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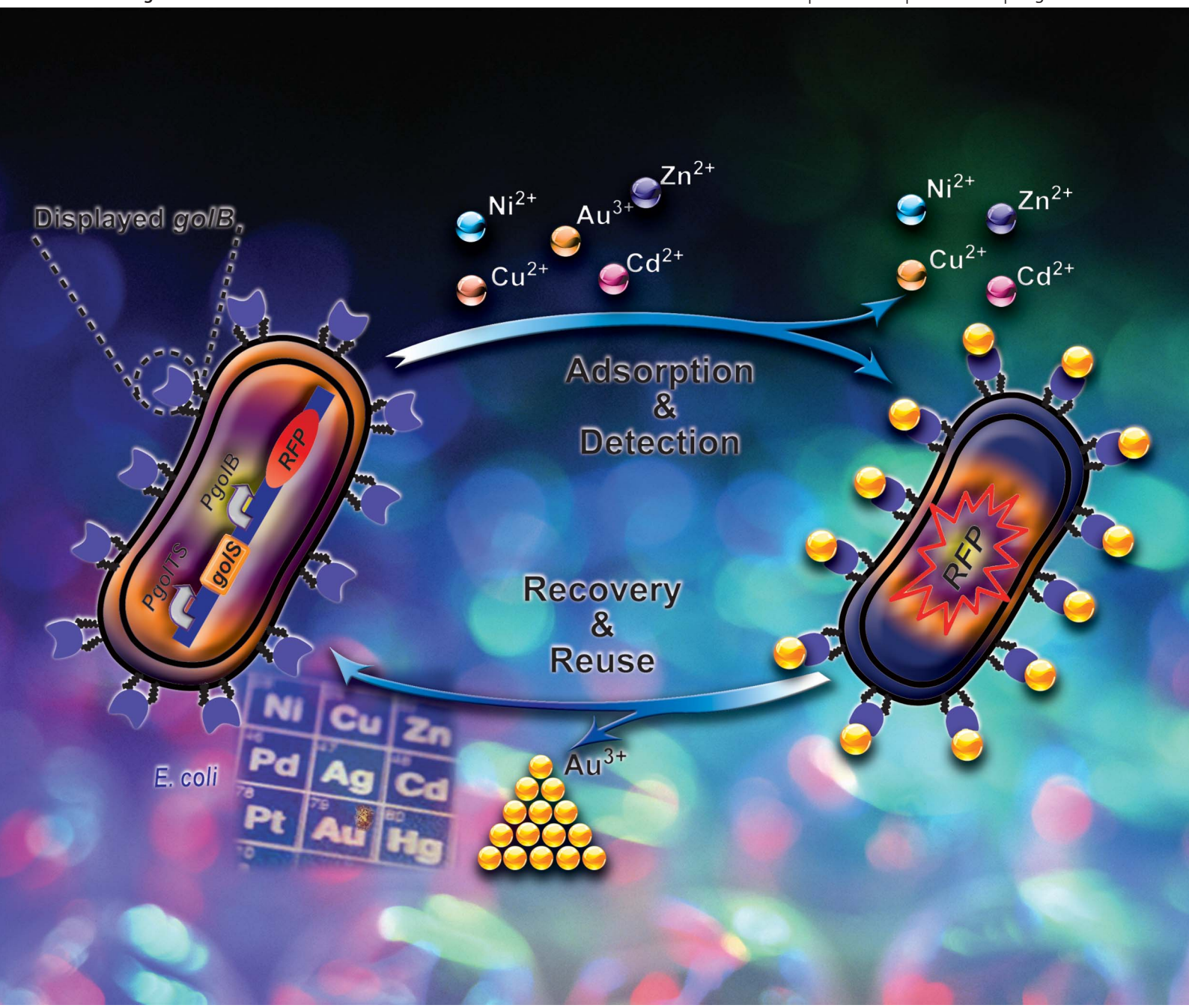

