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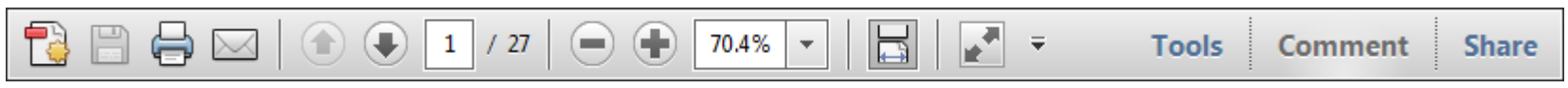

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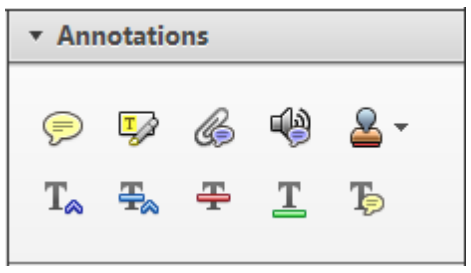

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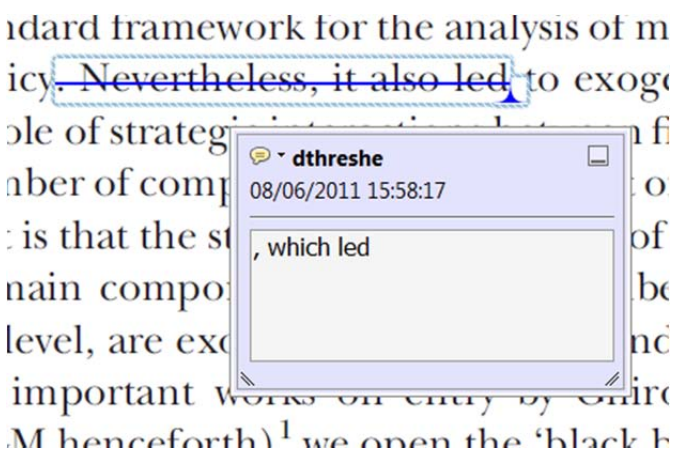

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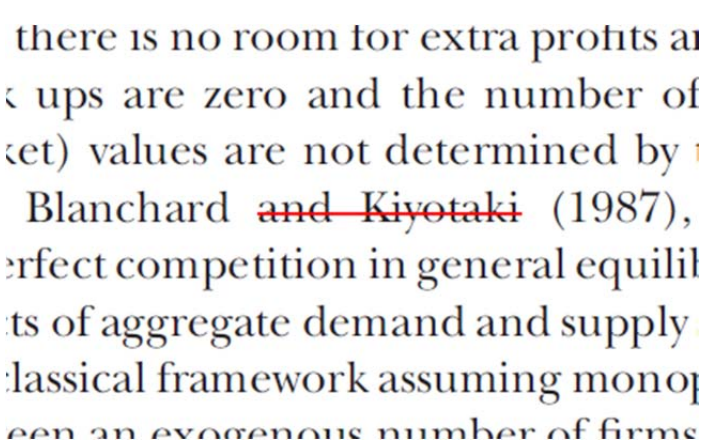

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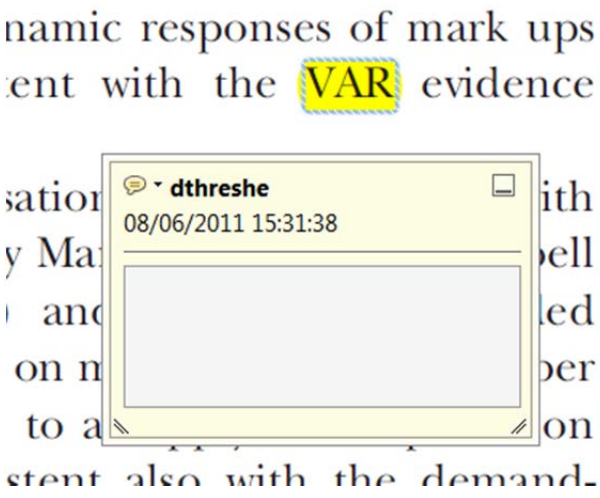

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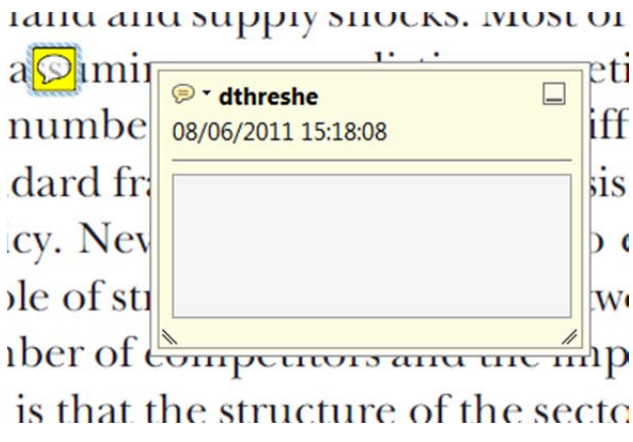

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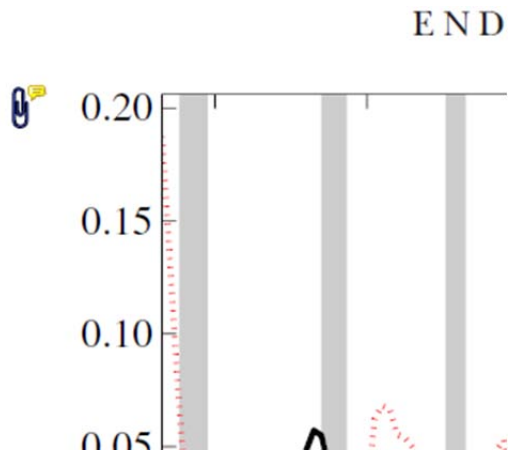

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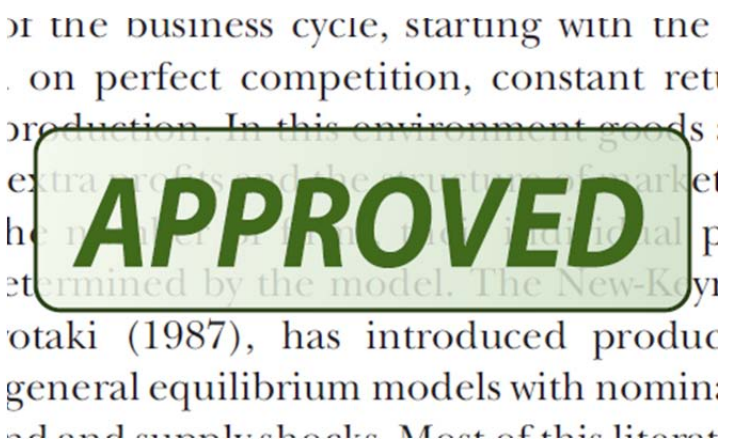

