Biomimetically Crystallized Protease-Resistant Zinc Phosphate decorated with Gold Atomic Clusters for Bio-Imaging

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Electronic Supplementary Information

1. Experimental Section

1.1. Materials

Gold(III) chloride solution (30 wt %, HCl, Sigma Aldrich, USA), bovine serum albumin (BSA, Sisco Research Laboratories Pvt. Ltd., India), sodium hydroxide (Merck, India), proteinase K (Sigma Aldrich, USA), zinc acetate dihydrate (Merck, India), sodium dihydrogen phosphate dihydrate (NaH₂PO₄.2H₂O), Merck, India), di-Sodium hydrogen phosphate dihydrate (Na₂HPO₄.2H₂O, Merck, India), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Himedia, India) and sinapinic acid (Sigma-Aldrich USA) were purchased and used as received without further purification. Milli-Q grade water (18.2 MΩ cm, Millipore) was used for all experimental work.

Cell Culture Studies: Human cervical carcinoma cells, (HeLa) for cellular study were acquired from the National Centre for Cell Sciences (NCCS), Pune, India. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (PAA Laboratories, Austria), L-glutamine (4mM), penicillin (50 units/mL), streptomycin (50 mg/mL, Sigma-Aldrich) and maintained in 5% CO₂ humidified incubator at 37 °C.

1.2. Synthesis of BSA stabilised gold nanoclusters (BSA-Au-NCs):

The synthesis of BSA stabilised Au nanoclusters was carried out following a report by Xie et al.¹ An aqueous solution of HAuCl₄ (2.5 mL, 10 mM) was added dropwise to an aqueous solution of BSA (2.5 mL, 50 mg/mL) under vigorous stirring at 37 °C. After stirring for 3 minutes, freshly made NaOH solution (350 μ L, 1M) was introduced to the reaction mixture and pH adjusted to ~12. The reaction was allowed to proceed under this condition for 12 h at 37 °C which resulted in the appearance of deep brown color solution, ensuring the completion of reaction.

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1.3. Preparation of Phosphate Buffer (pH=7.4) :

The phosphate buffer solution of pH=7.4 was made by mixing 18 mL of NaH₂PO₄ (0.02M, monobasic) and 82 mL of Na₂HPO₄ (0.02M, dibasic) stock solutions. The resulting solution was mixed thoroughly and pH was tested to be 7.4. The final volume of the solution was then adjusted to 200 mL.

1.4. Proteolytic digestion of BSA-Au-NCs:

The proteolytic digestion was carried out in phosphate buffer pH=7.4 by proteinase K at 37 °C.² In a 3 mL fluorescence cuvette, 100 µL of as synthesized BSA-Au-NC dispersion was added to 2.3 mL phosphate buffer at 37 °C. After 5 minutes of incubation at 37 °C, proteinase K (100 µL, 0.8 mg/mL) was added and the mixture was further incubated for 90 min. The proteolytic process was monitored by recording the time dependent emission spectra of BSA-Au-NC at an excitation wavelength of 500 nm. It may be mentioned here that protease treatment was carried out for 90 min because experimental results demonstrated very minute drop in PL intensity beyond 90 min of incubation, reaching a saturation at the end of 120 min. (Fig. S2) Control experiment, without the addition of proteinase K under similar experimental conditions, was also carried out and no change in luminescence intensity was observed. Proteinase K, a serine protease is known to cleave BSA into 313 fragments at the C-terminal site generating peptide fragments bearing free carboxylic group and amine group.

1.5. Formation of Au-NC decorated zinc phosphate nanocrystal (Au-NC-Zn₃(PO₄)₂):

Following proteolytic digestion of BSA-Au-NCs, the temperature of digested Au-NC suspension was then fixed at 27 °C. This was then followed by addition of zinc acetate

solution (300 µL, 68.3 mM) to the digested Au-NC dispersion. In brief, to 2.5 mL digested Au-NC dispersion, 50 µL of zinc acetate solution was added successively and the emission spectra recorded simultaneously at an interval of 1 min each. This led to gradual recovery of fluorescence which reached a saturation in emission maxima after final addition of 300 µL, $[Zn^{2+}] = 68$ mM. Immediately, after addition of zinc acetate salt solution, white suspension of zinc phosphate appeared which on standing for ~ 1 h settled down to form light brown powder of Au NC decorated with zinc phosphate (Au-NC-Zn₃(PO₄)₂). The powder so obtained was dried under vacuum for further characterization.

