Electronic Supplementary Information

SERS-based Nanostrategy for Rapid Anemia Diagnosis

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Instrumentation

The size of the cAgNPs before and after targeting were determined at 25 °C using dynamic light scattering (DLS; Zetasizer Nano-ZS, Malvern Instruments). The measurements were carried out in triplicate. Transmission electron microscopy (TEM) characterization was performed on a JEM 2010HT system (JEOL, Tokyo, Japan). UV-visible absorption spectra were recorded by SpectraMax i3X microplate reader (Molecular Devices, San Jose, CA95134 USA). Polystyrene (PS, 100% virgin polystyrene) 96-well microplates, including the lids (Cat. 701001) from Nest Scientific Company (Wuxi, China) were used for all the experiments. Perkin Elmer Optima 8000 ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry) was used to analyze Fe^{3+} and Ag^{+} ions in solutions, using Argon as an internal standard. Raman and SERS experiments were conducted on a Renishaw InVia Reflex confocal microscope (Renishaw, UK) equipped with a high-resolution grating with 1800 grooves/cm, additional band-pass filter optics, and a CCD camera. All measurements were carried out using a He-Ne laser (λ_0= 633 nm; laser power at the spot, 17 mW). For SERS detection, the integration for Raman measurement was 1s scans. The co-addition of 5 separate scans generated SERS the spectra. The laser was focused onto the sample by using a ×50 objective (N.A. 0.75), providing a spatial resolution of 1 μm^2. Each detection was repeated ten times at 8-10 different locations for every spot. The baselines were corrected for all spectra using inbuilt integrated Raman software except for the noise test. Wavelength calibration was performed by measuring silicon wafers through a ×50 objective, evaluating the first-order phonon band of Si at 520 cm^{-1}. The spectra were recorded using Renishaw WiRE 4.2 and analyzed with Origin Pro 9 software.

Molecular docking analysis

The freely available software Autodock (version 4.2) was employed for modelling the interaction between the protein and target ligand. The protein structure of oxy-Hemoglobin (Hb) (PDB ID 3B75) was downloaded from the protein data bank[1]. Q-Site Finder identified the Hb binding site and energy minimized for ligand docking to the cavity site of the protein, using Autodock.

For molecular docking studies, the PDB files obtained from the data bank often are existent with potential problems such as missing of hydrogen atoms, many molecules, added waters, and other related problems. Thus using (define) GUI of (define) ADT, the files were prepared and converted to the pdbqt file format. The Auto-Grid module was used to generate an auto-grid box near the active site of the Hb pocket in XYZ directions with a grid point spacing of 1Å. The autogrid enables prediction of bound conformation based on empirical force field Lamarckian Genetic Algorithm (LGA) runs that was performed with both molecules (Hb and MBN) to get free binding energy. Finally, docked structure analysis was performed using PyMOL and DSV, whereas Ligplot software was employed to analyze the 2D structure of the final docked structure [2, 3].

In vitro quantitation of target analytes

Solutions of Fe^{3+} (1 µM to 1 fM) and Hb (0.1 to 1,000,000 µg/mL) were prepared in DI water and 10 mM PBS buffer (pH 7.4), respectively. The change in SERS intensity at 2226 cm^{-1} was measured to develop a calibration curve, and limit of detection (LOD) was defined as S/N=3. Binding constants (K_d) were calculated according to the dose-response curve[4] as given below:

$$Y = A_1 + \frac{A_2 - A_1}{1 + 10^{(LOGx0 - x)p}}$$

Where p is the Hill slope, A_1 is the signal intensity ratio in the absence of target, A_2 is the signal intensity ratio at maximal concentration of the target and X is the logarithm of the target concentration resulting in 50% of A_2, which gives the K_d value; and p is a "slope factor" that determines the steepness of curve at center point, i.e., (LOGx0, $(A_1+A_2)/2$).

Reproducibility of cAgNPs

The SERS signal reproducibility were evaluated for Fe^{3+} (1 µM) and Hb (1 µg/mL in 10 mM PBS, pH 7.4) were added to the cAgNPs solution. Then the SERS scans were collected from 10 random spots on the drop cast sample. Each spot was scanned for 10 consecutive runs for same sample. The control experiment was also evaluated without trapped targets at identical conditions. The corresponding SERS intensity ratio at 2226

cm^{-1} was chosen to further evaluate the reproducibility and repeatability of signal. Results are represented as the mean of at least three independent experiments.

Collection of blood samples

All blood samples were collected from Kaifeng Central Hospital, Kaifeng, Henan Province China, using K3 EDTA tubes. All blood samples were provided after the legal consent of patients and healthy individuals. The collection of blood samples was performed while considering the significant factors of age, gender, and type of disorder in a clinical examination. A total of 25 blood samples were collected with known Hb levels provided from the Hospital`s clinical laboratory section after the approval of corresponding medical professionals. The choice of anemia patients was random, such that some presented with aplastic anemia while others were anemic due to other medical issues such as cancer. All subjects were aged >20 years, and no other age restriction applied. Subjects were approximately balanced in terms of gender and age. Normal blood samples from healthy donors were collected similarly.

