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## The efficient synthesis and purification of amyloid- $\beta$ (1–42) using an oligoethylene glycol-containing photocleavable lysine tag†

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**A short, monodisperse oligoethylene glycol-containing photocleavable lysine tag was developed to facilitate the efficient purification of hydrophobic and fibril-forming peptides. This new tag was used to prepare a modified A $\beta$ <sub>42</sub> peptide with increased solubility and decreased propensity to aggregate in aqueous media. The solubilising tag was readily removed by irradiation with UV light and permitted the preparation and isolation of A $\beta$ <sub>42</sub> in high purity and yield.**

Alzheimer's disease is the most common form of neurodegenerative dementia. Hallmarks of the disease include extracellular plaques and intracellular tau-enriched neurofibrillary tangles in the brain.<sup>1,2</sup> The plaques contain aggregated forms of the amyloid- $\beta$  (A $\beta$ ) polypeptide ranging in length from 39–43 amino acid residues, formed by proteolytic cleavage of amyloid precursor protein. The two main peptides produced are 40 (A $\beta$ <sub>40</sub>) and 42 (A $\beta$ <sub>42</sub>) amino acid residues in length. The longer A $\beta$ <sub>42</sub> has a greater propensity for aggregation *in vivo* and is often considered the more toxic.<sup>3</sup> Continued efforts to better understand the structure of A $\beta$ <sub>42</sub> in fibrils and plaques,<sup>4–6</sup> the toxicity of A $\beta$ <sub>42</sub> oligomers and assessing potential therapeutic molecules<sup>7</sup> all require ready access to pure synthetic A $\beta$ <sub>42</sub>.

Solid-phase peptide synthesis (Fmoc SPPS, Fmoc = 9-fluorenylmethoxycarbonyl) can be used to prepare A $\beta$ <sub>42</sub>.<sup>8</sup> Unfortunately, isolation of A $\beta$ <sub>42</sub> using conventional chromatographic techniques is extremely challenging. The propensity of A $\beta$ <sub>42</sub> to aggregate results in broad and asymmetrical peaks when attempting to purify the crude peptide by high performance liquid chromatography (HPLC). This makes fractionation of the target peptide from closely-eluting peptidic impurities difficult.<sup>9</sup>

Several strategies have been employed to improve chromatographic peak resolution and solubility of 'difficult peptides',<sup>9</sup> including the use of alkaline buffers to alter the net charge of the peptide<sup>10</sup> and high temperature HPLC.<sup>11</sup> Temporary chemical modification of hydrophobic peptides with iso-acyl dipeptides<sup>12</sup> and polycationic orthogonally cleavable tags also possess effective solubilising properties in acidic media, which is typically used during RP-HPLC purifications.<sup>13–17</sup> The addition of PEGylated or OEGylated (PEG = polyethylene glycol, OEG = oligoethylene glycol) functional groups can increase aqueous solubility over a wide pH range without increasing ionic charge and both are simple to incorporate during Fmoc SPPS.<sup>18</sup> It has been shown that *ortho*-nitrobenzyl (oNb) based photocleavable protecting groups are compatible with SPPS.<sup>19–21</sup> In this manuscript we describe a new amine-reactive, photolabile OEG tag that enhances the solubility of A $\beta$ <sub>42</sub> and enables its more efficient preparation.

Amine-reactive poly-disperse PEG reagents have been employed to switch on the function of proteins with light, but a small monodisperse OEG-containing substrate was considered more appropriate in this instance since a poly-disperse product would be difficult to isolate.<sup>22</sup> It was thought that this approach may also allow some control of fibril formation.<sup>23</sup>

An oNb-based substrate containing a *tert*-butoxycarbonyl (Boc) protected amino-OEG<sub>3</sub> chain (oNb-OEG<sub>3</sub>) was synthesised by adapting a method of Rossi and co-workers (Scheme 1).<sup>24</sup> Incorporation of an alkyne functional group at the benzylic position of the oNb derivative *via* a Grignard reaction gave **1**.<sup>17</sup> Copper(i)-catalyzed azide-alkyne cyclo-addition (CuAAC) between **1** and **2** gave **3** in a yield of 72%, that was then reacted with *para*-nitrophenyl chloroformate to produce **4** in 45% yield.

