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Regular Article

Inhibition of crude viper venom action by silver nanoparticles: A biophysical and biochemical study

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This investigation understands the interaction between lyophilized crude Viper snake venom (*Doboia russellie*) and Silver nanoparticles (SNPs) using biophysical and biochemical approaches. SNPs were synthesized by chemical reduction method and characterized using UV-Visible spectroscopy, Dynamic Light Scattering (DLS) and Transmission electron microscope (TEM). The average hydrodynamic size of SNPs was found to be 52 nm with 0.261 PDI. TEM image revealed the spherical shape of SNP. Interaction of SNPs and viper venom was resulted in the formation of complex which was confirmed by using DLS technique. Spectroscopic results showed an increase in absorbance intensity of venom upon interaction with SNPs which indicated interaction with venom proteins. Fluorescence spectroscopic data revealed the quenching in the fluorescence intensity of viper venom upon incubation with varying concentration of SNPs. The results obtained by biochemical assays (Protease and whole blood clotting test) revealed the inhibition of venom action due to presence of silver nanoparticles. The activity of protease enzyme was found to be decreased (10–13% reduction) in presence of silver nanoparticles. Prolonged clotting time (two fold) of viper venom upon interaction with SNPs compared to native crude viper venom was observed. The overall results confirmed the inhibition action of silver nanoparticles against viper venom.

Key words: russell's viper, fluorescence spectroscopy, dynamic light scattering, protease assay, whole blood clotting test

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Russell's viper (*Daboia russelii*) is one of the poisonous snakes commonly found in Asian countries and responsible for major snake bites in India [1,2]. Venom is a secretion of poisonous gland, injected in a prey with the help of hollow fangs. It is a complex mixture of various biologically, pharmacologically active proteins and polypeptides [3]. Its proteinaceous nature was confirmed in 1994 [4]. The current treatment available for snake bite is intravenous administration of anti snake venom (ASV) (passive immunization) which neutralizes snake venom. ASV is the polyvalent antibodies manufactured by using surrogate animals. These animals are immunized with the snake venom and then antibodies are collected from blood and purified, which seems to be a quite tedious process [5,6]. Treatment with Anti snake venom (ASV) is life saving but very risky, because the serum being

◀ Significance ▶

Snake envenomation is one of the most important public health problem in the worldwide, specifically in tropical and subtropical countries. It is a common occupational hazard mainly in rural peoples. There is significant morbidity and mortality reported worldwide. At present there is no reliable treatment established due various physiological/ biochemical problems. This preliminary investigation is being made to overcome these issues. We have shown inhibition of snake venom using metallic nanostructures. Various biophysical and biochemical principles have been used to understand the inhibitory action by the snake venom. So far this study focuses on interaction of silver nanoparticles with venom at fixed dimension of particles (52 nm). To the best of our knowledge, this is the first kind of report of inhibition of snake venom action by silver nanoparticles and other nanoparticles have not been used for similar studies.

used as antivenom is heterologous (produced in different species) and may cause sensitivity reactions in some patients [5]. Morais V. M. and Massaldi H. (2009) had discussed in detail about adverse reactions developed by snake antivenom [7]. Because of its heterologous nature, it may activate immune system and leads to the development of mild reactions (chills, nausea and fever) to severe reactions (hypersensitivity reactions or anaphylactic shock, complement system activation, pyrogenic reactions etc). Efforts are being made to reduce these symptoms by using immunoglobulin fragments and higher purification strategy, but this leads to the increase in the dose of antivenom and related cost. Another drawback is long storage duration, which causes protein aggregation and activates complement system. So, there is a need to focus on alternative treatment considering the limitation of available treatment.

Since nanoscience and nanotechnology are rapidly growing fields with significant potential to develop new generation of therapeutic agents, clinical tools and devices in field of medicine. The essence of nanoscience and nanotechnology is in the creation and use of molecules, molecular assemblies, materials and device in the range of 1–100 nm and exploitation of the unique properties and phenomenon of matter at this dimension scale. Silver nanoparticles (SNPs) are best gift of nanotechnology world to human beings with a great interest to the pharmaceutical industries. They have great impact on today's science and medicinal world because of its unique properties with wide application in different fields [8–10]. Interaction of silver nanoparticles with Bovine serum albumin (BSA) was well reported by Jessy M. (2011), Silver nanoparticle formed complex with BSA and showed hydrophobic interactions [11]. A lot of work has been undertaken to understand the interaction between nanoparticles and proteins, to explore protein- nanoparticle complex in medicine and pharmaceutical field. There are few reports which mention that nanoparticles are being developed as an anti venom action. Yu H. and co-workers (2011) designed synthetic polymer nanoparticles which can neutralize the bee venom (melittin) *in vivo*, named as plastic antidote [12]. Gomes A. *et al.* (2016) have mentioned the neutralization efficacy of gold nanoparticles conjugated with 2-hydroxy 4 methoxy benzoic acid (HMBA) extracted from *H. indicus* root. This study specified the use of GNP-HBMA for neutralization of viper venom with better protection [13].

