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Supporting Information

Zein film functionalized with gold nanoparticles and the factors affecting its mechanical properties

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Experimental Section

Materials. All chemicals obtained from Aldrich Chemical Co. were of the best available purity and used without further purification unless otherwise indicated. Milli-Q water was used for all preparations.

Instrumentation. UV-visible spectra were recorded on Agilent Technologies Cary 8454 spectrometer equipped with a UNISOKU Scientific Instruments for variable temperature experiments. CD spectra were recorded from 190 to 300 nm with a JASCO J-1500 CD spectrometer. Surface charge (zeta-potential) were analysed using a Beckman Coulter Delsa Nano C particle analyser according to protocol. Fluorescence measurements were performed using Jasco FP-8500 fluorescence spectrophotometer at 65°C with a data interval of 0.5 nm. The FT-IR spectra were measured in Bruker-Alpha Eco-ATR FTIR instrument in the frequency range of 400 - 4000 cm⁻¹ using solid samples prepared in KBr pellet. DSC measurements were conducted using a NETZSCH STA 449F3 instrument under a nitrogen atmosphere. 2.694 mg samples were sealed in an aluminium pan, which was heated from 27 to 507 °C at a rate of 10 °C/min. TEM investigation was carried out by using Tecnai G² Transmission Electron Microscope operating at an accelerating voltage of 200 kV. Samples for TEM analysis were prepared by casting a drop of zein-AuNP solution (in dil. EtOH) on a carbon-coated Cu TEM grid and dried in air. Atomic Force Microscopy (AFM) of zein protein film and AuNPs doped zein film was analysed using Park NX10 AFM. The analysis was done in tapping mode for an area of $1\mu m \times 1\mu m$. SEM images were recorded using cryo FEG SEM (JSM-7600F) instrument. Optical images were obtained by Olympus CX21i LED Binocular Microscope mounted with a magnus MIPS 5MP camera.

Synthesis of gold nanoparticle (AuNPs). To optimise the ratio of zein vs HAuCl₄, the AuNPs were prepared by heating the different ratios of zein protein with HAuCl₄ in 10 mM SDS aqueous solution. Appropriate amount of zein protein (0.2 %, 0.4 %, and 0.6 %) was first

dissolved in minimum amount of 90 % aqueous ethanol and mixed with a 15 mL SDS aqueous solution with its 10 mM overall final concentration. We have prepared the triplicate solutions for each zein protein solution (0.2 %, 0.4 %, and 0.6 % w/v). HAuCl₄ was added to each zein solutions in different concentrations (0.25, 0.50, and 1.0 mM) at room temperature in screw-capped glass bottles and mixed well. All the reaction mixtures were kept at 65 °C for 3 hours under static conditions till the colour of the solution changed from light yellowish to pink/purple in each case. The unreacted protein was removed by washing the reaction mixture using milli-Q-water at least three times. The AuNPs were centrifuged at 10,000 rpm (3 times) for 5 minutes after each successive washing respectively. To confirm the formation of AuNPs, in each case, we have recorded the UV-visible spectra and followed the characteristic SPR (Surface Plasmon Resonance) band of AuNPs at 550 nm for each combination. The UV-visible spectral studies confirmed that the zein vs HAuCl₄ ratio is 0.20 % w/v to 0.25 mM to obtain uniform sized AuNPs with high yield. We have prepared the above AuNPs by following the reported literature with some specific modification.^{1, 2}

Reactivity Studies. The formation of zein capped AuNPs was confirmed by following the characteristic SPR band (550 nm) using UV-visible spectroscopy. First, zein protein (10 mg) was dissolved in minimum amount of 90% aqueous ethanol solution mixture, and then SDS aqueous solution (10 mM, 14.4 mg) was added to make total volume 5 mL. To study the kinetics of AuNPs formation, we followed the reaction of zein protein solution with the gold aqueous solution. In this regards, 50 μ l of HAuCl₄ (25mM) was added to the prepared zein protein solution (2 mL, 10 mM SDS) and then followed by UV-visible at 65 °C. The blank for the above reaction was SDS solution (10 mM). UV-visible spectral measurements suggested that reaction complete in ~2 hours (Figure S1).