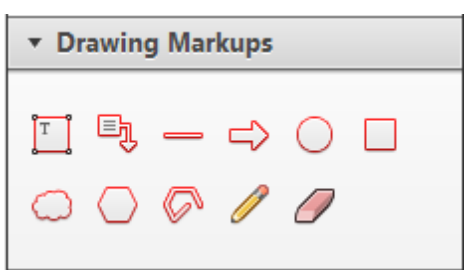

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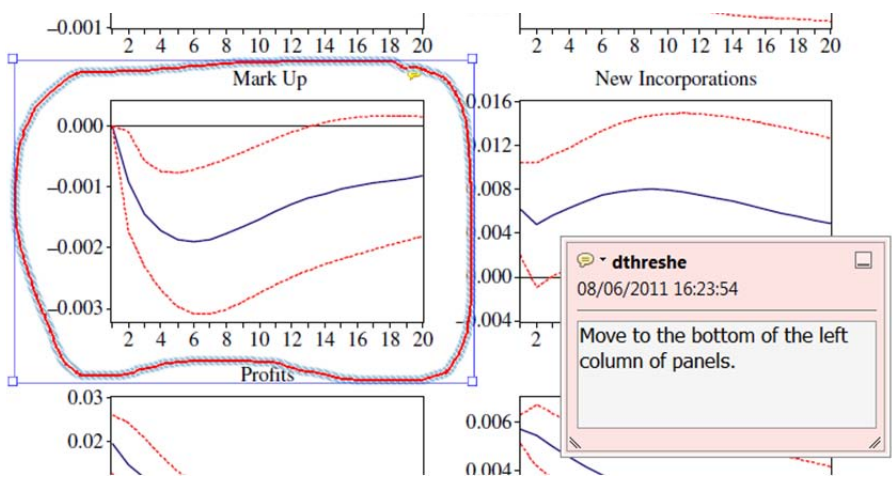

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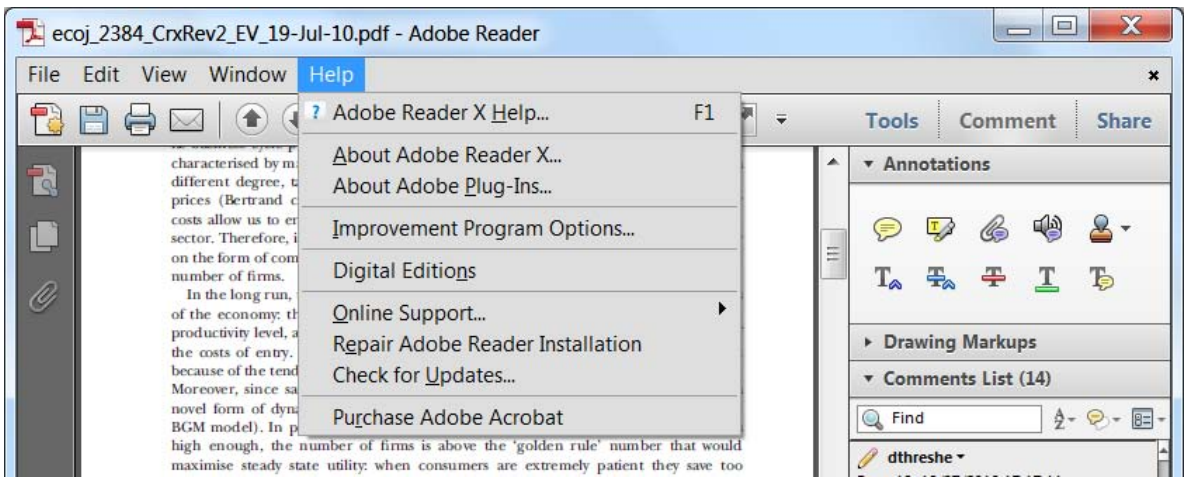

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Circular Dichroism and Electron Microscopy Studies In Vitro of 33-mer Gliadin Peptide Revealed Secondary Structure Transition and Supramolecular Organization

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ABSTRACT:

Gliadin, a protein present in wheat, rye, and barley, undergoes incomplete enzymatic degradation during digestion, producing an immunogenic 33-mer peptide, LQLQPF(PQPQLPY)$_3$PQPQPF. The special features of 33-mer that provoke a break in its tolerance leading to gliadin sensitivity and celiac disease remains elusive. Herein, it is reported that 33-mer gliadin peptide was not only able to fold into polyproline II secondary structure but also depending on concentration resulted in conformational transition and self-assembly under aqueous condition, pH 7.0. A 33-mer dimer is presented as one initial possible step in the self-assembling process obtained by partial electrostatics charge distribution calculation and molecular dynamics. In addition, electron microscopy experiments revealed supramolecular organization of 33-mer into colloidal nanospheres. In the presence of 1 mM sodium citrate, 1 mM sodium borate, 1 mM sodium phosphate buffer, 15 mM NaCl, the nanospheres were stabilized, whereas in water, a linear organization and formation of fibrils were observed. It is hypothesized that the self-assembling process could be the result of the combination of hydrophobic effect, intramolecular hydrogen bonding, and electrostatic complementarity due to 33-mer's high content of proline and glutamine amino acids and its calculated nonionic amphiphilic character. Although, performed in vitro, these experiments have revealed new features of the 33-mer gliadin peptide that could represent an important and unprecedented event in the early stage of 33-mer interaction with the gut mucosa prior to onset of inflammation. Moreover, these findings may open new perspectives for the understanding and treatment of gliadin intolerance disorders. © 2013 Wiley Periodicals, Inc. Biopolymers 00: 000–000, 2013.

Keywords: *33-mer gliadin peptide; circular dichroism; electron microscopy; supramolecular organization; gliadin intolerance*

Additional Supporting Information may be found in the online version of this article.

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INTRODUCTION

From biomolecules to complex biological systems, nature reveals to us as a complex and sophisticated machinery governed by recognition and supramolecular organization characterized by an exquisite spatial and temporal control. In this context, proteins carry out numerous functions playing a crucial role in the growth, repair, and metabolism of cells.[1] In the postgenomic[2] era, genetic encoding of proteins and linear sequencing of amino acids are well-established areas of understanding. However, the mechanism by which these chains of amino acids become preprogrammed to fold into their correct protein structure remains one of the mysteries of life.[3] There is a fine balance between the processes of protein folding that leads to proper biological function and misfolding, which may result in cellular pathology, such as Alzheimer's and Parkinson's[4] diseases and more recently with respect to cancer.[5] Critical to our understanding are the noncovalent interactions involved in the folding processes such as electrostatic attractions, van der Waals attractions, hydrogen bonds, and hydrophobic interactions. In addition to folding events, further understanding of molecular recognition can be attained because the same noncovalent interactions that enable a protein to fold into a specific conformation also allow proteins to bind to each other to produce larger structures such as enzyme complexes, ribosomes, protein filaments, viruses, and membranes.[6]

Inspired by the importance of protein folding in the balance between health and disease, we embarked on a project to shed light on the early stages of celiac disease, a complex immunological disorder with a prevalence of 1% among the healthy population triggered by the protein gliadin.[7] Gliadin is a protein present in wheat, rye, and barley, and the only treatment of this pathology is a life-long gliadin-free diet.[7] Early evidence for gliadin was shown by Khosla and coworkers[8,9] who studied in vitro gliadin and brush border membranes from rats and humans and demonstrated that a proteolysis-resistant peptide of 33 amino acids (33-mer) remained intact after 15 h of incubation. This unusual proteolytic resistance of 33-mer, LQLQPF(PQPQLPY)$_3$PQPQPF, was confirmed in vivo by a perfusion protocol in intact adult rats.[8] Structurally, the 33-mer gliadin fragment contains six partially overlapping copies of three distinct DQ2-restricted T-cell epitopes and is highly stimulatory toward T lymphocytes.[8] The immunological response in susceptible individuals associated with gliadin is well known; however, the initial stages before inflammation are still obscure.[10,11] It has been hypothesized that increased intestinal permeability is an early event in celiac disease pathogenesis; however, it is completely unknown what endows gliadin, especially such 33-mer fragment features as resistance to proteolysis, passage across membranes, and activity as stress triggers to the epithelium.[12]

To shed light on the molecular features of 33-mer gliadin peptide, we present circular dichroism (CD) spectra of 33-mer in aqueous solution at pH 7.0 and electron microscopy (EM) experiments in combination with partial charge electrostatics distribution (PCED) calculation and molecular dynamics (MD) simulation.