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EDGE ARTICLE

Engineering a gold-specific regulon for cell-based visual detection and recovery of gold†

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A gold-specific sensory protein GolS from *Samonella gol* regulon was incorporated into *E. coli*, which in conjunction with an engineered downstream red fluorescence protein allowed the highly sensitive and selective whole-cell detection of gold(III) ions by naked eyes. The putative gold-chaperone, GolB, in the *gol* regulon was next verified to be specific to gold(I) ions over other metal ions including copper(I). The subsequent display of GolB on *E. coli* cell surface permitted selective enrichment of gold ions from media containing various thiophilic metal ions. The cell surface-enriched gold(I) was further shown to be easily recovered and the gold-deprived bacteria were capable for re-usage. *E. coli* bacteria harboring these gold-specific elements from the *gol* regulon could be a valuable tool for visual detection and facile recycling of gold ions from environmental resources.

Introduction

Gold is a historically highly valued precious metal and we have witnessed a continuous climbing of gold prices in the new millennium, especially after the global financial crisis in 2008.[1] The detection and recovery of gold ions have attracted considerable attention not only because the gold is valuable and industrially important, but also because gold ions play key roles in biology, such as in bacteria resistance and medicine including anti-cancer drugs.[2] On the other hand, inappropriate disposal of this widely used rare metal has become an increasing environmental concern largely due to the toxic gold salts in waste water.

In most natural environments, gold is always accompanied by a small amount of copper or silver. Selective and effective distinguishing of gold from other metals is generally a vexing problem. Although several physico-chemical methods are available for heavy metal ions detection, including Au ions, these approaches generally rely on expensive instruments and complicated procedures, rendering them incompatible with practical usage outside of laboratory settings.[3,4] Gold-selective chemosensors largely rely on polymers, quantum dots, nanoparticles, or oligonucleotides,[5] but only a few of them can sense gold with high selectivity.[6–8] In contrast, biologists focused on living organisms that had evolved diverse mechanisms for maintaining homeostasis and resistance to heavy metals including gold.[9] Subsequently, highly selective and sensitive metal biosensors have been developed.[10–12] Furthermore, in addition to the sensing of Au ions, the current adsorption techniques are incapable of recovery or separation of the adsorbed metal ions, limiting their applications.[13]

Circumventing these obstacles requires an efficient and cost-effective system that can couple the detection and the recovery processes, which may achieve the selective detection and enrichment of target metal ions concurrently. Gold, a highly sought-after metal, is particularly attractive considering its high value and that it contaminates the environment. Engineering microorganisms for sequestering of heavy metal ions, including Hg(II) and Cd(II), has received growing attention, primarily due to the low-cost, high specificity and robustness of such systems. Among these techniques, using an engineered cell surface for toxic metal adsorption has several advantages: (1) metal ions in the environment can interact with the cell surface directly without the need to pass the membrane; (2) the method alleviates the burden for intracellular accumulation of toxic metal ions, which often results in less adsorption efficiency;[14] (3) the adsorbed metal ions can be readily removed without cell breakage, which is particularly valuable for the recovery of rare metal ions.[15] Despite these advances, however, there is currently no report on the utilization of this methodology for the selective recovery of precious metals, such as gold ions. Herein, we present selective gold-sensing and recovery using the gold-sensory protein GolS and gold-binding chaperone GolB. The verification of the newly identified putative gold binding protein GolB for specific Au(I) recognition was first demonstrated. The subsequent engineering of *E. coli* cell surface for displaying this unique

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protein allowed the selective sequestration and recovery of the precious gold metal (Fig. 1).

