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Measurement of the FMOC loading of protected amine-functionalised polymer beads

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Abstract

This paper presents an HPLC based procedure that has been developed for the determination of the 9-fluorenylmethoxycarbonyl (FMOC) content of protected amine-functionalised polymer beads. FMOC reagents are frequently employed both in the protection of amines and in their determination. The procedure utilises the stoichiometric base cleavage of dibenzofulvene from the protected amine using 1,8-diazabicyclo undec-7-ene. This stearically hindered base prevents the adduct formation that occurs with alternative base systems and measurement performance is enhanced by the incorporation of anthracene into the system as a non-reactive internal standard. HPLC separation of the reaction products permits the measurement of FMOC in the presence of additional chromophores that might otherwise impede direct measurement by UV–vis spectrophotometry. The procedure has been evaluated for the measurement of the FMOC content of protected amine-modified polymer beads employed in combinatorial solid phase synthesis.

Keywords: FMOC protection; FMOC measurement; Amine beads; Combinatorial chemistry; Internal standard; Solid phase synthesis; HPLC; DBU

1. Introduction

Over recent years, functionalised polymer beads have become the foundation of many new methodologies associated with solid phase synthesis and the generation of large libraries of compounds using the novel experimental design approaches of combinatorial chemistry. Amine-functionalised polymer beads have been commonly employed in such approaches as they offer a versatile functional group for attachment to the bead surface. They have become particularly popular in the synthesis of anchored peptides [1]. The stabilisation of the amine group for storage, or to block its activity, can be carried out in a number of ways. Reagents based on the 9-fluorenylmethoxycarbonyl (FMOC) moiety have found widespread application and are used in the protection of amine [2,3] and hydroxyl groups [4] during the combinatorial solid phase synthesis of compounds such as polypeptides or in the storage of amine-functionalised polymers. FMOC is one of the relatively few protecting groups for amines that is not cleaved under acidic conditions, that remains intact under neutral conditions but that can be readily cleaved under mildly basic conditions [2,5]. FMOC and its cleavage products are strong chromophores and their spectrophotometric analysis provides the foundation of a number of techniques employed in the measurement of functional group loadings on solid supports [6].

Whilst base-catalysed cleavage of FMOC from the molecule of interest releases dibenzofulvene (DBF) into solution [2], the product is normally found in the resulting solution as a base adduct [6,7] (Fig. 1).

To overcome this problem, the highly hindered base 1,8-diazabicyclo [7] undec-7-ene (DBU) has been introduced [8,9]. The DBF that is formed by the cleavage cannot form an adduct with the base and remains free in dilute solution (Fig. 2). It does however, polymerise in concentrated solution [2].

Early analytical methods based on FMOC cleavage measured the absorbance of the piperidine-DBF adduct, but as free FMOC and DBF are both also strong chromophores [6]

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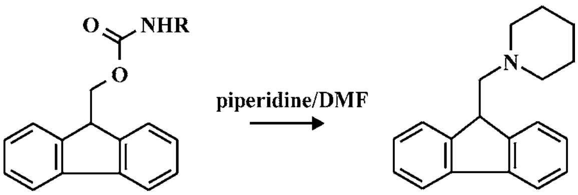

Fig. 1. Formation of the DBF-piperidine adduct.

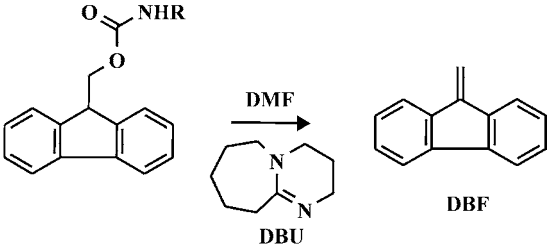

Fig. 2. DBU cleavage of the FMOC group.

it was necessary to ensure their absence to eliminate possible interferences. GC–MS analysis has shown the formation of 9-fluorenone from the piperidine/DMF cleavage of FMOC [7] and the presence of this compound has caused results obtained by this method to be highly variable. Similar investigations into the DBU/DMF based method have confirmed that DBF is the sole product of the deprotection, leading to significantly improved variability, and overall the method is more accurate than the corresponding piperidine-based procedure.

Whilst the reported GC-based analysis method exploits anthracene as the internal standard [7], the subsequently reported HPLC method [6] utilises nitrobenzoic acid. Anthracene, and its close structural neighbour naphthalene are relatively inert and ideally suited for use as reference materials and under conditions where the release of the FMOC group is to be measured in the presence of additional reagents. Nitrobenzoic acid is not an ideal choice of internal standard as it is reactive and may interfere with the FMOC deprotection step [6], or react with other components of the synthesis. In many laboratories routine HPLC is now more readily available than gas chromatography and an enhanced HPLC procedure was therefore required that did not employ the highly reactive nitrobenzoic acid internal standard. This paper reports on the use of essentially unreactive polynuclear aromatic hydrocarbons in the HPLC analysis of FMOC-modified amine polymer beads.

