Functionalised Carbon Nanotubes Enhance Brain Delivery of Amyloid-Targeting Pittsburgh Compound B (PiB)-Derived Ligands

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Supplementary Material

Materials

Phosphate buffered saline (PBS), nitric acid (HNO_3, 68-70%), sulphuric acid (H_2SO_4, 95-98%), methanol, dimethylformamide (DMF), ethyl acetate, hexane, dichloromethane (DCM), acetone and Whatman paper were acquired from Fischer Scientific (Loughborough, UK). Diethyl ether, tetrahydrofuran (THF), 2,2'-(ethylenedioxy)bis(ethylamine), Di-tert-butyl dicarbonate, benzyl 2-bromoacetate, trimethylamine, palladium on carbon, trifluoroacetic acid (TFA, 99%), monomethylphthalate, toluene, silica (98%), anhydrous magnesium sulphate (Mg_2SO_4), potassium permanganate ($KMnO_4$), sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), deuterated chloroform ($CDCl_3$), hydrochloric acid (HCl, 37%), *N*-hydroxybenzotriazole (HOBt), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 2,2'-(Ethylenedioxy)*bis*(*ethylamine*), hydrazine monohydrate, absolute ethanol, ninhydrin, phenol, pyridine and potassium cyanide, were obtained from Sigma- Aldrich (Gillingham, UK).

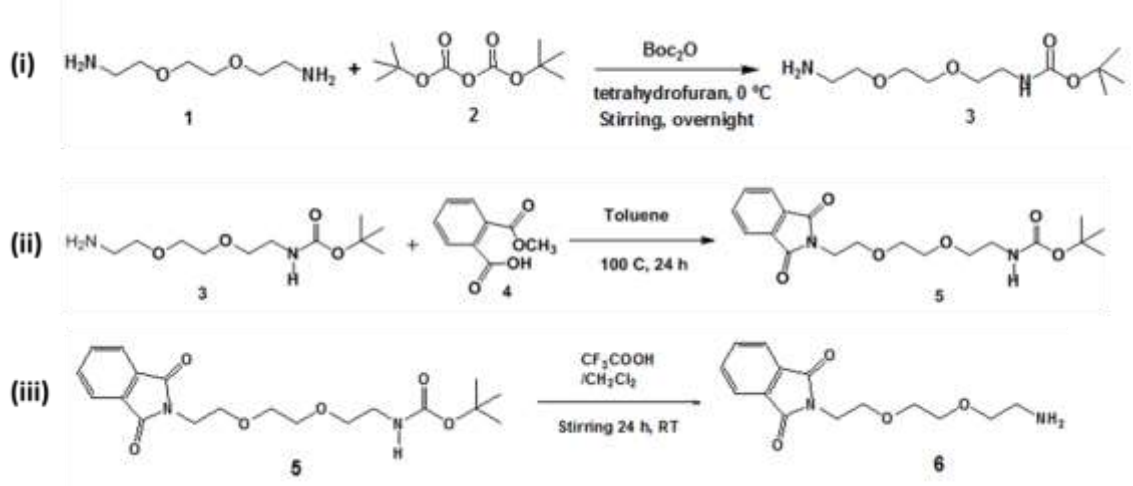
Synthesis of phthalimide-protected amine-terminated spacer

Glass equipment and apparatus

The rotary evaporation unit was formed of BUCHI vacuum controller V-850, rotavapor R-210, heating bath B-491 and vacuum pump V-700 (Fisher Scientific, UK). Magnetic hotplate stirred VMS-C4 Advanced was acquired from VWR (UK). Glass equipment included addition, separatory and Büchner funnels, 250 ml round bottom flasks, conical flasks (250 ml and 100 ml), Dean-stark and chromatography column. Filtration unit (XX1504700), PTFE filter 0.2 µm (FGLP02500) and thin-layer chromatography (TLC) silica gel were obtained from Merck-Millipore (UK).

