Research Article

DELIVERY OF PROTEIN USING NANOPARTICLE

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ABSTRACT

Nanoparticles are found to be useful as carrier for drug and gene delivery. In the present study, the most biocompatible-Gold nanoparticles were used for carrying serine protease.i.e. Trypsin. It is sensitive to the protease inhibitors present inside the cells and these proteases on binding with gold nanoparticle, loses its stability but retain its activity. The Gold Nanoparticle along with the protease was characterized by UV-Vis spectroscopy, TEM and FTIR. The interaction was confirmed between Trypsin and Gold Nanoparticles and its size was found between 20-28nm. Further, the activity of Trypsin bound to Nanoparticles and the ability of AuNP's as carrier were analyzed by *invitro* studies on *E.coli* HB101. Trypsin's activity on intracellular protein and specifically on ALPase enzyme showed a 25-29% decrease in protein content which directs that Trypsin bound to Nanoparticles has found to be active within the cell. These results suggest that, such proteolytic enzyme can be designed with nanoparticle to lyse the defective proteins produced by the cells due to genetic disorder.

Keywords: Gold nanoparticles, Trypsin, Alkaline phosphatase, Protein

INTRODUCTION

Nanomedicine, a branch of Nanotechnology involves the use of drugs and carriers to manipulate at the level of molecules and atoms. It's a promising field of research – to locate, diagnose and treat disease[1]. It involves the use of particles at nano scale to carry the medicinal agents to identify and highlight tumors which are undetectable by current diagnosis techniques. It is now majorly researched in delivering therapeutic agents to the target site[2].

Delivery systems based on lipid or bio-polymer nanoparticles[3] is manipulated to alter the important properties of drugs such as pharmacological and therapeutic effects[4]. The pharmacokinetics and biodistribution of the drug is majorly regulated by the delivery systems. Its most advantageous factor is to avoid the body's defence mechanisms[5], as the cells take up these nanoparticles. This has led to the development of complex systems which has the ability to get drugs into cytoplasm via cell membranes.

Many diseases are associated with the malfunctioning processes within the cell, and only those drugs can act which make their way into the cell for better efficiency. In order to increase the efficiency, various techniques are adopted to deliver drugs such as Triggered response, in which the drugs are in inactive form within the body and gets activated on sensing a particular signal. This technique is found to be more efficient as it acts only on the targeted site. Similarly in certain cases where drug has poor solubility, carriers containing both hydrophilic and hydrophobic environments are provided[6]. Also, tissue damage caused by the drug can be eliminated by regulating the drug release properties. Delivery systems can also be used to alter the pharmacokinetic of the drug to prevent patient from consuming high doses of drug due to drug clearance from body at quick phase.

Such drug delivery systems will reduce the effect on non-target tissue, thus solving the problem of poor distribution. Nanodrugs will have high potential to have its action in very specific mechanisms which will lead to new generation of drugs with more efficient in its action without side effects due to usage of low doses of drugs.

Drug delivery should have the following advantages: a) Efficient capping of the drugs, b) Delivery of drug to targeted site, and c) Release of drug without losing its property.

Chemotherapy Drug have lower tendency to reach the target site of the cell, as it get denatured or taken up by non-target cell on its path to target cell. To increase stability and specificity of drug, Nanoparticles are used as carrier. These Nanoparticles are found to be effective in delivering the drug even in low dosage form by protecting activity of drug from denaturants. Its specificity is increased by attaching suitable ligand which will direct its path to the target cell.

Many research work based on drug delivery using Nanoparticles has been increased mainly for overcoming the side effects of drugs due to HIGH dosage & AGGRESSIVE action leading to various side effects such as Cardiovascular disease, kidney failure, intestinal disorder, and genetic disorders. Each drug has list of adverse effects on continued usage.

The various application of nanotechnology in nanomedicine involves, Drug delivery[1, 4], Gene delivery, Molecular manufacturing, Biosensing device and it can be broadly classified into following categories, Nanobiopharmaceuticals, Oncology Imaging[7], Photodynamic Therapy[8], Surgical flesh welder[9], Visualization of drugs, Medical Imaging, Diagnostic Sensors, Neuro-electronic interfacing[10], Molecular nanotechnology[11], nanorobots[12], Nanonephrology.

The main objective is to study stable model for delivery of protease's and study its stability in bacterial system such as *E.coli* and its activity inside the cytoplasm.

