Structural conformation and self-assembly process of p31-43 gliadin peptide in aqueous solution. Implications for celiac disease

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Celiac disease (CeD) is a highly prevalent chronic immune-mediated enteropathy developed in genetically predisposed individuals after ingestion of a group of wheat proteins (called gliadins and glutenins). The 13mer α-gliadin peptide, p31-43, induces proinflammatory responses, observed by in vitro assays and animal models, that may contribute to innate immune mechanisms of CeD pathogenesis. Since a cellular receptor for p31-43 has not been identified, this raises the question of whether this peptide could mediate different biological effects. In this work, we aimed to characterize the p31-43 secondary structure by different biophysical and in silico techniques. By dynamic light scattering and using an oligomer/fibril-sensitive fluorescent probe, we showed the presence of oligomers of this peptide in solution. Furthermore, atomic force microscopy analysis showed p31-43 oligomers with different height distribution. Also, peptide concentration had a very strong influence on peptide self-organization process. Oligomers gradually increased their size at lower concentration. Whereas, at higher ones, oligomers increased their complexity, forming branched structures. By CD, we observed that p31-43 self-organized in a polyproline II conformation in equilibrium with β-sheets-like structures, whose pH remained stable in the range of 3–8. In addition, these findings were supported by molecular dynamics simulation. The formation of p31-43 nanostructures with increased β-sheet structure may help to explain the molecular etiopathogenesis in the induction of proinflammatory effects and subsequent damage at the intestinal mucosa in CeD.

Introduction

Gliadins and glutenins are proteins present in wheat, that form a viscoelastic structure, called gluten, through noncovalent interactions and disulfide bond exchange in the presence of water [1]. This protein complex has been extensively studied due to its technological properties in food manufacture, and because its consumption triggers celiac disease (CeD) in genetically predisposed individuals [2]. CeD is a highly prevalent chronic immune-
mediated enteropathy which occurs in 1% of worldwide population, characterized by damage to small intestinal mucosa due to an exacerbated immune response by overactivation of gluten-specific T lymphocytes [3].

Gliadins are the ethanol-soluble fraction of gluten which can be separated by gel electrophoresis at acid pH in α/β-, γ-, and ω-gliadins [4]. The primary structure of gliadins has several repetitive sequences rich in proline and glutamine residues. These proteins are resistant to degradation by gastrointestinal enzymes, and consequently, several long peptides may remain in the gut lumen [5]. Different mechanisms have been suggested for their translocation into the mucosa of the small intestine, where they exhibit immunogenic properties through binding to HLA class II molecules becoming T-cell epitopes [6,7]. Among wheat proteins, peptides from α-gliadins are the most studied. The peptide 56-87 from α-gliadin fraction, called 33mer, has been extensively studied as model of T-cell epitope because it induces a strong adaptive immune response in CeD patients [8], whereas the α-gliadin peptide p31-43 induces a non-HLA-mediated inflammatory response as well as several toxic effects evaluated by ex vivo and in vitro assays [9–12]. Using an experimental model developed by our group, the in vivo biological effects of p31-43 have been recently studied [13,14]. We observed that wild-type mice treated with p31-43 presented histological alterations and increased number of intraepithelial lymphocytes, production of proinflammatory mediators and cell death. We also demonstrated that biological effects depend on the activation of the NLRP3 inflammasome [14]. In the other hand, Villella et al. [15] showed that p31-43 is capable of inhibiting the cystic fibrosis transmembrane conductance regulator (CFTR) and reduces ATPase activity of the nucleotide-binding domain-1 (NBD1) impairing its function. This generates epithelial stress, tissue transglutaminase and inflammasome activation, NF-κB nuclear translocation, and IL-15 production. Considering that the activation of the inflammasome platform has been linked to the detection of nanostructures, fibrils, and particulate material and damage signals [16,17], we hypothesize that p31-43 self-assembling properties may be critical to its biological effects.

