

Fluorescence Enhancement Based Quantification of Human Serum Albumin from Biological Sample Using Indole Based Nanosuspension: Molecular Interactions and Molecular Docking Studies

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Research Article

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Abstract

Fluorescent 3-[(E)-(2-phenylhydrazinylidene) methyl]-1H-indole (PHI) was synthesized by condensation of indole-3-carboxaldehyde and phenyl hydrazine in presence of acetic acid and ethanol and after spectral characterization used further to prepare its aqueous nano suspension by reprecipitation method using polyvinylpyrrolidone (PVP) as stabilizer. The average particle size of nano suspension measured by Dynamic Light Scattering (DLS) was found 77.5 nm while FESEM microphotograph showed spherical morphology. The blue shift in the absorption spectrum and Stokes shifted fluorescence of nanosuspension of PHI compared to its monomer spectrum in dilute solution indicate formation of H-type aggregate by face to face overlapping of the molecules. The aggregation induced enhanced emission (AIEE) of PVP capped nanosuspension of PHI is increased appreciably by presence of aqueous solution of human serum albumin (HSA). A suitable mechanism of molecular binding interactions based on complex formation between PHI nanoaggregate and HSA through PVP is proposed. Fluorescence life time, zeta potential and particle size data of PHI nanoparticles (PHINPs) obtained in presence of different amounts of HSA are in support of molecular interactions leading to complex formation. The molecular docking studies showed that HSA and PVP capped PHINPs exhibit strong hydrogen bonding interaction. The fluorescence enhancement effect induced in PHI nanosuspension is used further to develop analytical method for quantitative estimation of HSA in aqueous biological sample solution.

Introduction

Pharmacodynamics plays crucial role in the drug-protein interaction [1]. Human serum albumin (HSA) is the most abundant drug carrier protein, with a well-known primary structure [2]. The distribution and bioavailability of free active concentration of administered drug is influenced by the binding interaction of drug and HAS [3]. It is shown that the binding of drug to HSA prolonged drug half-life by lowering the free drug concentration in the blood which is essential in the clinical care. Indole derivatives have been widely screened for anti-inflammatory activity and inhibition of multiple pathways in inflammation [4]. In addition, indole derivatives are reported to possess number of potent biological activities including analgesics, antipyretics, antifungal and antimicrobial [4]. Substitution of heterocyclic moiety at the 3-position of indole ring markedly influences the anti-inflammatory activity [5–7]. Varieties of nitrovinylindoles derived from indole-3-carboxaldehyde have shown to possess antifungal and amoebicidal activity [4]. 3-[E-(2-phenyl hydrazinylidene) methyl] 1H-indole (PHI), an indole based drug was prepared by condensation of indole-3-carboxaldehyde and phenyl hydrazine in high yield under mild laboratory condition. Synthesized PHI was characterized by ^1H NMR and ^{13}C NMR spectroscopy. However poor water solubility of PHI affects bioavailability. Formulation as nano suspension is an attractive and promising alternative to resolve the problem of low bioavailability of poor water soluble drugs [8]. In addition enhanced physical and chemical stability, possibility of dose reduction and safer dose are some of the additional advantages of using nano suspensions [9]. High pressure homogenization, media milling, microemulsion, melt emulsification and re-precipitation are currently used methods of preparation of nanosuspensions [10–14]. Of this reprecipitation method is simple,

economical and ecofriendly as water is dispersion medium. The other methods suffer from formation of large particles, generation of residue, toxicity of nonaqueous solvent and requirement of high amount of surfactant and stabilizer. With this in mind the suspension of PHI was prepared by reprecipitation method using polyvinylpyrrolidone (PVP) as stabilizer. Interest in PVP is because it acts as vehicle for dispensing and suspending drug and has low acute toxicity [15]. Additionally in examination of sensing performance of HSA by PHI suspension we found enhanced fluorescence signals when excited using characteristic excitation wavelength of PHI. Present paper reports execution of drug-protein interaction and application to develop analytical method for the quantification of HSA from blood sample. The binding interactions are supported by molecular docking study.

Experimental

Materials

Indole-3-carboxyaldehyde and phenyl hydrazine was procured from Sigma Aldrich (India). Polyvinylpyrrolidone (PVP, K-30) was obtained from Spectrochem Pvt. Ltd. Mumbai, (India). Human serum albumin (HSA) was purchased from HiMedia laboratories Pvt. Ltd. Mumbai (India). The serum samples were collected from the Health Centre of Shivaji University, Kolhapur (India). Ultrapure water was obtained by passing distilled water through a Millipore unit (India) and was used in all experiments.

Instrumentations

Nuclear magnetic resonance (NMR) spectrum of 3-[(E)-(2-phenylhydrazinylidene) methyl]-1H-indole (PHI) was recorded in deuterated dimethyl sulfoxide (d₆-DMSO) on Bruker AC-300 NMR spectrometer (300 MHz for ¹H NMR and 75 MHz for ¹³C NMR). Chemical shifts are reported using tetramethylsilane (TMS) as an internal standard. The particle size distribution and zeta potential of PVP capped 3-[(E)-(2-phenylhydrazinylidene) methyl]-1H-indole nanoparticles (PHINPs) in aqueous suspension was measured using a Malvern Zetasizer (NanoZS-90). Morphology of PVP capped PHINPs was examined using Field Emission Scanning Electron Microscopy (FESEM, FEI Quanta 650 F). UV-Vis absorption spectra were recorded on spectrophotometer Specord 210 plus model using 1 cm quartz cell. Steady-state fluorescence spectra of aqueous suspension of PVP stabilized PHINPs with and without HSA were recorded on Spectrofluorimeter (JASCO, Model FP-8300, Japan). Molecular docking study was performed by Auto Dock 4.2. Three dimensional structure of HSA (PDB ID: 1A06) was retrieved from RCSB Protein Data Bank.

Synthesis of 3-[(E)-(2-phenylhydrazinylidene) methyl] 1H-indole (PHI):

Indole-3-carboxyaldehyde and phenyl hydrazine (1 mmol of each) were added to a 96% ethanol containing 10 mL glacial acetic acid. The resulting mixture was heated under reflux and the reaction progress was monitored by thin layer chromatography [7]. 3-[(E)-(2-phenylhydrazinylidene) methyl] 1H-indole (PHI) was crystallized on cooling refluxed content and then filtered. The residue was washed with n-hexane, dried and recrystallized from ethanol solution. The synthesis route of PHI is shown in scheme 1. PHI is

obtained as off-white powder with 95 % yield and experimental melting point 197°C matches with literature value 198°C. The results of ^1H NMR and ^{13}C NMR further confirms the formation of desired product (ESI, Fig. S1 and S2).

Preparation of Nanosuspension of 3-[(E)-(2-phenylhydrazinylidene) methyl]-1H- indole (PHI) by Reprecipitation Method

2mL ($1 \times 10^{-5}\text{M}$) solution of PHI prepared in acetone was added through micro syringe into 100 mL 0.1wt % aqueous solution of PVP maintained at room temperature with vigorous stirring for about one hour. The content was then sonicated for 30 minutes to suspend the PHI nanoparticles (PHINPs) uniformly in aqueous dispersion medium. The aqueous suspension of PHINPs was also prepared by using different surfactants like sodium dodecyl sulphate (SDS), cetyltrimethylammonium bromide (CTAB). The particle size of nanosuspension given in Table S1 (supporting information) indicates nanosuspension prepared using PVP is of more fine particles.

Results And Discussion

Surface and Optical Properties of PVP Stabilized PHINPs

The histogram of particle size distribution of PVP stabilized PHINPs presented in Fig. 1 shows narrower particle size distribution in the range from 50-100 nm and the average particle size is 77.5 nm. Fig. 2 shows FESEM images of air dried film of aqueous suspension of PHINPs in different stabilizers and film prepared without stabilizer. It is seen that the image of the film prepared in PVP stabilizer reveals spherical monodispersed particles of average size 150 nm. However morphology of PHINPs prepared using other stabilizers seen irregular in SEM images. The observed larger size of the nanoparticle spheres in FESEM analysis as compared to the value obtained from DLS technique is due to agglomeration of nanoparticles which forms large size spheres during evaporation of water from aqueous suspension in the process of the preparation of thin film on silicon wafers.

