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Synthesis and characterization of cysteine functionalized silver nanoparticles for biomolecule immobilization

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Abstract A facile method for the aqueous phase synthesis of cysteine-functionalized silver nanoparticles by potato extract has been reported in the present work. These functionalized nanoparticles were then used for the covalent immobilization of a biomolecule, alkaline phosphatase, on its surface through carbodiimide coupling. Different reaction parameters such as cysteine concentration, reducing agent concentration, temperature, pH and reaction time were varied during the nanoparticles' formation, and their effects on plasmon resonance were studied using Ultraviolet–visible spectroscopy. Fourier transform infrared spectroscopy was used to confirm the surface modification of silver nanoparticles by cysteine and the particle size analysis was done using particle size analyzer, which showed the average nanoparticles' size of 61 nm for bare silver nanoparticles and 201 nm for the enzyme-immobilized nanoparticles. The synthesized nanoparticles were found to be highly efficient for the covalent immobilization of alkaline phosphatase on its surface and retained 67 % of its initial enzyme activity (9.44 U/mg), with 75 % binding efficiency. The shelf life of the enzyme-nanoparticle bioconjugates was found to be 60 days, with a 12 % loss in the initial enzyme activity. With a simple synthesis strategy, high immobilization efficiency and enhanced stability, these enzyme-coated nanoparticles have the potential for further integration into the biosensor technology.

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Introduction

Due to their attractive physical and chemical properties, nanoparticles have attained considerable importance over the past few years. Among the noble metals, silver nanoparticles (SNPs) have wider area of applications owing to its excellent bio-compatibility [1] and thus, can be used as antistatic, antibacterial and biosensor material [2]. One of the broader applications of these nanoparticles includes the support matrix for the enzyme immobilization. Immobilization is generally used to increase the stability, activity, reusability and shelf life of an enzyme in the solution, which further facilitate the different biotechnological processes. By providing a very large surface area and good electronic properties, these nanoparticles can act as a stable surface for the enzyme immobilization [3]. A large number of physical and chemical methods have been documented by various researchers worldwide, for the preparation of nanoparticles of varying size and shape, but owing to their toxicity, flammability and low synthesis rate, biological methods are usually preferred [4]. In either of the method employed for the silver nanoparticles' synthesis, stability of the nanoparticles remains the major concern. To increase the stability, different stabilizers and capping agents are generally introduced in the synthesizing reaction mixture [5–11]. Among the capped silver nanoparticles reported, cysteine-capped silver nanoparticles are gaining importance because besides providing stability to the nanoparticles, cysteine can also act as a linker for the biomolecule immobilization.

In the present study, silver nanoparticles were synthesized by the reduction of silver nitrate in aqueous solution,

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using potato extract as a natural reducing agent. The synthesized nanoparticles were stabilized and functionalized through cysteine amino acid, and its carboxyl group was then explored for the covalent immobilization of alkaline phosphatase (ALP) through carbodiimide coupling procedure [12]. Here, we investigated the role of different parameters such as pH, temperature, reducing agent concentration, cysteine concentration and reaction time, during the nanoparticles' synthesis and, its immobilization efficiency for the alkaline phosphatase has been discussed. The main focus of the present study was to rapidly synthesize the cysteine-functionalized silver nanoparticles, and further immobilization of alkaline phosphatase on its surface to achieve maximum stability, activity and reusability. The synthesized nanoparticles (bare capped and enzyme immobilized) were characterized by scanning electron microscope (SEM), fourier transform infrared spectroscopy (FTIR), particle size analyzer (PSA) and UV visible spectrophotometry.

Materials and methods

Chemicals

Silver nitrate, 4-Nitrophenyl phosphate disodium salt and *n*-butanol were obtained from Loba Chemie, while L-Cysteine HCL, 1-(3-Dimethylamino propyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-Hydroxysuccinimide (NHS) were purchased from Himedia. Fresh potatoes were purchased from the local market. Deionized (DI) water from Millipore was used for the reagent preparation and throughout the process.

Potato extract preparation

30 g fresh raw potatoes after thorough washing with the tap water were peeled off and cut into smaller pieces. The chopped potatoes were then boiled in 100 ml of deionized water for 15 min and filtered through Whatman filter paper No.1. Thus, the obtained potato extract was stored in the refrigerator and used within a week.

Enzyme purification

Alkaline phosphatase (ALP, orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) was partially purified from the cow milk employing some modifications [13]. In brief, 300 ml of raw milk equally diluted with water, was warmed at 37 °C in a water bath for 20 min. Cream was separated from it by centrifuging at 12,000 rpm, for 10 min at room temperature (RT). The cream thus obtained, was churned and suspended in DI water followed by 20 % (v/v) *n*-butanol treatment for 1 h, with constant stirring on a magnetic stirrer at RT. The aqueous phase containing alkaline phosphatase was then separated out by centrifuging at 12,000 rpm for 15 min at 4 °C.