MATERIALS AND METHODS

Materials

Tetrachloroauric acid 0.02% (HAuCl$_4$) (Sigma Aldrich, Product no: HT1004), Trisodium citrate (Na$_3$C$_6$O$_7$), Bovine Serum Albumin, Luria Bertani broth (Himedia, M1245), *E.coli* HB101 (Medox) and other chemicals used were of high purity and grade from Himedia.

Instrumentation

Biospectrometer – Basic (Eppendorf) was used for determining the size of gold nanoparticle, to plot standard graph for BSA and Trypsin, ALPase substrate assay; Cooling Centrifuge- C24BL (Remi) was used in *invitro* studies to maintain 0-4°C; PAGE (Medox) was used to analyze the protein bands; Magnetic stirrer – 2MLH (Remi) was used in preparation of gold nanoparticles. Computer aided Transmission Electron Microscope was employed a Philips instrument. PERKIN ELMER SPECTRUM RX1 1.60 spectrometer was used for FTIR analysis were samples was prepared as KBr pellets. Analyses were performed at the room temperature with 4 cm-1 resolution.

Methods

Synthesis of gold nanoparticle

The AuNPs was synthesized by citrate reduction method. An aqueous solution of HAuCl$_4$ (5mM, 80μl) was brought to a vigorous boiling with continuous stirring in a round bottom flask, fitted with a

reflux condenser and then trisodium citrate (38.8mM, 100µl) was added rapidly. The solution was boiled till the color of the solution changes from pale yellow to deep red. The solution was cooled to room temperature with continued stirring.

Coating of Trypsin to gold nanoparticle

A standard graph was plotted for Trypsin to determine the saturation point for Trypsin on gold nanoparticles. The prepared gold nanoparticle solution was diluted by a factor of 1 with the glycine buffer (75mM, pH 9). The saturation point of Trypsin on AuNP was in the ratio of 1:5 (AuNP:Trypsin) and it was added by stirring it to a portion of the dilute solution containing GNPs. The solution was incubated, before being centrifuged, to remove the unbound Trypsin remaining in solution. The precipitate obtained was subjected to wash cycles (2 times) involving rinsing with 50mM glycine buffer and centrifuged. Finally the GNP-Trypsin was suspended in the glycine buffer and then freeze dried.

Characterisation of nanoparticles

Gold nanoparticle synthesized was confirmed using UV absorption spectroscopy at 528nm wavelength. To determine the size, it was further characterized by using TEM and FTIR analysis.

Activity test for Trypsin coated on nanoparticles

The formation of Trypsin bounded AuNP's was confirmed by determining the amount of unbound Trypsin in the supernatant using a standard graph for Trypsin by Bradford assay. The activity of the bound Trypsin was determined using BSA as a substrate[8].

In Vitro test

In vitro analysis was carried out in *E.coli* cells. Nanoparticles are easily taken up by bacterial cells[13] yet analyses were carried out with competent cells. In order to analyze the variation in uptake of nanoparticles, two different conditions along with a control was used. The two different conditions were competent and normal cells. Competent cells were prepared by calcium chloride method as given by Joseph Sambrook and David Russell[14] and stored with 70% glycerol for further studies.

Nanoparticle incorporation within bacterial cells

E.coli cells were cultured using LB (Luria Bertani) broth till 0.4 O.D was achieved. Overnight culture was re-inoculated in 50ml LB broth and placed in shaker; culture was grown until the O.D reaches 0.3. Subsequently 1ml of the culture (0.3 O.D) was mixed with 100 µl of Au nanoparticles bounded to Trypsin (A), similarly 500µl of the *E.coli* culture was mixed with AuNP alone and considered as control (C). 1ml of fresh LB medium was also added to each sample, mixed and incubated at 37℃ for 24hr in a shaker at 100rpm. Competent cells were also incubated with AuNP-Trypsin (B) at 37℃. All the above mentioned cultures were incubated and invitro studies were carried out under the similar conditions.

Screening by protein extraction

Screening process was carried out by protein extraction using alkaline lysis method. Cultures obtained from the above were centrifuged to remove any trace of LB medium. The Pellet obtained was resuspended in shock buffer and incubated at room temperature for 10min. After incubation, the solution was centrifuged at 5000rpm and the pellet was resuspended in 1ml of ice cold water and placed undisturbed in cold condition for 10min. It was followed by centrifugation at 9000rpm and the supernatant containing protein was transferred to sterile centrifuge tube which was filtered using 0.45µm Nupore filter paper. Protein present in the filtrate was determined by measuring the absorbance at 280nm.

