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Facile synthesis of gold-silver alloy nanoparticles for application in metal enhanced bioluminescence.

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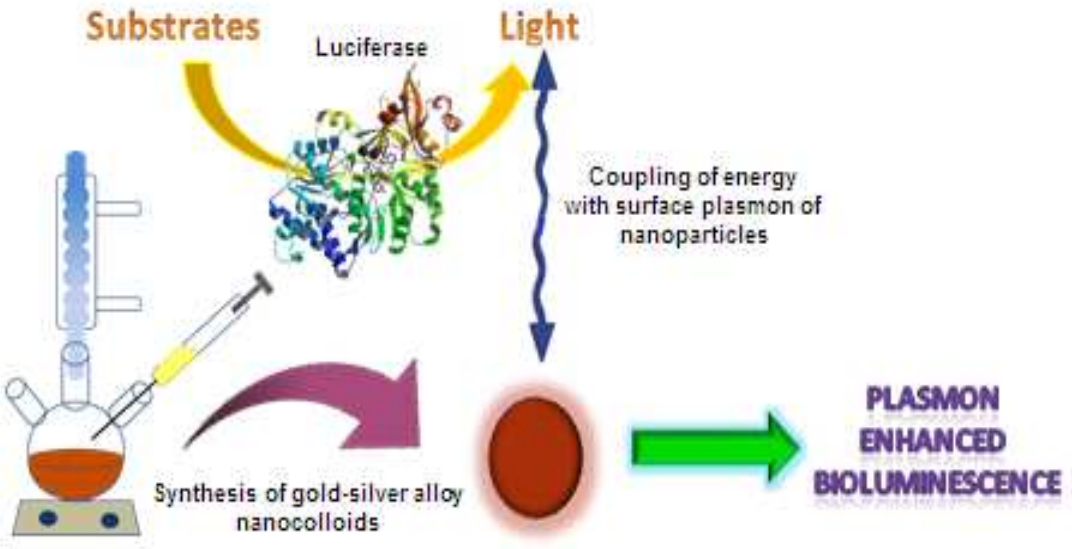
Abstract

In the present study we explored metal enhanced bioluminescence in luciferase enzymes for the first time. For this purpose a simple and reproducible one pot synthesis of gold silver alloy nanoparticles was developed. By changing the molar ratio of tri-sodium citrate and silver nitrate we could synthesize spherical Au-Ag colloids of sizes ranging from 10 to 50 nm with a wide range of localized surface plasmon resonance (LSPR) peak (450-550 nm). Optical tunability of the Au-Ag colloid enabled their effective use in enhancement of bioluminescence in luminescent bacteria *Photobacterium leiognathi* and in luciferase enzyme systems from firefly and bacteria. Enhancement of bioluminescence was 250% for bacterial cells, 95% for bacterial luciferase and 52% for firefly luciferase enzyme. The enhancement may be a result of energy transfer or plasmon induced enhancement. Such increase can lead to higher sensitivity in detection of bioluminescent signals with potential applications in bio-analysis.

Graphical abstract:



In the last decade, metal nanoparticles have attracted increasing interest compared to bulk metals because of their unique properties and their promising applications in catalysis, biosensing, electronic/optical devices, medical diagnosis, etc. [1-3] The development of water-dispersible metal nanoparticles (also called metal colloids) has drawn more attention, considering their direct catalytic or biological applications in aqueous media with less or no hazardous effects.[4-6] Among the various metal colloids, gold (Au) and silver (Ag) colloids have received special attention by virtue of their fascinating physical and chemical properties, their intriguing biological uses and catalytic activity in a number of reactions.[7-9] Recently much emphasis has been given to the fabrication of gold silver alloys and bimetallic core shell particles owing to their unique non-linear optical properties and tunable surface plasmon resonance[10]. Ag colloids display an absorption maximum at about 420 nm and the Au colloids show the same at around 520 nm. When gold-silver alloy nanoparticles were prepared, the plasmon maximum was between those of Ag colloids and Au colloids, showing blue-shift with an increasing amount of silver[11].