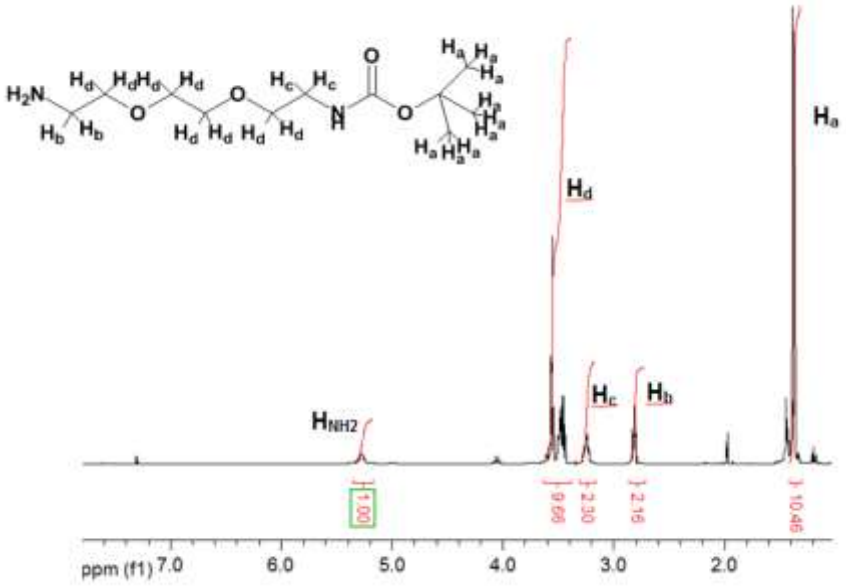
Synthesis

The phthalimide-protected amine spacer was synthesised as described in **Scheme S1** below**.**

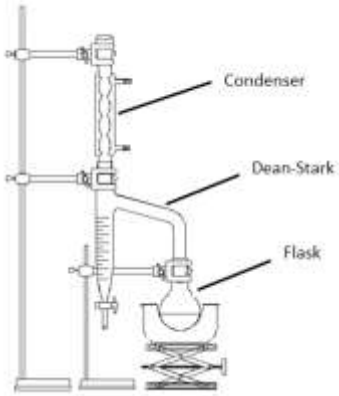


(i) Boc protection of diamine: A solution of Boc_2O (3.5 g, 16.2 mmol; in 50 mL THF) **(2)** was added dropwise (using addition funnel) to a solution of diamine (7.5 g, 51 mmol; in 150 mL THF) **(1).** The reaction mixture was stirred overnight, with temperature was kept at or below 0 ºC by using an ice bath. Subsequently, a silica TLC was run in order to check the formation of (**3)** using ethyl acetate as mobile phase and staining with $KMnO_4$. Ethyl acetate was evaporated under reduced pressure (rotavap, 40 ºC, 250 mbar) to reveal a yellow oil product. The reaction crude was re-suspended in 100 mL ethyl acetate and unreacted Boc_2O was removed by washing with 150 mL dH_2O in a separation funnel. The aqueous phase was further washed with ethyl acetate to ensure complete recovery of the mono and di-protected amine. Excess anhydrous magnesium sulphate ($MgSO_4$) was added to the organic fraction to remove water, followed by paper filtration.
To collect the mono-protected product, ethyl acetate was evaporated under reduced pressure (rotavap, 40 ºC, 250 mbar) and a silica gel TLC was run to separate mono-protected from diprotected amine and starting diamine (mobile phase: using ethyl acetate:hexane, 8:2). Elution of the **mono-protected amine** was achieved using ethyl acetate: methanol (7:3) as mobile phase (6 x 200 ml fractions). After evaporation of the mobile phase fractions, a yellow oil product is obtained (yield: 60 %), and NMR spectroscopy was used to confirm purity (solvent: deuterated chloroform, $CDCl_3$, 0.4 mg/ml).

^{1}H NMR ($CDCl_3$, ppm) δ: 1.39 (singlet, 9 H, 3C<u>H</u>$_3$); 2.8 (d, 2 H, -C<u>H</u>$_2$-NH-), 3.2 (d, 2 H, -C<u>H</u>$_2$-NH$_2$), , 3.4-3.7 (m, 8 H, -C<u>H</u>$_2$-), 5.2 (broad singlet, 1 H, N<u>H</u>).