Though biological effects triggered by p31-43 (LGQQQFPFPQQPY) are observed very rapid upon treatment, the surface cell receptor or the mechanism of peptide entry has not been identified [18]. In our previous study, we showed by CD and transmission electron microscopy (TEM) that p31-43 presents a polyproline II (PPII) structure and forms oligomers which may promote the activation of the inflammasome platform [14]. To gain structural details on p31-43, several spectroscopic and microscopic techniques were used, as well as coarse-grained (CG) dynamic simulation in order to understand at a molecular level, peptide interactions, and driven forces that leads to p31-43 self-assembly.

Results

Characterization of p31-43 oligomers by spectroscopic and atomic force microscopy techniques

In a previous report, we showed by TEM that p31-43 self-aggregates presenting a PPII structure, in agreement with CD determinations [14]. Subsequent NMR experiments performed by Calvanese et al. [19] confirmed that this peptide is able to adopt this structure. To further evaluate the self-assembly propensity of p31-43 and the characteristics of the oligomers [14], we decided to use a variety of biophysical tools. Firstly, we used a fluorescent probe containing a boron dipyrromethane scaffold (BODIPY) [20]. The molecule used in this work is able to sense the presence of particular hydrophobic patches that can be generated by the presence of oligomeric structures. This probe was used to evaluate the self-aggregation of the β-amyloid peptide, detecting oligomers prior to the formation of fibrils which could not be detected by other probes [21]. When this probe binds to a specific site, an increment of its fluorescence intensity is detected at approximately 540 nm when sample is excited at 525 nm. Thus, to analyze if p31-43 can self-assemble in solution, we measured fluorescence emission spectra at different peptide concentrations (10, 50, and 100 µM) in presence of 1 µM BODIPY. A concentration-dependent increase in the fluorescent signal was observed, suggesting that, at higher concentration, more binding sites were available. These results support the existence of p31-43 soluble oligomers in the range from 10 to 100 µM (Fig. 1A).

Furthermore, dynamic light scattering (DLS) was used to detect structures in solution. In this study, we evaluated p31-43 at 125 µM. This concentration was chosen because samples at lower concentrations lack of detectable dispersion. The solution presented high polydispersity, suggesting the presence of different oligomer populations in the sample. Two main populations were observed, one of 107 ± 7.5 nm of hydrodynamic diameter and big aggregates with an average diameter of 493 ± 31 nm (Fig. 1B). These findings prompted us to propose that, at this concentration, p31-43 oligomers are in equilibrium with larger nanostructures.

In our previous study, by using CD and TEM, we demonstrated that p31-43 presents a PPII structure...
and forms oligomers which may promote the activation of the inflammasome platform [14]. Herein, we explored the self-assembly process of p31-43 by atomic force microscopy (AFM) as complementary technique, which allows a deeper evaluation of the morphology of the oligomers because of its higher resolution achieved in a hydrated environment. This tool provides a topographic image of the sample [22], providing a visualization of p31-43 self-assembly with minimum interference [23–25]. This methodology was previously used to understand the self-association process of different proteins and peptides such as β-amyloid [26] and 33-mer gliadin peptide [27] and protein such as α-synuclein [28], the silk protein [29], elastomeric ones as resilin [30], and it has been widely used to characterize protein complexes, oligomers, and fibers [31]. In this case, 5, 50, and 100 µM p31-43 solutions were prepared to cover the same range of concentrations used in previous in vivo assays (6.5 and 65 µM). The samples were freshly prepared to avoid a time dependence effect [32].

At 5 µM final concentration, p31-43 self-assembled as isolated spherical oligomers randomly distributed on the mica surface with different heights (Fig. 2A). When a height distribution and statistical analysis of the whole sample deposited in mica was performed, we found that most of the structures were of 2.6 ± 2.08 nm height average (Fig. 3A), with a minor proportion of oligomers having higher values. The high standard deviation indicated the presence of different oligomers, as it was detected by cross section analysis of the aggregates (indicated with white arrows in Fig. 2A,B). Oligomers presented different heights that were divided into three groups: 1.2; 2.25; and 3.25 nm, suggesting that these species may be in equilibrium among each other. When the 50 µM p31-43 solution was analyzed, we observed diverse oligomer populations (Fig. 2C,D), indicating that different species are in equilibrium. Most oligomeric structures presented a mean height of 5 ± 3 nm (Fig. 3B). In this case, we observed three kinds of round shaped species with a height around 2.4, 4, and 10 nm. Interestingly, higher structures of 20–60 nm were detected. This suggests that some precipitation of soluble species may have been occurred. Finally, we analyzed a 100 µM solution were clusters of small height, as well as amorphous aggregates with approximately lengths of 250 nm were observed (Fig. 2E), having widths of 100–140 nm and heights of 5 nm (Fig. 2F). Considering that the height of these aggregates was in the range of the oligomers observed in the 50 µM sample, we propose that they were produced by lateral association of oligomers.

