A nanostructural investigation of glassy gelatin oligomers: molecular organization and interactions with low molecular weight diluents

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Abstract. The effects of low molecular weight diluents (namely water and glycerol) on the nanostructure and thermodynamic state of low water content gelatin matrices are explored systematically by combining positron annihilation lifetime spectroscopy (PALS) with calorimetric measurements. Bovine gelatin matrices with a variation in the glycerol content (0–10 wt.%) are equilibrated in a range of water activities ($a_w = 0.11–0.68$, $T = 298$ K). Both water and glycerol reduce the glass transition temperature, $T_g$, and the temperature of dissociation of the ordered triple helical segments, $T_m$, while having no significant effect on the level of re-naturation of the gelatin matrices. Our PALS measurements show that over the concentration range studied, glycerol acts as a packing enhancer and in the glassy state it causes a nonlinear decrease in the average hole size, $v_h$, of the gelatin matrices. Finally, we report complex changes in $v_h$ for the gelatin matrices as a function of the increasing level of hydration. At low water contents ($Q_w \sim 0.01–0.10$), water acts as a plasticizer, causing a systematic increase in $v_h$.

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Conversely, for water contents higher than \( Q_w \sim 0.10 \), \( \nu_h \) is found to decrease, as small clusters of water begin to form between the polypeptide chains.

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1. Introduction

Gelatin is one of the most versatile and widely used biopolymers with a vast array of applications across the biomedical [1, 2], pharmaceutical [3] and food industries [4]. Gelatin oligomers are derived from collagen, the principal protein component of white fibrous connective tissue [5], by hydrolytic degradation using either acids (type A gelatin) or alkali (type B gelatin) [6, 7]. The fundamental molecular unit of collagen is tropocollagen, which is approximately 300 nm long and 1.5 nm in diameter [5]. This consists of three extended left-handed polyproline-II-like peptide helices, coiled along a left-handed threefold axis, which are arranged parallel to each other and form a highly ordered right-handed superhelix along a common axis [5]. During the degradation process, the characteristic triple helical structure of tropocollagen is denatured, producing a heterogeneous material with a predominantly random coil conformation and a high polydispersity [5]. The main components of gelatin are single-chain species (\( \alpha \)-type, \( M_w \sim 1 \times 10^5 \) Da) and double-chain species (\( \beta \)-type, \( M_w \sim 2 \times 10^5 \) Da), although in some cases small amounts of a disordered form of tropocollagen may still be present [5]. The proportion of these components varies depending on the method of degradation. Gelatin may be dissolved in water by heating the solution to \( \sim 315 \) K and above this temperature the majority of the polypeptide chains are in a random coil conformation. When an aqueous gelatin solution with a protein concentration typically higher than 5 mg ml\(^{-1}\) is cooled down, thermo-reversible gelation occurs [8, 9]. In the gel state, the gelatin oligomers are strongly intertwined, forming extended physical cross links resulting from the partial reversion of the polypeptide chains into ordered triple helical segments, whose structure is similar to that of tropocollagen [9, 10]. These triple helical (tropocollagen-like) segments are joined along the chain contour by peptide residues in a random coil conformation, forming a transparent, elastic network (see figure 1).

Its thermo-reversible gelation which occurs at temperatures close to the human body temperature, as well as its ability to produce strong, clear films with adjustable morphologies, that are readily soluble in water, make gelatin an invaluable raw material for the manufacture of hard and soft capsules by the pharmaceutical industry [3, 4, 13]. These gelatin capsules are
Figure 1. Schematic representation of the partial re-naturation process in gelatin. (1) When an aqueous gelatin solution (protein concentration >5 mg ml\(^{-1}\)) is cooled down, the imino acid-rich segments of single-polypeptide chains begin to re-arrange into conformations that are similar to those in the collagen structure. A ternary structure commonly referred to as the ‘collagen fold’ is formed [5]. (2) Next, separate polypeptide chains in the ‘collagen-fold’ conformation associate into a 3D network. Finally, the 3D network structure is stabilized by lateral inter-chain hydrogen bonding within the helical regions [5]. Inset: the crystal structure of a collagen-like peptide showing the three extended left-handed polyproline-II helices (shown in different colours for clarity), coiled around each other to form a highly ordered right-handed superhelix (Protein Database code 1QSU [11]; the image was constructed using PyMOL [12]).

commonly used to encapsulate labile active compounds (e.g. drugs and essential nutrients) in order to protect them from undesirable external influences (in particular atmospheric oxygen [4]), facilitate their retention and achieve their targeted release when required [3, 4, 13]. Alongside the large number of useful properties inherent to gelatin described above, it also exhibits some significant shortcomings. Among these is the high brittleness of gelatin in the glassy state at very low water contents (<5 wt.%) [14], resulting in the undesired failure of gelatin capsules. In order to overcome this problem and achieve optimal mechanical and barrier properties, low water content gelatin films/matrices are often modified by the inclusion of small amounts of low molecular weight polyols, such as glycerol [13–15]. Glycerol has been reported to modify the mechanical properties of gelatin matrices, while altering their permeability towards gases and water vapour [14]. Furthermore, gelatin is highly hygroscopic, meaning that it can readily absorb water vapour from the atmosphere [15]. This can cause significant deterioration of the barrier properties of gelatin capsules, leading to the oxidation or premature release of the encapsulated active ingredients. The barrier properties of glassy gelatin encapsulating matrices may therefore be altered and optimized by adding small amounts of glycerol, while keeping the water content low.