RESULTS AND DISCUSSION

CD Studies of 33-mer Peptide Show a Conformational Transition and Self-Assembling Capabilities

CD experiments were carried out because it is a very sensitive technique to monitor changes of protein secondary structures.[13,14] A single CD experiment of 33-mer gliadin peptide has been reported[8] showing a polyproline II (PPII) conformation, but unfortunately, the authors did not state the concentration of the peptide solution. However, concentration is one of the important parameters in the study of protein secondary structure by CD.[13–17] In addition, simple inspection of 33-mer sequence showed that ~65% of the amino acids are proline and glutamine. It is known that fragments with high percentage content of proline and/or glutamine are able to self-associate, which are governed mainly by hydrophobic effect.[18–20] Moreover, glutamine can also interact strongly in water presumably via complementary hydrogen bonding interaction in addition to the aforementioned hydrophobic effect.[21,22]

Therefore, we decided to pursue an initial concentration-dependent experiment using as medium a previously reported buffer[8] as well as water (pH 7.0) in the concentration range from 10 to 613 μM at 5°C. Similar results were obtained under both conditions (water experiments are presented in the Supporting Information Figure S1). CD spectra showed marked dependence on peptide concentration above 46 μM, detecting a red-shift displacement of the negative band from 203 to 215 nm and hypochromic behavior of the molar ellipticity of the band from −18.000 to around −2000 deg cm^2 dmol^{-1} on increasing peptide concentration from 46 to 613 μM (Figures 1A and 1B). The observed trend is characteristic of a conformational transition from an unordered or extended structure F1

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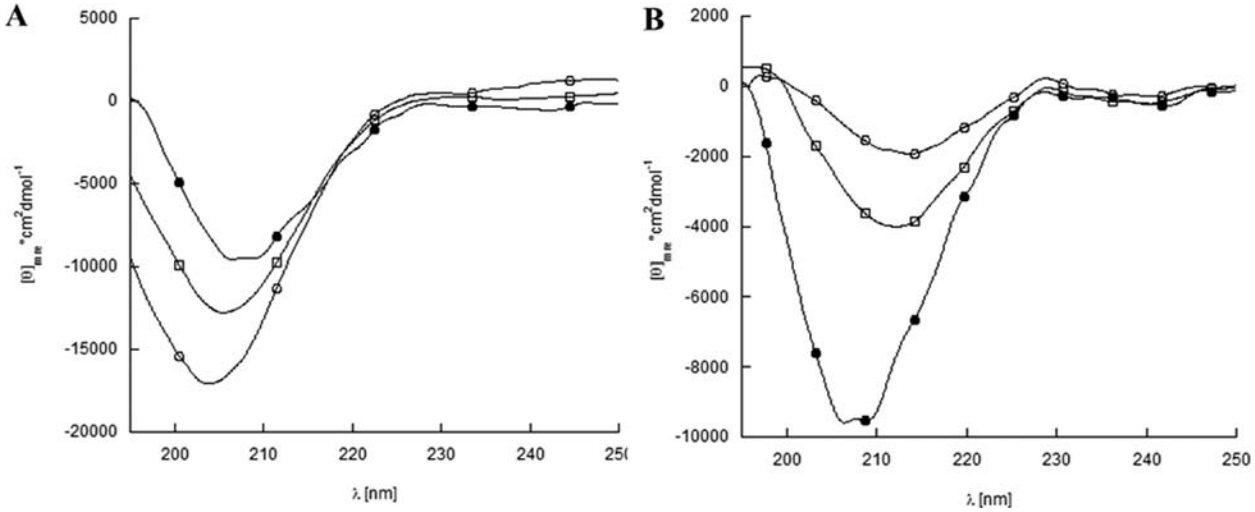

FIGURE 1 CD spectra of 33-mer in 1 m*M* sodium citrate, 1 m*M* sodium borate, 1 m*M* sodium phosphate buffer, 15 m*M* NaCl, pH 7.0 at 5°C. Concentration-dependent experiment: (A) 46 (○), 122 (□), and 197 μM (●) and (B) 197 (●), 418 (□), and 613 μM (○).

to a more folded one due to self-assembly.[23–28] The process was completely reversible because the same band shifts were observed for both concentration and dilution experiments. At 5°C, the negative band in the range of 203–215 nm (46 and 613 μ*M*, respectively) can be initially associated to a π–π^* transition of a highly populated PPII structure in equilibrium with other conformations (Figures 1A and 1B).[8,15,17,27] From 197 to 613 μM, a new small positive band at 228 nm was observed only at low temperature (Figures 2A and 2B). The 228 nm band could be associated to the n–π^* transition of PPII structure.[17] It has been reported that PPII conformation is always in equilibrium with other structures like β-turns, β-strands, and unordered conformations due to the close proximity of the respective dihedral angle values.[15] Conformational equilibrium can be evaluated by temperature-dependent experiments. At 197 μM (Figure 2A), increasing the temperature from −10 to 37°C showed one isodichroic point at 211 nm. This trend is indicative of a conformational equilibrium where PPII was the dominant conformer population in equilibrium with more folded structures. PPII conformation was maximum at the lowest temperature (−10°C), as evidenced by the strong negative band at 206 nm and the positive band at 228 nm,[17] whereas increasing the temperature to 37°C led to a small red shift of the negative band to 209 nm and the loss of the positive

