

Solid state differentiation of plasma thiols using a centrifugally activated mercaptobenzothiazole disulfide exchange indicator†

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Received (in Cambridge, UK) 22nd August 2006, Accepted 20th October 2006

First published as an Advance Article on the web 10th November 2006

DOI: 10.1039/b612092j

The solid state interaction of mono and macromolecular thiols at a disulfide heterocycle is shown to provide a versatile pathway for their speciation.

The concentration of anti-oxidant thiol (RSH) within plasma has been shown to be a potentially valuable semi-quantitative indicator through which the severity of various injuries or diseases can be assessed.^{1–6} While Ellmans Reagent (5,5'-dithio-(2-nitrobenzoic acid))⁷ has long been the mainstay of routine thiol analysis, it is unable to speciate between sulfhydryl albumin and the low, monomolecular species such as glutathione (GSH), cysteine (CSH) and homocysteine (HCSH) and, as a consequence, can only deliver a total plasma thiol (PSH) concentration. The latter is widely recognized as having clinical merit⁴ but there is considerable debate as to whether mono, macro or total thiol respond in equal measure to the onset of physiological stress.^{4,6,8–10} The ability to differentiate between the different components invariably requires more elaborate liquid chromatographic analysis and hence requires the referral of the sample to specialist laboratories with the time delays often negating the diagnostic advantage. The present communication presents a wholly new but integrated strategy for plasma thiol analysis that provides the clinician with ready access to a procedure that could distinguish between these groups. This could thereby offer the possibility of facilitating a better understanding of the PSH/RSH metabolomics.

The basic strategy is outlined in Fig. 1 and is highlighted by the passage of cysteine (I) through a centrifugal filter packed with a 2,2'-dithio(bis)benzothiazole (II) indicator. The latter is insoluble within aqueous solution and serves as a densely packed particulate filter. In principle, the disulfide should react with free sulfhydryl thiols through a mechanism analogous to that observed with Ellman's Reagent (ER) resulting in the formation of the mixed disulfide (III) and the release of the mercaptobenzothiazole anion (IV).

Spectroscopic investigation (UV-vis and ¹H NMR) of the filtrate revealed that the principal product in the filtrate was in fact the mixed disulfide (III) and not the free mercaptobenzothiazole (IV). It appears that the latter, like its disulfide parent, is insoluble within the buffer and is retained within the packing material. The addition of the amino acid functionality to the complementary thiazole component serves to aid solubilisation and, as such, is

preferentially released. Similar results were obtained for homocysteine and glutathione.

An assessment of the percentage conversion of the low molecular weight thiol into the mercaptobenzothiazole (MBT) conjugate was conducted. This employed a before and after spectroscopic methodology in which the filtrate was assayed with Ellmans reagent to pick up underivatised thiol. The conversion of the constituents (non albumin) being in order of $100 \pm 2\%$ (based on 20 μM cysteine). The product characterisation and experimental details UV spectroscopic profiles for additions of CSH (40 μM), GSH (40 μM) and bovine albumin (0.82 mg L^{-1}) are shown in Fig. 2. The principal absorption bands for CSH, GSH and HCSH moieties were observed at 312 nm and correspond predominantly to the MBT component of the conjugates. Significantly, the passage of albumin did not lead to any change in the absorption profile from that of the corresponding unfiltered control.

The molar absorptivities of monomolecular species (CSH, $\lambda_{\text{max}} = 312 \text{ nm}$, $\epsilon = 275 \text{ mol}^{-1} \text{ L cm}^{-1}$ and GSH, $\epsilon = 365 \text{ mol}^{-1} \text{ L cm}^{-1}$) are significantly less than that of ER ($\lambda_{\text{max}} = 412 \text{ nm}$, $\epsilon = 14150 \text{ mol}^{-1} \text{ L cm}^{-1}$) and can be attributed to the lack of the nitrothiolate chromophore. It can be inferred that the added complexity of the proposed procedure and poor sensitivity would fail to make this a realistic competitor to existing spectroscopic protocols. However, the prime advantage lies in the interaction of the thiol component with the particulate