Functionalized silver nanoparticles' synthesis and characterization

In 100 ml aqueous solution of silver nitrate (1 mM), 1 ml of L-cysteine (1 mM) was added and stirred on a magnetic stirrer for 10 min at RT. To this, 5 ml of potato extract was added slowly, followed by dropwise addition of sodium hydroxide (1 M) with constant stirring. The solution was then heated in a water bath till the color of the solution changes to dark brown. An instant color change from transparent to dark brown indicates the formation of cysteine-functionalized silver nanoparticles (CSNPs). The particles were separated by centrifugation at 12,000 rpm for 30 min and dried at 37 °C to obtain the powder form. Initial characterization of the nanoparticles was done by UV visible spectrophotometric method (NanoDrop 1000, v 3.8.1, Thermo Scientific), by studying their plasmon resonance in the wavelength ranges 250–750 nm. The binding of cysteine to the nanoparticles' surface was confirmed by FTIR spectroscopy (Shimdazu IRAffinity-1), while the approximate size was determined by particle size analyzer (Malvern, UK Nano ZS). Scanning electron microscope (SEM-JEOL-JSM-6480) was used to study the surface characteristics of these functionalized nanoparticles.

Effects of various parameters

Effects of five different parameters were studied during the silver nanoparticles' synthesis. It included:

Cysteine concentration

To investigate the effect of cysteine on the synthesis of nanoparticles, the reaction was carried out at seven different molar concentration of cysteine (0.5–10 mM) and the surface plasmon resonance (SPR) peaks of the resulting solution were measured spectrophotometrically.

Effect of pH

To study the effect of pH on the nanoparticles' synthesis, the reaction pH was varied from 8 to 13, and its effect on the SPR peaks was measured at each pH.

Effect of reaction temperature

The nanoparticles' synthesis was carried out at three different temperatures (4 °C, RT and 60 °C) to study their effects on

the SPR peaks. The absorbance of the resulting solution was measured spectrophotometrically at each temperature.

Effect of potato extract

To examine the effect of reducing agent on the nanoparticles' synthesis, the potato extract and silver nitrate were added in different volumetric ratio of 1:20, 1:50, 1:100 and 1:200. After the reaction, the SPR peaks were measured spectrophotometrically.

Enzyme immobilization

Before the immobilization of alkaline phosphatase, the cysteine-capped silver nanoparticles (10 mL) were treated with 100 µL of a freshly prepared mixture of aqueous NHS (30 mM) and EDC (15 mM) solution (1:1 volumetric ratio), to activate the carboxyl group of cysteine. After 1 h of RT incubation, the NHS-terminated silver nanoparticles were centrifuged and re-suspended in 3 mL of DI water. The CSNPs were then incubated with the alkaline phosphatase (3.84 mg) for 24 h at 4 °C. Finally, the ALP-CSNPs bioconjugates were washed twice with Tris–HCL buffer (0.5 M, pH 9.0), and re-dispersed in it. The percentage of the immobilized enzyme was calculated by measuring the protein content in the supernatant, before and after the immobilization process.

Enzyme assay for soluble and immobilized enzyme

The standard assay for ALP was carried out in 1 mL reaction volume, containing 300 µL of 5 mM 4-nitrophenyl phosphate (PNPP) substrate, 400 µL of 0.5 M Tris HCl buffer (pH 9) and 300 µL enzyme. The yellow colored *p*-nitrophenol produced after 30 min of incubation at 37 °C was measured spectrophotometrically at 405 nm, and the concentration was determined using its standard curve. One unit of the alkaline phosphatase activity was defined as the amount of enzyme which liberated 1 µmol of *p*-nitrophenol per min under the assay conditions.

Reusability and storage stability

After each assay cycle, ALP-CSNPs bioconjugates were collected by centrifuging the reaction mixture at 12,000 rpm for 20 min followed by thorough washing with the assay buffer and then, re-suspended in it. The reusability of the immobilized enzyme was studied by evaluating the activity of same bioconjugates for seven continuous days, under standard assay condition. The storage stability of the soluble and immobilized alkaline phosphatase was studied by assaying their activity once in a week, for up to 2 months, at 4 °C.

Statistical analysis

For the reaction parameters which were performed in triplicates ($n = 3$), a mean with a standard deviation was calculated and reported using Origin Pro (Version 8).