2. Experimental

2.1. Materials

2.1.1. Beads

FMOC-aminoethyl-photolinker-AM resin (NovaBiochem, Nottingham, UK; polystyrene-1% divinylbenzene, 100–200 mesh (150–75 μm). Manufacturer's quoted loading = 0.75 mmol g^{-1} (FMOC)).

2.1.2. Reagents

Acetonitrile (MeCN) and dimethylformamide (DMF) were HPLC Glass Distilled Grade (Rathburn, Walkerburn, UK). Anthracene (BDH, Poole, UK), 1,8-diazabicyclo [7] undec-7-ene (DBU) (Aldrich, Poole, UK), piperidine (Aldrich) and trifluoracetic acid (TFA) (Lancaster Synthesis, Morecambe, UK) were used as supplied.

2.1.3. Cleavage solutions

Two percent DBU in DMF (v/v) or 20% piperidine/DMF (v/v).

2.1.4. Internal standard solution

Anthracene (0.039 mM) dissolved in 2% DBU/DMF (v/v).

2.2. Apparatus

2.2.1. HPLC

Chromatography was carried out using a Beckman System Gold HPLC instrument consisting of a Module 126 programmable solvent delivery system and a Module 166 programmable ultraviolet detector set at 254 nm. Manual injection was employed through a Rheodyne 7125 injector fitted with a 200 μL loop. Data was processed by the Beckman System Gold Chromatography Software (version 8.1) and output on an Epson FX-800 printer.

The separations were carried out on a 5 μm Sphereclone ODS(2) column (Phenomenex, 150 mm × 4.60 mm i.d.) at room temperature and eluted at 1.0 mL min^{-1} with 70% (v/v) acetonitrile: water, containing 0.1% (v/v) trifluoroactic acid. The eluent was degassed with helium prior to use.

2.3. Analytical method development

A solution of 2% (w/v) DBU/DMF was applied to the sample for at least 5 min at room temperature to catalyse the liberation of the chromophore [6]. These previous studies have shown the DBF yield to be complete within 5 min of the reagent addition and to remain unchanged for at least 1 h. For this investigation a 1 h reaction time was found to be commensurate with a fixed reaction time and efficient utilization of the HPLC instrumentation.

2.4. Utilisation of an internal standard

The choice of internal standard can be influenced by the nature of the analysis. Particularly when samples are to be analysed that can be unstable, the internal standard that is chosen should be as inert as possible under the conditions of the analysis. For this work anthracene was chosen as the internal standard as is relatively inert and is not adsorbed by the polymer under the reaction conditions employed. Its retention time under the isocratic HPLC conditions employed in this work is approximately 8.5 min slightly longer than that of dibenzofulvene, the cleavage product of the FMOC group. The two compound peaks are close enough in the chromatogram to be comparable, whilst still remaining resolved from each other. The full chromatogram can be obtained within 10 min, permitting the analysis of six samples solutions per hour.

A known concentration of anthracene was incorporated into the cleavage solution by dissolving solid anthracene in 2% (w/v) DBU/DMF to first produce a concentration of 0.39 mM. This was subsequently diluted by one-tenth with 2% (w/v) DBU/DMF to give a final standard of 0.039 mM. This solution was used in the production of all calibration standards and samples.

2.5. Calibration

As any attempt to isolate the DBF monomer results in an insoluble polymeric species [2], calibration of the DBU cleavage product has to be carried out by the in situ production of DBF [6,7]. This is achieved by subjecting known concentrations of FMOC-protected compounds to the DBU cleavage. The reaction is stoichiometric, and chromatographic analysis is employed to ensure that the starting material is no longer present. The incorporation of an internal standard enhances accuracy and precision.

The DBF calibration was carried out by the in situ production of DBF from FMOC-valine. FMOC-valine reacts rapidly and quantitatively with DBU to produce DBF. A series of standard solutions of FMOC-valine in a reagent solution of 2% DBU/DMF (v/v) were prepared (3.89, 5.07, 23.22, 34.77, 34.89 μM) together with a blank sample. The DBF product resulting from the reaction of FMOC-valine with DBU was analysed by HPLC.

The standard solutions were prepared using the internal standard solution as the diluent. The solutions produced therefore also contained anthracene (0.039 mM). The calibration graph was prepared by ratioing the peak height absorbance of the DBF to the absorbance of the anthracene peak.

2.6. Bead analysis protocol

Beads were weighed to three decimal places (of a milligram) on a Mettler AE163 Microbalance. Ten measured aliquots of resin were placed into 5 mL volumetric flasks and the flasks were filled with 2% DBU/DMF containing 0.039 mM anthracene. These solutions were stored in the dark, at room temperature, for 1 h prior to analysis. After shaking, a 20 μL aliquot of sample solution was analysed by HPLC.

2.7. Comparison of bead analysis protocols

The FMOC loading of the beads was also determined using a conventional colorimetric method based around the DBF-piperidine adduct formed by the piperidine liberation of the FMOC moiety. The method was performed as follows:

Beads were weighed to three decimal points (of a milligram) on a Mettler AE163 Microbalance. Each portion of beads to be analysed was placed in a 5 mL volumetric flask, and the flasks were filled with 20% piperidine/DMF. Samples were stored in the dark for 1 h and then shaken immediately prior to their analysis. Analysis was performed on a Perkin-Elmer Lambda 2 S UV–vis spectrometer at 254 nm against a 20% piperidine/DMF blank.