Thermal Studies. The formation of zein capped AuNPs was carried out at different temperatures (20 - 80 °C) in order to find the best suitable temperature for the formation of

AuNPs. To determine the optimum temperature, 15 screw-capped bottles containing 20 mg of zein in 90% aqueous ethanol solution and SDS aqueous solution (10 mM) were reacted with 100 µl of HAuCl₄ (25 mM). The final volume of reaction mixture was fixed to 10 mL and 10 mM SDS concentration. All reaction mixtures were kept at different temperature ranging from 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, and 85 °C to 90 °C. UV-visible spectra of the reaction mixtures were recorded after 3 hours suggesting that the best suitable temperature to prepare the AuNPs is 65 °C (Figure S2).

Fluorescence studies. Inherent fluorescence property of zein is due to the presence of Tyrosine amino acid and the intensity decreases when it interacts with NPs.^{3, 4} The decrease in the fluorescence intensity is quantitative with respect to NPs concentration and therefore the binding constant (K_b) for the interaction of the protein with NPs can be calculated using Stern-Volmer or Hill equation.

Static quenching model suggests that the AuNPs-zein complex do not show any fluorescence and therefore the Stern-Volmer equation can be written as $(F_0/F) = 1 + K_{SV}$ [AuNPs]. Where F_0 = fluorescence of zein protein, F = fluorescence of zein protein at specific AuNPs concentration and K_{SV} is Stern-Volmer quenching constant. As the concentration of AuNPs increases the fluorescence intensity decreases continuously and at a specific concentration of AuNPs, we observed the lowest fluorescence intensity. At this point, we can consider the quenching as static quenching, where zein and AuNPs form a stable complex, K_{SV} becomes K_a (the association constant)^{5, 6}. The fluorescence intensity of zein and the AuNPs concentration can be correlated by Hill equation as $(F_0-F/F-F_{sat}) = \log K_a + n \log$ [AuNPs]. In this equation, F_{sat} = fluorescence intensity of zein at AuNPs saturation and the Hill coefficient (*n*) is the degree of cooperatively in zein protein binding to NPs surface.⁷ We have calculated the value of K_a and *n* from *y*-intercept and the gradient of the best line fit by plotting a double logarithmic plot of [($F_0-F/F-F_{sat}$)] versus log[AuNPs] (Figure S7).

In the present study, we have observed the decrease in fluorescence emission intensity (305 nm), characteristic of zein protein, upon addition of AuNPs which clearly suggest the strong interaction/binding of AuNPs with zein protein domain. Binding constant was calculated for the reaction of zein protein (0.25 μ M in 0.25 mM SDS) with different amounts of AuNPs (0.02 nM, 0.04 nM, 0.06 nM, 0.08 nM, 0.10 nM, 0.12 nM and 0.14 nM, Figure S7). From the binding curves, we have calculated the binding constant ($K_a = 9.22 \times 10^{10}$) and the Hill coefficient, *n*, (cooperativity of binding) was determined to be 1.0279 ± 0.1391 . For fluorescence quenching experiments, the excitation wavelength was fixed at 280 nm, and the emission spectra were collected in the range of 290 to 450 nm with a scanning speed of 100 nm/min. Both excitation and emission slit widths were kept at 10 nm.

Circular Dichroism (CD) studies. The change in the secondary structure of the zein protein was studied using CD spectroscopic experimental studies. The spectra were recorded using a quartz cuvette of the path length of 1.0 cm with a scan speed of 100 nm/ min and a data interval of 0.05nm. We have recorded the CD spectra for AuNPs, zein protein and zein film at room temperature in 10 mM SDS aqueous solution.

