exhibits structural features of a disordered liquid but exhibits mechanical properties of common elastic solids.

The change in slope in second-order thermophysical properties is similar to an Ehrenfest thermodynamic transition. [2] However, the current opinion [3] is that the glass transition does not meet all of the requirements of a true thermodynamic transition. A prominent indication for the absence of a true Ehrenfest transition is the dependence of the T_g on the cooling rate. There is a

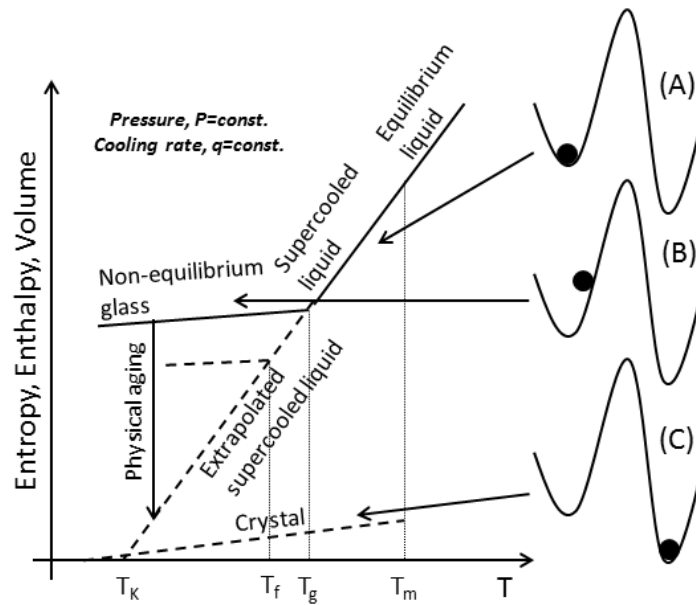


Figure 87 (Left) Schematic representation of thermodynamic properties in glass-forming systems. The following temperatures are shown: T_K , the Kauzmann temperature at which the supercooled liquid reaches the entropy of corresponding crystal; T_f , the temperature at which a glass with a fixed structure would be at thermal equilibrium; T_g , the temperature at which the super-cooled liquid changes into the glass at a specific cooling rate of the experiment; and T_m , the melting temperature. **(Right) Free-energy/Stability plots** of (A) the metastable supercooled liquid; (B) the non-equilibrium glass; and (C) the thermodynamically stable crystal. Adapted from [3].

link between the molecular mobility in the glass former and the cooling-rate dependent T_g . Another piece of evidence for the kinetic nature of the glass transition is the fact that it usually occurs over a broad temperature interval. Further, a second-order Ehrenfest phase transition has to obey:

$$\Pi = \frac{1}{VT} \frac{\Delta C_p \Delta k}{(\Delta \alpha)^2} = 1 \quad (5.1)$$

where V and T denote volume and temperature respectively, ΔC_p is the specific heat, Δk the volumetric compressibility and $\Delta \alpha$ the coefficient of thermal expansion differences at the transition temperature, respectively. Π is known as the Prigogine–Defay ratio [4] and it is well known [2] that this ratio is larger than unity at the glass transition.

Despite the evidence for the kinetic nature of the glass transition, this topic is still a matter of discourse. [3] Indeed, this discourse dates back to the seminal work by Kauzmann [5]. Kauzmann analysed thermodynamic properties of glass forming substances (including synthetic polymers) and observed that the specific heat of a super-cooled liquid is larger than that of the corresponding crystal. In this sense, there exists a temperature where the first-order thermodynamic properties of the glass will equal those of the crystalline form. This temperature is known as the Kauzmann temperature (T_K) and is shown in Figure 87. It has fascinated generations of scientists for the following implication: there would exist a glassy/disordered system and a corresponding crystalline/ordered system that both have identical entropies at $T_K > 0$ K.

Thermodynamically, glassy systems are classified as non-equilibrium systems. They spontaneously evolve towards the nearest available free energy minimum (cf. Figure 87(B)) which is that of the super-cooled liquid. This spontaneous evolution is known as ‘physical aging’ and is of tremendous scientific and technological importance. In other words, the physical aging of glasses results in decreases in the energy landscape occupied. It has been extensively studied since

the seminal publications by Simon [6] in 1931, and Kovacs [7] in 1963, and Struik [8] in 1978. To experimentally assess the evolution of the volume, the enthalpy and other glassy-state sensitive properties within the physical aging regime have been measured in order to estimate the time scales of glassy equilibration. For reviews on this topic, the interested reader may be referred to excellent reviews [9, 10].

The thermodynamic landscape of glasses (as opposed to the dynamic properties for which techniques are different) can be directly estimated from a measurement of a set of pressure–volume–temperature data using standard dilatometric techniques [11]. Methods based on calorimetry have also been heavily used in the characterisation of glassy-state systems. Almost all calorimetric experiments are carried out at ambient pressure and if the pressure is assumed constant during the experiment, the heat exchanged in an experiment equals the enthalpy of the system. Absolute values of thermodynamic functions cannot be found calorimetrically. However, the temperature variation of the enthalpy at constant pressure gives the specific heat $C_p = (\partial H / \partial T)_p$. In most cases, standard differential scanning calorimetry (DSC) is employed to study the thermodynamics of a glass. In this experiment, the specific heat is obtained under non-isothermal conditions. A heating or cooling rate is applied to a pan containing a sample and an empty pan that acts as a reference. The difference in the heat delivered to or removed from the sample and the reference provides information on the specific heat of the sample. Depending on the calibration of the calorimeter and the applied heating/cooling rate, the actual C_p of the sample might differ by up to 10 – 15%. [3]

Numerous relaxation phenomena that have been observed in native proteins can be analysed and interpreted within a ‘glassy’ dynamics framework [12-15]. Proteins are often considered as single-molecule systems and yet display features of glass-forming many-molecule systems (such as synthetic polymers). The superstructure of a protein, that contains many rearrangeable sub-units,

can be described by a potential energy hypersurface with many resembling features of the hypersurfaces typical of glass-forming many-molecule systems. Ever since Angell's important review on the glass formation from liquids and biopolymers [16], the treatment of water-containing native proteins as plasticised polymer systems can now be regarded as the generally accepted view. It is the citation history of Angell's review with more than 3,100 individual citations in peer-reviewed journals since its original publication in 1995 that indicates the importance of the topic.

The glass transition temperature of a polymer depends on the intermolecular forces between the chains of the macromolecules, that is the cohesive energy. T_g is markedly higher in structural proteins such as collagen, elastin and keratins, in cellulose and synthetic polyamides due to additional hydrogen bonding present between the molecular chains. [17] Furthermore, the breaking of hydrogen bonds by water molecules leads to a reduction in T_g , an effect reported for polyamides [18], elastin [19], gelatin [20], and cellulose [21] in early studies.

In this Chapter, I shall review empirical and thermodynamic treatments of the concentration-dependent glass transition temperature and their respective theoretical frameworks. The glass transition temperature of bovine serum albumin and water mixtures were investigated with a differential scanning calorimeter after quasi-equilibration of the samples at various water activities at 293.15 K. Different approaches to model or predict the concentration dependence of the glass transition temperature are tested. The importance of plasticisation is discussed in the context of moisture sorption isotherms and its relationship to the binary Flory–Huggins interaction parameter is explored.

5.2 THEORETICAL BACKGROUND

Numerous approaches to describe the concentration dependence of the glass transition temperature of binary polymer mixtures and solvent–polymer systems have been proposed on the basis of knowledge of some properties of the pure components. Whilst differing in their details, the majority of the empirical and semi-empirical approaches are based on the additivity of basic thermodynamic properties of the mixture’s constituents.

Gordon and Taylor [22] derived a theory assuming volume additivity (i.e. ideal volume of mixing), and a linear dependence between volume and temperature. By far, their expression is the most widely used equation to describe the concentration dependence of the glass transition temperature of amorphous mixtures:

$$T_{gm} = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2} \quad (5.2)$$

where subscripts 1, 2, and m refer to components 1 and 2 and their mixture, respectively. w_i denotes the weight fraction of component i and k is a dimensionless parameter that depends on the ratio of the thermal expansion coefficients (α_i) of the components as they go through the glass transition. It is formally given by $k = [V_2/V_1][\Delta\alpha_2/\Delta\alpha_1]$ where V_i is the specific volume of component i at its T_g . In most applications, Equation 5.2 is simplified. This simplification can be done by (1) employing the Simha–Boyer rule [23] which yields $k = V_2 T_{g2}/V_1 T_{g1}$ and eliminates the α_i terms, or (2) by using k as a curve fitting parameter [24].

Couchman and Karasz [25] proposed a model prescribed by some thermodynamic considerations. They relied on the observation that the glass transition events resembles the façade of a second-order phase transition in the sense that the entropy of mixing is continuous through the glass transition. This analysis yields:

$$\ln T_{gm} = \frac{x_1 \Delta C_{p1} \ln T_{g1} + x_2 \Delta C_{p2} \ln T_{g2}}{x_1 \Delta C_{p1} + x_2 \Delta C_{p2}} \quad (5.3)$$

where x_i are the mole fractions of component 1 and 2, and ΔC_{pi} the corresponding heat capacity difference between the heat capacity of the glass and the liquid of component 1 and 2, respectively. Upon inspection of Equation 5.3, it can be seen that when $k = \Delta C_{p2}/\Delta C_{p1}$ the Couchman–Karasz equation may be written as:

$$T_{gm} = \frac{x_1 T_{g1} + k x_2 T_{g2}}{x_1 + k x_2} \quad (5.4)$$

which is analogous to Equation 5.2.

Fox [26] proposed an empirical relation which can be obtained from the Gordon–Taylor equation assuming the Simha–Boyer rule holds good as well as $k \approx T_{g1}/T_{g2}$. By definition, $k <$

1. The expression for predicting the mixture glass transition temperature is then:

$$\frac{1}{T_{gm}} = \frac{w_1}{T_{g1}} + \frac{w_2}{T_{g2}} \quad (5.5)$$

where symbols have the common meaning. An even simpler relation can be derived from Equation 5.2 if $k \approx 1$ is assumed [27]. In this case, it reduces to the linear case:

$$T_{gm} = w_1 T_{g1} + w_2 T_{g2} \quad (5.6)$$

Equations 5.2 to 5.6 can all be derived from one expression. They are mathematically alike and, therefore, produce similar predictions. However, there are numerous examples of T_g -composition profiles (e.g. non-additive mixing) that cannot be adequately represented by expressions of this type.

Jenkel and Heusch [28] proposed an extension to Equation 5.6 in order to account for monotonic (strictly positive or strictly negative) deviations from simple linear combinations:

$$T_{gm} = w_1 T_{g1} + w_2 T_{g2} + k w_1 w_2 \quad (5.7)$$

where k is an empirical fitting parameter now.

Kwei [29] investigated the effect of hydrogen bonding on the glass transition behaviour of polymer mixtures. This work included systems that showed both positive and negative deviations from linear combination (e.g. S-shaped T_g -composition profile):

$$T_{gm} = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2} + q w_1 w_2 \quad (5.8)$$

where k and q are both empirical fitting parameters. Whilst this equation is empirical in nature, it has been argued that its parameters would contain physical/molecular information on the intermolecular interactions between the mixture components. [30]

Brekner, Schneider and Cantow [31] suggested a theory for the composition dependence of the glass transition temperature of compatible polymers based on concepts of polymer solution thermodynamics. They proposed a third order polynomial equation with respect to the volume fraction ϕ of the stiffer polymer present:

$$(T_g - T_{g1})/(T_{g2} - T_{g1}) = (1 + K_1)\phi - (K_1 + K_2)\phi^2 + K_2\phi^3 \quad (5.9)$$

where K_1 depends on the interaction energy difference hetero- and homo-contacts and includes nearest neighbour effects. K_2 accounts for the effect of immediate molecular surroundings on the interaction only including binary interactions.

Gibbs and Di Marzio [32, 33] developed a model that relates the glass transition to a second-order transition which would be present in the super-cooled amorphous phase even if the latter were allowed to come to internal (metastable with respect to the crystal) equilibrium. An expression for the entropy of a homogeneous mixture of long chain polymers of degree of polymerisation r_a with short chain polymers of degree polymerisation r_b was developed using a quasi-lattice model.

The number of ways to pack a binary mixture of molecules A and B is given by equation 10.10.5² of reference [34]. From this equation, Di Marzio and Gibbs obtained an expression for the configurational entropy:

$$\begin{aligned} \frac{S}{kr_A N_A} = & \frac{(z-2)}{2(1-v)} \ln \left[\frac{(z-2)r_B + 2v}{zr_B} \right] + \frac{v}{r_B(1-v)} \ln \left[\left(\frac{[z-2]r_B + 2v}{zv} \right) \frac{z_1 z_2}{2} \right] \\ & + \frac{v(r_B - 3)}{r_B(1-v)} (\ln[1 + (z-2)\exp\{-\Delta\epsilon_B/kT\}] + f_B \Delta\epsilon_B/kT) \\ & + (\ln \left[1 + (z-2)\exp\left\{-\frac{\Delta\epsilon_A}{kT}\right\} \right] + f_A \Delta\epsilon_A/kT) \quad (5.10) \end{aligned}$$

where

$$f_i = \frac{(z-2)\exp\{-\Delta\epsilon_i/kT\}}{1 + (z-2)\exp\{-\Delta\epsilon_i/kT\}} \quad i = A, B \quad (5.11)$$

The various quantities used in the above equation are defined as follows: N_A and N_B are the total numbers of A type and B type molecules, respectively; k is Boltzmann's constant; and T is the absolute temperature; the volume fraction of plasticiser v is defined by:

$$v = r_B N_B / (r_A N_A + r_B N_B) \quad (5.12)$$

r_A and r_B are really the number of lattice site occupiers per molecule. z_1 is the number of sites available to the second segment of the molecule after the first segment has been located on the lattice. z_2 is the number of sites available to the third segment of the molecule after the first and second segments have been located on the lattice.

The last two terms of Equation 5.10 represent contributions to the entropy arising from the multitude of energy-dependent conformations available to a molecules once its position in space

$$^2 g(N_a, N_b) = \left(\frac{\rho_a}{\sigma_a}\right)^{N_a} \left(\frac{\rho_b}{\sigma_b}\right)^{N_b} \frac{(r_a N_a + r_b N_b)!}{N_a! + N_b!} \left\{ \frac{(q_a N_a + q_b N_b)!}{(r_a N_a + r_b N_b)!} \right\}^{\frac{1}{2}Z}$$

has been determined by the location of the first three segments. [33, 35] The terms are energy-dependent because it was assumed that each segment can occupy $z - 1$ positions relative to the coordinate system formed by the previous three segments of the chain and that one of these is favoured by an energy $\Delta\epsilon_i$. This assumption had proven succesful when a tetrahedral lattice with a coordination number (z) of 4 was used. [33] f_i is the fraction of segments belonging to the i -type molecules which are in the $(z - 2 = 2)$ high energy wells.

In accordance with this theory, the second-order transition temperature is obtained by setting the entropy equal to zero. This yields an equation for the second-order transition temperature in terms of the variables r_B , v , $\Delta\epsilon_A$ and $\Delta\epsilon_B$. Then, $\Delta\epsilon_A$ should be determined from Equation 5.10 (for $S = 0$) applied to the case of the pure homopolymer ($v = 0$) with the second-order transition temperature of the pure homopolymer subsitituted for T .

Chow [36] published an explicit expression based on classical and statistical thermodynamics for predicting the glass transition temperature of polymer–diluent mixtures in 1980. The composition-dependent depression of the glass transition temperature for these systems was known for some time but since DiMarzio and Gibbs’s seminal article from 1963, there was no significant progress in interpreting T_g by statistical mechanics.