Analytical chemiluminescence (CL) and bioluminescence (BL) are well-established technologies that are widely applied in life sciences and food analysis. However the main disadvantage in the present techniques is its low light emission. To address this issue, studies involving these phenomena have been extended to nanoparticle systems in the past few years. Plasmon induced enhancement or metal enhanced luminescence is a similar phenomenon wherein emission from a bioluminescent chemical reaction couples to surface plasmons in metal nanoparticles thereby enhancing the signal.[12]

The bioluminescent chemical reaction induces electronically excited states. The emission is by the reaction itself without the need of an external excitation

source.[12] The effect is a result of the interaction of the plasmon field of the metal with the excited state of the bioluminescent molecular species. This might lead to photoluminescence enhancement and quenching depending on the distance between emitter and metal with concomitant changes in excited state lifetime.[13] There are many reports of bioluminescence resonance energy transfer (BRET) using nanoparticles[14, 15] but literature on bioluminescence enhancement due to metal nanoparticles are scanty except an article by Eltzov et al (2009) where they have reported enhancement of bioluminescence in *E.coli* in presence of silver island films.[16]

Our group has been studying the effect of metal nanoparticles especially gold nanoparticles for fluorescence and chemiluminescence enhancement.[17] In continuation of the studies herein, we report a fast, facile, economic method for the synthesis of the Au-Ag colloids in the absence of stabilizers, with the use of simple co-reduction method and their application for enhancement of bioluminescence. Spherical and mono-dispersed colloidal Au-Ag nanoparticles were successfully synthesized within only 5 min with this reduction route. Further, effect of gold silver alloys on luminescent bacterium *P. leiognathi* and luciferase enzymes were studied. To our knowledge this is the first report on metal enhanced bioluminescence studied on luciferase enzymes. All the reagents used in the experimentation were of analytical grade and extra pure. The water used in all the experiments was purified in three stage Millipore- Milli Q plus purification system. Glasswares used in the present work were properly washed with aqua regia, rinsed many times with distilled water and dried**.**

Spherical gold nanoparticles were synthesized according to the method described by Xia et al, [18] modified to our requirements. The effect of tri-sodium citrate

and pH on nanoparticle formation and stability was studied. Studies were also carried out on reaction times for nanoparticle growth (Refer ESI). Overall reaction in synthesis can be summarized as follows irrespective of reducing agent.[20]

$$(AuCl_4)^- + 3e^- \longrightarrow Au^0 + 4Cl^-$$

The particle formation takes place in three stages, nucleation, aggregation and capping. Typically in a solution citrate reduces the $HAuCl_4$ into three reaction intermediates i.e., tetrachloroaurate (III) ($(AuCl_4)^-$), the monoaqua-substituted complex ($(AuCl_3OH_2)$), and the deprotonated monohydroxo-analogue ($(AuCl_3OH)^-$).[21] $(AuCl_3OH)^-$ is hypothesized to be the dominating oxidizing Au(III) form which is reduced to Au(I) in the form of $(AuCl_2)^-$ by two electrons, and further to metallic gold (Au^0), The whole process is known as nucleation. Second step in the synthesis, is growth. Freshly formed gold atoms start coalescing to form bigger particles (around 3-5 nm) of several nuclei. Towards the end of second step the concentration of Au° passes the critical nucleation limit and immobilization of capping agents on the surface of newly formed AuNPs starts. Finally over the time remaining smaller particles dissolve and redeposit into larger particles contributing to the homogeneity of the AuNPs solution.

In the present work, addition of silver nitrate into the $HAuCl_4$/citrate mixture before adding to boiling water dramatically increased the rate of reaction from 45 minutes to less than 5 minutes (Figure S1 A&B, ESI) when compared to classical Turkevich method. Addition of different concentrations of silver nitrate (5.9×10^{-6} M, 1.18×10^{-5} M, 3.54×10^{-5} M, 4.72×10^{-5} M, and 5.9×10^{-5} M) to the reaction mixture resulted in blue-shift of the absorption maxima of synthesized particles from 530 nm to upto 454 nm, proving formation of alloy nanoparticles (Figure S2, ESI)

The higher the concentration of silver nitrate used (168 µM and 112 µM) the particles constitute more of silver atoms and resemble silver nanoparticles. The broadening of peak in case of citrate reduced silver nanoparticles has been reported earlier. Citrate reduction leads to larger particles with less homogeneity as compared to other reducing agents used[22].