The zeta potential distribution of PVP stabilized PHINPs shown in Fig. 3 gives -24.9 mV zeta potential due to electron rich 'O' atoms of PVP capping. The hydrophilic pyrrolidone part acted as head group while the hydrophobic $-\text{[CH}_2\text{-CH]}_n$ part is the tail. During the formation of nanosuspension the PHI molecules nucleate and aggregate to form dimeric species in aqueous medium. The absorption spectrum of nano suspension and that of dilute solution of PHI in acetone presented in Fig. 4 shows blue shift indicating formation of H-type of aggregates due to lateral π -stacking effect between neighboring PHI molecules in suspension [16-17]. However the fluorescence spectrum (D) of nanosuspension in Fig. 5, peaking at 406 nm is seen enhanced very strongly in comparison with the weak structured fluorescence of dilute solution of PHI appeared in the wavelength region of 400-500 nm (spectrum B). The broad, pronounced fluorescence spectrum of nanosuspension of PHI is also shifted towards blue of the fluorescence spectrum of its dilute solution. In addition Fig. 5, shows a large stoke shift of 7116 cm^{-1} between the excitation spectrum (C) and fluorescence spectrum (D) of nanosuspension of PHI. On the contrary the

stoke shift value of 3932 cm^{-1} estimated from excitation spectrum (A) and fluorescence spectrum (B) of dilute solution of PHI in acetone is small. These observations suggest that the fluorescence of nanosuspension is aggregation induced enhanced emission (AIEE). The results of pH dependence of fluorescence intensity presented in Fig. 6 show maxima at pH 7.5.

Recognition Test of Nanosuspension of PHI for HSA

The fluorescence spectra produced using 315 nm excitation wavelength of nanosuspension of PHI in presence of various biologically important molecules such as HSA, bovine albumin serum (BSA), D-penicillamine, guanine, sucrose, glucose, cyanocobalmine, hemoglobin, vit.B-1, glutathione each of 1.8 μM concentration are given in Fig. 7. The careful observation of the spectra reveals that the fluorescence of nano suspension of PHI is enhanced significantly by HSA as compared to enhancement induced by BSA, while other biomolecules decreases the fluorescence of nanosuspension. In addition to this the bar diagram in Fig. 8 indicating effect of fluorescence change estimated as, (F_0 is the intrinsic fluorescence of nanosuspension and F is the fluorescence in presence of biomolecules) supports to the observation of recognition of HSA by suspended nanoparticles of PHI. The blue bar in Fig. 8 shows enhancement effect by HSA and BSA. The enhancement produced by HSA is significantly large in comparison with BSA. The orange bar seen in the same figure reveals that the fluorescence enhancement induced by HSA is not affected even in presence of coexisting substances. In addition to these the comparison of the fluorescence spectrum of nanosuspension containing HSA with the pure spectrum without HSA shown a blue shift of about 2035 cm^{-1} while BSA does not produced any spectral shift. This observation led to consider binding between PHI nanoaggregate and HSA molecule only.

Studies on Binding Interactions between HSA and PHI Nanoparticles

The gradually increasing amounts of HSA solution in the concentration range 0.1-1.8 μM were added to definite amount of nanosuspension of PHI buffered at pH 7.5 taken in the different test tubes (ESI, Table S2). Fluorescence spectra of the nanosuspension monitored at excitation wavelength of 315 nm in presence of HSA and without HSA are shown in Fig. 9. From the spectra it is seen that the fluorescence intensity of PHI nanosuspension not only increases significantly but also the wavelength of maximum emission shifts from 406 nm to 375 nm as concentrations of HSA increases.

The fluorescence enhancement and observed wavelength shift effect are discussed on the basis of complex formation between PVP capped PHI nanosuspension through the electron rich 'O' atoms of PVP with HSA molecule. It is thought that PVP may acts as receptor between PHINPs and HSA. This proposed binding interaction mechanism further supported by the double logarithmic plot [18]. The binding constant ' K ' and binding sites ' n ' are estimated from the double logarithmic plot $\text{Log}_{10} (F_0 - F) / F$ Vs $\text{Log} [HSA]$ as shown in Fig. 10. This plot is based on equation 1 given below.

$$\text{Log}_{10} \frac{(F_0 - F)}{F} = \text{Log}_{10} K + n \text{Log}_{10} [HSA] \quad (1)$$

where F_0 and F are the fluorescence intensities of PHINPs in absence and presence of HSA concentration. The value of K obtained from the plot is $7.030 \times 10^5 \text{ Lit. mol}^{-1}$ indicates good stability of complex while the value of binding site (n) nearly equal to 1 indicates availability of one possible binding site on the surface of suspended nanoparticles.

Fluorescence lifetime determinations of nanosuspension of PHI in presence of HSA and without HSA are found in harmony with the proposed mechanism of molecular interaction leading to formation of complex. Fig. 11 shows the decay profile of PHINPs in absence and in presence of HSA solution. The life time 7.29 ns (Fig. 11[A]) of suspension without HSA found to increase in order of 8.02 ns, 8.52 ns and 10.61 ns (Fig. 11[B-D]) when concentration of HSA solution was 0.3 μM , 0.4 μM and 1.0 μM respectively. The molecular interactions and complexation lead to prolonged fluorescence lifetime [19]. Thus increase in lifetime of suspension with addition of HSA suggests stabilization of PVP-PHINP-HSA complex. The schematic representation of fluorescence enhancement of PHINPs upon interaction with HSA is graphically presented in scheme 2. The average particle size and zeta potential measurements further confirm complex formation between PHI nanoparticles and HSA. The zeta potential of as prepared nanosuspension -24.9 mV seen in Fig. 12 decreases to -16.7 mV and to -10.6 mV when concentration of HSA increased from 0.5 μM and to 1.0 μM , while at the same time the particle size seen increasing from 77.5 nm to 250 nm and to 481.1 nm respectively. The increase in particle size and decrease in zeta potential led to consider adsorption of HSA over the negatively charged surface of suspended particles [20]. Thus the observed fluorescence enhancement is attributed to the possible interaction with negatively charged oxygen atom of PVP with HSA as presented in scheme 2.

Exploration of mechanism of binding between PVP capped PHINPs and HSA by absorption spectroscopy

The absorption spectra of the PHINPs in presence of different amounts of HSA showed in Fig. S3 reveals the red shift in wavelength of maximum absorbance as indicating complex formation between PVP capped PHINPs and HSA. The PVP acts as receptor between them for enhancing the rate of charge transfer in complex formation.

Molecular Docking Studies

To provide further, the deeper insight into the interaction of HSA with PHINPs, a molecular docking technique is used. The experiment of docking studies carried out between human serum albumin (2bx8.pdb) and PVP-N-PHI is shown in Fig. 13. The three-dimensional structure of human serum albumin (2bx8.pdb) was extracted from RCSB PDB. Further the three dimensional structure of PVP-N-PHI was built by SPARTAN ver 6.0.1 Software and minimized by using HartreeFock (HF) method in the SPARTAN ver 6.0.1 Software [21-22]. After completion of minimization the PVP-PHINPs structure with less energy were used for the molecular docking with human serum albumin (2bx8.pdb). Finally, the docked complex of PVP-N-PHI with human serum albumin (2bx8.pdb) was analyzed by CHIMERA. The best conformation with least binding energy of 5.02KJ/mol was visualized as presented in Fig. 13.

The best possible solution obtained by the molecular docking study suggests that PVP capped PHI nanoparticle is able to interact with HSA by means of hydrogen bonding. The amino acid residues of human serum albumin (2bx8.pdb) such as TYR-150, GLN-196, HIS -242, LEU-238, ARG-257 are having strong hydrogen bonding interactions with the PVP-PHINPs. These interactions are predicted to occur at distances required for effective H-bonding ranging from 1.837Å to 2.774 Å (Table 1). Thus the formation of PVP-PHINP-HSA complex through hydrogen bonding interactions is strengthened by molecular docking analysis.