Gliadin p31-43 peptide presents a polyproline II conformation in equilibrium with extended structures

We aimed to evaluate whether the structure of p31-43 was affected by different physiological pHs that resemble the ones of the gastrointestinal tract. This is a relevant issue because despite the absence of ionizable side chains in the p31-43 sequence, the C-terminal carboxylates are generally expected to be protonated at pH values near 3.3. To this end, we assessed the secondary structure by CD in 20 mM phosphate buffer at pHs: 3.0, 5.0, 7.4, 8.0 at 50 µM final peptide concentration.
in Milli Q water (Fig. 4). In our previous work, we demonstrated that p31-43 presents a PPII structure at 4 °C [14] which was also observed for other gliadin peptides such as the 33-mer gliadin peptide [33]. As this conformation is always in equilibrium with other structures and it has been shown that PPII is more populated at low temperatures [34–36], we selected 4 °C as the one to study pH dependence on the peptide conformation. Herein, we confirm that p31-43 presents a PPII structure which remains stable at different pHs.

Aimed to get further details on the self-organization process of p31-43, we reanalyzed the conformations of the oligomers obtained performing CG simulations [14]. As shown in Fig. 5A, p31-43 peptides undergo spontaneous oligomerization in aqueous solution, as evidenced by a decrease in the radius of gyration from 10 nm, that is, half the maximum distance between peptides in the initial configuration of the simulation to 3.1 nm that corresponds to the final radius of the 50-mer. Once a compact state is reached, the N- and C-terminal moieties remain close to each other forming intra- and intermolecular salt bridges that optimize electrostatic interactions among different peptides (inset in Fig. 5A). The formation of salt bridges contributes to decrease the pKa of the C termini. Indeed, calculating the pKa of all carboxylates along the simulation for the former oligomer shows a clear shift to lower pH than expected for a single carboxylate in solution (Fig. 5B), explaining the absence of pH dependence observed in the CD experiments.

Next, we studied the peptide structure in a wider range of concentrations from 10 to 500 μM. We observed a characteristic PPII structure signal [34] determined by a negative band near 203 nm and a positive band at 225 nm, at 4 °C throughout all concentrations (Fig. 6A). Interestingly, when the p31-43 peptide concentration is increased, a hypochromic displacement of the negative band occurs, that is characteristic of a self-assembly process. In line with this, the
secondary structure content calculated along dynamic simulations suggested that the aggregation process induced a small increase in PPII conformation in two central prolines, as well as, in \( \beta \)-sheet conformation spread along the nonproline amino acids (Fig. 6C,D). A secondary structure analysis per amino acid of the monomeric and oligomeric states identified a structured segment located in the central portion of the peptide, which could account for the phenomenon observed in CD experiment. As the simulation progressed and the oligomer was formed, an increment of 28% of the PPII conformation was observed in residues Pro8 and Pro9, while more external amino acids experienced an increase in the \( \beta \) extended conformation stabilized by the formation of \( \beta \)-sheets among other peptides in the oligomer.