Results and discussion

GolS is a recently identified gold-specific MerR family transcriptional regulators from *Samonella* that can effectively differentiate Au(I) from the other two coinage metal ions, Cu(I) and Ag(I).[16,17] Inspired by the recognition of the *gol* regulon (Fig. 2a), we tried to develop a gold ion selective induced system in *E. coli* with the transformed *gol* cluster and red fluorescence protein to achieve gold ion selective sensing on bacterial cells. As shown in Fig. 2b, the gene of red fluorescence protein (RFP) was inserted after the *golS* promoter in the vector pSB1A2, allowing its expression to be driven by GolS, the gold-specific regulator.

The *E. coli* cells containing the engineering plasmid were cultured in LB medium until OD_{600} = 0.6–0.8, then a final concentration of 20 μM Au^{3+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, or Ni^{2+} was added to the medium. The induced *E. coli* cells were harvested by centrifugation and re-suspended in PBS buffer (pH 7.4). The results showed that in the presence of gold ions, RFP was significantly expressed and can be visualized by the naked eye (Fig. 2c). Next, the sensitivity of our engineered *E. coli* cells to gold was investigated. *E. coli* cells induced by gradient concentrations (0 μM, 0.25 μM, 1 μM, 5 μM, 10 μM and 20 μM) of $HAuCl_4$ (Au^{3+}) were re-suspended in PBS buffer (pH 7.4). The results demonstrated that even the induced concentration of gold ions reached as low as 0.25 μM (Fig. 2d, e), the red fluorescence of *E. coli* cells was still visible. Different from micromolecule chemosensors design and synthesis by organic chemists, by using this engineered *E. coli* we achieved highly selective and sensitive recognition of gold ions by a visible and ratiometric fluorescence change on bacterial cells.

To increase the display efficiency and minimize the potential interference with the function of proteins presented on bacterial cell surface, it is preferred to use a small-sized protein or a truncated domain from a large protein for surface display.[18] We therefore turned our attention to GolB, a recently identified small gold-specific protein in *S. typhimurium* that is highly induced in the presence of Au(I) over Cu(I), Ag(I), Zn(II), or Hg(II) ions.[16] At only less than 70 amino acids in length, GolB is a much smaller protein than GolS. Sequence alignment indicates that GolB contains a similar metal binding motif, MTCXXC, to that of the previously reported human copper chaperone AtoX1 and yeast copper chaperone AtX1 (Fig. S1†).[19] Furthermore, due to its metallo-chaperone nature, GolB may be more resistant to various misfolding stresses upon display on bacterial surface. We thus focused on introducing GolB protein on *E. coli* cell surface.

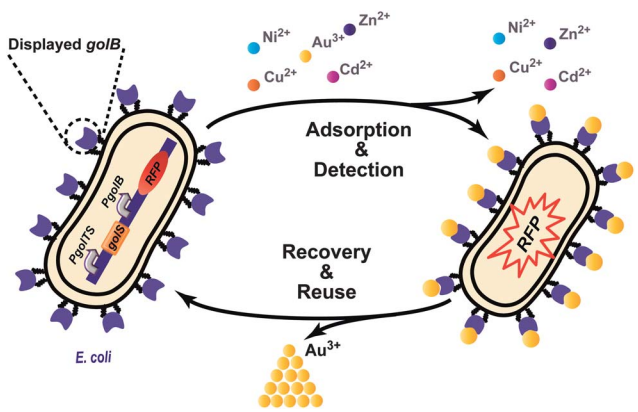

Fig. 1 The *E. coli* cell-based sensing and recovery of gold ions by gold inductive fluorescence protein expression and surface displayed GolB protein.