1.6. Synthesis of zinc phosphate hydrate:

A 6 mL of 100 mM $Zn(NO_3)_2.6H_2O$ solution was added to 20 mL Milli-Q water. This is followed by dropwise addition of 5 mL of 100 mM $Na_3PO_4.12H_2O$ solution under constant stirring at 27 °C. The mixture was stirred for 6 h and the resultant white precipitate was collected by centrifugation and finally redispersed in 20 mL Milli-Q water.

2. Characterization

UV-vis spectra were recorded using Perkin Elmer Lambda 750 UV-vis spectrophotometer. Fluorescence studies were carried out in HORIBA Jobin Yvon FluoroMax-4 spectrofluorimeter. FT-IR spectra were recorded in Perkin Elmer Spectrophotometer (Model- spectrum one) in the range 450-4000 cm⁻¹ for solid samples using KBr pellet method. The solid samples for FTIR were prepared by drying the samples (aqueous dispersion) in dessicator overnight. Matrix assisted laser desorption ionization (MALDI-TOF) spectra for the samples were obtained using Applied Bio systems 4800 Plus MALDI TOF/TOF analyser employing sinapinic acid as the matrix. Particle size and morphologies of all samples were obtained using JEOL JEM 2100 transmission electron microscope (TEM), operating at maximum operating voltage of 200 kV. Sample preparation for TEM was done by drop casting 7 µL of colloidal dispersion on the carbon coated copper grid and which was allowed to air dry. Powder X-ray diffraction (PXRD) data of as synthesized Au NC-Zn₃(PO₄)₂ was acquired using a Bruker AXS D8 Advance x-ray diffractometer with CuK_{$\alpha1$} radiation (λ^{\sim} 1.54060 Å), under 40 kV and 40 mA operating condition. Field emission scanning electron microscopy (FESEM) was carried out in a IGMA, Carl Zeiss FESEM. For sample preparation, 15 µL dispersion of Au NC decorated zinc phosphate nanocomposite was drop-casted on a pre-cleaned glass slide (wrapped with an aluminium foil), dried overnight in vacuum and further double coated with a gold film before sample mounting using SC7620 "Mini", Polaron Sputter Coater, Quorum Technologies, Newhaven, England. Brunauer-Emmett-Teller (BET) surface area and Barrett-Joyner-Halenda (BJH) average pore width of the samples were analysed using Beckman-Coulter SA 3100 N₂ adsorptiondesorption analyzer by degassing the samples at 80 °C for 3 h. Zinc and gold ion concentrations in the samples was determined using atomic absorption spectrometer (Varian, AA240, Netherlands). Size distribution analysis based on dynamic light scattering (DLS) and surface zeta potential was done using Zetasizer Nano ZS90 instrument (MODEL NO. ZEN3690, MALVERN), operating with red He–Ne gas laser (λ = 633 nm) at a temperature of 25 °C.



Fig. S1. TEM image of as synthesized BSA stabilised Au NC at (A) low and (B) high magnification.



Fig. S2. Time dependent PL spectra recorded for BSA-Au-NC dispersion incubated with Proteinase K at 37 C° in phosphate buffer at different time interval of incubation as mentioned in legend. Experimental results demonstrated very minute drop in PL intensity beyond 90 min of incubation, reaching a saturation at the end of 120 min. However, slight drop in PL intensity during longer time duration (120 min and beyond) could be due to aerial oxidative quenching in absence of BSA shell.



Fig. S3. Matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectra of (a) BSA only, (b) BSA-Au-NC only, (c) BSA-Au-NC after digestion with Proteinase K and (d) zinc acetate treated digested BSA-Au-NC (Au-NC-Zn₃(PO₄)₂).

Time-resolved photoluminescence study



Fig. S4. Photoluminescence lifetime decay curve of **(A)** as synthesized BSA-Au-NC (red), fitted (green line); BSA Au NC treated with proteinase K for 90 min (grey), fitted (pink line); **(B)** liquid dispersion of Au-NC-Zn₃(PO₄)₂, fitted line (white) and **(C)** solid powder of Au-NC- Zn₃(PO₄)₂, fitted line (white).

