RESEARCH ARTICLE

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Tuning Human Serum Albumin (HSA) Hydrogels through Albumin Glycation

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The changes of technological properties of albumin-based hydrogels induced by increasing degrees of post-translational modification of the protein are reported. Maillard-type modification of amino acids arginine and lysine of albumin is achieved through glyoxal as an α-dicarbonyl compound. The degrees of modification are fine-tuned using different molar ratios of glyoxal. Hydrogels are thermally induced by heating highly concentrated precursor solutions above the protein's denaturation temperature. While the post-translational modifications are determined and quantified with mass spectrometry, continuous-wave (CW) electron paramagnetic resonance (EPR) spectroscopy shed light on the protein fatty acid binding capacity and changes thereof in solution and in the gel state. The viscoelastic behavior is characterized as a measure of the physical strength of the hydrogels. On the nanoscopic level, the modified albumins in low concentration solution reveal lower binding capacities with increasing degrees of modification. On the contrary, in the gel state, the binding capacity remains constant at all degrees of modifications. This indicates that the loss of fatty acid binding capacity for individual albumin molecules is partially compensated by new binding sites in the gel state, potentially formed by modified amino acids. Such, albumin glycation offers a fine-tuning method of technological and nanoscopic properties of these gels.

1. Introduction

Human serum albumin (HSA) is the most abundant plasma protein in the human body, reaching blood concentrations of up to 50 mg mL^{-1}.[1] Due to its wide availability at comparatively low cost, it is ideally suited as a model protein for biochemical, chemical, and biophysical studies.[1]

Due to the propensity of albumins to form 3D networks, they are particularly suited for use in hydrogels.[2–7] Hydrogels made from synthetic or biological macromolecules have been deemed extremely promising soft matter for use in biology and medicine. In the search for new materials with high biocompatibility, proteins and hydrogels made thereof, have moved into the focus of research.[6–9]

In recent years, several studies have been published on albumins, their gel formation and the properties of gels.[5–7] To the best of our knowledge, no studies have been published on the gelation of modified albumins and their properties. It is known that albumin in the blood is modified to a certain extent; it is also known that these modifications can limit the biological activity of the molecules.[10]

Here, we present experimental data on hydrogels made from albumins that have been chemically modified to mimic in vivo modifications known as Maillard reactions in organisms. Our albumin modifications are so called AGEs (Advanced Glycation Endproducts).[10–13]

Such modified albumins play a role in vivo and we have recently used a combination of methods to characterize the functional (fatty acid binding capacity) differences of HSA in dependence of the glycation state.[14] To carry out these Maillard modifications in vitro, albumins were incubated with different concentrations of glyoxal. In general, the term *Maillard modification* encompasses reactions of α-dicarbonyls with amino acids. In the case of albumin, mainly the ϵ-amino group of lysine and the guanidino group of arginine, as well as the N-terminal end, are involved.[10–13,15] Since albumin gels are formed by transforming intramolecular alpha-helical into intermolecular beta-sheet regions, modifications of amino acid side chains may well alter these secondary structure contacts qualitatively and/or quantitatively. Here, we present evidence that different degrees of protein

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Table 1. Summary of MS results, n.d.: not detectable. G-H3 is the acidic conversion product of CMA and a measure of the amount of CMA.

Sample	CML [ng AGE/mg HSA]	G-H3 [ng AGE/mg HSA]	GOLD [ng AGE/mg HSA]
HSA	0,75 ± 0,07	1,34 ± 0,11	n.d.
G0	0,77 ± 0,05	1,41 ± 0,10	n.d.
G1	2,14 ± 0,15	7,06 ± 0,02	n.d.
G5	5,93 ± 0,19	25,46 ± 1,01	0054 ± 0001
G10	9,59 ± 0,79	39,28 ± 7,17	0,18 ± 0,02
G18	14,65 ± 0,68	52,87 ± 3,66	0,36 ± 0,03
G100	31,26 ± 2,33	78,30 ± 5,80	1,80 ± 0,16

EPR spectroscopic measurements were carried out to characterize the fatty acid binding capacity in the different gels prepared from the Maillard-modified albumins. To this end, 16-DSA (16-Doxyl stearic acid), a stearic acid derivative bearing a persistent nitroxide radical, was used. CW EPR spectra were thoroughly analyzed, and spectral simulations were carried out with the function "chili" in the EasySpin a toolbox for Matlab. This function uses the Schneider-Freed approach to describe intermediate tumbling in nitroxides in simulations of complex EPR spectra.[20]

modification result in a change of the secondary structure formation and the ensuing technical gel properties. We mimic natural modifications by reaction with glyoxal, varying the glyoxal content while keeping the procedure of HSA sample preparation constant. The different degrees of modification denote the amount of glyoxal added to a 1 mM HSA precursor solution: G0, G1, G5, G10, G18 to G100, i.e., the strongest modification G100 contains a potential 100 molecules of glyoxal per HSA.