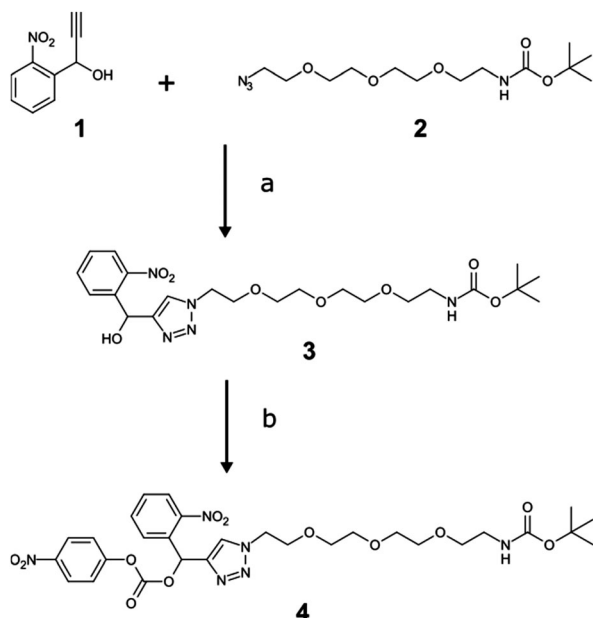
Although it would be simplest to incorporate the hydrophilic tag at the N-terminus, we chose to functionalise Lys<sub>28</sub> due to its close proximity to the hydrophobic C-terminus that is essential for aggregation. Residues 28–42 of A $\beta$ <sub>42</sub> were assembled on the solid support by microwave-assisted Fmoc SPPS, with monomethoxytrityl (Mmt) protection at Lys<sub>28</sub> (Scheme 2). The  $\epsilon$ -amino group was liberated after multiple treatments with dilute

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† Electronic supplementary information (ESI) available: Experimental details of organic and peptide synthesis, purification, photolysis and aggregation studies. See DOI: 10.1039/c7cc03147e





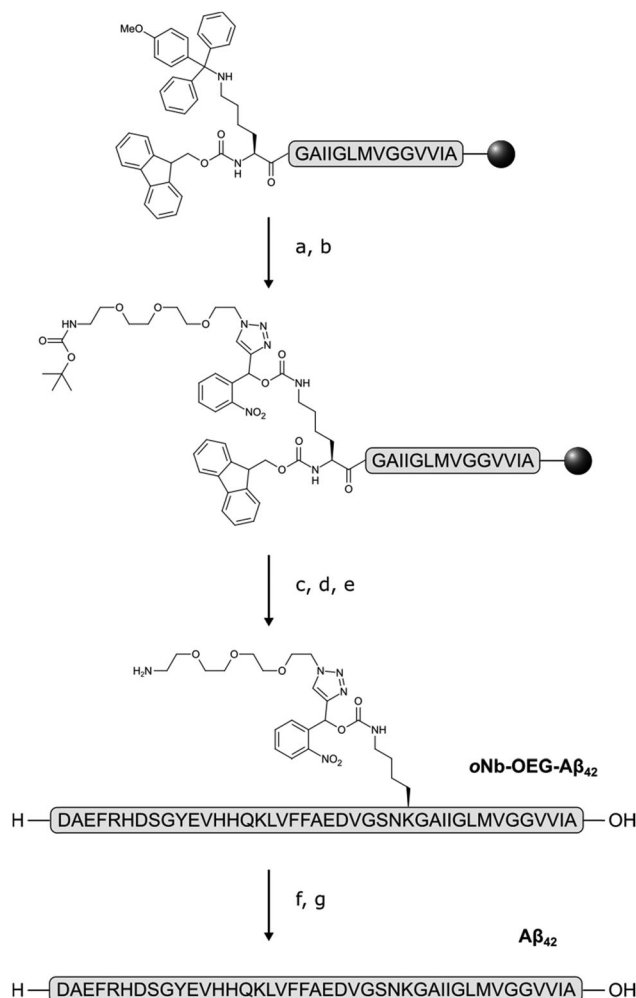
**Scheme 1** Synthesis of the oNb-OEG<sub>3</sub> tag. (a) CuSO<sub>4</sub>, sodium ascorbate, DMF/water; (b) *para*-nitrophenyl chloroformate, DCM.

trifluoroacetic acid (TFA), followed by acylation with *para*-nitrophenylcarbamate activated oNb-OEG<sub>3</sub> at high temperature. The remainder of the peptide was assembled by SPPS, followed by global deprotection and cleavage from the solid support.

