Signatures of Specificity of Interactions of Binary Protein Mixtures with Au Nanoparticles

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Electronic Supplementary Information

Experimental Section

UV-vis measurements

For sets II, III, IV and V the volumes of BSA were kept constant at 20.0, 40.0, 60.0 and 80.0 μL respectively and the volumes of α-amylase and buffer solutions were changed accordingly (so that the final volume of the added binary protein mixture was 160.0 μL). The final concentrations of BSA for sets I, II, III, IV and V were 0.316 μg/mL, 0.633 μg/mL, 1.266 μg/mL, 1.899 μg/mL and 2.532 μg/mL respectively and that of α-amylase varied from 0.024 μg/mL to 0.212 μg/mL (for sets I, II, III, and IV) and from 0.024 μg/mL to 0.188 μg/mL (for set V).

In a different set of experiment, the volume of α-amylase was kept constant at 20.0 μL (0.047 μg/mL) and that of BSA was varied from 10.0 μL to 130.0 μL (0.316 μg/mL - 4.114 μg/mL). However, the final volume of the binary protein mixture added to 3.0 mL of Cit-Au NP dispersion was 160.0 μL and this was adjusted using phosphate buffer as mentioned above. The different amounts of BSA and α-amylase solution present in the 160.0 μL binary protein mixture (used in the UV-visible measurements) are mentioned in Table S1 below.

In case of α-amylase-AMG mixture, the volume of AMG was kept constant at 50.0 μL (0.157 μg/mL) and that of α-amylase was varied from 10.0 μL to 110.0 μL (0.024 μg/mL - 0.259 μg/mL). The final volume of the binary protein mixture (added to 3.0 mL of Cit-Au NP dispersion) was adjusted to 160.0 μL using phosphate buffer. In a different set, the volume of α-amylase was kept constant at 50.0 μL (0.114 μg/mL) and that of AMG varied from 20.0 μL to 0.2 mL (0.046 μg/mL - 0.457 μg/mL). Here the final volume of the binary protein mixture added to 3.0 mL of Cit-Au NP dispersion was adjusted to 0.25 μL with phosphate buffer.

In case of GOD-POD mixture, three different sets of experiments were performed. In set I,

the volume of GOD was kept constant at 60.0 μL (0.903 μg/mL) and that of POD was varied from 10.0 μL to 120.0 μL (0.017 μg/mL - 0.206 μg/mL). In set II, the volume of GOD was kept constant at 30.0 μL (0.045 μg/mL) and that of POD varied from 10.0 μL to 140.0 μL (0.017 μg/mL - 0.240 μg/mL). In set III, the volume of POD was kept constant at 20.0 μL (0.034 μg/mL) and that of GOD varied from 5.0 μL to 160.0 μL (0.008 μg/mL - 0.241 μg/mL). For all the three sets, the final volume of the binary protein mixture added to 3.0 mL of Cit-Au NP dispersion was 180.0 μL (volume was appropriately adjusted using phosphate buffer).

For all the above sets, the UV-vis spectra of the Cit-Au NP dispersion before and after addition of binary protein mixture were recorded for each addition and all the experiments were performed in triplicate. Ratio of area under the extinction spectrum of Cit-Au NPs in presence of proteins to that of Cit-Au NPs only was calculated and plotted against the concentration of a protein in the final NP-protein solution. It may be mentioned here that all the UV-vis spectra were recorded in disposable plastic cuvettes with each cuvette being used for only one measurement.

Modes of addition of component proteins to Cit-Au NP dispersion

For combination I of proteins (30.0 μL α-amylase + 20.0 μL BSA + 110.0 μL sodium phosphate buffer) the following three modes of addition of proteins to the Cit-Au NPs were used: α-amylase and BSA as a mixture, α-amylase followed by BSA, and BSA followed by α-amylase. For the first mode of addition, 3.0 mL of Cit-Au NP dispersion was taken in a plastic cuvette and its UV-vis spectrum was recorded. To this 160.0 μL (30.0 μL α-amylase + 20.0 μL BSA + 110.0 μL buffer, mixed in an eppendorf) of binary protein mixture was added. The concentrations of BSA and α-amylase in the final solution were 0.633 μg/mL and 0.071 μg/mL respectively. The above solution was mixed well and kept for 5 min. UV-vis

spectrum was again recorded and the ratio of area of the final solution to the area of the Cit-Au NP dispersion was calculated. For second mode of addition, a mixture of 30.0 µL of α-amylase and 110.0 µL of buffer was added to 3.0 mL of Cit-Au NP dispersion in a plastic cuvette, mixed well, followed by the addition of 20.0 µL of BSA, they were mixed again and kept for 5 min. The UV-vis spectrum of the Cit-Au NP dispersion was recorded before and after addition of the proteins and the ratio of area of the final solution to the area of the Cit-Au NP dispersion was noted. For third mode, the order of addition of α-amylase and BSA was reversed, keeping the other conditions same. For combination II, the volumes of α-amylase and BSA were changed to 50.0 µL (0.118 µg/mL) and 60.0 µL (1.899 µg/mL) respectively in 160.0 µL binary protein mixture. All the three modes of protein addition as discussed for combination I were followed.

For combination III, the mixture of 50.0 µL α-amylase (0.118 µg/mL), 50.0 µL AMG (0.157 µg/mL) and 60.0 µL phosphate buffer was used. Three modes of addition of binary proteins to Cit-Au NPs were also applied here: α-amylase and AMG as a mixture, α-amylase followed by AMG and AMG followed by α-amylase.

For combination IV, the mixture of 30.0 µL (0.045 µg/mL) GOD, 25.0 µL (0.043 µg/mL) POD and 125.0 µL phosphate buffer was used. Three modes of addition of proteins applied were: GOD and POD as a mixture, GOD followed by POD and POD followed by GOD. All the experiments were performed in triplicates.

SDS-PAGE

A mixture of 2.8 mL of 7.4 µg/mL α-amylase, 1.6 mL of 0.1 mg/mL BSA, and 2.0 mL of sodium phosphate buffer was added to 120.0 mL of Cit-Au NP dispersion, mixed well and kept for 5 min. The final concentrations of α-amylase and BSA were 0.165 µg/mL and 1.266 µg/mL respectively. The above solution was centrifuged at 22000 rpm and 4°C for 30 min.

The supernatant was discarded and the pellet was re-suspended in sodium phosphate buffer. The NP-protein composite was recovered again by centrifugation under the same conditions. The composite was re-suspended in 150.0 μL of phosphate buffer. Protein loading buffer (final 1x concentration) was added to each of NP-protein solution, 0.074 mg/mL α-amylase solution, 0.1 mg/mL BSA solution, Cit-Au NP dispersion, and protein molecular weight marker (14.3 kDa – 97.4 kDa, Bangalore Genei). These solutions were boiled for 5 min and an equal volume was loaded in 12% polyacrylamide gel. Gel electrophoresis was performed at 100V for about 2 hours, until the dye front reached at the end of the gel. The gel was stained using silver staining method[1] and then photographed.