Sample	A ₁ (%)	τ (ns)	A ₂ (%)	τ (ns)	A ₃ (%)	τ (ns)	τ _{av} (μs)
BSA-Au-	0.93	5.71	5.93	205.59	93.14	1512.4	1.50
NC							
Digested	1.61	2.88	9.65	135.47	88.73	1004.3	0.99
BSA-Au-							
NC							
Au-NC- Zn ₃ (PO ₄) ₂ (liquid)	1.56	4.68	15.07	226.45	83.36	1229.5	1.19
Au-NC- Zn ₃ (PO ₄) ₂ (solid)	4.89	25	37.65	250.68	57.46	1343	1.22

Table S1. Decay parameters of BSA-Au-NC before digestion and following digestion, when

 excited with laser at 375 nm.

Discussions: The BSA-Au-NC underwent a drop in fluorescence lifetime from 1.50 μ s to 0.99 μ s (**Fig. S4A**) upon proteolytic digestion, whereas zinc acetate addition led to increase in the average lifetime to 1.19 μ s and 1.22 μ s as recorded for liquid dispersion (Au-NC-Zn₃(PO₄)₂) (**Fig. S4B**) and that for solid powder (**Fig. S4C**) respectively. Decay parameters of the BSA-Au-NC, digested NC and Au-NC-Zn₃(PO₄)₂ (liquid dispersion and solid powder) are tabulated in **Table S1**. Decay curves were fitted using tri-exponential fit function achieving χ 2 ~ 1. We then report the intensity-averaged lifetime (τ_{av}) as tabulated in Table S1 using the equation as below,

$$\tau_{\rm av} = \frac{\sum \alpha_i \tau_i^2}{\sum_{i} \alpha_i \tau_i}$$

where, α_i and τ_i are the pre-exponential factors and excited-state luminescence decay time of the *i*-th species, respectively.

3.1 UV-Vis Study:

BSA Au NC showed a characteristic absorption peak at ~ 280 nm attributed to the aromatic ring of tryptophan group in BSA (Fig. 1C (a)). The absorption increased following proteolytic digestion of BSA-Au-NC as seen in Fig. 1C (b). This is possibly due to change in the microenvironment surrounding the NC core upon digestion. The aromatic group of the tryptophan moiety, buried inside the hydrophobic environment in the protein was thus exposed to more polar aqueous environment, resulting in increase in extinction.³ Further, the peak nearly disappeared upon zinc acetate addition (Fig. 1C(c)). This might be due to complexation between oppositely charged zinc ion and protein fragments (containing tryptophan residues) thus stabilising the Au NC core.

3.2. Control experiments to validate the fluorescence recovery in digested Au NC:

The as synthesized BSA Au NCs were digested with proteinase K in water (pH=7.4), instead of buffer which has no phosphate constituent in it for 90 min as two different simultaneous experiment – case(i) and case (ii) as shown in Fig. S5 A and C respectively.

This is then followed by addition of zinc acetate (100 μ L, 68.3 mM) to the digested Au NC dispersion as obtained in **case (i)**, whereas digested Au NC dispersion as obtained in case (ii) was treated with as prepared zinc phosphate solution (300 μ L, 68.3 mM; Refer Experimental Section 1.6 for detailed synthesis of zinc phosphate hydrate) and luminescence spectra recorded. We did not observe considerable emission increment in case (i) as evident from Fig. S5B, however case (ii) exhibited considerable increment in luminescence (Fig. S5D) alike the one seen in the case corresponding to Fig. 1B. It is to be noted that phosphate only has no role in luminescence enhancement (data not shown). Therefore, it is the zinc phosphate which predominately plays the role in *luminescence recovery* rather than zinc ions only.



Fig. S5. (A) and (C) Photoluminescence spectra recorded for dispersion of BSA-Au-NC (a) in water (pH=7.4); after incubation with Proteinase K at 37 °C at (b) 5 min and (c) 90 min. **(B)** Photoluminescence spectra **(a)** of original BSA-Au-NC, **(b)** of digested BSA-Au-NC after 90 min incubation in water; upon addition of **(c)** 50 μ L and **(d)** 100 μ L of [Zn²⁺] =68mM to digested Au -NC dispersion whereas, **(D)** showing considerable increment in emission upon addition of varying concentration of **(c)** 50 μ L, **(d)** 100 μ L, **(e)** 150 μ L , **(f)** 200 μ L , **(g)** 250 μ L and **(h)** 300 μ L of zinc acetate hydrate to digested Au-NC dispersion as obtained in Fig. S5 C.