Results and discussion

Synthesis and optical characterization

In the present study, potato extract was used to reduce the silver nitrate in the aqueous solution to synthesize cysteine-functionalized silver nanoparticles, followed by the immobilization of the enzyme alkaline phosphatase on its surface (Fig. 1). The reducing properties of the potato extract can be attributed to its composition, which is mainly starch and different reducing sugars. In the first step, cysteine reacts with the silver ions and formed a complex by thiolate bonding [14] which also prevents any further surface interaction with potato extract constituents. This complex was then reduced by the addition of potato extract to form CSNPs, at alkaline pH and temperature of 60 °C. A very high temperature was avoided for the nanoparticles' synthesis to prevent any unwanted reactions like Maillard reaction or starch gelatinization in the aqueous medium, caused by the presence of starch or reducing sugars of potato extract. Within 15 min, a dark brown color was developed, which is a primary indication of the formation of silver nanoparticles. Also, it is reported that the silver nanoparticles generally exhibit a yellowish-brown color in the aqueous solution as a result of the surface plasmon resonance [15]. The reaction was further allowed to proceed for 30 min for the complete reduction, and the solution was finally cooled to RT. The nanoparticles were initially characterized by studying their surface plasmon resonance (SPR), a characteristic property of nanoparticles owing to which they strongly absorb electromagnetic waves in the visible region. Figure 2 shows the UV absorption spectra of cysteine-capped silver nanoparticles at different time intervals. It was observed that up to 30 min, the SPR peak intensity increased and after that, no significant changes occurred in the peak up to 90 min. It indicates that all the silver nitrate has been reduced to the silver nanoparticles, with a single sharp SPR peak at 405 nm (λ_{max}). From the spectral properties of silver nanoparticles in the solution, different types of information can be obtained. It gives an idea about the size of synthesized nanoparticles as an increase in the nanoparticle diameter causes the SPR peaks to broaden and shift it to a longer wavelength [16]. According to Mie's theory [17], spherical nanoparticles give rise to a single SPR peak while two or more peaks are expected in anisotropic particles

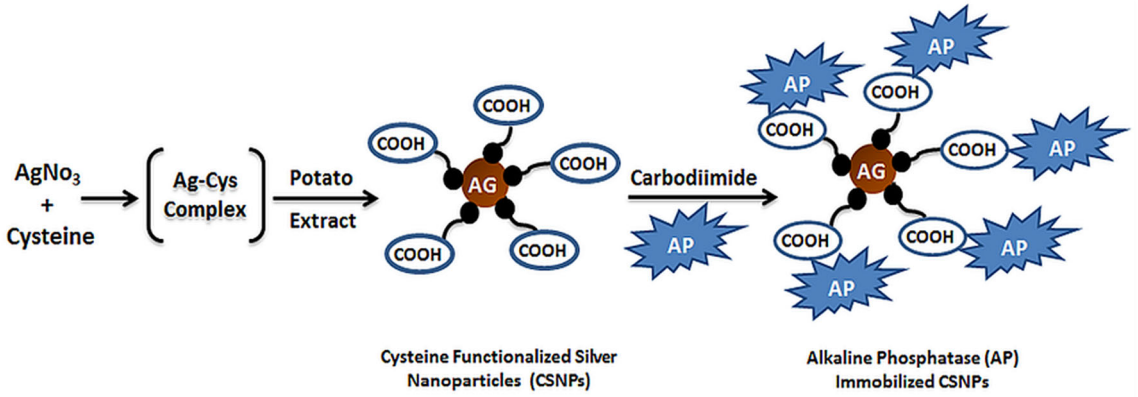

Fig. 1 Diagrammatic representation of the technique to synthesize cysteine-functionalized silver nanoparticles. The cysteine-functionalized silver nanoparticles were synthesized using potato extract as a natural reducing agent, and activated through carbodiimide to covalently immobilize alkaline phosphatase

