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Covalent β-lactoglobulin-maltodextrin amyloid fibril conjugate prepared by the Maillard reaction

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ABSTRACT

The surface modification of β-lactoglobulin amyloid fibrils (AFs) was investigated by performing the Maillard reaction with the free anomeric carbon of the maltodextrin in water at pH 9.0 and 90 °C. The bonding of maltodextrin to fibrils was confirmed by determining the free amino group content and the presence of final products from the Maillard reaction. The secondary structure of AFs was preserved as observed by circular dichroism analysis. Atomic force microscopy evidenced that prolonged heat treatment caused hydrolysis of the attached polysaccharide and consequently lowered the height of the fibrils from 8.0 nm (after 1 h) to 6.0 nm (after 24 h), which led to the reduction of hydrophilicity of resulting conjugate. Increasing the reaction time, however, resulted in the improvement of colloidal stability and decrease in turbidity ascribed to the increment of glycation degree, as well as, a decrease in the isoelectric point of the protein-based supramolecular object.

1. Introduction

Depending on the pH value and temperature, β-lactoglobulin can self-assemble into fractal clusters (pH > 6.0), spherical particles (pH value close to the isoelectric point) and fibrils (pH = 2.0) when heated above the denaturation temperature (Jung, Savin, Pouzot, Schmitt, & Mezzenga, 2008). Proteinic fibril aggregates, also referred as amyloid fibrils (AFs), have received considerable interest in several fields of research, e.g., medicine, bio-nanotechnology, supramolecular chemistry, food science and soft matter, as a novel kind of functional biomaterial, due to their unusually high aspect ratio, mechanical strength, well-defined nanostructure (Knowles & Mezzenga, 2016; Nyström, Fernández-Ronco, Bolisetty, Mazzotti, & Mezzenga, 2016; Rühs, Adamcik, Bolisetty, Sánchez-Ferrer, & Mezzenga, 2011; Wei et al., 2017), and since they occupy a minimum energy configuration on the protein folding landscape (Adamcik & Mezzenga, 2018). In particular, food amyloid fibrils generated from globular food protein precursors are receiving progressively more attention in the field of food science due to their functionality, surface active properties and the possibility to combine them with nutraceutical and mineral components so to target nutrition applications (Shen et al. 2017). For a comprehensive review on the properties and applications of food amyloid fibrils the reader is addressed to a recent review (Cao & Mezzenga, 2019).

Peptides, which are generated from protein hydrolysis, are the building units of AFs. They organize into β-strands (perpendicular to the fibrillar direction) and form cross β-sheets of indefinite length, which are in turn stabilized by hydrophobic interactions, hydrogen bonding, and π-π stacking of aromatic groups (Hu et al., 2018). By using single-molecule atomic force microscopy (AFM), Adamcik et al. (2010) showed that β-lactoglobulin fibrils had a contour length from *ca.* 0.5 μm to > 15 μm, and a height distribution centered at maxima of about 2.0, 4.0, and 6.0 nm, with the 4.0 nm distribution being the most frequent.

These supramolecular biopolymers form a stable dispersion as a result of electrostatic repulsions at pH ≪ pI due to the presence of positive charges exposed to the water medium, but undergo agglomeration and progressive precipitation near the protein isoelectric point (pI ~ 5.2) (Bolisetty, Arcari, Adamcik, & Mezzenga, 2015; Sagis et al., 2002). Thus, applications are restricted to pH values far from the pI. It has been demonstrated that adsorption of surfactants and polysaccharides on to the surface of protein particles inhibits their precipitation at the pI (Jung et al., 2008; Karbasi, Askari, & Madadlou, 2019). AFs are also interesting in terms of their multifaceted surface chemistry, where several available amino acids provide further

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functionality to the fibrils and can be used, e.g., for PEGylation (Rühs et al., 2011), phenolic modification (Hu et al., 2018) and polysaccharide complexation (Jones et al., 2011).

Maillard reaction is a spontaneous, organic solvent-free process, and can be considered as a food-grade method for modifying peptides and proteins (Karbasi & Madadlou, 2017), e.g., kidney bean vicilin (Tang, Sun, & Foegeding, 2011), whey protein isolate (Liu & Zhong, 2013b) and β-lactoglobulin (Jiang & Brodkorb, 2012). When limited to the initial stage, the Maillard conjugation of food proteins with carbohydrates can improve their functionalities such as solubility, heat stability, resistance to aggregation, as well as emulsification and foaming properties (Karbasi et al., 2019). Liu & Zhong (2013a) reported formation of nanofibrils from pre-conjugated whey protein isolate (WPI) with lactose, which were well-stable at pH 3.0–7.0; however, fibril formation yield was lower compared to the fibrillation of non-conjugated WPI. It is noteworthy that advanced glycation stages of the reaction result in severe cross-linking and polymerization of Maillard reaction products (MRPs) in an uncontrolled manner. Advanced glycation end-products (AGEs) have been found associated with the increasing propagation of diet- and inflammation-related illnesses such as hormonal disorders (Ravichandran et al., 2019), diabetic complications (Nowotny, Schroeter, Schreiner, & Grune, 2018), and food allergy (Teodorowicz, Van Neerven, & Savelkoul, 2017).

Maltodextrin is a starch-derived linear oligo/polysaccharide, which has been conjugated to whey proteins via the Maillard reaction for increasing proteins solubility at the pI (Martinez-Alvarenga et al., 2014). Maillard conjugation using maltodextrin was also exploited to tune the functionality of pea protein electrospun fibers (Kutzli et al., 2020). Conjugation of proteins (molecules, fibrils and spherical particles) with maltodextrin is favored over mono and disaccharides. Besides, while trimming the free amino groups as a consequence of the Maillard reaction influences the electrostatic repulsion between proteins, maltodextrin can establish an effective steric hindrance. Every maltodextrin molecule has one moiety (carbonyl group), which could involve in the Maillard reaction, while the rest of the saccharide chain would protrude into the surrounding solution. The protruded maltodextrin molecule can hydrate the conjugated protein, increasing its solubility, as well as repel neighboring proteins due to the volume exclusion.