To study the effect of silver nitrate in detail we have added different concentrations of silver nitrate ($5.9x10^{-6}$ M, $1.18x10^{-5}$ M, $3.54x10^{-5}$ M, $4.72x10^{-5}$ M, and $5.9x10^{-5}$ M) to the reaction mixture. Spectrophotometric analysis showed that with increasing silver nitrate concentration the maximum absorbance linearly increased (Figure S2). Silver nitrate is a shape guiding and catalytic agent, its addition leads to faster synthesis, thereby increasing the amount of nanoparticles synthesized in a given time (Figure S2) and lesser time to complete the reaction (Figure S1). Also silver has a higher surface enhancement factor, in comparison to AuNPs.

For bioluminescence generation *P. leiognathi*, a marine luminescent bacterium isolated from shrimps, was collected from the Indian coast of Arabian Sea. The bacteria were grown in the laboratory under optimized conditions as described previously[19] (Refer ESI). The cell pellet after centrifugation was used for interaction studies.0.1g of the bacterial pellet was resuspended in 10 mL of saline water (pH 8). 200uL of this bacterial suspension was mixed with different concentrations of Au-Ag alloys (10-50µL). The bioluminescence enhancement was recorded in a luminometer for 15 minutes. Bacterial solution without Au-Ag alloys serves as control.

In-vitro studies on the effect of gold-silver alloy on luminescence of luciferase enzymes were carried out by extraction of bacterial luciferase enzyme from *P.*

leiognathi cells. Firefly luciferase enzyme from *P. pyralis* (10-40 x 10^4 LU) was commercially procured and used for the luminescence assay.

For the assay 20 µL of bacterial luciferase was added to 40 µL of 50µM flavin mononucleotide (reduced) and 20 µL of 0.06% v/v dodecanal in a luminometric cuvette. The volume was made up to 200 µL with the assay buffer. For firefly luciferase enzyme same volume of enzyme was taken along with 20 µL of 100 µg/mL D-Luciferin and 40 µL of 10 µg/mL ATP solutions.

The luminometer used for the luminescence detection is equipped with a cuvette holder in front of the detector. The system is well insulated from external light. The cuvette was placed in the holder and the substrates injected into it sequentially using micropipettes. The blank reading was noted before addition of the final reactant. After adding the final reaction component, the cuvette was swirled for mixing and the luminescence was recorded after 5 seconds from the last injection (this dead time was kept constant for all).

Bioluminescence signals are generally weak in comparison with traditional fluorescence based probes. But advantage of the bioluminescence over fluorescence based methods is its simplicity. For bioluminescence no external excitation source is required and a simple luminometer is enough to detect the signals. In marine bacteria bioluminescence is developed as a defense mechanism, hence they are extremely sensitive to external changes. Any external stimulant activates the reporter luciferase genes resulting in emission.

The most commonly used systems are the *luc* gene from the firefly and *lux* genes from bacterial species of the genus *Vibrio*. Expression of the lux luciferase operon produces light without any additions, allowing thereby online monitoring of gene

expression, whereas the expression of firefly luciferase genes requires externally added substrate luciferin for luminescence.[16] The reactions proceed as follows:

Equation 1. Bacterial bioluminescence:

$$\text{Luciferin (dodecanal)} + FMNH_2 + O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin}^* \longrightarrow \text{Oxyluciferin} + \text{Light}$$

Equation 2. Firefly bioluminescence:

$$\text{Mg-ATP} + \text{D-luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin}^* \longrightarrow \text{Oxyluciferin} + \text{Mg-AMP} + \text{PPi} + \text{Light}$$

When Au-Ag colloid was introduced to a solution containing *P.leiognathi* cells, the presence of nanoparticles triggered luminescence. Figure 1 clearly shows that there is upto 2.5 folds increase in the intensity of luminescence in presence of nanoparticles. We assume this effect is due to the sudden metabolic changes brought about in the bacterial system due to the introduction of foreign element, which in this case are metal nanoparticles. However luminescence started decreasing at higher concentrations of nanoparticles due to the toxicity of silver ions on bacterial cells. Possible explanation for this phenomenon lies in the genetic makeup of the bacterium. Marine bacteria produce light under stress; hence very small external trigger also is effective enough to elicit luminescence. The use of silver nanostructures to enhance bioluminescence produced by sensitive bacteria under toxicity stress has been studied earlier.[16]

Though, studies clearly demonstrated the enhancement, experimenting with live bacterial cells has some drawbacks. Most important drawback is the difficulty in controlling the parameters. Live bacterial cells being dynamic in nature tend to change with different batches and also there is a possibility of acclimatization after

successive generation. Keeping this in mind we further concentrated on effect of Au-Ag colloids on luciferase enzyme extracted from bacterium *P. leiognathi.* The average relative light units (n=5) emitted by bacterial luciferase have been plotted in figure 2. From the figure it can be observed that the luminescence signal was doubled in presence of 3 µL of Au-Ag colloid. Higher concentration of nanoparticles caused rapid degradation of enzyme. The experiments were repeated with different batches of enzymes and found that the results are reproducible. The advantage of luciferase enzyme system is that, here the parameters are controllable once it has been optimized.