Enzymatic assay of α-amylase

Starch agar solution (3%) was prepared in Milli-Q grade water, autoclaved, and poured into a petri plate. After solidification of agar, five distinct wells were punctured into the agar plate and filled with 200 μL of sodium phosphate buffer, 7.4 μg/mL α-amylase solution, 0.1 mg/mL BSA solution, Cit-Au NPs only, and NP-protein composite (having the same concentration as in SDS-PAGE) respectively. The plate was incubated at 37 °C overnight, and α-amylase activity was tested by flooding the plate with iodine solution and observing zone of clearance around the wells. The same experiment was also repeated with three different combinations of α-amylase and BSA. The concentrations of α-amylase and BSA used were 1.266 μg/mL BSA and 0.094 μg/mL α-amylase, 1.266 μg/mL BSA and 0.165 μg/mL α-amylase and 1.2666 μg/mL BSA and 0.235 μg/mL α-amylase.

Fluorescence measurements

3.0 mL of sodium phosphate buffer solution was taken in a fluorescence cuvette and to it 160.0 μL (50.0 μL α-amylase + 50.0 μL BSA + 60.0 μL buffer) of binary protein mixture was added and its emission spectrum was recorded using a Fluoromax 4 spectrofluorometer with a xenon lamp. The concentrations of BSA and α-amylase in the final solution were

1.582 μg/mL and 0.118 μg/mL respectively. Excitation wavelength was set at 280 nm. The excitation and emission slit widths were fixed at 5 nm. Similarly, the emission spectrum of 160.0 μL of the above mentioned binary mixture of proteins in the presence of 3.0 mL of Cit-Au NP dispersion was recorded, the protein mixture being added drop wise to the Cit-Au NPs. Before recording the spectrum the sample was mixed well and left for 5 min. The emission spectra of the above mentioned binary protein mixture were also recorded in presence of 3.0 mL Cit-Au NPs following the addition of the individual proteins one after the other (rather than mixing them together before addition). In one case α-amylase was added before BSA and in another case the order of addition was reversed. The emission spectrum of 0.118 μg/mL α-amylase in presence of 3.0 mL Cit-Au NP dispersion was recorded and compared with its emission spectrum in absence of Cit-Au NPs (i.e. in buffer only). Similarly emission spectra of 1.582 μg/mL BSA in presence and absence of 3.0 mL Cit-Au NP dispersion were recorded. The above mentioned binary mixture of proteins was added drop wise to 3.0 mL of Cit-Au NP dispersion and the time-dependent fluorescence measurement was made. Spectra were recorded after 1, 3, 5, and 10 min from the time of addition of binary mixture of proteins to Cit-Au NP dispersion.

Sample preparation for FTIR measurements

The dispersion of Cit-Au NPs containing 0.118 μg/mL α-amylase and 1.899 μg/mL BSA was centrifuged at 22 000 rpm and 4°C for 30 min. The supernatant was discarded and the pellet was lyophilized (using Christ Alpha 1-4 LD lyophilizer). Dispersion of Cit-Au NP only was also lyophilized after centrifugation. The lyophilized products were analyzed by FTIR. FTIR spectra of trisodium citrate 2-hydrate, α-amylase, BSA, and the above lyophilized products were recorded, by forming disc with KBr, using a Perkin Elmer Spectrum One Spectrometer. The scan was performed in the range of 400-4000 cm^{-1} in transmittance mode.

DLS measurements

DLS measurements were carried out for GOD and POD mixture. Cit-Au NP dispersion was taken in a quartz cuvette and its particle size distribution was measured using a Zetasizer Nano ZS90 (Model No ZEN3690). To this mixture 0.045 μg/mL GOD and 0.017 μg/mL POD was added, mixed well and kept for 5 min. The particle size distribution of this solution was recorded again. Similar addition of GOD and POD binary mixture were made to fresh Cit-Au NPs keeping the concentration of GOD constant and varying the concentration of POD from 0.017 μg/mL to 0.240 μg/mL. In a different set the concentration of GOD was fixed at 0.903 μg/mL and POD was varied from 0.017 μg/mL to 0.206 μg/mL. The particle size distribution was recorded after the addition of each binary mixture to Cit-Au NP dispersion. Measurements were carried out at 25°C using a sample volume of 1.5 mL. Each sample was measured in duplicate and the wavelength of the HeNe laser used for the DLS instrument was 633 nm.

Results

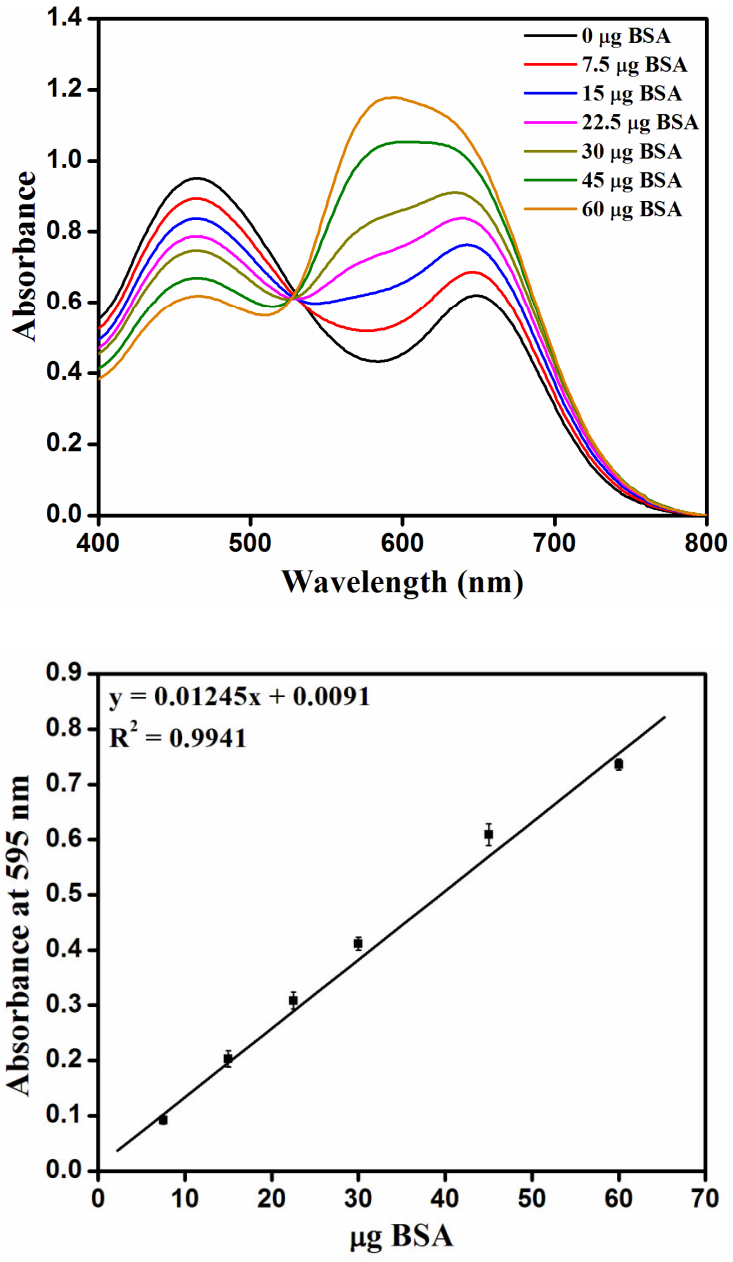

Fig. S1 Graphs representing Bradford assay of proteins. (**A**) UV-vis spectra of Serva Blue G dye in the presence of various amounts of BSA as mentioned in the legends. (**B**) Standard curve representing absorbance at 595 nm versus amount of BSA (in µg).

