Original Article

Chitosan-myristate nanogel as an artificial chaperone protects neuroserpin from misfolding

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Abstract

Background: Molecular chaperon-like activity for protein refolding was studied using nanogel chitosan-myristic acid (CMA) and the protein neuroserpin (NS), a member of the serine proteinase inhibitor superfamily (serpin).

Materials and Methods: Recombinant his-tag fusion NS was expressed in *Escherichia coli*. For confirmation of refolding of the purified NS, structural analysis was performed by circular dichroism and spectrofluorometric along with its inhibitory activity, which was assayed by single-chain tissue plasminogen activator. For evaluating NS aggregation during preparation, the samples were separated on a 7.5% (w/v) nondenaturing polyacrylamide gel electrophoresis. MA and chitosan covalently join together by the formation of amide linkages through the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-mediated reaction. The morphology and size of the prepared CM nanogel were characterized by transmission electron microscopy and scanning electron microscopy.

Results: Heating at different temperatures (25°C, 37°C, 45°C, 65°C, 80°C) results in a further rise in β-structures accompanied by a fall of helices and no significant change in random coils. Structural changes in NS in the presence of CMA nanogel were less than that in the absence of CMA nanogel. Mater nanogel effectively prevented aggregation of NS during temperature induced protein refolding by the addition of cyclodextrins. The nanogel activity resembled the host-guest chaperon activity.

Conclusion: These conditions, called conformational disorders, include Alzheimer's, Parkinson's, Huntington's disease, the transmissible spongiform encephalopathies, prion diseases, and dementia. Nanogels can be useful in recovery of the structural normality of proteins in these diseases.

Key Words: Cyclodextrin, misfolding, nanogel (chitosan-myristic acid), neuroserpin

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INTRODUCTION

Neuroserpin (NS) is a member of the serine proteinase inhibitor superfamily (serpin). It is expressed in neurons of the central and peripheral nervous systems in adults.[1,2] Like other members of the serpins, this protein has three β-sheets, nine α-helices, and a reactive center loop structure, which interacts directly with protease substrates. Changes in conformation of proteins that are principally expressed in the central and peripheral nervous system result in loss of protein function, inducing a wide range of disorders.[3-5] These conditions that are called conformational disorders (e.g. Alzheimer's, Parkinson's, Huntington's disease, the transmissible

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spongiform encephalopathies; prion diseases and dementia) involve deposits of the aggregated, misfolded proteins in the related organs or tissues.[6,7] Mutation in the NS gene or certain environmental conditions such as oxidative stress, may result in structural and functional changes of NS.[8-10] In this study, self-assembled nanogel was designed as an artificial molecular chaperones consisting of chitosan-myristic acid (CMA) (CM nanogel). CM nanogel consists of a backbone of chitosan and a hydrophobic moiety such as MA that form a nanoparticle in water by the intermolecular self-assembly. Its chaperone-like properties were evaluated using human NS, a protein involved in a conformational disorder.[11] Secondary and tertiary structural analysis along with an antiproteinase activity assay of NS was considered for evaluation of protective ability of the CM nanogel. The proposed mechanism of action of the nanogel as an artificial chaperone is the "host-guest" mechanism, based on transient binding of the hydrophobic surface of unstable protein (folding intermediates or partially denatured protein) with hydrophobic region inside the nanogel. The nanogel-protein complex dissociates by trapping other proteins or by the addition of cyclodexterin. The chaperone process results in the release of a properly folded protein.[12] Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit).[13] It is a biocompatible and biodegradable material and has a number of possible biomedical and pharmaceutical uses.