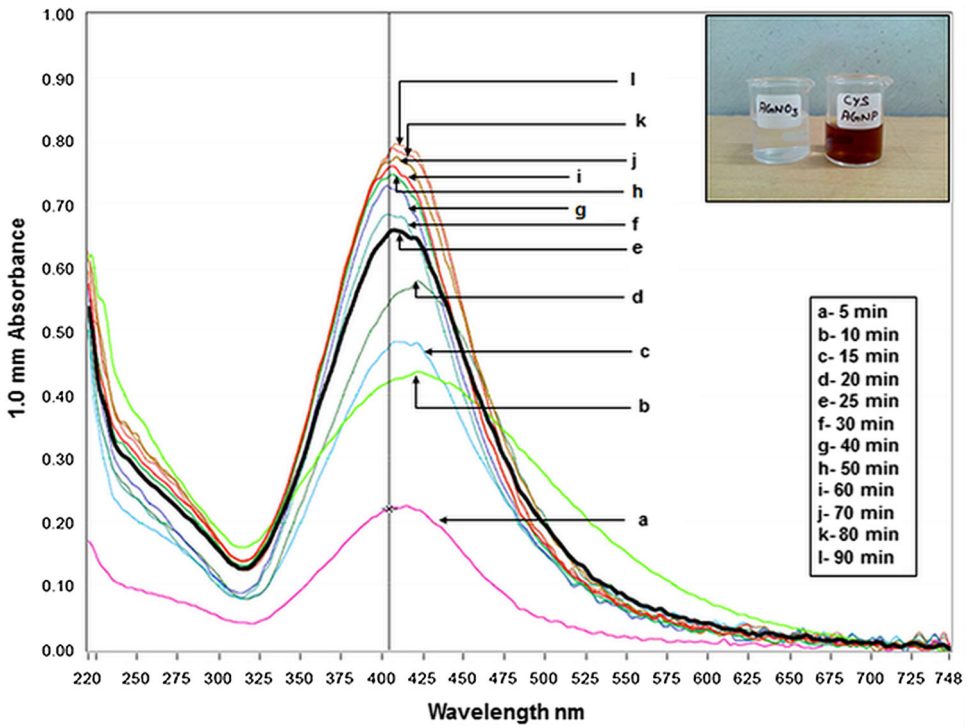

Fig. 2 UV–visible spectroscopy of cysteine-functionalized silver nanoparticles at different time intervals. The transparent solution of silver nitrate undergoes reduction by the potato extract to form a brown colored solution of cysteine-functionalized silver nanoparticles (*inset*)

depending on the shape of particle. From our SPR data, we can assume that the synthesized silver nanoparticles were smaller in size and isotropic in shape.

SEM, PSA and FTIR analysis

To confirm the surface binding of cysteine to the silver nanoparticles, FTIR analysis was performed by grinding the powdered sample with KBr pellets and analyzed in the range of 500–4,000 cm^{-1}. The thiol bond which has a characteristic vibration peak at 2,550 cm^{-1} was not observed in the cysteine-functionalized silver nanoparticles (Fig. 3). It indicates the association of metal surface with the cysteine-SH group via thiolate linkage [18]. Similar disappearance of thiol characteristic peak was also reported in cysteine-capped silver nanoparticles [19] and cysteine-capped gold nanoparticles [20]. The size and the morphology of CSNPs were further analyzed by particle size analyzer and scanning electron microscopy (Fig. 4). SEM analysis revealed that the synthesized nanoparticles were almost spherical in shape and dispersed evenly. The slimy appearance in Fig. 4b would possibly be due to the attachment of enzyme, alkaline phosphatase, on the CSNPs surface. The

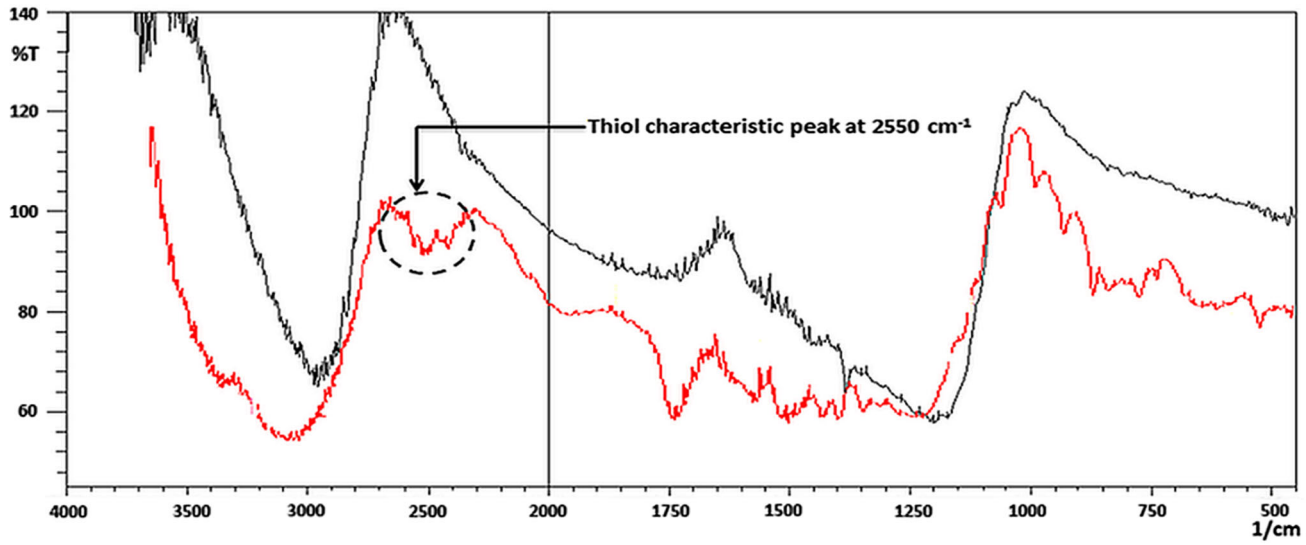

Fig. 3 FTIR analysis graph of cysteine (*lower spectrum*) and cysteine-functionalized silver nanoparticles (*upper spectrum*). The characteristic peak of cysteine thiol bond at 2,550 cm^{-1} (shown in *black dotted circle*) is not present in *upper spectrum*, indicating the formation of thiolate linkage between cysteine and nanoparticle surface

