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COMMUNICATION

Photocatalytical reduction of disulphide bonds in peptides on Ag-loaded nano-TiO_2 for subsequent derivatization and determination†

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We reported an alternative strategy to reduce disulphide bonds in peptides with Ag-nanoparticle loaded nano-TiO_2 (Ag/TiO_2) under UV irradiation. The feasibility of this strategy was adequately demonstrated using the model peptides oxidized glutathione, vasopressin and insulin, which contain various disulphide bonds, as well as by its application to the determination of Cd-induced phytochelatins in *Phaeodactylum tricornutum*.

Disulphide bond formation is an important post-translational modification related to protein folding and conferring stability on the 3D structure of proteins.[1,2] In addition, redox mutual-conversion between sulphydryl and disulphide is significant to some vital biological functions (such as protection of proteins against irreversible oxidation) and an abnormal sulphydryl redox ratio related to many diseases (such as cancer,[3] alcoholic liver disease,[4] Alzheimer's disease,[5] and acquired immunodeficiency syndrome[6]). Information on the sulphydryl and disulphide bond existing in a biomolecule are crucial not only because of the above mentioned issues but also because the quick oxidation of sulphydryls into disulphide under conventional environmental concerns, in which oxygen is always present, leads to difficulty in their determination.

Current analytical methods for the determination of cysteine-containing biomolecules generally involve a prereduction procedure to convert disulphide into free sulphydryls before their chemical labelling or derivatization for subsequent determination. They are determined based on signals from a product derived from the reaction of sulphydryls with the labels or derivatization agents. Information of the free sulphydryl and disulphide bond in the biomolecules can be obtained before and after suitable prereduction and subsequent determination. The most commonly used reductants, including phosphines (*e.g.* tris (2-carboxyethyl) phosphine,[7,8] triphenylphosphine[9] and tri-n-butylphosphine (TBP)[10]), strong reductants (sodium or potassium borohydride[11–13] and dithionite[14]) and sulphydryl-containing reductants (*e.g.* dithiothreitol[15–17] and 2-mercaptoethanol[18]), possess a high degree of S-nucleophilicity to meet the reduction of disulphides. Although they are effective for various reduction and subsequent determination purposes, their applications sometimes suffer from cross-reactions with labelling agents and/or require necessary separation before determination.[19]

The unique redox property of nano-TiO_2 has attracted much attention since it was used to split water and to realize various functional group transformations under UV illumination.[20,21] We have used the excited electrons (e^-) at the conduction band (CB) to reduce selenium(VI) and mercury(II) for further atomic fluorescence spectrometry while the oxidative holes (h^+) at the valence band (VB) are shown to be scavenged by electron donors.[22–24] On the other hand, noble metals such as silver, gold and platinum are reported to enhance the reducibility of CB e^- due to the enrichment effect of CB e^- on the metal surface and thus they reduce the possibility of e^-–h^+ recombination.[25] In this report, we synthesized Ag-loaded nanoTiO_2 (Ag/TiO_2) and coated it onto the surface of a glass fibre in order to demonstrate its feasibility for the reduction of disulphides in peptides under UV irradiation and for the subsequent UV-Vis determination of the peptides using a typical sulphydryl derivatization agent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Comparison of UV-H_2O, UV-HCOOH and UV-bare nanoTiO_2-HCOOH as well as UV-Ag/TiO_2-HCOOH systems was also performed. Moreover, the UV-Ag/TiO_2-HCOOH reduction system was applied to the determination of Cd-induced phytochelatins (PCs) in *Phaeodactylum tricornutum* (*P. tricornutum*). To the best of our knowledge, this is the first report of UV-Ag/TiO_2-HCOOH used for the reduction of disulphides in biological molecules following by subsequent UV-Vis determination.

To demonstrate this approach, a photocatalytical reduction unit was fabricated consisting of a curved-microchannel (1 mm I.D. × 300 mm in length) made of polydimethylsiloxane (PDMS), into which Ag/TiO_2 coated glass fibres (0.3 mm O.D.) (Fig. 1, average size of nanoTiO_2 was 10 nm; that of Ag nanoparticle loaded on nanoTiO_2 was 30 nm) were inserted (Scheme 1) (see details in the ESI†). Oxidized glutathione (GSSG), vasopressin (containing one disulphide) and insulin (three disulphides) were selected as model peptides. Typical DTNB assay (DTNB derivatives can be determined at 412 nm) was used for determination of the reduced and derivatized peptides to evaluate reduction efficiency (for detailed experiment operations, see the ESI†). The results obtained for GSSG indicated that the reduction efficiency (nearly 96%) of UV-Ag/TiO_2-HCOOH was much higher than those of UV-TiO_2-HCOOH (67%), UV-HCOOH (30%) and UV-H_2O (9%) in the corresponding optimized