Application of Nanosuspension of PHI for the Detection of HSA in Biological sample

A calibration curve as shown in Fig. 14 is constructed by plotting fluorescence intensity (ΔF) increase as a function of concentration of standard solution of HSA added in the known amount suspension of PHINPs. The calibration graph in Fig.14 is linear over the range of concentration of HSA from 0.0 μM to 1.8 μM . The correlation coefficient of the plot is 0.9902. The limit of detection calculated by using equation 2 is 0.032287 μM [23].

$$LOD = \frac{3.3\sigma}{K} \quad (2)$$

Where, 's' is the standard deviation of 'y' intercept of the regression line, 'K' is the slope of the calibration graph. An analytical method is developed for the quantification of HSA in the serum samples collected from the Health Centre of Shivaji University, Kolhapur. The blood samples were allowed to clot at room temperature for 15-30 minutes and then the clot was removed by centrifuging at 2000 rpm for 10 minutes in refrigerated centrifuge. The resulting supernatant liquid is serum. Serum consists of antibodies, antigens, electrolytes, hormones and proteins [24]. An appropriate amount of the serum samples were spiked with HSA to prepare two synthetic samples of concentration 0.3 μM and 0.7 μM , and diluted by distilled water so that fluorescence intensity of samplesolutions is in the working range of calibration curve. The results of analysis shown in Table 2 indicate that the amount of HSA found by proposed method is in close agreement with amount added with good percent recovery.

Conclusions

Biologically active 3-[(E)-(2-phenyl hydrazinylidene) methyl]-1H-indole (PHI), an indole based drug was prepared by condensation of indole-3-carboxaldehyde and phenyl hydrazine in high yield under mild laboratory condition. The aqueous nanosuspension of PHI prepared by simple reprecipitation method exhibit aggregation induced enhanced fluorescence. The DLS and FESEM estimations indicate that the nanoparticles of PHI having average diameter 77.5 nm with spherical morphology. The blue shift in the absorption spectrum and stokes shifted fluorescence of nanosuspension of PHI compared to its monomer spectra in dilute solutions indicate formation of H-type aggregate by face to face overlapping of the molecules. The aggregation induced enhanced emission (AIEE) of PVP capped PHI nanosuspension is increased appreciably by presence of aqueous solution of human serum albumin

(HSA). The possible mechanism of fluorescence enhancement with spectral shift towards blue is discussed on the basis of complex formation between PVPPHINPs-HSA. The binding constant value $7.030 \times 10^5 \text{ Lit. mol}^{-1}$ estimated from the fluorescence enhancement induced by HSA is favorable for its efficient biodistribution by blood plasma. Fluorescence life time, zeta potential and particle size data of PHI nanoparticles obtained in presence of different amounts of HSA are in support of molecular interactions leading to complex formation. The molecular docking studies showed that the human serum albumin and PVP capped PHINPs exhibits strong hydrogen bonding interactions. Further the proposed system is used for the detection of HSA from biological sample.

Declarations

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Conflicts of interest/Competing interests : The authors declare that they have no conflict of interest.

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Ethics approval/declarations: The submitted work is original and not published elsewhere. This manuscript is presented clearly and not split up into several parts to increase the quantity of submissions. All Authors are adhere to discipline-specific rules for acquiring, selecting and processing data. We acknowledged other works properly insuitable postion.

Consent to participate: All the authors are aware of the submission and agree to its publication.

Consent for publication: We wish to inform you that the data and ideas presented in the manuscript are of our own and are not under consideration for publication elsewhere.

Availability of data and material/ Data availability: Nil

Code availability (software application or custom code): Molecular docking study was performed by Auto Dock 4.2. Three dimensional structure of HSA (PDB ID: 1A06) was retrieved from RCSB Protein Data Bank.

Authors' contributions

All authors contributed to the study of synthesis of fluorescent nanoprobe, characterization and its biological application. The 3-[(E)-(2-phenylhydrazinylidene)methyl]-1H- indole (PHI) prepared and further analysis were performed by Dr. Sonali Babaso Suryawanshi. Dr. Netaji Keru Deasi and Dr. Anita Jivan Bodake helped for scintific and gramatic manuscript writing. Prof. Shivajirao Raghunath Patil has

communicated research work to esteemed journal 'Journal of Fluorescence'. All authors read and approved the final manuscript.

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Tables

Table 1 Hydrogen bonding interactions between human serum albumin (HSA) (2bx8.pdb) with PHINPs.

Sr. No.	Interaction between active site residues of human serum albumin (2bx8.pdb) with compound derivatives.	Distance (Å)
1	TYR 150.A HH — PVP 1.het O:	2.169
2	ARG 257.A HE — PVP 1.het O:	1.837
3	ARG 257.A 2HH2 — PVP 1.het O:	2.066
4	HIS 242.A HE2 — PVP 1.het C:	2.342
5	PVP 1.het C — LEU 238.A CD1:	2.774
6	GLN 196.A 2HE2 — PVP 1.het C:	2.603

Table 2 HSA recovery in serum samples by the standard addition method.

Samples	Amount of HSA added (µM)	Total found (µM)	RSD (n = 3) %	Recovery %
Serum 1	0.3	0.2963	1.059	98.95
Serum 2	0.7	0.6976	1.021	99.87

Supplementary Information

Supplementary Files were not provided with this version of the manuscript.

Figures

Statistics Graph (1 measurements)

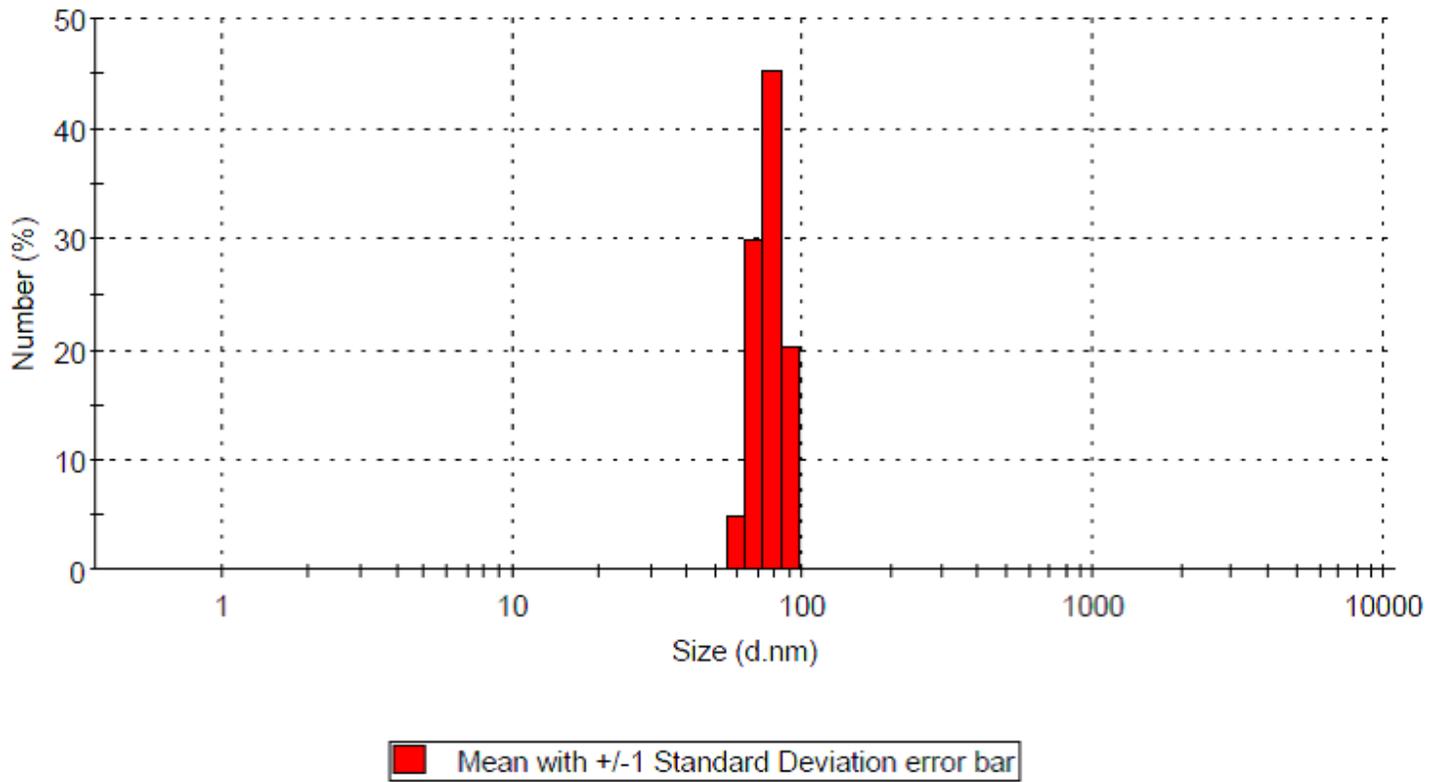


Figure 1

Particle size distribution histogram of PHINPs from DLS analysis.

