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ARTICLE TYPE

Diastase assisted green synthesis of size controllable gold nanoparticles

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Diastase, a natural enzyme was used for one pot aqueous synthesis of gold nanoparticles (AuNPs) of tunable size. During the synthetic process, diastase concurrently acts as both reducing and stabilizing agent, while no additional chemical reagents or surfactants are added. AuNPs formed was confirmed by using UV–visible spectrophotometer with the characteristic Surface Plasmon Resonance (SPR) band at 530 nm. The size of the diastase stabilized AuNPs can be easily controlled by changing the quantity of diastase. The produced AuNPs were characterized by using Powder X-ray diffraction (XRD), UV–visible spectroscopy, Fourier transformed infrared spectroscopy (FT-IR) and transmission electron microscopy (TEM). FTIR spectrum revealed the capping of diastase onto the AuNPs surface. Furthermore, the formed gold nanoparticles are stable for more than three months. In vitro cytotoxicity study by MTT assay on HCT116 and A549 cancer cells shows that cytotoxicity of as-synthesized Au nanocolloids depends on its size and dose.

Introduction

Gold nanoparticles have paid more attention due to their attractive shape and size associated optical, electronic and magnetic properties. Due to these properties, AuNPs are being used in electronics[1], sensors[2,3], catalysis[4] and in drug delivery systems[5]. It is interesting to note that the materials in nanometre range can exhibit a transition between molecular and solid states due to their unique properties which ascribed to their surface effects and the quantum confinement[6].

Two synthetic strategies are well known for metal NPs preparations, which includes the top down and bottom up approaches. However the latter is most effective and common where the metal ions are reduced to NPs in presence of a capping ligand by using a reducing agent. In recent times, a remarkable raise in the biological application of gold nanoparticle has been found due to their biocompatibility[7,8]. On the other hand, the properties shown by NPs depend on their morphology and dimensions and hence there is an increase in the demand for the size controlled synthesis of biocompatible NPs[9,10].

The synthesis of biocompatible AuNPs with extensive biomedical related applications have to be free from hazardous chemical substances used in the other chemical methods. Current trends in the preparation of nanoparticles using green methods draw the attention of many researchers due to its environmental friendly nature and its easy to prepare mode and exclution of toxic chemicals and solvents. Several reports have shown the synthesis of nanoparticles using variety of green reducing agents and microbes[11–13]. Our group has shown the reducing ability of plant extracts for the synthesis of size controlled gold nanoparticles[14].

Here in we report a green, one pot and size controlled synthesis of diastase stabilized gold nanoparticles in aqueous medium without using additional reducing agent. This work also demonstrates how size of the AuNPs can be tuned by simply changing the quantity of diastase.

Experimental Section

Materials

Hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot 3H_2O$, 99%), Diastase were purchased from Sigma-Aldrich (Banglore, India). Diastase powder was solubulised in double distilled water under sonication at 50°C for 5 minutes and used for further experiments for the preparation of AuNPs. Human lung carcinoma (A549) and Human colon carcinoma (HCT116) cell lines were obtained from ATCC (NCCS, Pune, India). Cisplatin (CDDP) was procured from HiMedia, Bangalore, India.

Preparation of AuNPs

0.6 mL of aqueous diastase solution (1%) was added to 2 mL of 0.5 mM $HAuCl_4$ and pH was maintained to 12 using NaOH and the mixture was heated for 5min to obtain a colloid D1. Further, to examine the effect of diastase quantity on size of AuNPs, the varied volumes of diastase solutions were added as 0.5 mL and 0.1 mL to obtain D2 and D3 colloids respectively.

Cytotoxicological evaluation

The cytotoxic effects of the D1, D2, D3 Au nanocolloids on A549 and HCT116 cancer cells were studied by MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with Penicillin (100 µg/mL), Streptomycin (100 U/mL) and 10% heat-inactivated foetal bovine

serum (FBS) in tissue culture flasks (T-25) in a humidified atmosphere at 37°C and 5% CO_2. Cells were seeded one day prior to the exposure of AuNPs.