Table S1 Combination of amount of α-amylase and BSA in their binary mixtures used for different sets of experiments in UV-vis measurements (the respective concentrations being mentioned in the manuscript). The concentrations of BSA and α-amylase stock solutions used were 0.1 mg/mL and 7.4 µg/mL respectively.

	α-amylase (µL)	**Buffer (µL)**
BSA = 10 µL	10	140
	20	130
	30	120
	40	110
	50	100
	60	90
	70	80
	80	70
	90	60

	α-amylase (µL)	**Buffer (µL)**
BSA = 20 µL	10	130
	20	120
	30	110
	40	100
	50	90
	60	80
	70	70
	80	60
	90	50

	α-amylase (µL)	**Buffer (µL)**
BSA = 40 µL	10	110
	20	100
	30	90
	40	80
	50	70
	60	60
	70	50
	80	40
	90	30

	α-amylase (µL)	**Buffer (µL)**
BSA = 60 µL	10	90
	20	80
	30	70
	40	60
	50	50
	60	40
	70	30
	80	20
	90	10

	α-amylase (µL)	**Buffer (µL)**
BSA = 80 µL	10	70
	20	60
	30	50
	40	40
	50	30
	60	20
	70	10
	80	0

	BSA (µL)	**Buffer (µL)**
α-amylase = 20 µL	10	130
	30	110
	50	90
	70	70
	90	50
	110	30
	130	10
	-	-

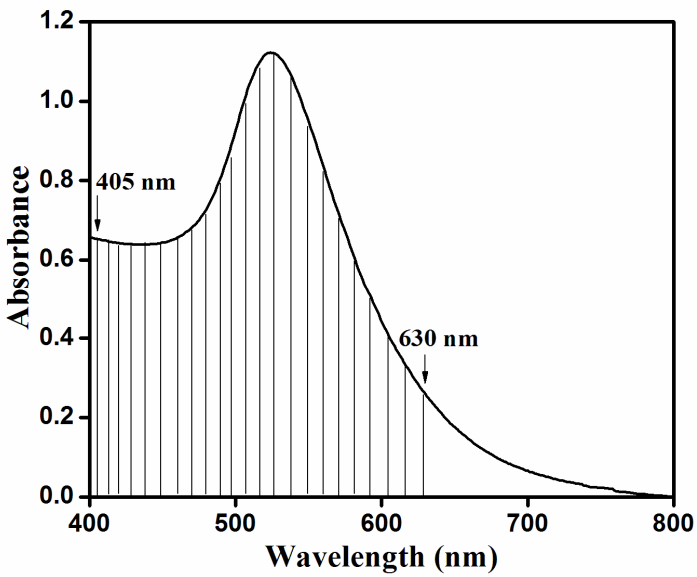

Fig. S2 A typical graph showing the area under the curve, after selecting the wavelength region, using the software associated with the UV-vis spectrophotometer.

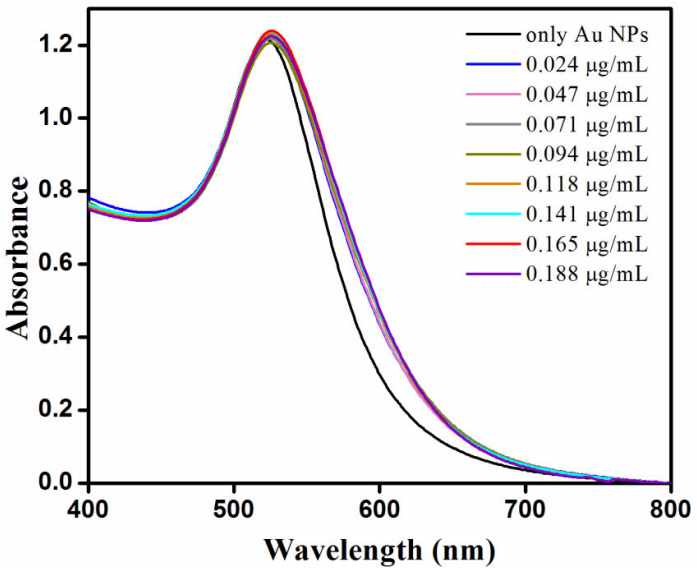

Fig. S3 SPR spectra of Cit-Au NP dispersion before and after addition of binary protein mixtures having different fractional content of α-amylase and BSA. Concentration of α-amylase was varied from 0.024 µg/mL to 0.188 µg/mL (the legends refer to different concentrations), keeping the concentration of BSA fixed at 2.532 µg/mL.

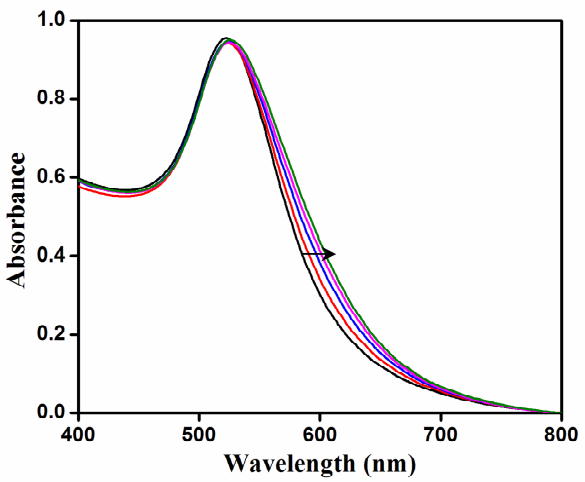

Fig. S4 SPR spectra of Cit-Au NP dispersion before and after addition of binary protein mixtures having different fractional content of α-amylase and BSA. Concentration of BSA was varied from 0.316 µg/mL to 2.215 µg/mL, keeping the concentration of α-amylase constant at 0.047 µg/mL. The arrow in the figure shows the increase in broadening of the SPR spectrum of Cit-Au NPs with increasing concentration of BSA.

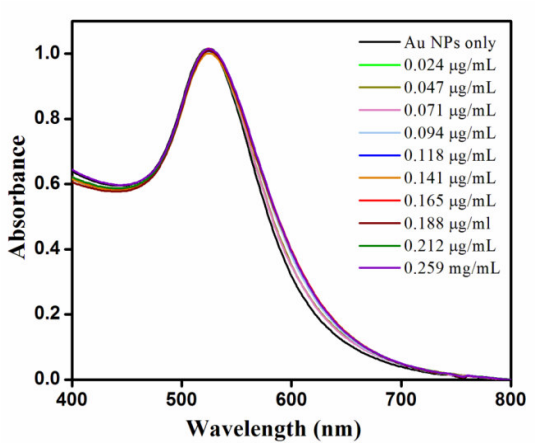

Fig. S5 SPR spectra of Cit-Au NP dispersion before and after addition of binary protein mixtures having different fractional content of α-amylase and AMG. Concentration of α-amylase was varied from 0.024 µg/mL to 0.259 µg/mL (the legends refer to different concentrations), keeping the concentration of AMG constant at 0.157 µg/mL.