MATERIALS AND METHODS

Expression, purification and refolding of human neuroserpin

Recombinant NS, 45 kDa protein that containing 394 residues, was expressed with a 6-histidine tag at the N terminus in the pQE81 L vector containing cDNA of human NS (a very generous gift from Professor David A. Lomas and Professor Didier Belorgey from Cambridge University, UK) in *Escherichia coli* M15 strains. A colony of the infected *E. coli* with the expression plasmid pQE81 L was cultured in 500 ml Luria-Bertani (LB) broth at 37°C in a shaking incubator overnight at 190 rpm. Ampicillin (50 μg/ml) and kanamycin (25 μg/ml) were used for selection and growth of the infected cells in the LB media culture. 1–2 mM IPTG was used as an inducer when the optical density of the media culture reached 0.5–1 at 550 nm in a spectrophotometer. After 4 h cultivation, the cells were harvested by centrifugation (4000×*g*, 15 min at 4°C) then resuspended in buffer A (50 mM Tris-HCl, 100 mM NaCl, 10 mM ethylenediaminetetraacetic acid, 10 mM phenylmethylsulfonyl fluoride, pH 7) and disrupted by sonication (10 s × 10 s with 10 s intervals, 60% amplitude, 0.5 cycle). The inclusion bodies were washed 3 times with buffer A + 2% (v/v) triton - ×100 and then solubilized in buffer A + 8 M urea. The expressed NS was purified using a cation exchanger resin-sulfopropyl A50 (SP A50) precharged with 100 mM nickel sulfate. The solubilized inclusion bodies were incubated in loading buffer (buffer A + 8 M urea + 20 mM imidazole) containing SP A50 precharged with nickel sulfate. The solution was loaded into a 5 ml syringe and then extensively washed with loading buffer until the optical density of the eluted fraction reached minimum and stable. NS that adhered to the SP A50 resin was eluted with elution buffer (buffer A + 8 M urea + 250 mM imidazole). For evaluation of the purity of the expressed NS, a sample of the eluted fraction was prepared for running on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) followed by staining with coomassie brilliant blue R-250. The expressed NS was refolded by drop-wise addition of the eluted sample to refolding buffer (20 mM phosphate buffer, pH 7.8, 100 mM NaCl) at 4°C. An ultrafiltration system with a cellulose acetate membrane (cutoff; 10 kDa) was used for concentrating the refolded NS. For confirmation of refolding of the purified NS, structural analysis was performed by circular dichroism (CD) along with its inhibitory activity, which was assayed by single-chain tissue plasminogen activator (sc-tPA). For evaluating NS aggregation during preparation, the samples were separated on a 7.5% (w/v) nondenaturing PAGE and then visualized by staining with coomassie brilliant blue R-250.

Inhibitory activity assay of neuroserpin

The proteinase amidolytic activity of the expressed NS was assayed using a protease and a chromogenic substrate-sc-tPA and H-D-isoleucyl-prolyl-arginine-*p*-nitroaniline dihydrochloride respectively. The expressed NS and sc-tPA were preincubated in amidolytic assay buffer (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.2% (w/v) bovine serum albumin and 0.1% (w/v) PEG8000) for 5 min at 37°C. The chromogenic substrate was then added at a final concentration of 1 mM. The reaction was further incubated at 37°C, and the velocity of amidolytic liberation of *p*-nitroaniline was determined by measuring the optical density of the reaction at 405 nm.

Preparation of myristic acid-chitosan nanogel

Myristic acid and chitosan covalently join together by the formation of amide linkages through the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-mediated reaction. Chitosan was dissolved in 1% acetic acid solution and sonicated using

a probe type sonifier (ulterasonic homogenizer VH-600) at 60 w for 20 min and then diluted with methanol. MA was added to the chitosan (1 MA group/100 glucosamine units) followed by a drop-wise addition of EDC in methanol while stirring at room temperature for 24 h. The 1:1 mol ratio of EDC to MA was used in this study.[14] After 24 h, the reaction mixture was poured into a methanol/ammonia solution while stirring. The precipitated material was washed with distilled water, methanol and ether successively and then dried under vacuum for 24 h at room temperature. For Fourier transform infrared (FTIR) analysis, the samples were milled with potassium bromide to form a very fine powder. Then the powder was compressed into a thin pellet. FTIR spectra were recorded on a Thermo Nicolet Nexus 670 FT-IR E.S.P. The morphology and size of the prepared CM nanogel were characterized by transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Spectrofluorometric studies

All fluorometric studies were performed on a Shimadzu spectrofluorometer with a 3 ml cuvette of 1 cm pass length. Emission spectra were recorded between 295 and 425 nm (excitation wavelength: 280 nm) in an excitation and emission bandwidth of 5 and 10 nm, respectively.