One of the main issues in the design of encapsulating gelatin matrices with optimal barrier properties is the molecular mobility through the matrix, which in the glassy state is governed
by the density of the molecular packing of the gelatin oligomer chains [4]. In amorphous and partially ordered soft matter such as the partially re-natured gelatin oligomers, the irregular arrangement of the polypeptide chains results in a certain amount of excess local free volume which arises due to the existence of static and dynamic structural disorder. This local free volume consists of a large number of sub-nanometre-sized free volume elements, commonly referred to as ‘holes’. The importance of quantifying the local free volume in a system cannot be overestimated since it plays an important role in phenomena such as self-diffusion, the diffusion of guest molecules [16, 17] and the glass transition [18]. Although modern theories of the glass transition and diffusion processes in soft matter are more complex than the original free volume concepts, they nevertheless show a close relation between molecular mobility (dynamic heterogeneity) and free volume (structural heterogeneity) [19].

Positron annihilation lifetime spectroscopy (PALS) is a unique and versatile technique which is capable of measuring the size and size distribution of the free volume elements directly at the sub-nanometre length scale [20, 21]. Over the last decade, this technique has been successfully used to study a wide range of biopolymers (e.g. starch [22, 23], cellulose [24] and their derivatives [25–29]) which are commonly used for the formulation of pharmaceutical excipients and encapsulants for bioactive compounds. Furthermore, positron lifetime measurements aiming to study the molecular organization in collagen [30, 31] and the sol–gel transition of gelatin oligomer hydrogels induced by changes in temperature and water content have also been reported by a number of authors [31–36].

In this paper, we explore the effects of water and glycerol on the density of the molecular packing and the thermodynamic state of low water content gelatin matrices by using PALS in conjunction with extensive calorimetric measurements. For this purpose, we prepared a series of cold-cast bovine gelatin films with a systematic variation in glycerol content (0–10 wt.%), equilibrated at a range of water activities ($a_w = 0.11–0.68$, at $T = 298$ K). We aim to provide novel insights for establishing a direct relation between the composition/level of hydration and molecular packing of these materials in order to eventually produce encapsulants with optimal barrier properties.

2. Materials and methods

2.1. Positron annihilation lifetime spectroscopy: revealing it at the molecular level

PALS experiments proceed by injecting a positron into the material being tested and measuring the length of time until that positron annihilates with one of the material’s electrons, producing $\gamma$-rays. The positron annihilation lifetime experiments described here were performed using a conventional fast-fast coincidence system [20, 21], with a Gaussian resolution function with a full-width at half-maximum (FWHM) of 0.175 ns. Full details of the system are described elsewhere [25, 37]. $^{22}$Na was used as a source of positrons since during its decay a prompt 1.28 MeV $\gamma$-ray is emitted simultaneously with the positron, which may be used to mark the ‘birth’ of the positron. The positron source used for the experiments in this study was prepared by evaporating carrier-free $^{22}$NaCl solution between two 7.5 $\mu$m thick sheets of Kapton foil. Gelatin films for the PALS experiments were cut into $1 \times 1$ cm$^2$ squares and were stacked on top of each other to produce a thickness of $\sim 1.1$ mm, enough to stop $\sim 99\%$ of the incident positrons. Film stacks were placed on either side of the source and the purpose-designed sample holder cavity was filled with film (the same composition) cut into small pieces equilibrated at
the same water activity as the sample, to minimize moisture transfer within the air-tight copper sample cell [25].

Initially, when the positron is injected into the sample it has a high kinetic energy but it thermalizes within a few picoseconds due to excitation and ionization of the medium molecules [20, 21]. After this, it may diffuse through the material (over a mean free path of a few nm) either as a free particle and self-annihilate, or capture a molecular electron forming a metastable positron–electron bound state, known as positronium. In fact, in molecular materials a significant fraction of the injected positrons annihilate from the positronium bound state. Ps may form in two different spin states: \textit{para}-positronium (\textit{p}-Ps, a singlet state with zero spin angular momentum) or \textit{ortho}-positronium (\textit{o}-Ps, a triplet state of unit spin angular momentum) with a relative abundance of 1 : 3 [20, 21]. In vacuum, \textit{p}-Ps self-annihilates to two 511 keV \(\gamma\)-rays in 0.125 ns, whereas \textit{o}-Ps due to spin considerations can only self-annihilate to an odd number of \(\gamma\)-rays (three being the most probable), with an average lifetime of 142 ns [20, 21]. In molecular materials, Ps localizes in the free volume holes between the molecules, where it remains throughout its lifetime. When confined in a free volume hole, the \textit{o}-Ps undergoes numerous collisions with the molecules of the medium; therefore, there is a finite probability that the positron of the \textit{o}-Ps bound state may annihilate with a molecular electron (with an opposite spin) rather than its bound partner via a two-\(\gamma\)-photon annihilation channel (emitting two 511 keV \(\gamma\)-rays). This process is known as ‘pick-off annihilation’ and it results in a reduction of the \(\textit{o}\)-Ps lifetime with hole size, from 142 ns in an infinite size hole (\(\textit{o}\)-Ps self-annihilation in vacuum) to the low-nanosecond range (1–4 ns) for sub-nanometre-sized holes [20, 21]. The \(\textit{o}\)-Ps lifetime is, therefore, environment dependent and delivers information pertaining to the size of the molecular free volumes in a material. The size of the local free volume holes can be related to the mean \(\textit{o}\)-Ps ‘pick-off’ annihilation lifetime, \(\tau_{po}\), using a simple quantum mechanical model, which assumes that the \(\textit{o}\)-Ps is localized in a spherical potential well of an infinite depth and a radius, \(r = r_h + \delta r\) [38, 39]:

\[
\tau_{po} = \left[ \sum_{i=0}^{1} f_i \lambda_i \right]^{-1} \left[ 1 - \frac{r_h}{r_h + \delta r_h} + \frac{1}{2\pi} \sin \left( \frac{2\pi r_h}{r_h + \delta r_h} \right) \right]^{-1} . \tag{1}
\]

Here, \(r_h\) is the radius of the free volume hole and the positronium wavefunction may overlap with the wavefunctions of molecular electrons within a layer \(\delta r\) of the potential well. \(f_i\) is the fraction of positronium with spin \(i\) (1/4 for \(\textit{p}\)-Ps and 3/4 for \(\textit{o}\)-Ps) and \(\lambda_i\) is the corresponding annihilation rate in vacuum (0.125 ns\(^{-1}\) and 142 ns\(^{-1}\) for \(\textit{p}\)-Ps and \(\textit{o}\)-Ps, respectively) [20, 21].