The effect of the Maillard reaction on the structural and functional properties of pre-formed β-lactoglobulin AFs has not been explored so far. Grafting maltodextrin onto AFs is non-trivial due to the "bottlebrush effect" which may either limit the grafting density or reduce fibrils length to decrease the entropic penalty of the stretched bottlebrush corona (Rühs et al., 2011). Therefore the evaluation of the maltodextrin-β-lactoglobulin amyloid fibrils presents also fundamental aspects, beside the possible ensued applications. We hypothesize that the surface decoration of β-lactoglobulin nanofibrils by maltodextrin increases steric repulsion, hydrophilicity and consequently colloidal stability of the protein dispersion around the pI. There are some worth mentioning applications for whey protein AFs from among which the first one is their usage in high-protein beverages, attributed to their predenaturized nature and densely packed structure, particularly in the presence of saccharides, which can lead to forming a sport drink. This usage can be practical through Maillard reaction which eliminates the obstacles caused by protein aggregation and final flocculation during heat processing and storage and also facilitates the utilization of carbohydrates. Besides, these particles can favorably be used as Pickering stabilizer in foams and emulsions, and also as delivery vehicles for bioactives. However, many food and drink products have a pH value between 4.0 and 7.0, which is near the pI of whey proteins. Conjugation of β-lactoglobulin AFs by maltodextrin is considered a potential approach to increase the utilization of these protein particles in acidic condition, likely enhancing the stability of the subsequently prepared emulsions and bioactive-loaded dispersions. Since controlling the reaction progress would be more facile by wet-heating method than dry-heating environment (Karbasi et al., 2019), Maillard reaction in a liquid system was used in the current research. The present study is, therefore, aiming at obtaining such coupling between AFs and maltodextrin, and to investigate the impact of conjugation on the particles' properties and colloidal stability of the obtained dispersions. Assessment of the influence of maltodextrin conjugation on the gastrointestinal digestibility, interface-related attributes, cytotoxicity and colonic fermentation of amyloid fibrils is an ongoing project in our lab.

2. Materials and methods

2.1. Synthesis of β-lactoglobulin amyloid fibrils

Purified β-lactoglobulin protein was prepared from BioPURE beta lactoglobulin (lot JE 003-6-922, Davisco Foods International) according to our previous study (Jung et al., 2008). Then, a 2% w/w purified β-lactoglobulin protein dispersion was adjusted to pH 2.0 using 1.0 M HCl (CAS: 7647–01-0) solution (VWR International company), filtered through a 0.45 μm Millipore cellulose acetate membrane filter (VWR International company), and heated at 90 °C in an oil bath for 5 h with magnetic stirring to avoid localized heating effects. During the incubation, the protein monomer unfolded, hydrolyzed and self-assembled into AFs. After heat treatment, the flasks were immediately cooled down by immersion in ice-water mixtures to quench the aggregation process. As already shown (Jordens, et al., 2014; O'Nuallain, Shivaprasad, Kheterpal, & Wetzel, 2005), due to the equilibrium between the peptides and the fully formed fibrils, dialysis after fibrillization is not effective for removing the unreacted peptides; however, after Maillard reaction we did perform dialysis in order to remove single peptides and unreacted saccharides.

2.2. Preparation of conjugates

Mixtures of AFs and maltodextrin (Sigma–Aldrich, Germany), with a nominal dextrose equivalent (DE) value of 4–7 (CAS: 9050–36-6) corresponding to a molar mass of 2600–4500 Da (M_n = 2583 Da by nuclear magnetic resonance (NMR) and M_w = 4516 Da by static light scattering (SLS), at a protein to polysaccharide molar ratio of 1:1, were dissolved in Milli-Q water at 2% w/w in AFs concentration. Based on AFM images and their statistical analysis (data not shown), as well as, previous studies (Mesquida, Riener, MacPhee, & McKendry, 2007; Mankar, Anoop, Sen, & Maji, 2011) we concluded that the AFs are stable at different pH values. So the pH of the dispersions was adjusted to 9.0 using 1.0 M NaOH (CAS: 1310–73-2), since lower pH values led to the gelation of the mixture after heating. Samples were heated at 90 °C for 1, 6, 12 and 24 h, then immediately cooled down in an ice-water bath, dialyzed (spectra/por® dialysis MWCO: 6–8 kDa) against Milli-Q water, and stored at 4 °C for further analysis. As controls, AFs dispersions were heated alone (without the addition of maltodextrin) at 90 °C for 1, 6, 12, and 24 h and then cooled down and dialyzed. Samples with maltodextrin – conjugates – and without were named "glycated AF" and "heated AF", respectively.

2.3. Determination of the glycation degree

The glycation degree (GD) of conjugated protein has been quantified by the orthophthalaldehyde (OPA) method. Concisely, 150 μL of sample dispersions mixed with 16.5 μL of OPA (CAS: 643–79-8) reagent (Sigma–Aldrich, Germany). After vortexing, samples were maintained in the dark at ambient temperature for 30 min, and then the absorbance was read at 340 nm by a Varian Cary 100 Bio UV–visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The corresponding blank was measured using Milli-Q water instead of AFs dispersions. An L-leucine (0–0.03 $g.L^{-1}$) standard curve was used to calculate the free amino group content.

The glycation degree (GD) was determined as follows:

$$GD(\%) = \frac{A_0 - A_t}{A_0} \times 100\% \quad (1)$$

where A_0 was the absorbance of the sample before glycation, and A_t was the absorbance of the sample after glycation for *t* min.

2.4. UV–visible spectroscopy

The UV–visible spectra of native AF, heated and glycated samples (pH 7.0) were recorded from 200 to 600 nm according to the literature (Kim & Lee, 2009) at room temperature (25 °C) to estimate the amount of intermediate MRPs (Amadori and Amadori-like products measured at 294 nm) and final MRPs (melanoidins and AGEs products measured at 420 nm).

2.5. Circular dichroism spectroscopy

Circular dichroism (CD) spectra of native AF, heated and glycated samples (diluted 100-fold in Milli-Q water at pH 7.0) were collected at 20 °C using a Jasco J-815 spectropolarimeter equipped with a Peltier-controlled cell holder in a precision quartz cell of 1 mm path-length from 190 to 250 nm with a bandwidth of 0.1 nm and a scan speed of 20 nm.min^{-1}. Spectra were background-subtracted, averaged over 10 scans, and smoothed using OriginPro 8G.

2.6. Atomic force microscopy

Morphologies of native AF, 24 h heated AF and glycated AFs, diluted to 0.2% w/w at pH 7.0, were investigated in dry state using AFM. A 20 μL aliquot of all dispersions was deposited onto freshly cleaved mica, incubated for 2 min, rinsed with Milli-Q water, and dried by compressed air flow. AFM experiments were performed by using a MultiMode VIII Scanning Probe Microscope (Bruker, USA) covered with an acoustic hood to minimize noise. AFM images were acquired in tapping mode under ambient conditions. In order to perform statistical analysis (contour length, persistence length, fibril height and quantitative heights distribution) of assembled AFs, AFM images were traced by using FiberApp, an open source tracking and analysis software written in MATLAB (Usov & Mezzenga, 2015).