After modifying the proteins, we have prepared respective gels according to methods that we and others have established in recent years.[5–7,14] Our approach then uses continuous wave (CW) electron paramagnetic resonance (EPR) spectroscopy to characterize the capacity of the gels and the respective low-concentration precursor solutions of all HSA samples to bind long chain (stearic acid) based fatty acids that carry a persistent radical. We have established this method to study the functional, solution state of albumin-based samples.[7,16–18] Especially the effect of changing/modifying amino acid residues can be studied from a functional, ligand-based point of view, as we could show before.[6,14,17,19]

2. Results

The above-described Maillard reaction mechanisms with glyoxal lead to the following modifications of Arg and Lys.[12] Mass spectrometric (MS) measurements were used to quantify the total number of modifications given in **Table 1** (see the Experimental Section and the Supporting Information). As acid protein hydrolysis was used to release AGEs from the albumin, chemically labile structures such as GODIC and GALA were totally degraded and CML was used as the sum parameter for CML and GOLA.

Likewise, CMA was accessed as the imidazolinone G-H3, which is its acidic conversion product. In contrast to GODIC and GOLA, GOLD remains intact as the single crosslink structure under these conditions.

All spectra clearly consist of three different spectral components, all of which are displayed in the inset in **Figure 1**: a freely tumbling 16-DSA species, a second species representing an immobilized (bound) component, and a third one consisting only of one line as expected for nitroxide spin probes undergoing heavy Heisenberg spin exchange, i.e., frequent radical-radical collisions, which usually is a sign of very high local concentration/aggregation.

All spectra were simulated with varying fractional weights of the three components (Figure 1) and the fractional weights of the bound species from the simulations are plotted in **Figure 2** as a measure of the fatty acid binding capacity. EPR spectroscopic measurement series, in which the 16-DSA-to-albumin ratio was varied systematically, were conducted for all albumins of

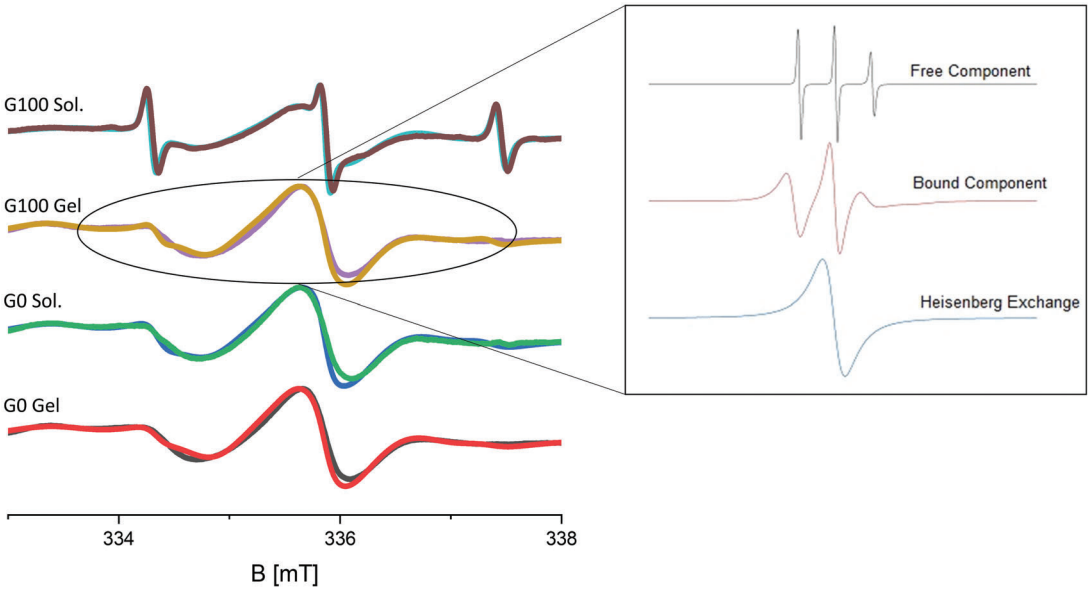

Figure 1. CW EPR spectra of the measured data (black) and spectral simulations (red, green, yellow and blue) of G0 and G100 in solution and gel state with explanation of the different spin systems used for simulation.