With ongoing advances in studies related to protein-nanoparticle interactions, nanoparticles could be conceived to interact with venom, which is majorly composed of mixture of proteins, along with other bio-macromolecules in minor amount. Understanding of such interactions can provide insights into their possible aptness as an alternative to existent anti-snake venom therapy and may be of crucial importance to develop new strategy for venom antidote in near future Therefore, present study is an attempt to understand the interaction between crude viper venom and silver nanoparticles using different biophysical approaches such as UV-Vis spectroscopy, fluorescence spectroscopy, DLS studies and neutralization potency was studied by using biochemical assays like protease assay, whole blood clotting test.

Materials and Methods

Chemicals

Lyophilised Russell's viper (*Daboia russelii*) crude venom was purchased from Haffkine institute, Parel, Mumbai, India. All chemicals [Silver Nitrate (Qualigens), tri-sodium citrate (SRL), Calcium chloride (Himedia), sodium deoxycholate (Himedia), Casein (SDFCL), Tris–HCl buffer (SRL), trichloroacetic acid (LOBA Chemie) and calcium chloride (SDFCL)] were used of analytical grade without any purification. The stock solutions of venom and SNPs were prepared in the saline (pH 7.4) and double distilled water respectively. Every experiment was carried out in triplicates.

Synthesis and characterization of silver nanoparticles (SNPs)

Silver nanoparticles (SNPs) were synthesized by using chemical reduction method [14] with some modifications. This method includes the use of silver nitrate, and tri-sodium citrate as a reducing and capping agent. 1 mM silver nitrate (100 ml) was heated up to 82°C on hot plate magnetic stirrer and 1% tri-sodium citrate was added drop wise till colourless solution turns to pale yellow. UV-Visible spectroscopy (Implen nanophotometer) was used to record the absorbance peak of SNPs. The sample was scanned from 200 nm to 600 nm wavelength. To record the hydrodynamic size, zetasizer nano ZS90 DLS (Malvern) system with 633 nm laser have been used. Morphology of SNP was studied using field emission Gun- transmission electron microscope 300 Kv (Tecnai G2, F30 Tecnai G2, F30)

Release of Silver (Ag^+) ion

The release of Ag^+ ions from Silver nanoparticles were monitored by colorimetric method with some modifications [15,16]. Varying concentrations of Ag^+ ions (2 ppm, 4 ppm, 6 ppm, 8 ppm) were prepared by diluting $AgNO_3$ (1 mM) in buffer solution (pH 7.4). Ten millimole of 3,3′,5,5′-tetramethylbenzidine (TMB) (120 µl) added in reaction mixture and the reaction was monitored after 30 minutes by directly scanning from 200 nm to 800 nm on UV-Vis spectrophotometer against blank (buffer+TMB). The linear calibration curve was obtained by plotting Absorbance at $\Delta A_{656\,nm-543\,nm}$ against concentration of Ag^+ ions [16]. Further release of Ag^+ ions were measured in presence of SNPs with time (upto 3 hrs).

Interactions of SNPs-Crude viper venom

Viper venom (40 ug/ml and 120 ug/ml in case of absorbance studies) was incubated with increasing concentrations of SNPs (0.062 nM, 0.124 nM, 0.186 nM, 0.248 nM SNP)

for 30 min at room temperature and samples (venom-SNP) were analysed by using following biophysical and biochemical approaches.

UV-Visible spectroscopic study

The absorbance maxima of viper venom and venom incubated with increasing concentration of SNPs were recorded on UV-Visible nanophotometer (Implen) in a wavelength ranges from 200 nm to 600 nm. Path length was set at 10 mm.

Fluorescence and synchronous spectroscopic measurements

The intrinsic fluorescence properties of crude viper venom and venom incubated with different SNPs concentrations were recorded on fluorescence spectrophotometer (Varian, Cary Eclipse) by using quartz cuvette. The parameters like excitation wavelength (280 nm), emission wavelength (281 nm to 600 nm), slit width (10 nm) and PMT voltage (600 V) were set prior to experiment. Synchronous fluorescence spectra of a crude viper venom and venom-SNP samples were taken by keeping $\Delta\lambda = 60$ nm for tryptophan. The initial excitation wavelength was set at 280 nm and scanned up to 600 nm. The slit width was set at 10 nm and PMT voltage was 600 V.

Dynamic light scattering study

The hydrodynamic radius of venom, SNPs and venom-SNP complexes were recorded on zetasizer nano ZS90 DLS system with 633 nm laser.

Protease assay

Protease assay was performed according to the method prescribed by Greenberg (1955) with some modification [17]. The assay involved reaction mixture of casein (200 µg/ml), Tris-HCl buffer (pH 8.0) and centrifuged viper venom (20–100 µg/ml) at 4500 rpm/10 min. Samples were incubated for three hours at 37°C. 100 µl of trichloroacetic acid (TCA) was added after incubation to stop the reaction. The reaction mixture was filtered with Whatman no. 1 filter paper so as to remove undigested casein. The filtrate (1.0 mL) was used for protein estimation by the method of Lowry *et al.* (1951) [18]. L-tyrosine was used as a standard. One unit of enzyme activity was defined as the amount that yielded 0.02 µmole of tyrosine/ hour [19]. The same experiment was performed for the samples by pre-incubating venom (20 µg–100 µg) with varying concentrations of SNPs (0.062 nM, 0.124 nM, 0.186 nM, 0.248 nM SNP).

