Nanoparticle-drug bioconjugate as dual functional affinity ligand for rapid point-of-care detection of endotoxin in water and serum

*Prasanta Kalita[#,‡], Anshuman Dasgupta[#,†,‡], Venkataraman Sritharan[] and Shalini Gupta[#,]**
[#]Department of Chemical Engineering, Indian Institute of Technology Delhi, Hauz Khas, New Delhi, India, 110016
[]Department of Molecular Diagnostics & Biomarkers, Global Medical Education and Research Foundation, Hyderabad, India 500004

Supplementary Information

Table of contents

Materials used:

Gold(III) chloride trihydrate ($HAuCl_4.3H_2O$), sodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$), tannic acid ($C_{76}H_{62}O_{48}$), polymyxin B sulfate (PMB), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), disodium hydrogen orthophosphate (Na_2HPO_4), trichloro(octadecyl)silane (OTS), glycine, toluene, glutaraldehyde (GLA), poly(methyl methacrylate) cuvettes, hydrogen peroxide (H_2O_2), ammonia (NH_3), purified form of lipopolysaccharide (LPS) extracted from *E. coli* 055:B5, and the silver enhancement kit were all purchased from Sigma Aldrich, India. Dithiolalkanearomatic PEG_6 hydrazide (DTH) was obtained from Sensopath Technologies, USA. All solutions were prepared in ultrapure deionized (DI) Milli-Q water (resistivity ~ 18 MΩ.cm; Millipore). The DI water was further autoclaved at 15 psi for 30 min and subsequently filtered through 0.22 µm PVDF membrane filters (Millipore, India) prior to all experiments. All the chemicals used were of analytical grade. Note: LPS is pyrogenic and toxic. Strict safety guidelines must be used at all times while working with it in the laboratory.

Glass silanization protocol

Microscope glass slides were hydrolyzed using acetone followed by a 1:1:5 (v/v) solution of H_2O_2:NH_3:H_2O for 1 h at 70 ^oC. The glass slides were then washed with DI water three times and dried with a blow of nitrogen gas. The dried glass slides were dipped for 14 h in a 10 mM OTS solution prepared in toluene followed by extensive washing with toluene to remove any excess OTS from the glass surface. The entire silanization protocol was performed in a clean and dry environment in the presence of nitrogen gas.

Synthesis of gold nanoparticles (AuNPs)

Citrate-stabilized AuNPs were prepared using a standard synthesis protocol given in the literature.[1,2] Briefly, $HAuCl_4.3H_2O$ was reduced with an aqueous solution of sodium citrate and tannic acid. 10 mL of 1 % (w/v) $HAuCl_4$ solution was diluted to 800 mL with ultrapure DI water (solution A) and another aqueous solution containing 40 mL of 1 % (w/v) sodium citrate and 100 µL of 0.1% (w/v) tannic acid was diluted to 200 mL (solution B). Both A and B solutions were mixed at 60 °C and kept for 4 h under constant stirring. The mixture changed its color from light yellowish to black to violet and finally to red. The mixture was removed from heat and immediately chilled in an ice bath to quench the reaction. The prepared AuNP suspension was then stored at 4 ^oC until further use. The stability of the AuNP suspension was checked for 3 consecutive months by UV-Visible spectroscopy and found to be stable. The size of the NPs was determined using transmission electron microscopy (TEM).

Covalent conjugation of polymyxin B sulfate (PMB) to AuNPs

The citrate groups on the AuNPs were ligand place exchanged with DTH molecules by taking 10 mL of 2 nM AuNP suspension and incubating it with DTH in $1:10^4$ molar ratio for 12 h at room temperature (~25 ^oC) under constant stirring (Rotospin, Tarson, India). The excess DTH molecules were removed from the suspension by washing thrice with HEPES buffer at pH 7.4 at speeds 8690, 11175 and 14900 rcf (Dynamica Velocity 14R) for 20 min each. The same HEPES buffer at pH 7.4 was used throughout the experiments. The final pellet was resuspended in 20 mL of 2.5 % (v/v) glutaraldehyde solution in HEPES buffer and kept incubated overnight at room temperature under constant stirring. The amine-reactive NP conjugates were again washed thrice as stated above and the pellet was resuspended in 8 mL of 20 nM PMB in HEPES, followed by 16 h of incubation under constant stirring. The unreacted aldehyde groups on the GLA were passivated with 0.05 mL of 20 mM glycine in HEPES to minimize nonspecific binding. The final conjugates were washed and resuspended in 6 mL of HEPES buffer. The

biofunctionalized AuNPs were found to be stable for at least 6 weeks and stored at 4 ^{o}C until further use. The conjugation of AuNPs and PMB was confirmed by UV-visible spectroscopy, dynamic light scattering (DLS) and zeta potential measurements.