Further the results were compared with the standard firefly luciferase enzyme procured from Sigma Aldrich, (Figure 3). The results were found to be interesting. Though there was increase in luminescence in presence of nanoparticles, it was low when compared to luciferase from bacterium (52% increase).

We noticed almost negligible change in the kinetics of bioluminescence of both bacterial and firefly bioluminescence systems. As seen in figures 1, 2 and 3, the slopes of the curves remain nearly the same after addition of the nanoparticles, barring a slight rise after addition of 10 µL and 50 µL Au-Ag colloid to bacterial cell suspension (Figure 1) and a slight dip in case of 3 µL colloid in bacterial luciferase system (Figure 2).

As has been previously mentioned, luminescence enhancement can also be a result of catalysis (as in chemiluminescence)[23] or plasmon-coupled enhancement.[12,16] Further investigation into the molecular interaction of the enzymes, reaction components and/or cells with the nanoparticles may illuminate the possibility of catalysis by the colloids. On the other hand, plasmon induced enhancement has only

been proven in case of coated colloids on solid matrices, a condition different from our present study. For dispersed nanoparticles distance between the bioluminescent source and the particles cannot be determined definitively, as such it is difficult to predict near and far-field radiation.

Energy transfer is a well-studied phenomenon in case of fluorescence and metal nanoparticles. In cue with the same principle, in bioluminescence it is prerequisite that the particles absorb in the same wavelength as that emitted by the luciferase system (490 nm for bacterial luciferase, 560 nm for firefly luciferase). The SPR absorbance regions for the synthesized Au-Ag colloids, therefore, justify higher enhancement in the bacterial system than in the firefly.

In conclusion, we have synthesized Au-Ag colloids using variable concentrations of silver nitrate during reduction of tetraaurochloric acid by tri-sodium citrate. The gradual shifting of the SPR peak from the wavelength characteristic of AuNPs to that of the AgNPs testify the incorporation of Ag atoms during nucleation and growth and thus formation of Au-Ag colloids. Stability of the colloids in different pH conditions has been studied. We have successfully shown the enhancement of bioluminescence generated from bacterial and firefly luciferases and whole marine bacterial cells in presence of Au-Ag colloids. Our results showed that similar to fluorescence, phosphorescence and chemiluminescence plasmon-enhanced bioluminescence signatures can also be used enabling much more sensitive detection of bioluminescent signals with potential multifarious applications in the biosciences. With enhancement ranging from 52% to 250% in our system, biosensing using the new principle can be taken up, which shall provide a definitive insight into the increase in sensitivity. Similar work (metal enhanced chemiluminescence and metal enhanced fluorescence) has already been attempted

by our group as well as other workers. The limit of detection (LOD) after enhancement was lowered to one-tenth (enhanced chemiluminescence)[24] and half (enhanced fluorescence)[17] of the original values. Such enhancement in case of firefly luciferase may enable performing assays to quantify ATP (measure of microbial load) and other metabolites and enzymes coupled to its the utilization or production, like NAD(P)H with enhanced sensitivity. For bacterial bioluminescence the effect of toxic substances on the enzyme may be studied. Processes producing or consuming molecular oxygen including reactions involving hydrogen production can also be monitored. The underlying principle of nanoparticle induced bioluminescence enhancement is yet to be established, which will be an interesting phenomenon to be investigated.

Acknowledgement

Authors thank Aeronautical Development Agency (ADA), Bangalore, for providing financial support for this work under National Programme on Micro and Smart Systems (NPMASS) scheme. The authors gratefully acknowledge the Director, CSIR-CFTRI for facilities, CSIR and UGC for research fellowship.