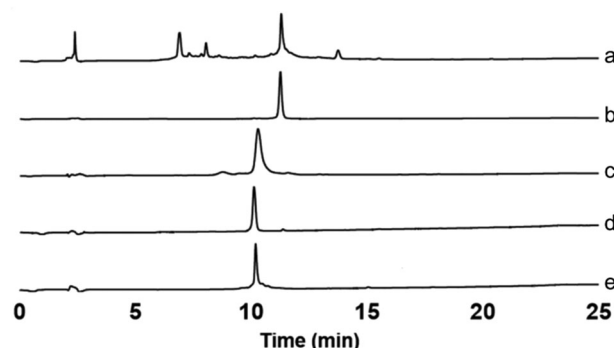
Electrospray ionisation mass spectrometry (ESI-MS) (see ESI†) and analytical RP-HPLC (Fig. 1a) indicated that the target peptide was the major species present in the crude reaction mixture. The purification conditions which gave optimal peak separation were alkaline buffers at 60 °C using a C18 column. It is very difficult to analyze or purify Aβ<sub>42</sub> with a C18 stationary phase so its use in this instance suggests that the oNb-OEG<sub>3</sub> tag does reduce the peptide's propensity to aggregate. The presence of the solubilising tag allowed isolation of oNb-OEG<sub>3</sub>-Aβ<sub>42</sub> in excellent yield (10%) and high purity (Fig. 1b). The solubilising OEG-tag could be readily removed by irradiation at 365 nm (20 min) (Fig. 2). The photolysis was performed in 80% aqueous 1,1,1,3,3,3-hexa-fluoroisopropanol (HFIP) to avoid premature aggregation prior to final purification. A shift in retention time was observed post-cleavage (Fig. 1c) as well as significant peak broadening, which is characteristic of native Aβ<sub>42</sub>. Importantly, no significant degradation caused by the photolysis was evident and the elution profile of the target material was well resolved from non-peptidic species originating from the oNb-OEG<sub>3</sub> tag.

The Aβ<sub>42</sub> peptide was purified and isolated in good yield and high purity (Fig. 1d) using semi-preparative RP-HPLC on a C4 column. The identity of the final product was confirmed by ESI-MS (see ESI†). The isolated yield from the photolysis reaction was 60% after a single HPLC purification.

The presence of amyloid fibrils is often determined by monitoring the fluorescence of thioflavin-T (2-[*p*-(dimethyl-amino)phenyl]-3,6-dimethyl-benzothiazolium chloride, ThT), which undergoes a 115 nm red shift in its excitation and emission profile following interaction with amyloid fibrils.



**Scheme 2** Synthesis of pure amyloid-β (1–42). (a) 10 × 1% TFA in DCM; (b) 3 eq. **4** & 6 eq. DIEA in DMF, 75 °C, 1 hour; (c) Fmoc SPPS; (d) 1% TIPS, 2% water, 2% thioanisole, 95% TFA, 3 hours; (e) RP-HPLC; (f) *hν* 365 nm, 80% HFIP/20% water, 20 min; (g) RP-HPLC.



**Fig. 1** Analytical RP-HPLC traces. (a) Crude oNb-OEG<sub>3</sub>-Aβ<sub>42</sub>; (b) purified oNb-OEG<sub>3</sub>-Aβ<sub>42</sub>; (c) crude (post-photolysis) Aβ<sub>42</sub>; (d) purified (post-photolysis) Aβ<sub>42</sub>; (e) control Aβ<sub>42</sub>.