While the lifetime of an individual positron may vary between 0 and \(\infty\), the lifetime spectrum of an ensemble of positrons annihilating from a single state is a single exponential of the form \(\exp(-t/\tau)\), where \(\tau\) denotes the mean lifetime of the positrons [20, 21]. When a positron is injected into a molecular medium, it can annihilate as a free positron, \(\textit{p}\)-Ps or \(\textit{o}\)-Ps, where each state has a characteristic average lifetime. The positron annihilation lifetime spectra of molecular materials are, therefore, commonly decomposed into three discrete exponential components [20, 21]:

\[
S(t) = \left[ R(t) \otimes \sum_i \left( I_i / \tau_i \right) \exp(-t/\tau_i) \right] + B , \tag{2}
\]
where \( i = 1, 2, 3 \) are the components attributed to the annihilation of \( p\text{-}Ps \), a free positron and \( o\text{-}Ps \), respectively, weighted by the relevant intensity, \( I_i \) (where \( \sum I_i = 1 \)). \( B \) is the background and \( R(t) \) is the resolution function, where \( t \) is time.

During the PALS experiments, the gelatin matrices were first of all heated to \( T > T_m \) (where \( T_m \), measured by DSC, is the temperature of melting/dissociation of the ordered triple helical segments) for several hours in order to erase their previous thermal histories and then spectra were collected in 10 K intervals during cooling temperature scans down to \( T_g - 60 \text{ K} \) (unless stated otherwise). Spectra at each temperature were collected over a period of 2 h to generate at least 2.5 million events per spectrum. The source correction was 10.1% of the total spectrum, with two components measured, 0.39 ns \((I = 94.9\%)\) and 3.32 ns \((I = 5.1\%)\), which were accounted for prior to the analysis. The lifetime spectra were analysed using the Life Time fitting routine (version 9.1) \cite{40}, which performs a weighted nonlinear least-squares fit of the model function given by (2) to the experimental spectra, giving in its output the lifetimes and intensities of all components.

2.2. Preparation of the gelatin matrices

Type B bovine gelatin \((M_w = 2.84 \times 10^5 \text{ Da}, \text{isoelectric point} = 5.0, \text{Russelot, Brazil})\) was used for the preparation of the matrices used in this study. Matrices/films with different compositions were prepared by dissolving well-defined amounts of gelatin and glycerol (> 99.9% purity, Merck, UK) in ultrapure water \((\text{Milli-Q, Millipore, Beford, MA, USA})\) at \( T = 333 \text{ K} \) to produce solutions with 7% \((w/w)\) of organic matter. The glycerol contents of the matrices were expressed as glycerol mass fractions on an anhydrous basis, \( Q'_g = m_g/(m_{gel} + m_g) \). Here, \( m_g \) and \( m_{gel} \) denote the mass of glycerol and gelatin, respectively. Blends were prepared with glycerol contents of \( Q'_g = 0, 0.02, 0.06 \) and 0.10. These glycerol mass fractions were corrected for the water contained in the glycerol. All materials were used without further purification. Cold casting was used to produce gelatin films from solution, whereby the solution was deposited on a Teflon mould and maintained at 280 K until the film was formed (typically within 10 days). Since the films were cast at a temperature below the temperature of re-naturation of the gelatin polypeptide chains to a triple helical motif, they contain a certain fraction of ordered triple helical regions and are, therefore, not completely amorphous. Once the films were fully set and dry, they were peeled off the substrate and cut into rectangular shapes. The films were then stored over \( P_2O_5 \) for 1 week, after which they were equilibrated at set water activities (at 298 K) in desiccators containing saturated salt solutions of known water activities \cite{41}. The interaction of water with the gelatin matrices involves a dynamic exchange of water molecules between the water vapour from the atmosphere within the desiccators and the water molecules located within the matrices. At a specific temperature in steady state, the ratio of the partial vapour pressure at the surface of the matrices, \( p \), and the saturation vapour pressure of pure water, \( p_0 \), is equal to the water activity, \( a_w = p/p_0 \) \cite{4}. The sorption of water vapour was followed gravimetrically until equilibrium was achieved (generally within 25 days). The water contents of the gelatin matrices were determined gravimetrically using an air-drying oven. The samples were kept at \( \sim 380 \text{ K} \) for 24 h and the water content was calculated as the water mass fraction on an anhydrous basis, \( Q'_w = m_w/(m_{gel} + m_g) \), where \( m_w, m_{gel} \) and \( m_g \) denote the masses of water, gelatin and glycerol, respectively. All water vapour sorption data for the gelatin matrices may be found in the supplementary data (stacks.iop.org/NJP/14/035016/mmedia).
Figure 2. DSC thermograms measured during heating temperature scans for a pure gelatin matrix equilibrated at $a_w = 0.44$: (1) was measured for the gelatin matrix as prepared by cold casting from the solution; (2) was acquired for the same gelatin sample shortly after cooling it from the 'molten state' at 40 K min$^{-1}$; (3) was measured after the gelatin matrix was subjected to the thermal cycle of the PALS experiment (described in the text) after initial preparation by casting from the solution. The glass transition temperatures ($T_g$ measured during the first heating scan and $T'_g$ measured during the second heating scan) and the temperature of dissociation/melting of the ordered triple helical segments, $T_m$, are shown for reference.