References:

[1] A. T. Bell, The Impact of Nanoscience on Heterogeneous Catalysis, Science 299 (2003) 1688, DOI: 10.1126/science.1083671

[2] S. Kundu, K. Wang, and H. Liang, Size-Controlled Synthesis and Self-Assembly of Silver Nanoparticles within a Minute Using Microwave Irradiation, The Journal of Physical Chemistry C 113 (2009) 134, DOI: 10.1021/jp808292s

[3] Y.Q. Wang, W.S. Liang and C.Y. Geng, Coalescence Behaviour of Gold Nanoparticles, Nanoscale Research Letters 4 (2009) 684, DOI: 10.1007/s11671-009-9298-6

[4] M.C. Daniel and D. Astruc, Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology, Chem Rev. 104 (2004) 293-346, DOI: 10.1021/cr030698+

[5] M.P. Pileni, Control of the Size and Shape of Inorganic Nanocrystals at Various Scales from Nano to Macrodomains, J. Phys. Chem. C 111 (2007) 9019, DOI: 10.1021/jp070646e

[6] S.K. Tripathy, Nanophotothermolysis of Poly-(vinyl) Alcohol Capped Silver Particles, Nanoscale Research Letters 3 (2008) 164, DOI: 10.1007/s11671-008-9131-7

[7] S. Pyrpassopoulos, D. Niarchos, G. Nounesis, N. Boukos, I. Zafiropoulou and V. Tzitzios, Synthesis and self-organization of Au nanoparticles, Nanotechnology 18 (2007) 485604, DOI:10.1088/0957-4484/18/48/485604

[8] S. Rucareanu, V.J. Gandubert and R.B. Lennox, 4-(N,N-Dimethylamino)pyridine-Protected Au Nanoparticles: Versatile Precursors for Water- and Organic-Soluble Gold Nanoparticles, Chem. Mater. 18 (2006) 4674, DOI: 10.1021/cm060793+

[9] M. Watanabe, H. Takamura and H. Sugai, Preparation of Ultrafine Fe–Pt Alloy and Au Nanoparticle Colloids by KrF Excimer Laser Solution Photolysis, Nanoscale Research Letters 4 (2009) 565, DOI: 10.1007/s11671-009-9281-2

[10] Y Cui, B Ren, J L Yao, R A Gu and Z Q Tian, Synthesis of Agcore Aushell bimetallic nanoparticles for immunoassay based on surface-enhanced Raman spectroscopy. J. Phys. Chem. B 110 (2006) 4002, DOI: 10.1021/jp056203x

[11] S. Link, Z. L. Wang and M. A. El-Sayed, Alloy formation of gold-silver nanoparticles and the dependence of the plasmon absorption on their composition. J. Phys. Chem. B 103 (1999) 3529, DOI: 10.1021/jp990387w

[12] C. D. Geddes, Metal-enhanced bioluminescence: an approach for monitoring biological luminescent processes (patent) (2012), Application no. 20120028270

[13] H. Mertens, A. F. Koenderink and A. Polman, Plasmon-enhanced luminescence near noble-metal nanospheres: Comparison of exact theory and an improved Gersten and Nitzan model, Physical review B 76 (2007) 115123, DOI: 10.1103/physrevb.76.115123

[14] Z. Xia and J. Rao, Biosensing and imaging based on bioluminescence resonance energy transfer, Current Opinion in Biotechnology 20 (2009) 37, DOI 10.1016/j.copbio.2009.01.001

[15] C-Y. Hsu a, C-W. Chen, H-P. Yu , Y-F. Lin and P-S. Lai, Bioluminescence resonance energy transfer using luciferase-immobilized quantum dots for self-illuminated photodynamic therapy, Biomaterials 34 (2013) 1204, DOI: 10.1016/j.biomaterials.2012.08.044

[16] E. Eltzov, D. Prilutsky, A. Kushmaro, R.S. Marks and C. D. Geddes, Metal-enhanced bioluminescence: an approach for monitoring biological

luminescent processes, Applied Physics Letters 94 (2009) 083901, DOI: 10.1063/1.3086283

[17] Abhijith K. S. and M. S. Thakur, Application of green synthesis of gold nanoparticles for sensitive detection of aflatoxin B1 based on metal enhanced fluorescence, Anal. Methods 4 (2012) 4250, DOI: 10.1039/C2AY25979F

[18] H. Xia, S. Bai, J. Hartmann and D. Wang, Synthesis of Monodisperse Quasi-Spherical Gold Nanoparticles in Water via Silver(I)-Assisted Citrate Reduction, Langmuir 26 (2010) 3585, DOI: 10.1021/la902987w

