This journal is © The Royal Society of Chemistry 2012

Electronic Supporting Information (ESI)

Immobilization of Engineered Enzyme on Gold-Carbon Nanotubes

Gordon Kuen-Chan Lee, Sui-Yi Kwok, Chih Hao Yu, Kin Tam, Hiu-Chi Chong, Yun-Chung Leung, Shik Chi Edman Tsang*

[a] *Department of Chemistry, University of oxford, Oxford, OX1 3QR, UK; E-mail: Edman.Tsang@chem.ox.ac.uk*
[b] *Department of Applied Biology and Chemical Technology, Lo Ka Chung Center for Natural Anti-Cancer Drug Development, and State Key Laboratory for Chirosciences, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China*

1. Experimental Section

E. coli strain BL21-DE3 harboring the expression plasmid was grown overnight at 37ºC in LB culture medium containing 80 μgmL^{-1} ampicillin. The inoculum was diluted 1:25 and grown to OD_{600} ~ 0.8 in a shake flask. The cells were then induced with 0.4 mM IPTG for 4 hours. The bacterial cells were collected by centrifugation, resuspended in 50 mM Tris, 0.1 M NaCl, 10 mM $MnCl_2$, pH 7.4, and disrupted by high pressure homogenization. The 6xHis-tagged *Bacillus caldovelox* arginase was purified by a nickel chelating FF sepharose (GE Healthcare) column (5.0 cm x 9 cm; bed volume of 176 mL) equilibrated with Buffer A (0.02 M sodium phosphate, 0.5 M NaCl, pH 7.4). The 6xHis-tagged arginase was eluted with a gradient of 0.15 to 0.25 M imidazole. The flow rate was 20 $mLmin^{-1}$. The elution fractions containing purified arginase were collected. The yield of purified arginase was about 200 mgL^{-1} cell culture. Enzymatic reaction catalyzed by the thermostable arginase was evaluated by the rate of forming products (ornithine + urea) from L-arginine. One unit of arginase is defined as the amount of enzyme that converts one micromole of L-arginine to urea per minute at pH 7.4, 37°C. For the chemistry of the bioassay, the enzyme activity of arginase was assayed based on colorimetric determination of the enzymatic reaction product, urea. Determination of urea was based on the Fearon's reaction. Carbamido compounds, with formular R-NH-CO-NH-R, such as urea and citrulline would react with diacetyl monoxime, when heating in acid solution, to form a color complex, diazine. The absorbance was measured at a wavelength of 540 nm. For the bioassay method, the activity of arginase was assayed by adding 300 µL enzyme solution to 150 µL of 20 mM sodium phosphate buffer at pH 7.4. L-arginine (300 µL, 720 mM) at pH 7.4 was added to the enzyme mixture. The reaction mixture was incubated at 37°C for exactly 5 minutes and enzymatic reaction was quenched by addition of 250 µL trichloroacetic acid. The reaction mixture (100 µL) was added to 3 mL of the chromogenic agents and boiled for 15 minutes and then allowed to cool to room temperature and absorbance was measured at 540 nm. 5 nm citrate stabilized gold nanoparticles were purchased from Aldrich.

2. Analytical Techniques

2.1 Transmission Electron Microscopy (TEM)

TEM images were obtained using a JEOL 2010 microscope with a high resolution pole piece. The samples were prepared by placing a drop of nanoparticle ethanol suspension onto a carbon-coated copper grid, and allowing the solvent to evaporate.

2.2 Circular Dichroism (CD) spectroscopy

The CD measurements reflecting conformations of native and immobilized Cyc-arginase were carried out using the Jasco J-810 spectropolarimeter. Each of three basic secondary structures of a polypeptide chain (helix, sheet, and coil) gives a characteristic CD spectrum. Thus, the CD spectrum can give the change of secondary structural features such as the proportion of α helical regions or β-sheet regimes or random coil regions upon the enzyme immobilization. CD signal is generally strong for α- helix and weak for β-sheets. In the unfolded state CD is nearly zero in the region 210-220 nm, which corresponds to the absorption band of peptide bond. Hence protein folding/unfolding can be conveniently followed by the CD spectroscopy.