3. Results and discussion

3.1. System performance

3.1.1. Chromatography

DBF and anthracene were well resolved with retention times of 7.3 and 8.1 min, respectively (Fig. 3).

3.2. Performance

3.2.1. Calibration graph

A typical calibration graph is shown in Fig. 4.

The coefficient of variation of the signal was calculated to be ±2%.

The detection limit of the method under the conditions described above was found to be 0.04 μM DBF in 2% DBU/DMF. This equates to 0.2 nmol of DBF in the 5 mL of solution used to prepare approximately 2 mg of resin beads.

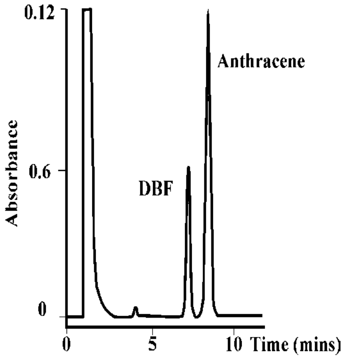

Fig. 3. HPLC separation of DBF and the anthracene internal standard.

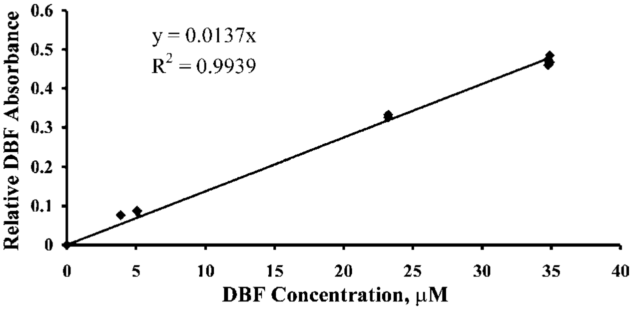

Fig. 4. A typical calibration graph of relative DBF absorbance (using anthracene as the internal standard).

The lowest observable FMOC loading using these estimations is calculated to be 0.1 μmol g^{-1} resin (FMOC).

3.3. Bead analysis

The analysis of 10 aliquots of FMOC-terminated resin gave an average result of 0.79 mmol g^{-1} (FMOC) with results ranging from 0.69 to 0.90 mmol g^{-1} (FMOC); the relative standard deviation was calculated to be 8.1% of the mean value. This compares well with the manufacturers' specifications for this batch of beads, which was given as an average loading of 0.75 mmol g^{-1} resin (NH_2). The manufacturers' stated values for this batch of beads were determined by the piperidine cleavage of the FMOC chromophore, followed by spectrophotometric determination of the piperidine-DBF adduct. Giving a relative standard deviation of ±8%, the method is significantly more reproducible than the piperidine/DMF cleavage procedures that have been reported to give poor reproducibility [6,7].

For comparative purposes the beads were also analysed by conventional spectrophotometric methodology. The FMOC content was found to be 0.69 mmol g^{-1} resin, which, given the relatively poor quoted precision of this piperidine/DMF cleavage procedure, is in good agreement with both the manufacturer's spectrophotometric results and results from the newly developed HPLC procedure reported here.

4. Conclusions

An HPLC-based analysis procedure has been developed for the measurement of the FMOC loading of amine-protected polymer beads. The procedure incorporates the best aspects of exisiting spectrophotometric and chromatographic methods, including the utilization of a stearically hindered base system permitting the direct measurement of the released dibenzofulvene and improved precision by the incorporation of an inert internal standard. HPLC separation of the reaction products permits the measurement of FMOC in the presence of additional chromophores that might otherwise impede direct measurement by UV–vis spectrophotometry.

Evaluated for the FMOC loading of commercially-available FMOC protected aminoethyl-modified styrene-divinylbenzene beads that are employed in combinatorial synthesis, the HPLC procedure compared favourably. There was a close correlation between results obtained by the HPLC procedure and those that were obtained using conventional spectrophotometric methodology based on the measurement of the piperidine-DBF adduct that is liberated using piperidine/DMF. The piperidine-based protocol is significantly less reproducible than procedures using DBF and the DBU procedure reported here, which, with the anthracene internal standard, exhibits significantly improved reproducibility compared to the piperidine based methods.

A number of extensions to this work can be envisaged. Firstly, the detection limit of the procedure, currently ca. 0.1 μmol g^{-1} of resin, could be significantly enhanced by the removal of the dilution step and the alteration of reagent volumes. This approach would make the approach potentially applicable to the analysis of single FMOC-derivatised beads. Whilst evaluated hare as a method of assessing the FMOC loading of protected beads, it should prove possible to extend the procedure to the measurement of the amine contents of amine-modified solids through the quantitative reaction of the amines with FMOC reagents. The procedure is also potentially suitable for homogeneous solution analyses.

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