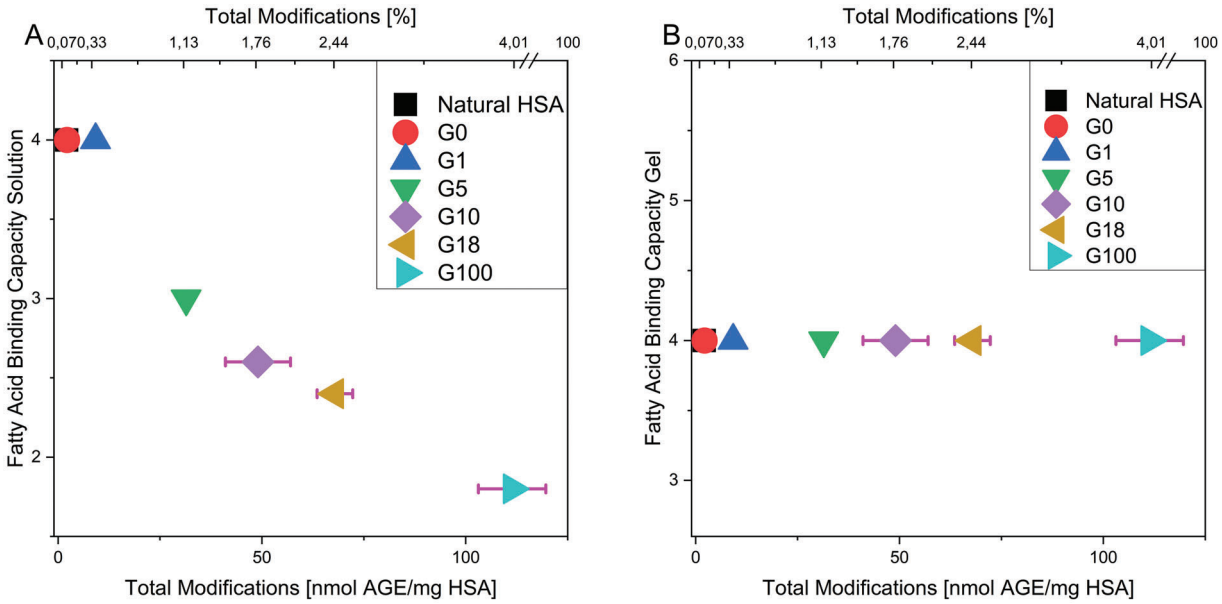

Figure 2. A) Total number of bound Fatty acids versus the number of total modifications from G0 to G100 in solution. B) Total number of bound Fatty acids versus the number of total modifications from G0 to G100 in formed hydrogels.

different degree of modification. Moreover, the measurements series obtained for all albumins in dilute solution and in the gel state. We defined the fatty acid binding capacity as the total number of bound fatty acids to the HSA molecule. We obtained it by comparing the spectra and the simulation of solution and gel state. All details and simulations are summarized in the Supporting Information. We further correlate the fatty acid binding capacity with the total number of modifications for the dilute albumin solutions (Figure 2A).

As can be seen in Figure 2A, the binding capacity decreases with an increasing number of protein modifications in aqueous solution. On the contrary, the binding capacity of all modified albumins in the gel state remains constant regardless of the degree of modification, as shown in Figure 2B.

The physical/mechanical stability of the gels and gelation times were then studied using rheological measurements. Detailed results from rheological measurements are summarized in the SI. For evaluation of the influence of the modifications on gelation, we correlate the gel formation time with the total number of modifications in **Figure 3**. To better judge the sample toughness, we correlate the mechanical stability with the total number of modifications in **Figure 4**.