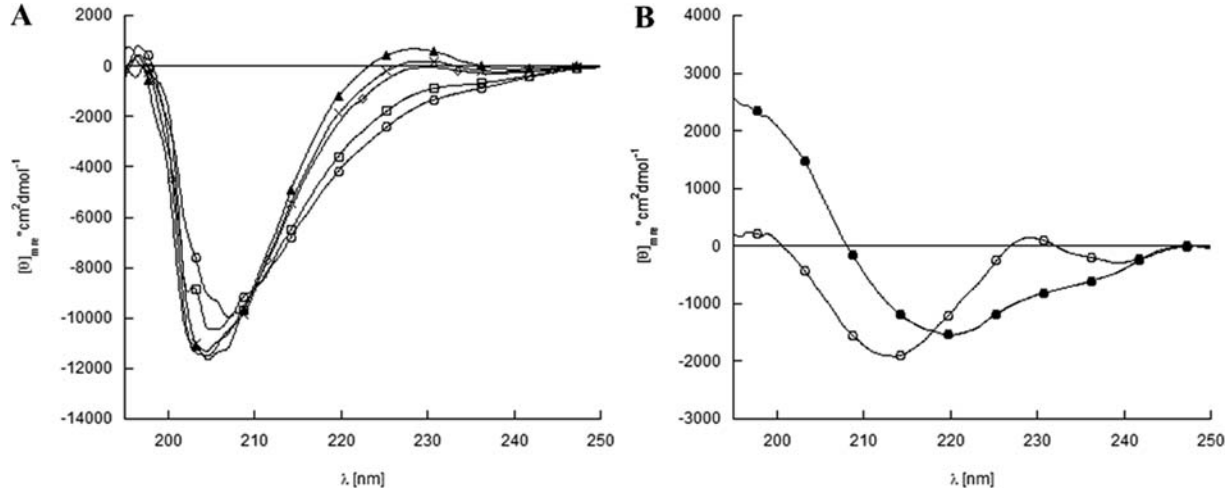

FIGURE 2 CD spectra of 33-mer in 1 m*M* sodium citrate, 1 m*M* sodium borate, 1 m*M* sodium phosphate buffer, 15 m*M* NaCl, pH 7.0. Temperature-dependent experiment at (A) 197 μM: 37 (○), 25 (□), 5 (¤), 0 (X), and −10°C (▲) and (B) 613 μM: 5 (○) and 37°C (●).

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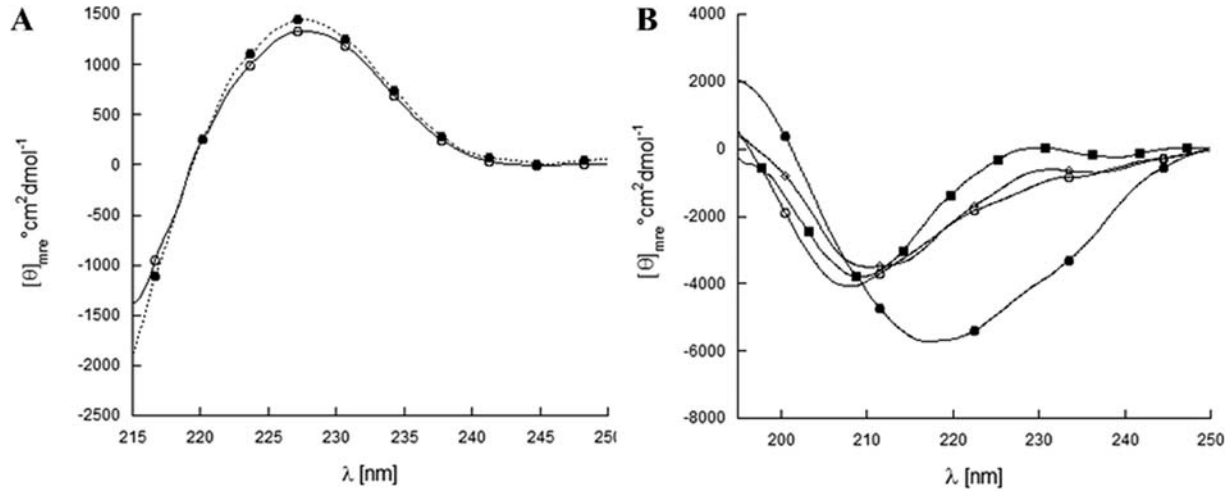

FIGURE 3 Conformational equilibrium modulation of (A) 33-mer peptide in a water solution of Gdn.HCl (8*M*) at 197 μM (○) and 613 μM (●) peptide concentration, 25°C. (B) Addition of TFE to 33-mer peptide solution at 418 μM, 5°C: 0 (■), 5 (¤), 20 (○), and 46 (●) of TFE.

band at 228 nm (Figure 2A). A previous evidence showing that Pro-Xaa model peptides are able to form turns[29,30] and the obtained CD spectra led us to hypothesize a Type II β-turn structure.[28,31,32]

At 613 μM, an equilibrium between two conformations was observed in the temperature variation experiment (Figure 2B). Meanwhile at 5°C, the CD signature was characteristic of PPII conformation, and at 37°C, the spectrum revealed a red-shifted negative band centered at 220 nm and a strong positive band at 195 nm, which are CD signatures of β-structure. Furthermore, the most important spectral finding is the onset of a strong positive band below 200 nm, indicating a dominant folded conformation at 37°C. PPII conformation unlike other secondary structures does not depend on intramolecular hydrogen bonding.[17] Thus, to confirm PPII conformation at 197 and 613 μM, we decided to dissolve 33-mer in a chaotropic agent such as guanidinium chloride (8*M*). At both peptide concentrations, CD spectra showed a similar increase of the
F3 positive band at 228 nm (Figure 3A). The decrease of the magnitude of the bands on increasing temperature and the responses of the CD of the sample to guanidinium chloride treatment support the hypothesis of the existence of PPII secondary structure, as previously reported.[8]

With the aim to reproduce the red shift observed at high concentration, a well-known structure induced cosolvent
AQ4 (TFE)[33,34] was added to a 33-mer solution at 418 μM. The choice of TFE is reasonable considering that this solvent favors intramolecular hydrogen bonding, promoting folded conformations such as helices and β-turns.[35] As it is presented in Figure 3B, the use of 46% TFE as cosolvent afforded reproduction not only of the red shift of the negative band from 210 to 220 nm and the strong positive band at 195 nm but also a strong negative band at 230 nm appeared, probably due to the arrangement of the Tyr side chains accompanying the increase in β-turn conformation in TFE-rich solvents.[32]

Similar spectral changes were obtained increasing the temperature or decreasing the dielectric constant of the solvent at high concentration. These trends confirm 33-mer capability of conformational transition from an extended PPII to a more folded structure of Type II β-turn depending on environmental conditions.[36,37] Both types of regular secondary structure could be stabilized by interactions with target molecules in an induced fit.[1] β-Turns are certainly involved in protein recognition processes, for example, as sites of protein phosphorylation and glycosylation[38] and in fiber structures of short prion peptides and amyloid fiber aggregates.[27,39] On the other hand, there are many more precedents for the involvement of PPII in protein–protein interactions. PPII helices are recognized by Class II major histocompatibility complexes[40] and two widespread protein domains: SH3[41] and WW.[42] Our CD data showed that the conformation equilibrium was shifted toward more ordered structures by probably a self-aggregation process.[20,27,28] This structural transition could represent an AQ5 unprecedented molecular trigger in gliadin intolerance disorders.