3.3. Formation mechanism of Au-NC-Zn₃(PO₄)₂ by TEM study:

The first stage of biomimetic crystallization involves the complexation of peptide fragments stabilising the gold NCs with the zinc ions, generating a biological matrix, which serves as a nucleation centre for primary zinc phosphate growth.⁴ Although the exact amino acid residues taking part in the complexation is not known presently, it is speculated that amino acid residues with high zinc binding affinity (e.g, imidazole nitrogen of histidine, carboxylic group from cysteine, arginine, asparatic acid and asparagine residues) might be selectively responsible for primary complexation. The synthesis of Au-NC-Zn₃(PO₄)₂ was carried out by sequential addition of zinc acetate solution

(300 µL, 68.3 mM) to digested Au-NC dispersion as previously discussed. The growth was monitored by extracting aliquots at different time of [Zn²⁺] addition and probing them through transmission electron microscopy studies. Initial addition of $[Zn^{2+}]$, (100 µL, 68.3 mM) to the digested Au-NC in phosphate buffer induced a metal mediated irregular crosslinking via electrostatic and coordinative interaction with the protein fragments stabilising the Au-NC cores. This is evident from the TEM image Fig. 2B recorded after initial addition of 100 µL (68 mM) zinc acetate solution at 4 min of reaction time. This led to partial emission recovery as depicted from Fig. 1B. Further, successive addition of zinc acetate i.e, 200 µL (68.3 mM) led to additional increase in emission intensity indicative of further reaction of zinc ion with the peptide fragments stabilising the Au-NC cores. TEM image obtained at this stage (8 min) of reaction represented an intermediate metastable phase (no characteristic SAED pattern seen, Fig. S6) with the same surface morphology (Fig. 2D) as that seen in the initial stage. It was further observed that the size of the structures increased with time and concentration of the zinc salt. This stage marked the first step towards the transformation from amorphous into the thermodynamically stable crystalline phase. This is in accordance with the Ostwald's rule⁵ where a least stable (amorphous) state transformed through an intermediate metastable state by small loss of free energy, before reaching the thermodynamically stable state. At this stage, appearance of white cloudy suspension was observed due to increasing zinc ion concentration in the medium. Hence, final addition of 300 μL of [Zn^{2+}]=68.3 mM lead to a supersaturated state during which thermodynamically stable zinc phosphate decorated with Au-NCs was formed that precipitated out within 20 min of reaction time. TEM image in Fig. 2E (manuscript) clearly depicted the final morphology of as synthesized Au-NC embedded zinc phosphate taken at 20 min of reaction time. The degree of supersaturation is governed by the metal induced nucleation rate which leads to precipitation. Thus overall process represents a fast transition from amorphous to crystalline state in solution within 20 minutes of reaction time at 27 °C.⁶



Fig. S6 Featureless SAED image obtained at 8 min of reaction time after addition of 200 μ L, 68.3 mM [Zn²⁺] to digested Au-NC dispersion.



Fig. S7. **(A)** Dark field scanning transmission electron microscopy (STEM) image of as synthesized Au-NC-Zn(PO₄)₃, EDX elemental mapping images of **(B)** Au as yellow dots, **(C)** O as green dots, **(D)** P as red dots and **(E)** Zn as purple dots as obtained from STEM image (yellow dotted box) in Fig. S7A.



Fig. S8. (A) SAED pattern of as synthesized Au-NC-Zn₃(PO₄)₂, **(B)** HRTEM image of as synthesized Au-NC-Zn₃(PO₄)₂ showing lattice plane corresponding to zinc phosphate; Inset: IFFT image representing the lattice spacing corresponding to hopeite (321) plane, and **(C)** Powder X-ray diffraction pattern of Au-NC-Zn₃(PO₄)₂.

3.4. Thermogravimetric and Differential Scanning Calorimetric Analysis :

The first endothermic region from 79 – 120 °C, can be attributed to loss of physisorbed water at an early stage from 79 – 100 °C followed by first dehydration step forming unstable trihydrate intermediate (due to loss of first crystal water molecule) confirming the existence of β -hopeite phase (Fig. S9). Although no clear gradation was seen for the water loss in TGA curve, a slight drop down at 230 °C marked the complete loss of second water molecule reaching the dihydrate state followed by loss of the rest crystal water.⁷ A weak transition at 375 °C in the TGA curve marked the start of decomposition of peptide fragments from BSA moiety and phosphate groups followed by sharp transition in the temperature range 520-579 °C indicating complete decomposition due to carbonization of $Zn_3(PO_4)_2.4H_2O$

polycrystal, clearly distinguished the four step water loss which matched with the characteristic thermogram of β -hopeite. The onset temperature of thermal decomposition appeared at 121 °C (Fig. S10) as evident from DSC thermogram with a broad endothermic peak appearing at 162 °C due to loss of one water molecule, corresponding to an unstable trihydrate structure. This was followed by a sharp peak at 184 °C, corresponding to loss of second molecule of water. Finally the dihydrate to anhydrous state transition was confirmed by appearance of two peak at 223°C and 265°C (Fig. S10) featuring the β -hopeite phase, which is mostly favoured at lower reaction temperature (< 30 °C).⁷