2.7. Tollens' test

Tollens' test assays the presence of aldehydes using silver (I), resulting in the oxidation of an aldehyde into the corresponding carboxylic acid and reduction of silver ions into metallic silver. Consequently, a grey or silver precipitate is generated after a series of color changes. (Huntley, Crews, & Curry, 2015). Three droplets of a maltodextrin-containing dispersion (4.4% w/w) adjusted to pH 9.0, before and after heat treatment at 90 °C for 24 h, were mixed with 1 mL Tollens' reagent of 5% silver nitrate, 2% ammonia, and 10% sodium hydroxide.

2.8. Visible-light turbidity and colloidal stability

The absorbance of AF dispersions was measured at 500 nm in a quartz cuvette in a UV–visible spectrophotometer (see above). Turbidity was calculated following the equation $\tau(\lambda) = 2.3\ A(\lambda)/L$, where A is the absorbance at 500 nm and L is the optical path length (L = 1 cm) (Narayanan, Xiong, & Liu, 2006). The colloidal stability of the solutions was determined at pH values between 2.0 and 7.0, which was adjusted by the addition of 1.0 M HCl. Samples were centrifuged at $173 \times g$ for 10 min and the absorbance of the supernatant was measured at 500 nm. To acquire an absorbance value below 1.2, in which the linear region of absorbance was preserved, dispersions were diluted in Milli-Q water of identical pH. Cuvettes containing pure water were used as a reference blank.

The colloidal stability was expressed as the ratio between the absorbance at 500 nm after and before centrifugation (Schmitt et al., 2010; Karbasi et al., 2019) as follow:

$$\text{Colloidal stability}(\%) = \frac{A_a}{A_0} \times 100\% \quad (2)$$

where A_0 was the absorbance of the sample before centrifugation, and A_a was the absorbance of the sample after centrifugation.

2.9. Electrophoretic mobility measurements

Electrophoretic mobility of the samples was determined by the Zetasizer Nano ZS dynamic light scattering device (Malvern Instruments, Worcestershire, U.K.) using plastic folded capillary cells (Malvern Instruments) possessing two metal electrodes at the capillary ends.

2.10. Statistical analysis

All measurements were performed on at least three freshly prepared samples and are reported as mean and standard deviation. The results were analyzed by one-way analysis of variance (ANOVA). SPSS software (version 16, IBM software, NY, USA) was employed to determine significant differences between mean values using Duncan's test at a p-level of 0.05.

3. Results and discussion

3.1. Synthesis and characterization of AF-maltodextrin conjugates

Generally, the Maillard reaction includes three stages. At the early stage, achromatic products, with no absorbance in the visible region of the spectrum, are generated, which correspond to the condensation of an aldehyde (C═O) and an amine (NH_2) group resulting in the formation of an imine group (N═C). This is the desired step to decorate our AFs with maltodextrin chains. Degradation of early ingredients into intermediate MRPs is a pH-dependent process; reductones and fission compounds such as diacetyl, pyruvaldehyde and acetol are synthesized at pHs ≥ 7.0, exploited in the current study, whereas acidic pH values result in the formation of hydroxymethylfurfural or furfural. Intermediate components endure condensation process to generate nitrogenous polymers (melanoidins and AGEs). Yellow and yellow/brown products are formed at intermediate and final stages of the Maillard reaction, respectively (Karbasi & Madadlou, 2017). Therefore, the development from transparent to brown color through yellow and orange is an indication for the extent of the reaction progress (Liu & Zhong, 2015).

The UV–visible spectra and pictures of glycated AFs and heated AFs samples as a function of the incubation time are shown in Fig. 1a and 1b, respectively. Just like for the normal spectrum of proteins, the first peak at 220 nm (Fig. 1a) is related to the carbonyl moiety from the peptide bonds found in the protein and the second peak at 280 nm is due to the constituent aromatic amino acids (i.e., tryptophan and tyrosine) (Noble & Bailey, 2009). The absorbance of glycated samples is higher in the ultraviolet region than in the visible region (Kim & Lee, 2009). While the spectra of AF alone and heated AFs were comparable, the absorbance of glycated AFs increased significantly, especially at the region from 294 to 420 nm, as a function of reaction time with a maximum absorbance after 24 h reaction (Fig. 1a). The color of glycated AF samples as well changed to yellow and yellow/brown over the reaction time (Fig. 1b). These changes indicate the formation of chromophores in the conjugate samples (Kim & Lee, 2009). The absorption value at these two wavelengths (294 and 420 nm) is ascribed to intermediate and final MRPs, respectively (Wu et al., 2014). Table 1 reports A_{294} and A_{420}, as well as, the free amino group content of AF samples. The content of intermediate MRPs increased as a function of