The actively growing A549 and HCT116 cells were seeded at 1×10^4 cells/well of a 96-well microtitre plate and incubated in DMEM/1% FBS with D1, D2, D3 Au nanocolloids with different volumes (0.4, 0.8, 1.2, 1.4 mL) for 24 h at 37°C, 5% CO_2 and a relative humidity of more than 80%. A control experiment was also carried out without AuNPs. Further to asses the toxicity levels of AuNPs, the test medium was discarded after 24 h of incubation and cells were incubated again with 20 µl of MTT solution (0.5 mg/mL MTT diluted in phenol-red free DMEM without FBS) for 1 h at 37°C, 5% CO_2. Subsequently, the MTT solution was replaced by 20 µl of DMSO in each well and the optical density was measured by using ELISA plate reader at 550 nm against a reference at 655 nm. Cell viability for each treatment was calculated as the ratio of the mean OD of 9 replicated wells relative to that of the negative control (only cell culture medium and cells were added). The absorbance values of negative control and treated cells were used for the determination of the cell viability. The percentage cell viability was calculated by assuming 100% cell viability for negative control: % cell viability = (OD of test/OD of control)*100 and expressed as mean percentage ± stdev (n=6). Alternatively, the cytotoxicological property of AuNPs was compared with standard drug Cisplatin (CDDP) as positive control.

Characterization

Initial characterization of different Au nanocolloids were done by using Jasco V-670 UV-visible double beam spectrophotometer. Spectral data was measured in the range from 200 to 800 nm, while the double distilled water was used for blank measurements. Origin 8.1. was used to plot the data obtained. In order to study the size and morphology of AuNPs obtained, the Au nanocolloids (D1, D2, D3) were observed under TEM (JEOL JEM 2100 HR-TEM), at an operating voltage of 200 kV. Samples were prepared by diluting 1 ml of colloid to 10 mL with distilled water and ultra sonicated for 5 minutes and placed a drop of it on a lacey copper grid with ultrathin Cu on porous carbon film which further allowed to dry under vacuum. The fully dried grids were then used for TEM analysis and a simultaneous measurement of selected area electron diffraction (SAED) analysis has been carried out. The solid AuNPs for XRD were obtained by centrifuging the colloids of D1 at 15,000 rpm. The X-ray diffraction (XRD) study for the AuNPs powder was carried out at room temperature using a Bruker D8 Advance diffractometer with Cu Kα radiation (λ = 1.54A°) over the angle range from 10° to 80°. Instrument was operated with a scanning rate of 4°/min, step size of 0.02° and calibrated with lanthanum hexaboride (LaB_6) before to the sample analysis. Purified AuNPs powder was analysed by using Attenuated Total Reflectance-FTIR (ATR-FTIR) spectroscopy (JASCO ATR-FTIR 4100). To study the surface functionalization, diastase was used as control. Dynamic light scattering (DLS) analysis for the diastase reduced Au nanocolloids was carried out by using Horiba Scientific Nanoparticci (SZ-100) instrument.

Results and discussion

In this work a natural enzyme, diastase was used as a bioreductant to prepare AuNPs of different sizes. The aqueous solution of diastase plays key role in the reduction and capping of the formed AuNPs. The effect of change in the quantity of diastase on the reduction time, size and morphology of AuNPs are discussed in details. The rapid reaction rate is due to the presence of NaOH, which is a stronger base causes the better hydrolysis of chloroauric acid and increases the rate of reaction[15]. Although, various amino acid residues of proteins have the ability to reduce gold ions into their nanoparticles, the kinetics of gold ions reduction at room temperature by proteins is very slow [16]. However, it has been reported that the enzymes having free exposed thiol groups have the ability to catalyse the synthesis of gold nanoparticles and others do not have[17]. Since diastase contains two free thiol group from its cysteine residue which possibly involved in the synthesis of AuNPs and further confers their stability[17]. Thiol group stabilized nanoparticles has been already reported[18] .

Initial conversion of Au^{+3} to Au^0 was observed with the change of colour from yellow to ruby red and is monitored by UV-visible absorption spectra. This intense colour of AuNPs was due to the surface plasmon resonance. Appearance of an intense absorption band at 530 nm under UV-visible spectrum showed an additional assurance of reduction, as the AuNPs colloids are well-known to reveal a distinct absorption band between 500-600 nm under visible region.

