Research Article

Simplistic Approach Towards Synthesis of Highly Stable and Biocompatible L-Cysteine Capped Gold Nanosphere Intermediate for Drug Conjugation

Rohan Kesarkar*[1], Madhuri Yeole[1], Bhagyashree Dalvi[2], Madhuri Sharon[3], Abhay Chowdhary[1]
[1]Department of Virology and Bionanotechnology, Haffkine Institute for Training Research and Testing, Parel, Mumbai, Maharashtra, India.
[2]Institute of Chemical Technology, Mumbai, Maharashtra, India.
[3]NSN Research Centre for Nanotechnology and Bionanotechnology, Ambernath, Maharashtra, India.
***Corresponding author's E-mail:** rohannk27@gmail.com

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ABSTRACT

For successful clinical translation of endless biomedical applications of gold nanoparticles (GNPs), decorating their surface with appropriate functional moieties becomes of cardinal value. Stability and biocompatibility of the nano-complex is also an important parameter that should be taken into consideration while designing a requisite nano-molecule. In the present study, we developed a facile method for impregnating a bi-functional linker L-cysteine onto gold nanoparticles. This linker was incorporated concurrent to the synthesis procedure of GNPs. UV-Visible and infra-red (FTIR) spectral analysis, along with zeta potential measurements confirmed that L-cysteine was successfully impregnated onto GNPs without affecting its stability. Half maximal cytotoxic concentration (CC_{50}) value of GNPs was also found to increase from 36.49ppm to 57.71ppm after conjugating with L-cysteine; improving its biocompatibility. This simplistic method of conjugating a linker during synthesis of GNPs is therefore likely to improvise the conjugation strategies for developing biocompatible and stable drug delivery and diagnostic systems using gold nanoparticles.

Keywords: Gold nanoparticles, L-cysteine, Dipole and quadrupole excitation, CC_{50}.

INTRODUCTION

Gold probably one of the first known metal to man has been long known not only for its beauty but also for its range of enigmatic physical and chemical properties. With an upsurge in nanotechnology, a burst of research activities related to gold nanoparticles (GNPs) have been seen in numerous biomedical applications in recent years. Facile synthesis, ease of functionalization, biocompatibility and inherent non-toxicity makes GNPs most favourable candidates for drug delivery and other diagnostic and therapeutic applications[1,2]. Recent studies have shown that GNPs possess potent anti-bacterial[3] and anti-viral activities against range of viruses including HIV[4]. Apart from an effective anti-viral activity, GNPs were also found to be efficient drug delivery systems capable of increasing the half life and efficacy of the drugs conjugated onto them[5,6].

For successful clinical translation, appropriate functionalization of these engineered gold nano conjugates are of critical value. Appropriate functional groups onto the surface of nanoparticles allow specific conjugation with target moiety.

Stability and biocompatibility of the nano-complex is also an important parameter that should be taken into consideration while designing an efficient therapeutic nano-molecule. Current functionalization strategies involve linker based chemistry, which involves chemical moieties with functional groups and act like intermediate bridge to conjugate GNPs with target molecule. Such chemical linkers are often less biocompatible and lead to de-stabilization of the stern layer around the nanoparticles which lead to their aggregation[7].

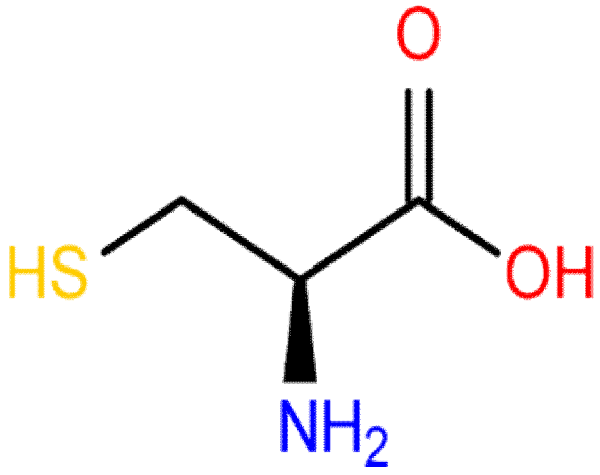

Figure 1: Structure of L-cysteine

Thus in the present study, we tried to explore L-cysteine as a linker for conjugating onto GNPs. L-cysteine is a known amino acid with thiol (-SH) and amine (-NH2) functional groups, which have high affinity for GNPs. Efforts were also taken to incorporate L-Cysteine during synthesis of nanoparticles itself, in order to preserve the stern layer and stability of the nanoparticles.