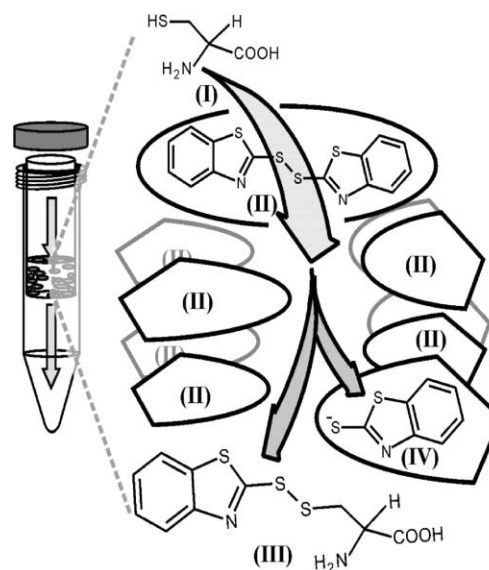


Fig. 1 Centrifugal Filter Device (CFD).

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† Electronic supplementary information (ESI) available: Product characterisation and experimental details. See DOI: 10.1039/b612092j

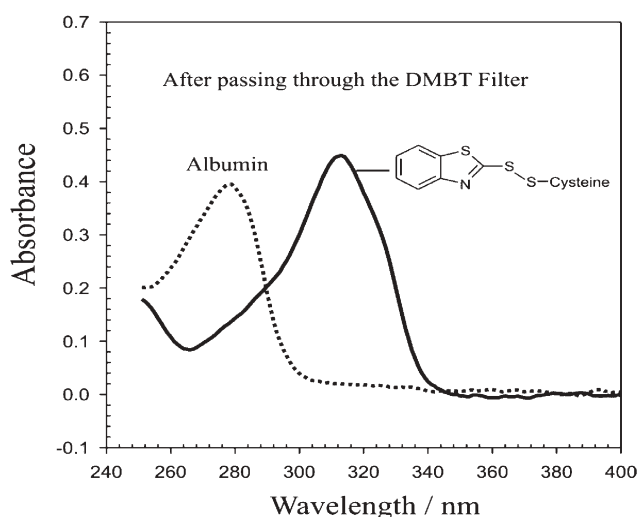


Fig. 2 Spectroscopic profiles of CFD processed MBT conjugates compared with that of an albumin sample.

packing. As 2,2'-dithio(bis)benzothiazole is essentially insoluble, the exchange is dependent upon the target reacting directly with the solid packing as it is forced through the filter. The key question in the present investigation revolved around whether sterically buried thiol functionalities, *ie* those within albumin, were capable of interacting with the solid. While such groups are accessible to freely diffusible derivatisation agents such as ER, it could be anticipated that the protein shield would prevent the intimate and specific contact required for reaction in the present case. The assay could therefore allow a specificity, as yet unattainable using conventional wet chemical techniques.

The absence of the MBT spectroscopic profile from the CFD processed albumin, Fig. 2, would suggest that the protein did not react. This preliminary result was followed up by passing a series of albumin solutions (0.02 to 0.82 mg L⁻¹) through the modified filter with the filtrates analysed for thiol through the addition of ER. In contrast to the previous experiments with the mono-molecular thiols where the conversion was essentially complete, a quantifiable response to ER was obtained indicating the presence of underderivatised albumin. The recovery of ER active albumin was typically 100 ± 1% (RSD = 1.5%, *N* = 3 based on 0.82 mg L⁻¹ AlbSH) and serves to confirm our supposition that direct and specific interaction at the solid interface is required for disulfide exchange to occur.