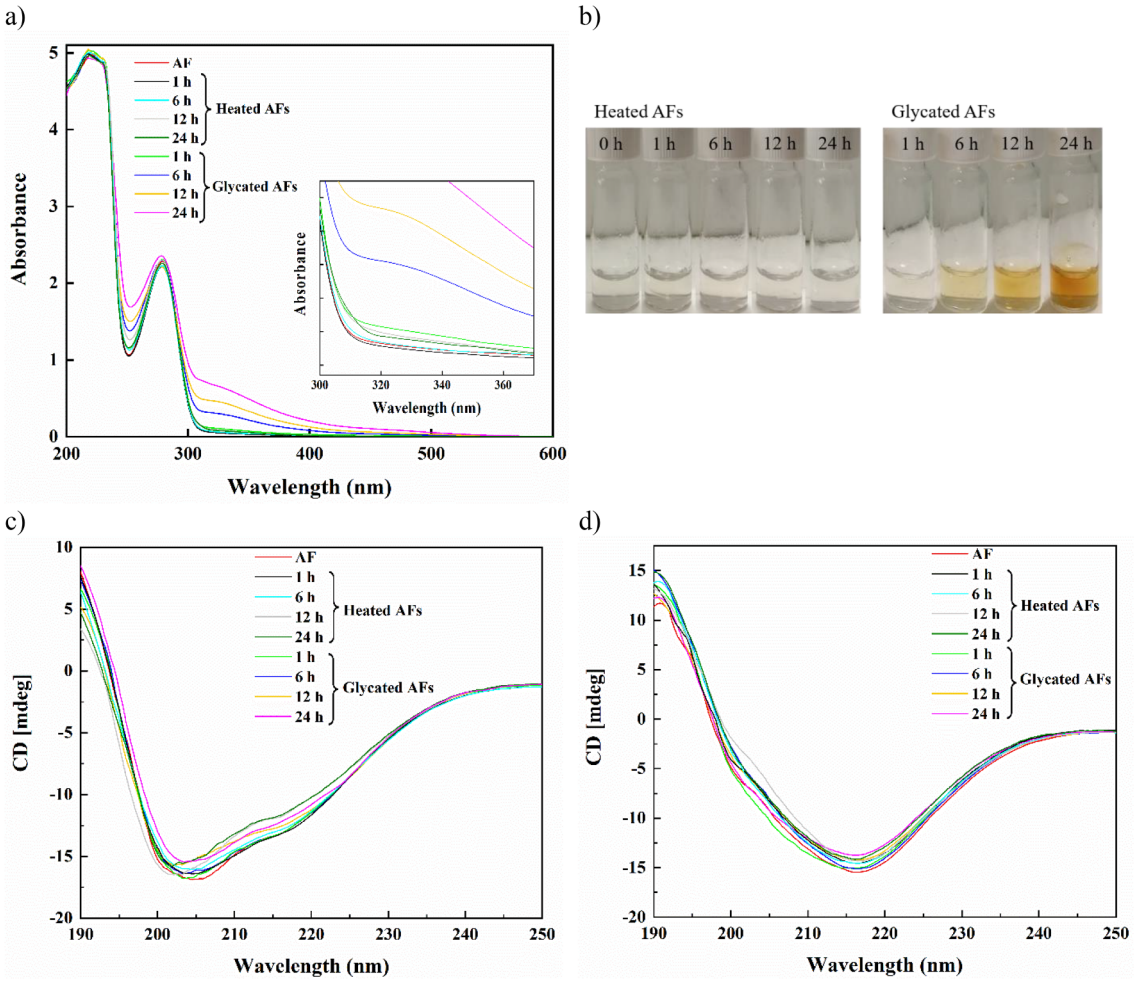

Fig. 1. β-Lactoglobulin amyloid fibrils (AFs) without (labeled as "Heated AFs") and with maltodextrin (labeled as "Glycated AFs") treated at pH 9.0 and at 90 °C for 0, 1, 6, 12 and 24 h. (a) UV–visible spectra of glycated AFs and heated AFs as a function of the heating time. Inset shows data with contracted y-axis and x-axis. (b) Aqueous dispersions of heated AFs and glycated AFs samples as a function of the heating time. (c) Far-UV circular dichroism before dialysis of glycated AFs and heated AFs as a function of the heating time. (d) Far-UV circular dichroism after dialysis of glycated AFs and heated AFs as a function of the heating time.

Table 1
Intermediate (A_{294}) and advanced (A_{420}) Maillard reaction products (MRPs) absorbance, free amino groups (FAGs), and glycation degree (GD) as a function of the heating time (t_{heat}) of β-lactoglobulin AFs without (heated AF) and with maltodextrin (glycated AF).

Sample	t_{heat} (h)	A_{294}	A_{420}	FAGs (g/L)	GD (%)
heated AF	0	1.0 ± 0.2^{b}	0.01 ± 0.01^{d}	1.71 ± 0.03^{a}	–
	1	1.0 ± 0.1^{b}	0.01 ± 0.01^{d}	1.70 ± 0.01^{a}	–
	6	1.0 ± 0.1^{b}	0.01 ± 0.01^{d}	1.70 ± 0.10^{a}	–
	12	1.0 ± 0.1^{b}	0.01 ± 0.01^{d}	1.70 ± 0.10^{a}	–
	24	1.0 ± 0.2^{b}	0.01 ± 0.01^{d}	1.64 ± 0.04^{ab}	–
glycated AF	1	1.1 ± 0.1^{b}	0.02 ± 0.01^{d}	1.50 ± 0.10^{bc}	11 ± 6
	6	1.2 ± 0.3^{b}	0.06 ± 0.01^{c}	1.50 ± 0.10^{cd}	15 ± 5
	12	1.2 ± 0.1^{b}	0.09 ± 0.03^{b}	1.42 ± 0.01^{cd}	17 ± 1
	24	1.6 ± 0.2^{a}	0.15 ± 0.02^{a}	1.40 ± 0.10^{d}	20 ± 7

heating time along with the increase of high molecular weight colored polymers, with maximum absorbance at 420 nm. Similarly, Jiang & Brodkorb (2012) found that the UV–vis absorption spectra of the α-lactalbumin- and β-lactoglobulin-ribose MRPs increased significantly with increasing heating time.

Such changes in the UV–visible absorbance indicate that maltodextrin was successfully conjugated with AFs, but the precise extent of the reaction can be evaluated by calculating the glycation degree (Jian, He, Sun, & Pang, 2016). Table. 1 reports the free amino group content and glycation degree of AFs in the presence and absence (control sample) of maltodextrin over a reaction period of 24 h. When AFs were heated alone, the content of free amino groups did not significantly change during the entire heating period ($p > 0.05$). On the contrary, in presence of maltodextrin, the glycation degree sharply increased (≈11%) over the first hour of reaction, followed by a slower increase upon further heating, which is ascribed to the decreased availability of reactive amino groups in the protein sequence (Jiang & Brodkorb, 2012). Reduced concentration of free amino groups showed that Maillard conjugation between an amino group from the protein and an aldehyde group from the polysaccharide moiety occurred (Bu et al., 2015). There are reports in agreement with our findings that the free amino group content of protein decreased upon conjugation with