Although GolB was found to be regulated by GolS and is required for gold resistance in *S. typhimurium*, its binding affinity and Au(I) selectivity against other metal ions, particularly Cu(I), remains to be verified.[16] We first examined the binding affinity between GolB and Au(I) using mass spectroscopy and UV-Vis spectroscopy. GolB-GST was overexpressed in *E. coli* and purified by GSTrap chromatography before being digested by thrombin to remove the GST-tag (Fig. S2†). The purified GolB protein was then incubated with Au(I) and applied in electrospray ionization mass spectrometry (ESI-MS), which yielded two peaks with masses of 7002.5 Da and 7199.5 Da. The first peak corresponds to the molecular weight of apo-GolB protein, while the second peak represents GolB in complex with one equivalent Au(I) (atomic mass of Au = 196.966 g mol^{-1}) (Fig. 3a). To further assess the binding between GolB and Au(I), the UV absorption of purified GolB was measured in the presence and absence of Au(I) ions. A decrease in the UV-absorption between 250 nm and 255 nm was observed upon the addition of aliquots of $Au(CN)_2$, and the binding ratio between GolB and Au(I) was 1 : 1, as obtained by the Job's plot method (Fig. S3†). Based on the Au(I)–S absorption at 250 nm and competitive titration with cyanide (Fig. 3b), the dissociation constant of GolB for Au(I) was determined to be 3.02×10^{-37} M (Table 1). The dissociation constant between GolB and Cu(I) was also determined according to a published protocol (1.61×10^{-17} M, Fig. S4†).[20,21] These data confirmed that GolB binds to Au(I) with a much higher affinity than Cu(I). Mutation of either cysteine residues in the MTCXXC motif of GolB abolished the binding between GolB and Au(I) or Cu(I), indicating the essential roles of these two cysteines on the recognition of Au(I)/Cu(I) ions. Besides these two cysteines, additional factors might contribute to GolB's selectivity for Au(I) over Cu(I) and we are currently investigating these features in our laboratories.

Next, we sought to display GolB on *E. coli* cell surface by fusing it with OmpA, an *E. coli* outer membrane protein.[22,23] The Lpp–OmpA fusion system has been previously used to display proteins as large as 263 amino acids on the surface of *E. coli*.[18] Following a similar protocol, we constructed a plasmid carrying the OmpA–GolB expression gene by connecting the N-terminal 1–159 amino acids of OmpA with the full-length GolB with a FLAG-tag at the C-terminus (Fig. 4a) using overlap PCR (Table S1†). A HA-tag was also created at the N-terminal of the whole fusion protein immediately following the OmpA signal peptide. This plasmid was transformed into *E. coli* and the expression of the fusion proteins was analyzed by SDS-PAGE. As shown in Fig. 4b, the expressed recombinant OmpA–GolB protein has a molecular weight near ~29kDa on the SDS-PAGE gel (calculated M_w = 29.2 kDa), and the identity of this fusion protein was further verified by immunoblotting analysis using the anti-HA and anti-FLAG antibodies, respectively. To further confirm that GolB was displayed towards the extracellular space of *E. coli*, rather than being buried in the outer membrane or