Circular dichroism experiments

Circular dichroism spectra were recorded on a JASCO J-715 spectropolarimeter (Welltech enterprises, Inc, USA) using 0.2 mg/ml NS in reaction buffer and at different temperatures (25°C, 37°C, 45°C, 60°C). The results were expressed as molar ellipticity [θ] (deg cm^2/dmol).[15] The molar ellipticity was determined as: [θ] = (θ × 100 MRW)/(cl), Where c is the protein concentration in mg/ml, l is the light path length in centimeters, and θ is the measured ellipticity in degrees at wavelength λ. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291} = 7820$ deg cm^2/dmol[13] and with JASCO standard nonhydroscopic ammonium (+)-10-camphorsulfonate, assuming $[\theta]_{290.5} = 7910$ deg cm^2/dmol.[14] Noise in the data was smoothed using the JASCO J-715 software, including the fast Fourier-transform noise reduction routine, which allows enhancement of most noisy spectra without distorting their peak shapes.[14,15] Results presented in this paper are the mean values of at least three repeated experiments in a typical run to confirm reproducibility.

RESULTS

Preparation of the native and oxidized forms of recombinant human neuroserpin

As shown in the previous study, NS was purely extracted by this method. The estimation of secondary structures content by analyzing the data from CD and also its inhibitory activity assay confirmed that NS properly refolded. No aggregation was observed during preparation of native NS. The inhibitory activity of NS for tPA decreased 40–60% after treatment in the reaction mixture at different temperatures.[16]

Data from spectrofluorimetric studies showed that the tertiary structure of NS altered remarkably. Reduction in intrinsic fluorescence intensity after heating showed that the NS was unfolded. On the other hand, a blue shift showed that protein aggregation was taking placed [Figure 1].

Circular dichroism results

The CD spectra of NS demonstrate minimum of around 210–216 nm indicating a high content of β-sheets in the native NS. The data are obtained from the JASCO protein secondary structure estimation program model SSE-338 (Welltech enterprises, Inc, USA). Quantitative analysis by JASCO software indicates that there are maximal content of β-structures and minimal content in helix conformation. The CD spectra of NS demonstrated that the program has given a good estimation of the secondary structures, especially helical structures. In addition, heating at different temperatures (25°C, 37°C, 45°C, 65, 80°C) results in a further rise in β-structures accompanied by a fall of helices and there is no significant change in random coils. Structural changes in NS in the presence of CMA nanogel were less than that in the absence of CMA nanogel.

Transmission electron microscopy experiments

The particle size and dispersion of the nanogel were confirmed by TEM experiments as shown in Figure 2. Particle size of the monodispersed nanogel was estimated to be <20 nm.

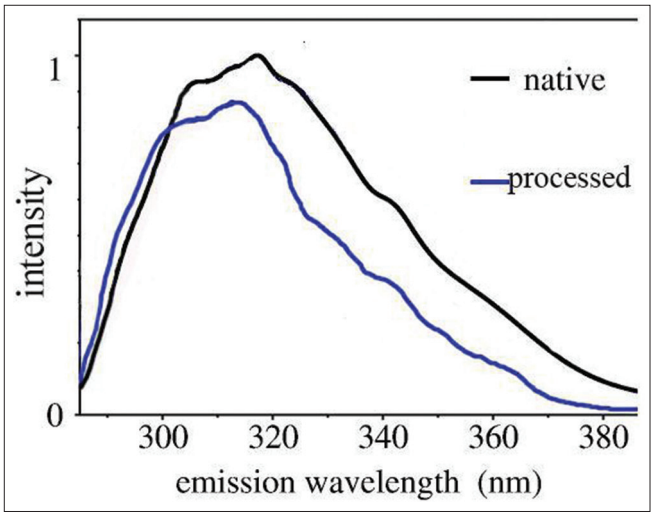

Figure 1: Spectroscopy before and after loading nanogel

Scanning electron microscopy experiments

The particle size and dispersion of the nanogel were confirmed by SEM experiments as shown in Figure 3. Particle size of the monodispersed nanogel that disperses in aqua solution was spherical shape.