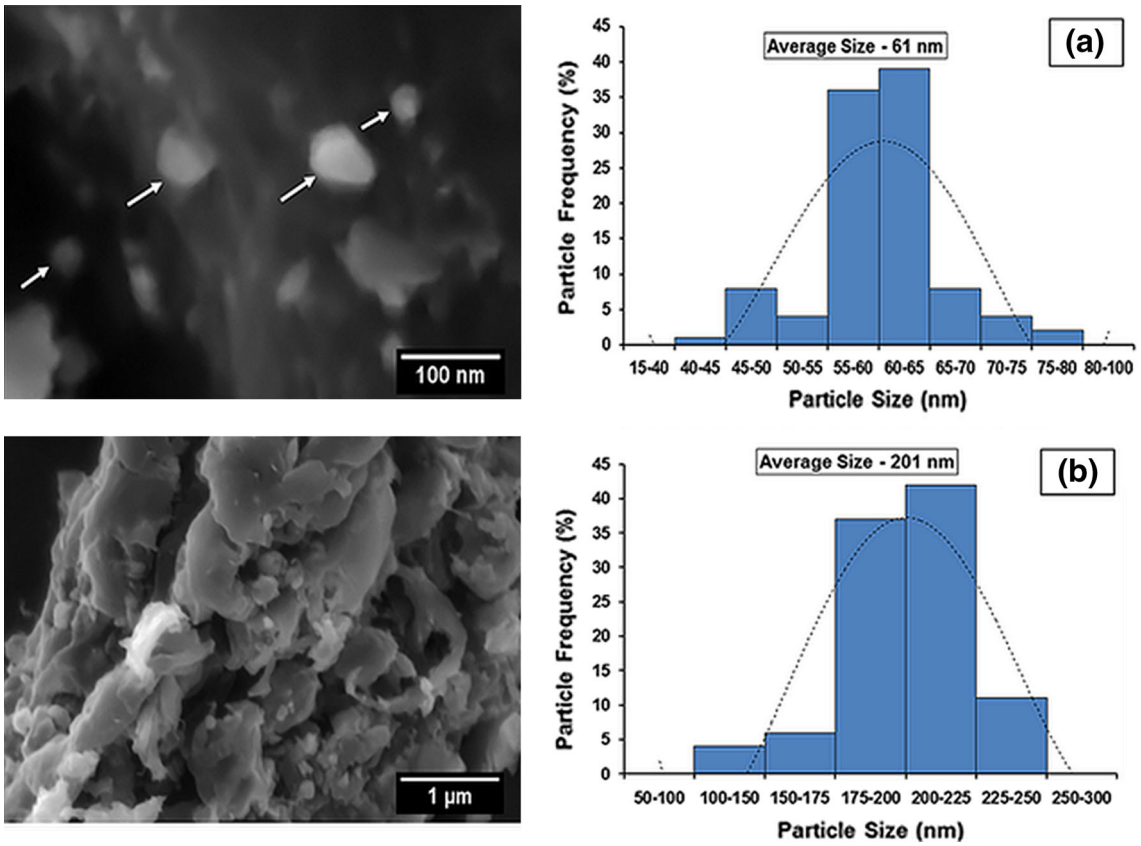

Fig. 4 Size distribution graph with SEM images of **a** CSNPs and **b** ALP-CSNPs, showing the variation in the size of cysteine-functionalized silver nanoparticles, before and after the enzyme immobilization

immobilization of enzyme on the nanoparticles' surface was further confirmed by PSA. It is evident that there is an increment in the particle size upon enzyme immobilization and the average size estimated to be 61 nm for CSNPs was increased to 201 nm for ALP-CSNPs.