Isolation of Hb from anemic blood

We used a previously reported protocol[5] for Hb isolation by sonication with minor changes. Briefly, the collected blood was mixed with an equal amount of saline (0.9% NaCl, w/v), followed by centrifugation at 1000 × *g* for 30 min in an Ependrop 5810R refrigerated centrifuge (Hauppauge, NY 11788s, USA). After centrifugation, the supernatant was discarded, and the pellet washed twice with sterile normal saline. The hemolysis of packed RBCs was carried out by sonication at 8 °C for 5 min (750W, equipped with a standard pin of 13 mm diameter at 40% amplitude). When complete hemolysis was achieved, the suspension of lysed cells was heated at 60 °C for 1 h in a water bath in the dark and centrifuged at 2000× *g* for 1 h. The hemoglobin-containing supernatant was collected, diluted with an equal volume of normal saline and then centrifuged at 2000 × *g* for 1h. The resulting centre layer containing hemoglobin solution was collected and filtered through carbon 0.22 μm Millipore membranes and freeze-dried for further use.

Supporting Figures

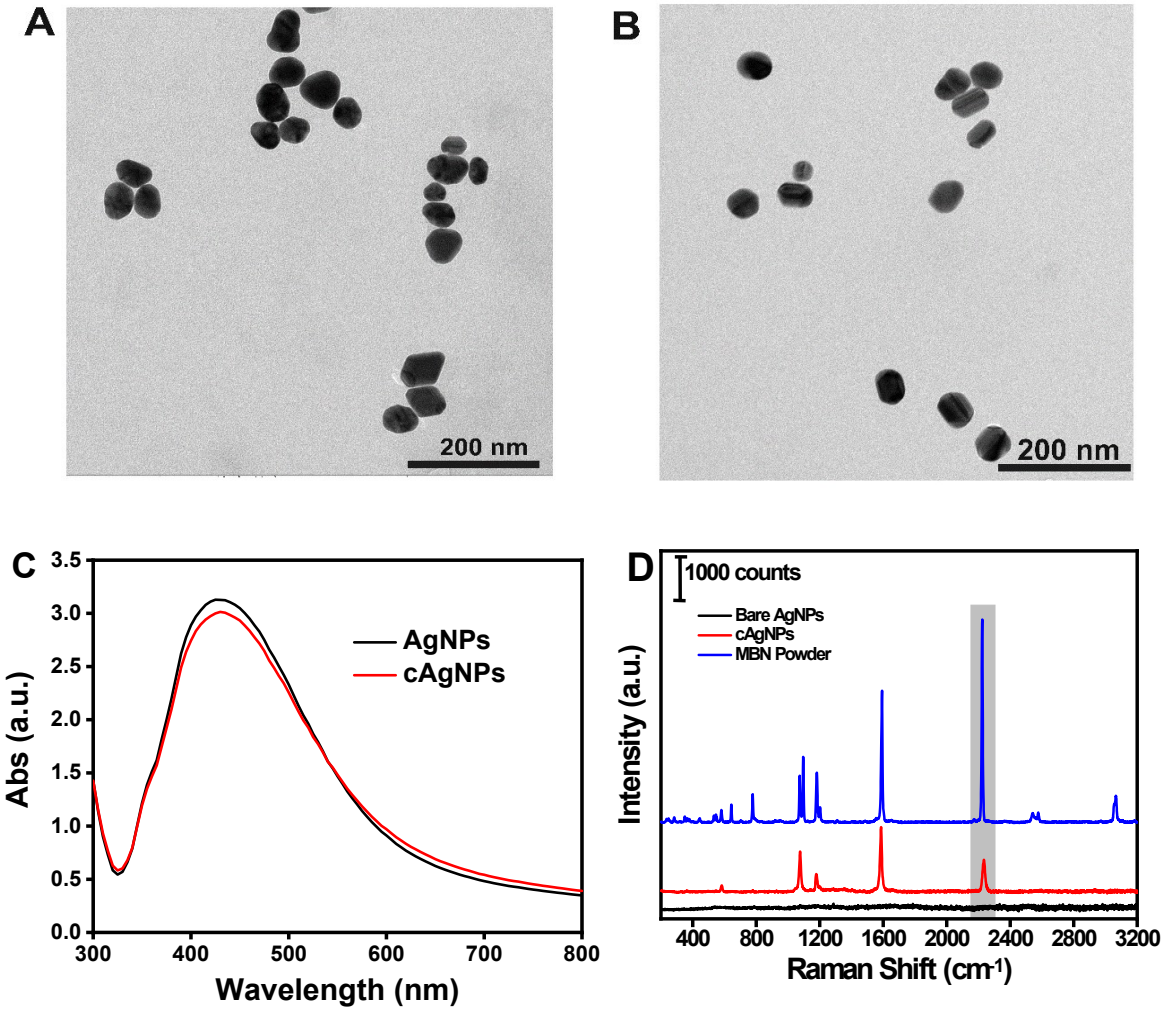

Fig. S1. TEM images of **(A)** bare AgNPs **(B) c**AgNPs (after modification with MBN. **(C)** UV-visible extinction spectra of AgNPs before and after modification with MBN in water. **(D)** Comparison of SERS spectra of bare AgNPs, cAgNPs and MBN powder.