2.3 Zeta Potential (ZP) measurement

Zeta potential indicates the surface charge of specie at different pH and represents the value of electrostatic potential at the interface between stern and diffuse layers. The zeta potential ζ (mV) which expresses the stability of a specie-liquid mixture is calculated from the electrophoretic mobility v_ε

$$\zeta = \frac{4\pi\eta v_\varepsilon}{D}$$

Where η is the liquid viscosity and D is the dimensionless dielectric constant of the liquid.

In this work, the zeta potentials of Cys-arginase, Cys-arginase-AuNP with and without attachment to PBPL/SWCNT were measured in order to determine the surface properties, respectively. The sample were all prepared in physiological pH solution at pH = 7.4. The zeta potentials were measured using a Malvern Zeta Master. The measurement of the electrophoretic mobility of the colloidal specie was used by a helium-neon laser light source in the Malvern instrument. Two coherent beams of red light crossed at the stationary level in the capillary cell with the sample suspension. The specie fluctuated in the applied field. The frequency of the fluctuations of the light scattered was related to the speed passing through the specie. The scattered light passing through the photomultiplier was then analysed by a digital correlator. The frequency component was extracted from the specie mobility.

2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to compare the relative concentrations of Cys-arginase after separations from immobilised forms based on a standardised procedure, which could reflect the interaction(s) between the enzyme and the carriers. The solution samples were mixed with concentrated loading buffer and incubated at 95°C for 5 minutes to ensure denaturation of the protein. The samples were then loaded on a commercial 10% NuPAGE Bis-Tris gel (Invitrogen) and separated by electrophoresis at 200 volts for 35 minutes along with protein markers in MES buffer. Coomassie blue was used for the protein staining.

2.5 Atomic Force Microscopy (AFM)

A Digital Instruments Multimode SPM atomic force microscope (AFM) was used with a Nanoscope IIIa controller, operating in tapping mode with an 'E' scanner, having a lateral range of 12 mm and a vertical range of 3.5 mm. Silicon probes (Nascatec GmbH model NST-NCHFR), with resonant frequencies of approximately 320 kHz were used. X, Y, and Z calibration of the AFM was accomplished by scanning a 10 µm pitch 200 nm 3D reference from Digital instruments. The SWCNT dispersions were dropped onto freshly cleaved mica surfaces using a Laurell Technologies WS-400 spin-coater (3000 rpm).

3. *Chemical Sources, Synthesis and Testing*

3.1 Gold Nanoparticle

A commercial gold colloid (G1402 from Aldrich) containing 3.0-5.5 nm particle (monodisperse) with the average particle size of 5nm was used. The particles were synthesised and stabilised with citrate/tannic acid in aqueous solution, which was confirmed to carry negatively charge by our Zeta potential measurement at the pH= 7.4. As seen from a typical enlarged TEM image, most of the particles were in truncated cubo-octahedral shape (S Figure 1a).

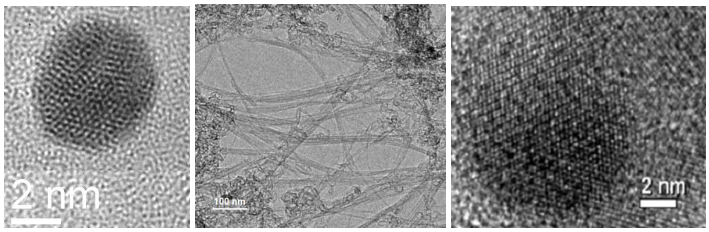
3.2 SWCNT and surface modification

Raw Chemical Vapour Deposition (CVD) SWCNT was supplied by Thomas Swan & Co Ltd. SWNT was purified using steam treatment [23] at 900 °C for 4h to remove amorphous carbon and graphitic particles. Then, the samples were refluxed in 6M HCl to wash away the metal particles, and collected by filtration using 0.2µm polycarbonate membrane, rinsed with distilled water, and dried under vacuum at 60 °C. A typical TEM image is given in S Figure 1b to show the morphology of the SWCNT. The 4-

(Pyren-1-yl) butanoylated polylysine (PBPL) conjugated on SWCNT was synthesised in aqueous medium according to the literature [23].