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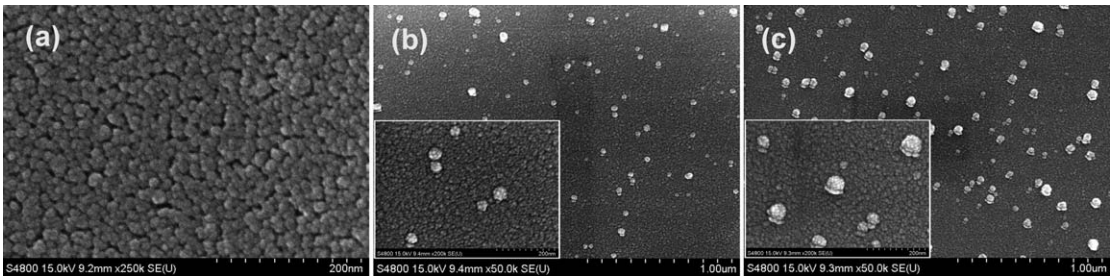
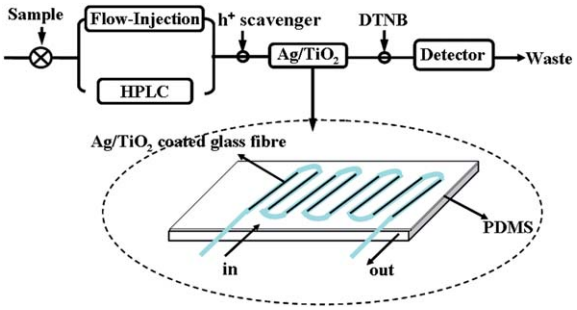


Fig. 1 SEM images of TiO_2 thin films. (a) TiO_2 films; (b) 0.25% Ag-loaded TiO_2 films; (c) 1.0% Ag-loaded TiO_2 films.



Scheme 1 Schematic diagram of the FIA/HPLC-(UV-Ag/TiO_2-HCOOH)-DTNB-UV-Vis. The on-line fluidic channel device consisted of two PDMS layers. Diameter and length of the channel was 1 and 300 mm. Thickness of the chip was around 4 mm. A master mould was made of polytetrafluoroethylene, and the curved protuberance was 1 mm in diameter and 300 mm in length. Sylgard 184 silicone oligomer and cross-linking agent (Sylgard 184 silicone elastomer curing agent) were thoroughly mixed at 10 : 1 (w/w) ratio and poured over the master mould. After curing at 65 °C for 1 h, the cured PDMS was carefully cut and peeled off from the master so as to get a PDMS layer with a curved microchannel (1 I.D. × 300 mm in length). TiO_2 or Ag/TiO_2 coated glass fibre (0.3 mm O.D.) was put into the channel; another freshly made PDMS membrane (~0.5 mm) was then bound onto it at 65 °C oven for 2 h. The final device was rinsed with 10 × the total volume (217 μL) of the device with ultrapure water using a syringe pump before use.

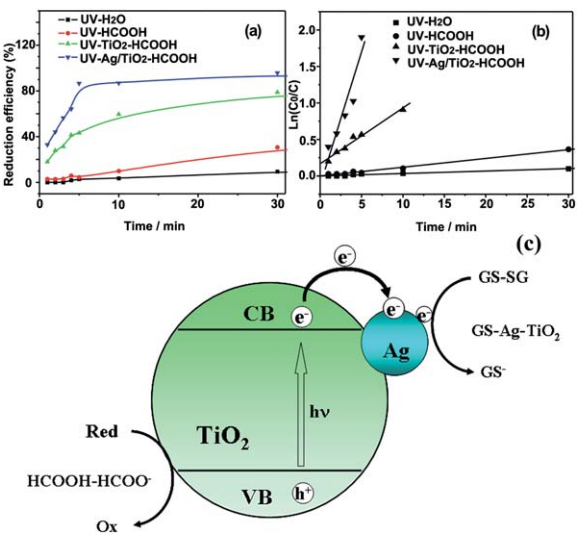


Fig. 2 The reduction efficiencies of GSSG (a), plots of $\ln(C_0/C)$ against t under various reduction systems (b) and possible reduction mechanism of GSSG on Ag/TiO_2 nanoparticles (c).

conditions (Fig. 2a and Fig. S1†). Considering that the reduction reaction is a pseudo-first- order reaction, the rate constant (k) can be determined from the slope of the plot of $\ln(C_0/C)$ against reaction time (t) according to $\ln(C_0/C) = kt + A$, where C_0 and C denote initial and remaining concentrations of the disulphide (Fig. 2b). The k values for GSSG were in the order UV-Ag/TiO_2-HCOOH (k = 0.37 min^{-1}) > UV-TiO_2-HCOOH (0.06) > UV-HCOOH (0.008) > UV-H_2O (0.004), implying that the reduction rate in UV-Ag/TiO_2-HCOOH was almost six times faster than that in UV-TiO_2-HCOOH. The improved reduction efficiency obtained under UV-Ag/TiO_2-HCOOH (Fig. 2c) was probably due not only to the transfer of CB e^- from TiO_2 to the Ag loaded nanoparticles,[26] thus reducing the probability of rapid e^-–h^+ recombination, resulting in the concentration of e^- on the Ag nanoparticles, but also the interaction of GSSG with Ag producing an enrichment effect towards GSSG.[27]