DISCUSSION

Structural changes in NS lead to aggregation and deposition of the unfolded protein in the nervous system which may cause neuronal damage.[17,18] Recently, chemical chaperones and free radical scavengers that protect proteins from structural or functional changes have attracted considerable interest as a beneficial therapeutic strategy. Osmolytes, arginine and sodium 4-phenylbutyric acid were proposed as chemical or pharmaceutical chaperones in refolding or protection of protein. Artificial chaperones consisting of pullulan and cholesterol nanogels were designed that protected the enzymes carbonic anhydrase B and citrate synthase from misfolding at higher temperatures.[19,20] The size of nanogel (CMA) was under 50 nm that can be used for drug delivery. These nanocages were studied with florescence spectroscopy with Atorvastatin as a molecular probe.[21] Atorvastatin is poorly soluble alone and strongly emits radiations, however, after self-assembly hydrophobic microdomains are formed in an aqueous solution that causes a reduction in the amount of emitted radiation. EDC is a "zero-length" cross-linker, which brings about the branches of an amide linkage between the carboxyl group of MA and the amino group of chitosan without leaving a spacer molecule.[16,22]

Figure 4 shows hydrogen-1 nuclear magnetic resonance (H^1NMR) spectra of the chitosan [Figure 4a] and the MA-chitosan [Figure 4b]. The new peak which originates from the H^1NMR spectra of MA-chitosan is mainly due to characteristic methyl protons ($-NH^+$ 3) of the reacted chitosan with MA;[22] this peak shows the presence of major functional groups linked to chitosan and can be used to determine the degree of substitution of MA groups.

Figure 5a corresponds to the FTIR spectrum of CMA while Figure 5b shows the one for myristc acid. The appeared numbers in these figures represent the stretching bond of the vibration modes. As it can be

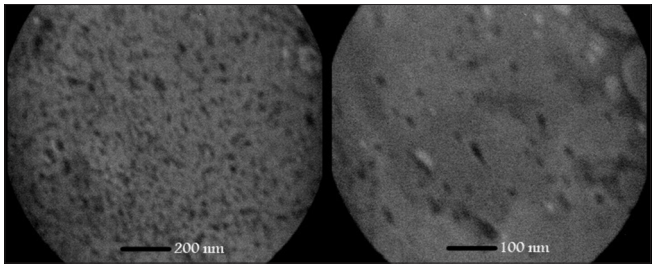

Figure 2: Transmission electron microscopy nanogel (chitosan-myristic acid)

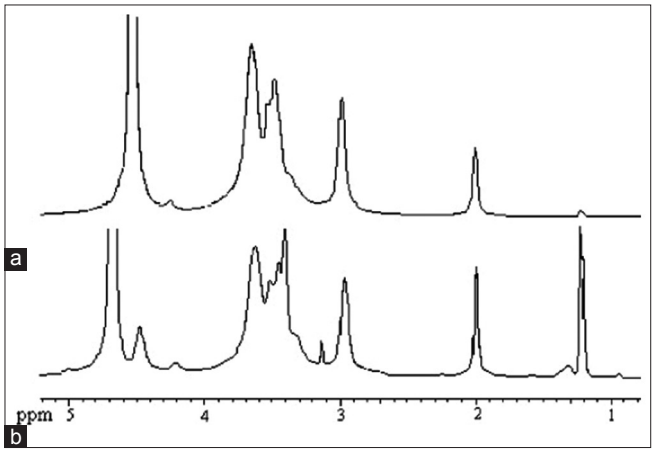

Figure 4: Hydrogen-1 nuclear magnetic resonance spectra of (a) chitosan and (b) myristic acid (MA)-chitosan in D_2O and CD_3COOD. The IR spectra were taken of MA-chitosan to study the binding of chitosan amine groups

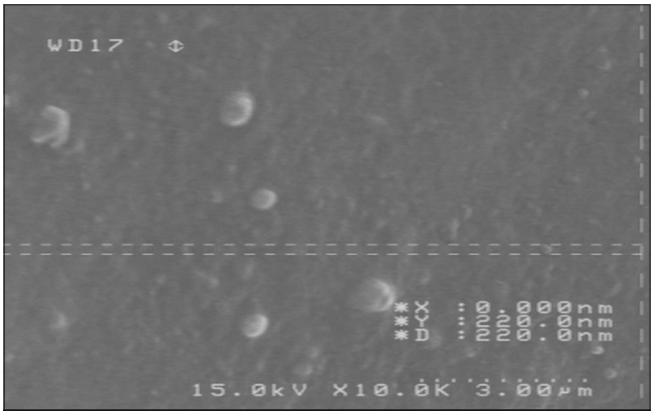

Figure 3: Scanning electron microscopy of nanogel (chitosan-myristic acid)