To know the effect of quantity of diastase on the bioreduction process, the volume of diastase in the synthetic mixture was changed while $HAuCl_4$ (0.5 mM) was maintained constant. From Figure 1, it is evident that a blue shift in the SPR was observed with increase in the quantity of diastase and absorption maxima for D1, D2 and D3 Au nanocolloids were 530 nm, 540 nm, 570 nm respectively. It is well known that SPR band is highly sensitive to the properties of the particles (size, shape), distance between the neighboring NPs, refractive index of the medium and

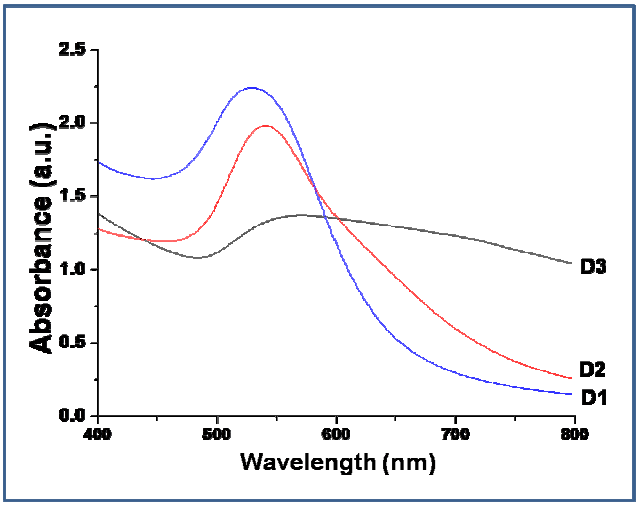

Fig. 1: UV–visible spectra of AuNPs synthesized at different quantities of diastase

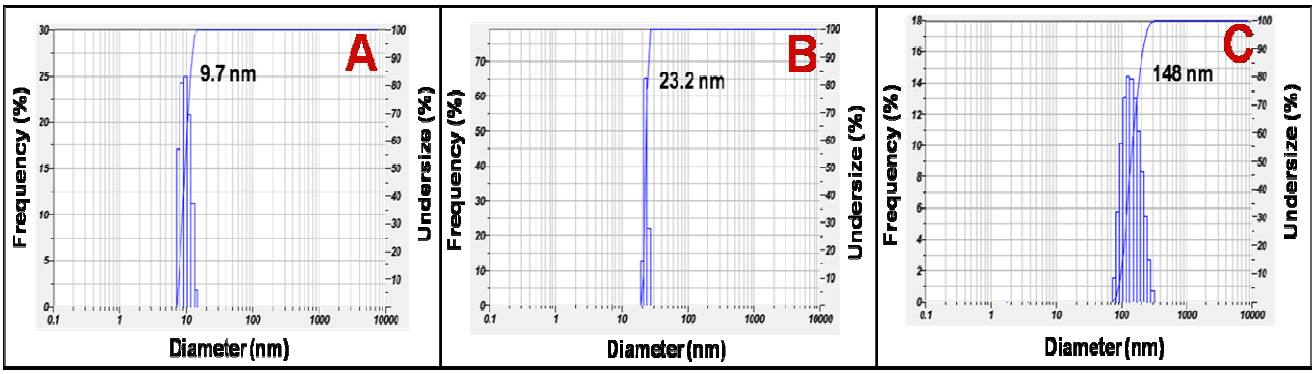

Fig. 2: Average particle sizes obtained for Au nanocolloids D1 **(A)**, D2 **(B)** and D3 **(C)**

the environment in which nanoparticles are dispersed. The blue shift observed with the increase in the diastase quantity is an indicative of the formation of smaller NPs (also confirmed by TEM). Figure 2 represented the size distribution of D1, D2, D3 nanocolloids obtained by using different volumes of diastase. It is noticed that the reduction in the size of nanoparticles was obtained with an increase in the quantity of diastase.