To study the effect of temperature over p31–43 structure, we performed CD experiments at four different temperatures increasing from 4 to 37 °C (Fig. 6B). We observed that the negative band had a hypochromic behavior with a displacement from 203 to 205 nm and the positive band at 225 nm reduced its signal. These features along with the presence of an isodichroic point at 210 nm indicate that the PPII structure was observed in...
is in equilibrium with extended structures such as β-turns and β-sheet, due to the close proximity of the dihedral angles in the conformational landscape [34,35]. To confirm this behavior, we used the BestSel server [37] which analyses CD spectra and gives an overall idea of the percentage of each structure in the whole peptide population at different conditions. The analysis of the 4 °C spectra by this server indicated that around 60% of the signal corresponded to structures as PPII, 18% are turns and 22% are anti-β strand. The same analysis for the 37 °C spectra showed that the PPII-like structures decreased to a 56% and the anti-β strand increase up to 26%. Moreover, as CD spectra could be considered the result of a combination of different structures, the mathematical spectrum subtraction of a protein at different conditions is an adequate tool to evaluate conformational transitions [38]. To confirm these findings, we subtracted the spectra between 37 and 4 °C and observed a negative band at 220 nm and a positive one at 200 nm, indicating that at physiological temperature, the p31-43 peptide has an increase of the β-sheet like conformation (Fig. 6E). At lower temperatures, the peptide had a PPII structure, whereas at 37 °C there was an increment of the β-sheet like one component. However, the PPII structure remained as the most representative.

The PPII conformation is stable in the presence of chaotropic agents such as urea and guanidinium chloride [39]. To evaluate whether these agents were able to modify the p31-43 structure, 50 and 200 µM p31-43 solutions were dissolved in presence of 8M urea. At both concentrations, a positive band was observed at 225 nm that confirms the PPII structure of p31-43 (Fig. 6C).

On the other hand, to confirm that the species were in conformational equilibrium, a 200 µM p31-43 solution was incubated in the presence of trifluoroethanol (TFE) (Fig. 6D). This cosolvent is used to favor intramolecular bonds, such as the ones present in α-helix and β-sheets [40]. A hypochromic displacement of the negative band from −11 000 to −6000 was observed when TFE concentration was increased from 0% to 50% together with a small red shift from 203 to 205 nm. Interestingly, the positive band at 225 nm decreased when TFE was added, also an isodichroic point a 210 nm was observed, suggesting that species may alternate between PPII conformation and extended structure. At TFE 50%, the positive band at 195nm and the red shift at 208nm suggested that p31-43 was in equilibrium with β-sheet like structures. Also, when the spectra of TFE 0% and 50% were analyzed using BestSel server, we detected a decrease of PPII-like structures from 60% to
50%, and an increase of the antiparallel β-structure from 23% to 29%. This result was also confirmed by the mathematical subtraction of CD spectra between 50% and 0% TFE, which showed a characteristic β-sheet like structure (Fig. 6F). However, these findings suggest that p31-43 has mainly a PPII structure in

![Graphs showing CD spectra](image-url)
equilibrium with β-sheet like structures, which depends on its self-assembly and the polarity of the environment.

**Self-assembly process of p31-43 depends on peptide concentration**

Protein oligomerization and aggregation is a highly time- and temperature-dependent process, among other factors. Therefore, we run a series of assays to study p31-43 self-assembling in a period of 7 days. For that, p31-43 solutions were incubated at 4 °C, to diminish the ongoing aggregation reaction and preserve the first stages of the peptide self-assembly as it was done for polyglutamine proteins [41]. In this study, we evaluated the morphological change of p31-43 oligomerization by AFM. A similar methodology was previously performed to evaluate the self-assembly process of proteins as the serum amyloid A1.1 [42], fibrils from the bacterial protein MinE [43], and the hen egg lysozyme [44]. We evaluated two peptide concentrations, 10 and 50 µM. At concentrations higher than 100 µM, a full coverage of the surface was observed; therefore, it was not possible to evaluate the system by AFM.

Analysis of a freshly prepared 10 µM sample deposited on mica surface showed three populations of oligomers. The smaller ones had an average height of 2.61 ± 0.54 nm, composed by 3–4 monomers, the intermediate ones of 4.71 ± 0.86 nm height with 5–6 monomers per oligomer, and higher ones of 6.259 ± 0.71 nm height composed by 8–10 monomers. The higher and the smaller ones were in minor proportion as shown in Figs 7A and 8A. It is worth to mention that the oligomers detected at 10 µM were bigger than the ones observed at 5 µM, thus confirming that the self-organization process depends on peptide concentration.