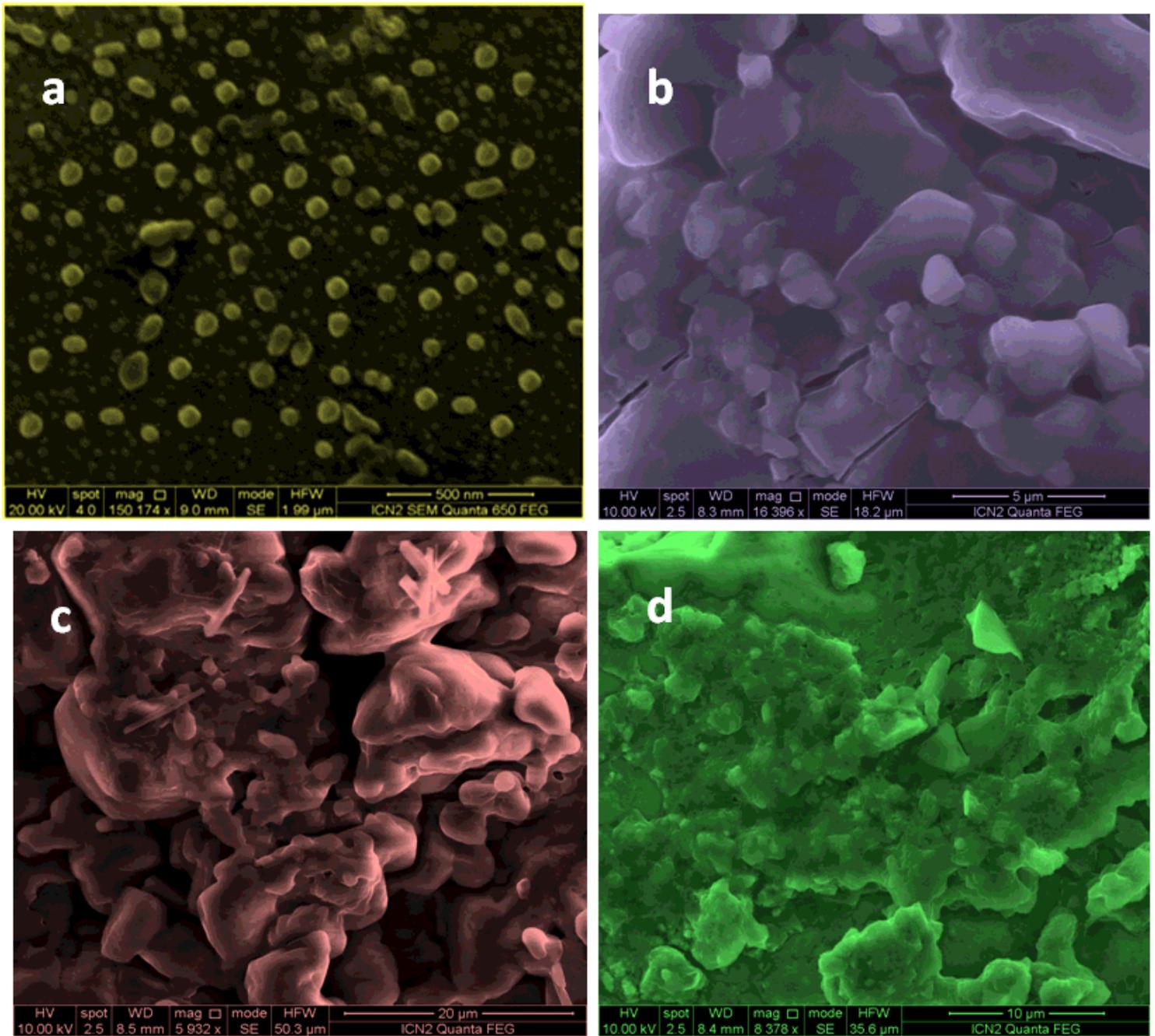


Figure 2

FE-SEM images of air dried film of PHINPs stabilized in PVP (image a), CTAB (image b) and SDS (images c) and without stabilizer (image d).

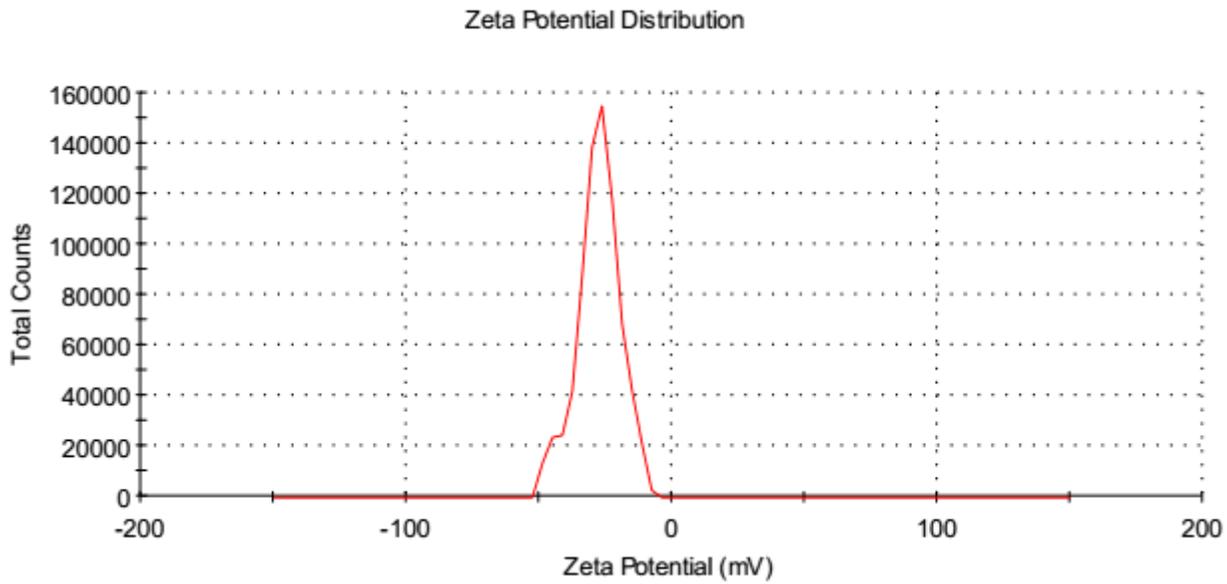


Figure 3

Zeta potential distribution of PVP stabilized PHINPs in aqueous suspension.

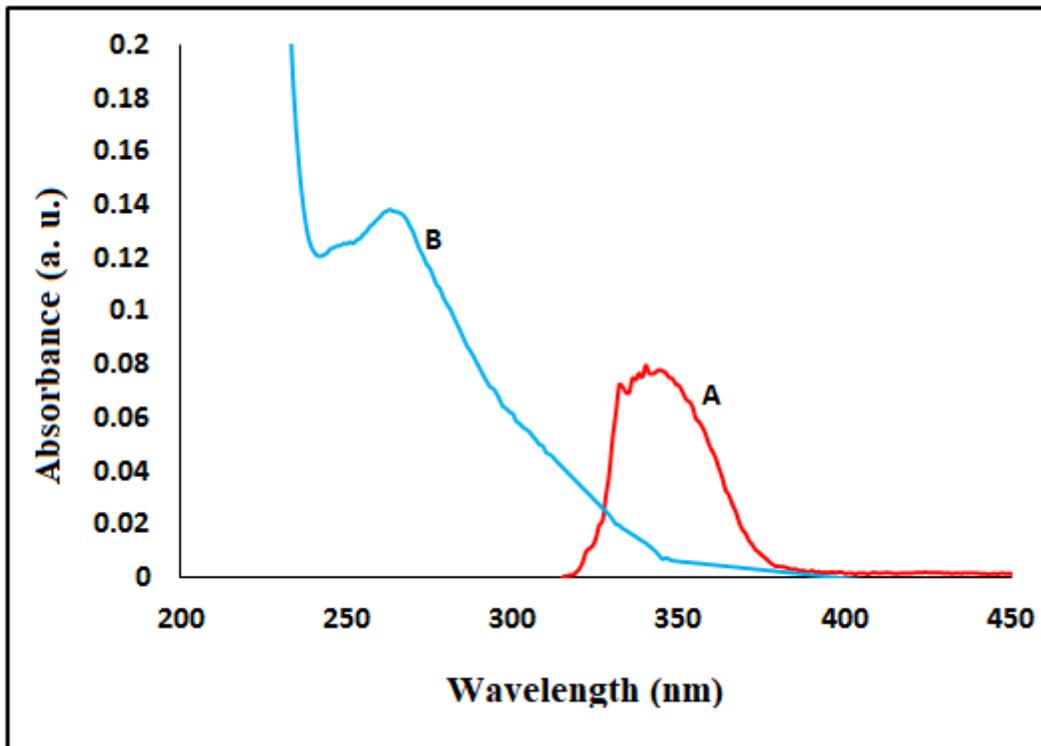


Figure 4

Absorption spectra of PHI in acetone (spectrum A) and PHINPs in aqueous suspension (spectrum B).