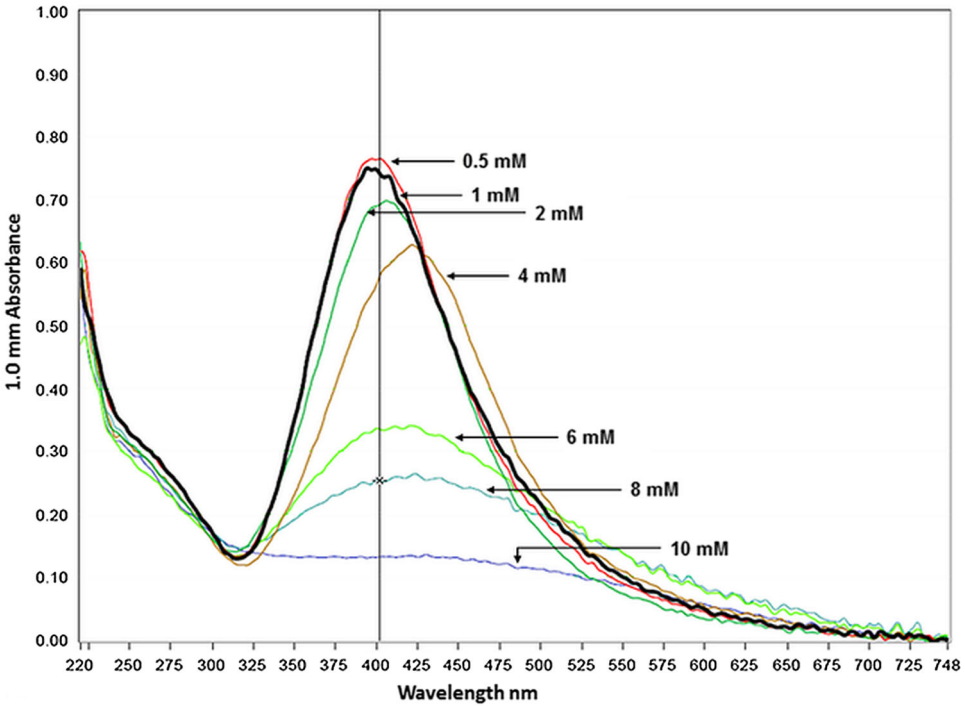

Fig. 5 Effect of varying concentration of cysteine on UV–visible spectra of synthesized CSNPs

Parameters effects

Cysteine concentration

Figure 5 shows the effect of varying concentration of cysteine (0.5–10 mM) on the SPR bands of the synthesized silver nanoparticles. It has been observed that, as the concentration of cysteine was increased from 1 to 10 mM (1, 2, 4, 6, 8 and 10), the SPR peaks broaden and shifted to a higher wavelength, which may indicate the aggregation of silver nanoparticles [21]. However, a sharper peak was observed at lower cysteine concentration (0.5 and 1 mM). The nanoparticles formed at 2 and 4 mM concentrations were not stable and showed a red shift within 48 h. At 6 and 8 mM, a broader peak indicates the formation of large sized unstable nanoparticles, while at 10 mM concentration, no peak was observed. The disappearance of peak at the higher cysteine concentration may be due to the acidic condition of the reaction medium contributed by the cysteine. It is reported that the acidic conditions usually suppress the nanoparticles' formation [22]. Also, at the higher concentration of cysteine, the tendency of aggregation of CSNPs increases, by interconnecting via hydrogen bonds. So 1 mM cysteine was chosen as an optimum concentration for further study. Similar aggregation has also been reported in the synthesis of silver nanoparticles [14, 23] and gold nanoparticles [24].

Effect of pH

The synthesis of CSNPs was also performed at different pH (8–13). The pH of the solution was varied by dropwise addition of sodium hydroxide (1 M) to the reaction mixture. No color was developed even after 24 h of incubation at pH 8.0 and 9.0. A rapid color development was observed on further increasing the pH, with the development of SPR peaks in the range of 400–410 nm (Fig. 6). At pH 10.0, light brown color was observed with a broader peak which narrowed down at pH 10.5. At pH 11, dark brown color was developed within 30 min with a sharp SPR peak, which indicates the formation of highly dispersed nanoparticles. The intensity of color and the height of peak remain almost constant on further increasing the pH. The results confirmed the role of pH as an accelerator in the nanoparticles' formation. The increased reduction at alkaline pH can be contributed to the presence of glucose in the extract, which undergoes mutarotation through a highly reactive open chain structure. The free aldehyde group of this open chain has the tendency to reduce silver nitrate at elevated temperature and in alkaline pH [25]. Such higher pH value for the formation of well-dispersed nanoparticles has also been reported for the silver nanoparticles, synthesized by albumin [26], synthetic oligopeptides [27] and *Cassia angustifolia* leaf extract [28].

Effect of reaction temperature

To investigate the effect of temperature on the reduction process, the reaction was carried out at three different temperatures (4 °C, RT and 60 °C). At 4 °C, no color change was observed up to 24 h of incubation while at RT, the color starts to develop after 90 min of incubation and took almost 16 h to complete (Table 1). At 60 °C,