UV visible spectroscopic study

The sizes of the AuNPs were measured after each conjugation step using a Shimadzu UV-2600 UV-visible spectrophotometer. The UV-visible spectra were recorded for dilute suspensions in a low volume quartz cuvette between 400 to 700 nm wavelengths at scan rates of 100 nm/min. The average size of the NPs was calculated using the following equations based on a spherical model and assuming that the particles in suspension are monodisperse[3]:

$$d = \exp(B1\frac{A_{spr}}{A_{450}} - B2)$$

$$A_{spr} = \varepsilon cL$$

where, d is the particle diameter, B1 and B2 are empirical constants with values 3 and 2.2, respectively, A_{spr} is the maximum absorbance recorded in the spectrum, A_{450} is the absorbance measured at wavelength 450 nm, ε is the extinction coefficient whose value of 5.51 x 10^{8} $M^{-1}cm^{-1}$ was taken from the literature,[4] c is the concentration of the AuNPs, and L is the path length of the light traversing through the medium. For a path length of 1 cm, the concentration of AuNPs was determined to be 2 nM and the average size was found to be 16 nm.

Transmission electron microscope (TEM) study

A dilute AuNP suspension was carefully dried on a carbon-coated copper TEM grid (Electron Microscopy Science) and imaged using the Tecnai G2 TEM. The optical micrographs were analyzed using the free software ImageJ and the average size of the NPs was determined to be 16 ± 4 nm by taking the weighted mean average from the particle size distribution (Figure S1).

Dynamic light scattering (DLS)

Dynamic light scattering measurements were performed on dilute AuNP suspensions using the Malvern NanoZS90 Zetasizer to determine the Z-average hydrodynamic radius of the AuNPs. The instrument uses a He-Ne laser source at wavelength 633 nm. All the measurements were taken at 25 ^{o}C by setting the detector angle at 173^{o} and the medium viscosity as 0.8872 mPa.s. Refractive index of the HEPES buffer medium was set at 1.339 and that of material at 0.200. All the samples were passed through a 0.22 µm polyvinylidene fluoride (PVDF) membrane and sonicated prior to the experiments. The sizes of the AuNP conjugates measured using DLS are shown in the table below and matched our expected theoretical estimates determined from the molecular configuration of the binding ligands (Figure S2).

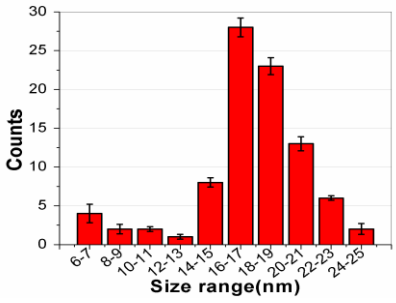


Figure S1. Size distribution of AuNPs determined after analyzing 87 randomly selected NPs from the TEM images. (2.8 px = 1 nm). The pixel resolution was large enough to discriminate the edges of the NPs.

	Theoretical size (nm)	Hydrodynamic size from DLS (nm)
AuNP	16 (from TEM analysis)	16
AuNP-DTH	24	27
AuNP-DTH-GLA	25.4	30
AuNP-DTH-GLA-PMB	32.8	38.5

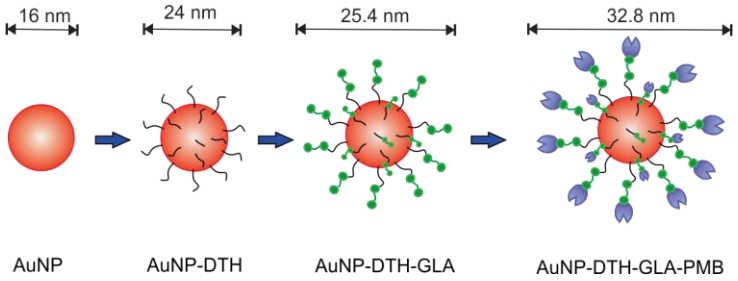


Figure S2. Theoretical size predictions of AuNPs after DTH, GLA and PMB functionalization steps made using Chemdraw and information given in product specification data sheets.

Zeta-potential measurements

Zeta potential experiments were carried out in the Malvern NanoZS90 Zetasizer. Diluted AuNP samples were loaded in a clear disposable folded capillary cell and measurements were taken in an automatic mode at 25 ^{o}C and 40 mV. The number of automatic runs for each sample was kept in the range of 10 to 100. Each reading was repeated thrice.