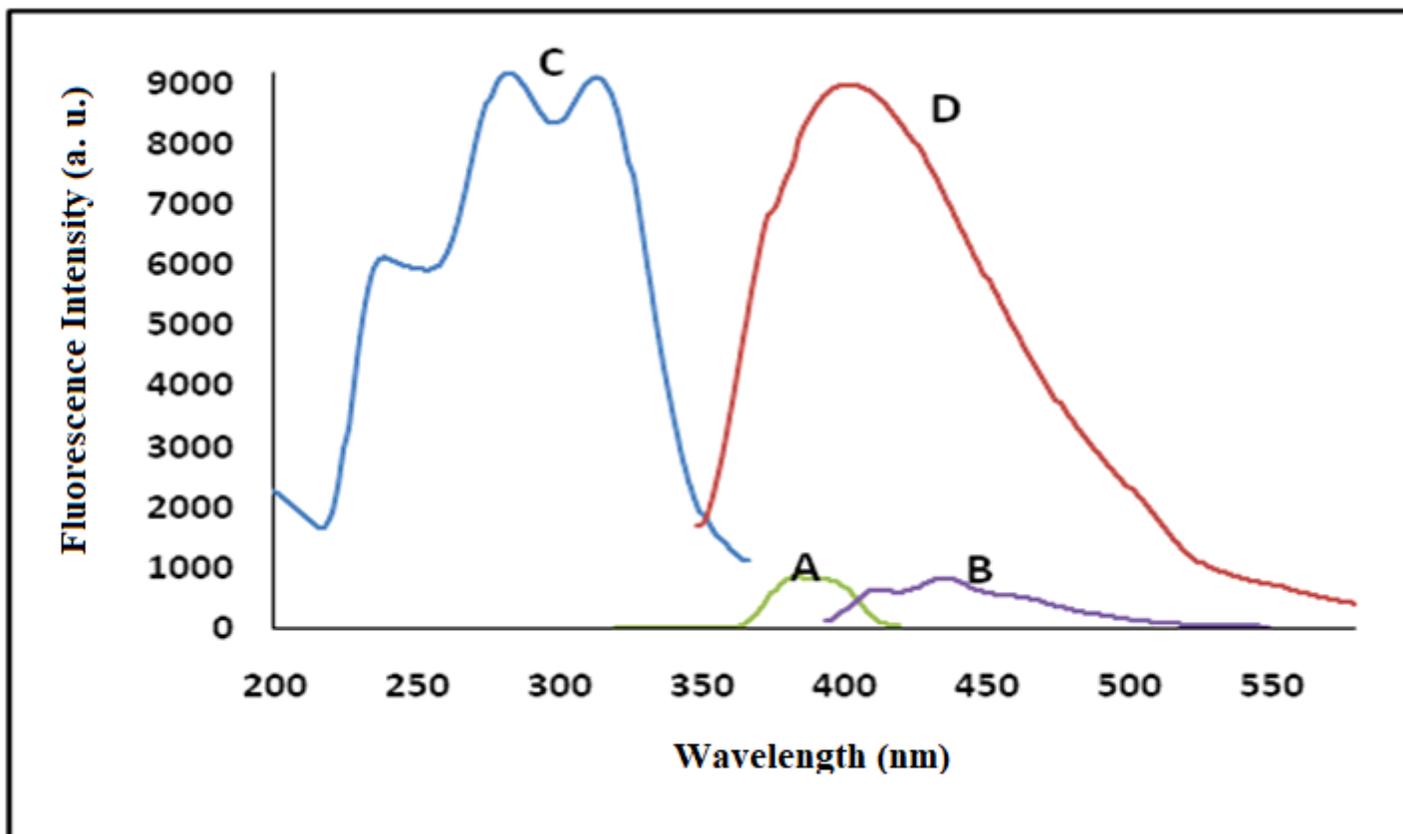


Figure 5

Fluorescence spectrum of PHINPs suspension (D) and dilute solution of PHI in acetone (B). (The excitation spectrum (A) of PHI solution and nanosuspension (C) are shown for comparison.)

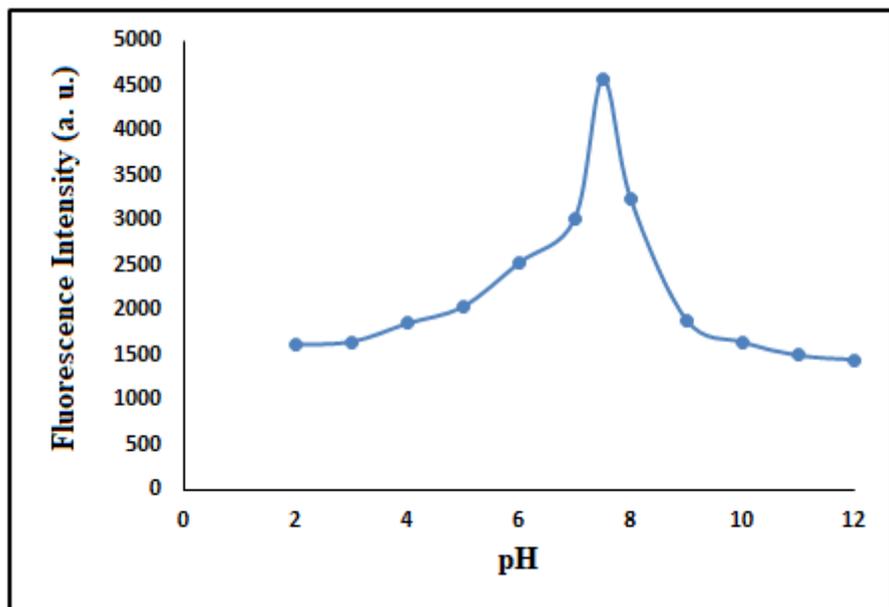


Figure 6

Effect of pH on the fluorescence intensity of aqueous nanosuspension.