In accordance with the work of DiMarzio–Gibbs, the glass formation is considered as a result of the system’s loss of configurational entropy here:

$$S_c = S^{liquid} - S^{glass} \quad (5.13)$$

For a pure polymer, the constant pressure heat capacity C_p and $S_c(0, T)$ are related by:

$$S_c(0, T) = \int_{T_{g,0}}^T \Delta C_p(T') d \ln T' \quad (5.14)$$

where ΔC_p denotes the heat capacity difference between the supercooled liquid and glass. For a polymer–diluent system, the configurational entropy is also a function of the number of diluent molecules N and $S_c(N, T)$ and is calculated from:

$$S_c(N, T) = \int_{T_g}^T \Delta C_p(N, T') d \ln T' \quad (5.15)$$

The Gibbs–DiMarzio theory sets $S^{glass}(0, T) = 0$ at the thermodynamic transition temperature T_2 , which implies that the energy contributions from vibration about the lattice sites are neglected. T_2 is the equilibrium transition temperature that signifies the lower bound of the experimentally obtainable glass transition temperature T_g . Gordon, Rouse, Gibbs and Risen [37] suggested that the composition dependence of the diluent on T_g and T_2 is the same. As a result of this, Chow assumes that $S^{glass}(N, T) = S^{glass}(0, T) = 0$. Assuming that the isobaric heat capacity increment ΔC_p is independent of composition and temperature, Equations 5.14 and 5.15 lead to:

$$\ln \left(\frac{T_g}{T_{g0}} \right) = -\frac{1}{\Delta C_p} [S^{liquid}(N, T) - S^{liquid}(0, T)] \quad (5.16)$$

From Equation 14.43 in reference [38], the entropy is related to the configurational partition function (Q) by the following equation:

$$S = k \ln Q + kT \left(\frac{\partial \ln Q}{\partial T} \right)_P \quad (5.17)$$

where k is the Boltzman's constant. Now, by substitution of Equation 5.17 back into Equation 5.16, Chow's original equation was obtained:

$$\ln \left(\frac{T_g}{T_{g0}} \right) = -\frac{k}{\Delta C_p} \left[\ln \left(\frac{Q_{N^{liquid}}}{Q_{0^{liquid}}} \right) + T \frac{\partial}{\partial T} \ln \left(\frac{Q_{N^{liquid}}}{Q_{0^{liquid}}} \right) \right] \quad (5.18)$$

The ratio of the partition functions may be evaluated by any one of the theories of mixtures such as the Bragg–Williams theory, the Flory–Huggins theory, and many refined theories including the Sanchez–Lacombe equation-of-state theory. In Chow's original work, the

Bragg–Williams approach was used as the interest was to determine the possible configurations of diluent molecules on polymer lattice sites rather than the arrangement of polymer molecules (which would better be described by the Flory–Huggins theory).

Consider N diluent molecules that are randomly distributed in a lattice of $N + L$ sites where L is the number of vacant sites and it is assumed that each diluent molecule occupies a single lattice sites. Then, one may write [38]:

$$Q_{0^{liquid}} = 1 \quad (5.19)$$

and

$$Q_{N^{liquid}} = \frac{(N + L)!}{N! L!} \exp\left(\frac{NL}{N + L} \frac{z\epsilon}{2kT}\right) \quad (5.20)$$

where z denotes the lattice coordination number, $\epsilon = \epsilon_{NN} + \epsilon_{LL} - 2\epsilon_{NL}$ and ϵ_{NN} , ϵ_{LL} , and ϵ_{NL} denote energies of each NN , LL , and NL pair. Substitution Equation 5.19 and 5.20 into Equation 5.18 yields Chow's final result:

$$\ln\left(\frac{T_g}{T_{g0}}\right) = \beta\{(1 - \theta) \ln(1 - \theta) + \theta \ln(\theta)\} \quad (5.21)$$

with the two non-dimensional parameters defined as $\beta = k(N + L)/\Delta C_p$ and $\theta = N/(N + L)$. Further, the number of diluent molecules N and the number of lattice sites $N + L$ can be written as:

$$N = m_d N_A / M_d \quad (5.22)$$

and

$$N + L = z m_p N_A / M_p \quad (5.23)$$

where N_A is the Avogadro number, m is the mass, and M is the molecular weight. Subscripts d and p denote the diluent and polymer, respectively. M_p denotes the molecular weight of the monomer

unit of the polymer. This expression allows rewriting the dimensionless parameters θ in terms of the diluent weight fraction ω as:

$$\theta = \frac{N}{N + L} = \frac{M_p}{zM_d} \frac{\omega}{1 - \omega} = \frac{V_p}{zV_d} \frac{\phi}{1 - \phi} \quad (5.24)$$

It is important to appreciate that the derivation is based on a temperature-independent ΔC_p and the application of the Bragg–Williams mixing theory requires θ to be small. Therefore, Chow suggests to approximate $\Delta C_p = M_p \Delta C_{pp}$ and gets

$$\beta = zR/M_p \Delta C_{pp} \quad (5.25)$$

where ΔC_{pp} denotes the excess transition isobaric specific heat of the pure polymer.

5.3 EXPERIMENTAL SECTION

5.3.1 Materials

Bovine serum albumin (BSA) (lyophilised powder, catalogue number 05470) was purchased at the highest commercially available grade from Sigma Aldrich (Gillingham, United Kingdom). Ultrapure deionised water (resistivity $\sim 16 \text{ M}\Omega \cdot \text{cm}$) was used for all sorption experiments.

5.3.2 Methods

Differential Scanning Calorimetry (DSC) was carried out with a *DSC Q2000* system (TA Instruments, New Castle, DE) using the *hermetic Tzero* pan technology (TA Instruments, New Castle, DE). Samples ($\sim 6 - 8 \text{ mg}$) were loaded into the pan, dried under a helium atmosphere until constant weight was achieved. The dry weight of sample and pan were recorded. Then, samples were equilibrated against a water vapour of controlled activity for 800 min at 293.15 K and, subsequently, weighed again. For the sample equilibration, a prototype humidity generator (Surface Measurement Systems, London, UK) was employed. Here, a dry air stream is mixed with a ‘wet’ air stream at controlled flow mix to achieve the desired water activity. Samples were sealed and inserted into the DSC system. The heat flow measurement was carried out at a scan rate of 2 K/min between 278.15 and 450 K . As thermal properties of ‘native’ BSA were of interest to this study, the cooling after heating and re-heating heat flow curves are neglected in the following.

5.4 RESULTS AND DISCUSSION

5.4.1 The glass transition in bovine serum albumin

In Figure 88, a typical heat capacity run as a function of temperature is shown for dry bovine serum albumin in the vicinity of the glass transition temperature. Within this temperature range, a rapid change towards a higher heat capacity is observed. The evolution of this change follows that of a second-order transition. The observed result is characteristic for a glass transition so that the glass transition temperature can be determined by a simple tangent construction around the inflection of the DSC curve. The T_g of dry bovine serum albumin is 418.1 ± 2.5 K and was reliably estimated as it lies below the protein's melting/decomposition temperature. There is no data published on the T_g of dry, native BSA but this value is typical [16] for globular proteins.

Similar experiments were carried out for the system water/bovine serum albumin with varying water content. Some of the resulting heat capacity runs as a function of temperature and water content are shown in Figure 89. An interesting feature of these DSC curves is the endothermic overshoot at T_g . Foltz and McKinney [39] and Petrie [40] suggested that the magnitude of the C_p endothermic peak at T_g , the so-called ' T_g -overshoot', is a quantitative measure of the enthalpy relaxations which had occurred prior annealing at a sub- T_g temperature. There have been various studies [41-47] that have investigated the effect of thermal and mechanical treatments on the T_g -overshoot. Berens and Hodge [48, 49] did not just test the effect of thermal and mechanical treatments but also that by vapours on the T_g -overshoot. They suggest that the development of the endothermic T_g -overshoot is a feature of physical aging. Observed effects can be illustrated on a schematic enthalpy–entropy diagram shown in Figure 90. For a sample that has been cooled into its glassy state along the path ABC and has been annealed at some sub- T_g

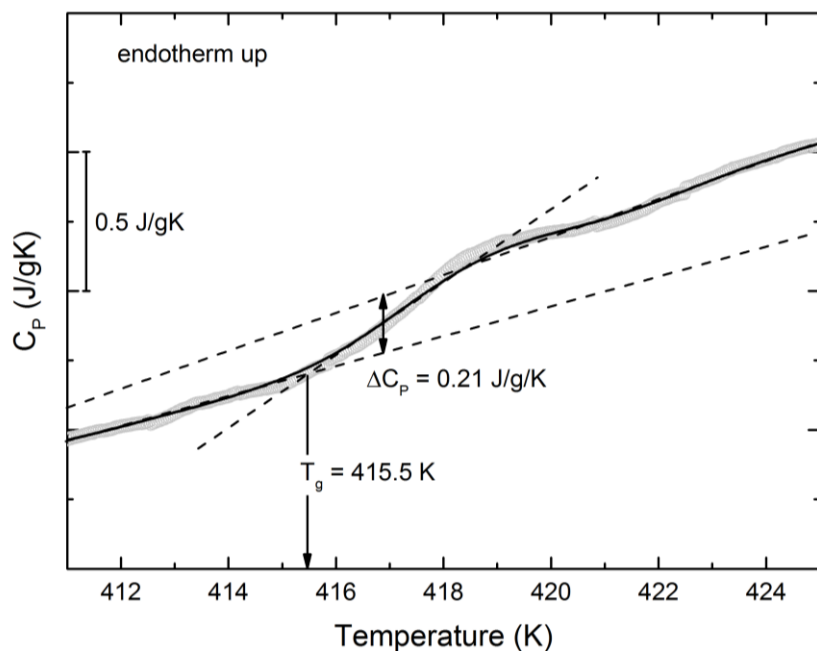


Figure 88 DSC curve for BSA in the vicinity of the glass transition under isochoric conditions. ($\circ$) Experimental data; ($-$) smoothed experimental data; ($--$) tangent construction.

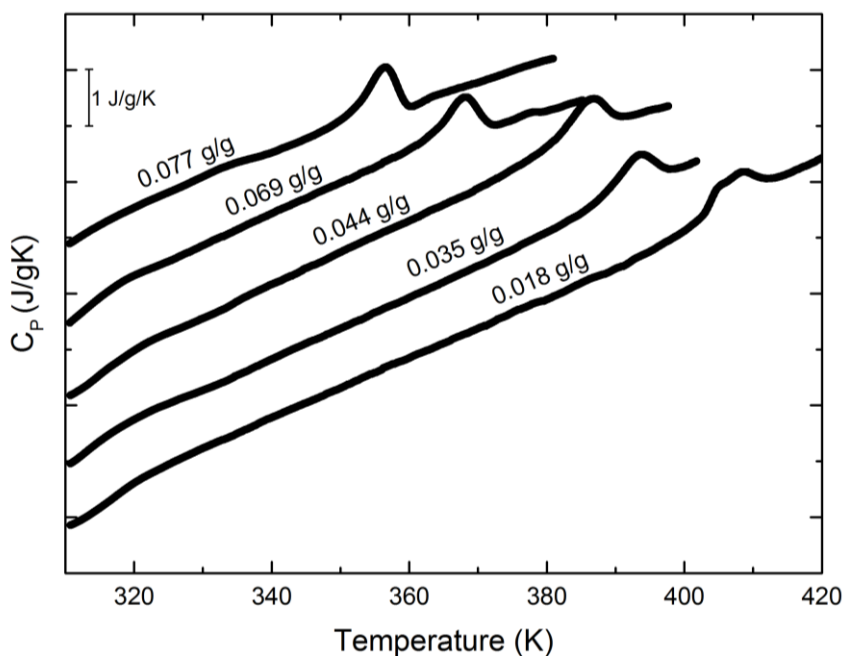


Figure 89 DSC curves for the system water/BSA at different water contents in the vicinity of the glass transition under isochoric conditions. Endotherm up. Data was obtained by differential scanning calorimetry

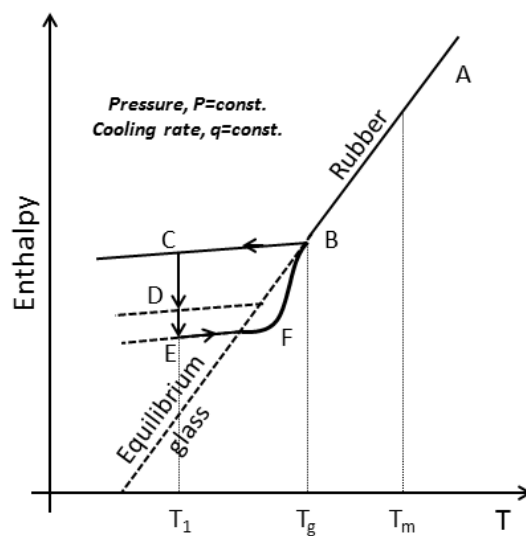


Figure 90 Schematic enthalpy–temperature diagram illustrating effects of annealing, mixing and aging. Conditions are isobaric and at constant cooling rate.

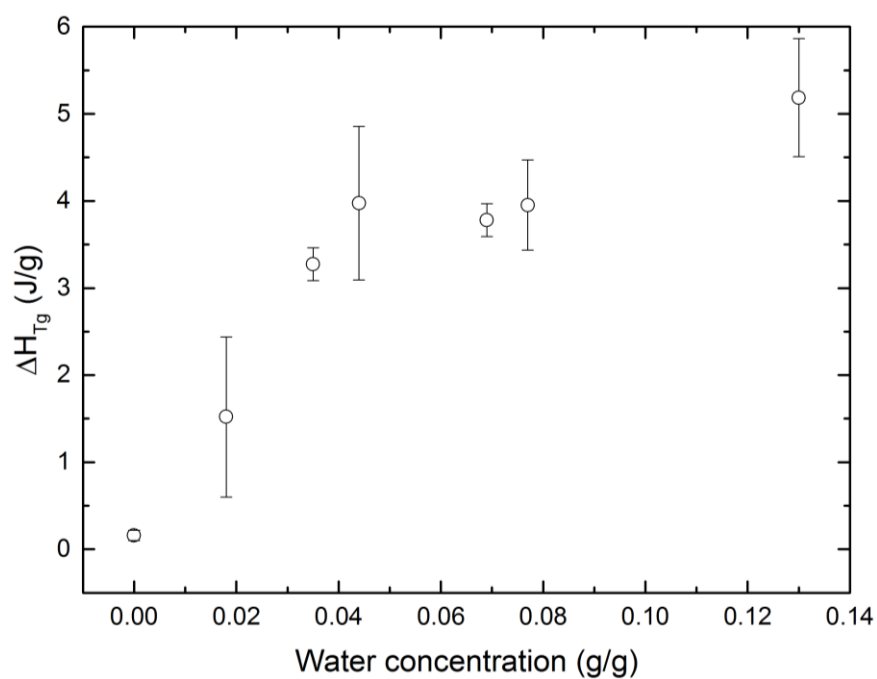


Figure 91 Endothermic enthalpy change due to T_g overshoot as a function of water concentration for the system water/BSA under isochoric conditions. Data was obtained by differential scanning calorimetry

temperature T_1 , the enthalpy reduces along the path CD upon mixing with a specific amount of water. Then, the enthalpy relaxes at constant composition and temperature along the path DE. A subsequent DSC scan along the path EFBA shows an endothermic peak that is superposed on the glass transition step; that is the T_g -overshoot. Figure 91 shows the enthalpy change associated with the glass transition overshoot as a function of water concentration. It was estimated against a sigmoidally extrapolated baseline. It was observed that an increase in water concentration increases the area under the C_p -overshoot suggesting that physical aging is more pronounced at higher water contents.

Figure 92 shows the ΔC_p at the glass transition as a function of water content. It was found that ΔC_p increases monotonically from a value of $0.21 \text{ J/g} \cdot \text{K}$ for dry bovine serum albumin. The shape of the data suggests, the higher the water content the greater the influence on ΔC_p .

The specific heat of a system can be written via its macroscopic properties according to $\Delta C_p = \Delta \langle \overline{\Delta S^2} \rangle / k_B$ where $\langle \overline{\Delta S^2} \rangle$ is the mean-square entropy fluctuation at the glass transition, k_B is the Boltzmann constant, and T is the temperature. This analysis would suggest that the glass transition process becomes more entropically favourable as the water content increases.

From the DSC curves as a function of temperature at different water contents, a plot of the glass transition temperature as a function of water content of bovine serum albumin can be prepared. This graph is shown in Figure 93. Below the data lies the glassy region whilst the rubbery region lies above this. The value of the T_g decreases with increasing water content. At first, this decrease occurs rapidly, then more slowly. The data in Figure 93 gives strong support to the concept of hydrated proteins as water-plasticised bio-polymer systems, which is line with other reports as reviewed in Chapter 1.1.