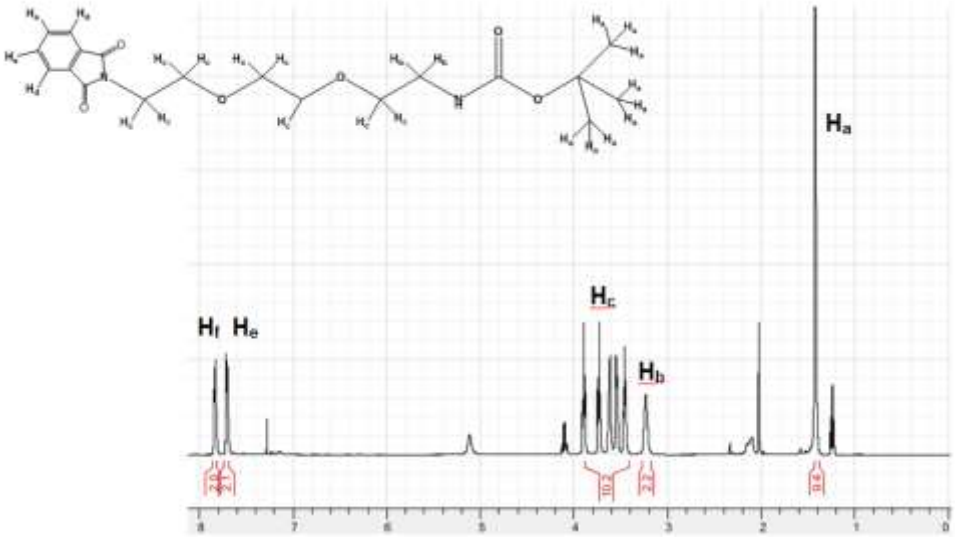


(ii) Addition of monomethyl phthalate Boc-protected amine: Monomethyl phthalate (2.58 g, 14.33 mmol) **(4)** and compound **3** (2.8 g, 12.9 mmol; in 150 mL toluene) were mixed in a 250 mL flask and a Dean-Stark (filled with toluene) and condenser were further attached (**image on the right**). The reaction was stirred at 100° C for 24 hours (in an oil bath). Toluene was evaporated under reduced pressure (rotavap, 40° C, 50 mbar), the reaction crude was re-suspended in 100 mL ethyl acetate and serial washes were performed in a separatory funnel (100 mL saturated Na_2CO_3, 100 mL saturated $NaHCO_3$, 100 mL dH_2O) to remove unwanted products.



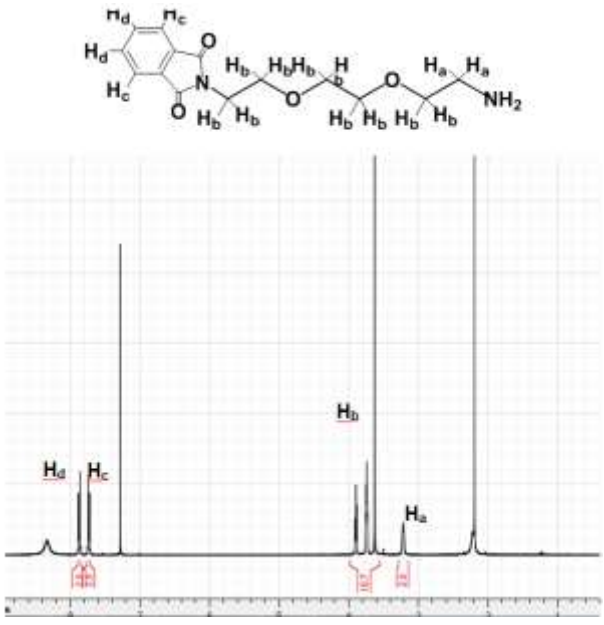
To obtain the final product **(5),** the reaction crude was re-suspended in 100 mL ethyl acetate and extracted by washing with 150 mL dH_2O in a separation funnel (compound in organic phase). The aqueous phase was further washed with ethyl acetate to ensure complete recovery of the desired product. Excess anhydrous magnesium sulphate ($MgSO_4$) was added to the organic fraction to remove water, followed by paper filtration. Ethyl acetate was then evaporated under reduced pressure (rotavap, 40 ºC, 250 mbar) and NMR spectroscopy was used to confirm purity (solvent: deuterated chloroform, $CDCl_3$, 0.4 mg/ml).

^{1}H NMR (CDCl$_3$, ppm) δ: 1.36 (s, 9 H, 3C<u>H</u>$_3$), 2.1 (s ancho, 1 H, OCO-N<u>H</u>), 2.8 (d, 2 H, -C<u>H</u>$_2$-NH-), 3.2 (d, 2 H, -C<u>H</u>$_2$-NH$_2$), 3.4-3.7 (m, 10 H, -C<u>H</u>$_2$-), 5.1 (s, 2 H, C$_{Ar}$-C<u>H</u>$_2$-O), 5.5 (s ancho, 1 H, N<u>H</u>-COO); 7.28 (m, 5 H, H$_{Ar}$).