![AFM images](image_url)

**Fig. 7.** Time-dependent morphological changes of p31-43 gliadin peptide at 10 µM. AFM images were obtained in tapping mode (5 x 5 µm²) by depositing the samples on the mica surface and drying by a nitrogen flux. The solutions were prepared in Milli Q water and incubated at 4 °C at different time periods: (A) freshly prepared sample (0 days), (B) 4 days, and (C) 7 days. Below each AFM image, it is presented the cross section of the structures detected. In blue (a) the highest, in red (b) the middle, and in green (c) the smallest structures detected. The sample at each condition was analyzed by duplicate.
Next, the sample was incubated for 4 days at 4 °C and deposited on the mica surface. At this condition, peptide oligomers self-associated generating larger isolated clusters with an average height of 5.56 ± 0.96 nm. These values indicate that the initial oligomers observed in Fig. 7A, associated to form plane-cluster-like structures (Figs 7B and 8B). These wider planar clusters (with approximately 32 monomers) may be generated by self-association of the smaller ones presented in Fig. 8C. This finding allowed us to hypothesize a colloidal behavior of p31-43 peptide.

Spherical oligomers were detected after a continuous incubation of 7 days. However, there were no planar-cluster assemblies (Fig. 7C). These spherical oligomers are higher than the planar clusters and had an average height of 22.2 ± 8.2 nm. This indicates that the oligomers observed in Fig. 7B might be produced by rearrangement of wider structures, producing higher stable spherical structures which have less surface exposure, as it was previously observed in other systems [45,46].

When the self-organization process was analyzed in 50 µM peptide solutions, the structures observed differed from the ones detected at 10 µM, confirming that this process is dependent on peptide concentration. In the freshly prepared sample, small and higher spherical oligomers were detected (Figs 9A and 10A). The smaller oligomeric structures average height was 4.1 ± 1.6 nm (approximately 6–8 monomers per oligomer) whereas the bigger ones were 18 ± 6.3 nm (10–14 monomers per oligomer). Also, some structures had heights of 50 nm, similar to Fig. 2. These could have been produced by some precipitation that might occur during the deposition. This was observed in all three samples prepared at different times.

After a 4-day incubation of the sample at 4 °C, the spherical oligomers arranged as a chain-like fractal network. Interestingly, in this structure, little individual oligomers were detected, since most of the peptide is forming these new linear structures. The smaller oligomers had an average of 8.07 ± 4.13 nm and the bigger ones were approximately 21.47 ± 5.47 nm height (12–20 monomers per oligomer) (Fig. 9B). For this structure, the fractal dimension (D_f) which described the self-similarity of the system was calculated, using the box counting method and we obtained a D_f of 1.4. This characteristic self-organization ability of p31-43 was also observed for the silicatein protein [47] and the 33-mer gliadin peptide [27,48].

After incubating the sample at 4 °C for 7 days, the linear chain network described above continued evolving forming longer linear structures like fractals with an average height of 14.53 ± 6.71 nm. Interestingly, no individual oligomers were distinguished in this structure, suggesting that they may have interacted with each other to form the small linear structures detected. However, some large clusters with a height of 40.33 ± 6.5 nm could be observed, which might have been generated by some precipitation (Fig. 9C). When D_f was calculated, a value of 1.7 was obtained, suggesting that, at this time, the structures were formed by a diffusion-limited aggregation (DLA) mechanism. By this theory, the peptide is described as a colloidal particle in which every particle collision leads to an irreversible association and formation of a fractal structure, which has a typical D_f value of 1.7–1.8 [29,47,49].