[19] R. Ranjan, N. K. Rastogi and M. S. Thakur, Development of immobilized biophotonic beads consisting of Photobacterium leiognathi for the detection of heavy metals and pesticide, Journal of Hazardous Materials 225 (2012) 114, DOI: 10.1016/j.jhazmat.2012.04.076

[20] C. Engelbrekt, P. S. Jensen and H. S. Karsten, Complexity of Gold Nanoparticle Formation Disclosed by Dynamics Study, J. Phys. Chem. C 117 (2013) 11818, DOI: 10.1021/jp401883h

[21] P.K. Sen, A. B. Bilkis and K. K. Sen Gupta, Kinetics and mechanism of the oxidation of glycolaldehyde by tetrachloroaurate(III), International Journal of Chemical Kinetics, 30 (1998) 613, DOI: 10.1002/(SICI)1097-4601

[22] Z. S. Pillai and P. V. Kamat, What Factors Control the Size and Shape of Silver Nanoparticles in the Citrate Ion Reduction Method?, J. Phys. Chem. B, 108 (2004) 945, DOI: 10.1021/jp037018r

[23] M. H. Chowdhury, K. Aslan, S. N. Malyn, J. R. Lakowicz and C. D. Geddes, Metal-enhanced chemiluminescence: Radiating plasmons generated from

chemically induced electronic excited states, Appl. Phys. Lett. 88 (2006) 173104, DOI: 10.1063/1.2195776

[24] Abhijith K. S., Vasanthragavan K and Thakur M. S, Gold nanoparticles enhanced chemiluminescence- A novel approach for sensitive detection of Aflatoxin B1, Anal. Methods 5 (2013) 4838, DOI: 10.1039/c3ay40694f