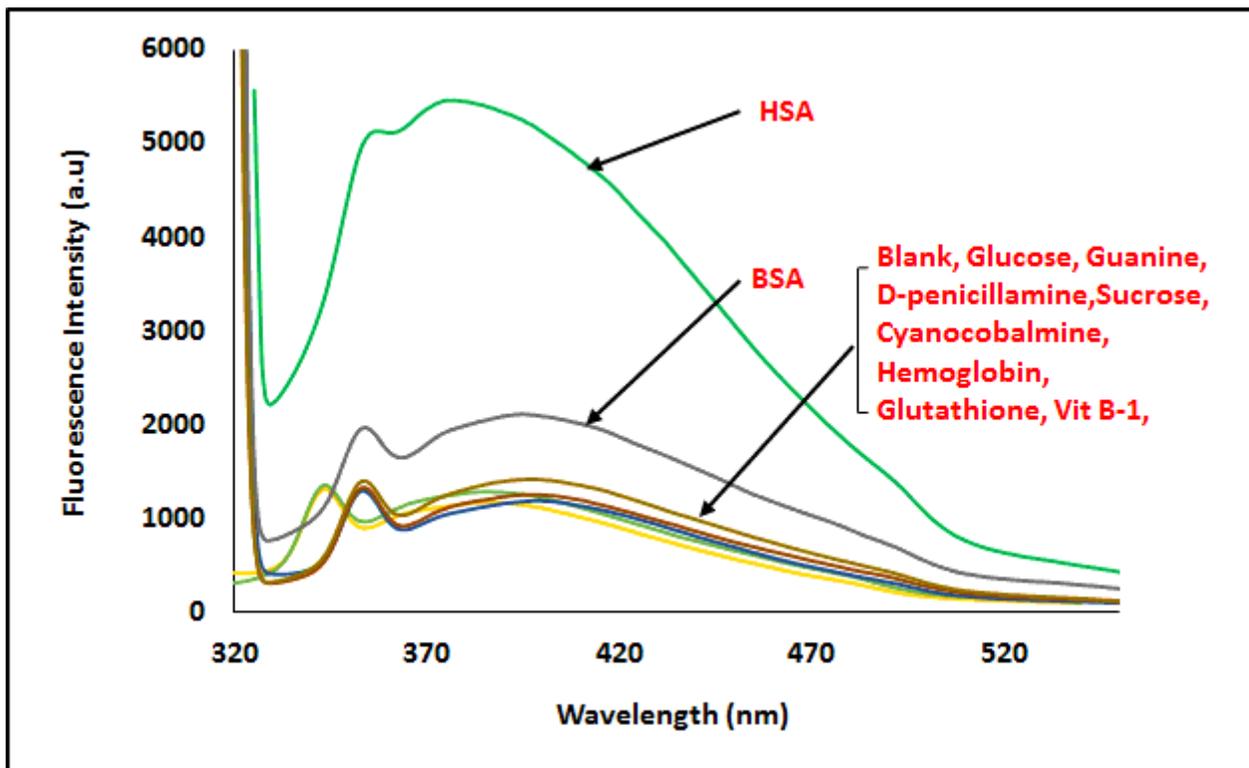


Figure 7

Fluorescence spectra of aqueous suspension of PHINPs in presence of various coexisting substances monitored at excitation wavelength 315 nm.

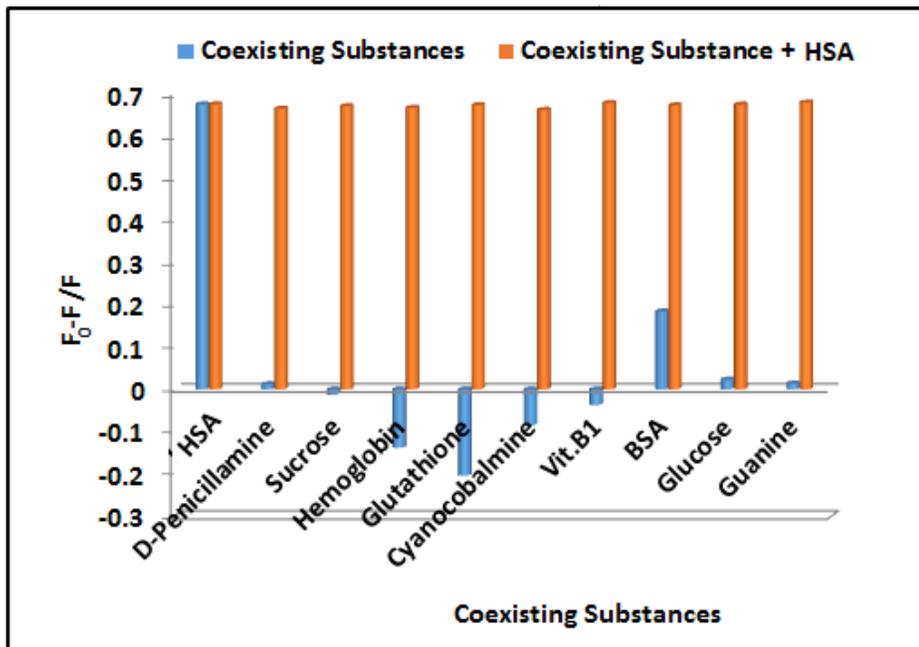


Figure 8

Bar diagram of fluorescence intensity change $(F_0-F)/F$ of the PHINPs in the presence and absence of HSA and several coexisting substances of concentration $1.8 \mu\text{M}$ (excitation wavelength = 315 nm).

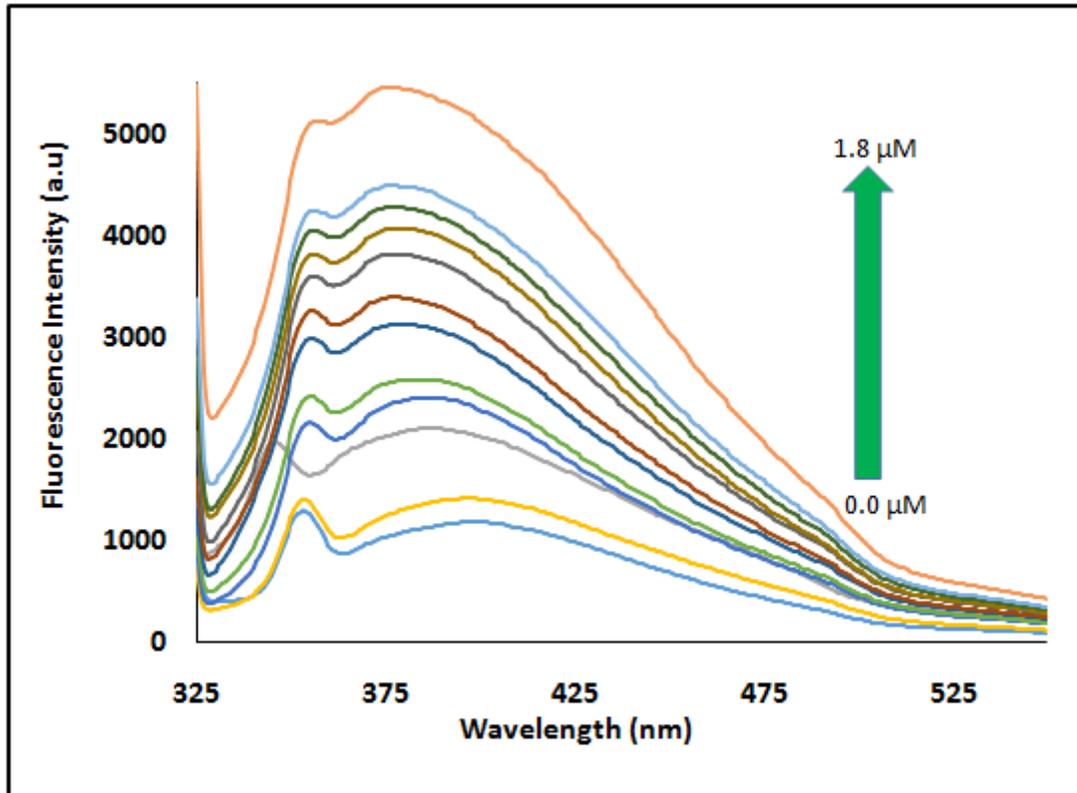


Figure 9

Fluorescence spectra of nano suspension of PHI in the presence of different amounts of HSA solution of concentration $0.0, 0.1, 0.35, 0.4, 0.5, 0.7, 0.8, 0.95, 1.0, 1.1, 1.2, 1.8 \mu\text{M}$ in phosphate-buffer of $\text{pH} = 7.5$ (Excitation at wavelength $\lambda_{\text{ex}} = 315 \text{ nm}$).

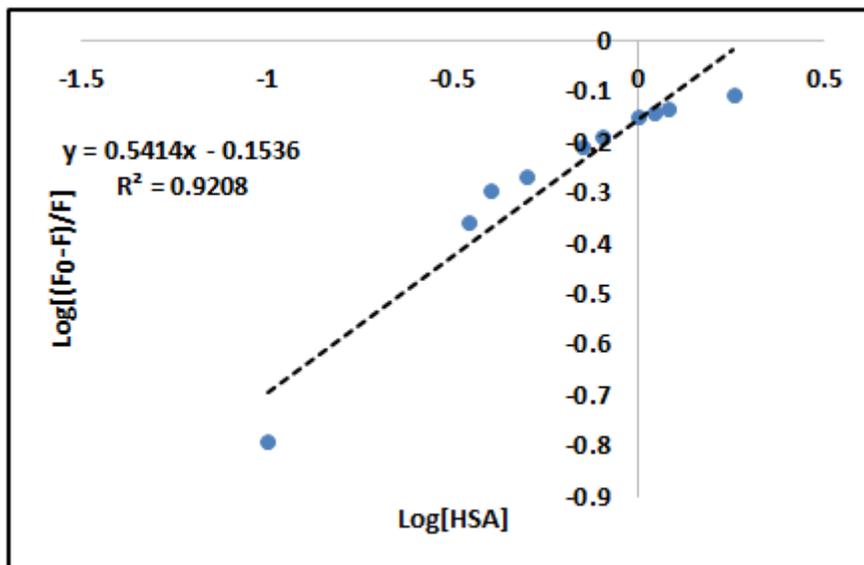


Figure 10

Double logarithmic plot of $\text{Log} [(F_0-F)/F]$ versus $\text{log} [\text{HSA}]$.