(iii) Deprotection of Boc group: Trifluoroacetic acid (10 mL) was added to a solution of compound **5** (2.8 g, 7.4 mmol; in 10 mL DCM0 mL) and the reaction mixture was stirred overnight at room temperature. Subsequently, DCM was evaporated under reduced pressure (liquid nitrogen was used to prevent damage from trifluoroacetic acid fumes) and the resulting oil was dissolved in the minimum amount of DCM and diethylether until the formation of a white precipitate. The precipitate (**phthalimide-protected amine spacer**, **6**) was filtered in a Buchner filter and keep under vacuum until drying. The final product was analysed by NMR spectroscopy (solvent: acetone).

^{1}H NMR (CDCl$_3$, ppm) δ: 3.2 (d, 2 H, -C<u>H</u>$_2$-NH$_2$), 3.6-4 (m, 10 H, -C<u>H</u>$_2$-), 7.74 (d, 2 H, H$_c$), 7.89 (d, 2 H, H$_d$).



Animal husbandry and welfare

Animal experiments followed the guidelines of the 3Rs (Replacement, Reduction, Refinement) for research involving laboratory animals. Male C57BL/6 mice were housed in a conventional animal room in groups of 4 to 5 animals per cage; mice distribution by each cage, and the position of the animal cages in the room, were randomized. Environmental conditions were maintained at 21ºC, 45-65% humidity, 15 room air changes/hour and 12-hour light/dark cycle. Animals were provided with water and food *al libitum,* as well as crushed corncob as bedding and shredded paper as enrichment. After intravenous injection under general inhalation anaesthesia (using Isoflurane) mice were carefully monitored for any signs of discomfort. Early humane end-points were defined for these experiments to avoid animal suffering. Animals were euthanized using a Schedule 1 accepted method at the experiment end-point or if any of the following symptoms were observed: weight loss superior to 20% normal body weight, weakness, inability to obtain feed or water.

Supplementary Figures

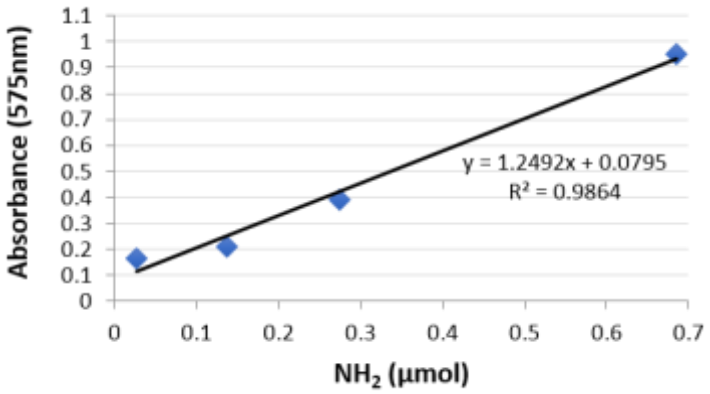


Fig. S1 Standard curve for quantification of amine groups by Kaiser test. Standards of known amine (NH_2) moles were prepared using 2,2- (ethylenedioxy) bis(ethylamine) (contains two free primary amines). Following reaction with the assay reagents (as described in Materials and Methods), the absorbance was measured at 575nm using the Lambda 35 UV/VIS Spectrophotometer (Perkin Elmer).