Whole Blood clotting test (WBCT)

Whole Blood clotting test [20] was performed with viper venom. Sterilized vacutainer tube containing sodium citrate (3.2%) was used to collect fresh blood prior to experiment from healthy volunteer. 100 µL blood was mixed with varying concentrations of venom. Clotting time was recorded upon addition of $CaCl_2$ after formation of gelatinous clot. Saline was used as control. Further inhibition study was done with the samples of pre-incubated venom with SNPs (0.062 nM, 0.124 nM, 0.186 nM, 0.248 nM SNP). Microscopic images (Bright field) of different blood clot samples (normal blood clot, clot formed by viper venom and clot formed by venom in presence of different concentrations of SNPs) were recorded by using the Olympus BX152 Microscope.

Results and Discussion

Characterization of Silver nanoparticles (SNP)

Silver nanoparticles were synthesized by using chemical reduction method where the Ag^+ ions from $AgNO_3$ co-precipitate to form a silver nanoparticle in presence of tri-sodium citrate which acts as a reducing as well as capping agent which prevents aggregation of silver nanoparticles. Pale yellow colour of the sample indicates synthesis of SNPs. UV-Visible absorbance spectroscopy is useful technique to characterise SNPs with its property to show Plasmon maxima in a range of 418–420 nm. Synthesized SNPs showed λ max at 420 nm wavelength (Fig. 1). DLS data confirmed the average hydrodynamic size of SNPs to be 52 nm (Fig. 2). TEM image revealed the spherical shape of SNP (Fig. 3). Concentration of the silver nanoparticle can be calculated using following formula [11].

$$C = N_{total} / NVN_A$$

Where, N_{total} = Total number of silver atoms added to the reaction solution,

N = Number of silver atoms present in each nanoparticle
V = Volume of the reaction solution in litres and
N_A = Avogadro's constant.

The concentration of silver nanoparticles in our system was

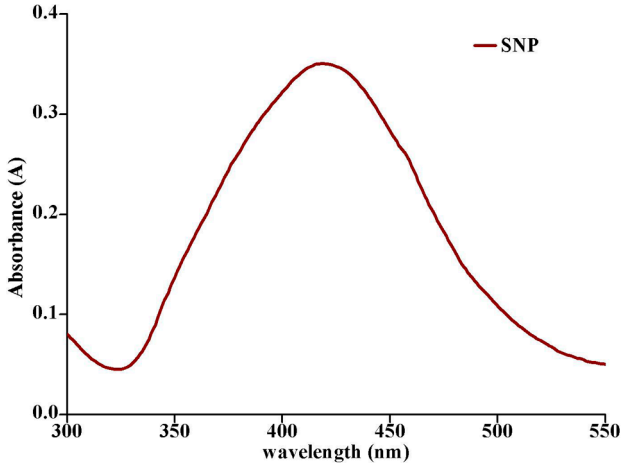

Figure 1 UV-Visible absorption spectra of silver nanoparticles.

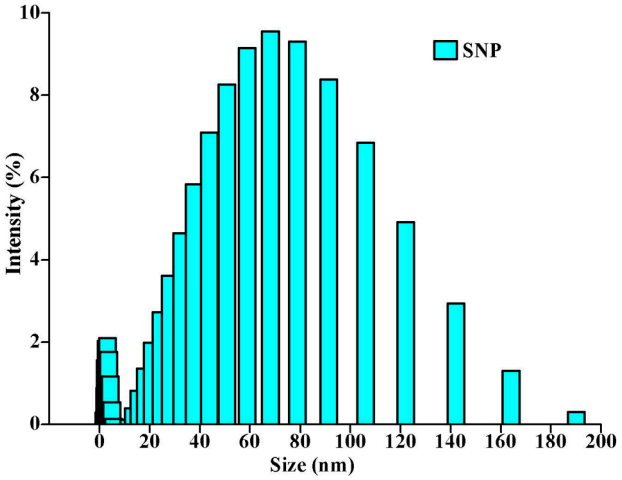

Figure 2 DLS spectra of SNP showing particle size distribution.

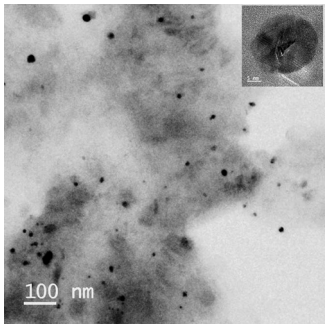

Figure 3 TEM image of SNP showing spherical shape.