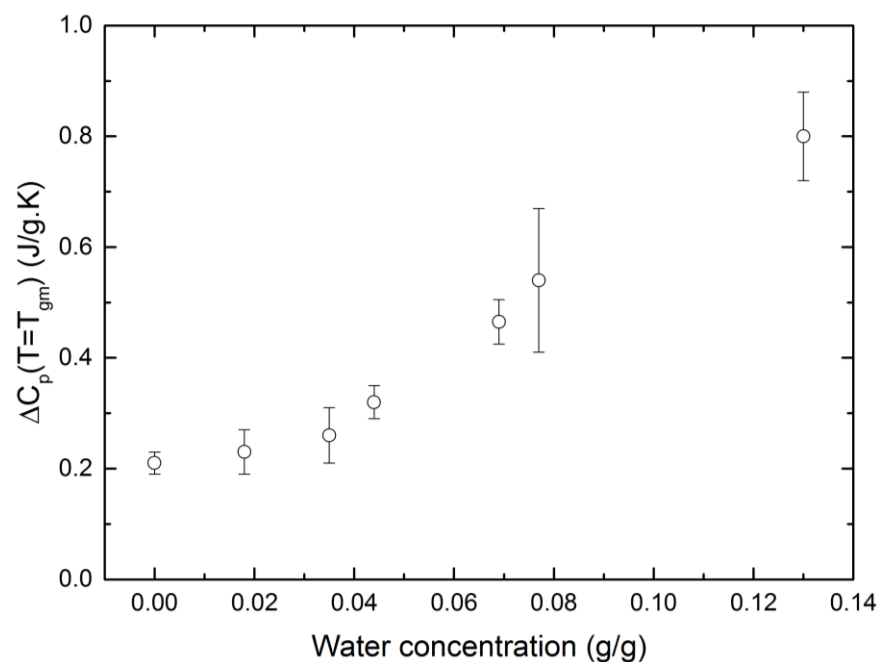


Figure 92 Heat capacity difference between the glass and rubber at the glass transition temperature for the system water/BSA as a function of water content under isochoric conditions. ($2 < n \leq 4$) Data was obtained by differential scanning calorimetry

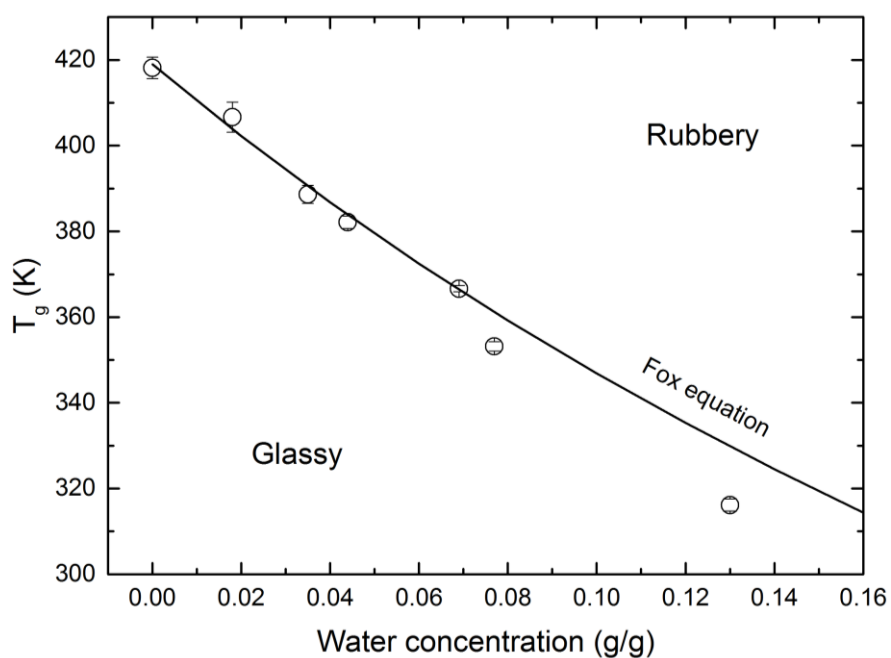


Figure 93 Concentration dependence of the glass transition temperature for the system water/BSA. ($2 < n \leq 4$) Data was obtained by differential scanning calorimetry

Whilst the results of Chapters 2 and 3 gave first evidence of the glassy nature and the ongoing structural and thermodynamic changes with time within a protein during water vapour sorption, and Chapter 4 can be seen as direct and, importantly, independent experimental evidence (independent in the sense that the technique employed is calorimetric and not gravimetric) for this phenomenon, it really are the results in this section, again independent from sorption calorimetric and gravimetric data, that ultimately lay weight to the overall understanding of hydration of amorphous proteins as presented in this study.

5.4.2 Modelling the glass transition in proteins

Figure 94 shows the use of the Gibbs–DiMarzio theory (Equation 5.10), the Chow expression (Equation 5.21), and the simple Fox relation (Equation 5.5) in predicting the mixture glass transition temperature of the system water/bovine serum albumin. All three equations represent the data well. Whilst the Fox equation is essentially a mixing rule, the Gibbs–DiMarzio theory and its extension by Chow are based on entropic considerations. Tables 6 and 7 list the model parameters for the Chow and Gibbs–DiMarzio theory, respectively.

The Gibbs–DiMarzio theory assumes no presence of holes (i.e. no free volume) on the basis of experimental evidence of their negligible influence on the transition [33] and employs Guggenheim’s zeroth approximation [34]. According to the zeroth approximation, the arrangement of molecules on the lattice is completely random; mixing is athermal. Further, a tetrahedral lattice ($z = 4$) was assumed as in the original work by Gibbs and DiMarzio, and perfectly flexible molecules for water were assumed ($\Delta\epsilon_B = 0$). The Gibbs–DiMarzio theory is ‘unhandy’ in the sense that it is a function of at least four variables/parameters that need determination; namely, r_B ,

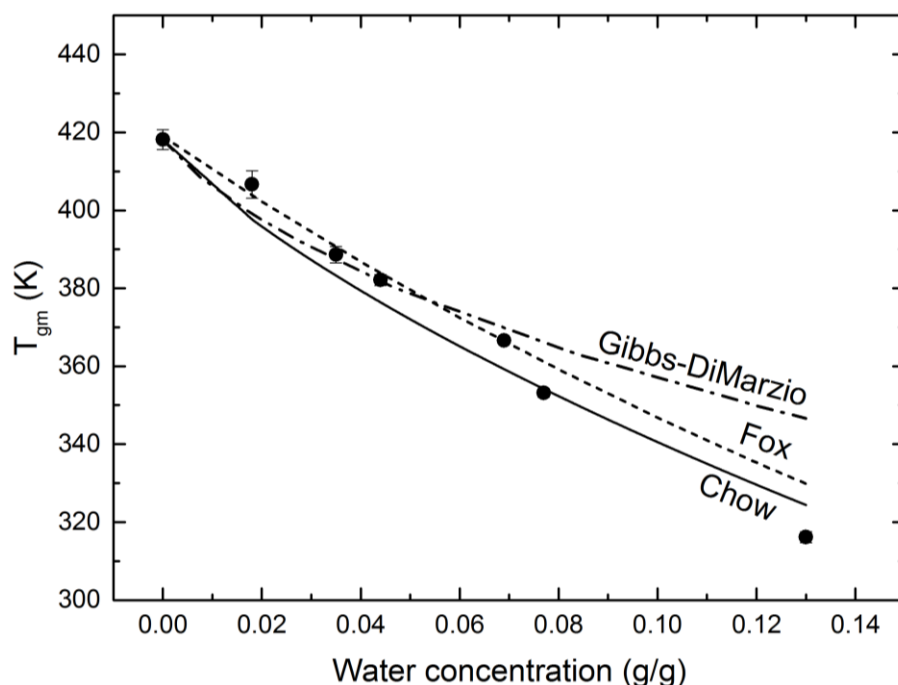


Figure 94 The concentration dependence of the glass transition temperature in water/BSA mixtures modelled by the Gibbs–DiMarzio (dash-dot), Fox (dash), and Chow (solid) theories.

v , $\Delta\epsilon_A$ and $\Delta\epsilon_B$. The definition of r_A is also difficult in the context of a protein. Here, r_A was set equal to 10^4 , a typical value for high polymers. The Chow theory is derived from the Gibbs–DiMarzio theory; however, an explicit expression for T_{gm} was given. A further advantage is its simple use and the fact, that the only parameter that is not accessible experimentally is the lattice coordination number. From the Bragg–Williams mixing theory, θ is required to be small. In other words, the ratio M_p/zM_d has to be sufficiently small. I therefore define $\bar{M}_p$ as the average molecular weight of the hypothetical repeated unit of the protein molecules. It is defined as the molecular weight of the protein divided by the number of residues. For BSA, this yields 114 g/mol which is a typical value for M_p of synthetic polymers (for which the Chow theory was derived). If all parameters in the Chow model are fixed apart from z , then z becomes a curvature factor. Whilst the representation of the experimental data is increased from $z = 4$ up to $z = 9$,

after $z = 9$ there is no improved representation. Another problem might arise from the assumption of a constant ΔC_{pp} . However, only a considerably small concentration dependence for ΔC_{pp} was found (cf. Figure 92).

Overall, it is a promising result that the system water/bovine serum albumin can be described by glass transition theories that have been derived for much simpler polymer–polymer and penetrant–polymer mixtures. It is an important finding in view of the need to advance theories to treat the phase behaviour (e.g. solubility) of protein and water mixtures.

Table 6 Model parameters for Chow theory (Equation 5.21) to describe the mixture glass transition temperature for the system water/BSA. The parameters presented represent the behaviour of the system as shown in Figure 94.

Parameter	Value
T_{g1}	$136 \pm 2 \text{ K}$
$\bar{M}_p$	114 g/mol
ΔC_{pp}	$1.13 \text{ J/g} \cdot \text{K}$
M_d	18 g/mol
z	9

Table 7 Model parameters for Gibbs–DiMarzio theory (Equation 5.10) to describe the mixture glass transition temperature for the system water/BSA. The parameters presented represent the behaviour of the system as shown in Figure 94.

Parameter	Value
$T(v = 0)$	418.15 K
$T(v = 1)$	136.15 K
$\Delta\epsilon_A$	$1.1073 \times 10^{-20} J$
$\Delta\epsilon_B$	0
z	4
r_A	10^4
r_B	2
z_1	4 (= z)
z_2	3 (= $z - 1$)

5.4.3 The glass transition and its relation to the water sorption isotherm

An interesting link is that between a polymer's water vapour sorption isotherm and its plasticisation by water. Vrentas and co-workers reported an extension to the Flory–Huggins model about 40 years ago [50-52] in which they link the vapour sorption behaviour of a glassy polymer and the plasticisation induced by the vapour itself. They established concepts to relate free volume theory [53] to account for non-ideal volumetric changes in a glassy polymer induced by the presence of a low molecular weight penetrant. These non-ideal volume changes occur as a direct result of structural changes within the polymer matrix with increasing penetrant concentration.

Assuming ideal behaviour of the gas or vapour phase over the polymer, the sorption isotherm for the glassy polymer–penetrant system for $T < T_{gm}$ at the mass fraction ω_1 of interest is given by:

$$a_w = \phi_1 \exp[\phi_2 + \chi \phi_2^2] e^F \quad (5.26)$$

and

$$F = \frac{M_1 \omega_2^2 (\hat{C}_{pg} - \hat{C}_p) \frac{dT_{gm}}{d\omega_1}}{RT} \left(\frac{T}{T_{gm}} - 1 \right) \quad (5.27)$$

where F is the volume change correction function, T is the experimental temperature, $\hat{C}_{pg} - \hat{C}_p$ is the specific heat capacity change upon glass transition and independent of T , and T_{gm} is the glass transition temperature of the polymer–penetrant mixture, which itself is a function of ω_1 but assumed constant here. For a rubbery polymer, i.e. $T \geq T_{gm}$, the Vrentas–Vrentas model takes the form of the standard Flory–Huggins model. The function e^F can thought of as a departure function describing the excess sorption due to plasticisation.

Figure 95 shows the e^F function as calculated from Equation 5.27 from the experimentally determined glass transition temperature depression by sorbed water as well as a hypothetical/experimental e^F which was calculated from the difference between the real isotherm of water/BSA at 293.15 K and its Flory–Huggins fit with a constant $\chi = 0.42$ (which is the value that gives the ‘best’ fit for the constant- χ Flory–Huggins theory). The model parameters of the Vrentas–Vrentas fit are shown in Table 8. Both e^F departure functions show a similar trend; though it is obvious that the Vrentas–Vrentas treatment may not produce a departure function that describes the actual isotherm with the given parameters used here. There is a much stronger concentration dependence of the $e^F(\phi_1)$ function than predicted by the Vrentas–Vrentas treatment.

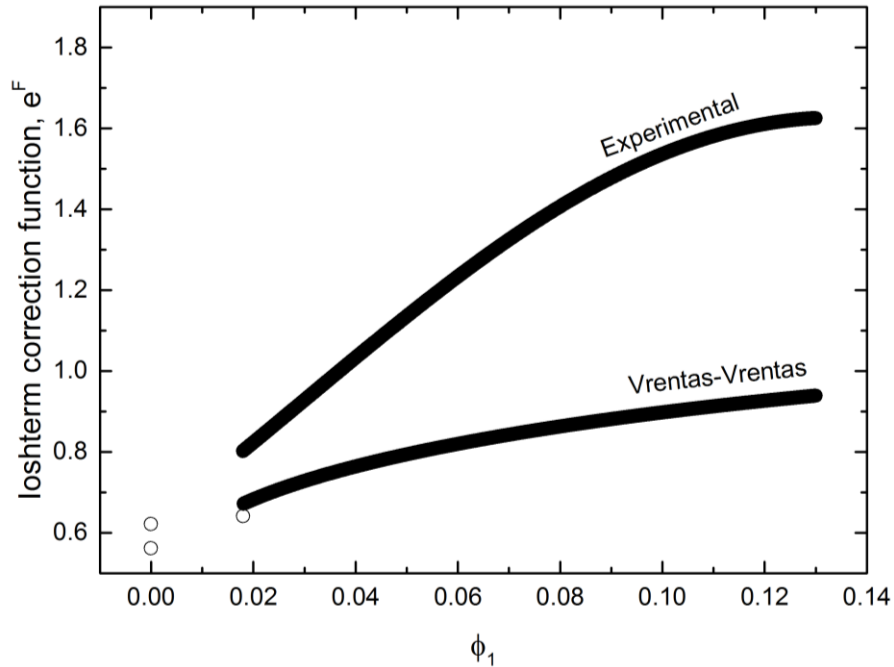


Figure 95 Vrentas-Vrentas correction function e^F according to Equation 5.27 and hypothetical departure function estimated from water/BSA isotherm and $T_g(\omega_1)$ at 293.15 K.

However, as χ was employed as a ‘fitting’ parameter when the standard Flory–Huggins isotherm was regressed, there is no theoretical basis for not reassigning a new value for χ . Figure 96 shows the Vrentas–Vrentas isotherm fit with $\chi = 0.85$ to the interval sorption data. This increase in χ demonstrates that there is a significant portion of sorption due to plasticisation (as the increased χ would suggest a poorer binary interaction). As the integral sorption data features an anomaly between 0.15 and 0.20 a_W , it was not attempted to model this data. The Vrentas–Vrentas treatment has been hugely successful for describing water vapour sorption in a wide range of amorphous solids. [54] However, it appears that plasticisation is not the sole reason for the isotherms to diverge so strongly from simple Flory–Huggins mixing.

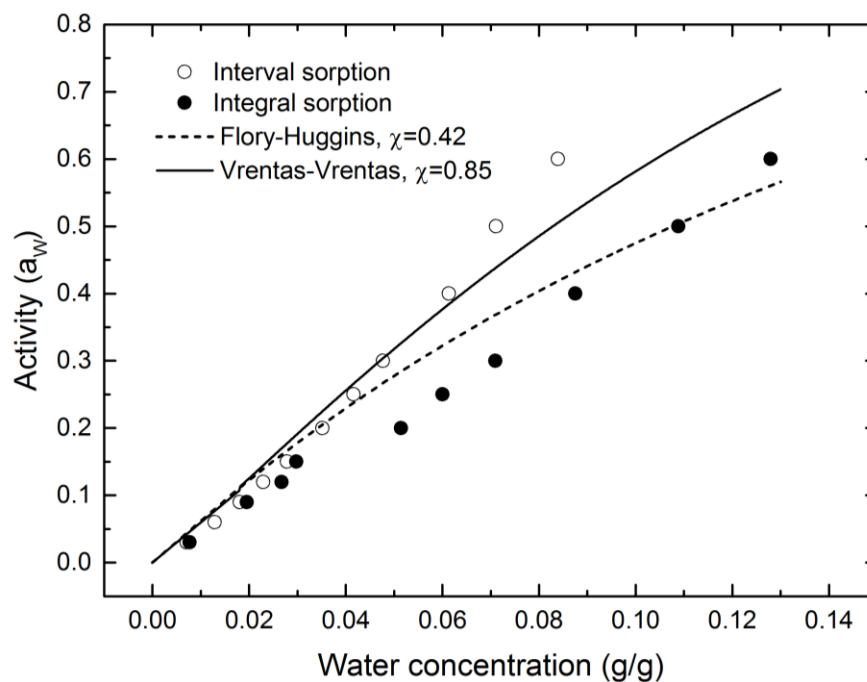


Figure 96 Interval and integral sorption data for the system water/BSA at 293.15 K.