Further, to know the effect of pH on the size of the NPs, synthesis of AuNPs has carried out using 0.6 mL of diastase of various pH. Figure 3 showed the role of pH in tuning the size of the AuNPs. It is interesting to note that the size of the NPs increases with decrease of pH from 12 to 2. The formation of smaller nanoparticles with diastase solution of pH 12 is indicated by the SPR absorption of nanocolloid at 520 nm.

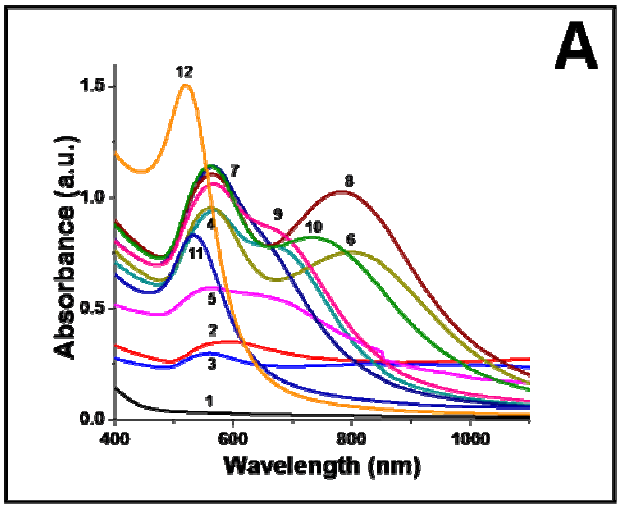

Fig. 3: UV-visible spectra of AuNPs synthesized with diastase of different pH

However, this peak is red shifted to 590 nm for the nanocolloid synthesized with the diastase of pH 2 and no NPs were formed with pH 1. On the other hand, the anisotropy is observed for the colloids prepared with the pH 10 to 4 diastase solutions, which is confirmed by the appearance of one extra peak between 650 to 800 nm (Table S1). This may be due to the decreased availability of anionic form of thiol groups ($-S^-$) of diastase with decrease of the pH of the environment. Although number of –SH groups are more in the acidic medium, the extent of capping is less because of the less availability of $-S^-$ groups in the acidic medium for capping to the formed Au nuclei and vice versa.

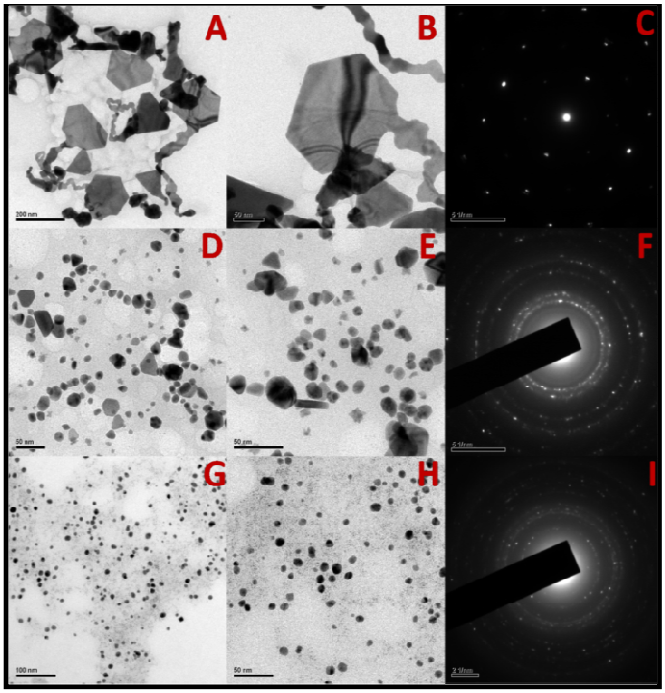

Fig. 4: TEM images, SAED pattern of Au nanocolloids D3 (A, B, C), D2 (D, E, F) and D1 (G, H, F)