Alkaline phosphatase assay

The protein extraction carried out by alkaline lysis method was further subjected to 60-90% of Ammonium Sulphate precipitation to precipitate alkaline phosphatase. The precipitate was centrifuged at high speed and the pellet obtained was dissolved in tris buffer of pH 8.5. The amount of ALPase extracted was determined by ONPP

substrate assay at 405nm. SDS-PAGE was performed for the alkaline phosphatase extracted by ammonium sulphate precipitation.

RESULTS AND DISCUSSIONS

Synthesis of gold nanoparticle

Gold nanoparticles were synthesized by citrate reduction method using tri sodium citrate as reducing agent and the size of AuNP's were confirmed by plasma resonance effect which gave maximum peak at 528nm and the size was found to be around 11-18nm[15] using TEM analysis.

Gold nanoparticles are mostly used in nanomedicine as drug or gene carrier due to its inertness and binding capability. The bound formed by AuNP's are thiol bonds. And this bound is formed to be major in AuNP's interaction with proteins. This leads to adsorption of protein on AuNP's, which causes protein aggregation and misfolded state[16]. Proteins remain as partially folded at the Au nanoparticle surface. Thus we have bounded gold nanoparticle with serine protease i.e. Trypsin which hydrolyses proteins. And this led to increase in size of AuNP's by ±4-6nm which showed shift in maximum peak from 528nm to 531nm, as shown in figure 1.

Coating of Trypsin to gold nanoparticle

A standard graph was plotted for Trypsin using Bradford assay in order to regulate Trypsin concentration left in the supernatant after adsorption on gold nanoparticle. The gold nanoparticle solution was diluted with the glycine buffer (75mM, pH 9) to maintain the pH around the pI of Trypsin which is 10.1-10.4. The adsorption of protein on nanoparticle is carried out around the pI of the protein but the activity of Trypsin is maintained at pH 8-9, thus pH 9 was maintained throughout the process. The saturation point for the adsorption of Trypsin was determined by plotting standard graph for varying range (0-10mg/ml).

The activity of Trypsin bounded to AuNP's was confirmed by proteolytic action on BSA and the value was found using the BSA standard curve. The amount of Trypsin bound to AuNP was determined by Trypsin standard curve by Bradford assay,

$$\% \text{ Bound Trypsin} = \{[A-B] / C\} *100$$

Where,

A = OD at 595nm of 100µl of Trypsin in 1mg/ml of glycine buffer

B = OD at 595nm of supernatant

C = OD at 595nm of 100µl of Trypsin in 1mg/ml of glycine buffer

Characterisation of nanoparticles

1) UV Visible spectrum

Figure 1, displays the UV-visible spectra for citrate capped gold nanoparticles. The pure Trypsin shows a maximum at 280 nm, but with the addition to colloidal gold, both the bands at 280nm and 528nm of pure Trypsin and Au colloids respectively decreases in intensity steadily with time. This reduction leads to emergence of an additional peak at 531nm (Figure 1). New peak is due to the protein aggregation on gold nanoparticles leading to the formation of gold-Trypsin complex. This complex formed results in reducing the activity of Trypsin due to adsorption, as thiol bonds are mainly formed between gold nanoparticles and protein.

2) Transmission Electrons microscopy (TEM)

TEM images of Gold Nanoparticle as given in Figure 2,

(**Fig 2a**), Gold Nanoparticle size was found using Computer aided TEM (Philips) at the magnification of 100nm and the size was found to be in the range of 11-17nm

(**Fig 2b**), Gold Nanoparticle was added with protease to interact and the TEM analysis confirmed the size of Trypsin bounded AuNP's as 22-25nm