In previous studies it was found in the crystal state[21,22] and the more functional, solution state[18,23,24] that healthy HSA can bind up to seven fatty acids in specific binding sites. The positions in HSA and recently also BSA[21,22] are known from crystal structures and have been corroborated in solution through various binding experiments.[8,25,26] As mentioned above, it is known that the Maillard reaction targets the ϵ-amino group of lysine and the guanidine moiety of arginine. The EPR data reflect a decrease in fatty acid binding capacity in solution when the number of total modifications increases (as seen in MS data). Hence, one may hypothesize that the main effect on the binding capacity upon modification arises from modified arginine and lysine residues

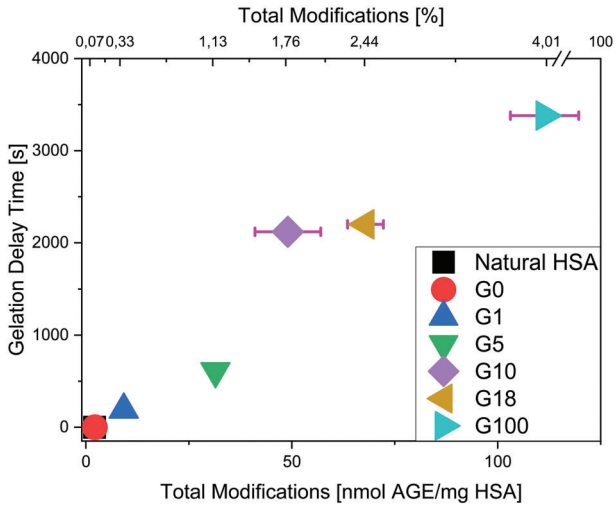

Figure 3. Gel formation time versus the total amount of modifications for all samples.

in or near the binding sites, as visualized in **Figure 5**. Glyoxal is a small molecule and can penetrate the tertiary structure of HSA, i.e., it should be possible to enter the binding sites. Albumin is a very versatile transport molecule in the bloodstream, it was shown that it can bind not only fatty acids but also other small, amphiphilic, hydrophobic, and even hydrophilic molecules such as drugs. So, one may envision that glyoxal can interact with the HSA molecule and initiate a Maillard reaction at these sites.[8,27]

The results of the rheological measurements can be considered, too, to complete the picture by inclusion of a more macroscopic, technological view. When plotting the gel formation time as a function of the total number of modifications, it is noticeable that the time until the gel forms increases with

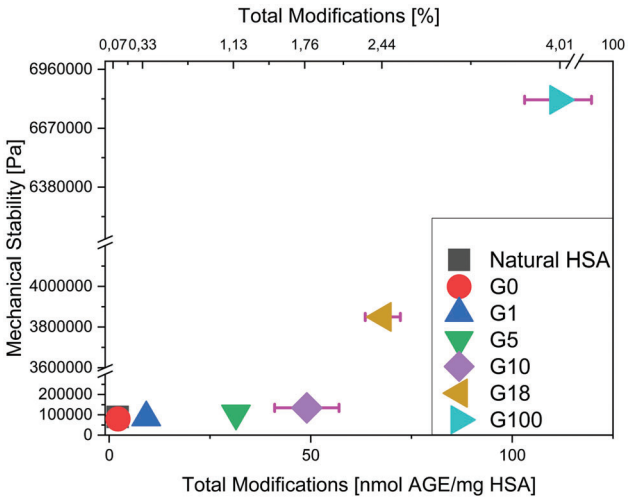

Figure 4. Mechanical stability versus the total amount of modifications for all samples the mechanical stability is defined as the maximum Value of the storage modulus subtracted with the corresponding loss modulus value everything with a value higher than 20 000 Pa is considered a robust hydrogel.

the number of modifications. It is known that large conformational changes occur during the gelation of albumins.[27,28] Here, mainly intramolecular α-helices are converted into intramolecular β-sheets, which can, e.g., be seen in the articles by Arabi et al.[7] In addition, it appears that the mechanical stability increases with the number of modifications (Figure 4)

As shown in **Figure 6**, the Maillard reaction with glyoxal results in different modifications, including two crosslink structures GODIC and GOLD. Earlier, the hypothesis was put up that the reduced fatty acid binding capacity is a consequence of the Maillard reaction taking place preferentially at or in the fatty acid binding sites, leading to crosslinks at these sites. We can further assume that the prolonged gel formation time is a result of intramolecularly crosslinked structures induced by the Maillard reaction. We have recently introduced the incubation/gelation time as a viable factor for the tuning of albumin gels[7] and since all hydrogels were thermally induced at 65 °C, three degrees higher than the denaturation temperature of native HSA (62 °C at pH 7.4) and the concentrations were constant, the lag time of gelation can be interpreted as a measure of hindrance to gel formation.[7] Furthermore, we see a drastic increase in physical toughness especially for the samples G18 and G100. They have the greatest