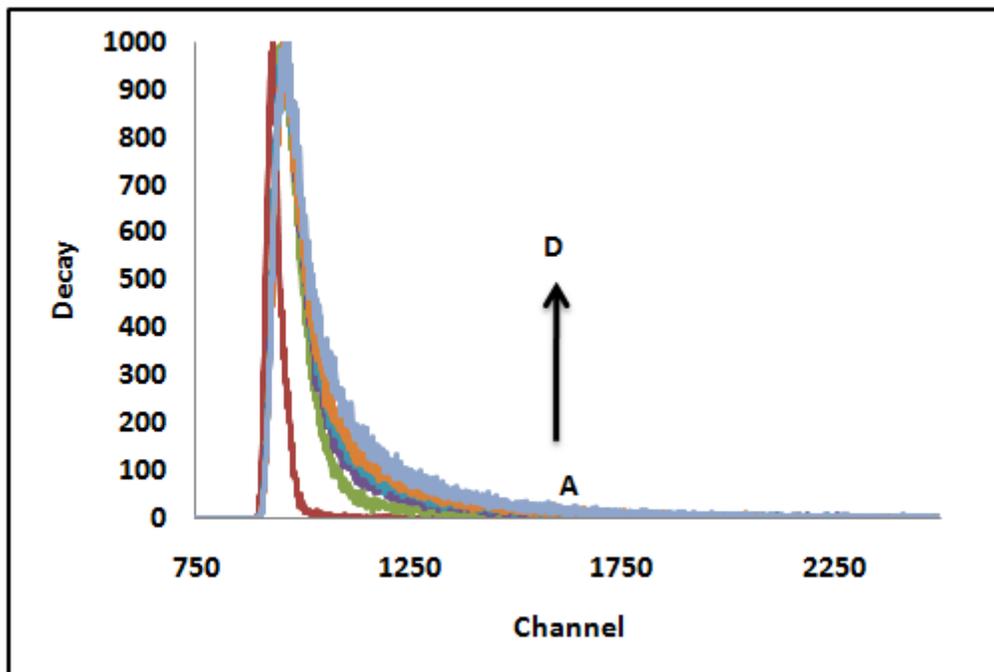


Figure 11

Fluorescence decay profile of PHINPs suspension (A) without and with HSA solutions of 0.3 μM, 0.5 μM and 1.0 μM concentration.

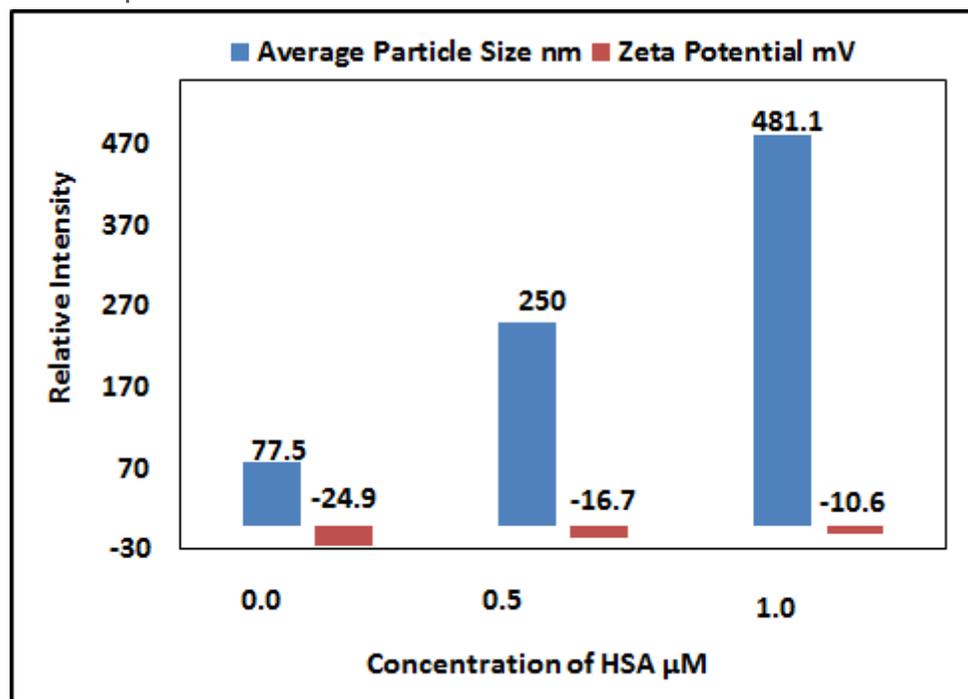


Figure 12

Representation of zeta potential and average particle size of PHINPs in absence and presence of 0.5 μM and 1.0 μM HSA solutions. (pH = 7.5).

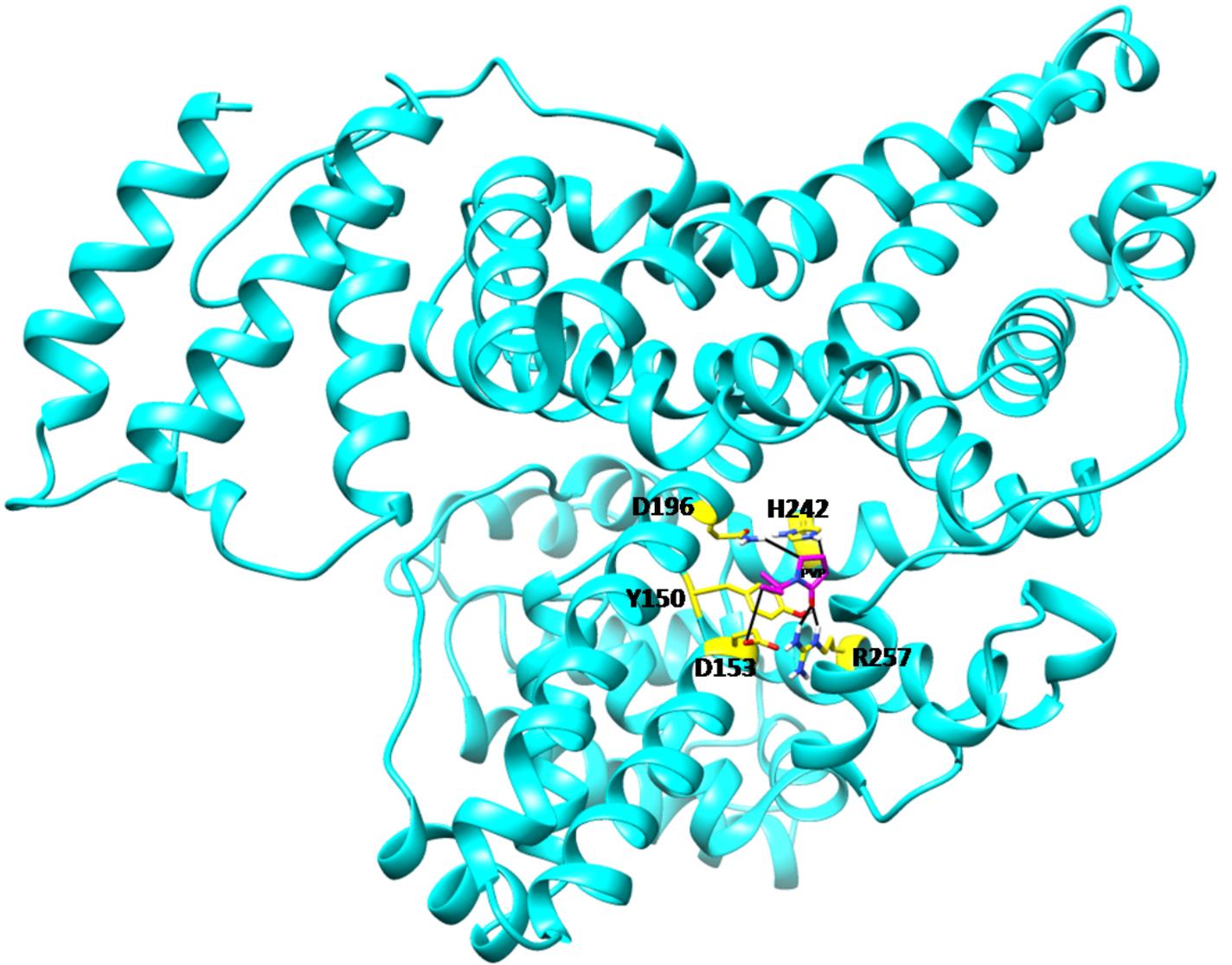


Figure 13

Docked complex of human serum albumin and PVP-PHINPs.