2.3. Thermal analysis by differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were made using a PerkinElmer Pyris Diamond DSC (PerkinElmer, UK), calibrated with a high-purity, annealed indium reference [42]. An empty aluminium pan was used as the reference and the measurements were carried out with approximately 10 mg of sample, sealed in hermetic aluminium pans. Heating temperature scans were carried out at a rate of 10 K min$^{-1}$ from $T_g - 60$ K to $T_g + 80$ K. The glass transition temperature, $T_g$, was determined from the midpoint of the change in heat flow measured during the heating temperature scan. The melting temperature, $T_m$, of the ordered triple helical regions was determined from the onset of the endothermal melting peak observed during the heating run and the enthalpy of melting of the triple helical regions, $\Delta H_m$, was determined by integrating the melting peak between the onset and endset temperatures. The DSC thermograms were analysed by using PerkinElmer Thermal Analysis software.

3. Results and discussion

In figure 2 we show DSC thermograms measured during heating temperature scans for a pure gelatin matrix equilibrated at a well-defined water activity, $a_w = 0.44$. First, we begin
by discussing thermogram 1, which was measured for the gelatin matrix as prepared by cold casting from solution.

Two endothermic events were observed during this temperature scan—a change in heat flow marking the glass transition temperature of the gelatin matrix, \( T_g = 324 \pm 0.5 \text{ K} \) followed by an endothermic peak associated with the melting/dissociation of the ordered triple helical segments. The melting/dissociation temperature of the triple helical segments, \( T_m = 358 \pm 0.5 \text{ K} \), was determined from the onset of the endothermal melting peak. Thermogram 2 was acquired for the same gelatin sample shortly after cooling it from the ‘molten state’ at 40 K min\(^{-1}\). This heating scan reveals only one endothermic event, namely the glass transition of the gelatin matrix \( T_g' \), indicating that the gelatin oligomers were completely amorphous after the DSC cooling cycle, i.e. lacking any degree of re-naturation. This confirms that the process of re-naturation of the polypeptide chains to a triple helical conformation occurs on timescales that are significantly longer than the timescale of the DSC cooling cycle [9, 10, 43]. Next, we checked if re-naturation occurred on the timescales of a typical PALS experiment by cooling a gelatin sample initially in the ‘molten state’ \( (T > T_m) \) down to temperatures below its glass transition. For this specific PALS experiment, the pure gelatin sample was heated from 270 to 380 K \( (T > T_m) \) by increasing the temperature in 10 K steps (each lasting 2 h), followed by annealing at 380 K for 4 h and a cooling run back to 270 K (again in 10 K steps).

The DSC measurement for a pure gelatin matrix subjected to this thermal cycle after initial preparation by casting from the solution is shown in thermogram 3. As in thermogram 1, we observe two endothermic events—a change in heat flow associated with the glass transition of the amorphous regions of the matrix, followed by an endothermic peak due to the dissociation of the triple helical segments. Both the glass transition temperature and the melting/dissociation temperature of the triple helical segments of the gelatin matrices were found to be the same (differences are within the experimental error) as in thermogram 1, i.e. the same before and after the thermal cycle of the PALS measurements. Furthermore, the enthalpy of dissociation/melting of the ordered triple helical segments, \( \Delta H_m = 25.4 \pm 2 \text{ J g}^{-1} \) (determined by integrating the endothermic peak between the onset and endset temperatures), which reflects the fraction of polypeptide chains in a triple helical conformation, was found to be the same in both cases. This suggests that the cooling rate during the PALS measurements was sufficiently low to allow the same extent of re-naturation of the gelatin polypeptide chains as when they were initially prepared by cold casting.

The changes in average hole volume measured for the pure gelatin matrix \( (a_w = 0.44) \) during the thermal cycle described above are shown in figure 3. During both the heating and cooling temperature scans, the average hole volume shows a strong temperature dependence with two linear branches, each of which can be fitted well with a linear regression. The point of intersection of the two branches may be identified as the glass transition temperature of the gelatin matrix \( (T_g = 325 \pm 2 \text{ K}, \text{ with no significant differences measured between the heating and cooling scans}) \), as confirmed by DSC. In fact, there is essentially a direct correspondence between the \( T_g \) values determined by PALS and those measured by DSC for all the gelatin–glycerol matrices in this study, as shown in the inset of figure 3. The small differences in \( T_g \) measured can be accounted for by the differences in temperature ramp rates used by the two techniques (10 K min\(^{-1}\) for DSC and quasi-isothermal in PALS) [37]. Looking at the temperature dependence of the average hole size measured for the gelatin matrices presented in figure 3, it is interesting to note that there is no signature of the dissociation of the ordered triple helical regions in the free volume measurements. We do not observe a sudden
Figure 3. Temperature dependence of the average hole size, $v_h$, measured during a heating (red data series) and a cooling (blue data series) temperature scan for a pure gelatin matrix equilibrated at $a_w = 0.44$ (full details of the thermal cycle can be found in the text). During both temperature scans, the average hole volume shows a strong temperature dependence with two linear branches and the temperature at which they intersect may be identified as the glass transition temperature of the matrix, $T_g$. The temperature of dissociation/melting of the ordered triple helical segments, $T_m$, for the gelatin matrix (determined by DSC) is also shown for reference. Inset: comparison of the glass transition temperatures measured by PALS and DSC for gelatin matrices with different compositions and water contents. There is almost a one-to-one correspondence between the values of $T_g$ measured by the two methods, as indicated by the red dashed line (gradient = 1, $R^2 = 0.98$).