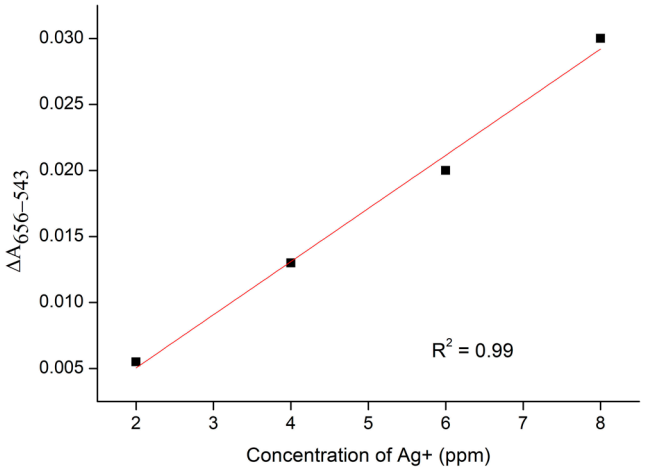

Figure 4 Calibration curve for quantification of Ag^+ ions.

found to be 1.24 nM per litre. These chemically synthesized silver nanoparticles having average size of 52 nm and spherical shape were used to interact with crude venom and formed complexes were characterized by different biophysical tools.

The release of Ag^+ ions was measured by colorimetric

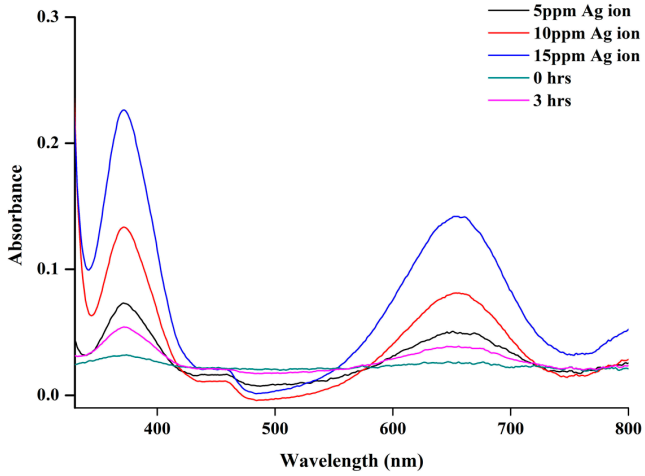

Figure 5 Absorption spectra of increasing concentration of Ag (5, 10, 15 ppm) in presence of TMB and SNPs sample at 0 hrs and 3 hrs.

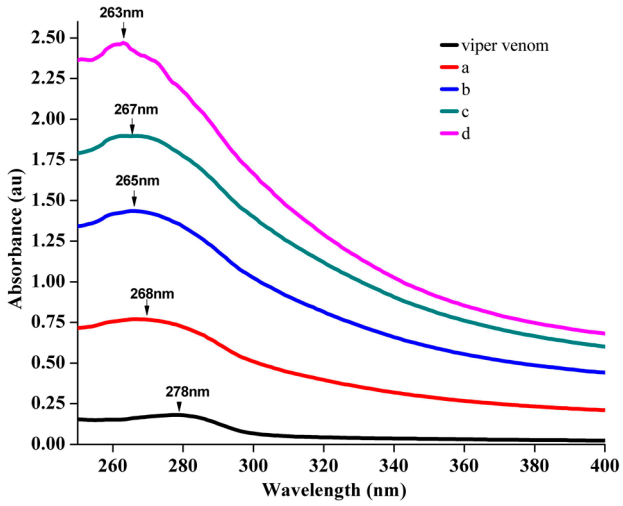

Figure 6 UV-Visible absorbance spectra of viper venom (*black solid line*) and viper venom in presence of different SNP concentrations viz. a-0.062 nM SNP, b-0.124 nM SNP, c-0.186 nM SNP, d-0.248 nM SNP.

method [15]. TMB undergoes oxidation in presence of Ag^+ which shows change in colour from yellow to blue in dose dependent. This method enables the detection of Ag^+ ions even in presence of SNPs. The typical absorbance spectra for oxidise TMB in presence of increasing concentration of Ag^+ ions (5, 10, 15 ppm) and SNP samples at 0hrs and 3hrs shown in Figure 5. The release of Ag^+ ions in SNPs solvent at the interval of 0, and 3 hrs were measured and at the end of 3 hrs it was found to be 0.5 ppm (2.75–2.25 ppm) which is very negligible may not interfere the reaction with venom.

UV-Visible spectroscopic studies

Figure 6 depicts UV-Visible spectra of crude viper venom and venom-SNP complexes. Viper venom showed absorbance maxima at 278 nm which is characteristic feature of

proteins present. Proteins generally show absorbance maxima in a range from 275 nm to 280 nm. Absorbance by protein in UV range is mainly due to its Tyr and Trp content along with Phe and disulfide bonds to small extent. It is sensitive to the change in the microenvironment of these amino acids or conformational change in polypeptide. The λ max of viper venom (278 nm) was found to be shifted towards lower wavelength (268 nm–263 nm) along with increasing absorbance intensity upon incubation with various concentration of SNP. This blue shift in spectra was result of increase in a polarity of solvent. This is indicative of interaction between SNPs and venom protein. These interactions might have resulted in conformational change of venom proteins with an increase in polarity of solvent, shifting amino acids in the hydrophobic environment of the protein (core of protein). SNPs formed a ground state complex with the venom proteins. Similar findings were reported by Jessy, M. *et al.* (2011) and Roy, S. *et al.* (2014) [11,21]. They studied the interaction of SNPs with BSA.