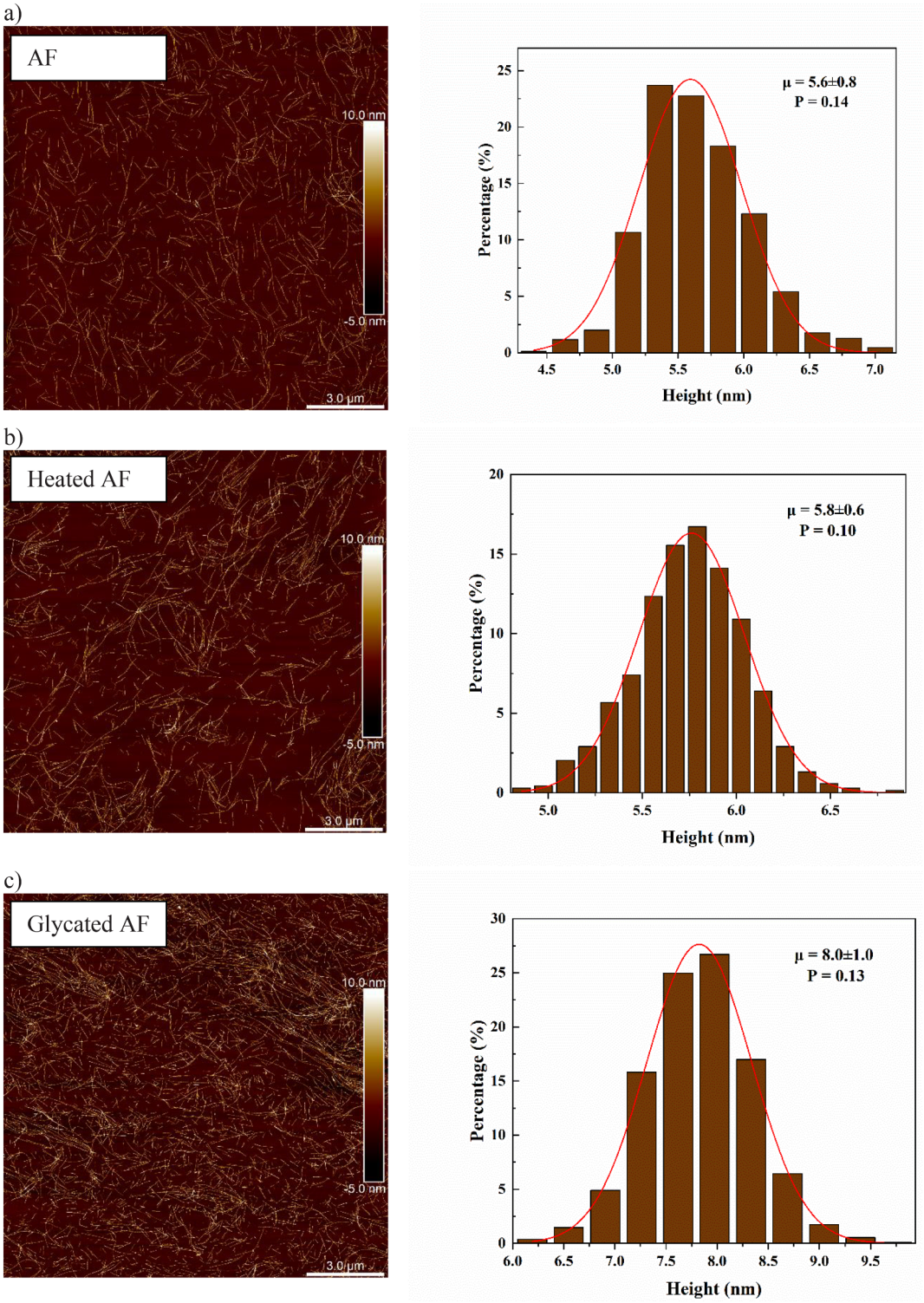

Fig. 2. β-Lactoglobulin heated AFs and glycated AFs samples at pH 9.0 and at 90 °C at different heating times. The AFM images at 0.2% w/w, and the corresponding average height distribution from the AFM images are shown in the left and right column, respectively. (a) AF, (b) 24 h heated AF, (c) 1 h glycated AF, (d) 6 h glycated AF, (e) 12 h glycated AF, and (f) 24 h glycated AF. P: Polydispersity index.

reducing sugars (Jian et al., 2016; Jiang & Brodkorb, 2012).

The far-UV CD spectrum arises from the peptide bonds absorption and yields valuable information about the secondary structure of proteins (Jian et al., 2016). Fig. 1c and 1d show the CD spectra of glycated AF and heated AF samples before and after dialysis, respectively. The CD spectra remained almost unchanged within all samples. Therefore, no changes occurred in the secondary structure of AFs upon the heating treatment and the Maillard conjugation, which is in agreement with the results of Kim, Choi, Shin, and Moon (2003) working on bovine serum albumin-galactomannan conjugates. Based on the study of Liu & Zhong (2013a), fibrillization after the Maillard conjugation of WPI with lactose, however, produced shorter nanofilaments with smaller magnitude of mean residue ellipticity than WPI fibrils. Fig. 1c shows a regular spectrum of β-lactoglobulin AFs, as also reported by Lara, Adamcik,

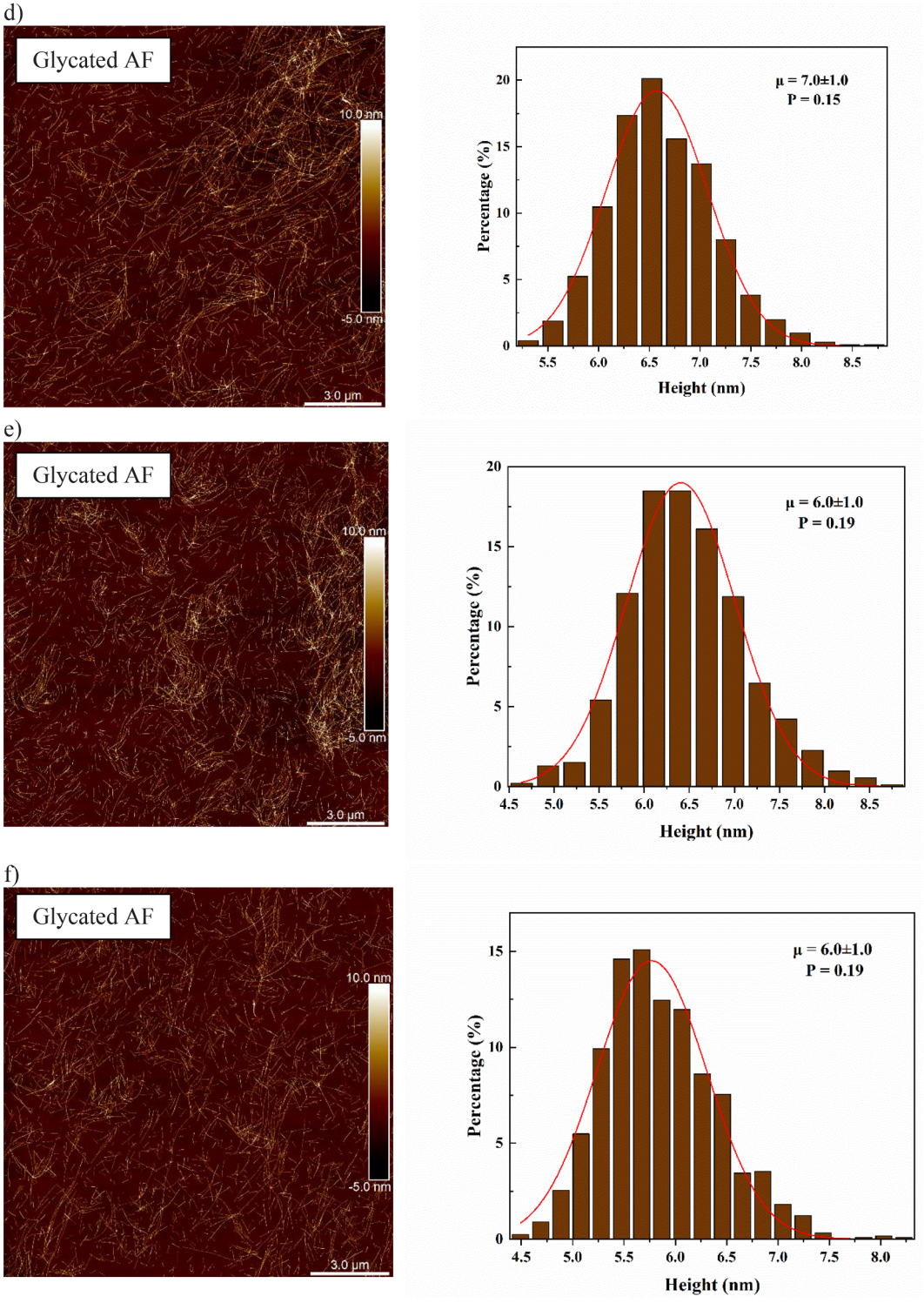

Fig. 2. (*continued*)