Additionally, to know the variations in the morphology and size with changing the quantities of diastase, the prepared Au nanocolloids (D1, D2 and D3) were subjected to TEM studies. The TEM images at different magnifications for D1, D2, D3 gold nanocolloids along with their SAED pattern are represented in Fig. 4. TEM images at various magnifications for D3 nanocolloids were shown in Fig. 4a and b. From TEM analysis it is clear that the abundance of hexagons and blunted angled triangular shaped AuNPs with an average particle size of 148 nm. However, the presence of tail like structures attached to the sharp edges of nanoplates may be the indication of surface capping of the nanoplates with diastase. TEM magnification images of nanocolloid D2 were revealed in Fig. 4d and e and the images also illustrated the presence of triangles, hexagons along with the more number of spherical particles with moderately lesser sizes compared to D3 nanocolloid and the average particle size was 23.2 nm. On the other hand, the TEM images of D1 colloids was shown in Fig. 4g and h, which represents the rich abundance of spherical AuNPs with smaller sizes (average particle size of 9.7 nm) when compared to D2 and D3. From the TEM results it is

clear that the uniformity in the size and shape of the NPs can be obtained by increasing the quantity of diastase. The SAED patterns of nanocolloids revealed the poly crystalline nature of D1, D2 colloids and single crystalline nature of D3 colloid respectively and the corresponding patterns of D2 was indexed as (111), (200), (202), (311) and (222) reflections (Fig. 3c, f and i). From the TEM analysis it is concluded the decrease in the particle size of AuNPs with increase in the quantity of diastase. However, the anisotropy can be overcome by raising the quantity of diastase to get uniform and smaller spherical NPs (0.6 mL of diastase). It is observed from UV-visible study that D1 and D2 nanocolloids were stable more than three months but D3 nanocolloid was stable for a week (data not shown).

The mechanism of size and shape tuning for the Au nanocolloids was shown in Figure 5. During the synthesis, diastase favors the development of blunted Au nanotriangles, hexagons when the quantity was 0.1 mL. In contrast, the formation of spherical nanoparticles was observed when the quantity of diastase was increased to 0.5 and 0.6 mL, indicating the role of diastase as protective controller for the spherical nanoparticles formation by reducing the Ostwald ripening and favors a diffusion-controlled process. At low quantities of diastase, the rate of nucleation was much higher while the rate of coating of AuNPs with diastase was relatively less, which results the formation of large blunted triangles. As the quantity of diastase increases, the rate of coating was gradually increases when compared with the nucleation rate, ensuing the effective formation of small spherical AuNPs. Since the diastase enzyme contains two free thiol groups that are arranged spatially closer to each other the possibility of attachment of the formed nanocrystals to both the thiol groups of diastase is higher[17]. Since, the size of the diastase is much lesser than the size of the NPs, formation of dense shell like structure with enzymes linked with the intermolecular interactions around the spherical nuclei is possible[19]. In case of colloid D1, the nanocrystals that are formed initially were well capped by diastase shell and are highly stable. In contrast the gold nanoparticles that are formed later were less protected by diastase and are less stable. Due to the room temperature coalescence, rapid reduction and assembly these spherical nanoparticles leads to the development of new anisotropic structures such as nanotriangles with high surface energy. The formed nanotriangles undergo a shrinking process inorder to minimize the surface energy resulting the formation of blunted nanotriangles[14]. However the similar kind of results were reported when honey[20], Cinnamomum camphora leaf extract[21] were used for the synthesis of AuNPs.