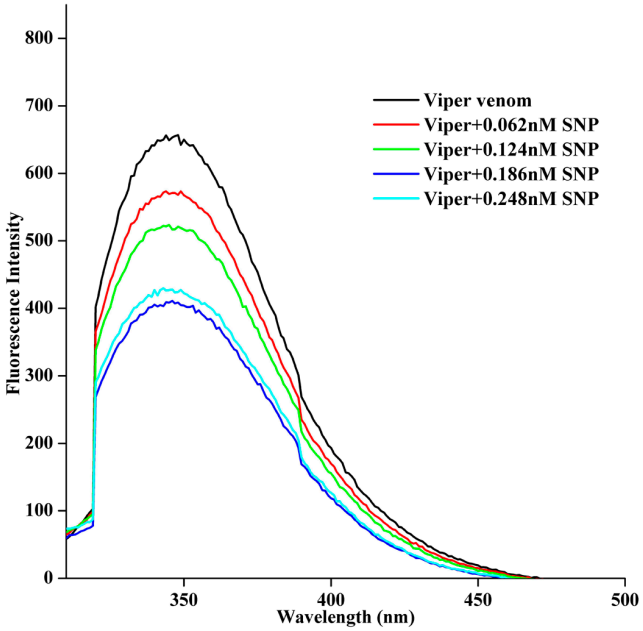

Figure 7 Fluorescence spectra of crude viper venom (*Black solid line*) and venom with increasing concentration of SNPs (0.062 nM, 0.124 nM, 0.186 nM, 0.248 nM SNP).

Fluorescence spectroscopy

Fluorescence spectroscopy deals with the analysis of fluorescence signals emerged from fluorescent molecule or fluorophore. It is dynamic technique to study the interactions at molecular level. It is being extensively used to study the protein-protein, protein-ligand, protein- nanoparticles interactions. It has wide applications in biochemistry, biophysics, and analytical science because of its high sensitivity. In a present study, we have analysed interactions of venom proteins (crude venom) with SNPs. Other bio-macromolecules present in venom like lipids and saccharides are essentially non-fluorescent. Proteins exhibits intrinsic fluorescence because of the presence of three amino acids viz. phenylalanine, tyrosine and tryptophan. Out of which tyrosine and tryptophan are of experimental use because of high quantum yield and thus can give good fluorescence signals [22]. Both amino acids get excited at 280 nm wavelength. Since these amino acids are very sensitive to change in microenvironment, they can be used to study the change in conformation of proteins, denaturation, substrate binding and ligand binding with protein. Enzymatic toxins present in snake venom like phospholipase A_2, acetylcholinesterase, proteases, vipoxin etc contain tyrosine and tryptophan residues [23]. Figure 7 depicts the emission spectra of crude viper venom (black line) and viper-SNP complexes. Crude venom sample showed the emission peak at 348 nm with higher intensity which was found to be quenched once interacted with SNPs. Quenching is referred as reduction in fluorescence intensity of fluorophore in presence of quencher [24]. It is function of different molecular interactions between quencher (SNP) and fluorophore (crude venom) which involve excited state reactions, rearrangements in molecules, energy transfer, ground state complex formation and molecular collisions. Here, we propose that interaction of SNPs and venom resulted in ground state complex formation. Lian, S. *et al.* (2009) observed the quenching in fluorescence intensity when viper and cobra snake venoms interacted with the 6-amino-4-aryl-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carbonitrile with the help of fluorescence spectroscopy [25].

Synchronous fluorescence

Synchronous fluorescence spectroscopy involves the simultaneous (synchronous) scanning of both the excitation and emission with constant wavelength interval between them (Δλ). Difference in the excitation and emission wavelength (Δλ = 60 nm) gives information about the microenvironment of tryptophan while difference of 15 nm (Δλ = 15 nm) reveals information about the microenvironment of tyrosine [26,27]. This information provides the conformational changes in protein. The synchronous spectra showed reduction in the fluorescence intensity of venom with increasing concentration of SNPs (Fig. 8). SNPs, upon interacting with crude venom, acted as quencher by causing a change in microenvironment of tryptophan amino acid, and ultimately altering the protein conformation.

Data obtained by using different Fluorescence spectroscopic modes (intrinsic, synchronous) revealed that interaction between silver nanoparticles and viper venom components resulted in conformational changes of venom proteins. Colloidal solution of viper venom has shown change in its polarity upon addition of SNPs. Researcher reported the fluorescence characteristics of complex formed by interacting silver nanoparticles, gold nanoparticles with BSA [11,28]. Formation of complex between Viper venom