Bioassay for LPS detection

The bioassays were performed on silanized glass substrates by immobilizing varying concentrations of LPS from 5 fg/mL to 50 ng/mL (see Fig. S3). The LPS powder was dissolved in water and serum and 10 µL of it was deposited on the glass slides by the drop cast method. The slides were kept inside a closed water bath system (Equitron) in a humid environment at 37 ^{o}C for 1 h during this immobilization step. The slides were then washed thrice with DI water and the trapped endotoxin was contacted with 20 nM of PMB-AuNP conjugates by flooding the slide with (> 100 µL) suspension for 40 min inside a humid chamber at temperature 37 ^{o}C. The excess unbound PMB-AuNPs were washed off with DI water and the slides were allowed to dry at room temperature (~ 25 ^{o}C) for 10 min. Finally, a silver enhancement procedure was carried out in which a solution containing a silver salt was freshly mixed with a reducing agent in 1:1 (v/v) ratio (proprietary item as instructed in the kit) and applied over the LPS spot for ~ 30 s. The slides were then washed with copious amounts of ultrapure DI water. In the end, concentration-dependent dark metallic silver spots were observed wherever the LPS was immobilized.

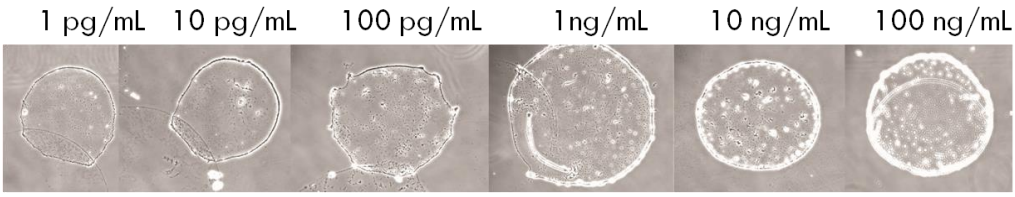


Figure S3. Fluorescence microscopy images of bound LPS on silanized glass slides for varying concentrations of LPS.

Optical quantification using a microscope

Quantification of the silver enhanced spots was performed using an Olympus BX53 optical microscope fitted with an Orca Flash 4.0 CMOS camera (Hamamatsu). Microscopic images were acquired in the brightfield mode at 4x magnification using a fixed transmitted light illumination keeping all other conditions and camera settings constant. The spot intensities in the images were quantified using the histogram tool in Adobe Photoshop 7.0.

Formula used for measuring standard deviation

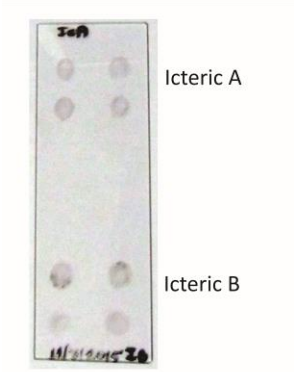
Mean, $\bar{U} = \sum_1^n \frac{U_i}{n}$

Standard deviation, $SD = \sqrt{\frac{\sum_{i=1}^{n}(U_i - \bar{U})^2)}{(n-1)}}$, where n, total number of data points.

Interference studies

Since the colorimetric bioassay was performed in serum itself, we checked to see if there was any interference in signal from colored pigments in blood. (i) We tested the efficacy of our technique on high icteric samples (patients suffering from jaundice) clinically identified as also suffering from sepsis. Figure S4 shows the results from two different patient samples both of which showed no interference due to pigments in the serum. (ii) We performed the bioassay on hemolysed serum to check its interference on the final signal. For this, serum and RBCs were separated by centrifugation and the serum was then spiked with 10 ng/mL LPS (final concentration). The collected RBCs were hemolysed by putting a small amount of DI water, mixing with a sharp edged stick and vigorous shaking in vortexer for 10 min. The hemolysed sample was then stored for 5 min to settle down the heavy components and the dark color supernatant was then mixed with the LPS-spiked serum.

(a)



(b)

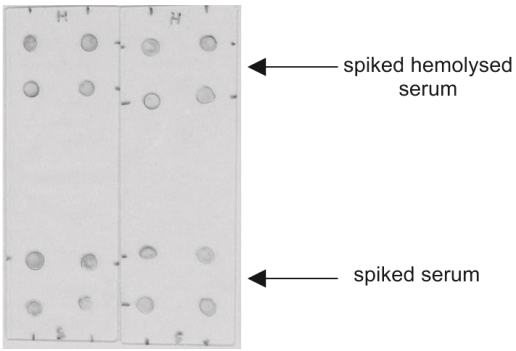


Figure S4. Dot blot assays performed to check interference by colored pigments in serum. (a) top-view CCD camera image of two icteric patient samples marked A and B. The four black spots in any one sample show quadruplet readings taken at different locations on the same slide. (b) top-view CCD camera images of eight spots obtained with 10 ng/mL endotoxin spiked hemolysed serum (top) and non-hemolysed serum (bottom). Experiments per slide were performed on the same day.