Table S1. Summary of major peaks position and assignment for the SERS bands observed for MBN.[6]

Raman Neat powder (cm^{-1})	**SERS**	**Peak Assignment**
260		Out-of-plane wagging of C-C skeleton vibration mode
330, 352		In-plane and out-of-plan deformation of Au-C≡N mode
	776	C-S bond stretching
	579	Wagging of Au-C≡N
953	1074	In-plane scissoring of C-C mode of ring
1200	1118	Symmetric C-N stretching vibration mode
1430		Symmetric CH_2 scissoring
1600	1594	Symmetric benzene ring stretching mode
1975		C-H vibration (overtone) mode
2539		
2226	2225	Symmetric -C≡N stretching mode
2850		C-H system stretching vibration mode
2920	2916	CH_2 stretching mode

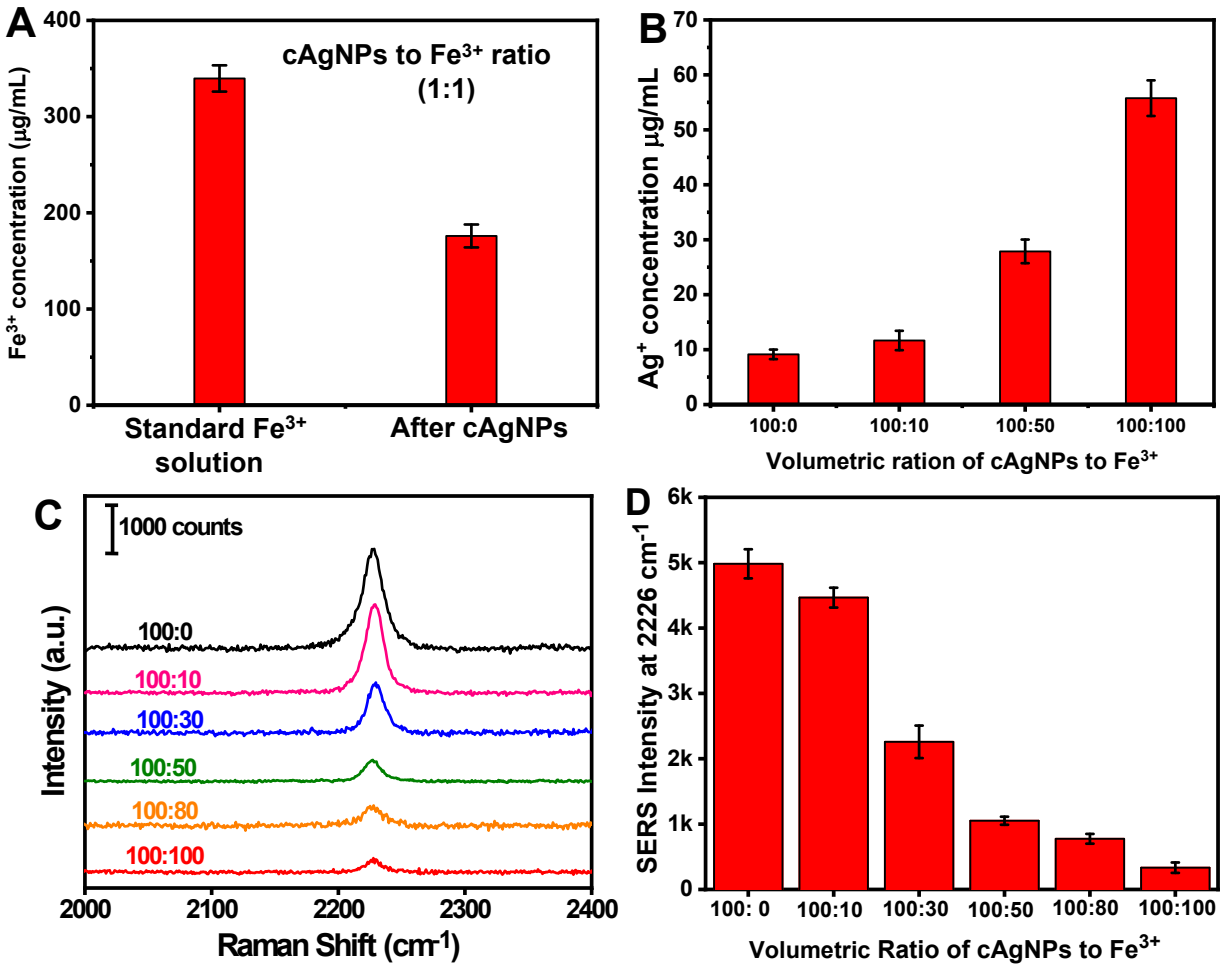

Fig. S2. (A) ICP analysis showing the etching process of standard Fe^{3+} solution before and after addition of cAgNPs in a fixed ratio. Fe^{3+} standard solution is 1 mM. **(B)** Consecutive release of Ag^{+} into working solution with increasing volume of target Fe^{3+} additions. Error bars were estimated from three replicate