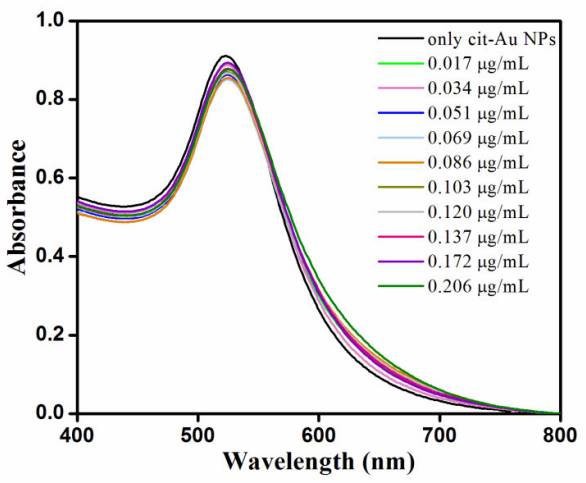

Fig. S6 SPR spectra of Cit-Au NP dispersion before and after addition of binary protein mixtures having different fractional content of GOD and POD. Concentration of POD was varied from 0.017 μg/mL to 0.206 μg/mL (the legends refer to different concentrations), keeping the concentration of GOD constant at 0.903 μg/mL.

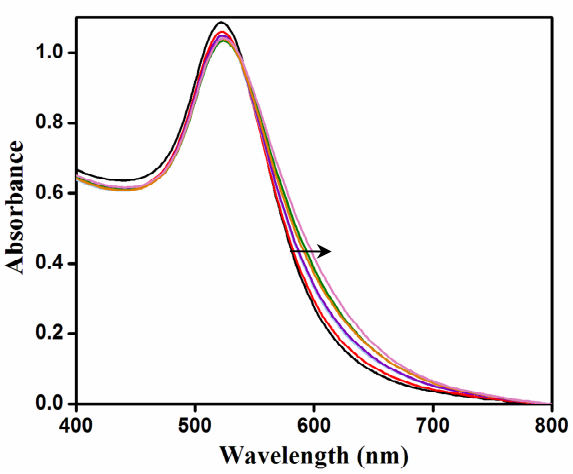

Fig. S7 SPR spectra of Cit-Au NP dispersion before and after addition of binary protein mixtures having different fractional content of GOD and POD. Concentration of POD was varied from 0.017 μg/mL to 0.146 μg/mL, keeping the concentration of GOD constant at 0.045 μg/mL. The arrow in the figure shows the increase in broadening of the SPR spectrum of Cit-Au NPs with increasing concentration of POD.

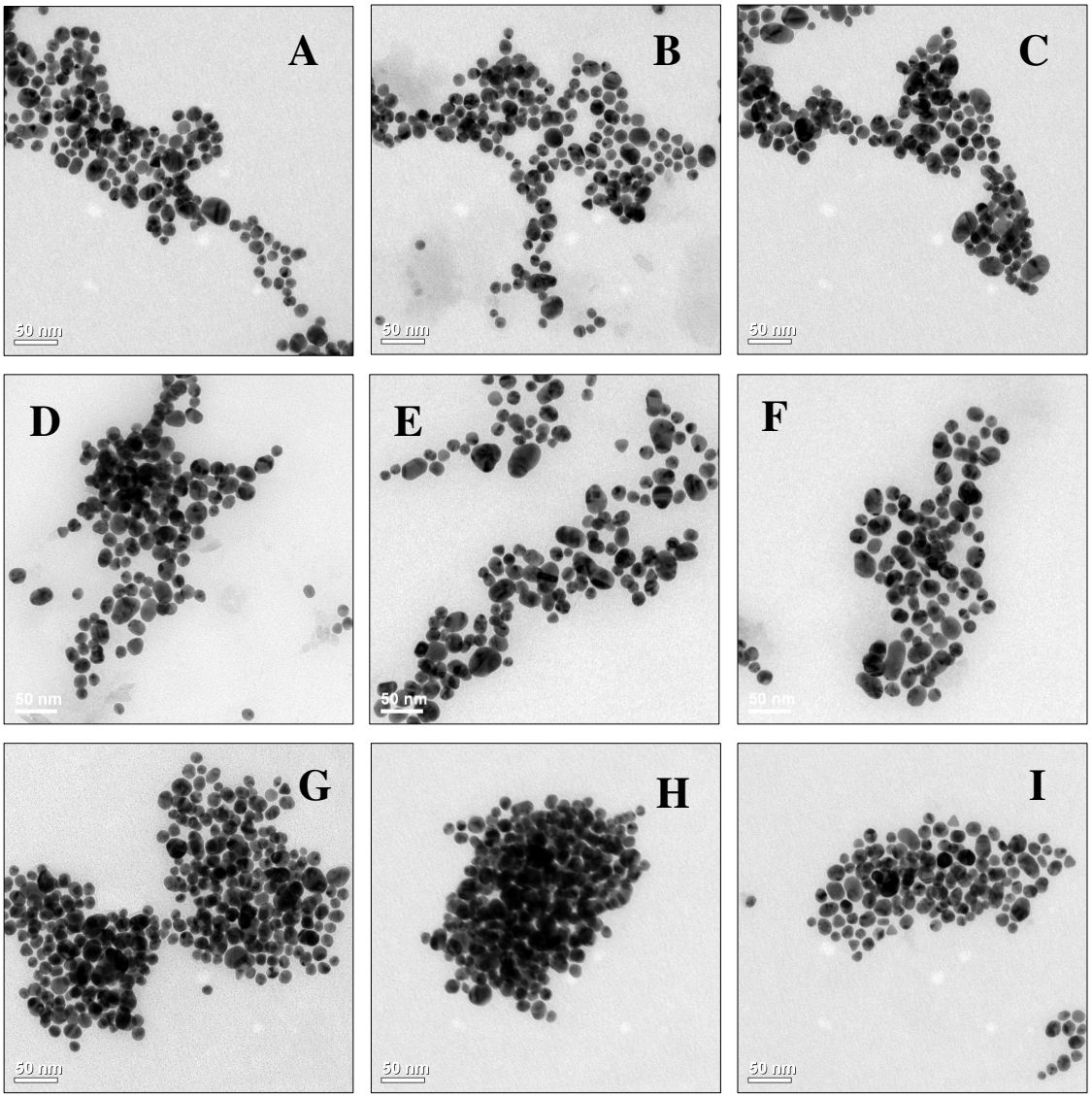

Fig. S8 TEM images of **(A, B, C)** Cit-Au NPs in presence of 0.071 µg/mL α-amylase and 0.316 µg/mL BSA, **(D, E, F)** Cit-Au NPs in presence of 0.071 µg/mL α-amylase and 1.899 µg/mL BSA and **(G, H, I)** Cit-Au NPs in presence of 0.094 µg/mL α-amylase and 1.266 µg/mL BSA. Scale bar is 50 nm in all.