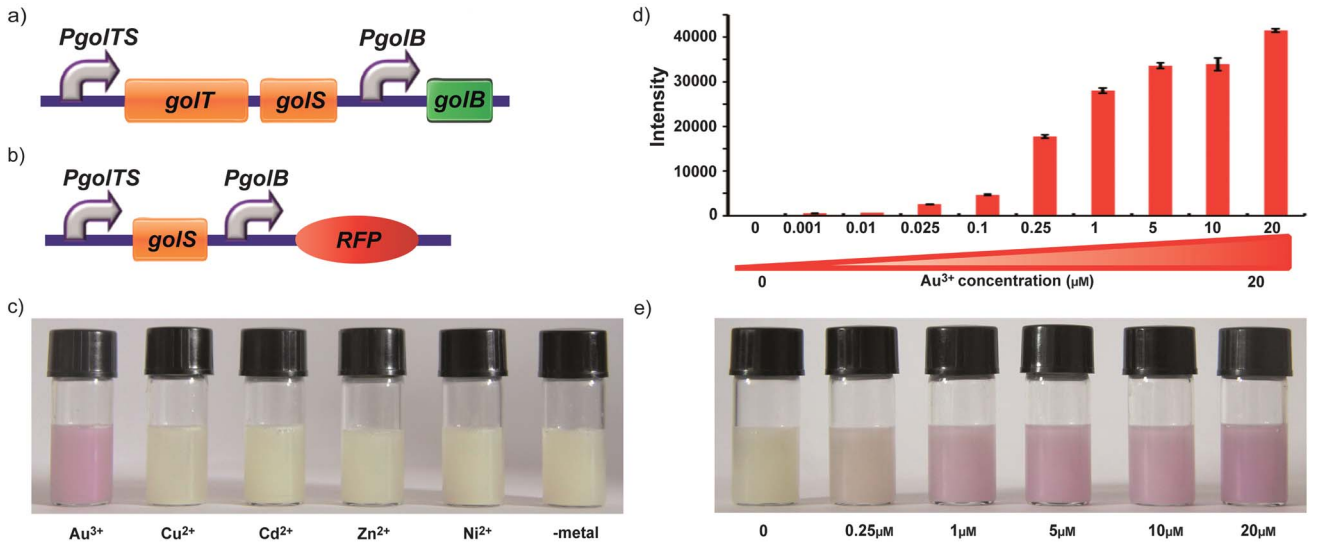

Fig. 2 a) Genetic organization of the *gol* locus in the *S. typhimurium* LT2 genome. b) Genetic organization of the gold inductive RFP expression plasmid in *E. coli*. c) A photograph of *E. coli* cells containing the gold-induced RFP expression plasmid with a concentration of 20 μM each of metal ions, or without metal ions induction and re-suspended in PBS buffer (pH 7.4). d) Fluorescence measurement of *E. coli* cells containing the gold-induced RFP expression plasmid after gradient concentrations of $HAuCl_4$ (Au^{3+}) induction and re-suspended in PBS buffer (pH 7.4). e) A visible photograph of d).

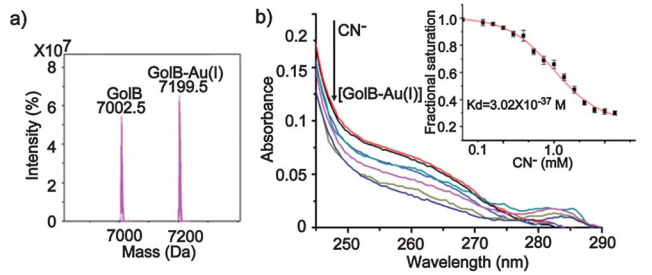

Fig. 3 a) ESI-MS of GolB apo-protein (found: 7002.5 Da, expected: 7006.9 Da) and GolB in complex with one equivalent of Au(I) ion (found: 7199.5 Da, expected: 7203.9 Da). b) The cyanide (CN^-) titration to Au(I)-loaded GolB and non-linear curve fitting to obtain the equilibrium concentration of CN^-, GolB and GolB-Au(I) complex for the calculation of dissociation constant between GolB and Au(I).

Table 1 Dissociation constants between GolB and Au(I) or Cu(I)

Protein	Au(I)	Cu(I)
GolB	3.02×10^{-37} M	1.61×10^{-17} M

towards the periplasm, we directly visualized the GolB protein displayed on *E. coli* cell surface by using FITC-labeled anti-FLAG antibody (FITC–anti-FLAG). *E. coli* cells carrying the OmpA–GolB expression plasmid was induced by arabinose and incubated with FITC–anti-FLAG before being fixed for visualization under a fluorescence microscope. The same batch of *E. coli* cells without protein induction was used as a control. Strong fluorescence was observed when the *E. coli* cells expressed the OmpA–GolB, whereas no fluorescence was detected for the uninduced condition (Fig. 4c). Taken together, these results confirmed that the GolB protein was displayed on *E. coli* cell