Quantification of PMB-AuNP binding with Fourier Transform Infrared (FTIR) spectroscopy

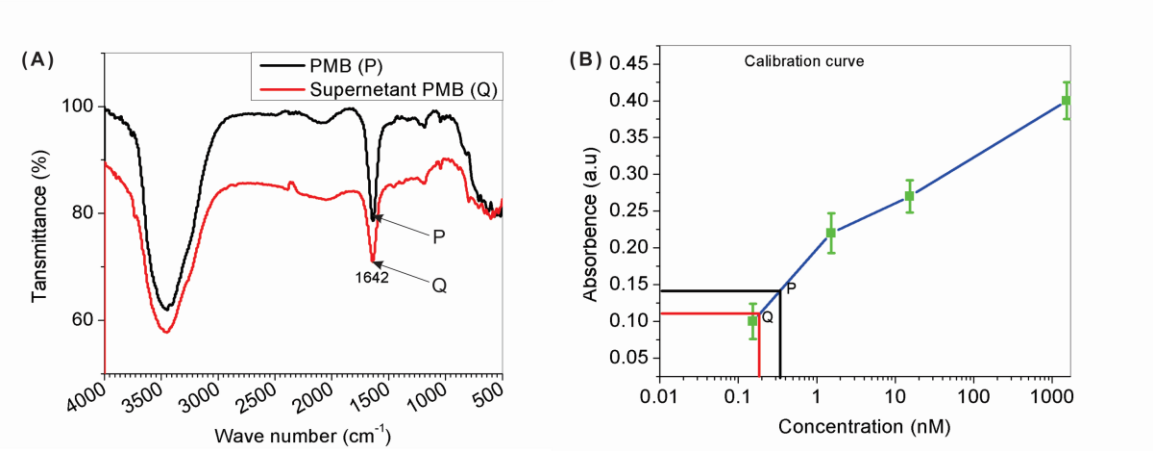


Figure S5. (A) FTIR spectra for a PMB solution before (black curve P) and after (red curve Q) conjugation with AuNPs. (B) An FTIR calibration curve at wave number 1642 cm^{-1} for varying concentrations of PMB in solution. The shift in the absorbance from point P to Q was used to estimate the average number of PMB molecules attached on the AuNPs.

An FTIR spectroscopy study was performed to quantify the average number of PMB molecules conjugated on the AuNPs. First, potassium bromide (KBr) pellets were prepared and the FTIR spectrum of each pellet was recorded for background subtraction. Then, 20 µL of PMB solution in water was added to the KBr pellets in varying concentrations and kept in a vacuum oven at 70 ^{o}C for 1 h. The FTIR spectra were again recorded in transmission mode after subtracting the background (blank pellet) and a calibration curve was drawn using the absorbance value of the peak at 1642 cm^{-1} for respective PMB concentrations (Figure S5B). The value of 1642 cm^{-1} was chosen since both the amide and the carbon-carbon bonds in the aromatic benzene present in the PMB molecular structure show a characteristic absorption peak at this wavenumber. The calibration curve was used to quantify the extent of PMB appendage by taking the FTIR spectra of the stock PMB solution before and after incubation with the AuNPs (Figure S5A). By measuring the amount of unconjugated PMB molecules in the supernatant after separating out the NPs by centrifugation, we could estimate the average number of PMB molecules per NP to be approximately 10.

References

1. J. W. Slot, H. J. Geuze, “A new method of preparing gold probes for multiple labelling cytochemistry.,” *Eur. J. Cell Biol.,* 1985, **38**, 87-93.
2. C. Y. Tsai, D. S. Lee, Y. H. Tsai, B. Chan, T. Y. Luh, P. J. Chen, P. H. Chen, “Shrinking glod nanoparticles: dramatic effect of a cryogenic process on tannic acid/sodium citrate-generated gold nanoparticles.,” *Materials Lett*., 2003, **58**, 2023-2026.
3. W. Haiss, N. T. K. Thanh, J. Aveyard, and D. G. Fernig, “Determination of size and concentration of gold nanoparticles from UV-vis spectra.,” *Anal. Chem.*, 2007, **79**, 4215–4221.
4. X. Liu, M. Atwater, J. Wang, and Q. Huo, “Extinction coefficient of gold nanoparticles with different sizes and different capping ligands.,” *Colloids Surf. B. Biointerfaces*, 2007, **58**, 3–7.