3) FTIR analysis

From the below FTIR graph, an interaction is found between gold nanoparticles and Trypsin which can be confirmed by sudden shift in CH$_2$ (1425.2cm^{-1} to 1395.6cm^{-1}) & NH$_2$ (1388.1cm^{-1} to 1395.6cm^{-1}), C=O (1654.6cm^{-1} to 1597.7cm^{-1}) and C-S (622.0cm^{-1}) bonds. The alcohol (OH) group present in amino acids of Trypsin shows peaks around 1074.6cm^{-1}. The major interaction between AuNP's and Trypsin is thiol bonds, thus there is sudden decrease in peak below 1000 as compared in figure 3.
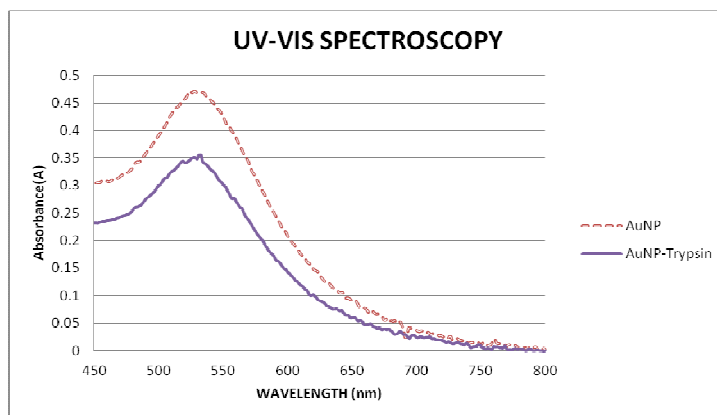

Fig. 1: Shows the UV-Vis absorption for AuNP with Trypsin - max. peak at 531nm and AuNP - max. peak at 528nm
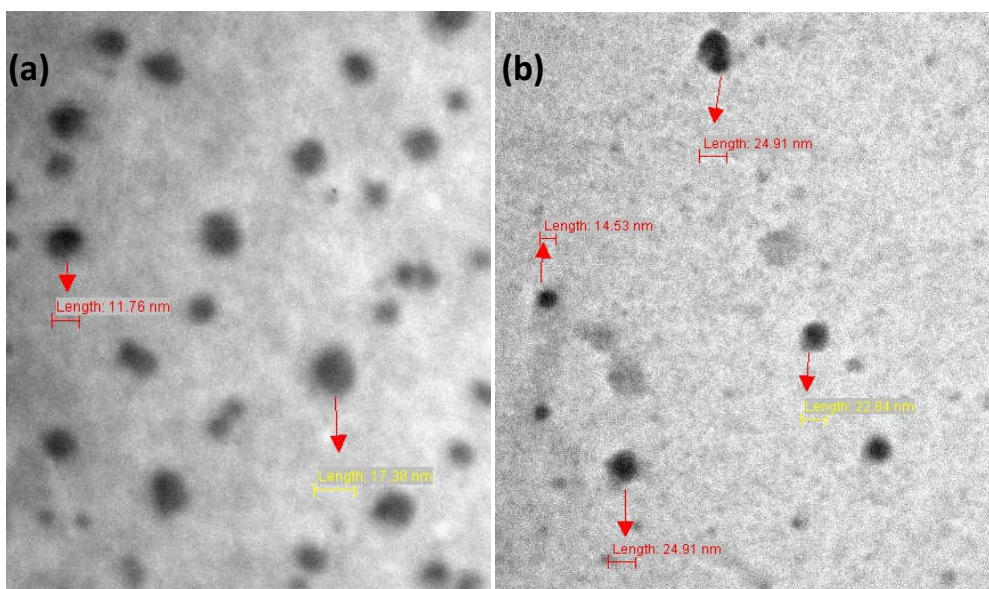

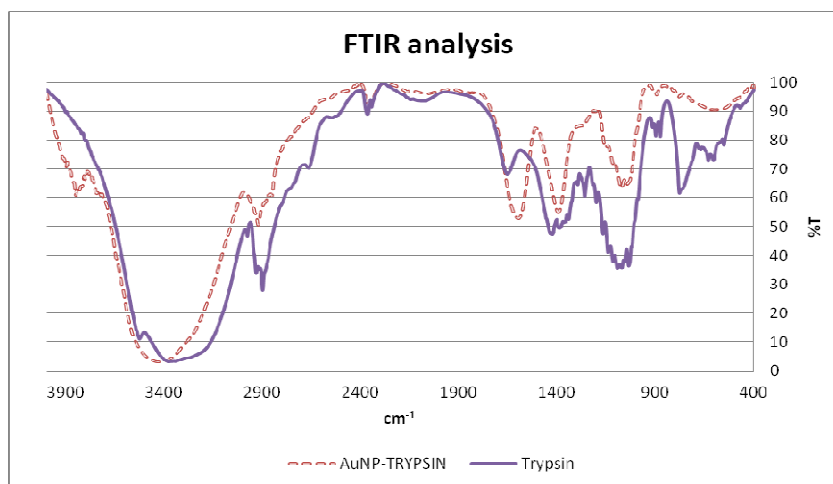**Fig. 2: Transmission Electron Microscope image of AuNP's(a) and Trypsin bounded AuNP's(b)**

Fig. 3: FTIR analysis of Trypsin and AuNP with Trypsin showing the shift in bond due to binding of Trypsin on Gold Nanoparticles.