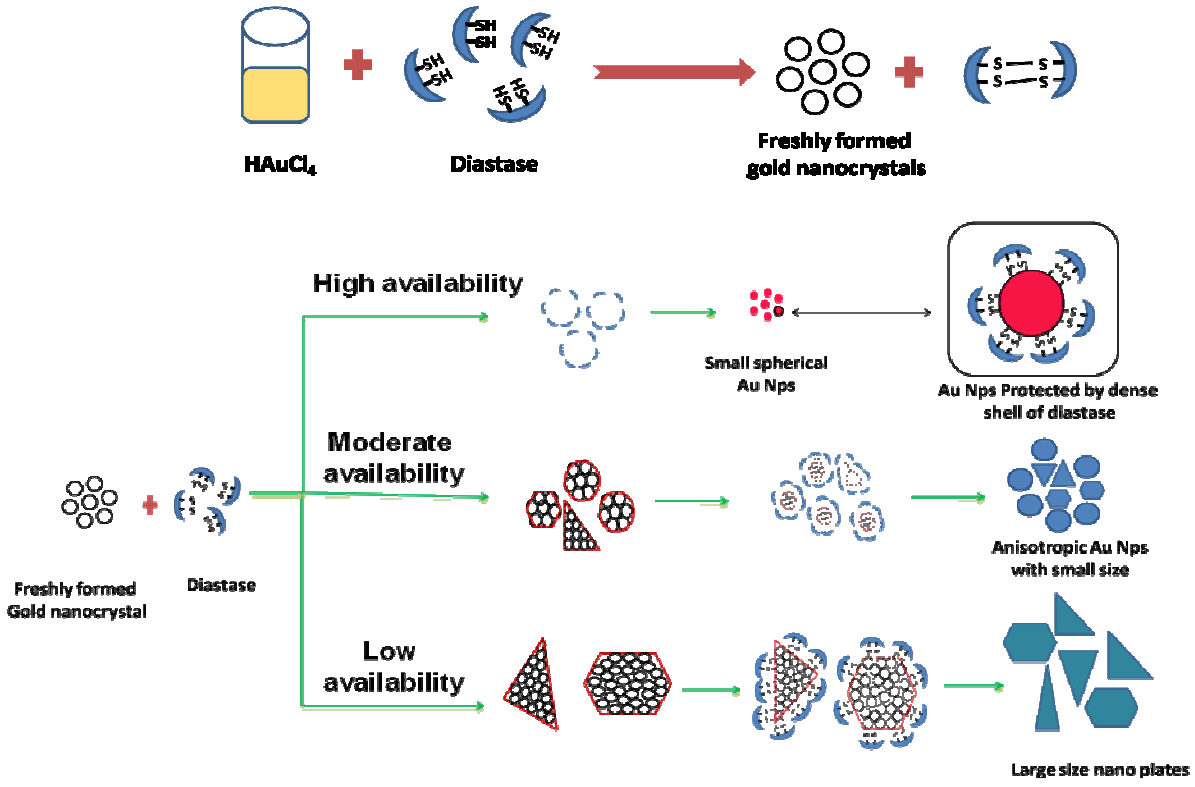

Fig. 5: Mechanism showing how diastase involved in reduction and tuning size, shape of AuNPs

The plausible mechanism of AuNPs formation using diastase could be the interaction of thiol moity of cysteine residues present in the diastase with the metal. Since diastase contains two free and surface exposed thiol groups, could react with the Au salt and forms NPs. The reducing ability of proteins is due to the presence of reducing amino acids (cysteine and histidine) in their structure[22]. However histidine is active only in organic environment due to its tertiary amine group[23]. Hence the free thiol groups present in the diastase is responsible for the reduction and stabilization. The mechanism involves the release

of electron and proton from the free thiol group of the diastase for conversion of Au^{+3} to Au^0 and the subsequent sulphur atoms contributed the disulphide bridge formation. The formed gold nuclei immediately stabilized by the free –SH groups of the diastase leftover to form Au-S bond, as the thiol groups have high affinity towards gold[17].

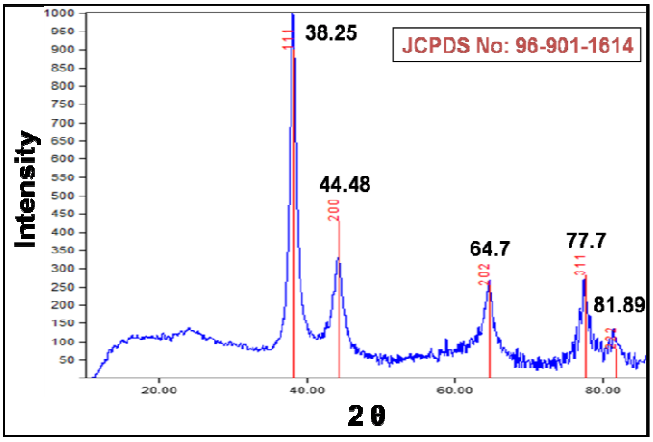

Fig. 6: XRD pattern of AuNPs obtained from D2 nanocolloid

Figure 6: XRD pattern of the purified and dried AuNPs. XRD showed different diffraction peaks at 38.25, 44.48, 64.7 and 77.7 which corresponding to (111), (200), (202), (311) and (222) planes of cubic AuNPs respectively (JCPDS No. 96-901-1614).