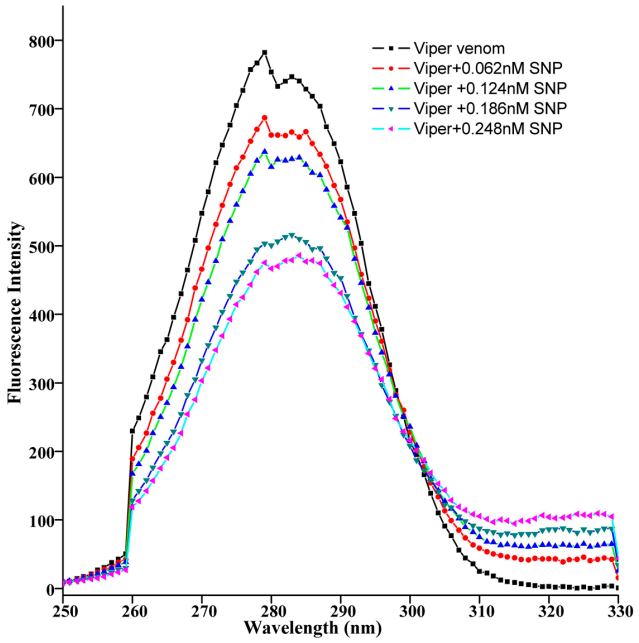

Figure 8 Synchronous fluorescence spectra at Δλ (60 nm) for viper venom and venom incubated with different concentration of SNPs (0.062 nM, 0.124 nM, 0.186 nM, 0.248 nM SNP).

components and SNPs was confirmed with dynamic light scattering technique.

Dynamic light scattering (DLS) studies

Dynamic light scattering method was used to confirm the interaction of viper venom with varying concentration of SNPs (0.062 nM, 0.124 nM, 0.186 nM, 0.248 nM SNP). Hydrodynamic size of viper venom suspended in physiological saline was found to be higher (526 nm). This is due to; most of the proteins and polypeptides (Phospholipase A_2, proteases etc.) present in venom form complexes (homo/heterodimers). These homo and heterodimers show synergistic interactions and thus exhibit higher level of activity or potency [29]. Upon incubation with the silver nanoparticles, the hydrodynamic size of venom-SNP complexes were found to be reduced to approximately 200 nm (Table 1). This may be due to adsorption of venom proteins on to the surface of silver nanoparticles leading to conversion of dimmers into monomers. Destruction of venom protein structure would results in suppression of activity of venom proteins. The size of venom-SNP complexes was found to be saturated to 202–214 nm. There is no significant change in size of complex observed even with increase in concentration of SNP.

Several reports have shown that proteins adsorbed on the surface of nanoparticles and forms corona. Tommy, C. *et al.* (2006) mentioned that proteins compete for adsorption on nanoparticles and its rate is dependent on the nature of nanoparticle surface and protein [30]. Stefano, P. B. *et al.* (2013) showed that BSA has strong adsorption on the surface of gold nanoparticles (GNP) [31]. Jessy, M. *et al.* (2016)

Table 1 DLS data of Viper venom (40 µg) and venom-SNP complexes

Samples	Z Average size (nm)
0.062 nM SNP + venom	208
0.124 nM SNP + venom	202
0.186 nM SNP + venom	214
0.248 nM SNP + venom	207
Crude viper venom (40 µg)	526
SNP	51.91

Venom incubated with increasing concentration of SNP viz. 0.062 nM, 0.124 nM, 0.186 nM, 0.248 nM SNP.

studied the interaction of gold nanoparticles with BSA. They observed that BSA has strong adsorption on negatively charged GNP and change its alpha helical structure [28].

Protease assay

Protease assay measure the activity of proteases by using spectroscopic approach. It is quantitative assay and most efficient way to study the activity of protease enzyme. Enzyme proteases could cleave protein by breaking peptide bonds and fragment it in to amino acids. The released amino acids can be quantified and correlated with proteases activity. In present investigation, casein was used as a substrate for venom proteases. Protease enzyme digests the protein casein, it releases tyrosine with other amino acids and peptide fragments. Folin's reagent was used to quantify free tyrosine which produces a blue colour chromophore, spectrophotometricaly. The chromophores generated are directly proportional to the tyrosine released from casein. Thus more the tyrosine released stronger the activity of the protease. Viper venom showed dose dependent protease activity. Enzyme activity of proteases of venom was found to be differed upon interaction with SNPs. At the concentrations of SNPs (0.124 nM and 0.186 nM), 9–13% inhibition in protease activity was observed. Snake venom proteases like serine proteases, metalloproteases, etc. are mainly involved in different biological activities like digestion, blood coagulation, immune system regulation and inflammation [23,32,33]. The inhibition of proteases enzymes would result in suppression of these biological roles attributed to it. Thus affects (inhibit) the digestion, blood coagulation and inflammation pathways of proteases in presence of silver nanoparticles. Ibrahim, M. A. *et al.* (2011) studied the inhibition of cobra venom in presence of *L. egyptiaca* and *N. rutica* extract [34]. Similarly, methanolic extract of *L. aspera* has shown inhibitory action on protease activity of cobra venom [35]. Janardhan, B. *et al.* (2014) studied the antivenom activity of extract isolated from *A. etracantha* leaves against viper venom and krait, where they observed inhibitory activity on phospholipases, phospholdiesterase while no inhibitory activity on protease observed [19].