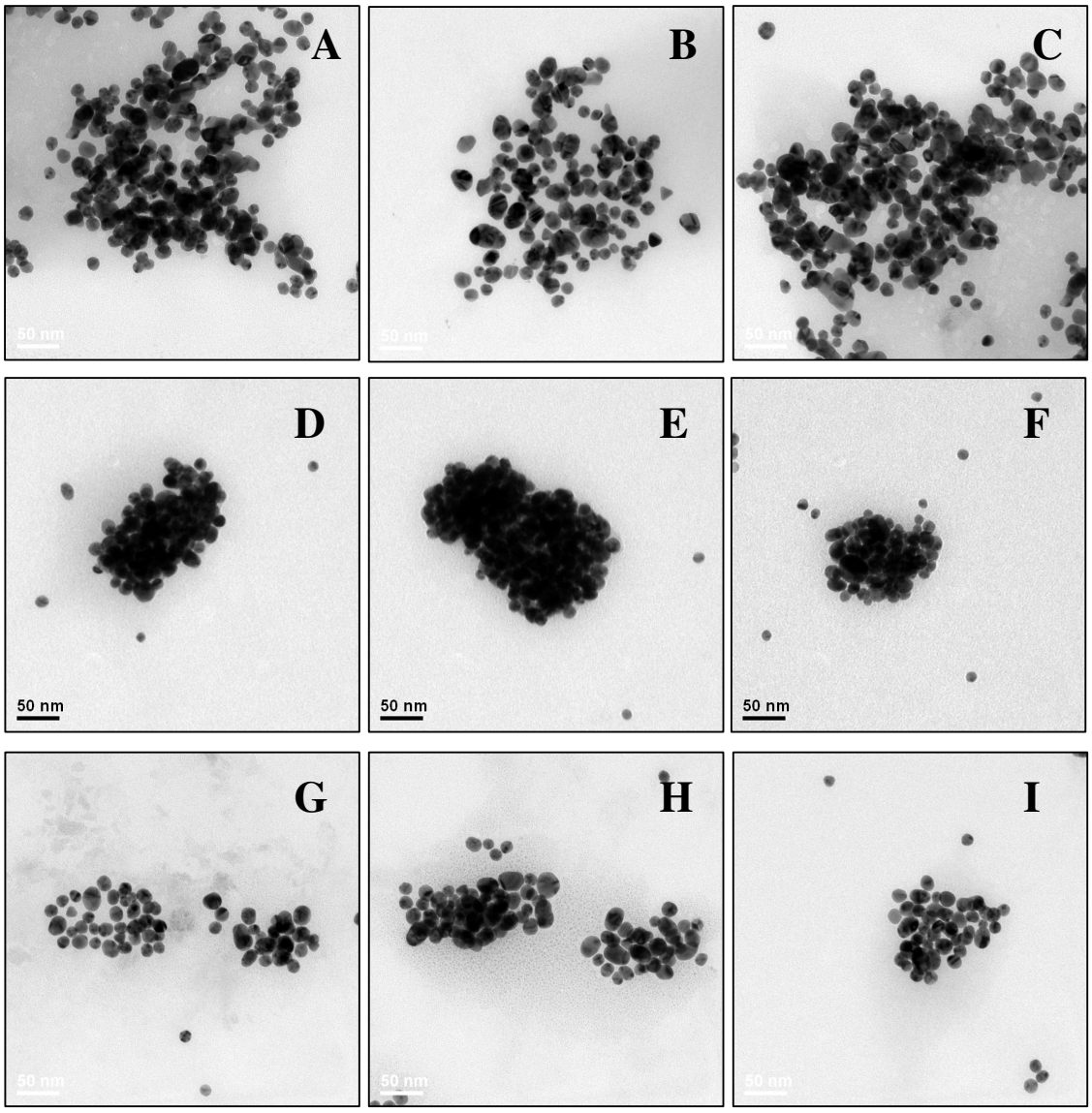

Fig. S9 TEM images of **(A, B, C)** Cit-Au NPs in presence of mixture of 0.118 µg/mL α-amylase and 0.157 µg/mL AMG, **(D, E, F)** Cit-Au NPs in presence of mixture of 0.045 µg/mL GOD and 0.043 µg/mL POD and **(G, H, I)** Cit-Au NPs in presence of mixture of 0.903 µg/mL GOD and 0.051 µg/mL POD. Scale bar is 50 nm in all.

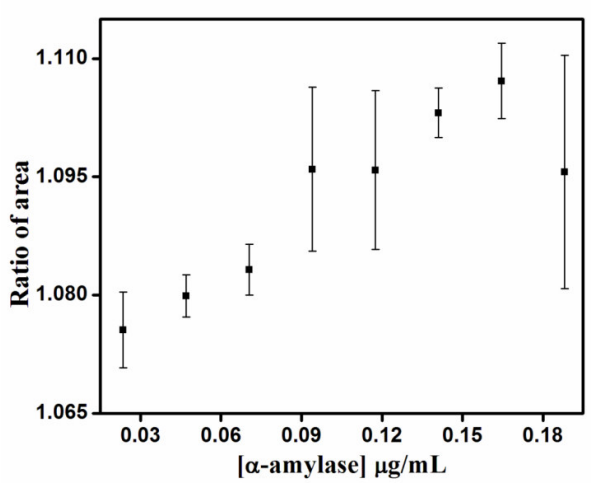

Fig. S10 Ratio of area under the UV-vis spectrum of Cit-Au NP dispersion in presence of different fractional content of α-amylase and BSA to that of Cit-Au NPs only plotted against α-amylase concentration (µg/mL). Concentration of α-amylase was varied from 0.024 µg/mL to 0.188 µg/mL, keeping the concentration of BSA fixed at 2.532 µg/mL. The error bars were calculated from the results of three independent experiments.

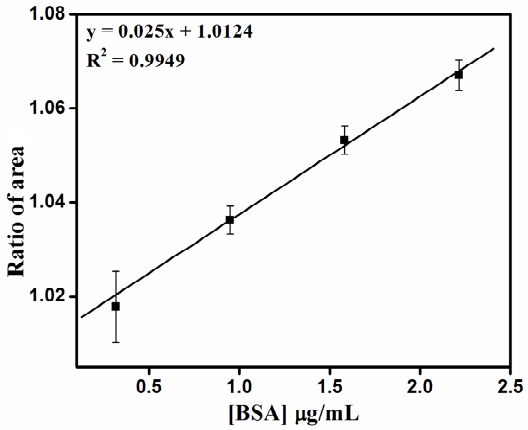

Fig. S11 Ratio of area under the UV-vis spectrum of Cit-Au NPs in presence of different fractional content of α-amylase and BSA to that of Cit-Au NPs only plotted against BSA concentration (µg/mL). Concentration of BSA was varied from 0.316 µg/mL to 2.215 µg/mL, keeping the concentration of α-amylase constant at 0.047 µg/mL. The error bars were calculated from the results of three independent experiments.