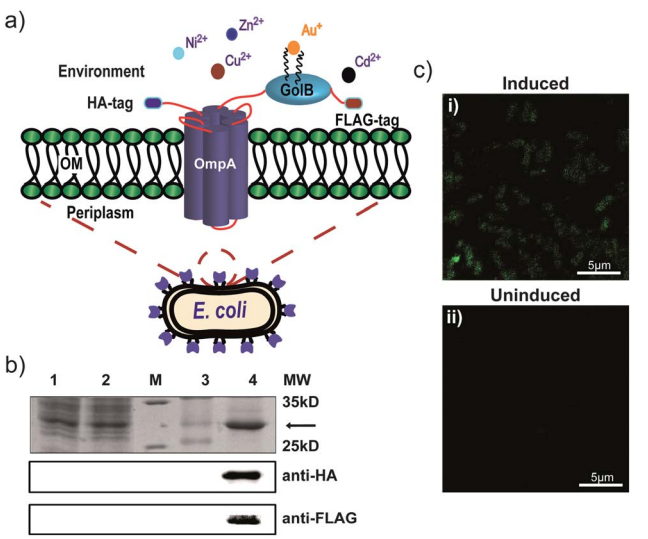

Fig. 4 a) An illustration of the gold(I)-specific protein GolB displayed on *E. coli* cell surface *via* the outer membrane protein OmpA. The blue columns represent the membrane-spanning domains of OmpA. GolB is displayed on the outer surface of the *E. coli* outer membrane. b) SDS-PAGE and immunoblotting analyses of surface-displayed GolB protein. The black arrow on the SDS-PAGE gel indicates the OmpA–GolB protein expressed in bacterial membrane fraction from induced bacteria expressing OmpA–GolB (lane 4). The membrane fraction from uninduced bacteria (lane 3), the supernatant protein fraction from induced (lane 2) or uninduced (lane 1) bacteria are used as controls. The presence of OmpA–GolB protein was further confirmed by immunoblotting analysis using anti-HA and anti-FLAG antibodies. c) Immunofluorescence labeling of *E. coli* cells using anti-FLAG antibody and FITC conjugated anti-mouse IgG antibody.

surface and is readily accessible by molecules from the extracellular environment.

We then utilized the engineered *E. coli* bacteria for gold adsorption. The GolB-displayed *E. coli* was incubated with 50 μM $AuCl_3$ (Au^{3+}) overnight and the treated cells were extensively washed with ddH_2O, followed by ICP-AES analysis. *E. coli* bacteria without induction or expressing GolB proteins with the two cysteine residues mutated to serine were used as controls. As shown in Fig. 5a, *E. coli* bacteria with the surface-displayed GolB were able to adsorb gold ions with a capacity of about 4.62 μmol g^{-1} cells, which is 12-fold higher than undisplayed samples and GolB mutant samples. Although our *in vitro* experiment showed that the purified Au(I)-binding protein GolB didn't bind to Au(III), the engineered bacteria were able to adsorb both Au(I) and Au(III) (Fig. S6†). Au(III) salts have been previously used by microbiologists to study the GolS regulon in living bacterial cells,[16] and we also suspect that Au(III) ions might be first reduced by the bacteria to Au(I), which is then adsorbed by the surface-displayed GolB protein.

Next, we demonstrated that these engineered *E. coli* cells could adsorb and selectively recover gold ions from LB medium containing a mixture of metal ions without apparent damage to the bacteria cells. As the bacterial outer membrane is a complex chemical structure, there must be some non-specific adsorption of other metal ions to interfere with gold selective recovery. Thus, the recovery experiment was performed on the metal-adsorbed *E. coli* cells in PBS buffer (pH 7.4) containing 10 mg L^{-1} papain, a cysteine-specific protease.[15,24] After incubation at 30 °C for 12 h with gentle shaking, gold ions bound with GolB on *E. coli* cell surface would be selectively removed by cleavage of the cysteine residues on GolB using papain. The gold ions removed from the bacterial surface were collected and applied in ICP-AES analysis. Nearly 89% of the gold ions were recovered from the total gold ions adsorbed on *E. coli* cell surface, which was much higher than other metal ions (Fig. 5b, c). Furthermore, as shown in Figs S7a and b†, the GolB-displayed *E. coli* bacteria showed a similar growth curve and gold tolerance after being reused for three times (for each time, the bacteria underwent gold-adsorption, papain treatment and then the recovery process), and the presence of GolB protein on the recovered bacteria surface was confirmed by SDS-PAGE analysis (Fig. S7c†). These results demonstrated that the GolB-displayed *E. coli* bacteria strain we developed was capable of being reused for highly selective recovery of gold.