Interval sorption data is fitted by Flory–Huggins theory and Vrentas–Vrentas theory.

Table 8 Properties of the water/BSA at 293.15 K as used in the Vrentas–Vrentas modelling.

Property	Value
T_{g1}	$136 \pm 2 \text{ K}$
T_{gm}	Use Equation 5.21
$\bar{M}_p$	114 g/mol
$\hat{C}_{pg} - \hat{C}_p$	$1.13 \text{ J/g} \cdot \text{K}$
M_d	18 g/mol
z	9
p_d^0	2333.7 Pa
χ	0.42

5.4.4 Relationship between glass transition temperature and binary interaction parameter

Lu and Weiss [55] suggested an equation that relates the glass transition temperature and the binary interaction parameter, χ , for miscible polymer blends or indeed polymer–solvent systems. In their original paper, they extended the theory by Couchman [56] by adding a term including χ . When independent values for χ can be obtained from solubility measurement or vapour sorption studies, the Lu–Weiss model contains no adjustable parameters. Their expression for the glass transition temperature of the mixture is:

$$T_{gm} = \frac{x_1 T_{g1} + k x_2 T_{g2}}{x_1 + k x_2} - \frac{\Delta H_m(T_{g2}) - \Delta H_m(T_{g1})}{(x_1 + k x_2)(\Delta c_{P1} - x_2 \delta c_P^g)} \quad (5.28)$$

with

$$k = \frac{\Delta c_{P2} - x_1 c_P^l}{\Delta c_{P1} - x_2 c_P^g} \quad (5.29)$$

and

$$\Delta c_{Pi} = c_{Pi}^l - c_{Pi}^g \quad (5.30)$$

where symbols have the common meaning. Introducing the van-Laar equation for the heat of mixing (as used in the Flory–Huggins theory):

$$\Delta H_m(T) = \chi R T \phi_1 \phi_2 \quad (5.31)$$

Equation 5.28 yields upon substitution:

$$T_{gm} = \frac{x_1 T_{g1} + k x_2 T_{g2}}{x_1 + k x_2} - \frac{\chi R (T_{g2} - T_{g1}) \phi_1 \phi_2}{(x_1 + k x_2)(\Delta c_{P1} - x_2 \delta c_P^g)} \quad (5.32)$$

In Equation 5.32, δc_P is usually small compared to Δc_{Pi} and, therefore, k can be approximated by:

$$k = \frac{\Delta c_{P1}}{\Delta c_{P2}} \left[1 + w_1 \frac{\delta c_P^l}{\Delta c_{P2}} + w_2 \frac{\delta c_P^g}{\Delta c_{P1}} \right] \quad (5.33)$$

In cases of strong interactions, such as systems that include hydrogen-bonding or other strong/directional interactions, a further simplification is possible via:

$$T_{gm} = \frac{x_1 T_{g1} + k_0 x_2 T_{g2}}{x_1 + k_0 x_2} - \frac{\chi R (T_{g2} - T_{g1}) \phi_1 \phi_2}{(x_1 + k_0 x_2) M_1 \Delta c_{P1}} \quad (5.34)$$

where

$$k_0 = \Delta c_{P1} / \Delta c_{P2} \quad (5.35)$$

From the concentration dependence of the glass transition temperature of the system water/bovine serum albumin (cf. Figure 93), the theoretical heat of mixing was predicted as shown in Figure 97 and compared against the calorimetrically determined heat of sorption subtracted by the corresponding heat of condensation. The relevant thermodynamic properties of amorphous low-density water were taken from [57] as $T_{g1} = 136 \pm 2 \text{ K}$, and $\Delta c_{P1} \approx 1 \text{ JK}^{-1} \cdot \text{mol}^{-1}$. The heat capacity step increase at T_{g2} was found to be about $\Delta c_{P2} = 3.38 \times 10^{-6} \text{ JK}^{-1} \cdot \text{mol}^{-1}$. Whilst a quantitative disagreement between the two ΔH_i is observed in Figure 97, the qualitative trend of the calorimetric measurements is retained. In this analysis, χ was allowed to vary with concentration according to Equation 2.12. Recalling the schematic of the thermodynamics of the glassy state (cf. Figure 87), this analysis suggests that the bovine serum albumin undergoes a volume contraction upon mixing with water. The sorption kinetics (cf. Chapter 3), exothermic heats of mixing (cf. Chapter 4), as well as the treatment by the Sanchez–Lacombe equation of state (cf. Chapter 6) corroborate this hypothesis. Volume contraction is suggested for exothermic mixing by the Sanchez–Lacombe equation of state.

Whilst the Lu–Weiss analysis is semi-empirical, it has to be pointed out that it is capable of representing the qualitative trend of the observed phenomena upon sorption of water in glass bovine serum albumin at the studied temperature.

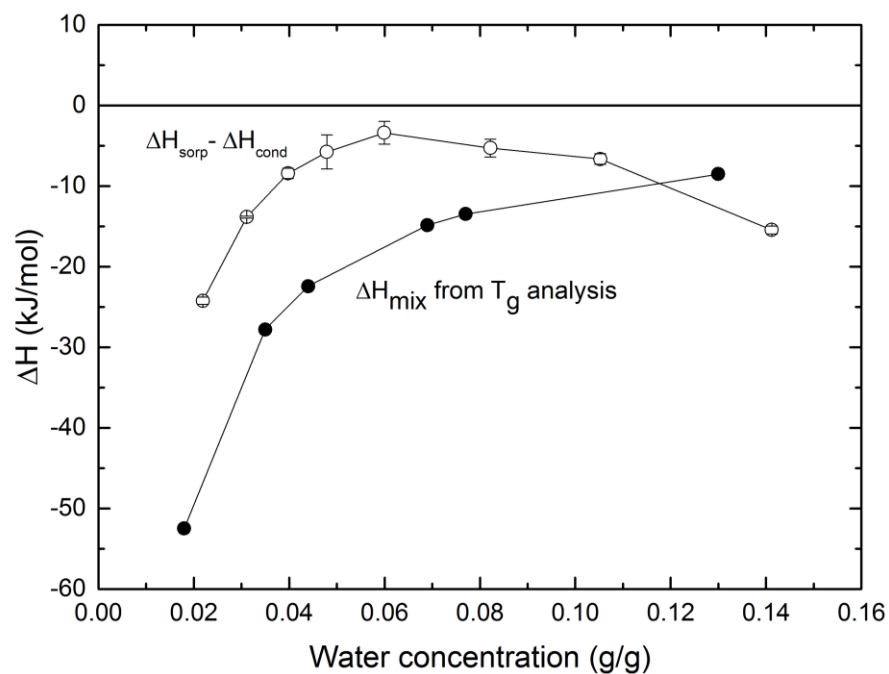


Figure 97 Heat of mixing ($= \Delta H_{\text{sorp}} - \Delta H_{\text{cond}}$) from calorimetric experiment and predicted heat of mixing from Equation 5.34 as a function of water concentration for the system water/bovine serum albumin at 293.15 K.

5.5 CONCLUSION

In this study, the glassy nature of bovine serum albumin was confirmed by differential scanning calorimetry. The glass transition temperature was determined for pure bovine serum albumin and for its concentrated mixtures with water. Water was found to strongly plasticise the protein's matrix and the resulting concentration dependence of the glass transition temperature was modelled with the Fox equation, Gibbs–DiMarzio theory, and the Chow equation. In all three cases, a good representation of the experimental data was achieved giving weight to the employed polymer–protein analogy. An endothermic overshoot upon glass transition was observed. It is suggested that this effect arises from physical aging/non-equilibrium effects of the protein glass. Furthermore, the specific heat capacity increases at the glass transition was found to monotonically increase with water concentration within the studied concentration range. The relationships between the mixture's glass transition behaviour and the observed moisture sorption isotherms (cf. Chapter 2) were studied by means of the Vrentas–Vrentas theory and the Lu–Weiss model. Whilst the former suggest a strong impact of plasticisation on the moisture sorption behaviour, the latter suggests a qualitatively (but not quantitatively) appropriate enthalpy of mixing of water with bovine serum albumin.

5.6 REFERENCES

- [1] Tamman, G. *Aggregatzustände*, Leipzig, **1922**
- [2] Schmelzer, J.W.P.; Gutzow, I.S.; Mazurin, O.V.; Priven, A.I.; Todorova, S.V.; Petrov, P.B. *Glasses and the Glass Transition*. Weinheim: Wiley-VCH **2011**
- [3] Cangialosi, D. *J. Phys. Condens. Matter* **2014**, 26, 153101-153120
- [4] Prigogine, I.; Defay, R. *Chemical Thermodynamics*, London: Longman, **1954**
- [5] Kauzmann, W. *Chem. Rev.* **1948**, 43, 219-256
- [6] Simon, F. *Z. Anorg. Allg. Chem.* **1931**, 203, 219-227
- [7] Kovacs, A.J. *Fortsch. Hochpolym. Forsch.* **1963**, 3, 394-508
- [8] Struik, L.C.E. *Physical Aging in Amorphous Glassy Polymers and Other Materials*. 1st ed. Amsterdam: Elsevier, **1978**
- [9] Hutchinson, J.M. *Prog. Polymer Sci.* **1995**, 20, 703-760
- [10] Cangialosi, D.; Boucher, V.M.; Alegria, A.; Colmenero, J. *Soft Matter* **2013**, 9, 8619-8630
- [11] Wunderlich, B. ed. **2005**. *Thermal Analysis of Polymeric Materials*, Berlin, Springer
- [12] Frauenfelder, H.; Sligar, S.G.; Wolynes, P.C. *Science* **1991**, 254, 1598-1603
- [13] Doster, W.; Cusack, S.; Petry, W. *Nature* **1989**, 337, 754-756
- [14] Rasmussen, B.F.; Stock, A.M.; Ringe, D.; Petsko, G.A. *Nature* **1992**, 357, 423-424
- [15] Green, J.L.; Fan, J.; Angell, C.A. *J. Phys. Chem.* **1994**, 98, 13780-13790
- [16] Angell, C.A. *Science* **1995**, 267, 1924-1935
- [17] Batzer, H.; Kreibich, T. *Polym. Bull.* **1981**, 5, 585-590
- [18] Papir YS, Kapur S, Rogers CE, Baer E. *J. Polym. Sci. Part A2* **1972**, 10, 1305-1319
- [19] Kakivaya SR, Hoeve CAJ. *Proc. Nat. Acad. Sci. U.S.A.* **1975**, 72, 3505-3507
- [20] Marshall AS, Petrie SEB. *J. Photograph Sci.* **1980**, 28, 128-134

- [21] Ogiwara Y, Kubota H, Hayashi S, Mitomo N. *J. Appl. Polym. Sci.* **1970**, 14,303-309
- [22] Gordon, M.; Taylor, J. *J. Appl. Chem.* **1952**, 2, 493-500
- [23] Simha, R.; Boyer, R.F. *J. Chem. Phys.* **1962**, 37, 1003-1007
- [24] Schneider, H.A.; Rieger, J.; Penzel, E. *Polymer* **1997**, 38, 1323-1337
- [25] Couchman, P.R.; Karasz, F.E. *Macromolecules* **1978**, 11, 117-119
- [26] Fox, T.G. *Bull. Am. Phys. Soc.* **1956**, 1, 123-128
- [27] Lesikar, A.V. *Polymers* **1976**,80, 1005-1010
- [28] Jenkel, E.; Heusch, R. *Colloid Polym. Sci.* **1953**, 130, 89-105
- [29] Kwei, T. *J. Polym. Sci.* **1984**, 43, 219-256
- [30] Lin, A.A.; Kwei, T.K.; Reiser, A. *Macromolecules* **1989**, 22,10, 4112-4119
- [31] Brekner, M.J.; Schneider, H.A.; Cantow, H.J. *Polymer* **1988**, 29, 78-85
- [32] Gibbs, J.H. *J. Chem. Phys.* **1956**, 25, 185-186
- [33] Gibbs, J.H.; Di Marzio, E.A. *J. Chem. Phys.* **1958**, 28, 807-813
- [34] Guggenheim EA. 1952. *Mixtures*. Clarendon Press Oxford
- [35] Flory P.J. *Proc. Roy. Soc.* **1956** A234, 60-73
- [36] Chow, T.S. *Macromolecules* **1980**, 13, 362-364
- [37] Gordon, J.M.; Rouse, G.B. Gibbs, J.H.;Risen, W.M. *J. Chem. Phys.* **1977**, 66, 4971-4976
- [38] Hill TL. **1960**. *An Introduction to Statistical Thermodynamics*. Addison-Wesley, London
- [39] Foltz, C.R.; McKinney, P.V. *J. Appl. Polym. Sci.* **1969**, 13, 2235-2245
- [40] Petrie, S.E.B. *J. Polym. Sci. Part. A-2* **1972**, 10, 1255-1272
- [41] Weitz, A.; Wunderlich, B. *J. Polym. Sci., Polym. Phys. Ed.* **1974**,12, 2473-2491
- [42] Brown, I. G.; Wetton, R. E.; Richardson, M. J.; Savill, N. G. *Polymer* **1978**, 19, 659-663
- [43] Richardson, M. J.; Savill, N. G. *Br. Polym. J.* **1979**, 11, 123-129

- [44] Prest, W. M., Jr.; O'Reilly, J. M.; Roberts, F. J., Jr.; Mosher, R. A. *Polym. Prep, Am. Chem. Soc., Div. Polym. Chem.* **1980**, 21 (2), 12.
- [45] Prest, W. M., Jr.; Roberts, F. J., Jr. *Ann. N.Y. Acad. Sci.* **1981**, 371, 67-86
- [46] Brady, T. E.; Jabarin, S. A. *Polym. Eng. Sci.* **1977**, 17, 686-690
- [47] Matsuoka, S. *Polym. Eng. Sci.* **1974**, 14, 162-166
- [48] Berens, A.R.; Hodge, I.M. *Macromolecules* **1981**, 15, 3, 756-761
- [49] Berens, A.R.; Hodge, I.M. *Macromolecules* **1981**, 15, 3, 762-770
- [50] Vrentas JS, Duda JL. *Macromolecules* **1976**, 9, 785-790
- [51] Vrentas JS, Duda JL. *J. Polym. Sci. (Polym Phys Ed)* **1977**, 15, 403-416
- [52] Vrentas, J.S.; Vrentas, C.M. *Macromolecules* **1991**, 24, 9, 2404-2412
- [53] Cohen MH, Turnbull D. *J. Chem. Phys.* **1959**, 31, 1164-1169
- [54] Hancock, B.C.; Zografi, G. *Pharm. Res.* **1993**, 10, 9, 1262-1267
- [55] Lu, X.; Weiss, R.A. *Macromolecules* **1992**, 25, 3242-3246
- [56] Couchman, P.R. *Macromolecules* **1987**, 20, 1712-1717
- [57] Handa, YP; Klug, DD. *J. Phys. Chem.* **1988**, 92, 12, 3323-3325

Chapter 6. Application of lattice fluid and perturbation theories to glassy proteins

6.1 INTRODUCTION

Predicting the pressure–volume–temperature (PVT) behaviour of polymers and their phase behaviour in solutions has received strong interest in the past couple of decades both experimentally and theoretically due to the growing industrial interest on polymer phase behaviour. Traditional approaches including the lattice [1] and lattice-fluid (LF) [2] theories largely fail to describe more complex systems adequately such as solutions of polyelectrolytes (because of their counter-ions), globular proteins (because of their compact structure), or non-linear chain-molecules (because of their molecular architecture). Moreover, these theories can neither account for non-equilibrium effects (as present in glassy polymers) *per se* nor can they reflect the structural complexity of associating (e.g. hydrogen-bonding systems) higher-order polymers.

