



# A simple method for the quantitative analysis of resin bound thiol groups

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**Abstract**—Non-aqueous solutions of Ellman's reagent [5,5'-dithio(2-nitrobenzoic acid), DTNB] can be used to quantify thiols supported on macroporous polystyrene and TentaGel resins. Organic solutions of Ellman's reagent may also be used as a qualitative tests for thiols on a wider range of solid supports. © 2001 Elsevier Science Ltd. All rights reserved.

## 1. Introduction

Whilst solid supported organic synthesis produces significant benefits in the parallel synthesis of arrays of compounds the development of the synthetic route is not without its problems notably the analysis of 'on bead' compounds. One important factor in this analysis is the degree of loading and whilst this can be determined by microanalysis<sup>1</sup> or spectroscopically<sup>2</sup> these are either slow or require expensive instrumentation. Consequently easy, inexpensive laboratory based alternatives are required. One simple protocol is to cleave a sample from resin, evaporate to dryness and record the mass of material liberated. This is relatively slow and has limitations in precision. Although there are a number of simple bead staining tests, akin to TLC in solution phase synthesis, that provide qualitative analysis of solid-phase reactions, relatively few of these have been adapted to provide simple low cost methods for the quantification of on-bead substrates.<sup>3–7</sup>

Arising from the well developed art of solid-phase peptide synthesis there has been considerable effort to develop simple laboratory methods for the analysis of amino terminated substrates with Fmoc analysis being predominant.<sup>8</sup> However, the analysis of other functionality remains less well established. Aldehydes and ketones may be assayed by conversion to the dansyl hydrazone,<sup>9</sup> whilst similar tests for alcohols, phenols and carboxylic acids have been reported.<sup>10,11</sup> Prompted

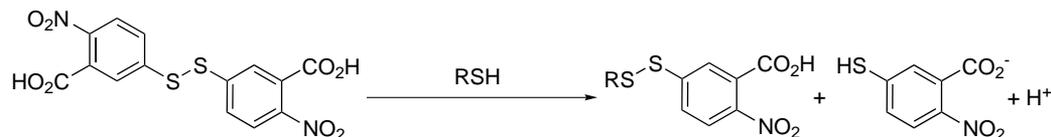
by the need for a readily accessible technique for thiol analysis,<sup>12</sup> we have undertaken a study of this area and now wish to report our endeavors to establish a simple, reliable and rapid test to quantify loading of resin bound free thiol groups.

## 2. Results and discussion

In connection with a project developing a range of functionalised solid supports for organic synthesis, we wished to develop a technique for the quantification of thiols, which would be as facile as the standard Fmoc procedure. Initial attempts to use Fmoc analysis whilst successful in a qualitative sense never gave the confidence to be used in a quantitative fashion and also suffered the drawback of having to prepare an appropriate Fmoc derivative. Consequently we sought other methods and chose to examine Ellman's reagent which is widely used in both qualitative and quantitative assays of cysteinyl thiols in proteins.<sup>13</sup> In these analyses the standard procedure is to use a dilute solution of Ellman's reagent in basic aqueous buffer (e.g. pH 8). The reaction of the disulfide reagent with thiols produces an anion which has a strong visible absorbance maximum at 412 nm ( $\epsilon=13600 \text{ M}^{-1} \text{ cm}^{-1}$  aqueous), Scheme 1. The intense yellow color forms the basis of a qualitative test whilst the measurement of absorbance values allows the concentration of thiols to be quantified (Beer–Lambert law).

To investigate the possibility of adapting Ellman's reagent to solid-supported thiols, we immobilized commercially available *N*-Fmoc-*S*(Trt)-cysteine onto a

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**Scheme 1.** Ellman's reagent.

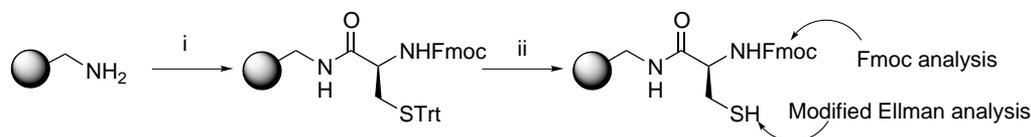
number of different resin supported amines via amide bond formation under standard PyBOP/DIPEA coupling conditions, Scheme 2. The *N*-Fmoc functional group allows a standard determination of cysteinyl loading to be obtained at all stages of the process.

Reflecting our initial project goals, and the fact that highly-crosslinked polystyrene has wide-solvent compatibility due to a permanent porous structure, allowing the use of a range of aqueous and non-aqueous solvent systems, we carried out our exploratory studies using Argopore aminomethyl resin. After removal of the trityl protecting group using TFA/Et<sub>3</sub>SiH, the reaction with Ellman's reagent was initially examined using the standard peptide analysis conditions involving aqueous solutions of the reagent.<sup>14</sup> However this failed to give reproducible results and consequently we turned to consider alternative more 'organic' solvent systems. After extensive screening, we found that the reaction is facilitated by highly polar protic solvents and that basic conditions are a necessity. Ultimately, for these macroporous resins, a solution of Ellman's reagent in methanol,<sup>15</sup> basified with DIPEA, gave excellent results.<sup>16</sup> Comparison of this new methanolic system with the aqueous buffered system in the solution-phase reactions of *N*-Ac-Cys-OMe gave identical results to that determined by Fmoc analysis within the limits of experimental error. Subsequently, in order to verify that this simple modification would provide an accurate method for a wide range of loadings, we generated a range of substrate loadings using sub-stoichiometric quantities of *N*-Fmoc-S-(Trt)-cysteine. Residual amino

functionalities were capped by acetylation with acetic anhydride/pyridine, before trityl deprotection with TFA/triethylsilane (95:5, 3 h, rt). In all cases spectrophotometric assays with Ellman's reagent in methanol gave values in good agreement with the Fmoc data, Table 1 Column A.