Jordens, and Mezzenga (2011). The shoulder at about 216 nm (Fig. 1c), presenting the characteristic β-sheet secondary structure as expected for amyloid aggregates, was more obvious after dialysis (Fig. 1d) in all samples, which is ascribed to the removal of unfibrillated short peptide fragments with random coil structure.

Structural morphology of AFs before and after glycation was characterized by AFM. Representative AFM images of AFs and measured height distributions of rigid AFs are shown in Fig. 2. Fibrils appear as rigid and semiflexible, long rods randomly oriented on the mica slide (Fig. 2, left column). The histogram of the fibrils displays a typical polydisperse distribution, as shown in Fig. 2 (right column). AFM imaging indicated that 1 h glycated AF had the greatest height (8.0 nm) compared to the other samples. The height of glycated AF samples decreased significantly as the heating time increased from 1 h towards 24 h for both rigid and flexible AFs (Fig. 3a), which is attributed to the hydrolysis of maltodextrin during the heat treatment in alkaline condition, as asserted by Tollens' test (Fig. SI-1). There are also other reports about the cleavage of glycosidic linkage in di- or oligosaccharides during glycation (Cämmerer, Jalyschko, & Kroh, 2002; Meydani, Vahedifar, Askari, & Madadlou, 2019). Therefore, the shorter the length

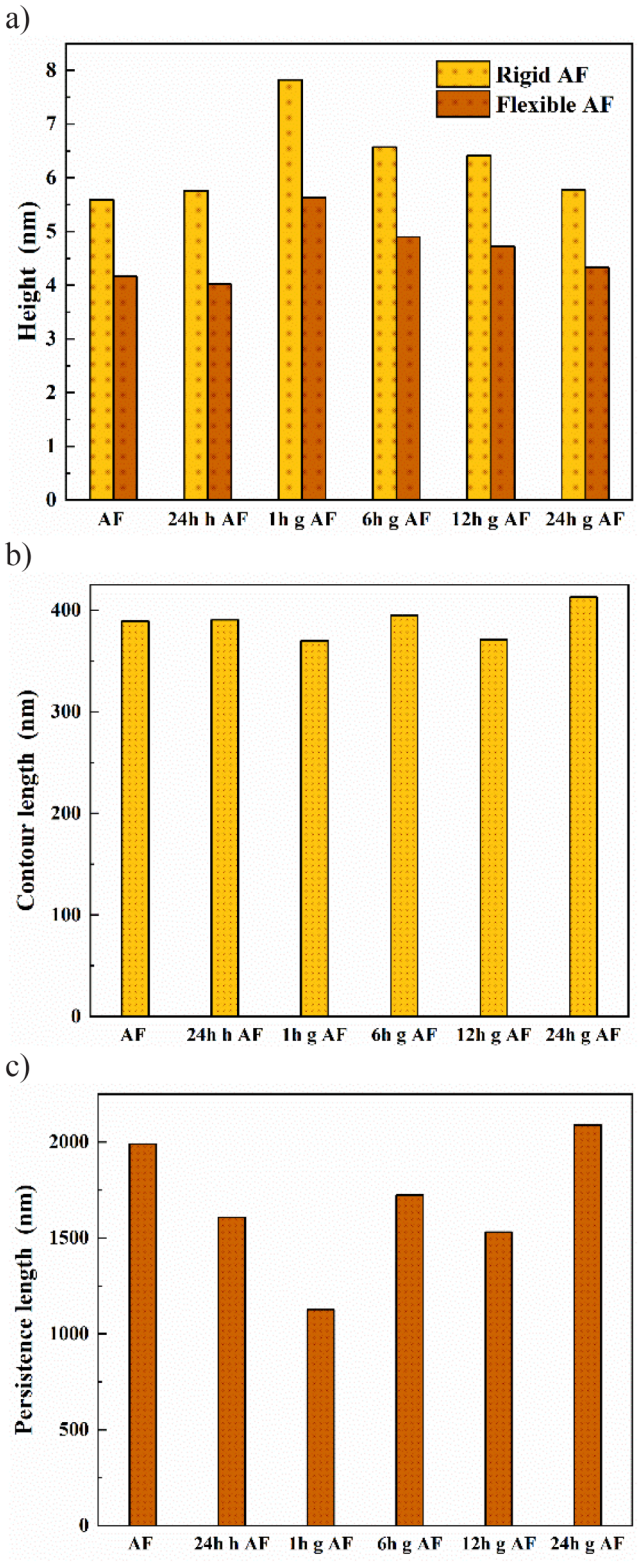

Fig. 3. β-Lactoglobulin heated AF and glycated AF samples at pH 9.0 and at 90 °C for different heating times. (a) Average height, (b) average contour length, and (c) average persistence length. *Note*: "h AF": heated AFs; "g AF": glycated AFs.

of attached polysaccharide, the lower the height of conjugated AFs (Fig. SI-2). Based on Fig. SI-2, it is expected that conjugation of a few polysaccharides to AF, after 1 h reaction, produces a structure similar to the supramolecular bottle-brush system, formed by ionic interactions and reported before (Rühs et al., 2011). Fig. 3b and 3c show that the incubation treatment and Maillard reaction did not remarkably change the fibril average contour length and persistence length of β-lactoglobulin AFs. Thus, contrarily to (Rühs et al., 2011), it is expected that the bottle-brush is with moderate to low grafting density, since high grafting density is usually associated to a reduction in AFs contour length (Rühs et al., 2011).

3.2. Turbidity and colloidal stability of dispersions

Images of dispersions adjusted to pH 2.0–7.0 before and after glycation are presented in Fig. 4I. All samples were transparent at pH 2.0, 3.0 and 7.0 and turbid at pH 5.0, as expected. The samples at pH 4.0 and 6.0 showed increasing and decreasing turbidity tendency, respectively, as the heating time increased. The samples were centrifuged for 10 min to investigate colloidal stability (Fig. 4II). As it can be observed, the extent of precipitation at pH 4.0 and 6.0 after centrifugation (Fig. 4II) indicates the same trend of turbidity, perceived before centrifugation (Fig. 4I). It could also be concluded that samples presented more aggregation at pH 4.0 than at pH 6.0, causing the amplified turbidity at pH 4.0 (Fig. 5a).