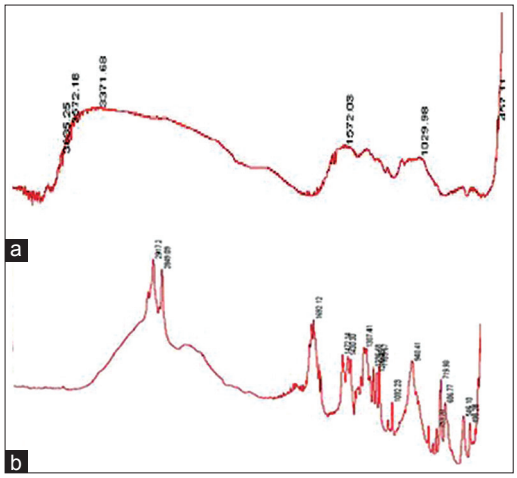

Figure 5: Fourier transform infrared (FTIR) spectra of chitosan-myristic acid (MA) (a) FTIR spectra of MA (b)

seen in Figure 5a, the stretching bond of C-H rings of the chitosan and the attached methyl groups to the ring has been appeared in 2876.96/cm. The peaks in 1598.10, 1153.65, and 1033.30 correspond to the bending mode of N-H, stretching mode of C-O, and stretching mode of C-N, respectively. In Figure 5b, it is seen that the stretching of O-H (carboxyl group) and C-H bonds, respectively in 2719.25 and 2849.09/cm, are overlapped and resulted in the broad peak of OH. In addition, the peak in 1692.12 and 1296/cm can be attributed to the stretching mode of C-O (carbonyl group) and stretching of the C-OH bond, respectively. 1572.03 is related to C-O in the functional group of meristic acid that converts to N-C-OH in attaching situation because C-O peak must be sharp, and the ratio shows that C-OH bond is a single bond.

The particle size of the nanogels was 10–50 nm, determined by (TEM) and (SEM). It was also demonstrated that they have same spherical shape in aqueous solution. Chitosan polymer is full of positive charges that cause them to disperse in aqueous solution. In this experiment, we use Atorvastatin as a probe for observing nanogel formation via florescence before and after the fluorophore is in the trapped state that occurs in nanogels.

It is well-known that chitosan is insoluble due to its crystalline structure, and that the crystalline structure can be destroyed by introducing hydrophilic groups or hydrophobic substitutes.

Atorvastatin is a hydrophobic drug, in prenanogel stage, when nanogels are forming, contemporary filtration atorvastatin trapped in the hydrophobic core but after filtration could not be trapped, and that is due to self-assembly phenomenon. Longer hydrophilic chains and bigger hydrophobic groups help stabilize the micelle structure and protect drug compounds from the environment. The CMA nanogels formed by the self-assembly of MA bearing chitosan have been shown to dissociate upon the addition of cyclodextrins. The CMA nanogels were able to form stronger complexes with heat-denatured NS than were native form such as molecular chaperones. The complexed proteins were selectively released in their refolded native form upon dissociation of the nanogels in the presence of cyclodextrin. Therefore, it was demonstrated that the nanogels possess heat shock protein-like activity that was similar to the mechanism of molecular chaperones that is, they were able to catch and release proteins as depicted below. It has been shown that the amount of β-structures of NS increased in at high temperatures, with the concentration of the protein, pH change or under oxidative stress and etc. One of the advances of this study is that secondary structures of native and heat-denatured NS were evaluated by CD in absence and in the presence of CMA nanogel at various temperatures as shown in Figures 6 and 7. Also in this research, a shift from α-to β-structures was observed when NS was heated. After adding nanogels to missfolding NS proteins, we had been a lots of structural changes in third and second structure in 37°C and 45°C, before we added nanogels to heated proteins, we added cyclodextrin until cover the MAs in nanogel and cause of release proteins of nanogels, In this condition, by increasing the temperature, structural changes of NS in the presence of CMA nanogel was less than that in the absence of CMA nanogel, also in present study showed that, the assembly of MA-chitosan in the water solution doesn't need emulsification. The micelles of MA-chitosan formed nanosize particles of 50–100 nm that this is similar to the results obtained in previous studies, which reported previously.[22,23] synthesized chitosan linked to MA and converted it to polysoap that could aggregate in aqueous solution; this resulted in the formation spherical nanocages. In fact, there are some ingenerate similarities among those