measurements. **(C)** SERS response of different combination of cAgNPs to different volumetric ratio of Fe^{3+} under identical conditions. **(D)** Dependence of the SERS intensity at peak 2226 cm^{-1} of the cAgNPs in the combination of different volumetric ratio of Fe^{3+} (v/v ratio, cAgNPs: 1 µM Fe^{3+}). Error bars were estimated from three replicate measurements.

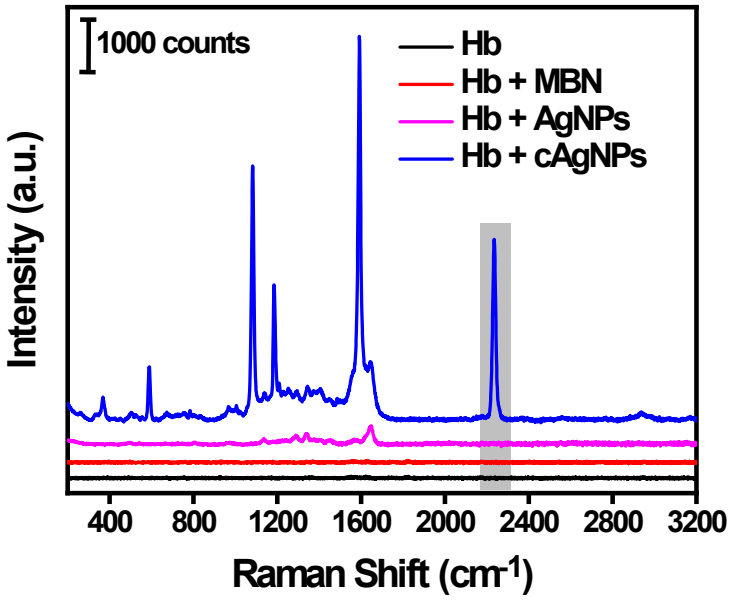

Fig. S3. Comparison of Raman spectra of Hb protein before and after addition of MBN solution, bare AgNPs, and cAgNPs at identical conditions. Sample: 1 µg/mL of Hb sample was prepared in 10 mM PBS (pH 7.4), whereas the other samples were prepared with 1:1 (v/v) ratio of Hb to Ag NPs, 10 mM MBN solution and cAgNPs, respectively.

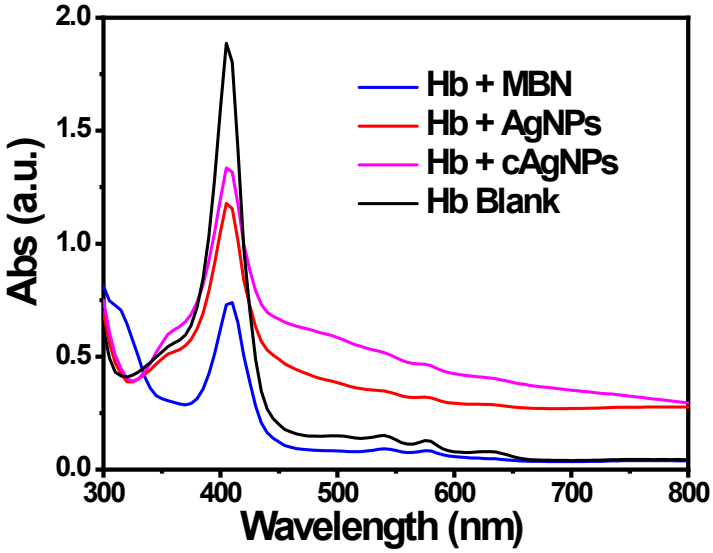

Fig. S4. UV-visible spectra of Hb before and after addition of bare AgNPs, MBN solution, and cAgNPs. Samples were prepared with 1:1 (v/v) ratio having 1 µg/mL Hb (prepared in 10 mM PBS, pH 7.4), AgNPs, 10 mM MBN solution, and cAgNPs, respectively.