**Fig. 8.** Characterization of the structures observed by AFM of the 10 µM solution of p31-43. In (A), it is presented in the size of the structures showed in Fig. 6A (freshly prepared solution, n = 100), (B) correspond to Fig. 5B (4 days incubated solution, n = 25). (C) In this image, it is shown by arrows the self-association of the different structures.
Celiac disease is a common gastrointestinal disease triggered by dietary gluten peptides. Though the specific immune response against a group of immunogenic peptides is well established, the role of gliadin peptides in the induction of inflammatory responses is less understood [12,13]. The p31-43 gliadin peptide, generated by digestion of gastrointestinal and brush border enzymes, is the most used model peptide to evaluate the innate immune response in CeD [9]. Using an experimental model in mice, we have shown that p31-43 induces local inflammatory responses and increased cell death in small intestine, which requires MyD88 and type I IFN pathways [13]. Further in vivo assays showed that the innate response depends on the NLRP3 inflammasome [14], and its capacity to sense signals derived from cell damage produced by nanostructures or by direct interaction with these species [16,17,50]. By CD and TEM, we showed that p31-43 is able to form oligomers in the multinanometer scale [14]. Since a cell surface receptor for p31-43 has not been identified [18], we hypothesize that structural features of p31-43 may be of key importance in the induction of different biological effects.

Fluorescence spectroscopy and DLS allowed assessing whether the self-organization process occurs in solution. A fluorescent probe called BODIPY, which was previously employed to detect oligomers of β42 amyloid peptide, was used [21]. The fluorescence signal of this probe in presence of p31-43 suggests that oligomers with hydrophobic patches are presented in solution. The oligomer size increased at higher peptide concentrations as it was detected in the spectra. In addition, DLS measurements confirmed that higher ordered and stable structures were present in solution.
This result is in concordance with our CD studies performed at a wide range of concentrations which showed a self-assembly behavior (Fig. 6A). This process was also previously described in elastomeric proteins such as lamprim [51], elastin II fragments [38,52], and the 33-mer gliadin peptide [27,33,48].

The AFM analysis confirmed that p31-43 oligomerizes and the size of the oligomers and the structures observed are dependent on peptide concentration. Results obtained at 5 and 50 µM showed spherical nanostructures with different heights that were in equilibrium with each other. However, at 50 µM some bigger oligomers and precipitation were detected. We hypothesize that, at these concentrations, p31-43 behaves as a colloid nanostructure where each spherical oligomer tends to interact with each other as it was observed in terminal fragments of serum amyloid A [42]. At 100 µM, the peptide generates clusters and amorphous aggregates in solution that may be deposited on the mica. These structures have an average height similar to the oligomers at 50 µM (Fig. 2), suggesting that p31-43 self-assembles to generate these structures. This behavior was also observed for the β-amyloid peptide [53] which is typical for colloidal structures at high concentration [49]. It is worth to mention that these structures are in concordance with our previous observations by TEM confirming that p31-43 self-assembles into oligomers and clusters.

CD analysis showed that p31-43 presented PPII secondary structure, which was also recently confirmed by NMR experiments [19]. Since this conformation lacks of intrachain hydrogen bonds and is fully hydrated in aqueous solution [54], PPII helix has the appropriate characteristics to be in equilibrium with other conformations. In fact, evaluation of the peptide at 200 µM solution at different temperatures showed a conformational transition from PPII to β-sheet like structures as it was inferred by both the use of BestSel and difference spectra, and supported by molecular simulations. The treatment of 200 and 50 µM solutions with urea returns p31-43 to a PPII conformation while TFE, a cosolvent that favors hydrogen bonds, produced 50% of a β-sheet like signal (Fig. 6B–D). These observations strongly suggest that the peptide is in equilibrium with extended and folded structures at physiological temperatures. Similar behavior was observed in other proteins and peptides such as the amyloidogenic exon-30 of tropoelastin [35]. The same self-association propensity was confirmed at the molecular level by the CG molecular dynamics simulations (MD) of a monomer in a box containing 50 replicas.

When the p31-43 was studied at different pHs by CD and MD, no structural transitions were observed, indicating that its structure it is not affected by changes in pH in the range of 3–8. Analysis of the oligomeric state obtained by simulations indicated that the formation of salt bridges between terminal zwitterionic moieties is determinant for pH stability, shifting C-terminal values to acidic conditions in almost one pKa unit. These findings suggest that the aggregation process might contribute to the resistance of properties of p31-43 would not be affected by changes in pH along the gastrointestinal tract and in the small intestinal mucosa.