The clinical efficacy of the system for discriminating between albumin, low molecular weight thiols and indeed other physiological components was then assessed through examining the plasma thiol profiles of three individuals (2 M, 1F). Plasma samples (500 µL) were passed directly through the filter without any form of pretreatment. The filtrate was first analysed using direct UV spectroscopic analysis as indicated previously (Fig. 2). The absence of any appreciable absorbance within the plasma at 312 nm should therefore allow any increase at this wavelength, subsequent to CFD processing, to be attributed predominantly to the presence of MBT conjugates. The magnitude of the latter providing an immediate estimation of low molecular weight thiol concentration. However, there is an inherent limitation to this approach in that the spectroscopic properties of the conjugate will differ depending

Table 1 Direct UV-vis analysis of plasma RSH concentration

Direct CFD Analysis	Subject		
	M1	M2	F1
[RSH _(GSH)]/mM	0.147	0.164	0.186
[RSH _(CSH)]/mM	0.142	0.164	0.193

^a Each based on 3 replicate measurements.

on the nature of the amino acid substituent. This is evident from the molar absorptivity data presented previously for the GSH, CSH and HCSH variants. The data obtained is presented in Table 1 and was analysed using the regression data from both GSH and CSH calibration runs, the difference between the two estimates in practice have a negligible bearing on the measurement with the differing determinations of total low molecular weight thiols being well within the experimental error for either process.

A series of secondary experiments were then conducted using ER before and after the passage of plasma through the modified filter device. The results are detailed in Table 2. Ellman analysis of the plasma before filtration yields the total plasma thiol concentration (PSH) with the subsequent filtrate providing [albuminSH]—given that the low molecular weight species will have reacted to form the ER inactive MBT conjugates.

It can be assumed that the subtraction of the albuminSH from the PSH result should yield the low molecular weight thiol concentration. The estimates shown in Table 2 are in excellent agreement with the direct UV spectroscopic determination. This again confirms that, despite the minor variation in UV-vis sensitivity between different MBT-SR conjugates, the procedure is indeed clinically robust.

It could be anticipated that chemical manipulation of the disulfide to incorporate a chromophore (such as the nitro group common to ER) or an electro-responsive redox substituent would provide a significant enhancement to the basic assay performance. The use of quinoid indicators to facilitate potentiometric detection—essentially acting as an electrochemical Ellmans test—is also possible, with the filter packing providing the all important differentiation between macro and mono molecular thiols.^{10–12}

The system also provides an effective means through which albumin can be assessed as this is increasingly being viewed as an important diagnostic in its own right.^{8–10} The standard adsorptive dye methodologies such as bromocresol green (BCG) assays that are currently used can be subject to interference^{13–16} and hence the approach advocated here could allow a more robust route to analysis. The clinical viability of the latter was assessed and the results obtained from the centrifugal system compared with the

Table 2 CFD-ER UV-vis analysis of plasma RSH concentration

CFD-ER Analysis	Subject		
	M1	M2	F1
(A) ER Before CFD			
[Total PSH]/mM	0.530	0.778	0.650
(B) ER After CFD			
[AlbSH]/mM	0.391	0.615	0.457
[RSH _(A-B)]/mM	0.139	0.163	0.193

^a Each based on 3 replicate measurements.

Table 3 Plasma albumin concentrations assayed using the solid state filter (CFD) compared with the bromocresol green (BCG) dye adsorption method

Plasma Albumin Determination	Subject		
	M1	M2	F1
UV/Vis-CFD g L ⁻¹	33	45	44
BCG Assay g L ⁻¹	33	42	39

^a Each based on 3 replicate measurements.

BCG test. The preliminary results are detailed in Table 3 and show a good correlation with the standard assay.

The system is clearly a versatile assay that can be used to effect the speedy, accessible, and accurate speciation of physiologically significant thiol groupings. It can thus aid the differentiation between mono and macromolecular species and provides distinct routes through which both can be easily quantified. The system is readily attainable and provides a simple route through which a key clinical need could be addressed. The solid state differentiation capabilities presented by the disulfide could also be exploited in a variety of other formats and is not limited solely to the centrifugal separation exploited here. It could serve as an selective coating in sensors or a pre or post column derivatising step in LC applications. It is clear however that the protocol presented thus far provides a novel, robust and generic foundation from which this further work can be pursued.

The authors thank the EPSRC for financial support and South West Surrey LREC for granting permission to conduct the clinical trial.

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