The surface capping of diastase molecules onto the AuNPs can also be confirmed by Fourier transform infrared (FTIR) spectroscopy. The FTIR spectrum of native diastase and the corresponding AuNPs are shown in Fig. 7. For pure diastase, the presence of a band at 1650 cm^{-1} indicates the alpha helix structure of the diastase.

Conversely, the FTIR spectrum of the dried AuNPs showed the presence of similar bands as in native diastase indicating the presence of diastase on the AuNPs surface. This shifting of bands corresponding to the C=O stretch of the peptide bond from 1650 to 1631 cm^{-1} from the native diastase to the AuNPs after reduction indicating the change in the secondary structure of enzyme capped on the AuNPs surface[24,25]. All this results indicated the surface stabilization of AuNPs with the diastase enzyme.

In vitro cytotoxicity

MTT assay was performed to determine the biocompatibility and potential of AuNPs for biomedical applications. The biocompatibility of AuNPs towards the human gastrointestinal cancer cells such as Panc-1, HepB3[26] and HeLa cells[7] was studied. In contrast, Shukla et al. and Connor et al. have reported the non toxic nature of citrated and biotinylated AuNPs in leukemic cells (K562), while the smaller AuNPs were more toxic[7,27]. In few reports, Au nanorods are found to be toxic when compared to the spherical AuNPs which are almost nontoxic[28–33]. This is due to the dependence of cytotoxicity on the shape, size and surface modification of NPs. On the other hand, the effect of AuNPs size on the *in vitro* cytotoxicity in HeLa cells is already

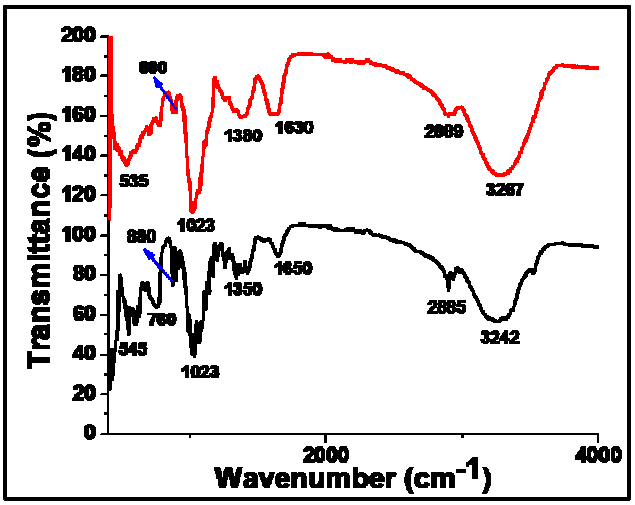

Fig. 7: FTIR Spectrum of pure diastase (black) and AuNPs (red) obtained from colloid D1

proved. Indeed, Hauck et al.[34] and Pan et al.[35] have shown that the size of the particle, not the capping agent is responsible for determining the toxicity of AuNPs. Moreover, there have been no systematic studies, to analyze the size and dose dependent cytotoxic behaviour of AuNPs synthesized by using diastase comparatively on two different cell lines.

The comparative cytotoxic behaviour of D1, D2 and D3 Au nanocolloids has been analysed on two different cancerous cell lines – HCT116 and A549. Figure 8a-b represents the cell viability data for D1, D2 and D3 Au nanocolloids and its comparative evaluation against positive and negative controls. From Figure 8 it can be concluded that smaller AuNPs were found to be more toxic for both the cell lines compared to the larger. It is observed that cell viability is more when treated with larger AuNPs and simultaneously A549 cells have shown more viability for all the three different sizes compared to the HCT116. Notably, the biocompatibility is observed below 30ppm for D1 Au nanocolloid and 35 ppm for D2, D3 Au nanocolloids for both the cell lines. Furthermore, to know the effect of capping agents on cell viability, a blank MTT assay was investigated with diastase. However, no significant toxicity was found with diastase, indicating the negligible role of capping agent on the toxicity of AuNPs. Additionally, a decline in the cell viability is observed with an increase in the concentration of AuNPs. The half maximal inhibitory concentration (IC_{50}) of D1, D2 and D3 Au nanocolloids are 107.5, 139.6 and 166.6 mg/L. All these results confirm that the toxic effects of as-prepared Au nanocolloids are size and dose dependent.