To get further insight into the self-assembly behavior of p31-43, we performed an AFM evaluation, assessment already used to understand protein aggregation, as it was done for the polyalanine expansion of PHOX2B aggregation [55]. We observed that p31-43 self-assembly process showed different behavior depending on the peptide concentration as described for the β-amyloid peptide [56] and the N-terminal...
serum amyloid A peptides [57]. We hypothesize that the peptide behaves as a colloidal nanoparticle that could self-associate depending on its concentration. At the lower concentration, the small colloidal particles, following a self-association process, produce planar clusters that then reorganize into spherical bigger oligomers. Since small oligomers co-exist with bigger ones and bigger structures are detected over time, we propose that this process is driven by an Ostwald ripening like process [58,59].

At 50µM p31-43 self-organize differently, the peptide structure changes from spherical oligomers to a chain network accompanied by some individual ones. After 7 days, this behavior progresses to large branched structures with a fractal morphology and a Df ~ 1.7 suggesting a DLA mechanism. This self-organization behavior was previously detected in 33-mer gliadin peptide [27,33,48] and whey proteins [60,61]. Peptide concentration could strongly influence the type of interactions established among monomers leading to differences in the self-assembly behavior [49]. Based on these findings, a scheme of self-assembly process is proposed (Fig. 11).

The driven forces of the self-organization of p31-43 may be explained by its high content of glutamines. In polyglutamine sequences, the formation of oligomers and higher ordered structures is a consequence of the hydrophobic effect and specific hydrogen bonds generated among monomers [62]. In the case of p31-43, an uncharged peptide, both intermolecular forces might be the key drivers of the self-assembly characteristic [63]. However, the formation of oligomers does not preclude that p31-43 may act also as monomer. Recent work by Villela et al. [15] showed that monomeric p31-43 binds to the NBD1 subunit of the chloride channel (CFTR) and inhibit its function. As consequence, autophagy is altered, and NFκB and inflammasome pathways are activated, producing the release of inflammatory mediators.

**Conclusion**

In conclusion, considering that the surface cell receptor or the mechanism of peptide entry has not been identified, and recently in silico analysis suggested that p31-43 does not bind to HLA class II molecules [19], our findings open a new perspective to understand the biological effects of this peptide in the induction of inflammatory and cell death pathways. As a monomer, it can mediate selective effects as, the recently shown, inhibition of the subunit of the chloride channel. Oligomers, in turn, may play a role in the multiple and fast non-HLA-mediated mechanisms, like altering vesicular trafficking and cellular stress [12], and our own work on the activation of NLRP3 inflammasome [14]. Altogether, these findings help to understand the innate immune response elicited as supplementary events in triggering CeD.

**Materials and methods**

**Sample preparation**

The p31-43 peptide was purchased from GeneCust (Schengen, Luxembourg). The peptide was dissolved in Milli Q water generating a mother solution of 600 µM which was stored at −20 °C. From this mother solution, the working samples were prepared before the measurements and stored on ice. Meanwhile, experiments were carried out, the samples were kept on ice to avoid effects of temperature on the peptide conformation.

The kinetic assay was performed by preparing peptide dilutions from the stock solution to the desired concentration (10 and 50 µM) and incubating them at 4 °C for 0 (fresh solution), 4, 7, and 14 days in a cold room, on ice, before deposition on the mica surface.

For reproducibility, three different peptide batches were used for the experiments presented in this work. The concentration-dependent experiments solutions at 10, 50, 200, and 500 µM in Milli Q water were prepared.

For the CD experiments in Urea 8 m, the peptide mother solution was directly dissolved in an adequate urea concentration solution to achieve the final urea concentration.
required. The sample was incubated 3 h to reach the equilibrium. The TFE titration experiment was performed on cuvette, and the concentration of the peptide was recalculated in each titration step the sample was incubated 10 min before data acquisition. The kinetic assay was performed by preparing peptide solutions from the mother one to the desired concentration and incubating it at 4 °C at 0 (fresh solution), 4, 7, and 14 days in a cold room and ice before the deposition on the mica surface.