Despite the long-standing engagement in thermodynamics of polymers containing liquids, our knowledge in some interesting and biologically important areas is still rudimentary. While polymer glasses are frozen liquids, proteins are macromolecules of a well-defined structure. Regardless of this difference, proteins and glasses share fundamental properties and ‘glassy dynamics’ may be used to describe pure-fluid protein properties. [3] Conceptually, bio-polymers are complex by nature. In view of the underlying central assumption of combinatorial mixing as the reference behaviour in the lattice and lattice-fluid theory, it is thought that these approaches may hold true without fundamental changes for associating systems such as proteins.

The LF fluid theory can be extended to account for non-directional association forces (i.e. not applicable to hydrogen-bonding systems). Among one of the first theoretical lattice treatments to

account for some level of association was the Panayiotou and Vera (PV) theory [4]. They introduced a correction term to account for non-random mixing of holes and mers. More recently, there has been strong interest in moving away from (quasi-)lattice models towards a continuous space. Tangent-sphere models have received increasing interest and have proven successful to predict the phase behaviour of progressively more complex macromolecules. [5] The essence of these approaches is to use a reference fluid which accounts for the repulsive forces and volume of the molecule. To model the interactions of real fluids, the free energy is expanded by the addition of attractive/association terms. Both the lattice fluid equation of state and the Statistical Associating Fluid Theory (SAFT) [6] have been extended to the non-equilibrium domain to account for the non-equilibrium nature of a glassy polymer. [7]

One of the main bottlenecks in employing more advanced thermodynamic treatments to describe the behaviour of pure-fluid (bio-)polymer and concentrated water/(bio)-polymer systems is the consistent estimation of the intermolecular force field and/or pure-component characteristic properties for the protein. Lattice fluid approaches are based on the idea that a combination of characteristic pure-component properties and a mixing parameter, both estimated over the relevant temperature and pressure ranges, can adequately describe the mixture's phase behaviour. Typically, the characteristic parameters are obtained from a regression of some experimental PVT data.

For quite some time now, a solution model approach was thought to be 'the best starting point' [8] for predicting the phase behaviour of bio-macromolecules and their mixtures with water.

Here, isothermal pressure–volume data for three amorphous model proteins measured at 298.15 K with a volume dilatometer using mercury as the hydrostatic fluid are presented. The suitability of the Sanchez-Lacombe EOS and a Perturbed Hard-sphere Chain EOS to describe data of such kind is tested and the influence of the strong association forces present in proteins onto the

suitability of some of these theoretical treatments are explored. Subsequently, the interval water sorption isotherm of bovine serum albumin at 293.15 K was modelled using a lattice fluid approach.

6.2 EXPERIMENTAL SECTION

6.2.1 Materials

Bovine serum albumin (BSA) (lyophilised powder, catalogue number 05470), *porcine* pancreas lipase (PPL) (lyophilised powder, catalogue number L0382), and *chicken* egg white lysozyme (lyophilised powder, catalogue number L6876) were purchased at the highest commercially available grade from Sigma Aldrich (Gillingham, United Kingdom). Ultrapure deionised water (resistivity $\sim 16\text{ M}\Omega \cdot \text{cm}$) was used for all sorption experiments. Mercury (purity $> 99.99\%$) was obtained from Mercury Recycling Limited (Manchester, United Kingdom).

6.2.2 Methods

See Section 2.2.2 for detailed methodology on Dynamic Vapour Sorption experiments used in this Chapter.

The pressure–volume isotherms presented here were measured with an Autopore IV 9500 (Micromeritics Co., Norcross, GA) dilatometer that uses mercury as the hydrostatic pressure fluid. Protein powder samples (typically 300 mg) were placed ‘as received’ into the sample penetrometer/holder. A 3 mL powder penetrometer was used (Micromeritics, Product number, 920-61716-01). The shaft of the penetrometer was greased with a silicon free high-vacuum sealant (H Grease, Apiezonm Manchester, UK) to achieve a vacuum-tight fit of the sample holder within the instrument. After insertion of the penetrometer into the apparatus, a vacuum was pulled for 12 hours to remove any residual moisture from the protein powder. Then, the hydrostatic pressure was increased in a step-wise fashion from 0.015 bar up to $2,275\text{ bar}$. The experiment was computer-automated with a typical pressurisation–depressurisation loop taking about six hours.

6.3 RESULTS AND DISCUSSION

6.3.1 The isothermal compressibility of glassy proteins

Figure 98 shows isothermal pressure–volume data for three amorphous model protein powders at 298.15 K. For all three systems, the experimental temperature is below their glass transition temperature (at atmospheric pressure). A plot of reduced experimental density, $\tilde{\rho}_{exp}$, which was defined here as:

$$\tilde{\rho}_{exp} = \frac{\rho}{\rho^*} \quad (6.1)$$

where ρ^* denotes the high-pressure close packing density is provided. The data suggests that all three materials are compressible with the actual extent differing between the three proteins. Particularly at near-ambient pressures that are so relevant to most protein applications and modelling attempts, it is observed that the matrix compressibility may not be neglected when treating a protein theoretically.

This finding implies that any theoretical treatment of a protein and for instance its binary mixtures with low molecular weight solvents (e.g. water) should allow for compressibility of the matrix. While this effect has been anticipated for quite some time, there has been no previous experimental investigation into the pure-fluid isothermal compressibility of a protein powder. From early on it was realised that the Flory-Huggins theory fails to describe the experimentally determined isotherm of water with hydrophilic (bio-)polymers. [8] The impact of structural changes on a protein's water sorption was first reported in the early 1970s [9]. Up-to-date, most efforts in making the Flory–Huggins theory somehow ‘work’ rely on the extensions by Vrentas and Vrentas [10, 11]

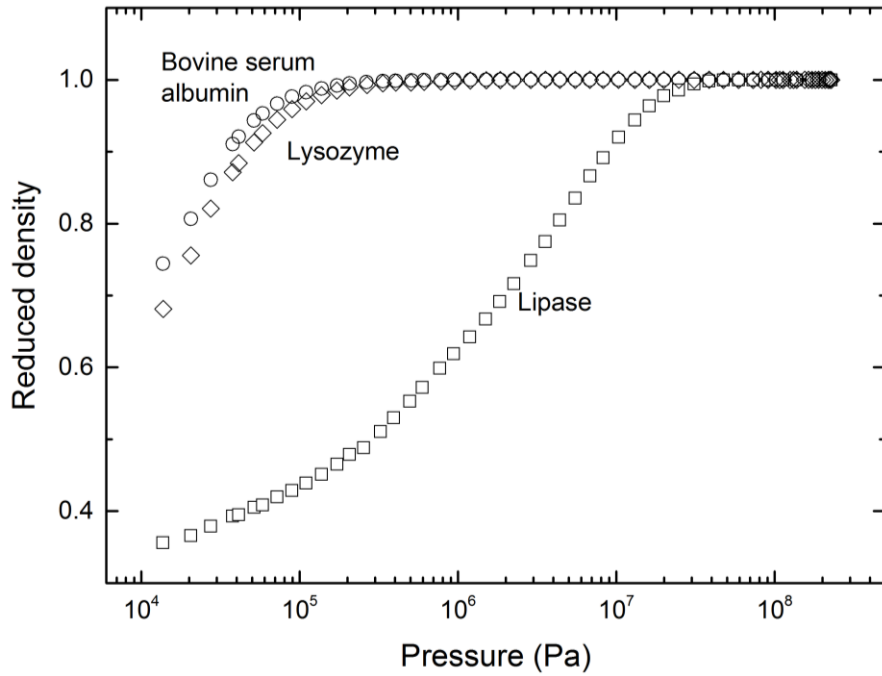


Figure 98 Isothermal pressure–volume data for BSA, lysozyme from chicken egg white, and PPL at 298.15 K. Experimental error smaller than symbol size. $n=2$ for bovine serum albumin and lysozyme, $n=4$ for lipase.

and Rosenbaum [12]. Rosenbaum's [12] theory has only been applied to one system, the keratin/water system. This result is most likely due to the somewhat empirical nature of the model. Rosenbaum's isotherm model is derived from the Flory-Huggins theory, which cannot account for pressure, and yet a compressibility term to estimate the volumetric changes induced by water sorption was incorporated. The excess free energy (and enthalpy) of dilution contribution, that Rosenbaum added to the Flory–Huggins isotherms is of the form of:

$$\Delta\mu_1^E = (1/2\beta) \frac{\partial(\Delta V^2/V)}{\partial n_1} = (1/2\beta) \tilde{V} \quad (6.2)$$

where symbols have the standard meaning and ΔV denotes the volume contraction upon mixing. In his treatment, this term is then divided by RT and subtracted from the standard Flory–Huggins isotherm equation (cf. Equation 2.11) While the Vrentas–Vrentas theory is popular and has been

widely and successfully applied to numerous hydrophilic (bio-)polymers, the assumption of a non-compressible lattice seem to be unsuitable in the light of the experimental results reported here.

6.3.2 Modelling the isothermal compressibility

Sanchez-Lacombe EOS. The lattice fluid (LF) theory derived by Sanchez and Lacombe [2] is an equation of state for chain-like fluids that is based on the Ising (lattice) fluid model. Compressibility is introduced via the introduction of vacant lattice sites. Their EOS for a polymer of r segments, that is randomly arranged on a lattice of constant size, is given by:

$$\frac{\tilde{p}\tilde{v}}{\tilde{T}} = \frac{1}{r} - \left[1 + \tilde{v} \ln \left(1 - \frac{1}{\tilde{v}} \right) \right] - \frac{1}{\tilde{v}\tilde{T}} \quad (6.3)$$

where the reduced ($\sim$) and characteristic (*) properties are defined by:

$$\tilde{p} = p/p^* = pv^*/\varepsilon \quad (6.4)$$

$$\tilde{T} = T/T^* = RT/\varepsilon \quad (6.5)$$

$$\tilde{v} = V/V^* \quad (6.6)$$

Here, ε is the mer-mer interaction parameter and v^* is the closed packed volume per lattice site related via $V^* = N(rv^*)$. A real fluid is completely characterised by the three molecular parameters: p^* , the hypothetical cohesive energy density at absolute zero; T^* , does not have a clear physical meaning but is proportional to the potential energy well depth; and V^* the hypothetical specific volume at closest packing. Equation 6.3 suggests that PVT data for polymer liquids are relatively insensitive to polymer molar mass. As polymer molecular weight increases ($r \rightarrow \infty$), the term $1/r$ becomes insignificant ($1/r \rightarrow 0$) suggesting a corresponding-state behaviour for polymer liquids.

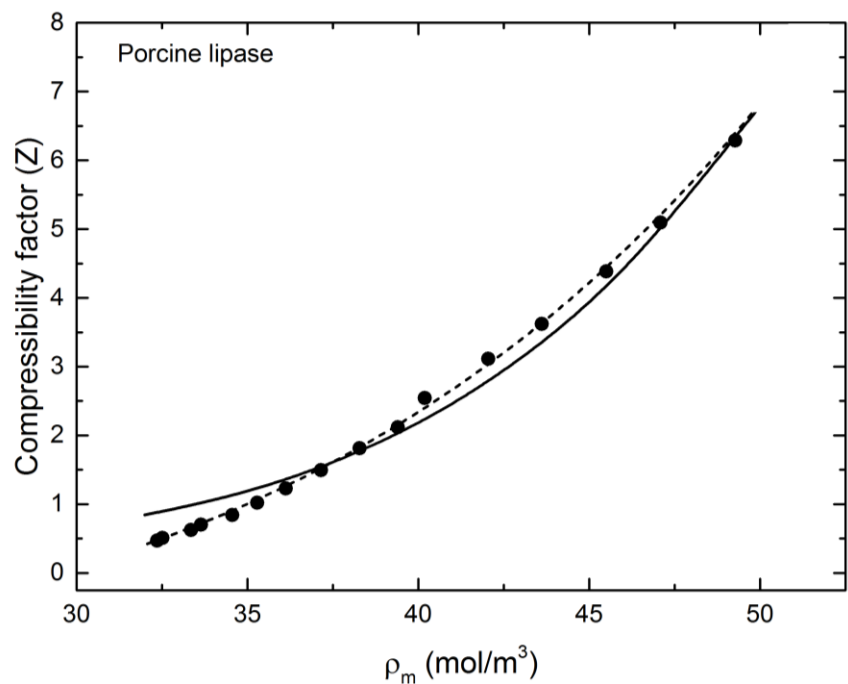


Figure 99 Isothermal compressibility factor Z for PPL glass at 298.15 K.

(●) Experimental data; (—) PHSC model fit; (---) SL EOS fit.

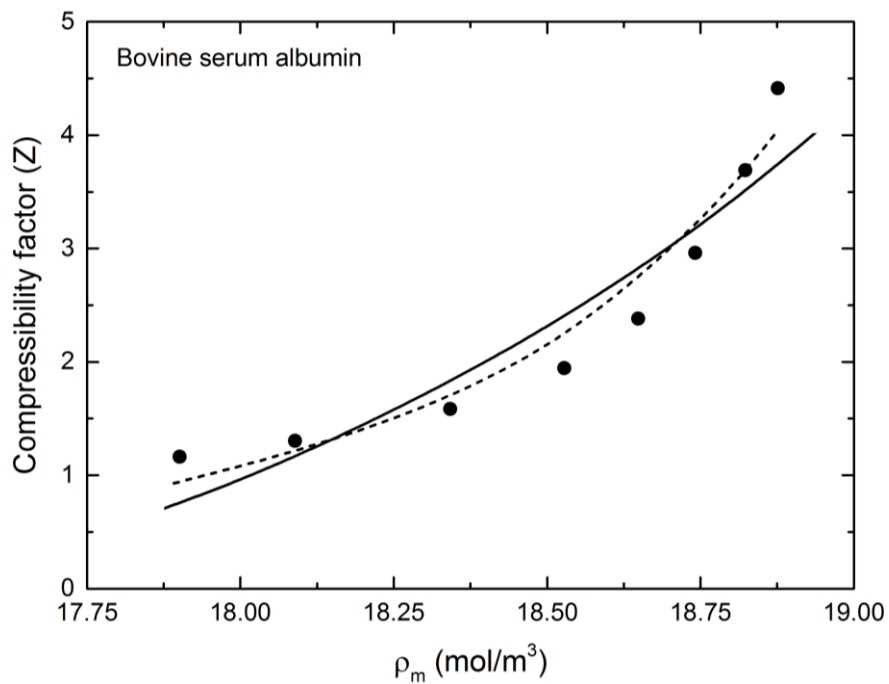


Figure 100 Isothermal compressibility factor Z for BSA glass at 298.15 K.

(●) Experimental data; (—) PHSC model fit; (---) SL EOS fit.

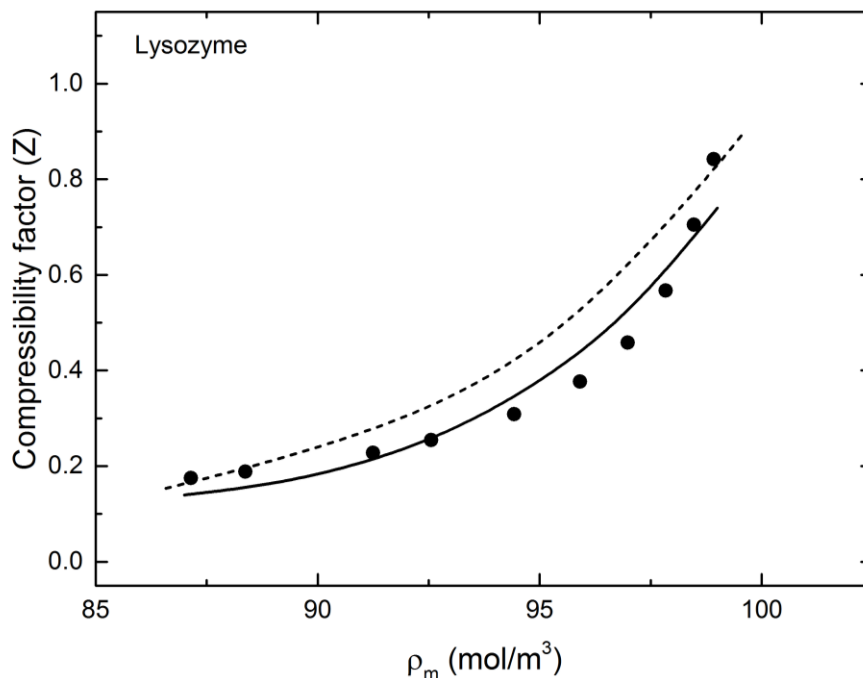


Figure 101 Isothermal compressibility factor Z for lysozyme glass at 298.15 K.