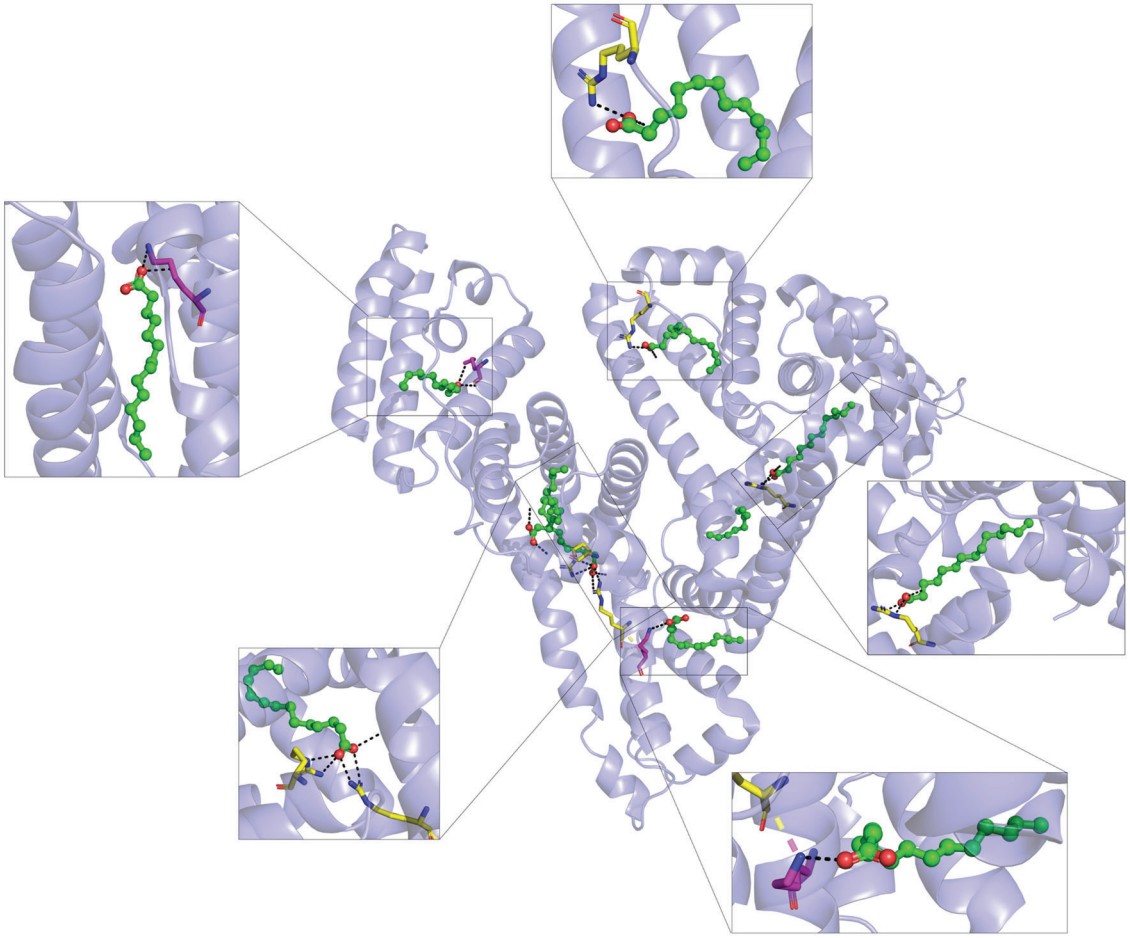

Figure 5. Crystal Structure of Human Serum Albumin (PDB: 2BXM) with the seven fatty acids in the respective binding sites. Arginine residues located in the binding sites are shown in yellow, lysine residues located in the binding sites are shown in magenta.