Whole Blood clotting test (WBCT)

Blood coagulation is one of the important tests used for

understanding the hemostasis system. Hemostasis means prevention of blood loss with the formation of blood clot (coagulation). The normal coagulation cascade of blood constitute of extrinsic pathway and intrinsic pathways. Extrinsic pathway starts with trauma to tissue releasing tissue factor (TF) which further activates the cascade by activating factor VII to VIIa which in turn activates Factor X to Xa. Factor Xa converts prothrombin to thrombin through activation of factor V in presence of Ca^{++} ions. Whereas intrinsic pathway begins with trauma to blood. In this coagulation cascade factor XII get activated to XIIa which subsequently activate factor XI to XI, IX to IXa and VIII to VIIIa. In presence of Ca^{++} ions Ca^{++} ions factor VIIIa converts factor X to Xa. Factor Xa converts prothrombin to thrombin with the help of activated factor V and subsequent formation of fibrin clot takes place from fibrinogen. Fibrin forms a mesh to avoid blood loss [36].

Viper venom contains Russell's viper venom factor V (RVV-V) and Russell's viper venom factor X (RVV-X) which directly activate factor V and X of coagulation cascade; thus converting factor V and X into Va and Xa respectively. Va and Xa then converts pro thrombin to thrombin in presence of phospholipids and calcium ion. Thrombin once activated converts fibrinogen into fibrin which forms mesh with RBCs to form blood clot [37]. If we compare this coagulation cascade with normal one; RVV-V and RVV-X skips initial phase of coagulation cascade which involves other factors like tissue factor (TF) and factor VII of extrinsic pathways or factor XII, XI, IX and VIII of intrinsic pathway. This result in faster coagulation of blood compare to normal coagulation cascade. Viper venom exhibits strong dose dependent coagulant action, with increase in dose of crude venom the clotting time of human blood decreases [38]. In present study, the whole blood clotting test was carried out for crude viper venom where clotting time was found to be decrease with increase in venom concentration (100 µg–500 µg). The clotting of blood in presence of saline (under physiological condition) showed normal clotting time (135 sec) and SNP also showed no interference in coagulation pathways (clotting time = 120 sec.). The clotting time was prolonged in presence of silver nanoparticles from 28 sec (100 µg/ml viper venom) to 62 sec (0.062 nM SNP + Venom) (Table 2). This might be due to silver nanoparticles interfering with clotting factors present in venom and suppressing its activity. RVV-X shows stable conformation due to presence of disulfide bridges, multiple hydrophobic interactions and hydrogen bonds. In presence of silver nanoparticles, the polarity of solvent increases which may hinder these interactions of RVV factor. SNPs may even break the H- bond and Disulfide bond which disturb the stability of RVV-X factor. This resulted in inactivation of RVV factors thus prolonged the coagulation time.

Similar results were reported by Vineetha, M. S. *et al.* (2017). They studied the neutralization of viper snake venom by *Canthium parviflorum* extract, where the clotting time was found to be prolonged in presence of extract [39].

Table 2 WBCT data of crude viper venom (100 µg) and viper venom-SNP complexes

Samples	Clotting time (Seconds)
0.062 nM SNP + venom	62
0.124 nM SNP + venom	54
0.186 nM SNP + venom	48
0.248 nM SNP + venom	38
Crude viper venom	28
SNP	120
Saline control	135

100 µg of venom incubated with increasing concentration of SNP viz. 0.062 nM, 0.124 nM, 0.186 nM, 0.248 nM SNP.

Microscopic study of whole blood clot

Whole blood clot is a clump having red blood cells (RBCs) and platelets (thrombocytes) entrapped in network of fibrins. RBC has phosphotidylserine expressed on its surface provide the surface for fibrin to accumulate and trigger the coagulation. During blood clot formation, fibrins are loosely arranged around the cells with formation of definite uniform pattern (Fig. 9: normal clot). This pattern of blood clot or its morphology may change due to several reasons like change in environmental conditions (pH or ionic strength), change in concentration of fibrinogen, thrombin, calcium ions and salt, number of RBCs and presence of other biomolecules that interfere with it [40–42]. Weisel, J. W. *et al.* (1992) studied the effect of change in fibrin, thrombin, salt concentration, platelet factor IV and other factors on the formation of blood clot and its turbidity where the turbidity is correlated with structure of clot. Structure of clot is also dependent on the rate of formation of fibrin mesh [43].

Figure 9 shows the morphological variations in blood clot in presence of viper venom and viper venom incubated with varying concentrations of SNPs compared to normal blood clot. The normal pattern of blood clot was found to be modified due to interference of viper venom with normal coagulation cascade. Here, we observed that the venom upon incubation with SNPs showed variation in the clotting morphology than venom alone. The clot obtained in presence of venom with 0.062 nM SNP concentration showed a pattern which closely resembles normal blood clot. With increase in concentration of SNPs, heterogeneity in clotting was observed. This may be due to oxidative susceptibility of fibrinogen upon exposure to oxygen in presence of metal. These results help to correlate with the results obtained with whole blood clotting test.