(●) Experimental data; (—) PHSC model fit; (— —) SL EOS fit.

Table 9 shows the regressed EOS parameters for the proteins of interest. While a good fit can be achieved for glassy bovine serum albumin (Figure 100) and lysozyme (Figure 101) at near-ambient pressures, the treatment is not consistent for the glassy lipase (Figure 99) as $\tilde{T} > 1$. Proteins are different (in terms of molecular architecture and its non-randomness) to the chain molecules the SL theory was derived for so that the corresponding-state theory may not hold good for protein-type systems.

Table 9 Sanchez–Lacombe pure-fluid equation of state parameters for some amorphous proteins regressed from isothermal pressure-volume data at 298.15 K.

Protein	P^*	T^*	V^*	Pressure range
	M N/m ²	K	m ³ /kg	M N/m ²
Porcine lipase	68	198	0.001104	0.4 – 12
Bovine serum albumin	1.5	489	0.000811	0.4 – 1.5
Lysozyme	1.4	315	0.000885	0.3 – 1.4

None of the prominent extensions to the SL theory that account for non-random mixing such as the Panayiotou–Vera theory [4] can account for the strongly directional attraction forces present in hydrogen-bonding polymers or biological macromolecules. The lattice-fluid theory in its original form may therefore not provide a suitable framework for such systems.

Perturbed Hard-Sphere Chain (PHSC) EOS. In the last twenty years, there has been increased interest in developing EOS models for polymers and polymer mixtures outside the lattice description. Tangent-sphere models portray chain-like fluids as freely-jointed tangent hard spheres. A hard-sphere chain (HSC) equation of state is used in these models and a perturbation term is added to account for the attractions in the form of:

$$\frac{p}{\rho k_B T} = \left(\frac{p}{\rho k_B T} \right)_{HSC} + \left(\frac{p}{\rho k_B T} \right)_{pert} \quad (6.7)$$

where p is the pressure, $\rho = N/V$ is the number density, N is the number of molecules, V is the volume of the system, and T is the absolute temperature. Among the most famous tangent-sphere models are the Generalized Flory (GF) theory as introduced by Hall and co-workers [13, 14] or the

perturbed hard-sphere-chain theory (PHSC) by Prausnitz and co-workers [15]. According to the PHSC first-order theory, the hard-sphere-chain equation of state is given by [15]:

$$\left(\frac{p}{\rho k_B T}\right)_{HSC} = 1 + r^2 b \rho g(d^+) - (r-1)[g(d^+) - 1] \quad (6.8)$$

or alternatively by a derivation based on Wertheim's first-order thermodynamic perturbation theory [16, 17] as given by [6]:

$$\left(\frac{p}{\rho k_B T}\right)_{HSC} = 1 + r^2 b \rho g(d^+) - (r-1) \rho \frac{\partial \ln(g(d^+))}{\partial \rho} \quad (6.9)$$

where the $g(d^+)$ is the radial distribution function, calculated from the Carnahan-Starling EOS [18], r is the number of tangent hard spheres per molecule, b may be interpreted as the second virial coefficient of hard spheres prior to bonding to form chains or the van-der-Waals (vdW) co-volume ($b = 2\pi d^3(T)/3$). For the perturbation term, a vdW attractive term was used [15]:

$$\left(\frac{p}{\rho k_B T}\right)_{pert} = -\frac{r^2 a \rho}{k_B T} \quad (6.10)$$

where a represents the strength of the attractive force between two non-bonded segments.

An approximate expression for the radial distribution function at contact ($g(d^+)$) was obtained from the solution of the Percus–Yervick [19] equation as:

$$g_{PY}(d^+) = \frac{1 - \eta/2}{(1 - \eta)^2} \quad (6.11)$$

or semi-empirically by Carnahan and Starling [19]:

$$g_{CS}(d^+) = \frac{1 - \eta/2}{(1 - \eta)^3} \quad (6.12)$$

where $\eta = b\rho/4 = (\pi/6)\rho d^3$ is the packing fraction. Equation 6.11 and 6.12 are known to yield surprisingly similar results. [15]

Equation 6.7 is not directly applicable to associating fluids, for which specific interactions (e.g. hydrogen-bonding) play an important role in their structure and thermodynamic properties. Specific or oriented forces are inconsistent with one of the basic assumptions of the PHSC theory. That is, the structure of a liquid is dominated by repulsive forces. One method is to add an association term to the EOS:

$$\frac{p}{\rho k_B T} = \left(\frac{p}{\rho k_B T} \right)_{HSC} + \left(\frac{p}{\rho k_B T} \right)_{pert} + \left(\frac{p}{\rho k_B T} \right)_{assoc} \quad (6.13)$$

A famous example of this methodology is the statistical associated-fluid theory (SAFT) by Chapman and co-workers [6], where the association term is a statistical mechanical perturbation theory for spheres with ‘sticky’ bonding sites. Due to the complexity of the associating forces in a protein and their profound effect on maintaining the protein’s native structure, it was thought that the PHSC theory with an ‘effective’ perturbation contribution would describe the *PVT* behaviour of the model proteins adequately. Further, the experimental data lies near the protein’s dense-fluid limit and, therefore, it is expected that the hard-sphere chain contribution may play a more significant role on the protein’s *PVT* behaviour than the perturbation or association term.

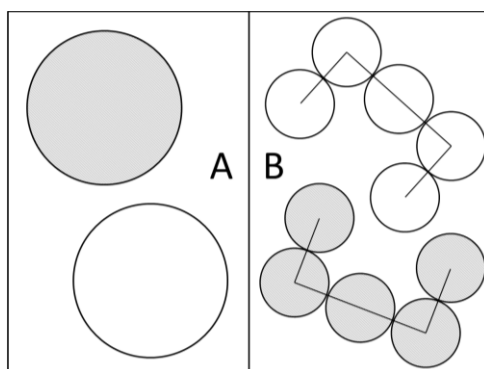


Figure 102 Equal-density system of (A) two single-sphere molecules (simple fluid approximation, $r = 1$) and (B) two chain molecules (molecular fluid approximation) of length r ($r = 5$).

In the PHSC theory, for $r = 1$, the theory becomes a two-parameter model with an attractive energy parameter $a(T)$ and an effective van der Waals covolume $b(T)$. Both parameters can be scaled by the depth of the pair potential at its minimum, ϵ , and separation distance at the minimum in the pair potential, σ . Molecular fluids (nonspherical or chain-like molecules) need an additional parameter, r , representing the number of segments per molecule, to describe molecular size.

Table 10 lists the regressed PHSC EOS parameters. A good representation of the experimental data was achieved for all three model systems. Typical values for r/M_w of common synthetic polymers (M_w 10^3 to 10^7 Da) lie between 0.01 and 0.10 mol/g. For these proteins, it was found that the pressure–volume isotherm can be modelled with $r \leq 3$ indicating that the proteins do not behave like a long-chain molecule. This result is not a surprising conclusion as all three proteins are globular. Indeed, it was discussed in Chapter 5, that proteins are often treated as single-body systems as opposed to many-body systems such as homo-polymers. Values for b and a are temperature-dependent and regressed values presented here are limited to 298.15 K (and the given pressure range).

The presented analysis suggests that a tangent-sphere model is fundamentally more suitable to describe the PVT behaviour of bio-polymers than a lattice-based approach. In principle, the complexity of interactions present in a bio-polymer could be captured in a tangent-sphere model. While it is beyond of the scope of this study, it has to be pointed out that it is the work on bio-oligomers or poly-peptide, which are structurally simpler than the model protein systems employed here, that will guide the way into more advanced theoretical treatments of thermophysical properties of bio-polymers.

Table 10 PHSC EOS parameters for some amorphous model proteins using the molecular-fluid approximation ($r \geq 1$) regressed from isothermal pressure-volume data at 298.15 K.

Protein	r	$b(T)$	$a(T)/k_B$	Pressure range
			K	bars
Porcine lipase	2	0.018009	10.572	0.4 – 12.0
Bovine serum albumin	3	0.012062	1.6674	0.2 – 1.4
Lysozyme	1.5	0.0021829	0.4015	0.4 – 1.7

Table 11 Pure component characteristic properties of water.

From reference [20].

Parameters	H ₂ O
T^* (K)	670
P^* (MPa)	2400
ρ^* (kg/m ³)	1050

6.3.3 Lattice fluid prediction of water sorption in BSA

In order to model or purely predict the sorption isotherms of water in glassy bovine serum albumin, the non-equilibrium treatment of the lattice fluid theory (NELF) can be employed. The NELF model has been successfully used to describe the solubility of non-condensable gases [21], condensable vapours [22], and liquids [23, 24] in glassy polymers. The NELF theory utilises the pure component characteristic properties of the penetrant and polymer together with an appropriate

mixing function. The fundamental idea is that the chemical potential of the penetrant in the pure penetrant phase is equal to that of the penetrant in the mixture. The characteristic properties for bovine serum albumin were obtained in Section 6.3.2. The lattice fluid pure component parameters for water are shown in Table 3 and were taken from the literature [20].

For binary mixtures (e.g. water/bovine serum albumin), the mixing rules as originally proposed by Sanchez–Lacombe apply and can be summarised as follows.

The molar lattice sites in the mixture have the volume:

$$v^* = \phi_1^0 v_1^* + (1 - \phi_1^0) v_2^* \quad (6.14)$$

where the concentration variable ϕ_1^0 is defined by:

$$\phi_1^0 = \frac{r_1^0 n_1}{r_1^0 n_1 + r_2^0 n_1} \quad (6.15)$$

The ratio of occupied sites of an individual molecules of species 1 in the mixture (r_1) is then defined as:

$$r_1 = r_1^0 (v_1^*/v^*) \quad (6.16)$$

The characteristic pressure of the mixture P^* is given by:

$$P^* = \phi_1 P_1^* + (1 - \phi_1) P_2^* - \phi_1 (1 - \phi_1) \Delta P^* \quad (6.17)$$

where the volume fraction ϕ_1 of the penetrant species in the mixture is defined as:

$$\phi_1 = \phi_1^0 (v_1^*/v^*) \quad (6.18)$$

The characteristic density of the mixture is defined in terms of weight fractions as:

$$\frac{1}{\rho^*} = \frac{\omega_1}{\rho_1^*} + \frac{1 - \omega_1}{\rho_2^*} \quad (6.19)$$

Further, the characteristic temperature is given by:

$$T^* = \frac{v^* P^*}{R} \quad (6.20)$$

The Gibbs free energy for a binary mixture of n_1 moles of a low molecular weight penetrant and n_2 moles of polymer is given by:

$$G = RT^*(r_1 n_1 + r_2 n_2) \left[-\tilde{\rho} + \frac{\tilde{P}}{\tilde{\rho}} + \frac{\tilde{T}}{\tilde{\rho}} \left((1 - \tilde{\rho}) \ln(1 - \tilde{\rho}) + \frac{\phi_1}{r_1} \tilde{\rho} \ln(\tilde{\rho} \phi_1) \right) \right] \quad (6.21)$$

In Equation 6.21, the densities are given by their equilibrium values. In order to allow for the non-equilibrium nature of a glassy polymer, the polymer density can be taken as an internal state variable. [25] This internal state variable can be related to the reduced density of the mixture by:

$$\tilde{\rho} = \frac{\rho_2}{1 - \omega_1} \frac{1}{\rho^*} \quad (6.22)$$

In addition, the internal state variable needs to be coupled to a time-evolution equation according to:

$$\frac{d\rho_2}{dt} = f(T, P, \omega_1, \rho_2) \quad (6.23)$$

For numerous glassy polymers, it has been shown [25] that the density can be assumed to be proportional to the external pressure of the vapour phase according to:

$$\rho_2 = \rho_2^0 (1 - kP) \quad (6.24)$$

where ρ_2^0 denotes the density of the dry polymer and k is a swelling coefficient.

From Equation 6.21 and 6.23, the chemical potential of the penetrant species in the mixture (μ_1^S) can be obtained and is given by:

$$\begin{aligned} \frac{\mu_1^S}{kT} = \ln(\tilde{\rho} \phi_1) - \left[r_1^0 + \frac{(r_1 - r_1^0)}{\tilde{\rho}} \right] \ln(1 - \tilde{\rho}) - r_1 \\ - \frac{\tilde{\rho}}{RT} [r_1^0 v_1^* (P_1^* + P^* - (1 - \phi_1)^2 \Delta P^*)] \end{aligned} \quad (6.25)$$

In the NELF model, the binary parameter ΔP^* is obtained from mixing rules and can be written as:

$$\Delta P^* = P_1^* + P_2^* - 2\psi \sqrt{P_1^* P_2^*} \quad (6.26)$$

where ψ is the dimensionless binary parameter and is related to the binary interaction parameter K_{12} by:

$$K_{12} = 1 - \psi \quad (6.27)$$

which can be regarded as an indicator for the attractive properties of the binary mixture.

The sorption isotherm can be calculated by assuming that the chemical potential of the vapour in the gas phase is equal to that of the vapour species in the mixture. The interested reader can find the solution strategy outlined elsewhere [26]. Figure 103 shows the fit of the lattice fluid theory of an interval water vapour sorption isotherm of bovine serum albumin. There was no experimental swelling data available. Therefore, the swelling parameter k was set to zero. This implies that the NELF model was actually employed in a quasi-equilibrium mode. However, as attractive

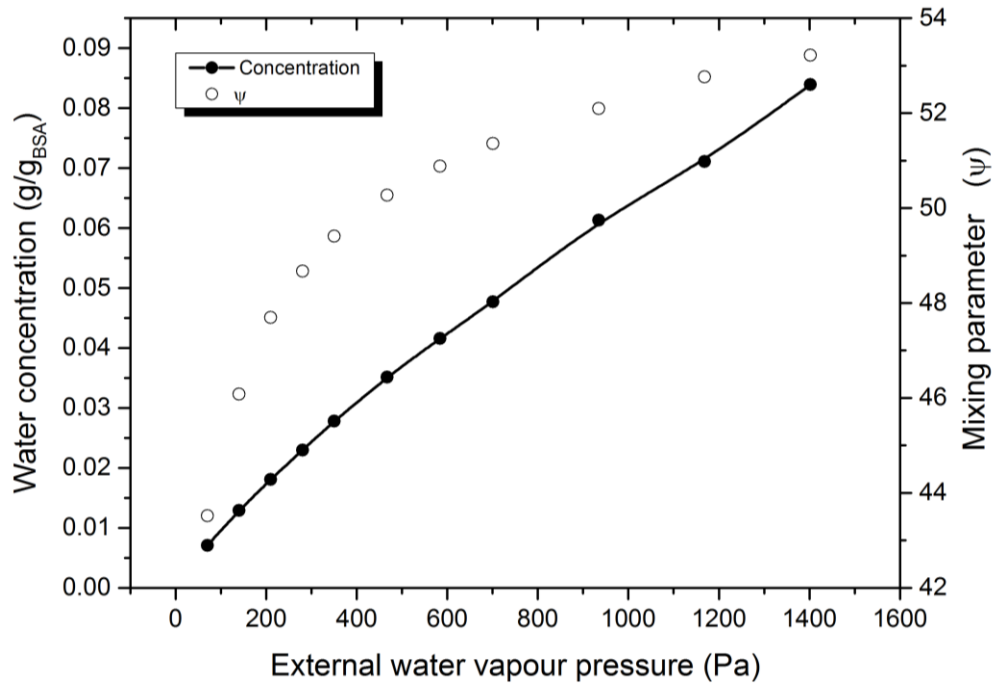


Figure 103 The NE-LF isotherm for water/BSA. Interval water vapour sorption isotherm of BSA at 293.15 K (primary axis) (—) Lattice fluid model fit. Lattice fluid mixing parameter as a function of external vapour pressure (secondary axis).

interactions are very strong and the lattice fluid theory for mixtures was found to be sensitive to the value of the mixing parameter, ψ was allowed to vary with external vapour pressure.