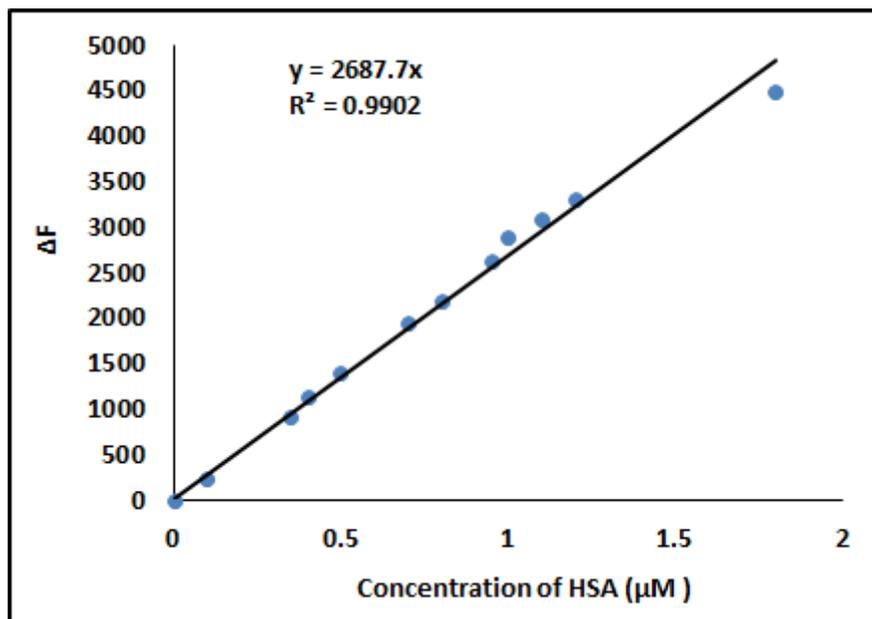


Figure 14

Calibration curve of fluorescence intensity change (ΔF) of PHINPs suspension versus concentration of HSA solution.

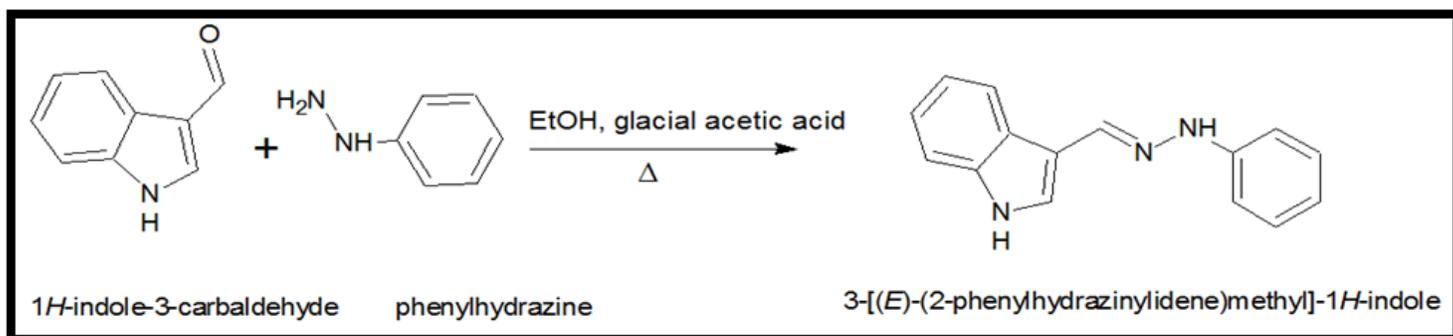


Figure 15

Scheme 1 Synthesis route of 3-[(E)-(2-phenylhydrazinylidene) methyl]-1H-indole (PHI).

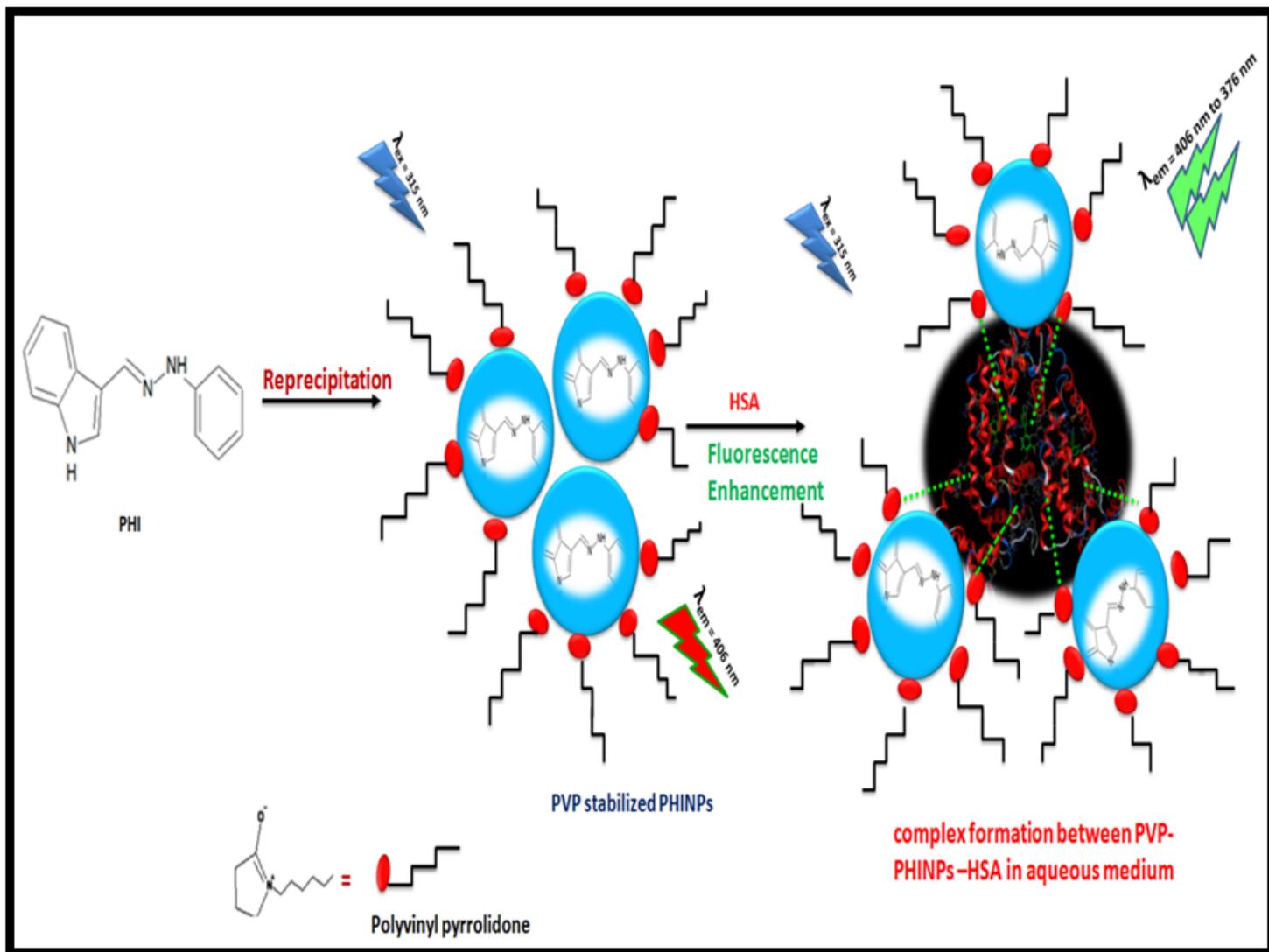


Figure 16

Scheme 2 Schematic presentation of molecular interaction forming complex between PVP capped PHINPs and HSA in aqueous suspension.