Partial Electrostatic Charge Distribution and MD Calculation of 33-mer Revealed Nonionic Amphiphilic Behavior Allowing Dimer Formation Due To Electrostatic Complementary

The PCED calculation is a well-known computational tool used in biophysical studies[43] given that electrostatics

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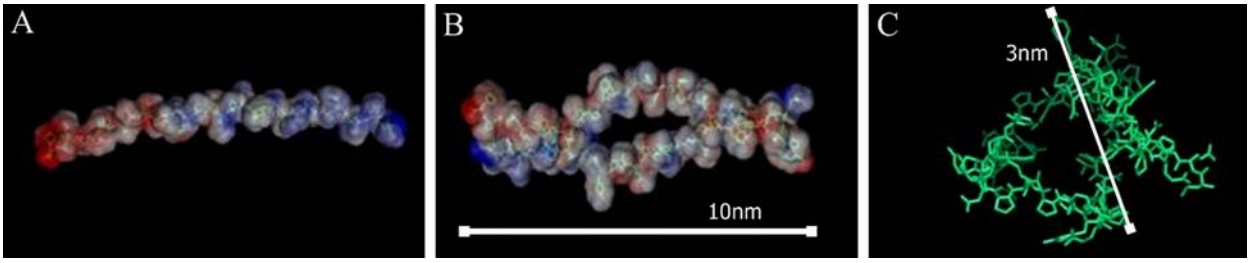

FIGURE 4 (A) Electrostatic profile of the PPII peptide model after molecular dynamics simulation. Partial charges were assigned with PARSE field force implemented in PDB2PQR software (see Materials and Methods section). Color represents only the positive (blue) or negative (red) partial charge distribution. (B) Model of a plausible dimer of the 33-mer showing electrostatic complementarity after molecular dynamics simulations. (C) Tilted view of the 33-mer dimer.

interactions influence or even dominate initial biochemical reactions. PCED and MD calculations of 33-mer fragment were performed to visualize the molecular nature of 33-mer self-assembling process. By this procedure, the 33-mer construct revealed a regular pattern of partial charge distribution along the peptide backbone, showing an amphiphilic nature even though in the absence of ionic amino acids (Figure 4A). Consistent with 33-mer high PPII propensity and previously established nuclear magnetic resonance and CD results of the short repeated fragment,[44] PQPQLPY, we propose a peptide model obtained by energy minimization and MD (Figure 4A). Based on the stability of the resulting conformation and partial charge distribution, it was possible to construct a dimer. The final model was structurally inspected and shown to maintain a high degree of PPII structure following MD simulation, which is in agreement with our CD results (Figures 4B and 4C).

PPII secondary structure is not only a protein motif important for protein–protein interaction[45] but is also implicated in structural stability, for example, the collagen triplex helix.[46] In several other cases, such as amelogenin,[23] lamprin,[24] and tropoelastin,[47] PPII leads to well-defined molecular recognition processes with formation of supramolecular assemblies, such as fibrils and nanospheres. In this context, it has been reported that α-gliadin protein[48] and some wheat gluten peptides[49] containing the 33-mer sequence are able to form fibrils in water under certain conditions. Based on our results, a first supramolecular evaluation of 33-mer is needed.

Supramolecular Organization of 33-mer Detected by Transmission Electron Microscopy and Scanning Electron Microscopy

Transmission electron microscopic (TEM) image of a negatively stained preparation of 33-mer solution provided sufficient structural details for general characterization. Colloidal nanospheres of around 35 and 66 nm of diameter and amorphous aggregates were detected (Figure 5A). Statistical evaluation of the nanospheres showed two families, one around 35 nm and the second around 66 nm. According to the relative size, the larger spheres could be formed by coalescence of the smaller spheres (Figure 5B). The amorphous aggregates could be agglomerates of the nanospheres or artifacts probably because of the presence of different salts[50] [compare buffer control of TEM and scanning electron microscopic (SEM) experiments; Supporting Information Figure S2]. To avoid the possible artifacts and because CD experiments showed no significant differences depending on the presence or absence of salts, 33-mer in water at pH 7.0 was evaluated. Under this condition, nanospheres, filaments, and fibrils were detected depending on the observation field. Filaments and fibrils appeared curvilinear and were complex with adjacent filaments and fibrils which organized into higher order structures, ranging from fibrils of 58 nm in diameter to clusters from 200 to 800 nm in width (Figure 6A). A field populated mainly by nanospheres of 25 nm in diameter was also found (Figure 6B).

At a higher magnification, we observed that the thickest fibrils arose from the product of a lateral association of thin fibrils and filaments (Figure 7A, see arrows). A few single filaments of 28 nm in width were also detected (Figure 7B). The internal structure of the filaments and fibrils were difficult to define; however, it seemed to be composed by small nanospheres (Figure 7C). Contact among nanospheres was also observed (Figure 7D).

Topographical information about the supramolecular structures was obtained by SEM experiments. Observation of 33-mer in 1 m*M* sodium citrate, 1 m*M* sodium borate, 1 m*M* sodium phosphate buffer, 15 m*M* NaCl, pH 7.0, showed random colloidal nanoparticles (Figure 8A). Meanwhile in water at pH 7.0 (Figures 8B and 8C and Supporting Information Figure S3), it was possible to observe random nanospheres in

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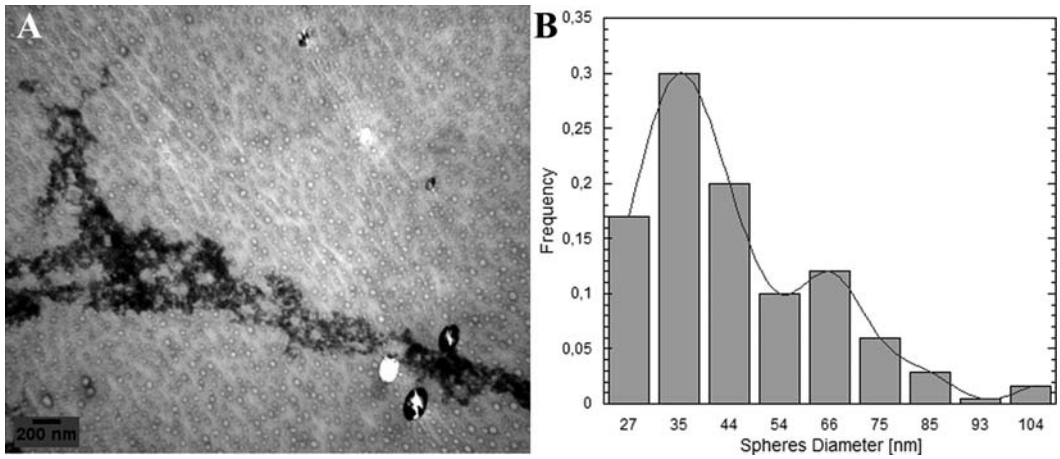

FIGURE 5 (A) TEM micrographs of 33-mer solution in 1 m*M* sodium citrate, 1 m*M* sodium borate, 1 m*M* sodium phosphate buffer, 15 m*M* NaCl, pH 7.0. (B) Statistical evaluation of the nanospheres.