This assumption was based on experimental evidence for the concentration dependence of the heat of sorption/mixing as presented in Chapter 4. The analysis of the mixing parameter suggests that the strongest changes of the protein–water interaction occur at low hydration level; a picture already established in previous chapters. However, it has to be pointed out that the observed large value for ψ arises from the small value of P^* of BSA and the employed mixing rule (Equation 6.26). A refined mixing rule or a value for P^* that was regressed from isotherms over a few temperatures might yield a more common value for ψ .

6.4 CONCLUSION

Isothermal pressure–volume data of three amorphous protein powders (bovine serum albumin, porcine lipase, and chicken egg-white lysozyme) were obtained at 298.15 K with a hydrostatic technique using mercury as the hydrostatic fluid up to pressures of about 2,275 bar. A pronounced pressure-dependence of the pure-fluid density for bovine serum albumin and chicken egg white lysozyme for pressures up to about 5 *MPa* and for the lipase up to about 50 *MPa* was observed.

Two of the most common polymer equation-of-state (EOS) theories, namely the Sanchez-Lacombe (SL) EOS and the perturbed hard-sphere chain (PHSC) EOS, have been applied to isothermal pressure–volume data of three amorphous protein powders. A good representation of the experimental data by both EOS models except for the SL EOS for porcine lipase, where the corresponding-states assumption is suggested to not hold good, was found. From this work (and its theoretical framework), it is suggested that tangent-sphere models/perturbation theories are more suitable to treat biological macromolecules theoretically. However, the bio-chemical complexity of a protein (and its influence on the association behaviour) does not allow treating the protein as ‘sticky’ molecule in a meaningful manner. Therefore, the SL EOS was tested to model the interval water vapour isotherm for bovine serum albumin at 293.15 K.

As more thermo-physical experimental data of model proteins systems will become available, our theoretical understanding is expected to meet the increasing demand by industry to control and predict the phase behaviour of biological macromolecules. The presented work points towards the importance to pursue advanced thermodynamic modelling in this field.

6.5 REFERENCES

- [1] Flory, P.J. Principles of Polymer Chemistry. Cornell University Press: London, **1953**
- [2] Sanchez, I.C.; Lacombe, R. *Macromolecules* **1978**, 11(6), 1145-56
- [3] Angell, CA. *Science* **1995**, 267, 1924-1935
- [4] Panayiotou, C.; Vera, J.H. *Polym. J.* **1982**, 14, 681-694
- [5] Lambert, S.M.; Song, Y.; Prausnitz, J.M. **2000**. Equations of state for polymer systems. In: Equations of State for Fluids and Fluid Mixtures, Part II. Ed.: Senger, J.V.; Kaiser, RF.; Peters, C.J.; White Jr, H.J.
- [6] Chapman, W.G.; Gubbins, K.E.; Jackson, G.; Radosz, M. *Ind. Eng. Chem. Res.* **1990**, 29, 1709-1721
- [7] Davis, E.M. Water sorption and diffusion in glassy polymers. PhD thesis, Drexel University, **2013**
- [8] Shamblin, S.L.; Hancock, B.C.; Zograf, G. *Europ. J. Pharmac. Biopharmc.* **1998**, 45, 239-247
- [9] Ikeda, S.; Kito, A.; Imae, T.; Maeda, H. *J. Colloid Interface Sci.* **1974**, 48, 256-262
- [10] Vrentas, J.S.; Vrentas, C.M. *Macromolecules* **1991**, 24, 2402-2412
- [11] Vrentas, J.S.; Vrentas, C.M. *Macromolecules* **1996**, 29, 4391-4396
- [12] Rosenbaum, S. *J. Polym. Sci. (Part C)* **1970**, 31, 45-55
- [13] Dickman, R.; Hall, C.K. *J. Chem. Phys.* **1986**, 85, 4108-4115
- [14] Hornell, K.G.; Hall, C.K. *J. Chem. Phys.* **1989**, 90, 1841-1855
- [15] Song, Y.; Lambert, S.M.; Prausnitz, J.M. *Ind. & Eng. Chem. Res.* **1994**, 33, 1074-1057
- [16] Wertheim, M.S. *J. Stat. Phys.* **1984**, 35,1/2, 19-34
- [17] Wertheim, M.S. *J. Stat. Phys.* **1984**, 35,1/2, 35-47
- [18] Carnahan, N.F.; Starling, K.E. *J. Chem. Phys.* **1969**, 51, 635-636

- [19] Hansen, J.P.; McDonald, I.R. **1990**. Theory of Simple Liquids (Ref. [1]),Sec. 5.5
- [20] De Angelis, M.G.; Sarti, G.C.; Doghieri, F. *J. Membr. Sci.* **2007**, 289, 106-122
- [21] Sarti, G.C.; Doghieri, F. *Chem. Eng. Sci.* **1998**, 53, 3435-3447
- [22] Galizia, M.; De Angelis, M.G.; Sarti, G.C. *J. Membr. Sci.* **2012**, 405-406, 201-211
- [23] Sarti, G.C.; De Angelis, M.G. *AIChE Journal* **2012**, 58, 292-301
- [24] Davis, E.M.; Minelli, M.; Giacinti Baschetti, M.; Sarti, G.C.; Elabd, Y.A. *Macromolecules* **2012**, 45,7486-7494
- [25] Giacinti Baschetti, M.; Doghieri, F.; Sarti, G.C. *Ind. Eng. Chem. Res.* **2001**, 40, 3027-3037
- [26] Doghieri, F.; Ghedini, M.; Quinzi, M.; Rethwisch, D.; Sarti, G.C. *Desalination* **2002**, 144, 73-

Chapter 7. Conclusions

7.1 SUMMARY

In this work, the water sorption and diffusion was measured for a number of glassy proteins including bovine serum albumin (BSA), *Bacillus sp.* protease, *Bacillus licheniformis* α -amylase, porcine pancreas lipase. Except for BSA, this has not been reported before. A Dynamic Vapour Sorption technique was employed in different experimental modes (i.e. interval, or integral sorption; cyclic or non-cyclic experiments) to capture the sorption and diffusion of the diffusant (water) in real time. Significant anomalies in terms of both water solubility and sorption kinetics were found for all systems studied here. Neither did the water solubilities show clear temperature trends nor was it possible to model or predict these complex water sorption isotherms of proteins by simple surface-based approaches or solution theories. Pronounced sorption–desorption hysteresis was observed. Specifically studied for BSA, it was found that full hydration of the protein is not achieved during interval sorption (or indeed an interval sorption–desorption cycle) even at long experimental time scales; though, the non-cyclic interval water desorption experiment, which started with a fully hydrated sample yielded an isotherm which was suggested to represent (quasi-)equilibrium. Upon reducing the time length per activity increment during interval water sorption, it was found that the mass uptake/water solubility of BSA increased with decreasing time length per activity interval. For shorter time lengths, where no mass-transfer equilibrium could be achieved, the mass uptake approached values for the long non-cyclic interval desorption experiment. A detailed comparison into the experimental modes of the sorption experiment for a water/protein system has not been reported before.

The interval and integral water sorption kinetics, specifically studied for BSA, showed non-Fickian behaviour. For the first time for a water/protein system, a relaxation–diffusion model was

employed to model sorption kinetics and the respective time constants were estimated for the interval water sorption experiment. Water sorption in BSA is found to be under relaxation control at experimental time scales. The kinetic sorption data for the interval sorption experiments at short time lengths per activity increment, that gave rise to an increase in water solubility compared to experiments at longer time lengths, were found to be significantly less affected by relaxation-induced anomalies. Indeed, it was suggested that the long-time scale relaxations could, therefore, be overridden by means of experimental design.

Exothermic heats of mixing, that were both temperature and concentration dependent, were measured with an isothermal flow calorimeter set-up in an experimental mode to mimic the long interval water sorption experiments of BSA. Enthalpy of mixing time evolution data was compared to the gravimetric sorption kinetic data, here for the first time, and was found to agree excellently. The enthalpy evolution data lead to the suggestion that competing exothermic and endothermic events occur during water sorption in BSA. The endothermic contributions to the overall heats of mixing may be related to the relaxations but, as of yet, a clear picture cannot be drawn. An analysis of the partial thermodynamic excess function of the process revealed typical energetics of hydrophilic hydration and that absorbed water begins to interact with itself at intermediate and high water activities. Furthermore, the calorimetric analysis over different temperatures suggests an energetic landscape of the sorption process that is different to that suggested by the so commonly used van-Laar/Flory–Huggins theory. It appears, it is not only the assumption of thermodynamic equilibrium in the development of the Flory–Huggins theory but also the assumptions behind both the entropies and enthalpies of mixing that make this theory in its original form unsuitable to describe water sorption in BSA and, indeed, proteins. Whilst phenomenological corrections of the Flory–Huggins theory were reported before, there has been

no report previous to this that is based on a combination of gravimetric and calorimetric experimental evidence.

The glassy, non-equilibrium nature of BSA and concentrated water/BSA solutions was further supported by insight from differential scanning calorimetry experiments. Whilst the glass transition temperature was found to be high for native and dry BSA, it sharply decreases upon increasing hydration. This concentration dependence of the glass transition temperature was modelled by using simple mixing rules and theories derived from statistical thermodynamics. Further, it was estimated that water contents achieved during non-cyclic interval desorption or integral sorption at high water activities are sufficient to depress the glass transition temperature of the water/protein mixture below the experimental temperature, thus making the mixture effectively rubbery. The connection between observed plasticisation and water solubility was explored by some polymer theories and it was found that the complex interactions and effects in water/protein system may not be appropriately represented by these theories.

Moving away from surface-based approaches, solution theory, or simple lattice fluid theories to describe the water solubility in glassy proteins requires knowledge of pure component equation-of-state type properties of the two mixture constituents. Therefore, pressure–volume isotherms were obtained for glassy BSA, porcine pancreas lipase, and chicken egg white lysozyme with a dilatometer at 298.15 *K* for the first time. The available experimental set-up did not allow to measure isotherms at different temperatures, though the observed pressure–volume isotherms were regressed to the Sanchez–Lacombe equation of state and a perturbed hard-sphere chain equation of state. The characteristic properties of BSA were then used to model a water vapour sorption isotherm by the non-equilibrium lattice fluid theory, here for the first time.

It is anticipated that this work would stimulate a revaluation of the design of water sorption experiments in glassy proteins and the interpretation of such data. This in turn is expected to

profoundly affect formulation engineering and Quality-by-Design approaches for a wide range of protein-based products. Ultimately, the findings of this study are of paramount importance to freeze-dried biological products both during development and regulatory approval. Recent efforts to achieve Quality-by-Design, the idea that a product is designed to meet specific objectives and is not empirically derived from performance of test batches, in the area of biologicals are greatly affected by the presented findings. As residual moisture, storage stability and bio-availability are intimately linked, it is suggested that more thought has to be given when determining the residual moisture content/water solubility of such products. How can decisions during development be made when there is a significant level of arbitrariness in the measurement and interpretation of the water sorption isotherm of a lyophilised biological?

Moreover, presented results touch upon regulatory frameworks. For instance, the Centre for Biologics Evaluation and Research of the U.S. Food and Drug Administration regulates freeze-dried products under Title 21 of the Code of Federal Regulations for Food and Drugs. Per regulation, each lot of dried biological product has to be tested for residual moisture. It has to meet and not exceed established limits as specified by an approved method on file of the respective license. As residual moisture is so strongly governed by non-equilibrium relaxations and affected by sample history both prior and during a water sorption experiment in solid, lyophilised biological products, it appears that both product design and regulatory approval considerations ought to consider these phenomena. Failing to do so would only continue what has been a line of tradition of some arbitrary water sorption uptake reports. It is not the natural origin of biological products and its variability that necessarily give rise to this behaviour but rather the inconsistencies in experimental design, and failures to describe the methodology accurately, that cause reports on water solubility data of amorphous, solid biologicals to vary considerably.

7.2 EPILOGUE

Having answered the question, ‘What have I done?’, in the previous section, it is now of interest to briefly explore the following question: ‘Why does it matter?’

The interaction between liquid water and proteins is ubiquitous; it is inherent to life’s equation. Much of the work on the complex interactions between water molecules and a protein are carried out within the dilute concentration regime. There, the topic of choice has long been the study of the protein hydration shell and its dynamics [1-4].

On the other side of the concentration spectrum stand non-dilute/concentrated protein/water solutions. Whilst these systems are also biologically interesting, particularly for structural proteins that, for instance, make up our hair or muscles, it is really a subject of chemical and bio-chemical engineering, and the physical chemistry of biological macromolecules. Traditionally, concentrated water/protein mixtures, such as those obtained during a water sorption measurement, were treated as solid-state systems. Gas adsorption and stoichiometric theories have been used for more than half a century now to explain hydration phenomena in protein powders. Therefore, a translation of knowledge gained from dilute mixtures to concentrated mixtures, and vice versa, was not possible due to the applied frameworks. A great field of current research is the establishment of the solution characters of protein/water mixtures containing as little water as a few weight percent, or indeed, proteins in the completely dehydrated state.

Much of the experimental work that forms the basis of the argument presented in this thesis was logically outlined in the sense that experiments were designed to complement each other and to move away from classical views of protein hydration towards more elaborate treatments that could include the solution character of the process. It was felt that there exists a great opportunity for the proposal of a paradigmic change on the view of protein hydration; a more advanced view that relies on the ever-advancing knowledge of solvent/polymer phase behaviour and equation-of-state

modelling approaches. In this notion, the thesis follows one idea: Having established the shortcomings and over-simplifications by surface-based approaches and simple solution theories (in Chapters 2 and 3), what does one needs to – via analogy – employ theories derived for polymer solution phase behaviour?

Whilst the experimental systems there would be simpler (e.g. in terms of molecular architecture, interactions, chemical stability), the theories are much more advanced allowing to describe molecular phenomena (e.g. relaxation, phase separation, association) present during solvent transport in these polymers. In most cases, the theoretical basis of these equations of state is knowledge over pure-component characteristic properties of the mixture's constituents as well as at least one mixture property. Therefore, Chapters 4 and 5 explore two mixing phenomena/properties, whilst Chapter 6 presents pure characteristic properties of glassy proteins. The pure-fluid phase behaviour of water is well studied. In theory, this knowledge would then suffice to describe the energetics of water transport as well as the solubility, or density changes upon mixing for instance. A great bottleneck here is the development of appropriate 'mixing rules' though. The strong directional interactions in concentrated aqueous protein solutions may only be captured in theories that allow for association such as the SAFT model [5].