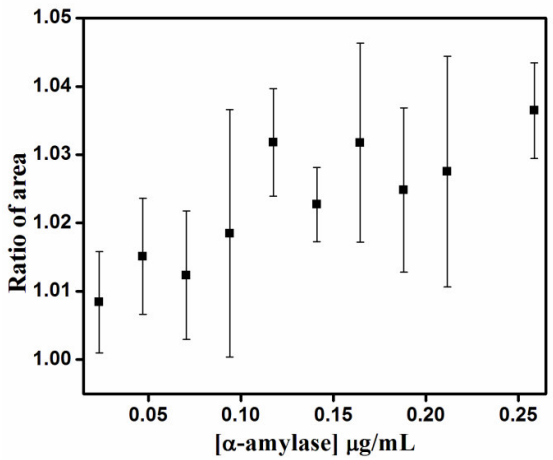

Fig. S12 Ratio of area under the UV-vis spectrum of Cit-Au NPs in presence of different fractional content of α-amylase and AMG to that of Cit-Au NPs only plotted against α-amylase concentration (μg/mL). Concentration of α-amylase was varied from 0.024 μg/mL to 0.259 μg/mL, keeping the concentration of AMG constant at 0.157 μg/mL. The error bars were calculated from the results of three independent experiments.

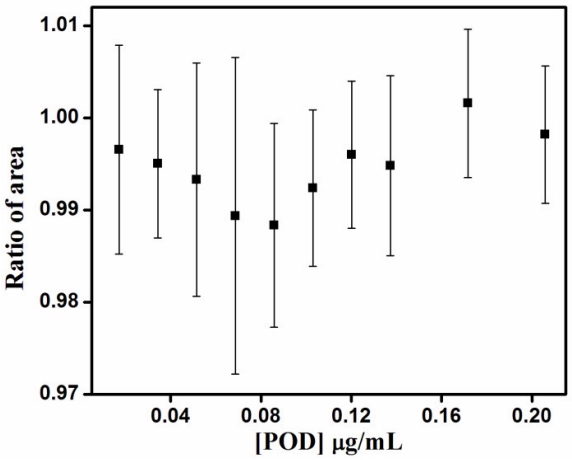

Fig. S13 Ratio of area under the UV-vis spectrum of Cit-Au NPs in presence of different fractional content of GOD and POD to that of Cit-Au NPs only plotted against POD concentration (μg/mL). Concentration of POD was varied from 0.017 μg/mL to 0.206 μg/mL, keeping the concentration of GOD constant at 0.903 μg/mL. The error bars were calculated from the results of three independent experiments.

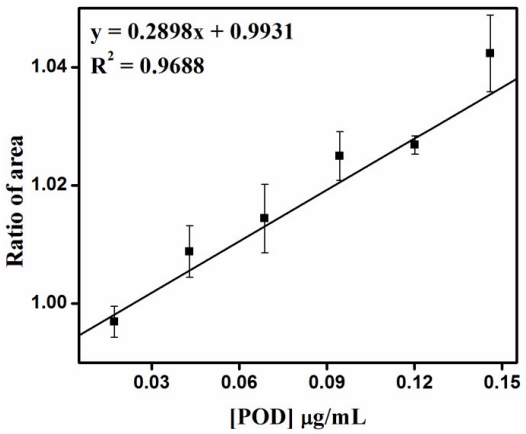

Fig. S14 Ratio of area under the UV-vis spectrum of Cit-Au NPs in presence of different fractional content of GOD and POD to that of Cit-Au NPs only plotted against POD concentration (µg/mL). Concentration of POD was varied from 0.017 µg/mL to 0.146 µg/mL, keeping the concentration of GOD constant at 0.045 µg/mL. The error bars were calculated from the results of three independent experiments.

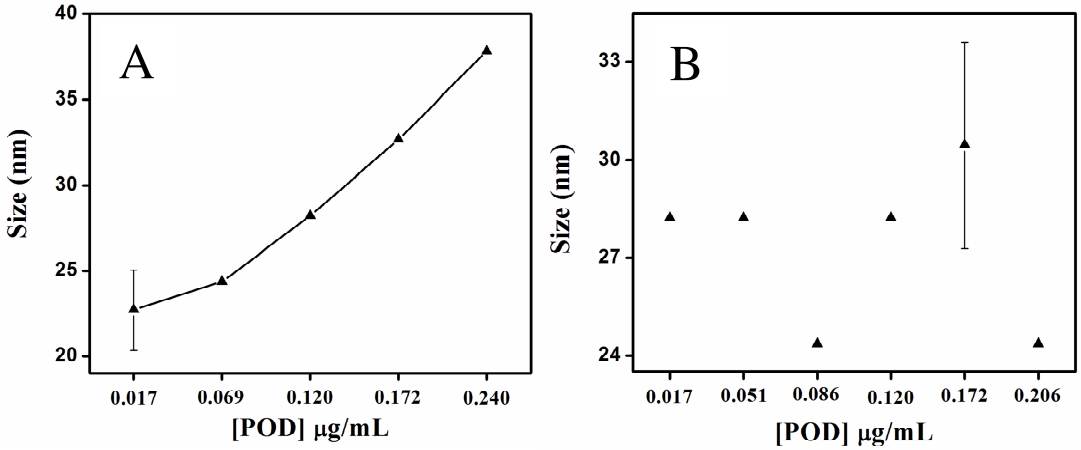

Fig. S15 DLS analysis of Cit-Au NPs in presence of binary mixtures of GOD and POD. Concentration of POD was changed keeping the concentration of GOD constant. The size referred here corresponds to the maximum of the particle size distribution curve. (**A**) is for low concentration of GOD i.e 0.045 µg/mL and (**B**) is for high concentration of GOD i.e 0.903 µg/mL. The error bars were calculated from the results of two independent measurements carried out for the same sample.

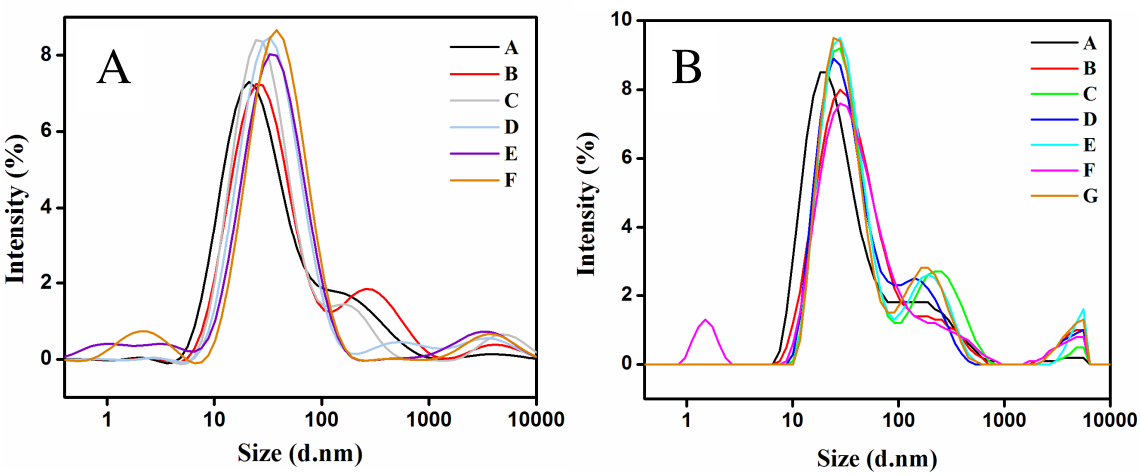

Fig. S16 **(A)** Particle size distribution curves of Cit-Au NPs in presence of GOD and POD in the linear regime. The legends refer to (**A**) Cit-Au NPs only, (**B**) Cit-Au NPs + 0.045 µg/mL GOD + 0.017 µg/mL POD, (**C**) Cit-Au NPs + 0.045 µg/mL GOD + 0.069 µg/mL POD, (**D**) Cit-Au NPs + 0.045 µg/mL GOD + 0.120 µg/mL POD, (**E**) Cit-Au NPs + 0.045 µg/mL GOD + 0.172 µg/mL POD and (**F**) Cit-Au NPs + 0.045 µg/mL GOD + 0.240 µg/mL POD.