Fig. S9. Thermogravimetric analysis of Au-NC-Zn₃(PO₄)_{2.}



Fig. S10. Differential scanning calorimetry thermogram of Au-NC-Zn₃(PO₄)₂.

3.5. FTIR analysis:

BSA-Au-NC shows characteristic band at 1632 cm⁻¹ (amide I), arising due to C = 0 stretching vibration along with out of plane C - N stretching vibration. (Fig. S11 A) Band at 1545 cm⁻¹ (amide II) corresponds to out- of-phase combination of N - H in plane bend combined with C - N stretching vibration. The peak in the range 1200-1400 cm⁻¹ (amide III) is attributed to in-phase combination of N - H bend and C - N stretching vibration (1259.7 cm⁻¹) with minor contribution from C - 0 in-plane bending.¹⁰ The band appearing at 1744.7 cm⁻¹ corresponds to C = 0 stretch of COOH group of amino acids. The band at 1405.1 cm⁻¹ and 1607 cm⁻¹ can be assigned to the symmetric stretching mode v_{str} in COO^- group and aromatic (C = C) bond respectively,^{11,12} whereas band at 1450.5 cm⁻¹ can be ascribed to CH₂ bending mode of the alkyl chain in amino acid. (See enlarged view - from a select region in pink dotted box of Fig. S11 A(i)) While the peak appearing at 1169.7 cm⁻¹ is assigned to C - 0 stretch due to tyrosine residues,¹² a broad band seen in the range 500-850 cm⁻¹ and 1000-1120 cm⁻¹ is ascribed to O-H and N-H stretching vibrations along with peaks in the range

2850-2966 cm⁻¹ attributed to C-H stretching vibration.¹³ The IR spectral band obtained after digestion of BSA Au NC showed slight shift in the peak position, however bands remained unaltered with appearance of new peaks in the spectra (Fig. S11 A (ii)). The amide (I), (II) and (II) bands are still preserved in the digested Au NC due to restricted cleavage site present in BSA. Interestingly, new peak appeared at 519.5 cm⁻¹, 935.6 and 2408.6 cm⁻¹ respectively. The peak arising at 519.5 cm⁻¹ as in Fig. S11 (ii) is due to the disulphide (S-S) bond from cysteine residues present in BSA, stabilising the Au NC core.¹⁴ The shift in peak position and appearance of new peak can be understood, due to the disruption of protein conformation upon proteolytic digestion altering the α -helix and β -sheet content in BSA moiety. Interestingly, FTIR spectra so obtained for as synthesized AuNC-Zn₃(PO₄)₂ appears albeit complex, however band assignment was carried out as follows: Fig. S11B displayed band at 1648 cm⁻¹, 1553.9 cm⁻¹ and in the range 1200-1400 cm⁻¹ which are assigned to amide (I), amide (II) and amide (III) band respectively, that are still preserved in the composite. Also, C-H stretching (symmetric and asymmetric) vibration peak appeared in the range 2800-3100 cm⁻¹ as already mentioned. While the sharp peak appearing at 1634.4 cm⁻¹ corresponds to the internal bending vibration (v_3) of crystal water, peaks appearing at 3743.6 cm⁻¹, 3342.3 cm⁻¹, 3385.6 cm⁻¹ and 3133.3 cm⁻¹ corresponds to (O-H) stretching vibrational modes from the crystal water (two molecule of water), supporting the hydrated nature of the as made Au-NC-Zn₃(PO₄)₂. The four corresponding O-H peak obtained along with red-shift in stretching frequencies indicated the presence of β -hopeite phase and internal hydrogen bonding in the crystal respectively, as also mentioned in literature.⁷ The peak appearing in the range 500-1200 cm⁻¹ are all ascribed to PO_4^{3-} stretching modes. While, three peak observed at 996.2, 1071.8 and 1129.0 cm⁻¹ refers to v_3 vibrational mode of PO₄³⁻ group corresponding to $v_{as}(P-O)$ vibration, peak appearing at 538.7, 557.1 and 619.4 cm⁻¹ represents the triple degenerate v_4 vibrational mode corresponding to δ_{as} (O-P-O) bending vibration. Additionally, peak at 957.1 cm⁻¹ represents the P - O vibration arising in P-O-Zn with (P - O - P) symmetric and antisymmetric stretch occurring at 761.1 and 863.7 cm⁻¹ respectively.^{7,13} Lastly, that the zinc ion in the system underwent complexation through the hydrolysed carboxylate anion (obtained following digestion) at pH=7.4 was further justified by appearance of peak at 478.3 cm⁻¹ corresponding to Zn - O stretching vibration.¹⁵