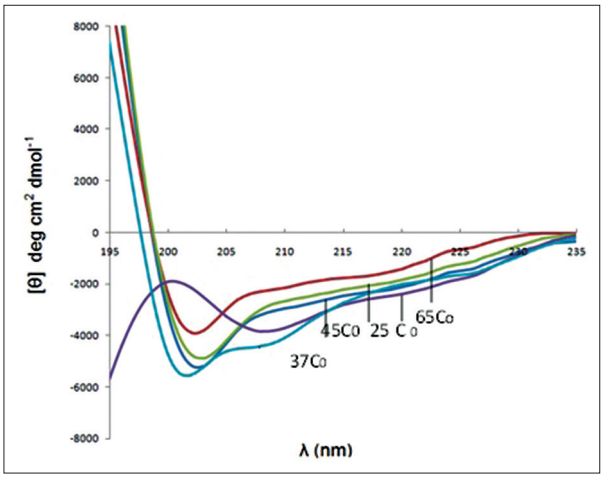

Figure 6: Ultraviolet circular dichroism spectra of neuroserpin and nanogel at various temperatures

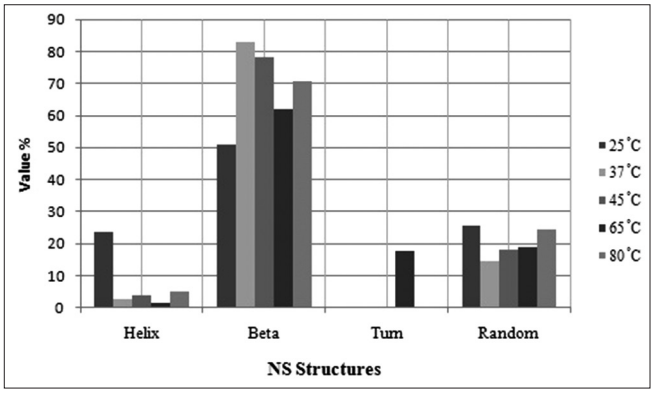

Figure 7: Neuroserpin structures in percent under various temperature conditions

chitosan-based poly soaps,[24] polymeric surfactants,[25-27] hydrogels,[28] vesicles[29] and micelles. According to the CD experiments, thermal denaturation of the protein results in a further rise in β-structures accompanied by a fall in helices and no significant changes in random coils. It is necessary to note that CD deconvolution results are similar to those for X-ray crystallography for mouse NS.[13] Also, the serpin superfamily folds into a conserved structure, so we can conclude that the program has given a good estimation of the secondary structures, especially helix structures. This means that treatment of the protein at higher temperatures has no denaturing effect on the secondary structure of NS, but acts as a transmitter of helix to β-structures.[14,15]

Our results of CD experiments showed that CMA nanogel have a protective effect on the structure of native NS at high temperatures. Inside nanogels are hydrophobic surface so proteins tend inter them after adding nanogels under temperature condition because of diameter increased and after adding cyclodextrins, NS release in hydrophilic environment and it has been changed to hydrophilic surface, On the other hand in the presence of nanogels, such aggregation was significantly prevented. The interesting chaperone-like activity of CMA nanogel for preserving the native structure of NS is shown; however, this should be confirmed by further studies. In conclusion, we conclude that CMA nanogel, a biocompatible biomaterial, protects NS from denaturation. So it could be candidate as a drug for prevention of progression of some conformational diseases such as dementia.

