Sequential Surface Functionalization by Polyoxometalates and Lysine Renders Non-toxic Gold Nanoparticles Strong Antibacterial Agents

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ABSTRACT

We demonstrate sequential surface functionalization of tyrosine synthesised gold nanoparticles (AuNPs) with polyoxometalates (POMs) and lysine. Antibacterial activities of these AuNPs and their nanoconjugates were evaluated toward Gram negative bacteria *Escherichia coli*. AuNPs were found to be non-toxic to the *E. coli* but after their surface functionalization with POMs, these AuNPs showed antibacterial activity due to the oxidative stress to the bacterial cells induced by POMs present on the surface of AuNPs. Lysine modified AuNPs-POMs nanoconjugates exhibited significantly higher antibacterial activity as cationic amino acid lysine directs these nanoconjugates toward negatively charged bacterial cells and provoke rupture of cell wall/membrane. Short incubation time was enough to observe the bactericidal action. By varying the surface functionlization of non-toxic AuNPs with POMs and lysine, we have shown the different levels of antibacterial action and their mode of action.

Keywords: surface functionalization, gold nanoparticles, polyoxometalates, lysine, antibacterial

1 INTRODUCTION

Inorganic nanomaterials have been used in antimicrobial applications and their size, shape, composition and surface charge play crucial role in controlling their toxicity against pathogenic microorganisms. In the context, silver nanoparticles (AgNPs) are typically found to exhibit significantly higher level of toxicity,[1-3] whereas AuNPs is typically considered biocompatible as demonstrated by us and others previously [1, 4, 5]. In general, cytotoxicity of metal ions/nanoparticles is believed to be arisen through cation induced cell wall rupturing, uncoupling of the respiratory chain from oxidative phosphorylation, denaturation of thiol containing membrane proteins and DNA damage [2, 6-8]. In an interesting study, Rotello *et al* demonstrated that cationic AuNPs may also be moderately toxic by causing cell lysis, whereas anionic AuNPs were found nontoxic [9]. This clearly indicates that even most biocompatible nanomaterials such as gold can be made toxic by tuning its surface properties.

Therefore, in this study we demonstrate sequential surface functionalization of biocompatible AuNPs with POMs and lysine. It is important to know that POMs are negatively charged clusters consisting of early transition metals and oxygen atoms formed by self-assembly processes [10, 11] and in the field of medicine POMs are known for their antibacterial, antiviral and anticancer activities [12, 13]. AuNPs were synthesized using tyrosine amino acid as reducing and stabilizing agent and surface functionalized with POMs and lysine to step up their toxic nature in a controlled manner. Antibacterial activities of these nanomaterials were evaluated against model Gram negative bacteria *E. coli*. By varying the surface functionlization of non-toxic AuNPs with POMs and lysine, we have shown the different levels of antibacterial activity. Major advantage of this approach is the very less requirement of the toxic material used for surface modification or functionalization and the AuNPs act as carrier. The size, shape, composition, metal content, surface chemistry and surface charge of these nanomaterials were characterized by UV-visible spectroscopy, TEM, AAS, ICPMS and zeta potential measurements. Nano SEM was used to study morphological changes in bacterial cells after treatments with AuNPs and their functionalized nanoconjugates.

2 EXPERIMENTAL SECTION

2.1 Reagents and Materials

Tetrachloroauric acid, L-tyrosine, phosphotungstic acid (PTA) and potassium hydroxide were purchased from sigma-aldrich and phosphomolybdic acid (PMA) was purchased from che-supply pty. Ltd and used as received. *E. coli* was bought from Southern Biological (risk group:1). Nutrient agar and Luria-Bertani broth were obtained from oxoid and US biological respectively, and used to grow and maintain the bacterial culture as per the standard protocol.

2.2 Tyrosine mediated gold nanoparticles synthesis and their surface functionalization

In a typical experiment, aqueous solution consisting of 10^{-4} M L-tyrosine and 10^{-3} M KOH were allowed to boil. Under alkaline boiling conditions, 2.0×10^{-4} M gold ions

($AuCl_{4-}$ ions) were added and this solution was further boiled 3 times in order to increase the metal concentration by the factor of 3. This AuNPs solution was dialyzed to remove free ions of KOH, unreduced chloroaurate ions and unbound amino acid present in solution, if any [14].