Fig. S11. FTIR spectra of (**A**) – (**i**) BSA-Au-NCs; (**a**) shows the expanded view of BSA-Au-NC spectra in the range $1000 - 1800 \text{ cm}^{-1}$ and (**ii**) digested BSA-Au-NCs, (**B**) as synthesized Au-NC-Zn₃(PO₄)₂; expanded view of Au-NC-Zn₃(PO₄)₂ in the range (**b1**) 1300-1700 cm⁻¹ and (**b2**) 500-900 cm⁻¹.

3.6. Control experiments validating role of peptide fragments as structure directing agent.

Case (i) Native BSA-Au-NC dispersion (5 mg/mL, 100 μ L) was taken in phosphate buffer (2.3 mL) solution, and then zinc acetate (300 μ L, 69.3 mM) were added to the buffer mixture at 37 °C successively (Refer experimental section 1.5) and mixed rapidly. In this case, the Au-NC seems to remain scattered in the zinc acetate-phosphate buffer matrix with undefined morphology as revealed from TEM image Fig. S12A. In addition, no characteristic SAED features (Fig. S12B) corresponding to zinc phosphate formation were seen.

Case (ii) 100 μ L (50 mg/mL) native BSA solution in 2.3 mL phosphate buffer medium was mixed and then digested with proteinase K at 37 °C for 90 min. This was then followed by sequential addition of zinc acetate salt solution (300 μ L 68.3 mM). Fig. S12C shows the TEM image of the zinc phosphate obtained by digestion of native BSA in phosphate buffer, which is similar to the one obtained from digestion of BSA-Au-NC (Fig. 2E). Fig. S12D is the magnified view from a select region of Fig. S12C.

Case (iii) Zn^{2+} ions (300 µL, 69.3 mM) were added successively to the phosphate buffer solution (2.3 mL) at 37 °C and mixed rapidly. Interestingly, in this case, micron sized and star shaped structures with plated wings were formed as revealed from FESEM images (Fig. S12E). Fig. S12F is the magnified view of star shaped zinc phosphate nanostructure. XRD analysis of the same evidenced the formation of orthorhombic zinc phosphate tetrahydrate in situ.

Hence, the peptide fragments bearing specific amino acids plays important role in directing the size and morphology of the zinc phosphate from micrometer to nanometer size range. Plausibly, the structure directing property of amine moieties might arise from the coulombic attraction between the positively charged amine moieties e.g., histidine¹⁶ (system pH= 6.26) present in the peptide fragments and negatively charged phosphate group. Also, contribution from metal – amino acid coordination and hydrogen bond cannot be neglected.



Fig. S12. (A) Low magnification TEM image (Inset: High magnification image from select region of **(A)**) and **(B)** SAED image (scale size = 2 nm⁻¹) of 300 μ L zinc acetate dihydrate (68.3 mM) treated BSA-Au-NC in phosphate buffer (pH=7.4), **(C)** TEM image of zinc phosphate (Zn₃(PO₄)₂) obtained after 300 μ L zinc acetate dihydrate (68.3 mM) treated digested BSA solution at 37 °C for 90 min, **(D)** high magnification image from select region of **(C)**, **(E)** FESEM image of Zn₃(PO₄)₂ obtained by treating phosphate buffer solution (pH=7.4) with 300 μ L, [Zn²⁺] = 68mM showing star shaped structure and **(F)** High magnification image of Zn₃(PO₄)₂ from a select region of **(E)**.



Fig. S13. DLS-based particle size distribution obtained for (a) BSA-Au-NC (red curve), (b) digested BSA-Au-NC (green curve) and (c) Au-NC- $Zn_3(PO_4)_2$ (blue curve).