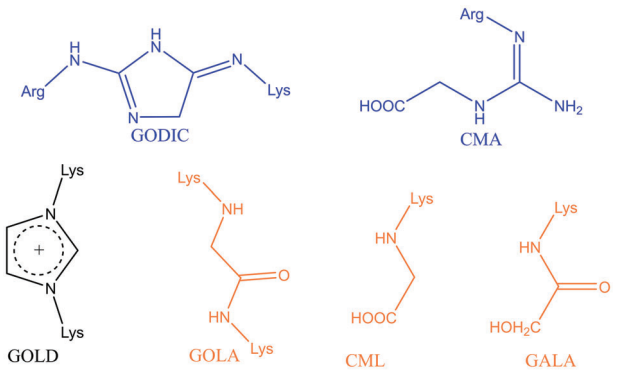

Figure 6. structure formula of established arginine modifications CMA N_ϵ-Carboxymethylarginine, GODIC Glyoxal-Lysine-Arginine-crosslink lysine modifications, CML N_ϵ-Carboxymethyllysine, GALA Glycolic acid lysylamide, GOLA Glyoxal-lysine-amide, GOLD Glyoxal-lysine-dimer.

amount of the crosslinking modification GOLD and also have the longest delay times.

We see a significant decrease in the fatty acid binding capacity of HSA in solution and from the strongly reduced fatty acid binding capacity determined by EPR spectroscopy (Figure 2A), we conclude that the Maillard reaction targets the amino acid residues in or near the binding sites. When we chemically modify the fatty acid binding sites it interferes with the proper uptake (and potentially transport) function, leading to a decrease in binding capacity. It is known that the strong immobilization takes place at the interfaces of α-helices,[7] but if amino acids at these sites are modified it explains the decrease in bound spectral component from which we derive the binding capacity. Note that unspecific attachment of amphiphilic substances such as 16-DSA on hydrophobic patches, e.g., on the protein's surface can nonetheless take place. Contrary to the observation in solution, we see that the binding capacity in the gel state remains constant. Also, this finding can be rationalized, as in the gel state HSA builds a 3D protein network that immobilizes small and intermediately sized amphiphilic molecules such as 16-DSA in a different way, with new hydrophobic and water-depleted volume fractions that are formed through intermolecular contacts. Apparently, despite the AGEs, the newly formed pockets are significantly water-reduced to attach 16-DSA molecules. Therefore, the strong reduction in specific binding sites does not nominally reduce the binding capacity with the increase of total modifications.

3. Conclusion

Taken together, after incubating HSA with different concentrations of glyoxal, Maillard-modified albumins of different modification degrees were achieved. Then their microscopic or rather nanoscopic properties were measured using EPR spectroscopy and their macroscopic properties using rheology. We found that the fatty acid binding capacity of individual albumin proteins in dilute solution decreases with the increase in modifications and the gel formation time increases with the increase in modifications. This led us to conclude that in aqueous solution the decrease in the fatty acid binding capacity stems from mainly modifications of arginine and lysine residues in or around the fatty acid binding sites. Furthermore, the intermolecular crosslink structures may hinder the conformational changes which are necessary for gel formation, which results in an increased gel formation time. An interesting aspect that arises from this is that although the fatty acid binding capacity in solution and the gel formation time have suffered, the structure of the hydrogel allows the fatty acid binding capacity in the gels to remain constant despite or potentially due to Maillard modifications. This binding capacity in the Maillard-modified albumin gels seems to be upheld by newly forming binding sites with intermediate binding strength (see Figure 2A,B), which may be due to new local anchoring groups for fatty acids. These new anchoring groups could stem from the Arg and Lys derivatives that are shown in Figure 6. Since the number of fatty acid binding sites in the gels remains constant, while the number of canonical binding sites in the individual molecules decreases, the new binding sites could be situated at the Maillard-modified interfaces of secondary structure elements of different HSA molecules. One may speculate that other amphiphilic molecules (such as drug molecules that we have tested amply) may also bind to the new binding sites in the gels, even without a carboxylic acid headgroup of fatty acids. This is subject to further research in our labs. If we take this theory further and assume that HSA gels could be used to deliver drugs locally and in a sustained manner in the future, one has to consider aging processes like the Maillard reaction that occur naturally in the human body. One can only speculate, but we assume that modification patterns arise in vivo that are similar or even the same as those we have studied here in vitro, which additionally leads us to conclude that Maillard modifications do not pose a risk to the structural integrity of the hydrogels.