change in the temperature dependence of the average hole volume at temperatures above $T_m$, nor do we observe any systematic changes in the $o$-Ps intensity (which reflects the probability of $o$-Ps formation) as a function of temperature over the whole temperature range studied. In the final analysis of the data presented in figure 3, the $o$-Ps intensity was, therefore, fixed to its average value ($I_{o-Ps} = 21.4\%$) in order to improve the stability of the fit when (2) was fitted to the experimental spectra. Furthermore, we do not observe any drastic changes in the temperature dependences of other annihilation parameters (e.g. the free positron annihilation lifetime and intensity) calculated using a three-discrete-lifetime component fit. It is possible that there may be two separate $o$-Ps lifetimes, one for $o$-Ps annihilating in the amorphous regions of the gelatin matrices where the polypeptide chains assume a random coil conformation and one for $o$-Ps annihilating in the ordered ‘tropocollagen-like’ triple helical regions [20, 21]. The statistics required to analyse the lifetime spectra in terms of four discrete lifetimes (free positron, $p$-Ps and two $o$-Ps lifetimes) can, however, be prohibitively high (about eight times as high as the statistics of the present study). Collection of high statistics data may also pose experimental problems such as radiation damage to the sample, potential instrumental drift and unfeasibly long experimental timescales. Furthermore, the two $o$-Ps lifetimes may be very difficult to resolve even with high statistics spectra, as their values are often very similar [20, 21].

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It is also clear from figure 3 that the average hole sizes measured during the cooling temperature scan are all systematically lower than those measured during the heating run prior to the annealing and re-naturation steps. It is likely that the differences in hole size result from annealing of the amorphous regions of the gelatin matrices, where the polypeptide chains are in a random coil conformation. A hysterisis in the average hole size has previously been reported for a number of carbohydrate oligomer matrices, but the magnitude of the effect was significantly more pronounced due to the differences in matrix preparation methods and due to the completely amorphous nature of those samples [25, 37]. Finally, it is interesting to note that the average hole volumes measured for the gelatin matrices are significantly larger than those reported in our previous papers for carbohydrate oligomer matrices equilibrated at the same water activity. For example, at $T = 298 \text{K}$ the average hole volume of gelatin matrices equilibrated at $a_w = 0.44$ is $v_h = 91.5 \text{Å}^3$, compared to $v_h = 57.3 \text{Å}^3$ measured previously for a carbohydrate oligomer ($M_w = 1.9 \times 10^4 \text{Da}$) [27]. These differences in molecular packing observed are too large to be attributed to the disparities in molecular weight of the two biopolymer matrices [25], and are most likely to be caused by the differences in chemical composition and the structure of the constituent molecules in each case. Furthermore, the differences in water vapour sorption behaviour of the two biopolymers may also contribute to the looser molecular packing of the gelatin oligomer matrices. The gelatin matrices were found to absorb significantly more water than the carbohydrate oligomer matrices over the entire range of water activities studied. For example, when equilibrated at $a_w = 0.44$, the water content (on a dry basis) of the pure gelatin matrices was found to be $Q'_{w} = 0.131$ (see the supplementary data (stacks.iop.org/NJP/14/035016/mmedia) for more details), compared to $Q'_{w} = 0.093$ for the carbohydrate matrices [27]. Water is a strong plasticizer\(^6\) for biopolymer matrices and, as shown by our previous studies, it can decrease the density of their molecular packing, causing an increase in the average molecular hole size [4].

In order to elucidate the effect of water on the molecular packing of the gelatin matrices in figure 4(a), we show the temperature dependence of the average molecular hole size for pure gelatin matrices equilibrated in a range of water activities.

As the water activity of the gelatin matrices increases from $a_w = 0.11$ to 0.33, we observe a significant increase in the average hole size (in both the glassy and the rubbery states), as water ‘loosens’ the molecular packing of the gelatin oligomer matrices. However, when the water activity of the samples increases further, we start observing a systematic reduction in the average hole size of the matrices, which is significantly more pronounced in the glassy state. The effect of glycerol on the molecular packing of the gelatin matrices is less complex compared to that of water, as illustrated in figure 4(b), which shows the temperature dependence of the average molecular hole size for matrices with different compositions, equilibrated at $a_w = 0.11$. Over the entire concentration range studied, glycerol acts as a packing enhancer, causing systematic reductions in the average hole size of the gelatin matrices. Furthermore, both water and glycerol also cause a systematic depression of the glass transition temperature of the gelatin matrices (figures 4 and 5). In figure 5, we show all glass transition data for the gelatin matrices with different compositions and water contents, measured by DSC. The depression in the glass transition temperature of gelatin matrices as a function of increasing glycerol and water contents may be

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\(^{6}\) A review of the phenomena of plasticization and anti-plasticization, their manifestation in terms of changes in mechanical properties and relaxation dynamics, as well as the changes in local free volume occurring at the molecular level, may be found in [27, 28].