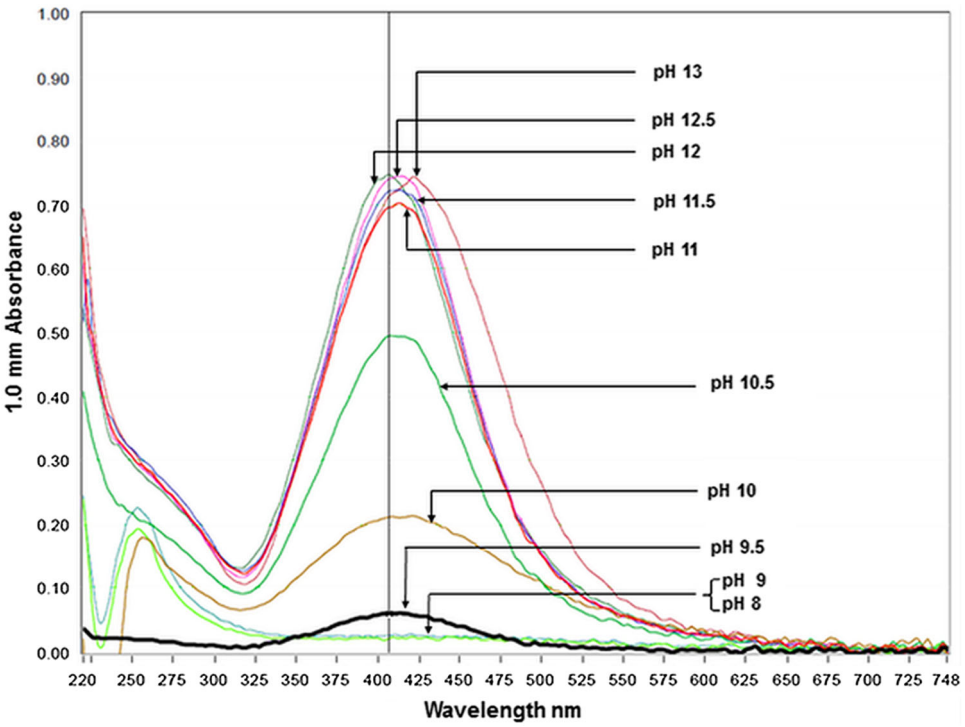

Fig. 6 Effect of varying pH on UV–visible spectra of synthesized CSNPs

Table 1 Effect of varying reaction temperature on the synthesis of cysteine-functionalized silver nanoparticle

Parameters	Reaction time								
Reaction temperature	30 min	60 min	90 min	120 min	4 h	12 h	16 h	18 h	24 h
4 °C	–	–	–	–	–	–	–	–	–
RT	–	–	+	+	++	++	+++	+++	+++
60 °C	+++	+++	+++	+++	+++	+++	+++	+++	+++

No peak (−)

Low intensity peak (+)

Medium intensity peak (++)

High intensity peak (+++)

reduction occurs rapidly and the SPR peak began to develop and almost completed within 30 min. With further increasing the temperature, no significant changes in the reduction time were observed. From the data, we can conclude that the reduction process is a temperature-dependent process and a higher temperature favors a higher rate of nanoparticles' synthesis. So, 60 °C was chosen as an optimum temperature for the reduction process. Similar results for accelerated synthesis of silver nanoparticles at higher temperature have also been reported by Jiang et al. [29].

Effect of potato extract

To investigate the effect of potato extract concentration on the synthesis of silver nanoparticles, potato extract and silver nitrate solution were mixed in a different volumetric

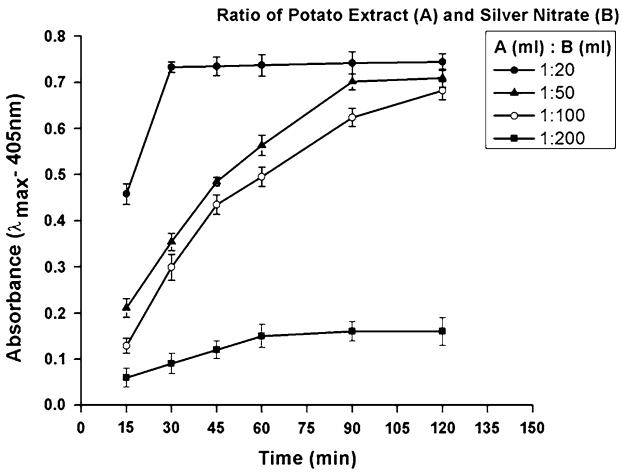

Fig. 7 Effect of varying ratios of potato extract (**a**) and silver nitrate (**b**) on the intensity of SPR peaks. Each observation is an average of three repeats ($n = 3$)

Table 2 Immobilization efficiency of alkaline phosphatase (ALP) on cysteine-functionalized silver nanoparticles (CSNPs)

Parameters	CSNPs (mL)	ALP offered (mg)	Bound ALP (mg)	Activity (U)	Specific Activity (U/mg)	Recovery (%)
Soluble ALP	–	3.84 ± 0.02	–	36.25 ± 1.92	9.44 ± 0.36	100
Immobilized ALP	1	3.84 ± 0.02	2.87 ± 0.04	18.10 ± 2.22	6.31 ± 0.49	67

Each observation is an average of three repeats ($n = 3$)