The presence of the solubilising tag in Aβ<sub>42</sub> results in less fluorescence from ThT after incubation for 48 hours when compared to a sample of native Aβ<sub>42</sub> prepared from the



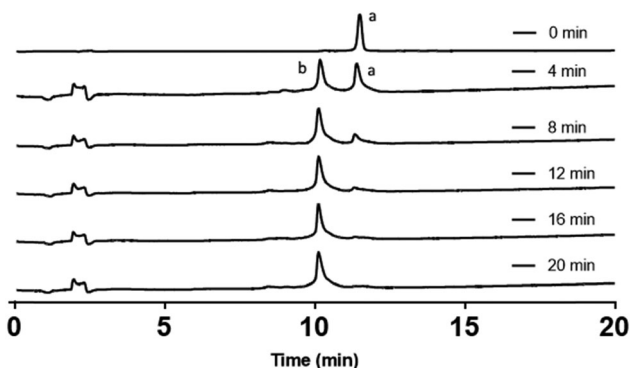


Fig. 2 Analytical RP-HPLC traces of a time course for the photolysis reaction. Peak (a) oNb-OEG-A $\beta_{42}$ ; peak (b) A $\beta_{42}$  obtained after photolysis of oNb-OEG-A $\beta_{42}$ .

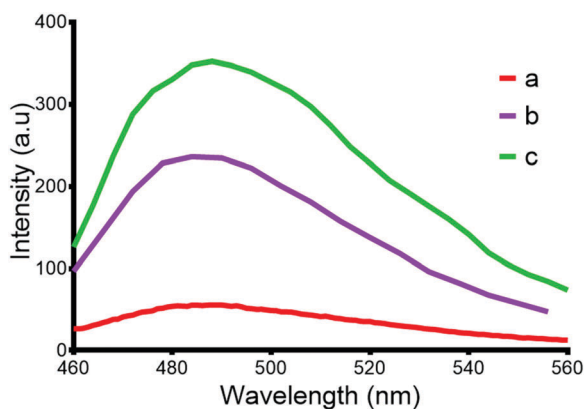


Fig. 3 ThT fluorescence curves after incubation for 48 hours at 37 °C in PBS ( $\lambda_{\text{ex}}$  = 444 nm). (a) ThT only in PBS; (b) oNb-OEG-A $\beta_{42}$  (20  $\mu\text{M}$ ); (c) A $\beta_{42}$  peptide (20  $\mu\text{M}$ ) obtained by photolysis of oNb-OEG-A $\beta_{42}$ .

photolysis of oNb-OEG-A $\beta_{42}$  (Fig. 3). This is presumably due to the tag hindering the formation of amyloid fibrils. The structural morphology of the aggregates after photolysis of oNb-OEG-A $\beta_{42}$  was investigated by transmission electron microscopy (TEM), and confirmed the formation of A $\beta_{42}$  fibrils (Fig. 4b). In contrast, the TEM image of oNb-OEG-A $\beta_{42}$  does not show well defined fibril structures characteristic of native peptide aggregates (Fig. 4a). Given these results, it appears that

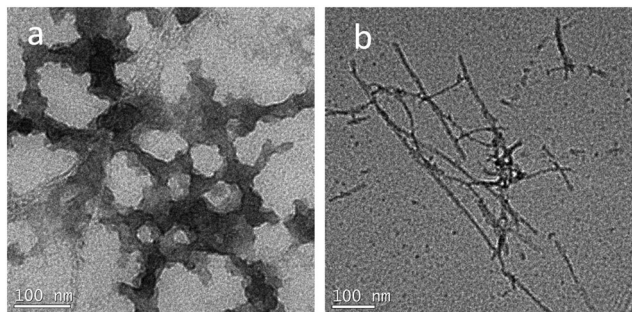


Fig. 4 TEM images after incubation for 48 hours at 37 °C in PBS, negatively stained with 2% uranyl acetate (a) oNb-OEG-A $\beta_{42}$  (10  $\mu\text{M}$ ); and (b) A $\beta_{42}$  (10  $\mu\text{M}$ ) prepared by photolysis of oNb-OEG-A $\beta_{42}$ .

strategic placement of the oNb-OEG<sub>3</sub> tag is effective in suppressing amyloid fibril formation. This could explain why oNb-OEG-A $\beta_{42}$  was obtained at such a high purity.

In conclusion, a photocleavable hydrophilic tag, oNb-OEG, has been prepared and incorporated into A $\beta_{42}$  at Lys<sub>28</sub> using Fmoc SPPS. The presence of the tag improved the solubility of this hydrophobic peptide in aqueous media and suppressed the formation of aggregates and fibrils. The increased solubility enabled the synthesis and isolation of oNb-OEG-A $\beta_{42}$  in good yield and high purity. The tag could be readily removed by photolysis. It is likely that this strategy could be used for the synthesis of other hydrophobic and fibril-forming “difficult” peptides.<sup>9</sup>

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