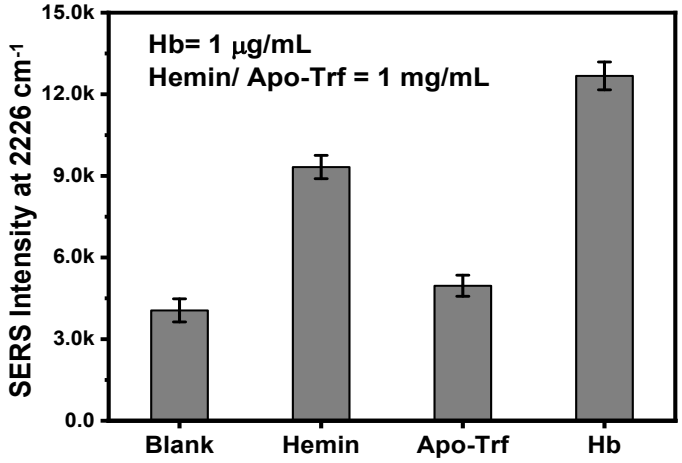

Fig. S5. Bar Chart of cross reactivity against various metalloprotein observed at Raman peak 2226 cm^{-1}. Sample: Proteins and Hemin having 1 μg/mL and 1 mg/mL concentration was dissolved in 10 mM PBS buffer (pH 7.4), while Blank sample: 10 mM phosphate buffer (pH 7.4).

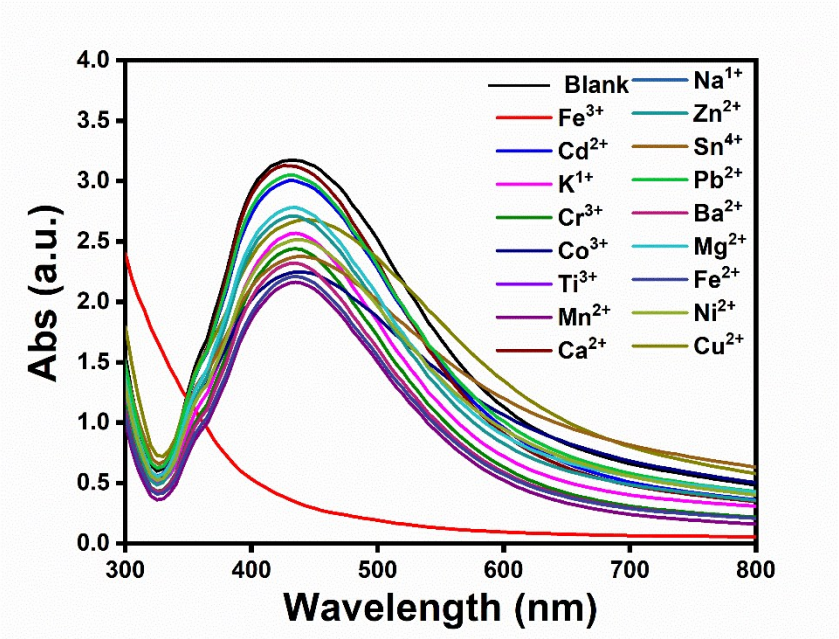

Fig. S6. Comparison of the UV-visible absorption spectra of cAgNPs without and with individually added various metal ions (1 mM) and 1 µM Fe^{3+} into cAgNPs in 1:1 ratio v/v and incubated at room temperature for 15 min.