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Figure 4. (a) Temperature dependence of the average molecular hole size measured for pure gelatin matrices equilibrated at a range of water activities: white series: $a_w = 0.11$; light blue series: $a_w = 0.22$; dark blue series: $a_w = 0.33$; grey series: $a_w = 0.44$; and black series: $a_w = 0.68$. (b) Temperature dependence of the average hole volume measured for gelatin matrices with different glycerol contents, equilibrated at a well-defined water activity ($a_w = 0.11$). Green series: $Q'_g = 0.00$; blue series: $Q'_g = 0.02$; yellow series: $Q'_g = 0.06$; and red series: $Q'_g = 0.10$. Both water and glycerol cause systematic reductions in the glass transition temperature, $T_g$, of the gelatin oligomer matrices, as shown.

modelled semi-empirically using the Gordon–Taylor equation for a ternary system [44]:

$$T_g = \frac{Q_{gel}T_{gel} + k_gQ_gT_{g,g} + k_wQ_wT_{g,w}}{Q_{gel} + k_gQ_g + k_wQ_w}. \quad (3)$$

Here, $Q_x$, $T_{gel}$ and $k_x$ are the mass fractions, glass transition temperatures and Gordon–Taylor coefficients (whose values depend on the change in the thermal expansion coefficient of the components as they undergo a transition from the glassy to the rubbery state) for the three components ($x = gel$-gelatin, $g$-glycerol and $w$-water) [44]. When modelling the experimental data, the values for the glass transition temperatures of the separate components were taken as $T_{gel} = 467$ K ($T_g$ of anhydrous gelatin), $T_{gel} = 189$ K [45], $T_{g,w} = 165$ K [46, 47] and $k_g$ and $k_w$ were considered as fitting parameters. In figure 5 it is observed that the Gordon–Taylor equation for a ternary system provides a very good fit to the experimental glass transition data, indicating that phase separation did not occur during the preparation of the matrices [4, 44]. In addition, the values of the fitting coefficients, $k_g = 3.9 \pm 0.5$ and $k_w = 8.1 \pm 0.6$, fall within the range reported for similar biopolymer systems [27, 48]. Here, we note that the Gordon–Taylor equation was originally derived based on assumptions concerning the ideality of mixing of copolymers [44], which are not necessarily fulfilled in our gelatin–polyol–water system. However, in the framework of the present study this equation was only used to provide a semi-empirical fit to the experimental data, in order to obtain the critical water content, $Q^*_w$, at which the glass transition temperature of the gelatin matrices is equal to the experimental temperature, 298 K (in order to distinguish between matrices which are in the glassy and rubbery states). $Q^*_w$ was found to decrease as a function of increasing glycerol content from $Q^*_w = 0.22$ for the matrix containing 0 wt.% glycerol to $Q^*_w = 0.11$ for
Figure 5. Glass transition temperatures (measured by DSC) of gelatin oligomer matrices with various compositions as a function of increasing water content. The solid lines represent the fits of the semi-empirical Gordon–Taylor equation for a ternary system [44] to the experimental glass transition data. The Gordon–Taylor fitting coefficients were found to be $k_w = 8.1 \pm 0.6$ and $k_g = 3.9 \pm 0.5$ for water and glycerol, respectively. The dashed red line representing $T_g = 298$ K is added for reference. Green series: $Q'_g = 0.00$; blue series: $Q'_g = 0.02$; yellow series: $Q'_g = 0.06$; and red series: $Q'_g = 0.10$. Inset: critical water content ($Q_w^*$) and critical water activity ($a_w^*$) at which $T_g = 298$ K for the gelatin matrices as a function of increasing glycerol content. The values of $Q_w^*$ and $a_w^*$ were determined from the fits of the Gordon–Taylor equation to the experimental glass transition data and the water vapour isotherms of the gelatin matrices.

The matrix containing 10 wt.% glycerol (see the inset of figure 5). The critical water activities, $a_w^*$, corresponding to $Q_w^*$ are also shown in the inset of figure 5. With increasing glycerol content, the value of $a_w^*$ decreases from 0.80 for the matrix containing 0 wt.% glycerol to 0.30 for the matrix containing 10 wt.% glycerol, confirming the plasticizing effect of glycerol on the gelatin matrices. Furthermore, as shown in table 1 both glycerol and water also lead to a reduction of the temperature of melting/dissociation of the ordered triple helical ‘tropocollagen-like’ segments of the gelatin matrices, in agreement with the literature [14, 15]. Finally, it is worth mentioning that the enthalpy of dissociation/melting of the ordered triple helical segments, $\Delta H_m = 25.4 \pm 2$ J g$^{-1}$ (calculated from the integral of the endothermic peak associated with the melting of the triple helical regions), was found to be independent of the water and glycerol contents (the variations in $\Delta H_m$ were found to be within the error margin of the measurement). Our observations are in agreement with the measurements reported by Vanin et al [14] and Coppola et al [15] for similar gelatin matrices, which show that the enthalpy of melting/dissociation for these systems is independent of the water and glycerol contents (up to 70 wt.% for both diluents). The degree of re-naturation/fraction of ordered triple helical regions ($x_h = \Delta H_m/\Delta H_{m,c}$, where $\Delta H_{m,c} = 62.05$ J g$^{-1}$ is the enthalpy of melting/dissociation of a tropocollagen unit [5]) was, therefore, found to be the same, $x_h = 0.41$, for all gelatin matrices in this study. This is within the range of degree of re-naturation reported by a number
Table 1. Melting/dissociation temperatures of the ordered triple helical segments for gelatin matrices with various compositions and water contents measured by DSC. The reported values are an average of measurements taken in triplicate (the standard deviation of the measurements was typically 0.5 K).