Under the flow injection mode, the reduction efficiencies of GSSG, vasopressin and insulin before and after UV-Ag/TiO_2- HCOOH reduction with 1,4,7,10-tetraazacyclododecane-1,4,7-trisacetic

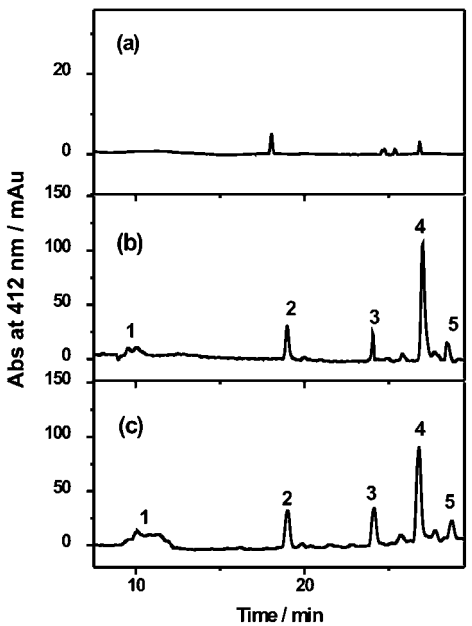


Fig. 3 Typical chromatograms without reduction (a), and with TBP (b) and UV-Ag/TiO_2-HCOOH (c). The identified peaks by HPLC-ESI-MS were GSH (1), PC_2 (2), PC_3 (3), PC_4 (4), PC_5 (5) (Fig. S5†). Mobile phase A, 0.02% TFA-H_2O; mobile phase B, 0.02% TFA-ACN; elution program: 0–25 min, B 2–25%; 25–30min, B 25%; flow rate, 0.15 mL min^{-1}; derivatization conditions: 1.8 mmol L^{-1} DTNB, flow rate 0.05 mL min^{-1}; detection wavelength, 412 nm.

Table 1 Levels of GSH and PCs in *P. tricornutum*[a]

	PCs[b]				
Reduction methods	GSH	PC_2	PC_3	PC_4	PC_5
TBP	30.1 ± 3.7	28.9 ± 3.0	20.2 ± 2.1	61.9 ± 15.6	7.2 ± 0.3
UV-Ag/TiO_2-HCOOH	32.5 ± 7.1	31.8 ± 6.4	19.7 ± 1.3	56.1 ± 11.2	7.4 ± 0.2

[a] Results from triplicate runs. [b] PCs, nmol g^{-1} FW; FW, fresh weight; the determination of the PCs with different number of Cys according to a molar concentration of Cys unit corresponding to the absorption value.

acid-10-maleimidoethylacetamide (MMA-DOTA) as a blocking agent (Fig. S2†) and/or DTNB as the derivating agent were confirmed by ESI-MS studies and DTNB UV-Vis determinations (Fig. S2, S3 and S4†). Detection limits (3σ) for GSSG, vasopressin and insulin reached 10.3, 89.0 and 17.8 μmol L^{-1}, respectively. The RSDs (%) for GSSG(0.1 mmol L^{-1}, $n = 11$), vasopressin (0.4 mmol L^{-1}, $n = 5$) and insulin (0.4 mmol L^{-1}, $n = 5$) were 3.9%, 4.7% and 3.1%, respectively. The linear ranges for GSSG, vasopressin and insulin were 0.05 to 0.4 mmol L^{-1} ($y = 1.0141x + 0.0924$; $R^2 = 0.9983$), 0.4 to 1.0 mmol L^{-1} ($y = 0.1176x + 0.0029$, $R^2 = 0.9960$) and 0.2 to 1.0 mmol L^{-1} ($y = 0.5868x + 0.0003$, $R^2 = 0.9950$), respectively.

Finally, we applied UV-Ag/TiO_2-HCOOH together with DTNB derivatization to the determination of Cd-induced PCs in *P. tricornutum*.[28,29] Typical chromatograms obtained are shown in Fig. 3. Compared with the traditional reduction agent TBP, similar results were obtained (Table 1), suggesting that the practical reduction efficiency of UV-Ag/TiO_2-HCOOH towards cysteine-enriched PCs is comparable to that of TBP.

In summary, UV-Ag/TiO_2-HCOOH offered an alternative for the reduction of disulphides in peptides, and was both more clean and more flexible to various subsequent labelling and determination systems not limited to DTNB assay without considering such things as cross-reactions with labelling agents or necessary separations of the chemical reduction agents before determination when the chemical reduction agents are applied. Further investigation of the applications of this photocatalytical reduction system to proteins in more complex biological samples are ongoing in our laboratory.

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