Table S2. Particle size (hydrodynamic diameter) distribution and the corresponding zeta-potential of BSA-Au-NC, digested BSA-Au-NC and Au-NC- $Zn_3(PO_4)_2$.





Fig. S14. (A) N_2 adsorption and desorption isotherms and **(B)** differential pore volume against pore diameter plot obtained from N_2 gas desorption isotherm of as synthesized Au-NC- $Zn_3(PO_4)_2$.

Table S3. Specific surface area	, specific pore vo	lume and pore size di	stribution of Au-NC- Zn ₃ (PO ₄) ₂ .
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Sample	Surface Area	Pore size distribution	Pore volume
	(m ² /g)	(nm)	(cc/g)
Au-NC-Zn ₃ (PO ₄) ₂	6.394	2-17	0.015

3.7. Cell viability assay:

Cell viability assay was carried out by seeding HeLa cells (5 × 10³ cells/well) in a 96 well microplate and grown in DMEM medium under 5% CO₂ humidified incubator overnight. Then cells were treated with varying concentrations of BSA-Au-NC (1–4.9 μ M with respect to gold) and Au-NC-Zn₃(PO₄)₂ (50–200 μ M, with respect to zinc). For cytotoxicity test of the composite on normal human cell line, cell viability assay was carried out by seeding HEK 293 cells (5 × 10³ cells/ well) in a 96 well microplate and thereafter cells were treated with varying concentration of Au-NC-Zn₃(PO₄)₂ (50–200 μ M, with respect to zinc). It may be mentioned here that the gold and zinc contents were estimated by atomic absorption spectroscopic (AAS) measurements. After 24 h incubation, MTT based cell viability assay was carried out. The absorbance at 570 nm due to formazan formation in DMSO was recorded using a TECAN microplate reader. The assay was carried out in triplicates for each aforementioned concentration of composite. Cell viability was calculated as follow:

% viable cell =
$$\frac{(A_{570} - A_{690}) of treated cells}{(A_{570} - A_{690}) of control cells} \times 100$$

where A_{570} corresponds to absorbance of formazan and A_{690} arises due to background interference.



Fig. S15. Cell viability of (A) HEK 293 cells and (B) HeLa cells treated with varying concentration of Au-NC- $Zn_3(PO_4)_2$ for 24 h.



Fig. S16. Cell viability of HeLa cells treated with varying concentration of BSA-Au-NC for 24 h.

3.8. Confocal laser scanning microscopic (CLSM) analyses:

For confocal analyses, 1 X 10^5 HeLa cells/well were seeded onto a microscopic coverslip placed in a 35 mm culture plate and allowed to grow overnight. The cells were then treated with as synthesized BSA-Au-NC (4.9 μ M), digested Au-NC (4.9 μ M), Au-NC-Zn₃(PO₄)₂ composite (204.9 μ M with respect to total zinc and gold present) and further incubated for 4 h in humidified atmosphere containing 5 % CO₂ at 37 °C. For endocytosis inhibition experiment, HeLa cells were first treated with 0.1% sodium azide for 10 min followed by treatment with Au-NC-Zn₃(PO₄)₂ nanocomposite (204.9 μ M with respect to total zinc and gold present) and then incubated under the same condition as described above. For confocal analyses of the composite on normal human cell line, 1×10^5 HEK 293 cells/well were seeded onto a microscopic coverslip placed in a 35 mm culture plate and thereafter treated with Au-NC-Zn₃(PO₄)₂ composites (204.9 μ M with respect to total zinc and gold present) following above described procedure. After this, the cells were washed with PBS and were fixed with 0.1% formaldehyde and 70% chilled ethanol. Finally the fixed cells were mounted on a glass microscopic slide and properly sealed. The samples were then imaged under Zeiss LSM 880 confocal microscope. ($\lambda_{exc} = 405$ nm).

3.9. Quantitative analysis of the luminescence intensity of the confocal Images

The cellular uptake of Au-NC-Zn₃(PO₄)₂ by HeLa cells in absence and presence of 0.1 % sodium azide (NaN₃) were expressed by the mean luminescence intensity (calculated from quantitative image analysis of CLSM images (3 different cell images) using Image J software). To compare the amount of luminescence in respective HeLa cells incubated with (i) Au-NC,(ii) digested Au-NC and (iii) Au-NC-Zn₃(PO₄)₂, the confocal images so obtained in three different cases (i-iii) were also independently analysed using Image J software (considering at least 5 different cells). Then each HeLa cells were selected and background subtracted corrected total cell fluorescence (CTCF) were measured using Image J software. The mean luminescence intensity was then obtained by dividing the CTCF by cell area under consideration. The values obtained from the measurements in each case were then represented as mean ± standard deviation.¹⁷