Figures and legends

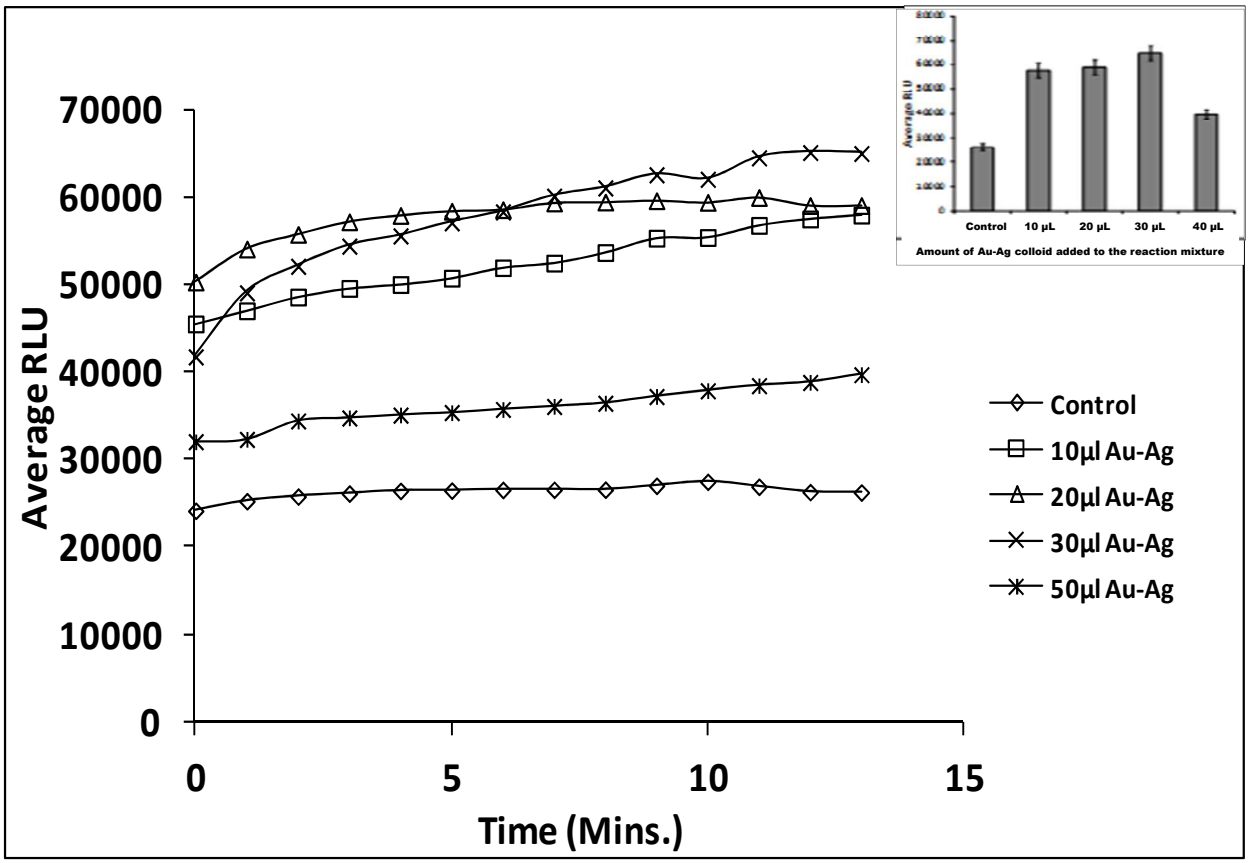


Figure 1: RLU observed over a period of 13 minutes after Au-Ag colloids (10 to 50 µL) have been added to cell suspension of *P.leiognathi* (n=5). The emission is seen to be stable over time. Maximum enhancement occurs on addition of 30 µL of colloids (around 250%). The inset shows the column graph for the average RLU over the studied time.