| a_w  | T_m (K) |
|------|---------|
|      | 0.11    | 0.22    | 0.33    | 0.44    | 0.68    |
| Pure gelatin | 386.6 | 377.5 | 374.0 | 358.9 | 357.0 |
| Glycerol  | 376.7 | 371.7 | 367.0 | 356.5 | 351.7 |
| 0.06    | 370.7 | 367.5 | 362.6 | 351.9 | 350.1 |
| 0.10    | 365.0 | 361.6 | 359.7 | 350.4 | 348.8 |

of authors for bovine gelatin matrices with similar molecular weights and thermal histories [14, 15]. Since the fraction of ordered triple helical segments in the gelatin matrices is independent of the level of hydration and the composition of the gelatin matrices, it is reasonable to assume that the changes in average hole volume observed as a function of increasing water and glycerol contents (figure 4) are due to changes in molecular packing occurring in the amorphous parts of the gelatin matrices where the polypeptide chains assume a predominantly random coil conformation. The interpretation of the PALS data presented in figure 4 is, however, complicated since at a well-defined temperature, the water contents of the various matrices equilibrated at a well-defined water activity vary depending on the exact matrix composition (see the supplementary data (stacks.iop.org/NJP/14/035016/mmedia) for more details). For this reason, in figure 6 we plot the changes in (a) the average hole volume, \(v_h\), and (b) the \(\alpha\)-Ps intensity, \(I_{\alpha-Ps}\), measured at \(T = 298\) K for gelatin matrices with well-defined compositions as a function of increasing water content. Similar changes in \(v_h\) (and \(I_{\alpha-Ps}\)) upon hydration have been reported for a number of systems, including hydroxypropyl methylcellulose [49], methyl cellulose plasticized with poly(ethylene glycol) [50], poly(vinyl alcohol) [51], DNA [52] and Nafion membranes [53].

It can be seen from figure 6(a) that as the water content of the gelatin matrices with various compositions increases from \(Q_w \sim 0.01\), we observe a pronounced increase in the average hole volume, which reaches maximum size at \(Q_w^* \sim 0.10\) (depending on the exact matrix composition), after which it begins to decrease upon further sorption of water, up to water contents of \(Q_w \sim 0.17\). The initial increase in the average hole volume of the gelatin matrices upon sorption of water indicates that water acts as a plasticizer for the gelatin matrices [4, 25, 27, 28], thereby increasing the separation between the polypeptide chains. A number of previous studies have shown that initially, upon hydration, water molecules bind to the polar side groups of the gelatin polypeptide chains, which alters the hydrogen bonding interactions between them [54, 55]. In turn, this can lead to swelling of the gelatin matrices, accompanied by an increase in the average hole size as the random coil segments of the polypeptide chains are pushed apart. There is, however, a limit to the extent of re-arrangement of the random coil segments imposed by the extended physical crosslinks formed by the partial reversion of the polypeptide chains into ordered triple helical segments. Therefore, as the water content increases further (above \(Q_w^* \sim 0.10\), water molecules become accommodated between the polypeptide chains without being able to cause further swelling of the matrix. The average hole sizes measured for the plasticized gelatin matrices are larger than the average hole size measured...
Figure 6. (a) Average molecular hole size and (b) the o-Ps intensity as a function of increasing water content for gelatin matrices with well-defined glycerol contents, measured at $T = 298$ K. Green series: $Q_g' = 0.00$; blue series: $Q_g' = 0.02$; yellow series: $Q_g' = 0.06$; and red series: $Q_g' = 0.10$. The chequered points represent matrices which are in the rubbery state.

for pure bulk water ($v_h = 81.6 \, \text{Å}^3$ at $T = 298$ K [56]). This indicates that the decrease in $v_h$ as a function of increasing water content (measured for $Q_w > Q_w'$) may be attributed to the formation of small ‘pockets’/clusters of water between the polypeptide chains [49, 57]. This would, in turn, result in the overall o-Ps lifetime measured for the sample being a sum of the weighted contributions of an o-Ps in the plasticized gelatin matrix and the o-Ps in bulk water. Since o-Ps probes the local molecular organization at the sub-nanometre length scale, water clusters of size less than one nanometre would be probed by o-Ps as bulk water. In a number of our previous papers we have also shown that clustering of water molecules at higher water contents also occurs in carbohydrate oligomers [25, 27], although for those systems, clustering was only observed in the rubbery state [27]. Furthermore, we have shown that at water contents above $Q_w \sim 0.4$ the hole volume measured for such carbohydrate oligomer matrices becomes independent of the water content and it is very close to the value of $v_h$ measured in pure bulk water [28]. Since the focus of the present study is on the changes in molecular packing of the gelatin matrices in the glassy state, we have not extended our PALS measurements to higher water contents. It would not be unreasonable to assume, however, that the changes in free volume for gelatin oligomers at higher water contents (where gelatin is in a gel or solution state) would be similar to those reported for the carbohydrate oligomers. As for the carbohydrate oligomers, once the clusters of water in the system become so large that the weighted contribution of o-Ps annihilating within them tends to unity, the hole volume measured would be very close to that measured for pure bulk water [26].

As mentioned previously, the o-Ps intensity was found to be independent of temperature. For this reason, this parameter was fixed at its average value (measured over the entire temperature range) in order to improve the stability of the fit when (2) was fitted to the experimental temperature-dependent lifetime spectra for matrices with different compositions and water contents. As it turns out, the o-Ps intensity is also independent of the matrix composition; however, it changes significantly as a function of increasing water content, as illustrated in figure 6(b). The o-Ps intensity initially decreases linearly from $I_{o-Ps} \sim 24.9 \pm 0.2\%$
Figure 7. Average molecular hole volume as a function of increasing glycerol content for glassy gelatin matrices with well-defined water contents: $Q_w = 0.02$ (pink series), $Q_w = 0.04$ (purple series), $Q_w = 0.06$ (cyan series) and $Q_w = 0.08$ (white series), measured at $T = 298$ K. The hole volume data presented for these matrices with well-defined water contents were derived by interpolation of the data reported in figure 6(a). The solid lines, included as a guide to the eye, represent fits of the experimental data to a previously proposed semi-empirical model [26, 59].