Table 1: Percentage Uptake of Au nanoparticle bound to Trypsin by *E.coli* cells

Sample	O.D. @ 531nm after 5 min	O.D. @ 528nm	O.D. @ 531/528nm after 24h (Supernatant)	% Uptake of AuNP-Trypsin/AuNP
A	0.332	-	0.184	44.58
B	0.333	-	0.213	36.04
C (CONTROL)	-	0.798	0.389	51.25

For TABLE 1, A = *E.coli* cells with AuNP-Trypsin; B = Competent *E.coli* cells with AuNP-Trypsin; C = *E.coli* cells with AuNP alone.

In Vitro Studies

Nanoparticle incorporation within bacterial cells

Cells incubated with AuNP's and AuNP-Trypsin for 24hrs were centrifuged to determine the uptake of nanoparticles using Biospectrometer–Basic (Eppendorf) at 531nm, and the values were as shown in Table-1.

The result shows that almost 35-45% of AuNP-Trypsin was taken up by cells, as plotted in figure 4. This variation in uptake of AuNP-Trypsin between the normal cells (A & C) and competent cells (B) is due to the difference in cell density. As the

competent cell culture contains low number of live *E.coli* cells compared to the normal cells and it takes long lag time compared to the normal cells, there is reduction in uptake of nanoparticles. But the uptake of AuNP's alone by the Sample C (51.25%) is almost close to the uptake of AuNP's bound to Trypsin by Sample A (44.58%). This shows that the uptake of Nanoparticles by the *E.coli* cells is similar in both conditions but the uptake was slightly being reduced in the case of Gold nanoparticles bound to Trypsin.

Further protein extraction was carried out and the absorbance was measured at 280nm to determine the amount of protein.
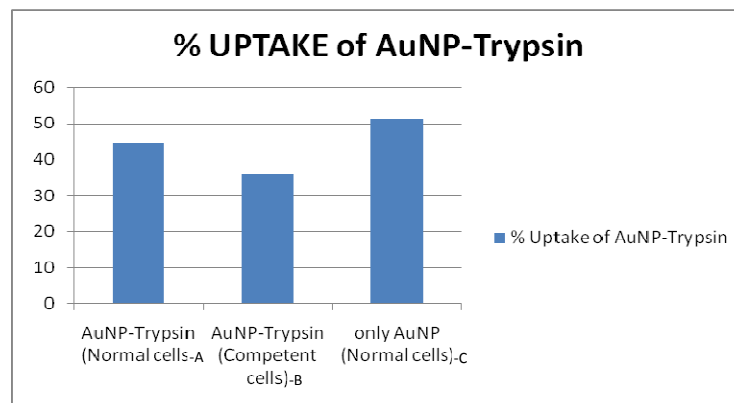
Fig. 4: Percentage Uptake of Gold Nanoparticles bound to Trypsin by *E.coli* cells

Table 2: Estimation of protein by Absorbance method @ 280nm

Sample	O.D. @ 280nm	Protein concentration (mg/ml)	% of Protein
A	1.63	0.82	71.93
B	1.74	0.86	75.44
C (CONTROL)	2.42	1.14	100
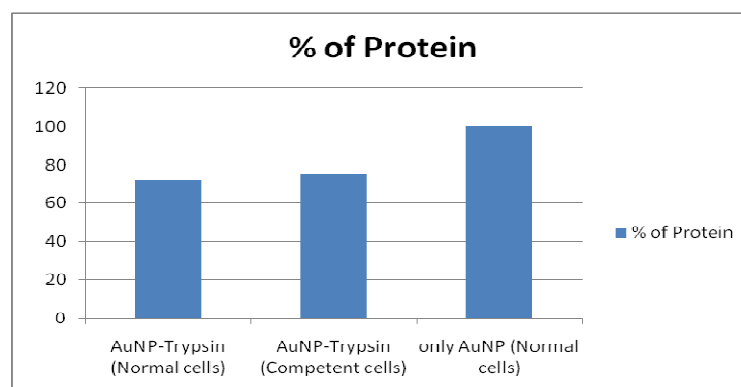
Fig. 5: Percentage of protein extracted from *E.coli* cells after incubation with Au nanoparticles bound to Trypsin.