4. Experimental Section

EPR Spectroscopy: The sample solutions were filled into 50 µL microcapillaries and then sealed with CRITOSEAL. After 12 h of incubation at 65 °C, the samples were measured at 37 °C in the Miniscope MS400 (Magnettech GmbH, Berlin). The frequency was set to ≈9.4 GHz, the magnetic center field B_0 was set to 336 mT, the sweep width to 150 mT, the sweep time to 60 s, the modulation amplitude to 0.05 mT, the microwave (MW) power to 20 mW, the phase to 180°. Always one scan was recorded with 4096 measuring points at a gain of 200.[29]

Rheology: Two milliliters of protein solution were put on the sample plate of the rheometer Physica MCR 301 (Anton Paar, Graz, Austria) and measured with the measurement system CP-50-2-SN 22751. The parameters were: gap width d 0.209 mm, temperature 65 °C, measuring point duration 10 s, Amplitude 0.5 %, and angular frequency 0.1 rad s^{-1}. Rheological characterization was used to determine the mechanical stability and the gelation delay time. Mechanical stability was defined as the value of the storage modulus subtracted with the value of the corresponding loss modulus. Everything with a value above 20 000 Pa is considered a robust hydrogel. The gelation delay time is defined as the time when the storage modulus arises significantly above the loss modulus.

LC-MS/MS: For the MS measurements (**Figure 7**), 100 µL of each sample shown in Table 1 (from G0 to G100) with a concentration of 200 mg mL^{-1} were reconditioned according to the prescription of Henning et al.[10,12,13] The LC apparatus (Jasco, Pfungstadt, Germany) consisted of a pump (PU-2080 Plus) with degasser (LG-2080-02) and quaternary gradient mixer (LG-2080-04), a column oven (Jasco Jetstream II) and an autosampler (AS-2057 Plus). Mass spectrometric detection was con-

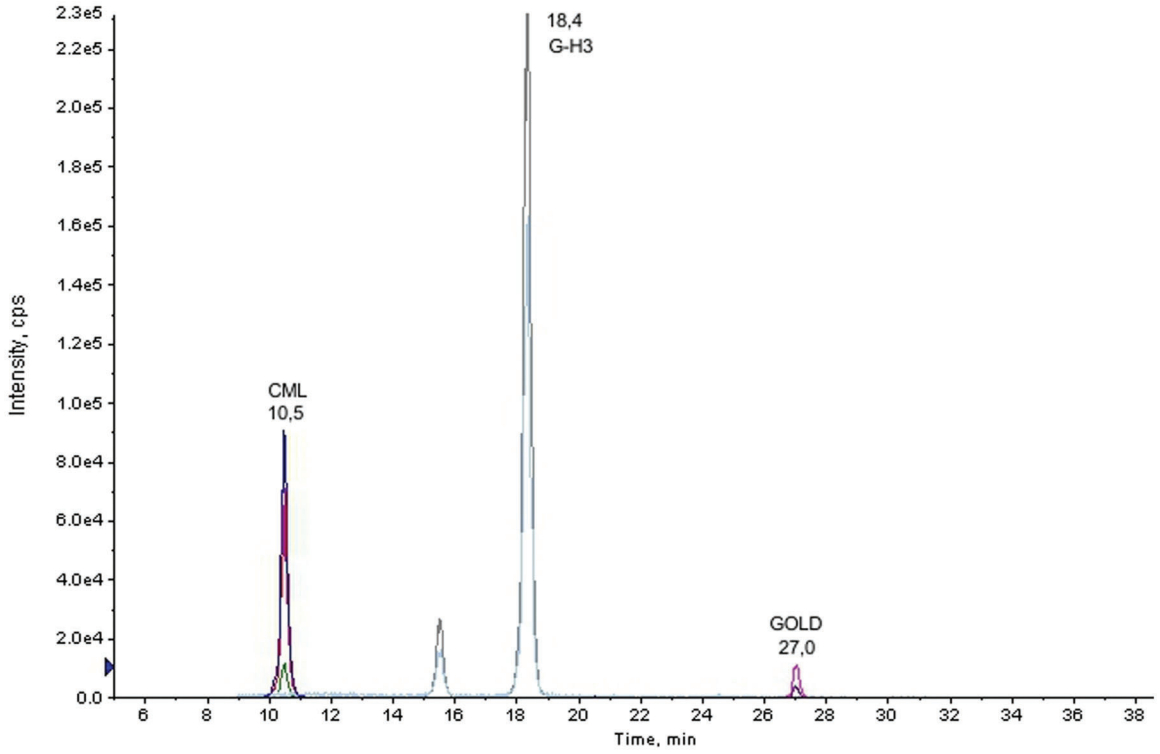

Figure 7. LC-MS/MS chromatogram of an albumin sample (G100) after acid protein hydrolysis. For each target analyte with specific retention time the most abundant fragment ion was used as quantifier and two more fragment ions were selected as qualifier.