3.3 Magnetic iron oxide nanoparticle and FePt nanoparticle

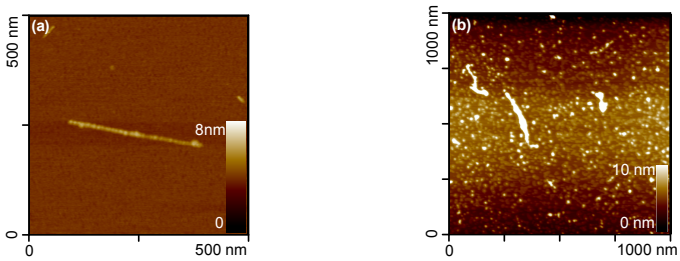
The average size of 9.4 nm iron oxide nanoparticle was prepared from iron (II) and iron (III) salts precursors, followed by co-precipitation using ammonium solution [22]. The sample was washed extensively using ultracentrifuge at 50,000 rpm to remove impurities. S Figure 1 c shows the typical enlarged TEM images of the iron oxide. Similarly, 4 nm iron platinum nanoparticle was first prepared according to Yu et al. (*Advanced Materials*, **2006**, 18, 2312- 2314) and then transferred to aqueous phase by adding 5mL of 20% wt tetramethylammonium hydroxide (TMAOH) to 100 mg of the FePt nanoparticle with agitation for 30 min, followed by extensive washing to remove impurities.

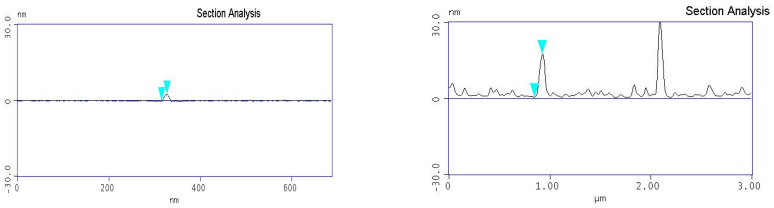


S Figure 1: Enlarged TEM images of typical 5nm citrate-stabilized cubo-octahedral gold nanoparticle (a); purified SWCNT after steam pre-treatment (b); a typical 9.4nm iron oxide nanoparticle (c).

3.4 Topological studies of PBPL/SWCNT with and without Arginase-AuNP by AFM

The topologies of PBPL/SWCNT conjugate with and without arginase-gold nanoparticles were studied by atomic force microscopy (AFM) operating in tapping mode. S Figure 2(a) shows the AFM image of the PBPL/SWCNT conjugate. AFM height analysis revealed an average step height of 2.5 nm of PBPL/SWCNT conjugate [23]. S Figure 2(b) shows the AFM image of the arginase-AuNP-PBPL/SWCNT onto a freshly cleaved mica surface. AFM height analysis revealed an average step height of 16-30 nm suggesting a 13-25 nm gain in height, which is consistent in scale with AuNP (c.a. 3-5 nm) carrying a deformable globular protein molecules (ca. 10-20 nm nodes on top of a tubular structure with occasionally aggregates. This topological feature is very similar to those AFM images of globular blood proteins on SWCNT (C. Ge et al., PNAS, 2011, 108, 16968-16973).





S Figure 2. Atomic force microscopy images of (a) PBPL/SWCNT conjugate on freshly cleaved mica; (b) global shape arginase-AuNP- PBPL/SWCNT conjugate on freshly cleaved mica.