Protein extraction

Protein extraction was carried out by alkaline lysis methods, and the amount of protein extracted was measured at 280nm using Biospectrometer. The concentration of protein determined is shown in Table-2,

Table 2 and figure 5, shows decrease in protein content when compared between samples A & C and B & C. The protein extracted from Sample C refers to 100% content of protein that should be present in all 3 Samples, as only AuNP's were added to it. The decrease in Sample A & B indicates the action of Trypsin on the

intracellular protein, the difference between A & B is mainly due to low cell density of Sample B. This shows that the proteolytic action of Trypsin which was bounded to Au nanoparticles is active even after instability caused by aggregation of proteins on Nanoparticles. This also shows that any drug or enzyme or protein can be bound to Au nanoparticles and used as drug carrier to the target site.

Alkaline phosphatase assay

Alkaline phosphatase, a periplasmic enzyme[17], of *Escherichia coli* is encoded by the *PhoA* gene consisting of two identical subunits as a zinc-containing protein. Intramolecular disulfide bridges are present between the two subunits. The complete protein of *PhoA'* is converted into protease resistant and enzymatically active conformation only after entering into the periplasmic space; its precursor is an N-terminal 20-residue signal sequence. But those that lack functional signal sequence and synthesized in the dsbA-deficient cell are prone to be reduced and degraded by the protease action[18].

Trypsin is one of the proteolytic enzymes to which the *PhoA* product is resistant, but they are susceptible to the Trypsin degradation if they lack the signal sequence or remain attached to the cytoplasmic domain of other proteins. This shows that there is need for this protein to be exported to the periplasmic space for proper folding and its enzymatic active form[19].

Alkaline phosphatase was taken as target since these proteins are resistive to protease cleavage in active form[20]. The presence of active Trypsin can diminish or denature the monomer of ALPase by proteolysis before forming the active form in the periplasm region, but this is prevented by the protease inhibitors present inside the cell wall. It is here, where the Au nanoparticles play its role as carrier and protective agent for the serine protease's i.e. Trypsin. When the Trypsin are bounded to Au nanoparticles, their activity and specificity changes[21] but it remains active and its activity were found to be lower compared to the unbound Trypsin. *In vitro* test was carried out in *E.coli*, to study and analyze whether the bound Trypsin was active when internalized by the cells, if so, then its effect should decrease the amount of active ALPase formed within the cells. ALPase are produced in higher concentration under phosphate deficient condition mostly in the stationary phase[22, 23] hence cells were grown under such condition. ALPase enzyme was extracted by alkaline lysis followed by ammonium precipitation which was carried out at 60-90% of $(NH_4)_2SO_4$ and the enzyme were assayed by para-nitrophenylphosphate as substrate[24]. The analysis done in 3 types of AuNP treated cells and the results obtained were shown in Table-3,

Table 3: p-NPP assay for ALPase enzyme extracted from *E.coli* cells

Sample	O.D. @ 405nm		p-nitrophenolate (concentration)(μM)		ALPase activity(UNIT)	
	5min	10min	5min	10min	5min	10min
A	0.310	0.673	1.824	3.959	0.365	0.396
B	0.312	0.692	1.835	4.07	0.367	0.407
C (CONTROL)	0.335	0.856	1.97	5.035	0.394	0.5035

The p-nitrophenolate has extinction coefficient $1.7 \times 10^4 M^{-1} cm^{-1}$ at 405nm[24], the enzyme activity was considered that one micromole of paranitrophenol obtained per minute at 25^0C from one unit of enzyme.

From the Table 3, it is well clear that Au nanoparticles bound to Trypsin has acted upon the cytoplasmic proteins resulting in decrease of protein content compared to control cells (Table 2 & Fig 6) which was incubated with Au nanoparticles alone. The ALPase extracted from three samples also shows that Trypsin bounded to Au nanoparticles had decreased the formation of active ALPase by denaturing/proteolysis of the monomer required for the formation of active ALPase in the periplasmic region, as given in Table 4 and figure 10. The result also shows that the effect is almost same in both competent cells and normal cells which confirm that Au nanoparticles are taken up by cells under normal growth conditions without any specific requirements.