Dialyzed AuNPs were surface functionalized with two POMs namely phosphotungstic acid (PTA) and phosphomolybdic acid (PMA) by addition of 10^{-4} M PTA or PMA. In principle these nanoconjugates should possesses negative surface charge, which further confirmed by zeta potential measurements (Table 1). After addition of POMs and aging for 24 hours, these gold-POMs nanoconjugate solutions ($AuNPs^{PTA}$ and $AuNPs^{PMA}$) were again subjected to dialysis to remove uncoordinated PTA or PMA. Subsequently these nanoconjugates were capped with cationic amino acid lysine and dialyzed to remove free lysine molecules.

2.3 Antibacterial assays

In order to determine the antibacterial activity of AuNPs, $AuNPs^{PTA}$, $AuNPs^{PTA@Lys}$, $AuNPs^{PMA}$ and $AuNPs^{PMA@Lys}$ colony counting method was performed against *E. coli*. At the beginning of the experiment approximately 10^4 CFU/ml bacterial cells were incubated with different concentrations (doses) of nanomaterials for 15-20 minutes, and 100 µl aliquots were plated on nutrient agar plates. Colonies formed after 20 hours incubation (at 37ºC) were counted and these corresponds to the number of live bacteria in each suspension at the time of aliquot withdrawal.

3. RESULTS AND DISCUSSION

In our previous report we have demonstrated that tyrosine reduces the metal ions using the phenol group [15]. In current study, we further demonstrate sequential surface functionalization of tyrosine reduced AuNPs with POMs and lysine to step up their antibacterial activity toward *E. coli*. The pH of these AuNPs solution was found to be around 8.40, well above the isoelectric point of tyrosine (pI~5.66). Hence, these nanoparticles should have a negative surface charge in principle, which was further confirmed by zeta potential measurements and given in Table 1. Here, as we add PTA or PMA in the AuNPs solution; the pH of the solution dropped around 3.6 to 4.5 (due to highly acidic nature of POMs) and at this pH these AuNPs have high positive surface charge. Therefore, POMs bounds electrostatically to the AuNPs surface. Further, we functionalized these $AuNPs^{PTA}$ and $AuNPs^{PMA}$ conjugates with cationic amino acid lysine to direct these nanomaterials toward negatively charged bacterial cell.

UV-visible spectral analysis of AuNPs and after their surface functionalization with POMs/lysine were carried out before and after dialysis (only dialysed spectrum are presented in Figure 1). There were no considerable changes in the position of the plasmon absorption of these nanomaterials even after dialysis, except the absence of pi-pi* absorption band around 280 nm of the aromatic rings of tyrosine.

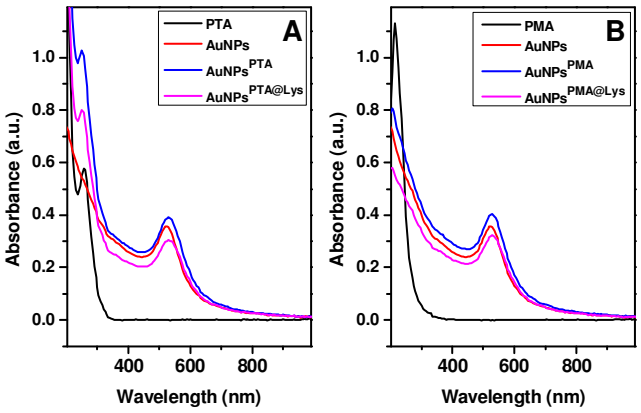
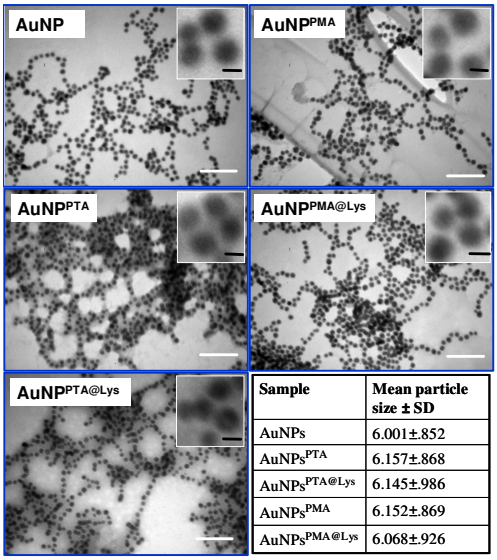


Figure 1: UV-visible spectra (A) PTA, AuNPs, $AuNPs^{PTA}$, $AuNPs^{PTA@Lys}$ and (B) PMA, AuNPs, $AuNPs^{PMA}$, $AuNPs^{PMA@Lys}$.