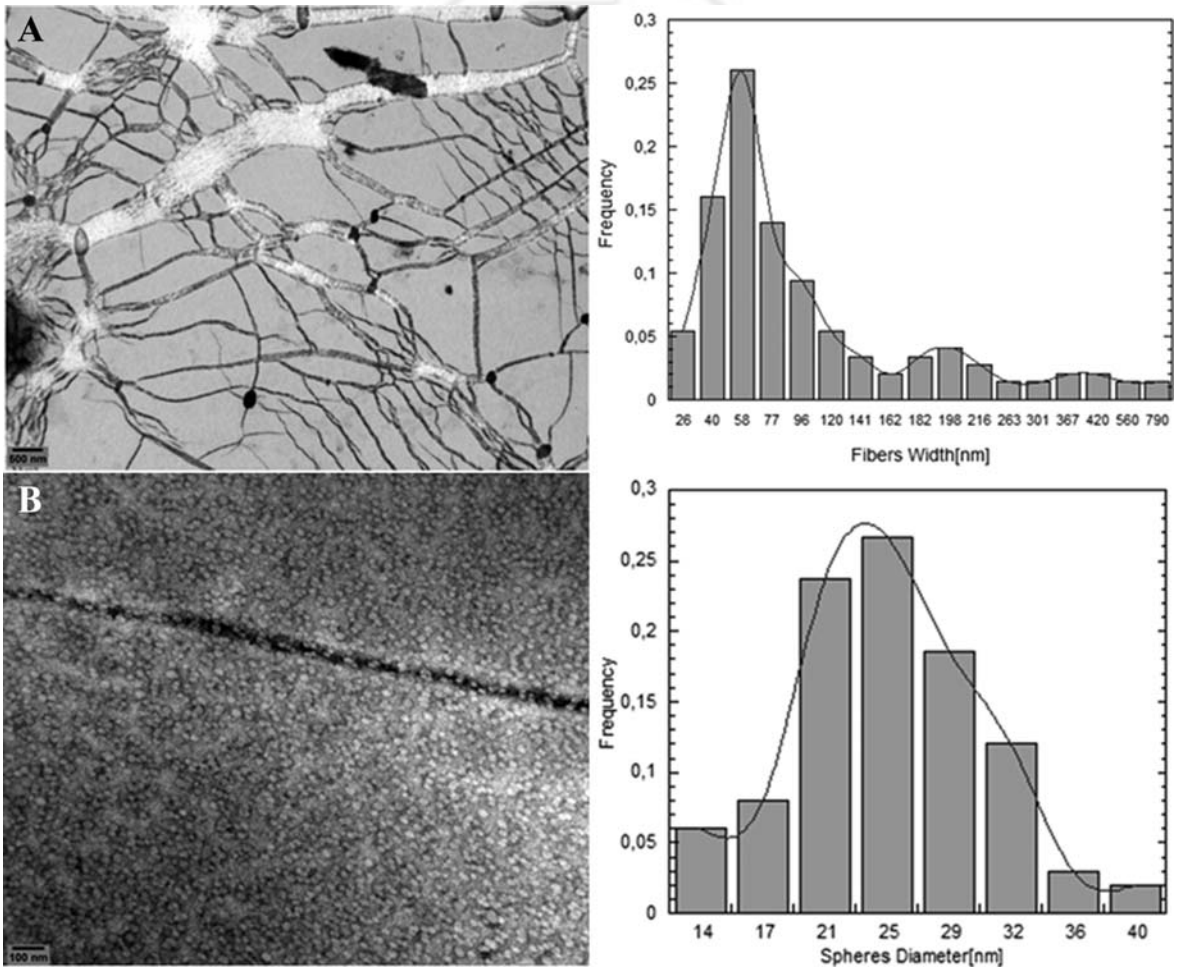

FIGURE 6 TEM micrographs of 33-mer solution in water, pH 7.0. (A) Field populated by filaments and fibrils with its statistical evaluation. (B) Field mainly populated by nanospheres and statistical diameter distribution.

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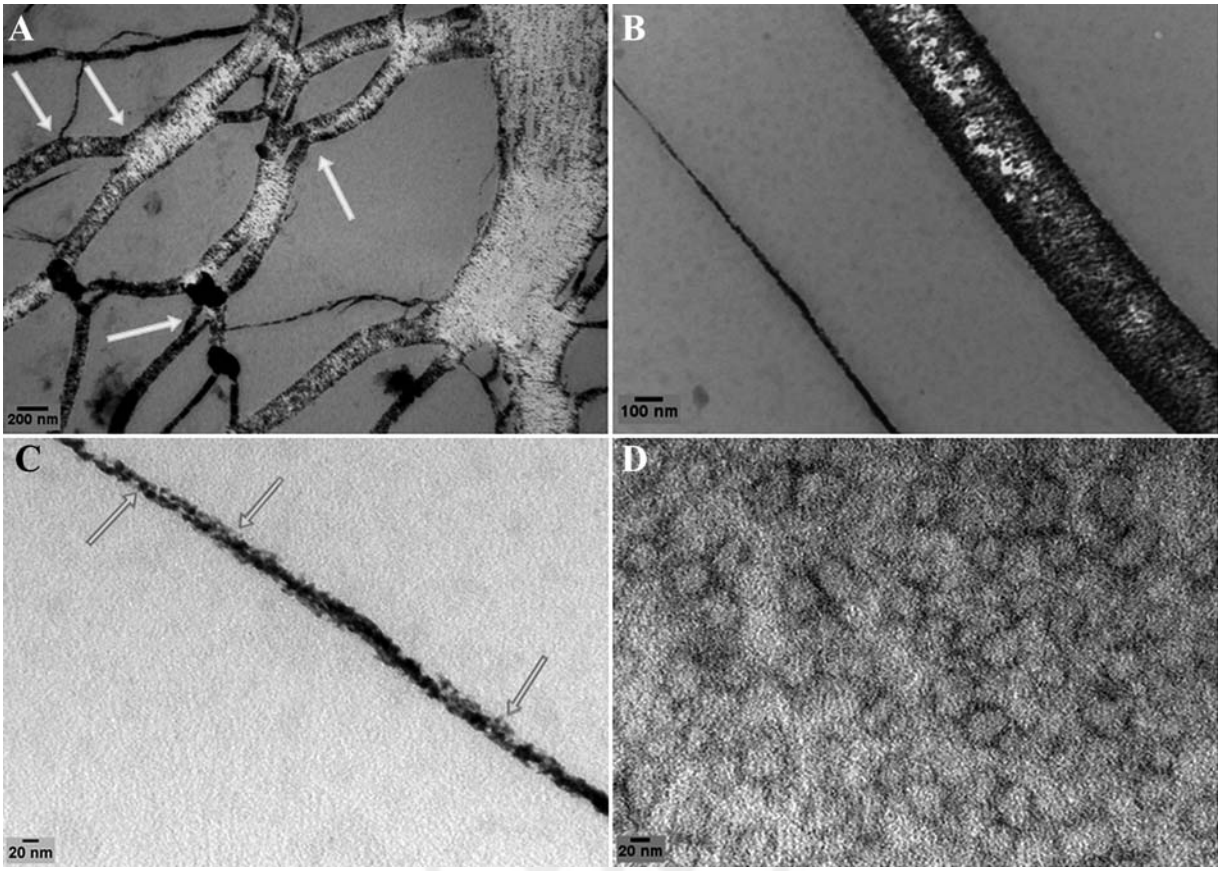

FIGURE 7 TEM micrographs of 33-mer in water, pH 7.0, showing the different quaternary nanostructures in detail. (A) The arrows show that lateral association of filaments contributes to the formation of fibrils. (B) In this micrograph, it is possible to distinguish the background full of nanospheres, a filament and the internal structure of the fibril. (C) The arrows show that the filaments seem to be composed by small nanospheres. (D) Micrograph showing that the nanospheres tend to interact between each other.

the background and linear supramolecular assemblies composed of interacting nanospheres. The morphological pattern was similar to those observed by TEM. It seems that the presence of 1 m*M* sodium citrate, 1 m*M* sodium borate, 1 m*M* sodium phosphate buffer, 15 m*M* NaCl stabilized the spherical aggregates, probably through electrostatic forces, inhibiting further interaction toward fibrils.[51]

On the basis of our results, we have proposed an initial supramolecular organization mechanism that was previously assumed for zein and related peptides,[52] spider silk proteins,[53] and also for amelogenins molecules, which are associated with the dental enamel biomineralization.[54] In general, spherical colloidal assemblies are forced into contact when the respective streamlines of the solvent get closer than the diameter of the particles, such behavior has been shown to be the dominant cause for aggregation of particles in the micrometer range.[55,56] We proposed that the calculated 33-mer dimer is engaged in one plausible initial step in the self-assembling process. Once the colloidal nanoparticles are formed, their intrinsic characteristics and the medium condition contribute to random assembly, as it would be expected for globular particles or spontaneous linear organization and formation of fibrils (Figure 9).[57–59] This mechanism is consistent with a recent study F9
wherein colloidal nanoparticles of α-synuclein protein involved in Parkinson's disease were reported to be able to linearly organize leading to the formation of fibrils.[60,61]

Considering that gut environment is highly dynamic,[62] the feasibility of the 33-mer to form colloidal nanospheres which can further organize into linear aggregates depending on environmental conditions would be relevant; however, in vivo studies are required to validate the real relevance.