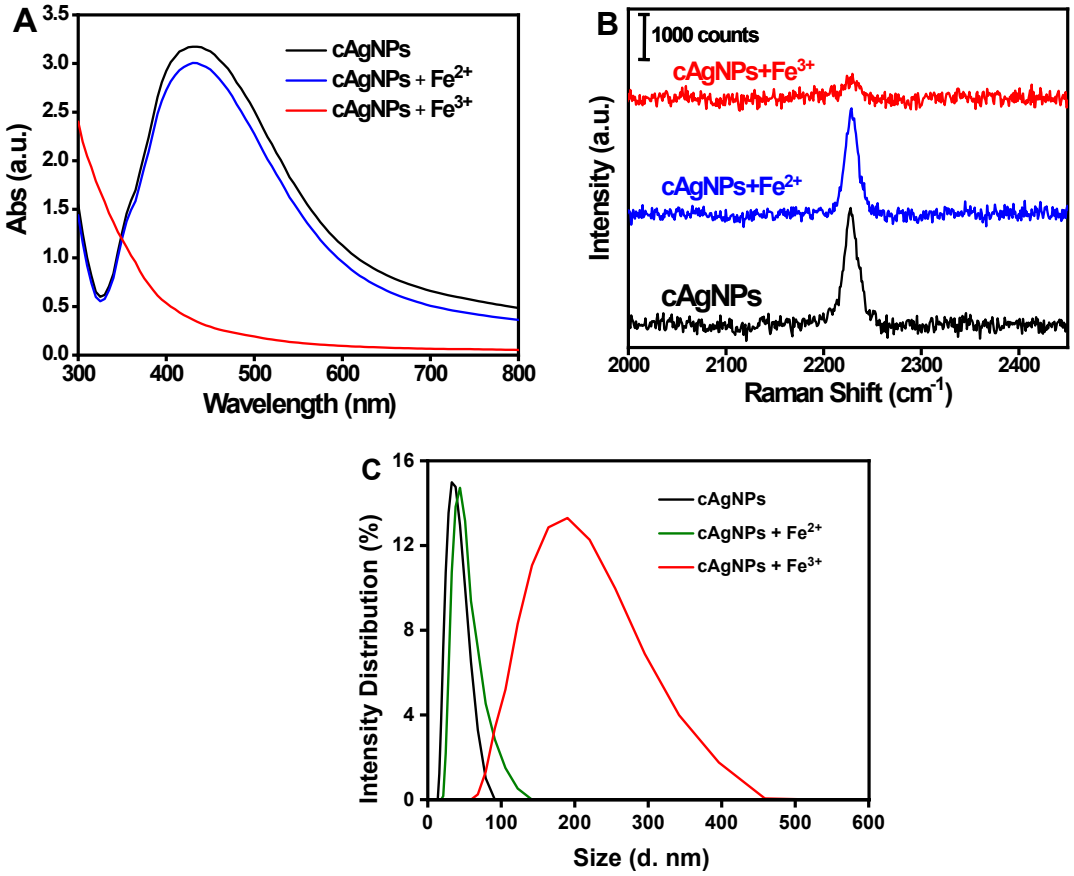

Fig. S7. (A) UV-visible spectra of cAgNPs with target Fe^{3+} ions, non-target Fe^{2+} ions. **(B)** Raman spectra of blank cAgNPs before and after addition of the target metal ion Fe^{3+}. Sample: Samples were prepared with 1:1 ratio (v/v) of cAgNPs to Fe^{3+} (1 μM). **(C)** DLS characterization of bare cAgNPs with Fe^{2+} and Fe^{3+} ions. Samples: 1 mM Fe^{2+}, and 1 μM Fe^{3+} was mixed in 1:1 ratio (v/v) of cAgNPs to sample solution.

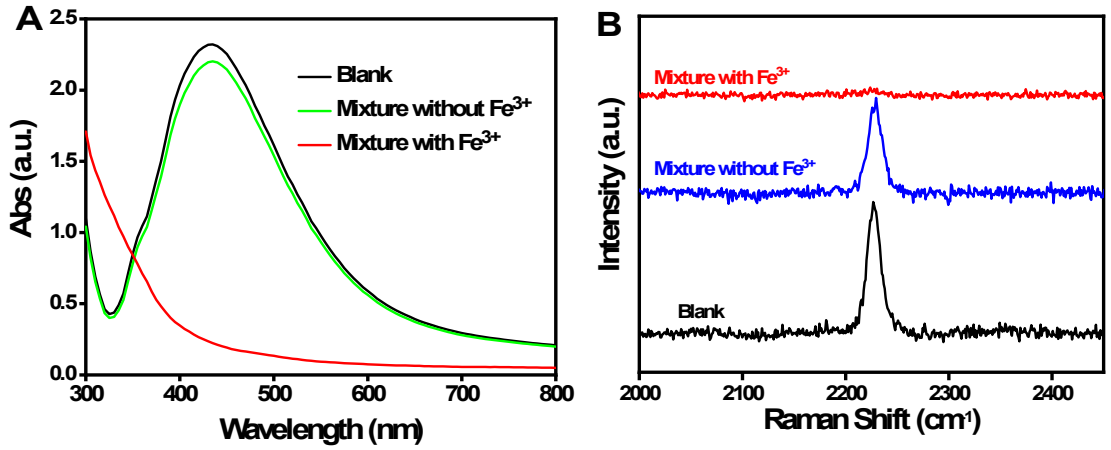

Fig. S8. (A) and (B) Comparison of the UV–visible absorption and SERS spectra of cAgNPs with and without addition of Fe^{3+} ions to the mixture of different metal ions, respectively. The concentrations of all the salts was kept 1 mM of each including Fe^{3+} and cAgNPs to metal ions ratio was 1:1 (v/v), while blank sample was just cAgNPs without addition of any salt.

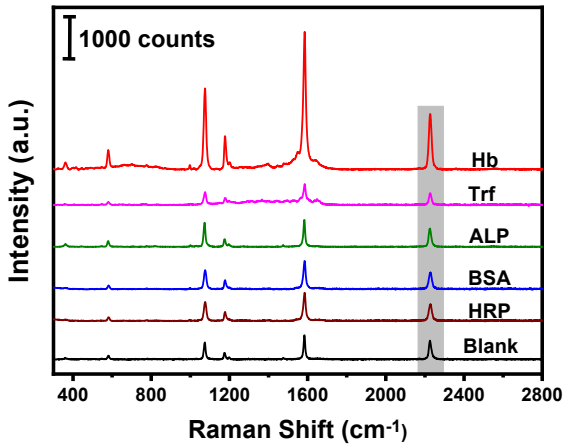

Fig. S9. Comparison of SERS spectra for cAgNPs against other metalloprotein(s) at identical conditions. Sample: The Target Hb with 1 μg/mL and other interfering proteins having 1 mg/mL concentration was dissolved in 10 mM PBS buffer (pH 7.4), while Blank sample: 10 mM PBS (pH 7.4).