Sample	Mean particle size ± SD
AuNPs	6.001±.852
$AuNPs^{PTA}$	6.157±.868
$AuNPs^{PTA@Lys}$	6.145±.986
$AuNPs^{PMA}$	6.152±.869
$AuNPs^{PMA@Lys}$	6.068±.926

Figure 2: TEM images of AuNPs, and their functionlized $AuNPs^{PTA}$, $AuNPs^{PTA@Lys}$, $AuNPs^{PMA}$, $AuNPs^{PMA@Lys}$ (scale bar is 50 nm, inset scale bar 5 nm).

AuNPs showed their SPR band around 523 nm and after POMs functionalization and lysine binding SPR band showed minor red shift due to the surface modification of AuNPs and small degree of aggregation. Functionalized $AuNPs^{PTA}$, $AuNPs^{PTA@Lys}$, $AuNPs^{PMA}$ and $AuNPs^{PMA@Lys}$ nanoconjugates showed their SPR bands around 530, 528, 532 and 532 nm, respectively. Additional absorbance bands were observed in the case of functionalized nanomaterials, which indicated the presence of PTA and PMA on the surface of AuNPs as dialysis will remove free PTA and PMA. In the case of pure PTA and PMA, bands were observed at 257 and 215 nm, while corresponding bands in

functionalized AuNPs^{PTA}, AuNPs$^{PTA@Lys}$, AuNPs^{PMA} and AuNPs$^{PMA@Lys}$ nanomaterials were observed at 251, 251, 205 and 205 nm, respectively. The observed blue shift was due to the interaction between AuNPs and POMs (PTA or PMA).

TEM images of AuNPs their POMs/lysine functionlized conjugates are shown in Figure 2. These nanoparticles are spherical in shape and average size of these nanoparticles were around 6 nm. It can be clearly seen that there was only slight aggregation after POM/lysine binding, which is agreement with UV-vis spectroscopic results. AAS was used to estimate metal concentration of gold present in nanoparticles; while ICPMS analysis was carried out to determine the concentrations of molybdenum (Mo) and tungsten (W) present in nanoconjugates system. Table 1 lists the AAS, ICPMS and zeta potential measurements after dialysis of nanomaterials. Here, we believe that the metal content is mainly due to being in the nanoparticles/ nanoconjugates form as dialysis removes free metal ions.

Sample Name	Metal concentration (ppm)			Zeta potential (milliVolts)
	Au	W	Mo	
AuNPs	100.2			- 29.0
AuNPs.PTA	103.7	52.0		- 35.5
AuNPs.PTA.Lys	79.3	29.0		- 30.9
AuNPs.PMA	106.9		7.20	- 46.3
AuNPs.PMA.Lys	85.6		2.60	- 17.9

Table 1: AAS (Au), ICPMS (W and Mo) and zeta potential measurements of AuNPs and their nanoconjugates.

To evaluate antibacterial potential of AuNPs and their respective nanoconjugate systems, the parent solutions were further diluted to obtain stock solutions containing the overall Mo or W concentrations 25 µM in nanoconjugate solutions. From these stock solutions, different concentrations such as 10 µM, 5 µM, 2µM and 1 µM of Mo or W were introduced in the bacterial culture containing approximately 10^4 CFU/ml of *E. coli*. Figure 3 shows that with the increasing amount of dose, bacterial cell viability decreases due to the adverse effect on *E. coli*, which indicates that antibacterial activity of these nanomaterials is dose dependent.

We demonstrate that comparatively PMA functionalized AuNPs were more toxic against *E. coli* then PTA functionalized AuNPs. PMA based nanoconjugates were found to be more active due to it's redox potential, which induced more oxidative stress in the bacterial cell (this fact was confirmed through cyclic voltametric studies and growth kinetics of UV irradiated gold nanoconjugates, results are not presented in this manuscipt). Further, we demonstrate that actibacterial activity of AuNPs^{PTA} and AuNPs^{PMA} enhanced significantly after lysine functionalization as cationic amino acid lysine facilitated initial electrostatic attraction between bacterial cell and nanomaterials and induced cell wall repture.