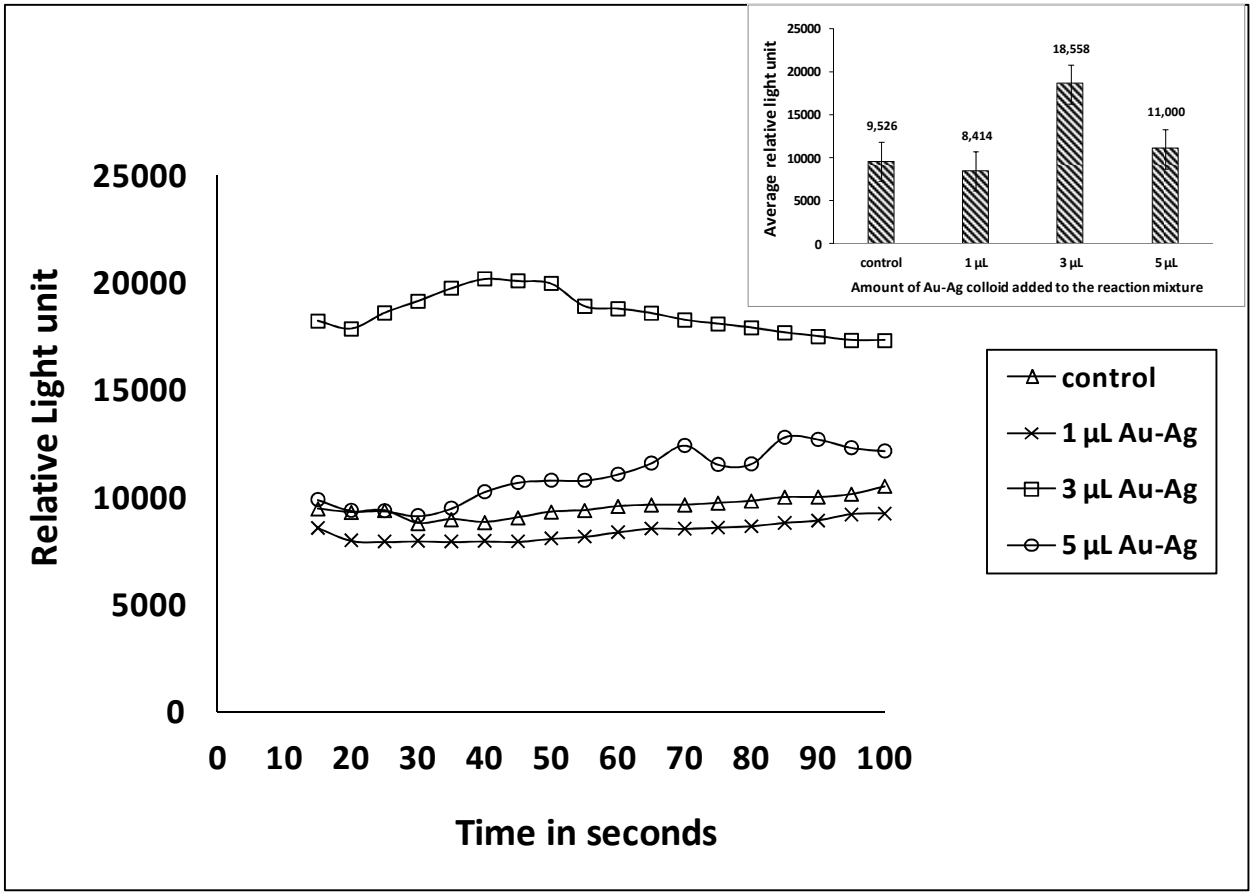


Figure 2: Variation in RLU after adding Au-Ag alloy (1, 3 and 5 µL) to reaction mixture observed over a time period of 5 to 100 s after reaction initiation (n=5). Enhancement is observed only for 3 and 5 µL of added colloids, where 3 µL shows enhancement of 95 % . Inset shows the average of the measured RLU.

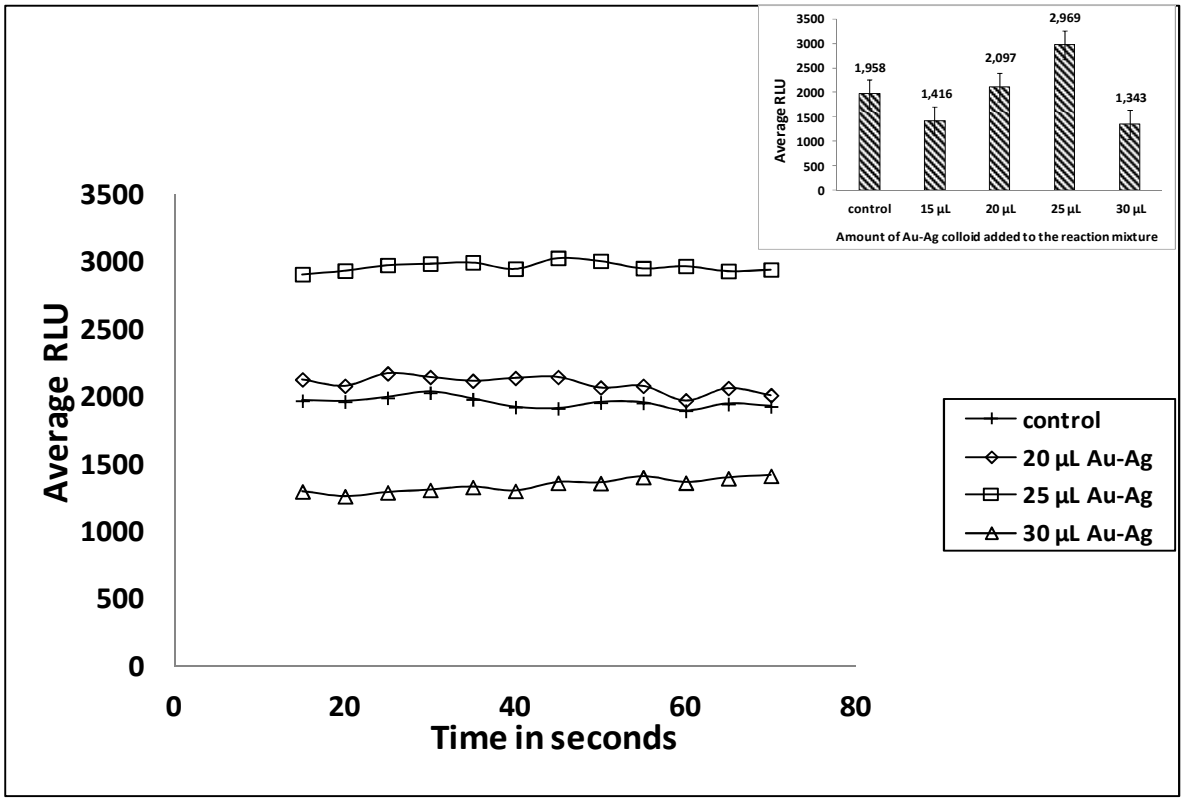


Figure 3 RLU observed over a period of 15 to 70 seconds after reaction initiation of firefly bioluminescence with 20 to 30 µL of added Au-Ag colloid (n=5). In the initial 15 seconds there is drastic decline in the observed RLU, which stabilizes only after. Highest level of enhancement has been observed for 25 µL of nanoparticles (52% increase) shown clearly by the average RLU plot in the inset.