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REFERENCES

1. Osterwalder T, Contartese J, Stoeckli ET, Kuhn TB, Sonderegger P. Neuroserpin, an axonally secreted serine protease inhibitor. EMBO J 1996;15:2944-53.
2. Schrimpf SP, Bleiker AJ, Brecevic L, Kozlov SV, Berger P, Osterwalder T, *et al.* Human neuroserpin (PI12): CDNA cloning and chromosomal localization to 3q26. Genomics 1997;40:55-62.
3. Krueger SR, Ghisu GP, Cinelli P, Gschwend TP, Osterwalder T, Wolfer DP, *et al.* Expression of neuroserpin, an inhibitor of tissue plasminogen activator, in the developing and adult nervous system of the mouse. J Neurosci 1997;17:8984-96.
4. Whisstock J, Skinner R, Lesk AM. An atlas of serpin conformations. Trends Biochem Sci 1998;23:63-7.
5. Butterfield DA, Stadtman ER. Protein oxidation processes in aging brain. Adv Cell Aging Gerontol 1997;2:161-91.
6. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. Biochem J 1997;324:1-18.
7. Forster MJ, Dubey A, Dawson KM, Stutts WA, Lal H, Sohal RS. Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain. Proc Natl Acad Sci U S A 1996;93:4765-9.
8. Aksenov MY, Aksenova MV, Butterfield DA, Geddes JW, Markesbery WR. Protein oxidation in the brain in Alzheimer's disease. Neuroscience 2001;103:373-83.
9. Varadarajan S, Yatin S, Aksenova M, Butterfield DA. Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. J Struct Biol 2000;130:184-208.
10. Mohsenifar A, Lotfi AS, Ranjbar B, Allameh A, Zaker F, Hasani L, *et al.* A study of the oxidation-induced conformational and functional changes in neuroserpin. Iran Biomed J 2007;11:41-6.
11. Telling GC, Parchi P, DeArmond SJ, Cortelli P, Montagna P, Gabizon R, *et al.* Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. Science 1996;274:2079-82.
12. Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, *et al.* Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci U S A 1993;90:10962-6.
13. Prusiner SB, McKinley MP, Bowman KA, Bolton DC, Bendheim PE, Groth DF, *et al.* Scrapie prions aggregate to form amyloid-like birefringent rods. Cell 1983;35:349-58.
14. Agnihotri SA, Mallikarjuna NN, Aminabhavi TM. Recent advances on chitosan-based micro-and nanoparticles in drug delivery. J Control Release 2004;100:5-28.
15. Suh JK, Matthew HW. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: A review. Biomaterials 2000;21:2589-98.
16. Segura-Sánchez F, Montembault V, Fontaine L, Martínez-Barbosa ME, Bouchemal K, Ponchel G. Synthesis and characterization of functionalized poly(gamma-benzyl-L-glutamate) derivates and corresponding nanoparticles preparation and characterization. Int J Pharm 2010;387:244-52.
17. Schippers PH, Dekkers HP. Direct determination of absolute circular dichroism data and calibration of commercial instruments. Anal Chem 1981;53:778-82.
18. Takakuwa T, Konno T, Meguro H. A new standard substance for calibration of circular dichroism: Ammonium d-10-camphorsulfonate. Anal Sci 1985;1:215-8.
19. Briand C, Kozlov SV, Sonderegger P, Grütter MG. Crystal structure of neuroserpin: A neuronal serpin involved in a conformational disease. FEBS Lett 2001;505:18-22.
20. Kelly SM, Jess TJ, Price NC. How to study proteins by circular dichroism. Biochim Biophys Acta 2005;1751:119-39.
21. Brunel F, Véron L, Ladavière C, David L, Domard A, Delair T. Synthesis and structural characterization of chitosan nanogels. Langmuir 2009;25:8935-43.
22. Schatz C, Viton C, Delair T, Pichot C, Domard A. Typical physicochemical behaviors of chitosan in aqueous solution. Biomacromolecules 2003;4:641-8.
23. Berth G, Cölfen H, Dautzenberg H. Physicochemical and chemical characterisation of chitosan in dilute aqueous solution. Analytical Ultracentrifugation VI. Berlin Heidelberg Springer; 2002. p. 50-57.
24. Popa-Nita S, Alcouffe P, Rochas C, David L, Domard A. Continuum of structural organization from chitosan solutions to derived physical forms. Biomacromolecules 2010;11:6-12.
25. Gohy JF, S Antoun, Jerome R. Self-aggregation of poly-(methyl methacrylate)-block-poly (sulfonated glycidyl thacrylate) copolymers. Polymer 2001;42:8637-45.
26. Timoshenko EG, R Basovsky, Kuznetsov YA. Micellesationvs aggregation in dilute solutions of amphiphilic heteropolymers. Colloids Surf 2001;190:129-34.
27. Chen XG, Lee CM, Park HJ. O/W emulsification for the self-aggregation and nanoparticle formation of linoleic acid-modified chitosan in the aqueous system. J Agric Food Chem 2003;51:3135-9.
28. Yang L, Alexandridis P. Physicochemical aspects of drug delivery and release from polymer-based colloids. Curr Opin Colloid Interface Sci 2000;5:132-43.
29. Yoshioka H, Nonaka K, Fukuda K, Kazama S. Chitosan-derived polymer-surfactants and their micellar properties. Biosci Biotechnol Biochem 1995;59:1901-4.

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