CONCLUSION

By a combination of biophysical techniques, we have shown that 33-mer is able to self-assemble in a concentration-

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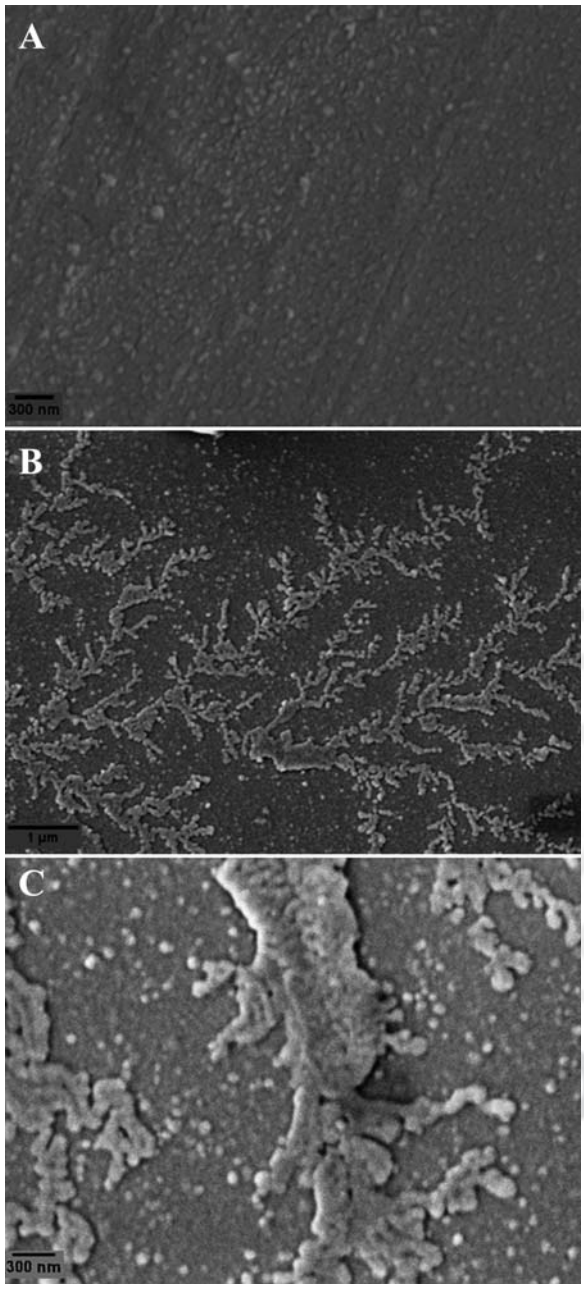

FIGURE 8 SEM micrographs of 33-mer solution metalized with gold: (A) in 1 m*M* sodium citrate, 1 m*M* sodium borate, 1 m*M* sodium phosphate buffer, 15 m*M* NaCl, pH 7.0 and (B) in water, pH 7.0. Statistical evaluation is presented in the Supporting Information Figure S3. (C) A higher magnification micrograph of 33-mer in water showing that interacting nanospheres composed the fibrils.

dependent manner through structural transition. Initially, PPII structure was dominant enabling further intermolecular interaction toward a more folded conformation, such as Type II β-turn, on increasing peptide concentration. The same structural transition at constant concentration was observed in TFE, a solvent known to provide a membrane mimicking environment.[63] As mentioned, both secondary structures are known to be important in protein–protein interaction, thus the structural transition could serve as a signaling command at the molecular level.

The 33-mer high percentage of proline and glutamine amino acids and the nonionic amphiphilic character detected by the PCED calculation could be a determining factor in the self-assembling process. Furthermore, a molecular model of 33-mer dimer is presented as one possible initial step in the interaction.

Finally, supramolecular organization was observed by EM experiments showing formation of colloidal nanospheres. The spherical aggregates were randomly distributed in 1 m*M* sodium citrate, 1 m*M* sodium borate, 1 m*M* sodium phosphate buffer, 15 m*M* NaCl, pH 7.0. However, linear organization of the nanospheres occurred in water pH 7.0, leading to the formation of fibrils.

Owing to the importance of protein aggregates[4,64] and nanoparticle well-known immunological role[65] in the balance between health and disease, the only existence of 33 mer nanospheres and fibrils allow us to think some new hypothesis related to the early steps of gliadin intolerance disorders mediated by 33-mer peptide. First, the unusual proteolytic resistance of 33-mer could be due to the structurally robust core within 33-mer nanospheres unable enzymatic degradation, as previously reported in other protein aggregates such as lysozyme fibrils.[66] Second, once 33-mer small nanospheres (14–35 nm) were formed, they could be transported across the epithelium cells due to its size probably by endocytosis pathway (enter) and budding (exit) by a similar mechanism observed by virus and other bionanoparticles.[67] Third, the multivalent surface display of the epitope on the nanospheres/fibrils would be the source of the strong immunomodulator activity and the subsequent break of the balance between tolerance and disease.[68] Finally, accumulation of 33-mer aggregates on epithelium surface could lead to the formation of 33-mer fibrils and clusters bigger than 0.5 μm. This event could be a supramolecular signal to start phagocytosis, the principal component of the body's innate immunity machinery. Recently, it has been reported that the overall process of phagocytosis is a result of the complex interplay between shape and size.[69] After recognition, the phagocytic cells start the internalization process; however, if the phagocytosis is frustrated due to the size and geometry of the particle, the toxic agents of the macrophages can be released into the environment. As these agents are also toxic to host cells, they can cause extensive damage to healthy cells and tissues,[70] as those observed in celiac disease intestinal lesion. After withdraw of gliadin from diet, the epithelium health is restored, showing the direct or indirect importance of