To $I_{o-Ps} \sim 21.1 \pm 0.3\%$ as the water content increases from $Q_w \sim 0.01$ to $Q_w \sim 0.10$, followed by a less pronounced linear increase to $I_{o-Ps} \sim 22.4 \pm 0.2\%$ for the gelatin matrix with the highest water content. The water content at which the minimum in $I_{o-Ps}$ is observed in figure 6(b) appears to occur at a similar water content as the maximum in $v_h$ (figure 6(a)), although it is independent of the matrix composition. These changes in $I_{o-Ps}$ measured for the gelatin matrices are very similar to the changes in $I_{o-Ps}$ reported previously by Trotzig et al [49] for hydroxypropyl methylcellulose matrices. It is possible that the initial decrease in $I_{o-Ps}$ is due to the reduction of the probability of $o$-Ps formation due to the shielding of the molecular electrons (and the free positron) as a result of an increase in the local polarity when the small, polar water molecules are absorbed by the gelatin oligomers [58]. The $o$-Ps intensity measured at 298 K for pure, bulk water is 26.9% [56], which is higher than the $I_{o-Ps}$ measured for the plasticized gelatin matrices. The subsequent increase in $I_{o-Ps}$ for water contents greater than $Q_w \sim 0.10$ (where the water molecules form clusters) is, therefore, most likely to be due to $I_{o-Ps}$ becoming a weighted average of $I_{o-Ps}$ in the plasticized gelatin matrix and $I_{o-Ps}$ in the water clusters, whose size increases as the gelatin oligomer becomes increasingly more hydrated.

In figure 7 we show the effect of glycerol on the molecular packing of glassy gelatin matrices with well-defined water contents. The hole volume data presented in figure 7 for the matrices with well-defined water contents are derived by interpolation of the hole volume data reported in figure 6(a), rather than direct measurements due to experimental reasons [26]. Following the procedures outlined in section 2, the water content of the matrices is varied by equilibrating them at defined water activities. As the amount of water absorbed by the
matrices at a given water activity is governed by the matrix composition (see the supplementary data (stacks.iop.org/NJP/14/035016/mmedia) for more details), this will result in a series of samples with (slightly) different water contents. In order to obtain data series at well-defined water contents we have, therefore, interpolated the data points from the extensive free volume measurements presented in figure 6(a). Over the entire concentration range studied, glycerol acts as a packing enhancer for the gelatin matrices and we observe a nonlinear reduction in the average hole size of the matrices, indicative of non-ideal mixing of the disparate components. The initial rapid decrease in the average hole size may be related to the reduction in the molecular frustration of the polypeptide chains upon the addition of the smaller polyol molecules, as they reduce the glass transition temperature of the matrices to lower temperatures and water contents [4, 26, 59]. In addition, glycerol is also likely to interfere with the hydrogen bonding between the polypeptide chains, thus allowing them to pack more efficiently, leading to the observed decrease in average hole size [27]. Glycerol has been previously shown to have a similar effect on the molecular packing of glassy carbohydrate oligomer matrices [27, 59], where we proposed a simple semi-empirical model to predict the changes in molecular organization as a function of the increasing polyol content [26, 59]. The reported decrease in hole volume for these carbohydrate matrices was, however, significantly less pronounced compared to that of the gelatin matrices. For example, a decrease in hole volume of the order of $\sim 5 \, \text{Å}^3$ was observed as the glycerol content increased from 0 to 10 wt.% for the carbohydrate oligomer matrices ($Q_w = 0.08$) [27], compared to a decrease of $\sim 16 \, \text{Å}^3$ for the gelatin matrices ($Q_w = 0.08$), over the same range of glycerol concentrations. This difference in the effect of glycerol on the molecular packing of the two bio-oligomers is most likely to be explained by the differences in the chemical composition, the number of proton donors/acceptors available for hydrogen bonding, the molecular weight and polydispersity of the two matrices.

4. Final remarks

Our study provides novel insights into the changes in molecular organization and thermodynamic state of glassy gelatin oligomer matrices which occur as a result of a change in the level of hydration and the addition of glycerol, a polyol commonly used by the pharmaceutical and food industries as a humectant. We provide a basis for establishing the composition–structure–property relationships for the glassy state of this biopolymer, which would eventually enable the rational control of its macroscopic properties at the molecular level, and the design of optimal encapsulating matrices.

We measured systematic reductions in the glass transition temperature, $T_g$, and the temperature of dissociation/melting of the ordered triple helical ‘tropocollagen-like’ segments, $T_m$, as a function of increasing water and glycerol contents. However, the level of re-naturation (fraction of ordered triple helical regions) of the gelatin matrices was found to be independent of the level of hydration and composition of the matrices, suggesting that the changes in local free volume observed reflected the changes in molecular packing occurring in the amorphous parts of the gelatin matrices. Although the glass transition behaviour of the gelatin matrices was reflected in the free volume measurements, we did not observe any signature of the dissociation of the ordered triple helical regions as the gelatin matrices were heated to temperatures above $T_m$.

We reported complex changes in the average hole size (and $o$-Ps intensity) of the gelatin matrices as a function of increasing water content in the water content range, $Q_w \sim 0.01–0.18$. 

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At low water contents \((Q_w \sim 0.01–0.10)\), the average hole size of the gelatin matrices was shown to increase rapidly as a function of increasing water content, as the water molecules bind to the polar side groups on the polypeptide chains, thereby altering the local hydrogen bonding of the gelatin oligomers. For water contents higher than \(Q_w \sim 0.10\), water molecules were found to form clusters. In the presence of such clusters, the average hole size of the matrices was found to decrease systematically as a function of increasing water content, as the lifetime of \(\sigma\)-Ps becomes the sum of the weighted contributions of its lifetime in the plasticized gelatin matrix and that in bulk water. Finally, over the concentration range studied, glycerol was found to act as a packing enhancer, causing significant reductions in the average hole size of the gelatin matrices both in the glassy and rubbery states. In the glassy state, we observed a nonlinear reduction in the average hole size for gelatin matrices with well-defined water contents, which is indicative of non-ideal mixing of the disparate components.

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