Subsequently, we sought to extend this methodology to other supports having poorer compatibility with highly polar protic solvents. TentaGel resins have a broad solvent compatibility and are known to tolerate aqueous-organic solvent systems, albeit with reaction rates affected by the choice of solvent. Neither of the single solvent systems (aqueous or methanolic) yielded loading values that were close to the Fmoc loadings. Consequently, mixtures of various organic co-solvents with either the methanolic or aqueous conditions were investigated in order to improve swelling and diffusion rates. These investigations produced a THF/methanol 1:1 solvent mixture, basified with DIPEA, as an effective system.

After reaction of the resin and Ellman's reagent in THF/MeOH the solution was subsequently diluted with methanol to give a predominately methanolic solution, in which  $\lambda_{\max}$  and  $\epsilon$  are virtually the same as in 100% methanol. Comparison of this binary system with methanol in the solution-phase reaction with *N*-Ac-Cys-OMe gave identical results. In a similar fashion to that described above a range of loadings of TentaGel aminomethyl bound *N*-Fmoc-S-(Trt)-cysteine was pre-



**Scheme 2.** Reagents: (i) *N*-Fmoc-S-Trt-(*L*)-Cys, PyBOP, DIPEA, DMF then Ac<sub>2</sub>O, Py; (ii) TFA, Et<sub>3</sub>SiH, DCM.

**Table 1.** Comparison of Fmoc and Ellman's reagent for analysis of resin bound thiols<sup>a</sup>

A Macroporous resins (Argopore <i>N</i> -Fmoc-cysteine)		B PEG-Graft gel resins (TentaGel <i>N</i> -Fmoc-cysteine)	
Fmoc loading <sup>b</sup> (mmol/g)	Thiol loading <sup>c</sup> (mmol/g)	Fmoc loading <sup>b</sup> (mmol/g)	Thiol loading <sup>c</sup> (mmol/g)
0.052	0.050	0.040	0.042
0.12	0.12	0.11	0.10
0.24	0.25	0.22	0.17
0.36	0.36	0.32	0.31
0.50	0.48	0.44	0.50

<sup>a</sup> All values are the average of repeat experiments.

<sup>b</sup> Determined by classical Fmoc analysis.

<sup>c</sup> Determined by modified Ellman's assay, see footnotes for experimental data.

pared and analyzed in THF/MeOH to give values in good agreement with the Fmoc data, Table 1 column B.<sup>17</sup> We then turned to examine simple lightly crosslinked polystyrene resins (1–2% DVB, 200–400 Mesh). However, although providing a very rapid method for the detection of free resin bound thiol using the THF/MeOH solvent combination (akin to a simple bead stain),<sup>18</sup> we were unable to find a solvent combination to provide satisfactory quantitative data for these highly hydrophobic resins.

In conclusion, Ellman's reagent can provide a simple, cheap and rapid laboratory based method for the quantitative analysis of free macroporous or PEG graft resin bound thiols. Further enhancements to the process can be achieved through a secondary treatment with excess dithiothreitol. This cleaves the disulfide bond and releases a second equivalent of thionitrobenzoate allowing a duplicate value to be obtained from the same resin sample.<sup>19</sup> Although providing a convenient qualitative test for thiols linked to lightly crosslinked polystyrene, attempts to generate quantitative analyses are not reliable and are the subject of current efforts. Results of these studies will be reported in due course.

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14. Ellman's reagent ( $2.5 \times 10^{-2}$  M, in pH 8.0 aqueous phosphate buffer), 200  $\mu$ l, 5  $\mu$ mol and *N*-Ac-Cys-OMe ( $4.4 \times 10^{-3}$  M, in water), 50  $\mu$ l, 0.222  $\mu$ mol were made up to 5.0 ml with water. A412=0.617.
15. Ellman's reagent ( $2.5 \times 10^{-2}$  M, in methanol), 200  $\mu$ l, 5  $\mu$ mol, *N*-Ac-Cys-OMe ( $4.4 \times 10^{-3}$  M, in water), 50  $\mu$ l, 0.222  $\mu$ mol and 50  $\mu$ l DIPEA were made up to 5.0 ml with methanol, A412=0.613.
16. 5–10 mg of resin (<5 mol) is weighed accurately into a 5 ml volumetric flask. 2 ml of a solution of Ellman's reagent ( $2.5 \times 10^{-3}$  M, in methanol), 5  $\mu$ mol, is added followed by 5  $\mu$ l DIPEA. The flask is shaken on an orbital shaker for 30 min, and diluted to 5.0 ml with methanol. The solution is subsequently diluted to give a concentration of the analyte between  $5 \times 10^{-5}$  and  $5 \times 10^{-3}$  M and the absorbance at 412 nm measured against a methanol reference. Solutions of Ellman's reagent in methanol were found to have long shelf-lives, but the addition of DIPEA to these standard solutions is not recommended, as this results in the slow development of a yellow-coloration which interferes with the assay.
17. 5–10 mg of resin (<5 mol) is weighed accurately into a 5 ml volumetric flask. 1 ml of THF is added and the resin allowed to swell for 10 min. 1 ml of a solution of Ellman's reagent ( $5.0 \times 10^{-3}$  M, in methanol), 5  $\mu$ mol, is added, followed by 5  $\mu$ l DIPEA. The solution is then treated in the same way as for methanol.
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