Preparation of Zein protein film. Zein protein film was prepared by mixing 0.75 gm of zein and 0.25 gm of glycerol in 10 ml of aqueous ethanol (80% v/v) with freshly prepared AuNPs suspension (1.5 mL, 15% v/v). After proper mixing, 2 mL of this solution was placed in a 15 cm diameter polystyrene petri-dish and gently move to make a layer on the bottom of the plate. After making the uniform layer of the zein-AuNPs solution, we covered the petri-dish with aluminium foil having small holes punched in it and kept on a vibration free surface in an incubator at 37°C for 12 hours. The protein film formation with an average thickness of $\sim 0.02 - 0.10$ mm which was easily peeled off to do different experiments on it.

Gel Electrophoresis: Gel electrophoresis was carried out in order to find the charge on the nanoparticle. 0.2% sucrose gel was made in 1x TBE buffer and kept at 5°C to solidify.

Nanoparticle was loaded with the help of loading dye. The gel was run at 90V in TBE buffer for 45 minutes.

Mechanical Properties: We have also performed the basic experiments to determine the tensile strength and elongation/strain at failure.⁸⁻¹⁰ These two physical parameters are the most important properties of a polymer to be used in industrial applications. We have prepared the AuNPs doped zein thin film with various concentrations of AuNPs (0.025 nM, 0.05 nM, 0.075 nM, 0.10 nM, 0.125 nM, 0.15 nM) and the tensile strength was measured by Universal Testing Machine (UTE-20) and the strain at failure determined by Texture Analyzer (TAXT2, Stable Microsystems, Godalming, U.K.). Both the physical parameters increases as the AuNPs concentration reached to 0.075 nM, however, decreases drastically upon increasing more amount of AuNPs.

Young's modules:

Nano-indentation studies were conducted using AFM to evaluate Young's modulus of the AuNPs doped Zein film. The elastic modulus of zein film was calculated by fitting DMT (Derjaguin, Muller, Toropov) model to the AFM Tip – Sample contact section of the force-distance (f-d) curve and by measuring the adhesion forces between the tip and zein film using the following equation: ^{11, 12}

$$F_{Tip} = \frac{4}{3} E_r \sqrt{Rd^3} + F_{adh} = k(x) \tag{1}$$

Where, E_r , F_{adh} , d, R, k and x are the reduced modulus, adhesion forces between the tip and the counter sample, deformation on the counter sample surface at peak force, tip radius, cantilever spring constant and vertical displacement of the cantilever, respectively. Thus, the reduced modulus (E_r) can be calculated by the given equation.¹²

$$\frac{3(F_{Tip}-F_{adh})}{4\sqrt{Rd^3}} = E_r \tag{2}$$

$$\frac{1}{E_r} = \frac{1 - v_s^2}{E_s} + \frac{1 - v_{Tip}^2}{E_{Tip}}$$
(3)

Where, E_{Tip} , v_{Tip} and v_s are Young's modulus, Poisson's ratio of AFM tip and Poisson's ratio of the counter sample, respectively. Since, $E_{Tip} \gg E_s$ therefore the contribution of the second term in Eq. (3) is negligible and the poisons ratio used for zein is 0.35. Nano-indentation studies in AFM was conducted using a CONTSCR cantilever from Park having ~25kHz resonant frequency and 0.2N/m spring constant was used for the study.