MATERIALS AND METHODS

Reagents

Chloroauric acid, L-Cysteine, MTT and all other chemicals were purchased from Sigma Aldrich, USA. All experiments were performed in de-ionised water. Glasswares for

nanoparticles synthesis were washed with aqua regia and distilled water to remove traces of metal contaminants.

Ethical Approval

All procedures were conducted in accordance with the guidelines approved by the Institutional Ethics Committee - No. HITRT/IEC/12/2011, dated 24th January 2011.

Methodology

Synthesis of L-Cysteine Conjugated GNP (GNP-L-Cys)

With a prime aim to functionalize GNPs without affecting its stability, we adopted a modified technique where L-cysteine was incorporated onto GNPs during its synthesis. Briefly, 20µl of 0.5% L-cysteine was added to 10ml of 1% sodium citrate and kept stirring at 170°C on a magnetic hot plate. Upon boiling, chloroauric acid ($HAuCl_4$) at a final concentration of 100ppm was added and kept stirring till initiation of colour change. Another set of GNPs were also prepared following the above procedure but, without addition of L-Cysteine and considered as blank GNP solution.

For obtaining a comparative significance of the procedure, a set of blank GNPs after synthesis, were interacted with 0.5% of L-cysteine under same conditions and observed for visual and spectroscopic changes.

Characterization of GNPs

Characterization of the nanoparticles was done primarily by visual observation followed by UV-Visible spectroscopy and zeta potential measurements. Further understanding into deeper intricacies of nanoparticles was obtained by Fourier Transformed infrared spectroscopy (FTIR), Scanning Electron microscopy (SEM) and elemental analysis by Energy dispersion of X-ray (EDAX).

Biocompatibility of GNP-L-Cys

Effect of GNPs on mitochondrial metabolism of the cells was assessed as a parameter to inspect their biocompatibility. Quantification of mitochondrial toxicity was determined by tissue culture based MTT method. Briefly, Vero cell lines, maintained in MEM medium and supplemented with 10% (v/v) Fetal bovine serum were seeded into 96 flat bottom well plates at concentration of $4x10^5$cells/ml at 37°C and 5% CO_2. With confluency observed, medium was changed and different dilutions of blank GNP and GNP-L-Cys were added into wells respectively. After overnight incubation the cells were washed once with PBS and incubated with MTT dye (5mg/ml) for 4hrs at 37°C. After 4hrs of incubation, Dimethyl sulfoxide (DMSO) was added to each well and read at 570nm using a microplate reader (Biotek India Ltd.). Transition of colour change from yellow to purple is directly proportional to cellular mitochondrial metabolism and cell viability. Percentage cell viability was determined by following equation:

$$Percent\ (\%)\ Cell\ Viability = \frac{(S-N)}{(P-N)} \times 100 \qquad (1)$$

Where, S = Sample reading, N= Negative control (cells with DMSO) reading and P = Positive control (only cells) reading.

50% cytotoxic concentration of nanoparticles synthesized (CC50) was calculated by analyzing the above data using GRAPH PAD PRISM software, version 5.0.

RESULTS AND DISCUSSION

Nanoparticle Characterization and Functionalization

Transition of gold salt into nanoparticles was seen as a change in solution from transparent to wine red colour. Spectroscopic measurements of the blank colloidal gold (Figure 2A) showed an intense peak at 528nm. This peak results from the resonance obtained from the interaction of electromagnetic waves with the vibrating electrons of gold confined into a narrow space due quantum confinement effect. This phenomenon is known as surface Plasmon resonance (SPR)[8] and provides a preliminary data on size, shape, anisotropy and change in surface chemistry of nanoparticles.

In our study, we tried to orchestrate GNPs with L-Cysteine (L-cys). L-cysteine is natural amino acid known to be precursors of many vital components of metabolic system and hence a cardinal evidence of being non-toxic. L-cys is also highly cell permeable and thus likely to increase the uptake profile of the associated conjugate nanoparticle. Moreover, the linker has a –SH group and hence does not require pre-thiolation like the widely used polyethylene glycol. Another advantage of using small amino acids as linkers is that they offer a certain degree of physical barrier along with small hydrodynamic radius. For further *in vivo* applications such small hydrodynamic radii proves beneficial for efficient transmembrane permeation and excretion; suitable for passive targeting. Although polymeric ligands like PEG are well known for increasing the circulation times and opsonisation of the conjugate along with stability and biocompatibility but they are known to encapsulate the nanoparticles to a greater extent[9]. This might interfere with the innate anti-viral or anti-bacterial activity of the gold nanoparticles and thus hamper the overall efficacy of the conjugate molecule.