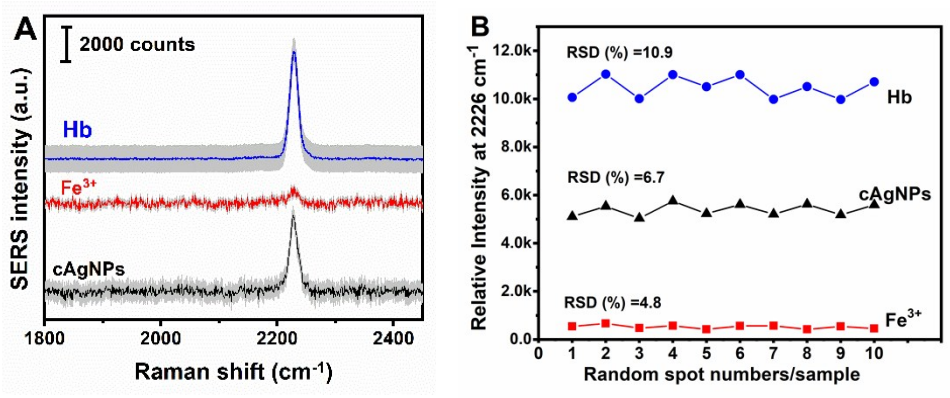

Fig S10. **(A)** SERS spectra of the main Raman vibrations of cAgNPs, before and after targets capturing (i.e., Fe^{3+} and Hb) in aqueous solution in 10 burst collected line-scan spectra for single SERS substrate. The averaged SERS spectra with relative standard deviations (RSDs) are indicated by shaded area. **(B)** Signal repeatability test. Signal variation for cAgNPs before and after target captured (i.e., Fe^{3+} and Hb) at different concentrations. The signal was read from 10 random points on one spot of the cAgNPs SERS substrate. The concentration of Fe^{3+} and Hb were 1 μM each in aqueous and PBS (10 mM, pH 7.4) solutions.

Fig. S11. Representative photographs showing the color change of lysed blood from anemic patient (Hb ≤ 10 g/dL) and healthy (Hb ≥12 g/dL) donors.

Table S2. Comparison of the Hb concentrations (g/dL) measured by the current methods with the conventional assay provided by the hospital.

# of Samples	Gender	Age (year)	Hb level observed in hospital (g/dL)	Observed Hb values via current approach (g/dL)±SD*	
				UV-visible Spectroscopy	SERS
1	F	36	8.8	8.71±0.554	8.62±0.721
2	M	62	8.9	8.68±0.846	8.51±0.871
3	M	73	7.4	7.52±0.687	7.56±0.589
4	F	59	8.5	8.58±0.426	8.58± 0.678
5	M	65	7.2	6.78±0.724	6.73±0.587
6	M	76	11.3	11.14±0.915	11.01±0.468
7	F	34	9.9	9.63±0.387	9.61±0.687
8	F	44	9.9	9.47±0.798	10.1±0.591
9	F	55	10.2	8.94±0.857	8.72±0.726
10	F	20	7.7	7.72±0.587	7.82±0.671
11	M	85	8.1	7.99±0.944	7.87±0.854
12	M	60	8.7	8.32±0.354	8.48±0.587
13	F	69	7.3	7.23±0.758	7.71±0.987
14	M	74	7.4	7.39±0.692	7.41±0.735
15	F	47	9.6	9.64±0.867	9.92±0.454
16	F	33	8.6	8.55±0.919	8.59±0.767
17	M	30	8.0	8.15±0.494	8.31±0.834
18	F	74	9.0	9.11±0.763	9.26±0.528
19	F	60	7.1	6.99±0.587	7.02±0.735
20	F	49	6.9	7.02±0.596	7.16±0.819
21	F	32	13.0	13.74±0.754	13.45±0.857
22	M	22	14.1	14.3±0.735	14.07±0.935
23	M	60	13.8	13.6±0.695	13.70±0.458
24	F	28	12.0	12.2±0.857	12.65±0.587
25	F	83	12.6	12.58±0.797	12.71±0.476

*±SD indicates the error of propagation within the triplicate run of experiments.