References

- 1 A. Mahal, P. Khullar, H. Kumar, G. Kaur, N. Singh, M. Jelokhani-Niaraki and M. S. Bakshi, ACS Sustainable Chem. Eng., 2013, 1, 627.
- 2 K. K. Li, S. W. Yin, X. Q. Yang, C. H. Tang and Z. H. Wei, J. Agric. Food Chem., 2012, 60, 11592.
- 3 Fluorescence and Phosphorescence of Proteins and Nucleic Acids, S. V. Konev Plenum, 1967.
- 4 J. M. Slocik, R. R. Naik, M. O. Stone and D. W. Wright, Journal of Materials Chemistry, 2005, 15, 749.
- 5 S. P. Boulos, T. A. Davis, J. A. Yang, S. E. Lohse, A. M. Alkilany, L. A. Holland and C. J. Murphy, Langmuir,
- 2013, **29**, 14984.
- 6 Principles of Fluorescence Spectroscopy, J. R. Lakowicz, 2006.
- 7 J. N. Weiss, FASEB J., 1997, 11, 835.
- 8 Q. Wang and G. W. Padua, J. Agric. Food Chem., 2005, 53, 3444.
- 9 Y. Y. Han and L. J. Wang, RSC Advances, 2016, 6, 112317.
- 10 T. B. Rouf, G. Schmidt and J. L. Kokini, J. Mater. Sci., 2018, 53, 7387.
- 11 W. C. Oliver and G. M. Pharr, Journal of Materials Research, 1992, 7, 1564.
- 12 O. Krivosheeva, M. Sababi, A. Dedinaite and P. M. Claesson, Langmuir, 2013, 29, 9551.



Figure S1. (a) Time course of the formation of AuNPs monitored at 550 nm in the reaction of 50 μ l of HAuCl₄ (25mM) with zein protein solution (2 mL, 10 mM SDS) at 65 °C. (b) UV-visible spectral changes showing the increase in the absorbance at 550 nm (absorbance band due to SPR) upon addition of constant amount of HAuCl₄ (100 μ L, 25 mM) to zein protein solution (20 mg in 10 mL) at various temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90°C).



Figure S2. (a) UV-visible spectral changes showing the increase in the absorbance at 550 nm (absorbance due to the SPR band) by addition of varying amounts of zein protein to the HAuCl₄ (0.25 mM) solution in various concentrations (0, 0.02, 0,04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18, 2.0 % w/v). (b) Spectral calibration curve for the formation of peak at 550 nm as a function of various amount of zein protein in increment of 0, 0.02, 0,04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.16, 0.18, 2.0 % w/v (see also Experimental Section).



Figure S3. (a) CD spectral changes showing the increase in the negetive absorbance at 208 nm of zein protein solution at various temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90 °C). (b) CD spectra of zein protein at 80 °C (light gray line) and zein protein with AuNPs (red line) at 45 °C recorded in aqueous medium.



Figure S4. IR spectral changes of zein protein (blue line) in KBr. Red line shows solution IR spectrum of zein coated nanoparticle formed.



Figure S5. Apparent zeta potential of zein coated nanoparticle indicating a surface charge of - 35.8.



Figure S6. Gel electrophoresis of AuNPs (Purple), showing the movement of NPs towards Anode (+), suggesting that the negative charge is present on AuNPs surface.



Figure S7. TEM images of AuNPs coated with zein protein. Figure a & b are low and high magnification images from a region of sample.



Figure S8. (a) Plot of log $[F_o-F/F-F_o]$ vs log [**AuNP**] derived from the fluorescence spectra and the red line depicting the best linear fit. The binding constant K_a and the Hill coefficient n, derived from the intercept and slope is respectively 9.22 x $10^{10} \pm$ and 1.0279 ± 0.1391 . (b) plot of F_0/F vs gold nanoparticle concentration and the red line depicting the best linear fit.



Figure S9. IR spectra of zein coated AuNPs (red line) and zein film doped with AuNPs (pink line).



Figure S10. (a) AFM image of zein protein film without AuNPs. (b) AFM image of zein protein film with AuNPs. (c) and (d) surface roughness line profile for zein protein film without (a) and with (b) AuNPs.



Figure S11. UV-visible spectra of AuNPs doped zein protein film (red line) and zein protein film without AuNPs (black line). The inset shows the difference of absorbance of zein film with AuNPs and only zein film.



Figure S12. Force-Distance curve obtained from Nano-indentation studies of AuNPs doped zein protein film by AFM. Red line indicates approach of the tip and blue line indicates withdrawal of the AFM tip from AuNPs doped zein protein film.