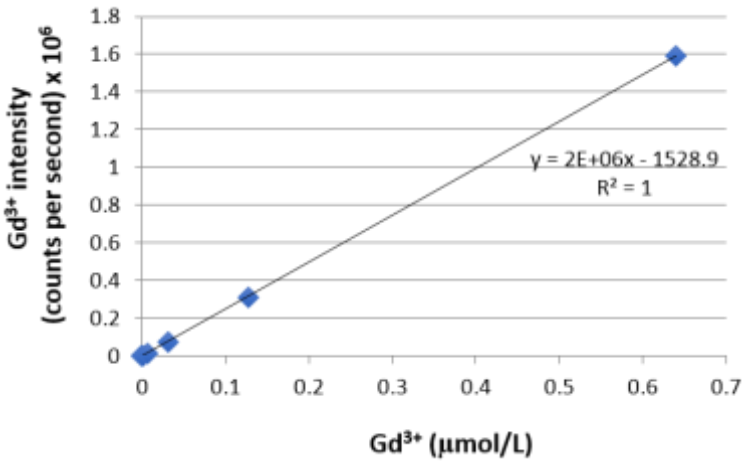


Fig. S2 Standard curve of gadolinium (Gd^{3+}) measured by ICP-MS. Gd^{3+} dilutions were prepared in 10% nitric acid to obtain a standard curve in the range of 10^{-3}-10^{3} parts per million (ppm) (1 ppm ~ 10^{3} µg/L ~ 6.36 µmol/L, MW=157.25 g/mol). The instrument response (represented as Gd^{3+} intensity) was measured in counts per second (cps) using a ICP mass spectrometer (NexION 350D, Perkin Elmer, USA). In the figure are included the standard curve concentrations that cover the sample range.

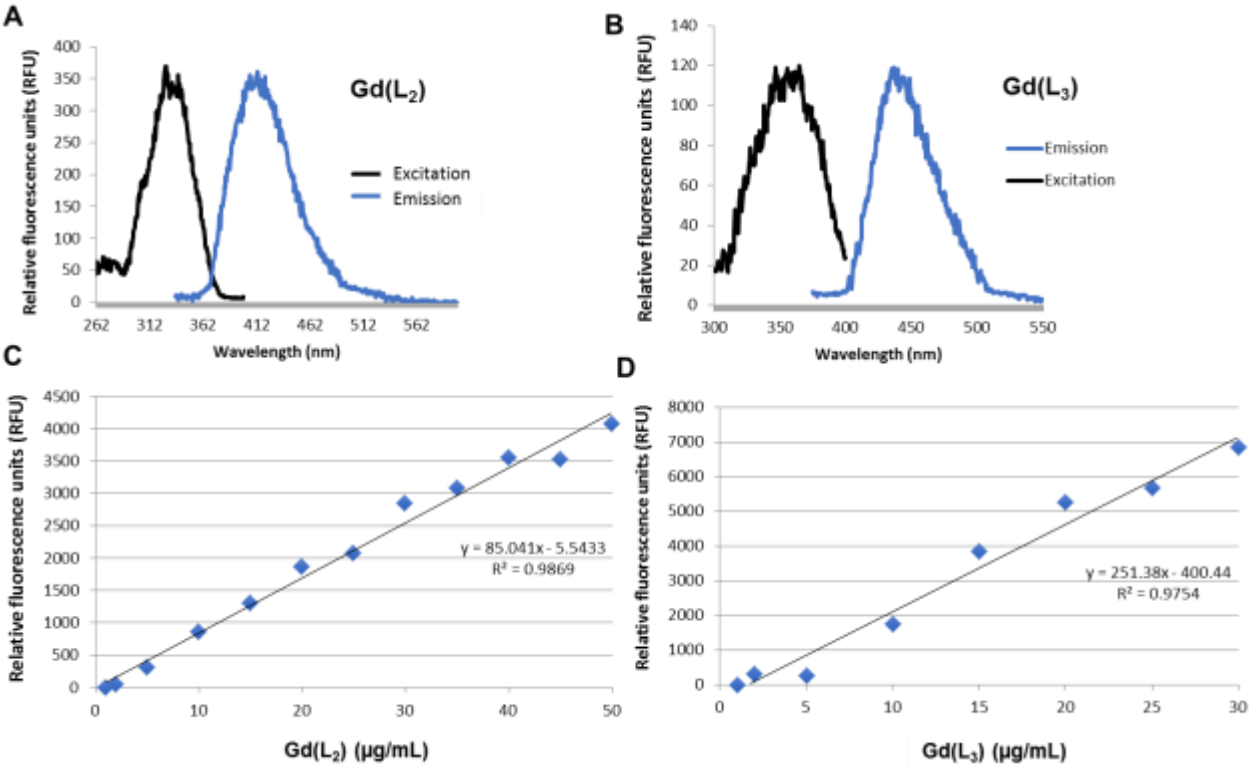


Fig. S3 Fluorescence excitation/emission spectra and standard curves of $Gd(L_2)$ and $Gd(L_3)$. The fluorescence spectra of (**A**) $Gd(L_2)$ and (**B**) $Gd(L_3)$ were determined using a cuvette LS50B Luminescence Spectrophotometer (Perkin Elmer). The standard curves of (**C**) $Gd(L_2)$ and (**D**) $Gd(L_3)$ were measured using the FLUOstar Omega microplate reader (BMG Labtech). Fluorescence intensity values are presented as relative fluorescence units (RFU) of two different experiments.