Conclusion

Snake bite is major health issue in Asian countries with lack of considerable treatment. The only treatment available is administration of ASV (passive immunisation) with some drawbacks. With increasing utilization of nanoparticles in

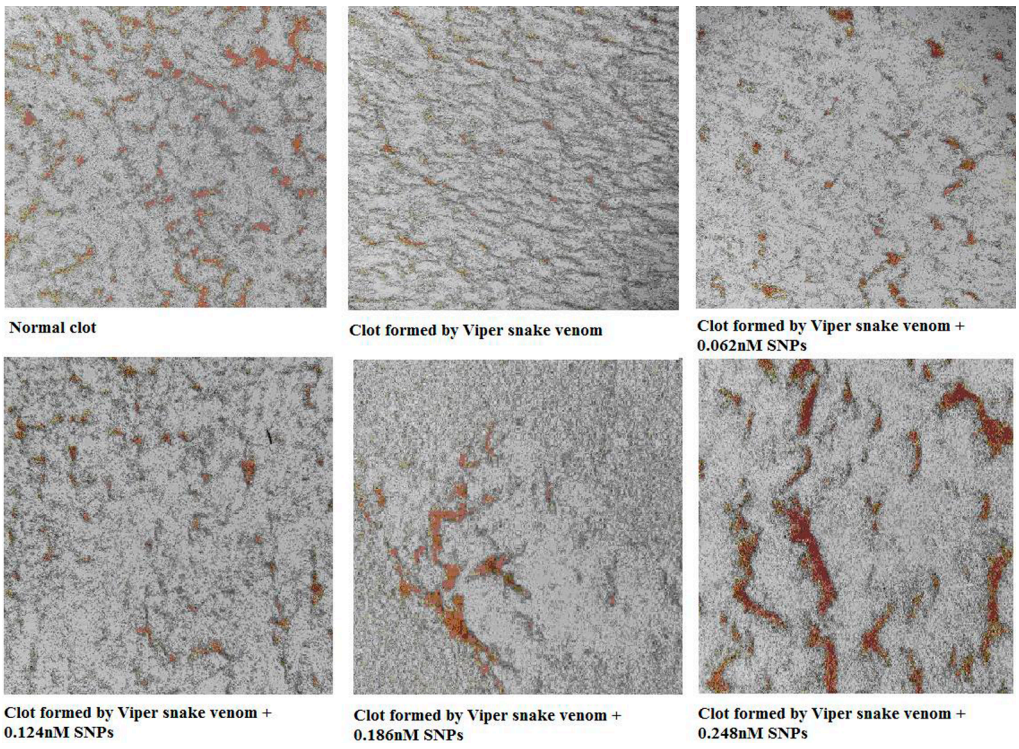

Figure 9 Microscopic images (bright field) of normal blood clot, clot formed by Viper snake venom and clot formed by viper snake venom in presence of varying concentrations of SNPs.

pharmaceutical field and also on the basis of the result obtained in present study, nanoparticles seem to be a good substitute for ASV. This is very primitive step towards the development of nanoparticles-based snake venom inhibition therapy. It could bring an expected outcome if studied systemically. This investigation monitored the interaction of silver nanoparticles with crude viper venom by using different biophysical, biochemical tools and also measured release of Silver ions during experimental condition. Results obtained by UV-Visible spectroscopy showed the formation of ground state complex between viper venom and SNPs. Different fluorescence spectroscopic modes revealed the interaction between SNPs and crude viper venom with decrease (quenching) in the fluorescence intensity. DLS study showed the formation of complex between SNPs and crude viper venom with decrease in hydrodynamic size of complex compared to the size of native viper venom. Venom components might have adsorbed on the surface of SNPs and making Viper venom proteins more compact in nature results in modification of its activity. The functional activity of the SNP-venom complex was studied by protease assay and whole blood clotting test. Significant reduction in the activity of protease enzyme was observed (9–13%), the clotting time of blood was found to be prolonged (2 fold) in presence of silver nanoparticles compared to crude venom. Morphology of blood clot was found to be altered in presence of snake venom. RVV proteases are mainly the major components responsible for pro coagulation of blood, can be conquered with the help of SNPs.

So far this study focuses on interaction of silver nanoparticles with venom at fixed dimension of particles (52 nm). To the best of our knowledge, this is the first kind of report of inhibition of snake venom action by silver nanoparticles and other nanoparticles have not been used for similar studies. The effect of changes in particle diameter and surface characteristics has not been investigated in this study. However, it is possible that size and shape dependent characteristics may influence the suppressive effect of venom due to change in physico-chemical characteristics.

Thus, present study suggests that inhibition of viper snake venom activity in presence of SNPs. This study could be explored to understand the practical applicability of SNPs as an alternative therapy for neutralization of snake venom. We believe SNPs have a potential to take a centre stage in advancement of treatment for snake poisoning in near future.

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Conflicts of interest

V. H., D. P. and P. D. declare that they have no conflict of interest.

Author contribution

V. H. and D. P. worked on the preparation of all chemicals and performed all the experiments V. H. has written the manuscript. P. D. is the corresponding author and designer of the research. He directed entire project and co wrote the manuscript. All authors read and approved the final manuscript.

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