3.5 Site-directed mutagenesis, protein expression and purification

The gene for the 6xHis-tagged *Bacillus caldovelox* arginase (*BcARG*) was cut from the pUC57/*BcARG* plasmid using NdeI and BamHI restriction enzymes. The insert fragment was subcloned into the pET3a expression plasmid vector (Strategene) to form the plasmid pET3a/ *BcARG*, which was used as a template for site-directed mutagenesis according to the QuikChange® site-directed mutagenesis kit (Strategene). The codon for Ser161 was mutated to the codon for Cys161 using the following mutagenic primers:

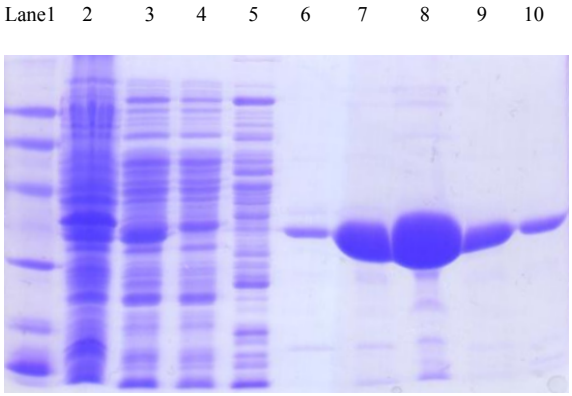
Primer 1: 5’ CAAATCGGCGGATACTGCCCCAAAATCAAGC 3’

Primer 2: 5’ GCTTGATTTTGGGGCAGTATCCGCCGATTTG 3’

The plasmid containing the mutated arginase gene was transformed into competent *E. coli* Top 10 cells. The sequence of the mutated gene was confirmed by DNA sequencing. The plasmid was then transformed into *E. coli* BL21-DE3 cells for protein expression. The identity of the S161C mutant was confirmed by electrospray ionization mass spectrometry (ESI-MS).

E. coli BL21-DE3 harboring the expression plasmid was grown overnight at 37ºC in LB medium containing 80 μgmL^{-1} ampicillin. The inoculum was diluted 1:25 and grown to OD_{600} ~ 0.8 in a shake-flask. The cells were then induced with 0.4 mM IPTG for 4 hours. The bacterial cells were collected by centrifugation, re-suspended in 50 mM Tris, 0.1 M NaCl, 10 mM $MnCl_2$, pH 7.4, and disrupted by high pressure homogenization.

The 6xHis-tagged *Bacillus caldovelox* arginase was purified by a chelating FF sepharose (GE Healthcare) column (5.0 cm x 9 cm; bed volume of 176 mL) equilibrated with Buffer A (0.02 M sodium phosphate, 0.5 M NaCl, pH 7.4). The 6xHis-tagged arginase was eluted with a gradient of 0.15 to 0.25 M imidazole. The fractions containing purified arginase were collected. The yield of purified arginase was about 280 mgL^{-1} cell culture. Specific activities of the wild-type 6xHis-tagged *Bacillus caldovelox* arginase and the S161C mutant were found to be ~336 units mg^{-1}, suggesting the change of Ser to Cys at the 161 position did not alter the enzymatic activity.