Fig. S17. A typical CLSM image of Au-NC-Zn₃(PO₄)₂ treated HeLa cells (**A**) DIC, (**B**) fluorescence with corresponding (**C**) depth projection showing the internalization of Au-NC-Zn₃(PO₄)₂ by HeLa cells. (Excitation source: 405 nm laser; Magnification – 63x). Moreover, the results exclude the possibility of any Au-NC-Zn₃(PO₄)₂ nanocomposite being present on the surface of the treated cells.



Fig. S18. Typical CLSM image of HeLa cells treated with Au-NC-Zn₃(PO₄)₂ nanocomposite for 4 h in (A1-2) absence and (B1-2) presence of 0.1% sodium azide (NaN₃). (Excitation source: 405 nm laser; magnification – 63x) (C) Cellular uptake of Au-NC-Zn₃(PO₄)₂ expressed by the mean luminescence intensity (calculated from quantitative image analysis of CLSM images (3 different cell images) using Image J software) of HeLa cancer cells incubated with Au-NC-Zn₃(PO₄)₂ in absence and presence of 0.1% sodium azide (NaN₃). Quantitative analysis of confocal images obtained for HeLa cells showed a decrease in cellular uptake of Au-NC-Zn₃(PO₄)₂ composite by 70 % in presence of NaN₃ as compared to its absence. The data are represented as mean ± standard deviation.



Fig. S19. A typical CLSM image of HEK 293 cell treated with Au-NC-Zn₃(PO₄)₂ for 4 h (A) DIC, (B) fluorescence, (C) merged with corresponding (D) depth projection showing the internalization of Au-NC-Zn₃(PO₄)₂ by HEK 293 cells. (Excitation source: 405 nm laser; Magnification – 63x)



Fig. S20. Mean luminescence intensity of HeLa cancer cells incubated with BSA-Au-NC, digested Au-NC and Au-NC-Zn₃(PO₄)₂, obtained from CLSM images using Image J software (5 different cell images). The data are represented as mean \pm standard deviation.

3.10. Stability of AuNC- Zn₃(PO₄)₂ against protease degradation:

The protease assay was carried out in phosphate buffer (pH=7.4) by Proteinase K at 37 °C. The as synthesized Au-NC-Zn₃(PO₄)₂ was centrifuged at 20627 relative centrifugal force (rcf) for 2 minutes at 20 °C and the pellet so obtained was re-dispersed in 2.4 mL phosphate buffer solution. After 5 minutes of incubation of the dispersion at 37 °C, proteinase K (100 μ L, 0.8 mg/mL) was added and the mixture was incubated for 120 min. The proteolytic process was monitored by recording the time dependent emission spectra of BSA-Au-NC at an excitation wavelength of 500 nm.



Fig. S21. Stability of Au-NC-Zn₃(PO₄)₂ against protease degradation. Emission spectra (λ_{ex} = 500 nm) recorded for (i) as synthesized Au-NC-Zn₃(PO₄)₂, proteinase K treated Au-NC-Zn₃(PO₄)₂ at (ii) 0, (iii) 10, (iv) 30, (v) 90, (vi) 120 min of incubation at 37 °C.

3.11. Stability of Au-NC-Zn₃(PO₄)₂ in human blood serum:

Blood serum required for the experiment was obtained by centrifugation of the human blood sample collected from healthy individual, following the standard guidelines laid by Indian Institute of Technology Guwahati, Guwahati, India. The as synthesized Au-NC- $Zn_3(PO_4)_2$ was centrifuged at 20627 rcf for 2 minutes at 20 °C and the pellet so obtained was redispersed in phosphate buffer. Then, equal volume (1:1) of the composite in buffer and as obtained blood serum was mixed and spectra recorded immediately. The resultant dispersion was then incubated at 37°C and emission spectra were recorded for the composite at different time intervals at λ_{ex} =500 nm. As control, emission spectra of the blood serum only was also recorded at the same excitation.



Fig. S22. Stability of Au-NC-Zn₃(PO₄)₂ in human blood serum. Emission (λ_{ex} = 500 nm) spectra of Au-NC-Zn₃(PO₄)₂ in human blood serum at different intervals of time. Inset: emission spectra of human blood serum only (λ_{ex} = 500 nm).

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