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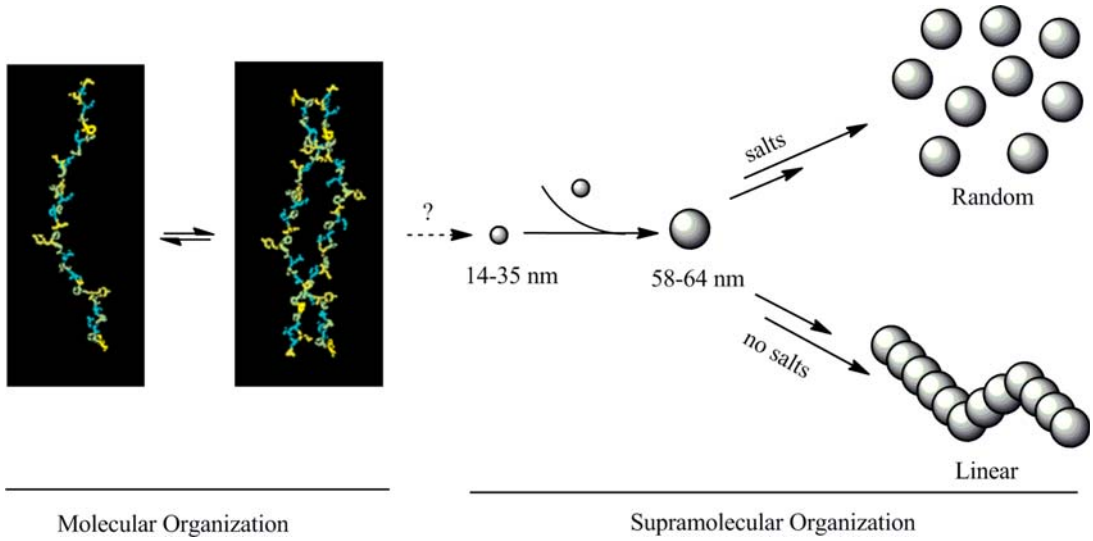

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FIGURE 9 Schematic representation of the hypothetical mechanism of 33-mer supramolecular organization.

this protein and probably of 33-mer in the pathological process of microvilli destruction and celiac disease.[7]

Currently, our research efforts are directed toward understanding the supramolecular mechanism involved in solution and on surface and the role of 33-mer nanospheres in vivo in order to test our hypothesis. Meanwhile, considering the importance of protein assemblies in disease,[71,72] our findings may open new perspectives for the understanding and treatment of gliadin intolerance disorders.

MATERIALS AND METHODS

Materials

To obtain reproducible results, three synthetic samples of 33-mer peptide LQLQPF(PQPQLPY)$_3$PQPQPF (3911 Da) with more than 95% purity were purchased from Biochem Shanghai in a lyophilized form at different periods of time. Peptide synthesis protocol was completed according to the protocol reported in the Supporting Information. Purity and mass integrity were reexamined before and after experiments to ensure no chemical modification occurred during handling. Reverse-phase HPLC analysis was performed using a Venusil XBP C18 250 × 4.6. Binary gradients of solvents A (CH_3CN 0.1% TFA) and B (H_2O 0.1%) were used at a flow rate of 1.0 mL min^{-1}. The injection volume was 10 µL. HPLC peaks were detected by monitoring the UV absorbance at $\lambda = 220$ nm and the identity were confirmed by MS. The retention time of 12.023 min was obtained under the following experimental conditions: CH_3CN/H_2O with 0.1% TFA, 28–100% A at 25 min. HPLC-MS: *m/z* 1958.61 $(M^+ + 2H)^{2+}$, 1305.16 $(M^+ + 3H)^{3+}$. Accurate mass determination was performed by ESI nano-MS: measured ion mass: 978.26472 (deviation 0.10) and calculated ion mass: 978.26463 (deviation 0.11); molecular formula obtained $(C_{190}H_{273}N_{43}O_{47})H_4^{+4}$ (Supporting Information Figures S4, S5, and S6).

Solutions for all determinations were prepared by complete dissolution of the peptide in Milli-Q water or 1 m*M* sodium citrate, 1 m*M* sodium borate, 1 m*M* sodium phosphate buffer, 15 m*M* NaCl with the pH adjusted at 7.0. Buffer selection was made in order to compare our results with previous studies with this peptide.[8,9,33] The pH adjustment was done with a solution of HCl or NaOH (0.1*M*) until the pH 7.0 was attained. Then the solution was filtered through a 0.2-μm Nylon membrane prior to use.

Methods

Circular Dichroism. CD spectra of peptide solutions at different concentrations were recorded on a Jasco J-810 CD spectrometer using a Peltier system as temperature controller. Usually, three scans were acquired in the range of 190–250 nm at a selected temperature (between −10 and 37°C with an incubation time of 5 min). A scanning speed of 50 nm min^{-1} was applied, and 1-mm and 0.1-mm quartz cuvettes were used. Water and buffer solutions were analyzed under the same conditions and subtracted from the spectra. Smooth noise reduction was applied eventually when it was necessary using a binomial method. The data were expressed as the mean residue molar ellipticity in deg cm^2 dmol^{-1}. Monitoring the samples at different concentration in solution from 1 h to 1 month was completed to show the absence of time-dependent evolution of results.

Graphics were represented using the program Kaleidagraph (v 3.5 by Synergy Software).

Model Building. Initial peptide was built using AVALONE free software, adding amino acids one by one in a perfect PPII helix structure ($\Phi = -75°$, $\Psi = 160°$).

Electrostatic modeling was performed using PARSE force field implemented in PDB2PQR software. All MD simulations were performed with GROMACS using the OPLS/AA/L force field. They were run in explicit water using the SPC model. Monomeric and dimeric peptides were solvated in a cubic box of 15 nm × 15 nm × 15 nm. Periodic boundary conditions were applied, and a 0.9-nm cutoff was

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used for Lennard-Jones interactions. All-bonds constraint was used. Long-range electrostatic interactions were handled using the Particle-Mesh Ewald summation method with a Fourier spacing of 0.12 and a fourth-order interpolation. All simulations were performed in the NVT ensemble at 310 K. Peptide and solvent were coupled to the same reference temperature bath with a time constant of 2 ps using the Berendsen method. An integration step size of 2 fs was used, and coordinates were stored every 10 ps.

With a single peptide, a 5-ns simulation was run in explicit water in order to reach a less energetic structure. Then, a dimer was built with two relaxed peptides, considering charge complementarity, minimizing energy, and followed by another 5-ns simulation that shows a stable structure. The final model was structurally checked, and it maintains a high degree of PPII structure.

Electron Microscopy. Experiments were performed in Milli-Q water or 1 m*M* sodium citrate, 1 m*M* sodium borate, 1 m*M* sodium phosphate buffer, 15 m*M* NaCl with the pH adjusted at 7.0. The same nanostructures were observed without dependence of concentration, but the most abundant leading to the best results were obtained at 613 μM (2.4 mg mL^{-1}). Specimen was prepared in duplicate, and grids were thoroughly examined to get an overall statistical evaluation of the structures present in the sample. Control experiments of water and buffer alone were performed showing no fibrils or spheres. Monitoring the samples at different concentration from 1 h to 5 months was performed to obtain good reproducibility and to demonstrate the absence of time-dependent evolution of results.

Statistical evaluation was completed using Image J software.

TEM Experiments. A 33-mer peptide aliquot (5 μL, 613 μM, 2.4 mg mL^{-1}) was deposited onto the copper grid (200 mesh) coated with Formvar. After 5 min of interaction, the excess fluid was removed. The sample was negatively stained with 2% of uranyl acetate in water. After 2 min, excess fluid was removed, let dry, and observed in a JEOL 100CX II microscope operating at 100 kV.

SEM Experiments. A 33-mer peptide aliquot (20 μL, 613 μM, 2.4 mg mL^{-1}) was deposited on uncoated cover slips. After 5 min of interaction, the excess of fluid was removed and let dry in a Petri dish. The resulting specimens were metalized with Au(0) using sputter coater Pelco 91000 and observed via a JSM-35 CF equipped with secondary electron detector (EVO 40).

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