Colloidal properties of non-glycated and glycated AF samples, determined by attractive hydrophobic and repulsive electrostatic interactions, were further studied and the results shown in Fig. 5b. The aim of this experiment was to determine whether the glycated AF samples would remain intact or not, after changing the pH of the medium. This is important from a practical point of view, as it would define the range of industrial products, where these particles could be used (Karbasi et al., 2019). Wet Maillard reaction (i.e. performed in aqueous solution) does not commonly improve whey proteins solubility as it causes extensive denaturation and aggregation of proteins (O'Mahony, Drapala, Mulcahy, & Mulvihill, 2019). However, in the present study running the reaction at wet condition was not expected to result in improper effects on solubility because AFs are pre-denatured and obtained from a heating process in aqueous solution. Colloidal stability results (Fig. 5b) were complementary to those of turbidity (Fig. 5a). Glycated AF samples had higher stability against aggregation than the non-glycated counterparts at pH 4.0, demonstrating that maltodextrin decoration increased the hydration of AFs. This is in agreement with the steric repulsion theory of particles against aggregation, when solvent conditions permit the extension of polymer chains grafted onto their surface and increase excluded volume interactions (Israelachvili, 1992). Similarly, Karbasi et al. (2019) found the increased colloidal stability of one-hour whey protein microgel-maltodextrin conjugate at pH 4.0 compared to their conjugated counterparts heated for longer periods, unheated microgel and heated microgels (without maltodextrin). Prolonging the heating time for periods longer than 1 h caused a significant precipitation and reduced the subsequent colloidal stability of the conjugated AFs at pH 4.0, which is ascribed to hydrolysis of maltodextrin and to less steric hindrance caused by the polysaccharide chains (as evidenced by AFM results).

Samples had lower tendency to aggregate at pH 6.0 compared to pH 4.0 (Fig. 5b). Additionally, the glycated AFs had higher colloidal stability than maltodextrin-free heated and non-heated AFs at this pH 6.0. The longer the heating time of glycated AFs, the higher the colloidal stability of AFs at pH 6.0, which could be attributed to the larger number of maltodextrin chains grafted to AFs, heated for a longer time (Table 1), and to the shift of pI to lower pH values. Electrophoretic mobility profiles indicate the electrostatic interactions of samples at pH 2.0–7.0, and are presented in Fig. 5c. The pI of β-lactoglobulin nanofibril was estimated to be at pI ~ 5.15 (through interpolation of data points), which agrees with the reported pI value in the literature of 5.18 (Jones et al., 2011). After the Maillard conjugation, the isoelectric point shifted slightly to lower pH values and decreased as the heating time increased (Fig. 5c). This observation was previously described by other authors (Liu & Zhong, 2013b; Tang et al., 2011). The electrophoretic

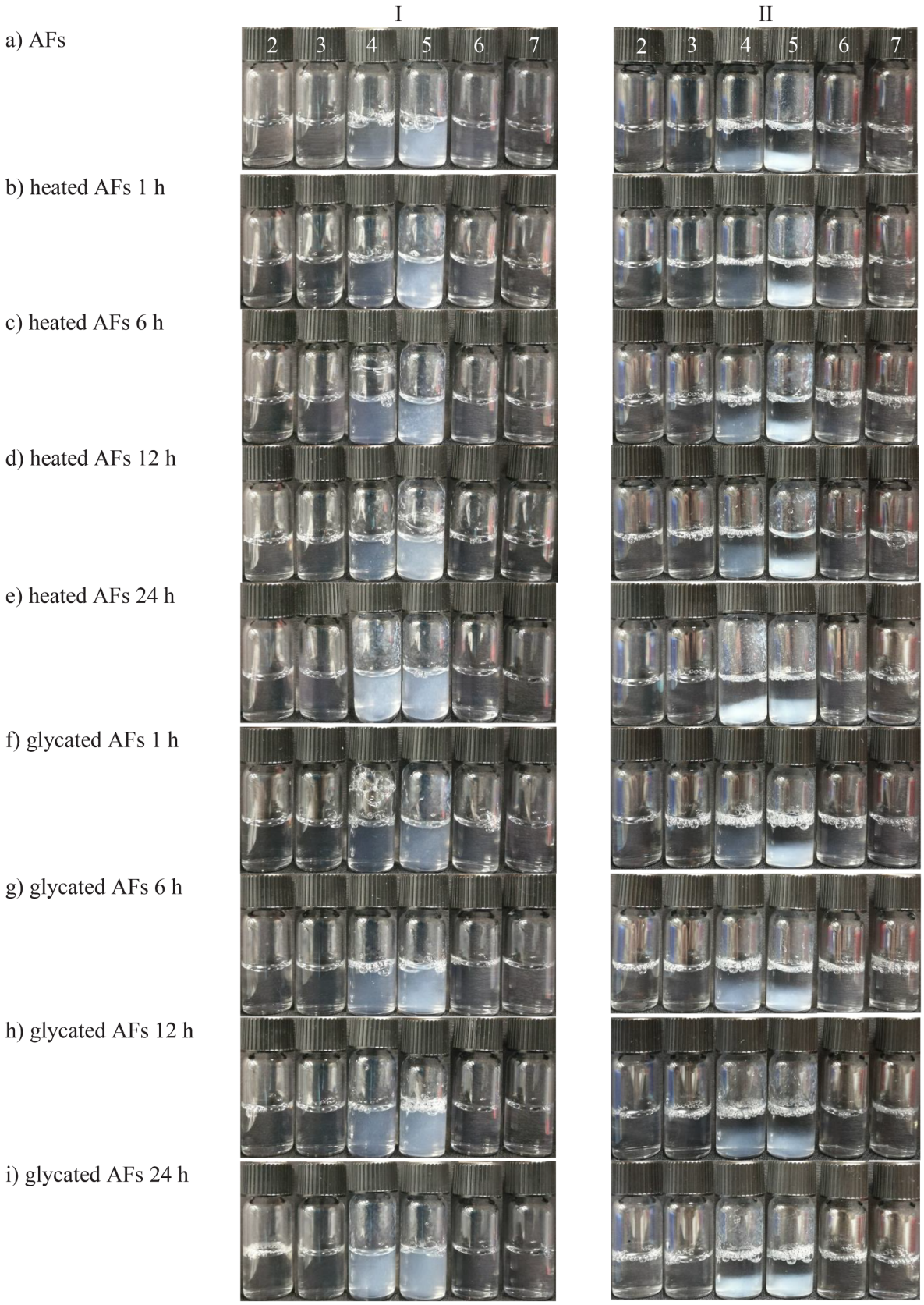

Fig. 4. Dispersions of β-lactoglobulin heated AFs and glycated AFs samples adjusted to pH 2.0–7.0 (from left to right) (I) before centrifugation, and (II) after centrifugation. (a) AFs, (b-e) heated AFs samples after 1, 6, 12 and 24 h. (f-i) glycated AFs samples after 1, 6, 12 and 24 h.