We also analyzed the gold deposition form on the cell surface. The transmission electron microscopy (TEM) was employed to check for the presence of gold particles on the cell surface (Fig. 5d). While there were no metal precipitates detected on the

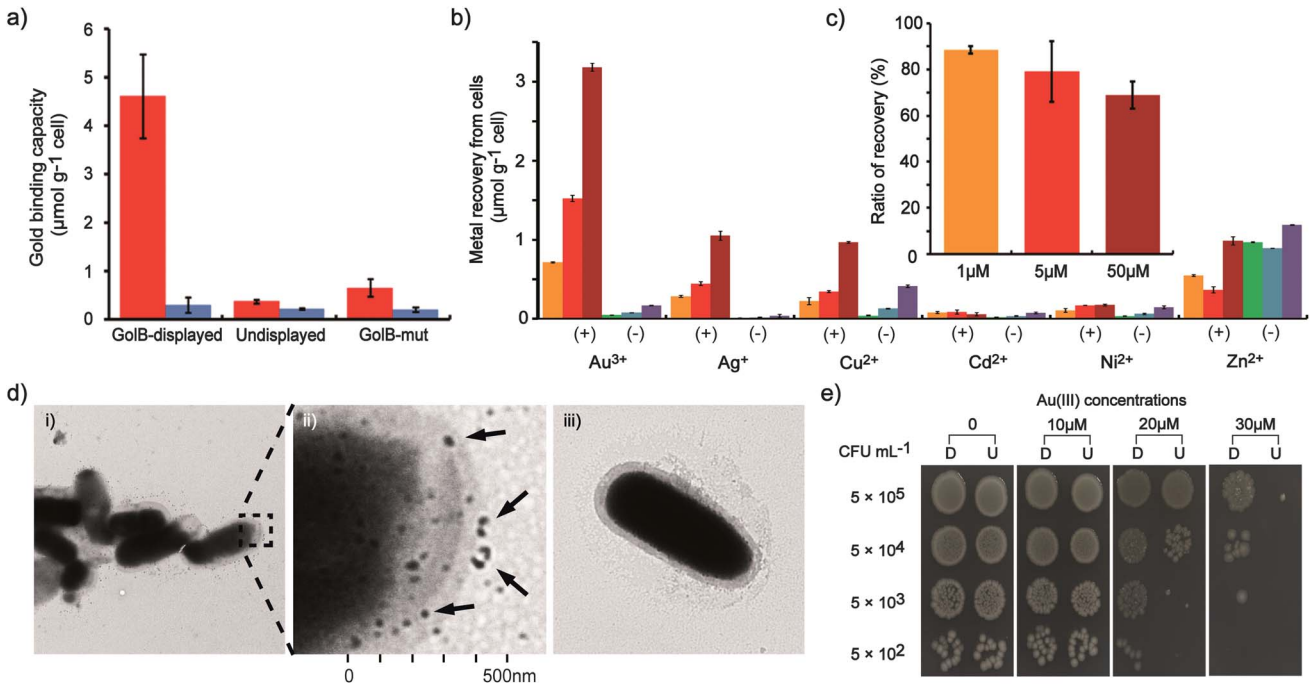

Fig. 5 a) Adsorption of gold ions by *E. coli* surface-displayed GolB protein. The *E. coli* bacteria (DH10B strain) containing the OmpA–GolB plasmid with (red columns) or without (blue columns) induction were grown in LB medium containing 50 μM $HAuCl_4$ (Au^{3+}). The amount of gold ions was measured by ICP-AES and the mean values of three independently performed experiments were reported. Error bars correspond to the standard deviations. b) ICP-AES analysis of metal ions selective adsorption and recovery by *E. coli* surface-displayed GolB protein. The *E. coli* bacteria containing the OmpA–GolB plasmid with (+) and without (−) induction were grown in LB medium containing gradient concentrations (1 μM yellow/green, 5 μM orange/blue and 50 μM red/purple) of metal ions mixture. c) The ratio of recovery gold ions from GolB protein surface-displayed *E. coli* in LB medium containing gradient concentrations of metal ions mixture. d) Deposition of Au at GolB protein surface-displayed *E. coli* cell membranes by TEM: i) the *E. coli* bacteria (DH10B strain) containing the OmpA–GolB plasmid with 0.002% arabinose induction was grown at 37 °C overnight in LB medium containing 50μM $HAuCl_4$ (Au^{3+}). The *E. coli* bacteria were directly inspected by TEM without fixation or staining; ii) a partial enlarged close-up of i), the electrondense gold particles are indicated with arrows; iii) the induced *E. coli* cells with GolB undisplayed were also grown at 37 °C overnight in LB medium containing 50μM $HAuCl_4$ (Au^{3+}) and inspected as control. e) A plate sensitive assay measuring the gold tolerance of *E. coli* cells with GolB displayed (D) and undisplayed (U) on the bacterial surface. All plates were incubated at 37 °C for 18 h before being read.