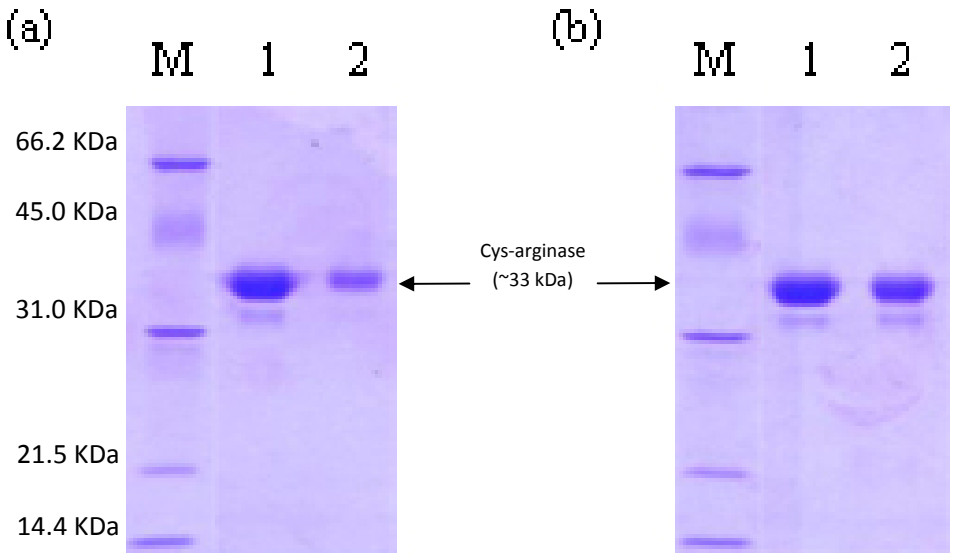


SDS-PAGE analysis of the Cys-arginase purification and expression. A 15% acrylamide gel from Laemmli buffer system was stained with Coomassie blue R-250. Lanes: (1) low range molecular weight markers 97, 66, 45, 31, 21, 14 kDa; (2) cell-free supernatant; (3) filtered cell-free supernatant; (4) flow-through from the nickel affinity column; (5) wash from the column; (6 – 10) Fractions collected from the column during elution with elution buffer (0.02 M sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4).

3.6 Immobilization of Cys-arginase

The immobilization of Cys-arginase onto the citrate stabilized gold nanoparticle (AuNP) was conducted as follows: A 600 µL of AuNP in aqueous solution was incubated with 600 µL of aforementioned engineered *Bacillus caldovelox* arginase (3 $mgmL^{-1}$, in Tris-Cl buffer, pH 7.3) for 20 min at room temperature (15-25°C). The immobilization of Cys-arginase onto the magnetic nanocarriers was conducted as follows: A 25 µL of magnetic nanoparticle (12.2$mgmL^{-1}$ iron oxide or 28.4$mgmL^{-1}$ iron-platinum) was mixed with 75 µL of aforementioned engineered *Bacillus caldovelox* arginase (0.56 $mgmL^{-1}$, in Tris-Cl buffer, pH 7.3) for 20 min at room temperature (15-25°C). After the immobilization, the mixture was gently centrifuged to give a clear supernatant and solid residue. Similarly, the purified and re-dispersed arginase-AuNP (sonicated) was incubated with SWCNT/PBPL conjugate under the same manner. The contents of Cys-arginase in both supernatant and residue for the immobilized enzyme were assayed, as described later.

In parallel to the immobilization study of Cys-arginase to AuNP and PBPL/SWCNT we have also carried an investigation on the immobilization of the engineered-enzyme to the magnetic nanoparticles (iron oxide or iron-platinum). The resultant complexes were subjected to SDS-PAGE analysis. S Figure 3(a) indicates that most of the Cys-arginase molecules are complexed with iron oxide nanoparticles (but with reduced activity, see S Table 1). Figure 3(b) indicates that a substantial amount of the arginase did not form complex with iron platinum nanoparticles. We attributed to the the fact that thiol group of arginase can form a strong covalent linkage with exposed iron species in iron oxide but not with the iron platinum, which is known to be passivated by TMAOH groups. S Table 1 shows the normalized activities of the arginase-oxide nanoparticle, and arginase-iron platinum nanoparticle per mg of the pure enzyme. As compared to free Cys-arginase the arginase-iron oxide nanoparticle and arginase-iron platinum nanoparticle showed a significant drop in activity. This suggests that iron oxide and iron platinum nanoparticles prepared by the literature methods were not suitable to be used as the enzyme carrier.



S Figure 3. (a) SDS-PAGE showing the formation of arginase-iron oxide nanoparticle. Lane M is the low-range protein marker; Lane 1 is Cys-arginase; Lane 2 is the remaining free arginase after the removal of arginase-iron oxide nanoparticle; (b) SDS-PAGE showing a substantial amount of arginase did not form complex with iron platinum. Lane M is the low-range protein marker; Lane 1 is Cys-arginase; Lane 2 is the remaining free arginase after the removal of arginase -iron platinum nanoparticle.