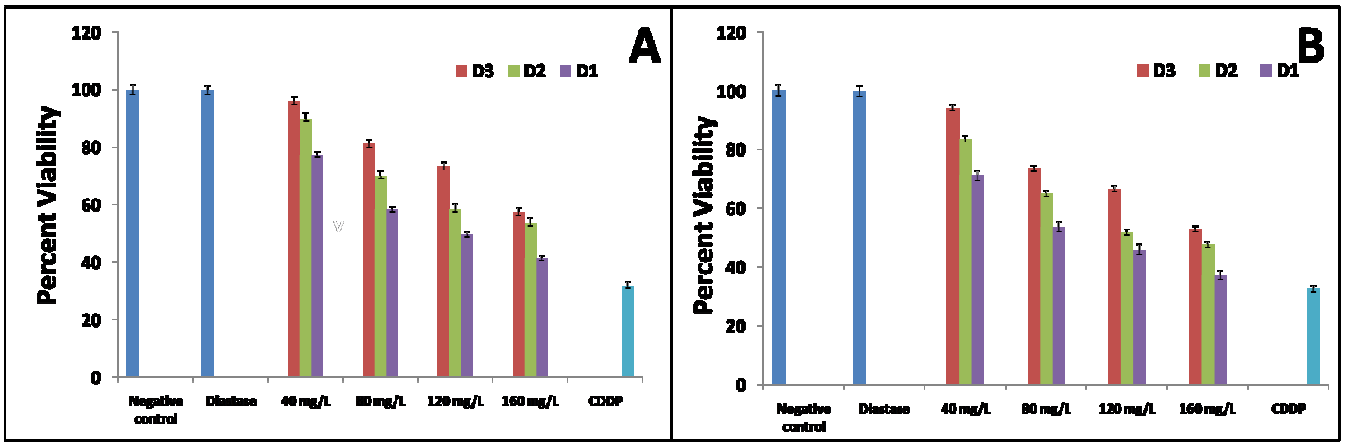

Fig. 8: Cell viability induced by diastase reduced AuNPs towards A549 **(A)** and HCT116 **(B)**

Acknowledgements

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Conclusions

In this paper we have showed the efficiency of diastase as reducing and stabilizing agent for the size controlled synthesis of AuNPs. The size distribution of the ensuing AuNPs can be effectively tuned by changing the quantity of the diastase used. The availability of thiol groups of enzyme may plays key role for tuning size and shape of the AuNPs. Additionally, the biocompatibility of diastase may offer the synthesized AuNPs towards biomedical applications. *In vitro* cytotoxicity study has shown that the toxic effects of as-prepared Au nanocolloids are size and dose dependent towards HCT116 and A549 cancer cell lines.

Notes and references

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Diastase assisted green synthesis of size controllable gold nanoparticles

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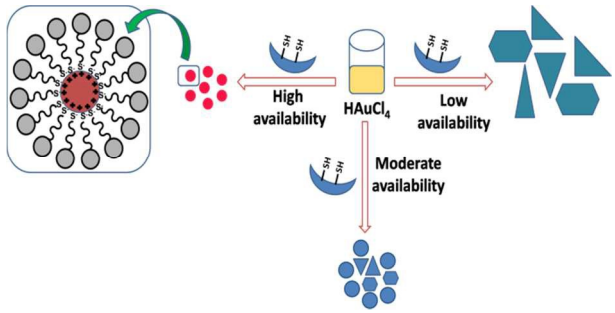

Availability of S^- groups with varying volume and pH of diastase tunes the size of AuNPs.