surface of the negative control cells, electron-dense gold particles with an average size of 20 × 50 nm were observed on the cells displaying GolB protein upon the treatment of 50 mM Au^{3+} ions overnight. Interestingly, we found that these GolB-displayed *E. coli* bacteria also exhibited an enhanced tolerance to gold ions. The resistance of *E. coli* bacteria to gold ions was examined by using the plate sensitivity assay (Fig. 5e). *E. coli* bacteria expressing the surface-displayed GolB protein were able to grow on LB agar plates containing 0–30 μM Au(III) ions respectively. By contrast, *E. coli* cells without the surface-displayed GolB protein were not able to survive in the presence of 30 μM Au(III) ions. This result suggested that the surface-displayed GolB could adsorb gold ions from the culture and thus protected bacteria cells from gold-induced toxicity. Indeed, the GolB-displayed bacteria collected from the gold-containing LB agar plates showed an enriched level of gold ions as confirmed by ICP-AES (Fig. S8†).

Conclusions

In summary, we have, for the first time, developed a whole-cell *E. coli* based gold-sensing system employing gold specific regulon to control the expression of RFP in the presence of gold. The high selectivity and sensitivity towards gold from these engineered *E. coli* cells provided us a new way to design novel metal biosensors. Compared to chemical and protein methods, cells are generally robust and can be reused and regenerated with ease, this method should find broad applications. Furthermore, we have developed a bacteria surface display system for enrichment and recovery of gold ions *via* a highly selective gold(I) binding protein-GolB. The specificity and affinity of this putative gold(I)-binding protein GolB was first verified and subsequently displayed on *E. coli* cell surface *via* fusion with a bacterial outer membrane protein. *E. coli* bacteria cells carrying the surface-displayed GolB were able to effectively adsorb gold ions from the environment and the enriched gold ions can be readily and selectively recovered. The enhanced gold tolerance of *E. coli* bacteria with GolB displayed is particularly useful in media of high gold ion concentration. Besides chemical treatment, the engineering of small size metal-binding protein on the cell surface could provide an alternative method for effectively recycling specific metal ions from waste water.

Acknowledgements

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