S Table 1. Weight normalized enzymatic activity of Cys-arginase, residue arginase-iron oxide nanoparticle and arginase-iron platinum nanoparticle per gram of pure enzyme

	Specific activity (Units/mg)
Cys-arginase	$3.4 \pm 0.2 \times 10^2$
Iron oxide nanoparticle	0
Arginase-iron oxide nanoparticle	$1.5 \pm 0.2 \times 10^2$
Iron platinum nanoparticle	0
Arginase-iron platinum nanoparticle	$0.7 \pm 0.3 \times 10^2$

3.7 Bioassay test for recombinant arginase

Enzymatic reaction catalyzed by arginase is shown below:

$$\text{Arginine} \xrightarrow{\textbf{arginase}} \text{Ornithine + Urea}$$

One unit of arginase is defined as the amount of enzyme that converts one micromole of L-arginine to urea per minute at pH 7.4, 37°C. For the chemistry of the bioassay, the enzyme activity of arginase was assayed based on colorimetric determination of the enzymatic reaction product, urea. Determination of urea was based on the Fearon's reaction. Carbamido compounds, with formular R-NH-CO-NH-R, such as urea and citrulline would react with diacetyl monoxime, when heating in acid solution, to form a color complex, diazine. The absorbance was measured at a wavelength of 540 nm. For the bioassay method, the activity of arginase was assayed by adding 300 µL enzyme solution to 150 µL of 20 mM sodium phosphate buffer at pH 7.4. L-arginine (300 µL, 720 mM) at pH 7.4 was added to the enzyme mixture. The assays for both supernatant (the unattached free enzyme) and solid residue after separation of the immobilized enzyme were carried out in a similar manner with the activity normalization in term of the unit activity per mg of pure enzyme. The reaction mixture was incubated at 37 ^{o}C for exactly 5 minutes and enzymatic reaction was quenched by addition of 250 µL trichloroacetic acid. The reaction mixture (100 µL) was added to 3 mL of the chromogenic agents and boiled for 15 minutes and then allowed to cool to room temperature and absorbance was measured at 540 nm.

3.8 Stability and clinical applications of immobilised arginase

This enzyme is thermostable and robust and can be produced from the thermophilic bacterium Bacillus caldovelox hence is clinically important. At this stage we do not have much information on the possible alteration in its stability upon immobilisation. However, after interaction with the NP and immobilization on the nanotubes, we have found that the activity remained virtually the same before and after storage at room temperature for more than a week. Hence, there is no evidence on the subtle change in their stability upon immobilisation.

This present paper has focused on the novel chemical method for this enzyme immobilisation and the future study on the arginine transformation in blood proteins matrix will be conducted. Since such study of enzyme-NP and immobilised conjugate for arginine transformation in blood (in the presence of blood proteins) in situ is important regarding the clinical applications. However, our previous in-vivo study has clearly demonstrated that pegylated arginase (formed by linking a PEG polymer to the Cys at the 161 position of this enzyme) is stable in mice. The injected pegylated arginase in the blood stream of mice can attenuate the blood arginine level to the undetectable level for a week. This indicates that the Cys 161 residue is a suitable location for conjugation or immobilization without seriously affecting the enzyme activity. It is expected that the enzyme-conjugate will display similar effects, which are yet to be confirmed. We anticipate that the oral route for administration is unlikely to work, as the immobilised enzyme may be absorbed by the GI tract but this conjugate is targeted for the intravenous route of administration at an efficacious dose (with a suitable formulation).

While this paper was under review two related papers on enzyme immobilization on carbon materials have been found:

Immobilization of trypsin on graphene oxide for microwave-assisted on-plate proteolysis combined with MALDI-MS analysis Guobin Xu , Xiaoyi Chen , Jianhua Hu , Pengyuan Yang , Dong Yang and Liming Wei Analyst, 2012, DOI: 10.1039/C2AN35093A http://xlink.rsc.org/?doi=10.1039/C2AN35093A

Multiple functionalization of single-walled carbon nanotubes by dip coating Michael Holzinger , Jessica Baur , Raoudha Haddad , Xu Wang and Serge Cosnier Chem. Commun., 2011,47, 2450-2452 http://xlink.rsc.org/?doi=10.1039/C0CC03928D

ACKNOWLEDGMENT

Apart from the acknowledgments in the main text we thank the Research Grants Council of Hong Kong (PolyU 5620/08M and PolyU 5617/09M) for supporting work in the YCL lab.