Functionalization of GNPs with L-cys in a traditional manner i.e. after synthesis resulted in appearance of dual peaks in UV-vis spectral analysis as shown in Figure 2B, followed by complete dampening and aggregation of the gold nanoparticles in 15 minutes after conjugation. One peak was near the original absorption maxima of blank GNP with a distinct red shift, whereas the new peak was in the range of 700-800 nm. According to Majzik the first peak located near the resonance peak for single particles is attributed to quadrupole plasmon excitation in coupled spheres, whereas the second peak at a longer wavelength is attributed to the dipole plasmon resonance of gold nanoparticles[7]. This aggregation resulted as a consequence of replacement of stabilizing citrate layer with cysteine and further electrostatic attraction between positively charged NH3+ and negatively charged COO-

groups present in cysteine but adsorbed onto different GNPs (Figure 2B).

Functionalization of GNPs by the modified protocol i.e. during synthesis itself, demonstrated a decrease in peak intensity with a prominent blue shift from 528 nm to 521nm (Figure 2A). No dual peak or complete dampening in SPR peak was noticed even after 24 hours after adsorption. Blue shift in SPR is an indication of change in the surface chemistry of cGNPs due to adsorption of L-cysteine. This nano-linker intermediate was stable at 4°C for more than 60 days without any spectral shift in its peak (SPR).

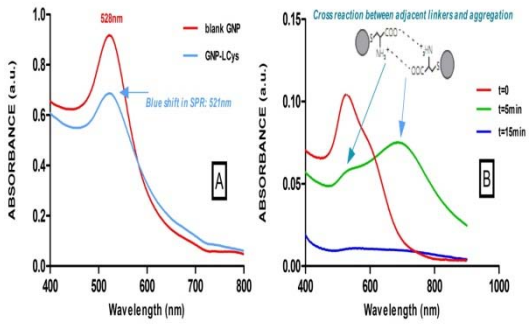

Figure 2: UV-Visible Spectrum of (A) GNP functionalized with L-cys during synthesis, (B) GNP functionalized with L-cys after synthesis. Spectrum displays aggregation behaviour of GNPs w.r.t. time.

Zeta potential measurements (Table 1) suggested an increase in the values when L-cys was impregnated onto GNPs during the synthesis; with an increase in the size of the nanoparticles. Nanoparticles with zeta potential values greater than +25mV or less than -25mV typically have higher degree of stability[10].

Table 1: Zeta potential of blank GNPs, and GNPs functionalized with L-cys by different protocols.

Sample	Charge (mV)	Particle Size (nm)
Blank GNPs	***-19.64***	***27.28***
GNP –Lcys (after synthesis)	***-21.3***	***74***
GNP-Lcys (during synthesis)	***-11.7***	***39.53***

A zeta potential value is a key parameter which indicates stability of the suspension. Table 1 clearly suggests that, functionalization of GNPs during their synthesis itself, does not affect or replace their stabilizing layer and avoid subsequent aggregation. Zeta readings thus go in good agreement with UV-vis spectroscopic analysis. Incorporation of the linker during the synthesis of GNPs therefore, does not hamper its stability and proves to be a beneficial method for functionalization of nanoparticles.

SEM analysis of GNPs functionalized during the synthesis process (Figure 3B) highlighted uniform distribution of small but stable clusters of nanoparticles. Thus it can be postulated that, incorporation of linker (L-cys) was concurrent with the nucleation and growth process of gold nanoparticles with subsequent stabilization with citrate layer. Thus synthesis and functionalization process of gold nanoparticles went hand in hand, resulting in a stable and functionalized form of gold sol. SEM images of GNPs functionalized after the synthesis process (Figure 3A) showed marked aggregation of nanoparticles and were in agreement with the data of UV-Vis spectroscopy and zeta measurements.

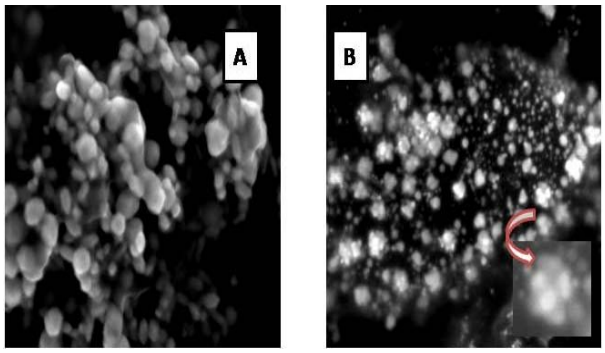

Figure 3: SEM images of (A) GNP functionalized with L-cys after synthesis, (B) GNP functionalized with L-cys during synthesis.