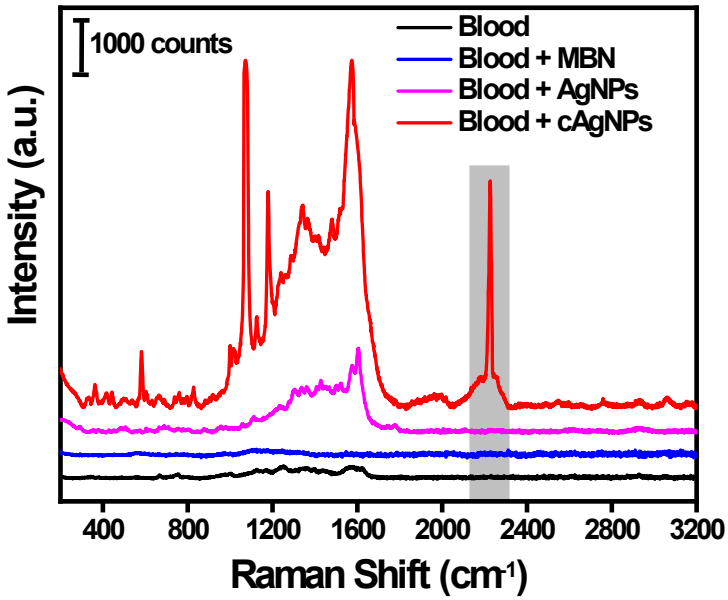

Fig. S12. Raman spectra of lysed blood before and after addition of MBN solution, bare AgNPs and cAgNPs. Sample: Samples were prepared with 1:1 (v/v) ratio of lysed blood to AgNPs, 10 mM MBN solution and cAgNPs, respectively.

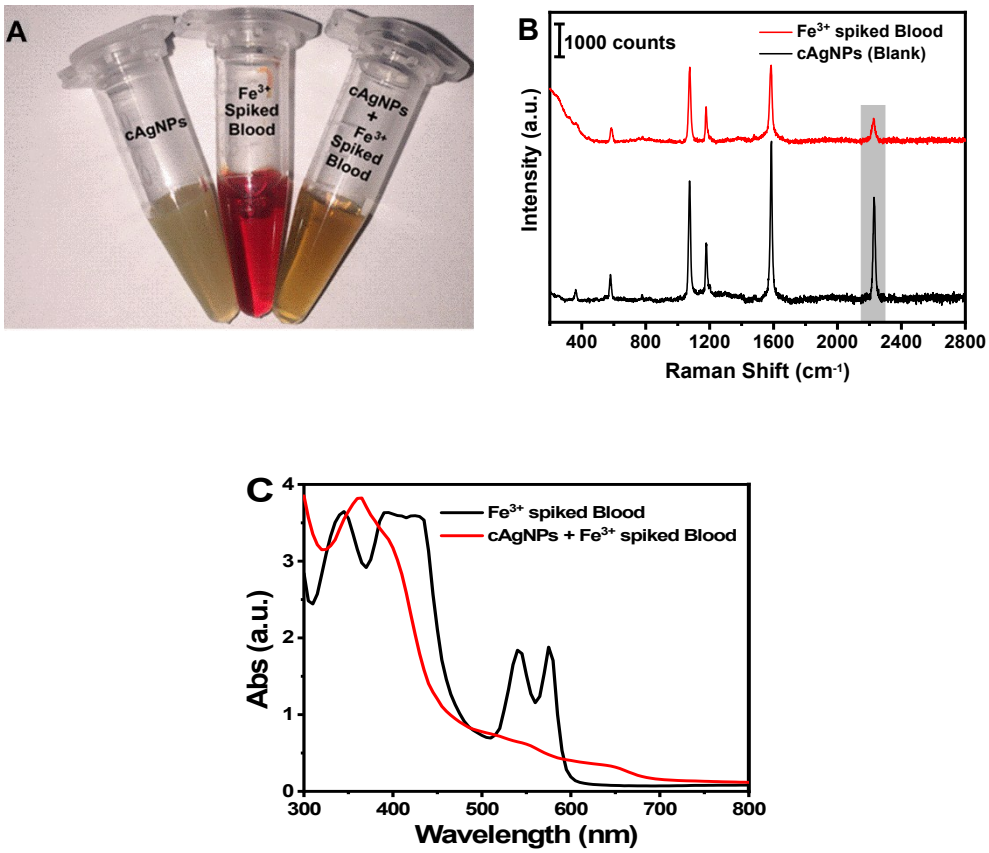

Fig. S13. (A) Representative photographs showing the color change response of the cAgNPs, Fe^{3+} spiked lysed blood before and after addition of cAgNPs. **(B)** Comparison of Raman spectra of lysed blood spiked with Fe^{3+} and cAgNPs. **(C)** UV-visible spectra of Fe^{3+}spiked blood before and after the addition of cAgNPs. Sample: Samples were prepared with 1:1 (v/v) ratio of Fe^{3+}spiked blood to cAgNPs. Fe^{3+} 1 µM concentration was spiked into the lysed blood.

Supporting References

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