Table 4: Percentage of ALPase after *in vitro* analysis

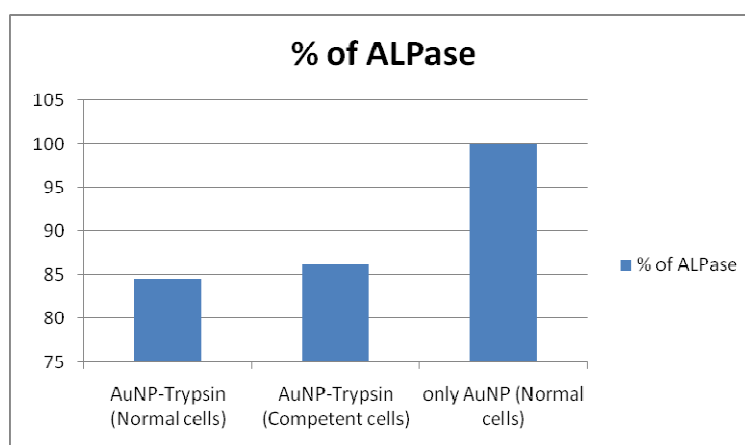| Sample	Avg. ALPase (UNIT)	% of ALPase
A	0.38	84.63
B	0.387	86.19
C (Control)	0.449	100

Fig. 6: Percentage of ALPase obtained after protein extraction

The SDS PAGE showed thinner (slightly visible) bands for alkaline phosphatase due to its low concentration instead of those obtained for the control cells which were thick, proving that Trypsin-AuNP's had entered and acted upon the ALPase monomers.

CONCLUSIONS

AuNP's as suitable carrier has been proved and are being studied as carrier for various drug for different disorders. Even gene therapy has evolved for various genetic disorders; still we need to improve for better treatment[25]. In Certain Cancer, Gene therapy can only impart changes to the defective gene but can't overcome the metabolic problems imposed by the defective protein synthesized by the same defective gene. These defective proteins remain in the affected cells and hinder the normal metabolic and building process as seen in Osteogenesis imperfecta where defective pro-collagen disturbs and results in bone fragility and other symptoms. For such disorder's, various research are carried out to deliver drugs which are toxic to normal healthy cells. Hence, it requires thorough understanding of carrier such as Au nanoparticles to deliver these toxic drugs to the target cells and result in better treatment with better patient compliance.

In this paper, we have studied the delivery of protein using Au nanoparticle as carrier for a serine protease which showed positive results in carrying the Trypsin in less active form and protects Trypsin from protease inhibitors present inside the cell. Proteolytic enzyme's can be designed to cleave specifically the defective proteins, which can be carried by Nanoparticles. It's an alternative to the above mentioned treatment, where the toxic drug is replaced by proteolytic enzyme's which are specific in its action. Thus, such treatment will be found to be more reliable and not fatal. The present research is a model to develop and study the factors associated in using Nanoparticles as protease carriers.

The major factors were pH, Trypsin activity, aggregation of Trypsin and Trypsin stability. Isoelectric pH, determines the interaction between AuNP's and the proteases. As pH changes, protease may lose its activity or no interaction will occur between AuNP's and Proteases.

Trypsin activity was reduced due to thiol bond formation with AuNP's where active site of Trypsin are blocked by AuNP's. Aggregation of Trypsin causes multilayer adsorption of protease on AuNP which indirectly reduces Trypsin activity, Nanoparticle uptake by cells, less binding of Trypsin and stability of AuNP-Trypsin complex. Stability of Trypsin is mainly due to the pH and its interaction with AuNP's. In this study, the pH was maintained at 9, which reduced the interaction with AuNP's. Thus only 69% of Trypsin was bond to AuNP's even at the ratio 1:5 (AuNP:Trypsin).

It shows that controlling such parameter's in formation of Nanoparticles bounded biological compounds can lead to stable carrier formation. Such carriers would enroot the treatment for Genetic disorders associated with defective Proteins. This type of treatment can be of patience compliance and won't kill neither the Healthy cells nor the Defective cells but only the defective Proteins.

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