(B) Particle size distribution curves of Cit-Au NPs in presence of GOD and POD in the non-linear regime. The legends refer to (**A**) Cit-Au NPs only, (**B**) Cit-Au NPs + 0.903 µg/mL GOD + 0.017 µg/mL POD, (**C**) Cit-Au NPs + 0.903 µg/mL GOD + 0.051 µg/mL POD, (**D**) Cit-Au NPs + 0.903 µg/mL GOD + 0.086 µg/mL POD, (**E**) Cit-Au NPs + 0.903 µg/mL GOD + 0.120 µg/mL POD, (**F**) Cit-Au NPs + 0.903 µg/mL GOD + 0.172 µg/mL POD and (**G**) Cit-Au NPs + 0.903 µg/mL GOD + 0.206 µg/mL POD.

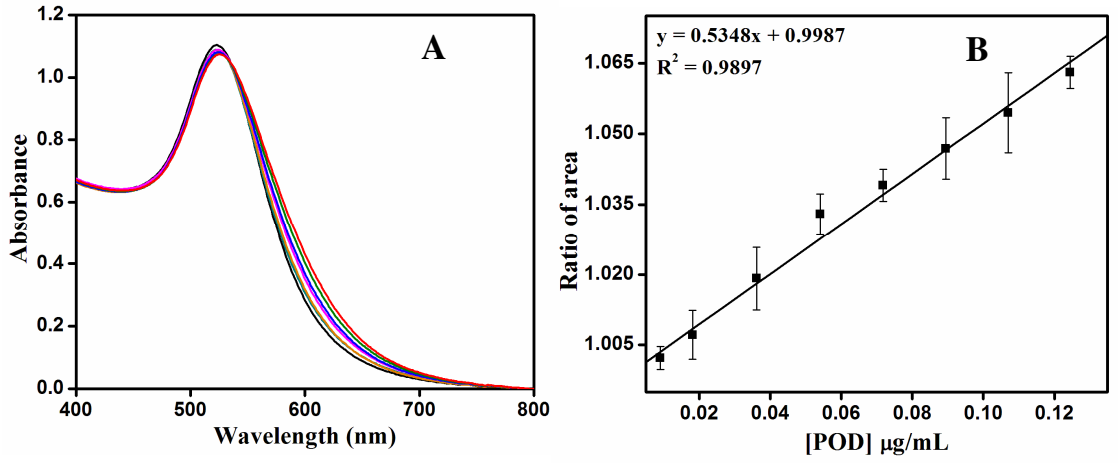

Fig. S17 (A) SPR extinction spectra of Cit-Au NP in the presence of increasing POD concentration. (B) Plot of the ratio of area under the extinction spectrum of Cit-Au NPs in presence of POD to that of Cit-Au NPs only as a function of concentration.

POD sensitivity: 0.009 µg/mL to 0.124 µg/mL.

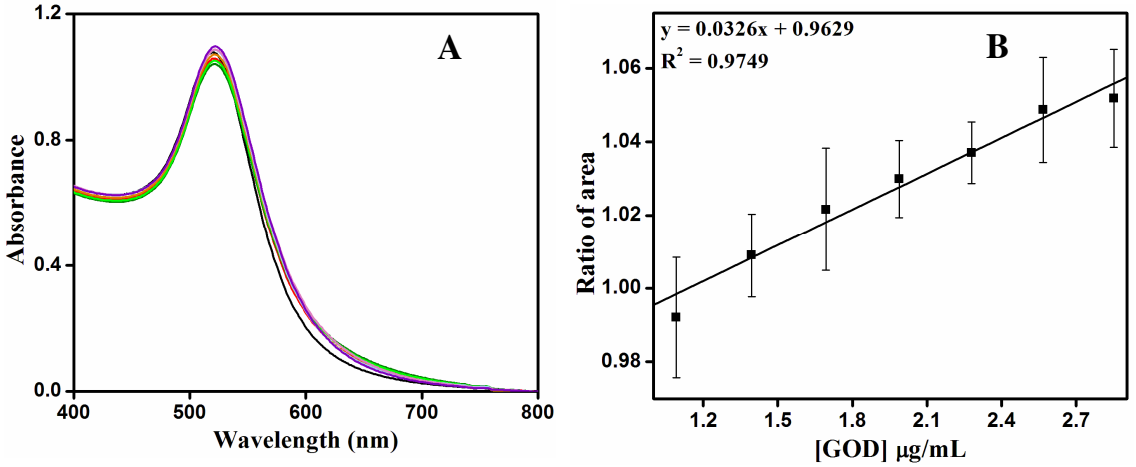

Fig. S18 (A) SPR extinction spectra of Cit-Au NP in the presence of increasing GOD concentration. (B) Plot of the ratio of area under the extinction spectrum of Cit-Au NPs in presence of POD to that of Cit-Au NPs only as a function of concentration. GOD sensitivity: 1.091 µg/mL to 2.851 µg/mL.

Table S2 The sequence of addition of α-amylase and BSA to the Cit-Au NPs dispersion to reach the final constant composition of the mixture and corresponding ratio of area under the extinction curves.

Composition of the final NP-protein solution	Ratio of area
3.0 mL Cit-Au NPs + (0.633 μg/mL α-amylase + 0.071 μg/mL BSA)	1.028 ± 0.010
(3.0 mL Cit-Au NPs + 0.633 μg/mL α-amylase) + 0.071 μg/mL BSA	1.025 ± 0.003
(3.0 mL Cit-Au NPs + 0.071 μg/mL BSA) + 0.633 μg/mL α-amylase	1.043 ± 0.008

Table S3 The sequence of addition of α-amylase and BSA to the Cit-Au NPs dispersion reach the final constant composition of the mixture and corresponding ratio of area under the extinction curves.

Composition of the final NP-protein solution	Ratio of area
3.0 mL Cit-Au NPs + (0.118 μg/mL α-amylase + 1.899 μg/mL BSA)	1.037 ± 0.007
(3.0 mL Cit-Au NPs + 0.118 μg/mL α-amylase) + 1.899 μg/mL BSA	1.039 ± 0.005
(3.0 mL Cit-Au NPs + 1.899 μg/mL BSA) + 0.118 μg/mL α-amylase	1.048 ± 0.006

Table S4 The sequence of addition of α-amylase and AMG to the Cit-Au NPs dispersion to reach the final constant composition of the mixture and corresponding ratio of area under the extinction curves.