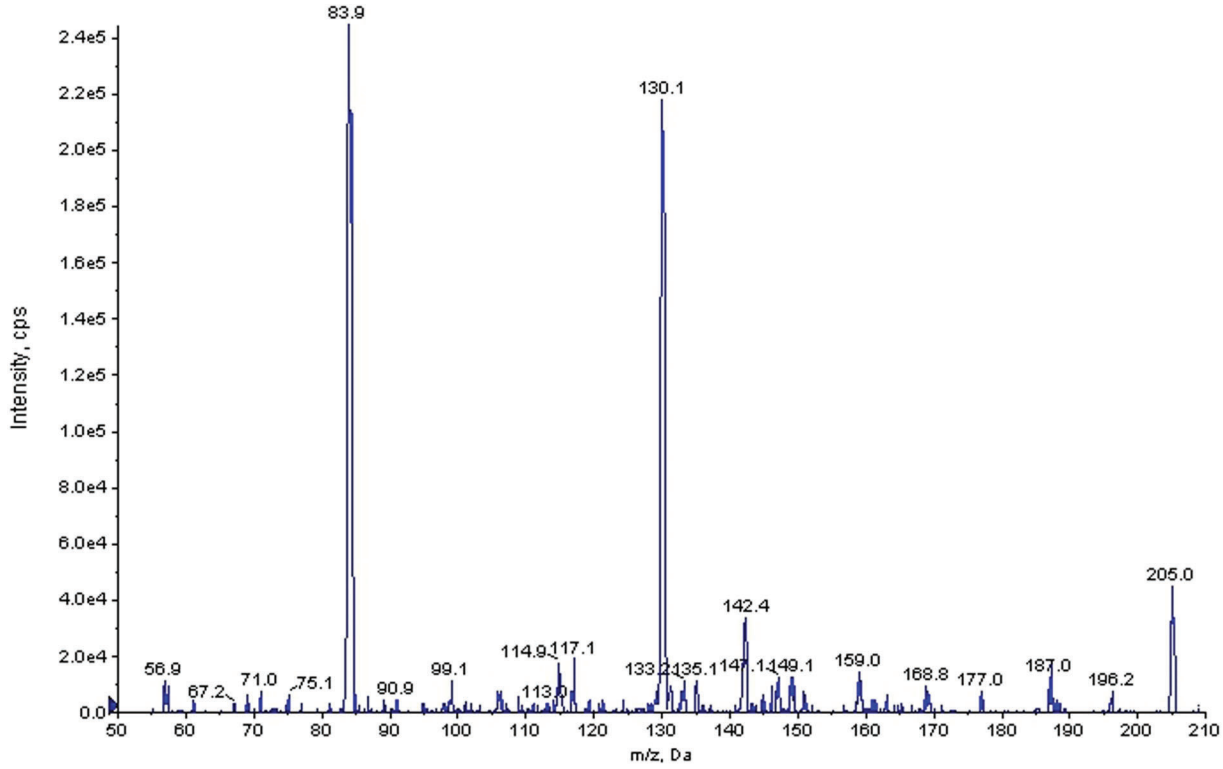

Figure 8. Verification of CML: Mass spectrum of collision-induced dissociation of m/z 205.0 [M+H+] Protein Modification.

ducted on an API 4000 QTrap LC-MS/MS system (AB Sciex, Concord, ON, Canada) equipped with a turbo ionspray source using electrospray ionization (ESI) in positive mode. Compound specific orifice potentials (*DP*, declustering potential) and fragment specific collision energies (*CE*) and collision cell exit potentials (*CXP*) are listed in the Supporting Information.

Protein Modification: 1 mM HSA solution was incubated with different concentrations of glyoxal: from 0 mM as a blank, 1 mM, 5 mM, 10 mM, 18 mM and 100 mM glyoxal. In the following, the samples are named after their glyoxal concentration starting from G0, G1, G5, G10, G18 to G100. After mixing the HSA with the glyoxal, the solution was incubated at 37 °C for 24 h and the reaction takes place according to the mechanism introduced by Henning et al.[12] After the incubation, remaining unreacted glyoxal was removed via dialysis and the sample was lyophilized. As an example, **Figure 8** shows the mass spectrum of a CML-modification. For details, see the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

drug delivery, ESR/EPR spectroscopy, intermolecular interactions, Maillard-reaction, posttranslational modification, protein folding

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