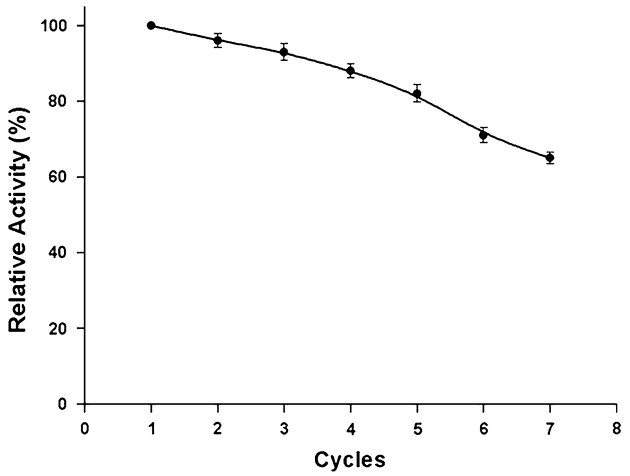

Fig. 8 Variation in the activity of immobilized enzyme after multiple cycles of reuse. Each observation is an average of three repeats ($n = 3$)

ratio of 1:20, 1:50, 1:100 and 1:200. Figure 7 shows the intensities of SPR peaks of silver nanoparticles at varying ratios. It was observed that the reduction occurred at all ratios but the reduction rate varies. At 1:20, a sharper peak was formed within 30 min with λ_{max} at 407 nm. At 1:50 and 1:100 ratios, almost same peak was observed with λ_{max} at 412 nm but the reduction rate was slow, and completed by 90 and 120 min, respectively. At such low concentration of reducing agent, the reduction rate decreases but it has no impact on the SPR peaks in terms of height and λ_{max}. On further decreasing the reducing agent concentration to 1:200, no significant peak was observed. So, the optimum ratio of 1:20 shows fast reduction and SPR peaks correspond to smaller sized silver nanoparticles.

Enzyme immobilization

NHS/EDC coupling is a widely used method for the covalent immobilization of enzyme on the carboxyl surface [30–32]. In the present study, the carboxyl groups of cysteine-functionalized silver nanoparticles were activated using NHS (30 mM) and EDC (15 mM), and then used for the covalent immobilization of enzyme ALP. It was observed that the enzyme was successfully immobilized and showed good catalytic activity (Table 2). About 75 % of the initial enzyme (2 mg/mL) was immobilized on the nanoparticles' surface with a slight decrement (approx. 33 %) in the specific activity. A comparable binding efficiency of 74 % for lipase on polydopamine-coated magnetic nanoparticles [33], 95 % for cellulase through adsorption on superparamagnetic nanoparticles [34], 66–72 % for glucose oxidase on thiophene acetylated and 94–100 % on carbodiimide-activated magnetic nanoparticles [35] has also been reported by the researchers. The results indicate the significance of nanoparticles to act as an efficient base for the enzyme immobilization. The percentage recovery of the enzyme activity was found to be 67 %, which is higher than those reported by other workers [36, 37]. It suggests that binding of ALP on the silver nanoparticles may allow it to obtain a thermodynamically stable state for a better activity and stability. Different industrial processes such as starch saccharification, inverted syrup production, protein digestion, alcohol synthesis can easily be developed with higher enzyme loading, employing these enzyme-coated nanoparticles. Further, using silanization process, these enzyme-immobilized nanoparticles can be attached to any glass or electrode surface, and thus, provide new approach for the fabrication of other enzymatic biosensors.

Stability of immobilized enzyme

Reusability experiment illustrated that the ALP-CSNPs bioconjugates can be recycled for at least 7 batch reactions with a loss of approximately 35 % of the enzymatic activity (Fig. 8). The decrease of activity may be caused by the loss of nanoparticles during isolation from the assay medium after the reaction [33]. An apparent increase in the shelf life of immobilized enzyme was also observed as compared to soluble ALP. After 60 days, there is only 12 % loss in the catalytic activity of immobilized enzyme stored at 4 °C, while in the case of soluble enzyme, a 20 % decrement in its initial activity was calculated. It indicates that the immobilization imparts stability to the enzyme which is a primary concern during any type of immobilization method.

Conclusion

The present work described a facile method for the synthesis of cysteine-functionalized silver nanoparticles, using

potato extract as a cost-effective reducing agent. The addition of potato extract leads to silver nitrate reduction to a brown colored solution of silver nanoparticles, within 30 min. The synthesized nanoparticles were having a very narrow size distribution and the average size was estimated to be 61 nm. These functionalized nanoparticles were then employed for the covalent immobilization of alkaline phosphatase on its surface. Under optimal conditions, 75 % of the enzyme covalently bound to the nanoparticles' surface, yielding a recovery of 67 % enzymatic activity. The shelf life of the immobilized enzyme was found to be 60 days and it showed hardly 35 % loss of its initial activity after seven times reuse of the same bioconjugates. With so effective and economical immobilization of ALP on silver nanoparticles, these enzymes-nanoparticles complex can further be used for the development of biosensors based on alkaline phosphatase, and provides an approach for the fabrication of other enzymatic biosensors.

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