Fluorescence spectroscopy

The emission spectra of p31-43 in presence of BODIPY probe was performed in a JASCO FP 8300. In this case, Milli Q water solution of the peptide at the concentration 10, 50, and 100 μM were freshly prepared and BODIPY probe was added at 1 μM final concentration. Also, we used water as control. Then, the emission spectrum of each sample was obtained by exciting the samples at 525 nm wavelength and the slits used were 5nm for the excitation and emission.

Dynamic light scattering

Samples of p31-43 gliadin peptide were dissolved on water and filtered with a 200 nm filter to eliminate big oligomers/aggregates and dust. Then, the measurements were performed by triplicate using a Zetasizer (Nano-ZS) from Malvern Instruments (Malvern Panalytical, Malvern, Worcestershire, UK) and the analysis of the autocorrelation functions obtained was performed by DISPERSION TECHNOLOGY SOFTWARE from Malvern.

Atomic force microscopy

All images to characterize the peptide self-assembly were obtained in Tapping mode AFM withMultiMode Scanning Probe Microscope (Nanoscope V; Bruker, Santa Barbara, CA, USA) equipped with a Nanoscope V controller (Veeco, Santa Barbara, CA, USA), using probes doped with silicon nitride (RTESP; Veeco with tip nominal radius of 8 –12 nm, 271e311 kHz, force constant 20–80 N m−1). Typical rate scanners were 1 Hz.

Water aqueous solutions of the peptides at concentrations from 10, 50, and 100 μM were deposited on freshly cleaved mica and left to interact with the surface for five minutes at room temperature. Then, the samples were dried by a Nitrogen flow. The height of the observed architectures was determined by cross section using Nanoscope Analysis 1.40 software (Bruker).

Circular Dichroism

CD spectra of peptide solutions at were recorded on a Jasco J-810 CD spectropolarimeter using a Peltier system as temperature controller. For this experiment, five scans were acquired in the range of 190–250 nm at a selected temperature (from 4 to 37 °C with an incubation of 10 min for each measurement). The scanning speed of 50 nm per minute was used. The spectra were obtained employing a 1- and 0.1-mm quartz cuvettes. Blank scans were subtracted from the spectra. The data were expressed as the mean residue molar ellipticity in deg cm2 dmol−1. Smooth noise reduction was applied eventually when it was necessary using a binominal method. Graphics were represented using the program Origin (OriginLab, Northampton, MA, USA).

Molecular simulations

MDs were performed at the CG level using the SIRAH force field [14,64] at 37 °C and 1 atm with no added salt to mimic the conditions of circular dichroism experiments (see above). Oligomerization was simulated by introducing 50 replicas of the isolated, zwitterionic, peptide in different conformations randomly selected along a MD trajectory of an isolated peptide. The simulation of the oligomer was performed for 5 μs. To ensure no aggregation bias in the initial spatial distribution, isolated peptides were placed in random conformations within a computational box leaving at least 4 nm between each replica. Secondary structure and atomistic structures from CG conformers were calculated using SirahTools [65]. Secondary structure contents per amino acid were averaged over the last 100 ns of the trajectory. pKa values on C-terminal moieties were calculated with PropKa [66] using the pdb2pqr server [67] on 10 conformers taken from the last 100 ns of simulation.

Fractal dimension

Fractal Dimension is a measure of the complexity of a system. It is a scaling rule that is used to compare how pattern details change when the scale varied from minor to major resolution. Mathematically, it is expressed as

$$N \propto \varepsilon^{-D_f}$$

where $D_f$ is the fractal dimension, $N$ is the number of pieces observed in each new resolution, and $\varepsilon$ is the scale used for each pieces.

The FRACLAC plugin from IMAGEJ (Charles Sturt University, Albury, Australia) was employed for the determination of $D_f$ using the box counting procedure. The basic procedure is to lay systematically a series of grids of decreasing caliber (the boxes) over an image and record data (the counting) for each successive caliber. The program gives access to $D_f$ obtained by the application of several mathematical algorithms.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

MFGC and MGH performed spectroscopic experiments. EP performed AFM. SP and EB performed molecular dynamic simulation. FC, MGH, and MFGC designed the experiments. VI, MGH, and MFGC analyzed the data. MFGC, MGH, SP, VI, and FC wrote the manuscript.

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