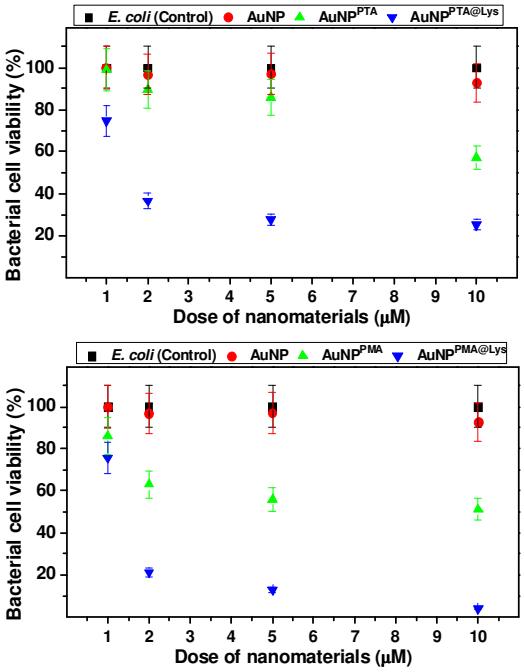


Figure 3: Antibacterial activity of AuNPs, AuNPs^{PTA}, AuNPs$^{PTA@Lys}$, AuNPs^{PMA} and AuNPs$^{PMA@Lys}$ by colony counting method against *E. coli*. Doses of W or Mo in 10^4 CFU/ml bacterial suspensions were as follow: (a) 10 µM, (b) 5 µM, (c) 2µM and (d) 1 µM.

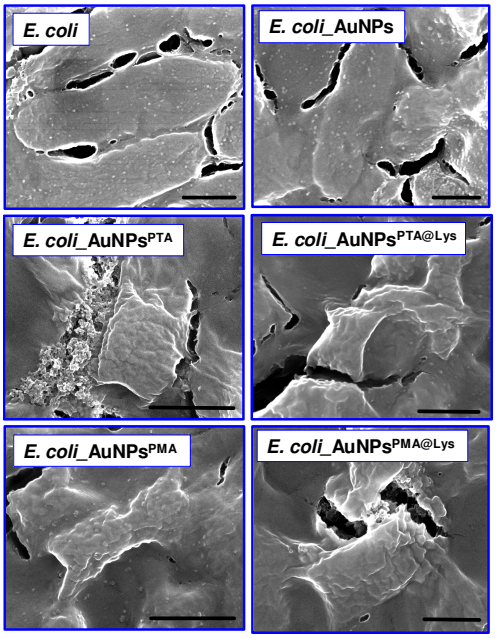


Figure 4. SEM micrographs of untreated *E. coli* and after their treatments with AuNPs, AuNPs^{PTA}, AuNPs$^{PTA@Lys}$, AuNPs^{PMA} and AuNPs$^{PMA@Lys}$ (scale bar 1 µm).

Functionalized nanomaterials initially interacted with basic elements of the bacterial cell wall/membrane and attached with the bacterial cell. This attachment of nanomaterials with bacterial cells created small pores and stress and facilities cell wall cleavage and this finally lead to bacterial cell death. Morphological changes and bacterial cell wall/membrane disruption in *E. coli* after treatment with AuNPs and their different nanoconjugates were visualized by nano-scanning electron microscopy (Nano-SEM) and shown in Figure 4. The SEM images of the bacterial cells treated with nanomaterials reveal distinct morphology compare to the control (without any treatment). After 20 minutes incubation with nanomaterials the integrity of most of the bacterial cells was lost, indicating irreversible cell damage and ultimate cell death.

4 CONCLUSIONS

It is clearly shown that tyrosine amino acid can be used as a reducing and stabilizing agent to synthesis AuNPs. Further these AuNPs were functionalized with polyoxometalates and lysine. Potential antimicrobial effects of these nanoconjugates with different surface functionalization were evaluated against *E. coli*. AuNPs do not have any antibacterial activity toward *E. coli* but POMs and lysine functionalized AuNPs have significant antibacterial activity due to the presence of POMs on the surface of AuNPs, which create oxidative stress in *E. coli* and lysine helps in initial electostatic attractions and cell wall lysis. Antibacterial activity was found to be dose dependent and higher dose enhanced antibacterial activity of nanoconjugates. Nano SEM results showed that treated *E. coli* cells were damaged, showing cleavage and cell wall lysis. In this study, we have shown that biocompatible AuNPs could be poteitial carrier for drugs due to their high surfce area and non-toxic nature.

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