Hopefully, the next few years will see a rising interest both by experimentalists and theoreticians to close the gap between theoretical modelling capacities and experimental data on the phase behaviour of concentrated aqueous protein solutions. Ultimately, this would then lead to a situation where one could predict the solubility of water in a protein at a given activity, temperature, and sample history (which might be treated by using density as an internal order parameter). Current models do not have predictive power *per se*. Another hope in this field is to construct phase diagrams of water/protein systems over the entire concentration range. This is not possible currently. It would be an exciting opportunity to link knowledge obtained from dilute

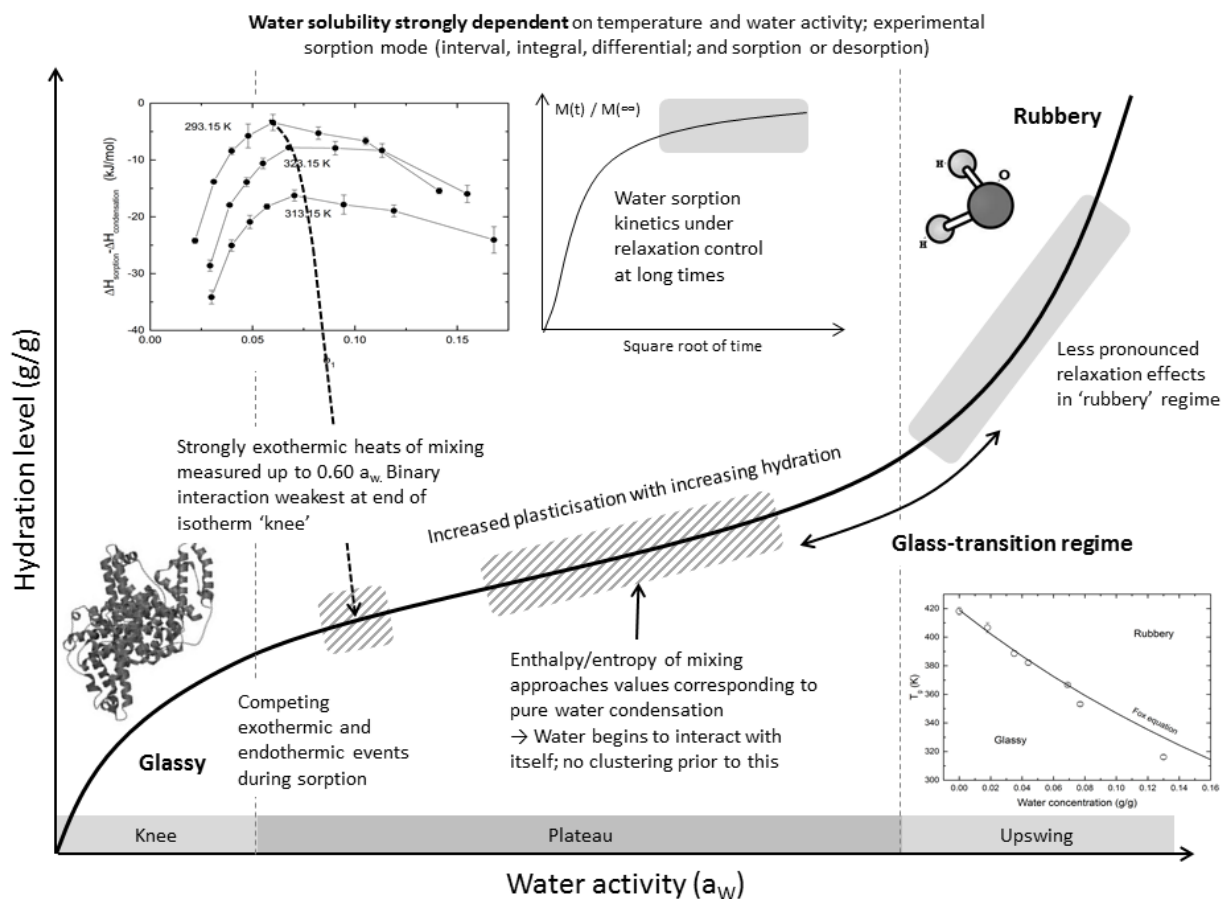


Figure 104 Schematic overview of the revised picture of hydration and water transport in a glassy protein. The hydration picture presented here summarises the outcomes of this Ph.D. study.

protein solutions to that obtained from concentrated protein solutions. For this, a theoretical framework is needed that spans the whole concentration range. It is one of the aims of the work presented in this thesis to outlay the experimental and theoretical hurdles in developing such unified solution theory across the complete concentration.

It is interesting now to compare the findings presented in the thesis to the picture of the chemistry of protein hydration as presented in Table 3 (cf. section 1.2). Figure 104 summarises some findings that the author would like to add to the picture of protein hydration. Most importantly, it has to be appreciated that the hydration picture presented in Table 3 does not

consider the kinetic nature of many processes occurring in an amorphous protein during water sorption. Furthermore, there is no mention of sample history and the dependence of the water solubility/mass uptake on the experimental path. It is obvious now that the water transport in a glassy protein, and the underlying molecular mechanisms, are different depending on the mode of sorption and experimental time scales.

In Table 3, it was presented that water mobility is low whilst protein motions are frozen at low hydration levels ($0 - 0.07 \text{ g/g}$). It is thought that this view needs to be corrected both in the light of previous and herein presented findings. Rasmussen and co-workers [6] studied dynamic properties of ribonuclease A down to very low temperatures. They found that ribonuclease A loses its catalytic function below the low-temperature 220K glass transition but would bind substrate or inhibitor rapidly at 228 K. Enzyme flexibility and function were found to be linked; clearly there could be no enzyme function if protein motions were completely ‘frozen’. This view is supported by the pronounced volume relaxations as manifested in both gravimetric and calorimetric kinetic interval water sorption data (cf. Chapters 3 and 4).

The presence of a two-dimensional phase transition, a transition in ‘surface’ water from disordered to ordered and/or clustered states, as suggested in Table 3, at a hydration level of about 0.07 g/g is partially supported by the calorimetric data presented in this study. Depending on the temperature, it was found that the enthalpy and entropy of mixing of water/BSA approach those values for pure water condensation at the respective temperature. Though, it is not possible to judge at this stage what state is exactly occupied by the water molecules; spectroscopic studies would be required. By the solvent–polymer analogy, it is also in question whether water is really ‘clustering’ as any plasticiser would actually be operating in an anti-clustering manner.

Furthermore, it was previously suggested that internal protein motions and water mobility would only increase in the plateau-region ($0.07 - 0.25 \text{ g/g}$) of the water sorption isotherm. (cf. Table 3).

On the basis on differential scanning calorimetry experiments, it is now suggested here that this statement needs to be extended. Indeed, it is the region of low hydration levels ($< 0.07 \text{ g/g}$) where water has a strong plasticising effect, thus increasing protein matrix flexibility. This suggestion is in line with the observed protein relaxations as manifested in the kinetic water sorption data.

Another important change to the picture of protein hydration suggested on the basis of the work presented in this thesis is about the notion of ‘monolayer coverage’. It should be apparent to the reader now that the dynamic changes occurring within a protein during water transport prohibit the use of surface-based approaches to treat the water sorption/desorption process in glassy proteins. Whilst stoichiometric analyses may still have a place in the realm of enzyme functionality and stability studies, where, for instance, the oxidation of some amino acids may result in a loss of bio-activity of the enzyme, the overall framework of analysis should really be that of a solution approach. Indeed, it is suggested to move even further than this position. Classical solution theories such as the Flory–Huggins theory (and its extensions) should not be employed on the basis of presented calorimetric data as the experimentally determined enthalpy/entropy landscape suggests a different picture of the hydration/mixing process as suggested by the Flory–Huggins theory. The fundamental problem here lies within the non-equilibrium nature of the glassy protein and the strong, directional forces between the protein and water molecules, both with themselves as well as in the sense of the binary interaction.

In the previous section, it was already mentioned how the herein revised picture of protein hydration (in the ‘solid’ state) affects both Quality-by-Design consideration as well as regulatory frameworks for solid-state biological products. The many vital discussions with the industrial co-sponsor of this research project, Procter & Gamble Co., gave greater insight into this topic. Without going into detail and touching upon sensitive information, it can be said that there is a

great industrial driving force in developing ‘better’ biological formulations. It is difficult to say what should be understood by the term ‘better’, but considerations are certainly centred on product safety, storage stability, performance during use and its economical profile. The situation is further complicated by the delicate inter-relations between these considerations. Therefore, there is great interest both industrially and academically [7-10] to achieve improved physical and chemical stability of lyophilised biologicals. Much of the formulation engineering in industry is constrained by short times-to-market (note, this is not true for the pharmaceutical industry) of new products and often product stability is tested in accelerated aging studies. In accelerated aging, a sample of a biological product is taken and exposed to a harsh physical (high temperature, high water activity) or chemical (e.g. oxidising species) environment and the residual product activity decay is measured over time. More than often, formulation engineering is an art than a science. In this sense, the revised understanding of protein hydration might also aid in the transition of moving away from ‘quality by extensive sampling’ to Quality-by-Design.

7.3 FUTURE STUDIES

Future studies on water transport in glassy proteins should extend to explore the importance of the experimental mode of water vapour sorption. By doing so, the notion of sample history prior and induced during the experiment should be reevaluated. The set of proteins studied should be extended to include both simpler proteins (e.g. poly-peptides) as well as more advanced biological products (e.g. antibodies). As the calorimetrically determined heats of sorption contain total thermodynamic information of the system, it is crucial to continue the initiated experimental work.

On the basis of those findings, further efforts should be pursued to measure sets of pressure–volume isotherms over a few relevant temperatures to then advance equation-of-state based modelling approaches, and those based on a non-equilibrium framework, to not just describe the behaviour of a pure protein but also to model the phase behaviour of aqueous protein solutions. A promising theoretical framework seems to be the Statistical Associating Fluid Theory (SAFT) as it can account for direct association; a phenomenon present in water–protein mixtures.

A mode of sorption that was neglected in this study is that of differential sorption. A paper presented at the annual meeting of the American Institute of Chemical Engineers (AIChE) in November 2014 by Ferruccio Doghieri (Paper 32g, Relaxation Behavior of Glassy Polymers: Experimental Sorption and Nonequilibrium Thermodynamic Model) stimulated the idea of carrying out some initial differential water desorption experiments of BSA. Figure 105 shows some dimensionless differential water desorption isotherms with activity ramping rates ranging over two orders of magnitude. (The methodology for these experiments is presented in the Appendix 7.5.1) All desorption experiments started with a fully hydrated protein sample at a partial vapour pressure of water of 2,217 Pa. At this point, the water content that correlates to ‘full hydration’ is about 0.43 g/g. The mixture is in the rubbery state. As the vapour pressure

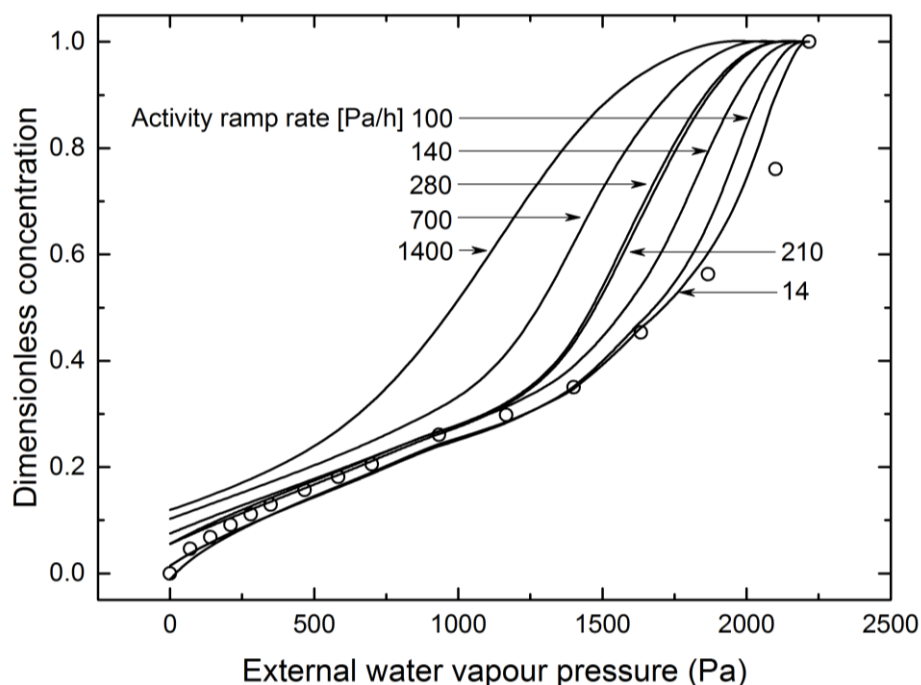


Figure 105 Dimensionless differential water desorption isotherms of BSA at 293.15 K and different activity ramping rates. (○) Corresponding interval desorption isotherm.

decreases, the water content decreases dynamically. The difference between zero water content and the observed content at the end of the experiment can be regarded as a measure of how far the experiment is out of mass transfer equilibrium. Somewhere at intermediate vapour pressures, the water/BSA mixture will go through a desorption-induced glass transition. While mass transport seems to depend significantly on the experimental ramping rate, it is in the glassy regime where this dependence is lost. It is suggested here, that this arises from glassy relaxations in this regime that control the overall transport process.

A further interesting feature observed in Figure 105 is the grouping of the desorption isotherms at intermediate and low vapour pressures. The experiments at a vapour pressure ramping rate of 140, 210 and 280 Pa/h and the ones at 14 and 100 Pa/h group together. This brings up the question whether the initially rubbery protein (at full hydration) can be kinetically locked upon

desorption into different but distinct conformational states depending on the desorption rate. Per analogy of the cooling of a polymer liquid into its super-cooled glassy state, one might think that this is possible. For synthetic polymers, the cooling rate not just determines the glass transition temperature but also the volume of the glass as well as the energetic landscape occupied by the system.

Water acts as a plasticiser of the protein conformation; it allows conformational rearrangement to occur. Solid-state NMR studies by [11] and [12] indicated that the distribution of conformational states occupied by a protein is much broader for the dry state than for the hydrated protein. As the protein's conformational flexibility decreases during desorption/dehydration, the protein molecule might become 'trapped' into a local free energy minimum corresponding to the conformational state the protein occupied as water molecules were removed. [13] Now, as dehydration continues, one might expect that protein molecules become 'trapped' in successive local free energy minima corresponding to a variety of conformational states (static disorder).

This brief digression nicely outlines how powerful a gravimetric sorption experiment can be. Combining gravimetric techniques with spectroscopic *in-situ* techniques would allow chemical foot-printing of the water sorption process; thus, creating an almost complete picture of hydration in glassy proteins. As for proteins of biological function, an interesting study would be to link these kinds of results to residual enzyme activity levels.

7.4 REFERENCES

- [1] Wuthrich, K.; Otting, G.; Liepinsh, E. *Faraday Discuss.* **1992**, 93, 35-45.
- [2] Otting, G.; Liepinsh, E.; Wuthrich, K. *Science* **1991**, 15(254), 974-980.
- [3] Tarek, M.; Tobias, D.J. *Biophys. J.* **2000**, 79(6), 3244-3257.
- [4] Modig, K.; Liepinsh, E.; Otting, G.; Halle, B. *J. Am. Chem. Soc.* **2004**, 126(1), 102-114.
- [5] Chapman, W.G.; Gubbins, K.E.; Jackson, G.; Radosz, M. *Fluid Phase Equil.* **1989**, 52, 31-38
- [6] Rasmussen, B.F.; Stock, A.M.; Ringe, D.; Petsko, G.A. *Nature* **1992**, 357, 423-424
- [7] Carpenter, J.F.; Pikal, M.J.; Chang, B.S.; Randolph, T.W. *Pharm. Res.* **1997**, 14(8), 969-975
- [8] Constantino, H.R.; Langer, R.; Klibanov, A.M. *Nature Biotech.* **1995**, 13, 493-496
- [9] Manning, M.C.; Patel, K.; Borchardt, R.T. *Pharm. Res.* **1989**, 6, 903-918
- [10] Hageman, M.J. *Drug. Dev. Ind. Pharm.* **1988**, 14, 2047-2070
- [11] Kennedy, S.D.; Bryant, R.G. *Biopolymers* **1990**, 29, 1801-1806
- [12] Gregory, R.B.; Gangoda, M.; Gilpin, R.K.; Su, W. *Biopolymers* **1993**, 33, 513-519
- [13] Gregory, R.B. Protein Hydration and Glass Transition Behavior. In: Protein-solvent interactions. (Gregory, R.B. ed.) Marcel Dekker **1995**, New York

7.5 APPENDIX

7.5.1 Methodology: Differential water desorption in BSA

Materials

Bovine serum albumin (BSA) (lyophilized powder, catalogue number 05470) was purchased at the highest commercially available grade from Sigma Aldrich (Gillingham, United Kingdom). Ultrapure deionised water (resistivity $\sim 16 \text{ M}\Omega \cdot \text{cm}$) was used for all sorption experiments.

Methods

See Section 2.2.2 for detailed methodology on Dynamic Vapour Sorption experiments used in this Chapter.

Differential desorption: After initial drying, the sample was exposed to a water activity of $0.95a_w$ for $1,600 \text{ min}$. In a subsequent step, the water activity was ramped down to zero at a controlled rate.

Jet

By Tony Hoagland

Sometimes I wish I were still out
on the back porch, drinking jet fuel
with the boys, getting louder and louder
as the empty cans drop out of our paws
like booster rockets falling back to Earth

and we soar up into the summer stars.
Summer. The big sky river rushes overhead,
bearing asteroids and mist, blind fish
and old space suits with skeletons inside.
On Earth, men celebrate their hairiness,

and it is good, a way of letting life
out of the box, uncapping the bottle
to let the effervescence gush
through the narrow, usually constricted neck.

And now the crickets plug in their appliances
in unison, and then the fireflies flash
dots and dashes in the grass, like punctuation
for the labyrinthine, untrue tales of sex
someone is telling in the dark, though

no one really hears. We gaze into the night
as if remembering the bright unbroken planet
we once came from,
to which we will never
be permitted to return.
We are amazed how hurt we are.
We would give anything for what we have.

Tony Hoagland, *Donkey Gospel: Poems*. Graywolf Press, 1998