Composition of the final NP-protein solution	Ratio of area
3.0 mL Cit-Au NPs + (0.118 μg/mL α-amylase + 0.157 μg/mL AMG)	1.032 ± 0.004
(3.0 mL Cit-Au NPs + 0.118 μg/mL α-amylase) + 0.157 μg/mL AMG	1.037 ± 0.006
(3.0 mL Cit-Au NPs + 0.157 μg/mL AMG) + 0.118 μg/mL α-amylase	1.087 ± 0.014

Table S5 The sequence of addition of GOD and POD to the NP to reach the final constant composition of the mixture and corresponding ratio of area under the extinction curves.

Composition of the final NP-protein solution	Ratio of areas
3.0 mL Cit-Au NPs + (0.045 µg/mL POD + 0.043 µg/mL GOD)	1.001 ± 0.004
(3.0 mL Cit-Au NPs + 0.045 µg/mL POD) + 0.043 µg/mL GOD	1.001 ± 0.001
(3.0 mL Cit-Au NPs + 0.043 µg/mL GOD) + 0.045 µg/mL POD	1.017 ± 0.004

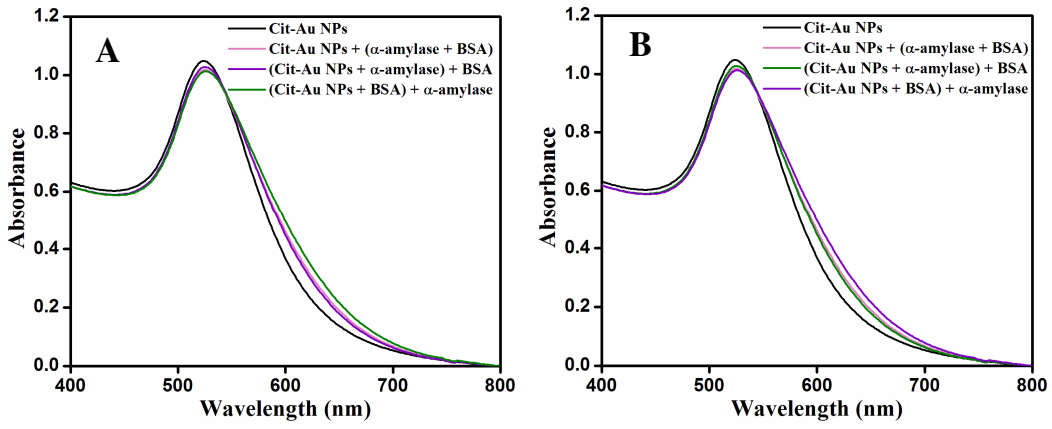

Fig. S19 SPR extinction spectra of Cit-Au NPs in absence and presence of binary mixture of proteins. **(A)** in presence of 0.633 µg/mL α-amylase and 0.071 µg/mL BSA and (B) in presence of 0.118 µg/mL α-amylase and 1.899 µg/mL BSA. The two proteins α-amylase and BSA were added following three different modes as mentioned in the text of the manuscript.

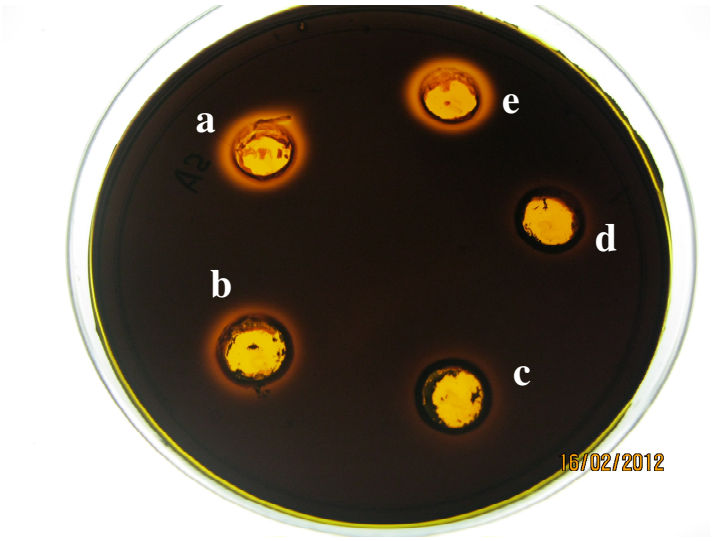

Fig. S20 Starch-agar plate assay for enzymatic activity of (**a**) pure α-amylase solution of 0.165 µg/mL, (**b**) composite of Au NPs containing 1.266 µg/mL BSA and 0.235 µg/mL α-amylase (**c**) composite of Au NPs containing 1.266 µg/mL BSA and 0.094 µg/mL α-amylase (**d**) composite of Au NPs containing 1.266 µg/mL BSA and 0.165 µg/mL α-amylase and (**e**) pure α-amylase solution of 0.235 µg/mL.

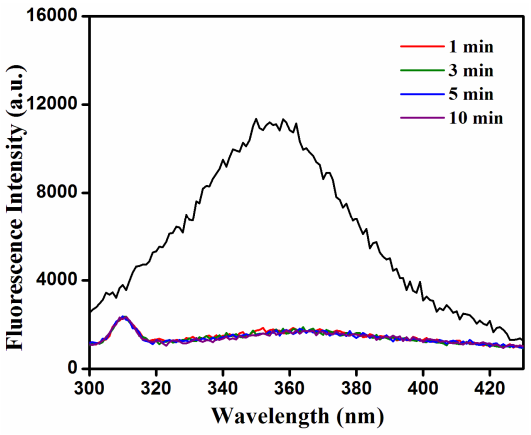

Fig. S21 Fluorescence spectra of binary mixture of α-amylase (0.118 µg/mL) and BSA (1.582 µg/mL) in the presence and absence of Cit-Au NPs at different times following sample preparation.

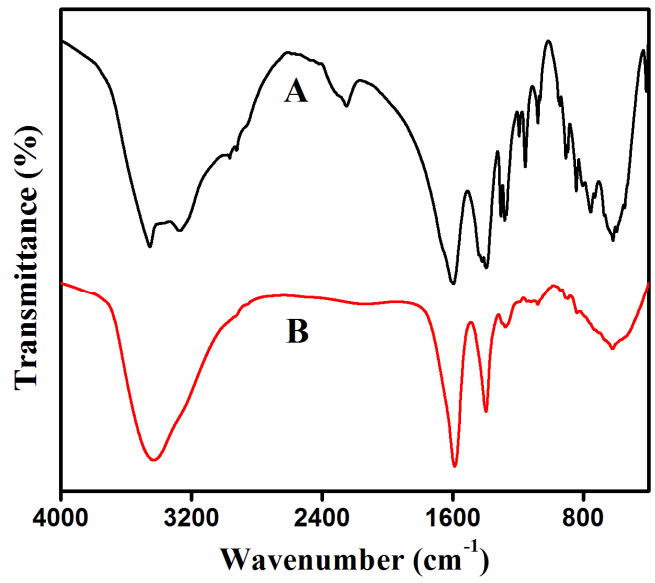

Fig. S22 FTIR spectra of (A) trisodium citrate 2-hydrate and (B) Cit-Au NPs only.

Reference

1. L. A. Echan, D. W. Speicher, *Current Protocols in Protein Science*; John Wiley & Sons, Inc., 2007.