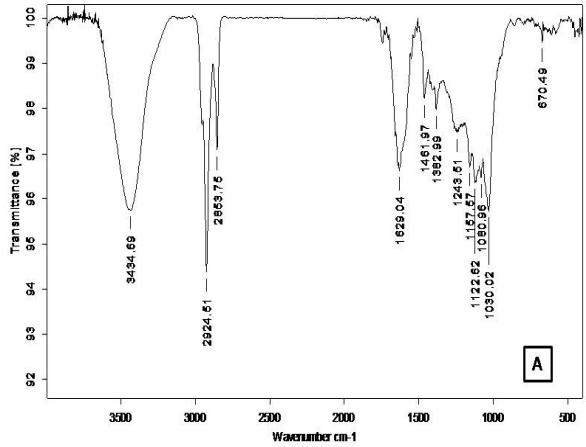

Figure 4A: FTIR spectra of GNP-Lcys.

The infra-red spectrum of GNP-L-Cys was collected in the course of the reaction in the spectral range of 4000cm^{-1} to 400cm^{-1}. FTIR spectra of GNPs synthesized with L-cysteine (Figure 4A.) showed presence of many peaks typical of L-cysteine. The most prominent peak was at 3434.69 cm^{-1}, which represents N-H stretch of primary and secondary amines. Mild peaks from 1243 – 1122 cm^{-1}can are speculated to be of C-N stretch of aliphatic amines. Whereas, a narrow peak at 1629.04 cm^{-1} correspond to the C=O stretch of carboxylic acid. Peaks at 2924 and 2853 cm^{-1} suggests C-H stretch of alkanes while, a small peak cluster at 670cm^{-1} represents the (=C-H) bend of alkanes. All these functional groups reveal presence of amine group (-NH2) and carboxylic group (-COOH) of L-cysteine and confirm its incorporation onto the cGNPs during the synthesis. The absence of thiol group at 2500cm^{-1} suggests that the –SH group of L-cysteine has reacted with the gold during the formation

of GNPs, rendering –NH2 and –COOH groups free for further attachment of the drug onto GNPs.

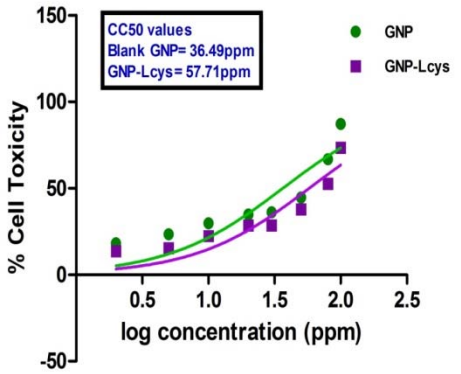

Figure 5: CC_{50} values of Blank GNP and GNP-Lcys as obtained from MTT assay using GRAPH PAD PRISM software.

Percent cellular toxicity with respect to increase in log concentrations of different gold nanoparticles is shown in Figure 5. Both the nanoparticles displayed high cell survival at lower concentrations, which progressively worsened at higher concentrations. However, GNPs after functionalization with L-cysteine demonstrated higher CC_{50} values (mentioned in Figure 5) as compared to blank GNPs, suggesting that GNP-Lcys were more biocompatible than GNPs alone.

Elemental analysis as executed by EDAX (figure 6) confirmed the presence of elemental gold in the colloidal solution synthesized.

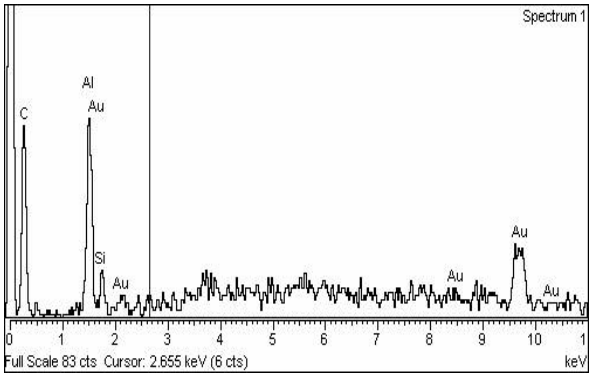

Figure 6: Elemental mapping of colloidal solution by Energy Dispersive X ray Spectroscopy (EDAX) to find presence of elemental gold in nanoparticles synthesized.

CONCLUSION

Incorporation of the amino acid linker during synthesis of gold nanoparticles did not affect the stability of GNPs. Biocompatibility of the nanoparticles was also found to be increased after conjugation with the amino acid. We were thus successful in developing a simplistic method for changing the surface chemistry of the gold nanoparticles without affecting its stability and biocompatibility. This method is therefore likely to improvise the conjugation strategies for developing biocompatible and stable drug delivery and diagnostic systems using gold nanoparticles.

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