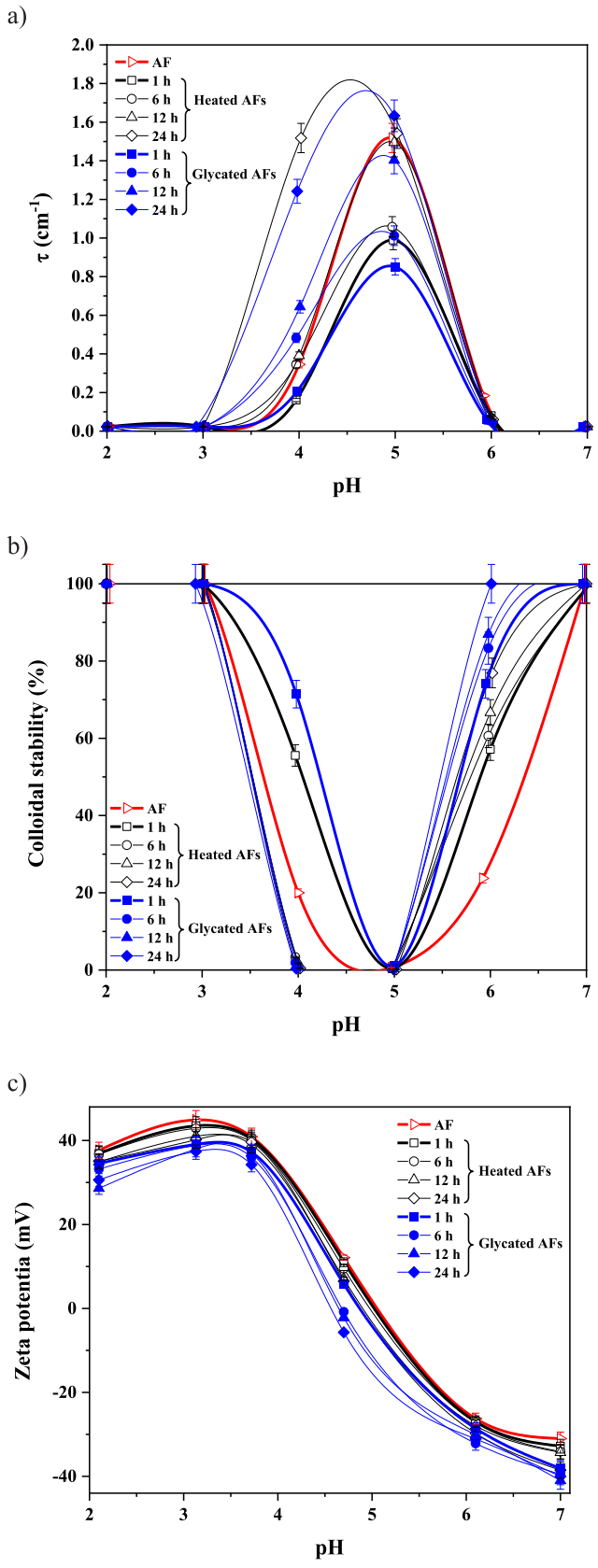

Fig. 5. β-Lactoglobulin heated AFs and glycated AFs samples adjusted to pH 2.0–7.0. (a) Turbidity measured at 500 nm, (b) colloidal stability, and (c) electrophoretic mobility.

mobility of glycated AF samples was lower than that of maltodextrin-free AFs, at pHs below pI. The amount of lysine (pK_a = 10.5), as the most participating amino acid in the Maillard reaction, is remarkably reduced after glycation. Therefore, the decreased pI, as well as, positive electrophoretic mobility at pHs below pI is likely due to the reduced amount of lysine residues (Liu & Zhong, 2013b). Recently, it was demonstrated that free amino groups consumption via acetylation decreases the pI of WPI (Madadlou, Floury, & Dupont, 2018). The utmost magnitude of positive electrophoretic mobility was observed at pH 3.0, attributed to the pK_a of carboxyl groups (i.e., C-terminal and aspartic and glutamic acid residues). Larger volume of HCl was used to reduce pH at pH values lower than pH 3.0, which causes increased ionic strength without significantly reducing the number of carboxylate groups. The increased ionic strength leads to the decreased electrophoretic mobility of fibrils at pH 2.0 compared to that of pH 3.0 (Liu & Zhong, 2013a).

4. Conclusions

β-Lactoglobulin amyloid fibrils are self-assembled semiflexible particles with a multitude of applications such as drug and nutraceutical delivery, texture modifiers and builders, biosensors, and biosorbents. Aggregation and precipitation of AFs at pH values from 4.0 to 6.0 close to the isoelectric point of the protein, however, restrict their applications. In our study we have investigated the glycation of AFs with maltodextrin through the Maillard reaction in aqueous conditions and the effect of the reaction time on the properties of the colloidal stability. The glycation extent of the AF-maltodextrin mixture rapidly increased during the first 1 h of reaction, while longer reaction times had smaller effects on the final glycation degree. Additionally, the amount of advanced glycation end-products from the Maillard reaction increased significantly as a function of conjugation time. Heat treatment and Maillard conjugation did not cause significant changes in the β-sheet content, contour length and rigidity of AFs, and these supramolecular bio-conjugates preserved their structures throughout the entire process. We found that 1 h glycated sample, showed the greatest cross section compared to maltodextrin-free AF, heated AF and other conjugates. For this sample the colloidal stability at pH 4.0 was significantly increased due to the surface-decorating polysaccharides steric repulsion. Such conjugate forms a pre-denaturized supramolecular bottle-brush system, modulating the colloidal stability of the protein-based objects and opening for a broader range of applications such as high-protein drinks, particularly in the presence of saccharides.

CRediT authorship contribution statement

Mehri Karbasi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Antoni Sánchez-Ferrer:** Conceptualization, Methodology, Validation, Formal analysis, Writing - review & editing, Supervision, Project administration. **Jozef Adamcik:** Investigation, Writing - review & editing. **Gholamreza Askari:** Writing - review & editing, Supervision. **Ashkan Madadlou:** Conceptualization, Writing - review & editing. **Raffaele Mezzenga:** Conceptualization, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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