Bioconjugation of Biotinated PAMAM dendrons.

Lei Tao,[a] Geng,[a] Gaojian Chen,[a] Yingjian Xu, [b] Jin Vincent Ladmiral[c] , Giuseppe Mantovani and David. M. Haddleton*[a]

[a] Department of Chemistry, University of Warwick, CV4 7AL, Coventry, UK

[b] Chemistry Research Laboratory, University of Oxford, OX1 3TA, Oxford, UK

[c] Division of Organic Material Chemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan.

CONTENTS

1.0 General

1.1 Reagents

1.2 Analysis

2.0 Synthesis of biotin terminated PAMAM dendrimer

3.0 Preparation of the Avidin-biotinated PAMAM dendrimer bioconjugate

4.0 HABA test of the avidin-biotinated PAMAM dendrimer.

1.0 General

1.1 Reagents

Biotin (Sigma-Aldrich, 99.0%), Avidin (Sigma), ethylenediamine (Aldrich, 99.0%) were used as received, methyl acrylate (MA, Aldrich, 99.0%) was stored at 4^{o}C and used as received. All other reagents and solvents were obtained at the highest purity available from Aldrich Chemical Company and used without further purification unless stated.

1.2 Analysis

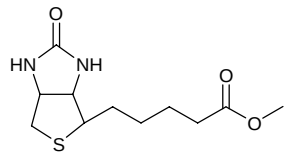
Analytical TLC was performed using pre-coated silica gel 60 F254 and developed in the solvent system indicated. Compounds were visualized by use of UV light (254 nm) or a basic solution (10.0% w/w K_2CO_3 in water) of $KMnO_4$. Merck 60 (230-400 mesh) silica gel was used for column chromatography. NMR spectra were obtained on a Bruker DPX400 spectrometer. All chemical shifts are reported in ppm (δ) relative to tetramethylsilane, referenced to the chemical shifts of residual solvent resonances (^{1}H and ^{13}C). The following abbreviations were used to explain the multiplicities: s = singlet, dd = doublet of doublets, t = triplet, bs = broad singlet, m = multiplet. Infrared absorption spectra were recorded on a Bruker VECTOR-22 FTIR spectrometer using a Golden Gate diamond attenuated total reflection cell. Polydispersity (PDI) were measured using size exclusion chromatography (SEC), on a system equipped with two PL gel 5 μm mixed D-columns (300 x 7.5 mm) and one PL gel 5 mm guard column (50 x 7.5 mm) (Polymer Laboratories) with differential refractive index detection using chloroform/triethylamine 95:5 at 1.0 mL min^{-1} as the eluent. Poly(MMA) standards ($1{\cdot}10^{6}$-200 g mol^{-1}) were used to calibrate the SEC. The Reverse phase HPLC system was fitted with a Phenomenex Jupiter 5μ C18 300A column and a UV

detector continuously measuring the relative absorbance of the mobile phase at 280 nm. The mobile phases are phase A (10% acetonitrile, 99.95% H_2O, 0.05% TEA) and phase B (99.96% acetonitrile, 0.04% TEA) respectively. The gradient of the mobile phase is 100%-50% phase A (27 min), 50% phase A (35 min), 100% phase A (36 min). The UV absorption data was collected from Perkin Elmer Lambda 25 UV/vis spectrometer.

MALDI TOF spectra were recorded using a Micromass, TofSpec 2E, MALDI-TOF mass spectrometer.

Synthesis of biotin terminated PAMAM dendrimers

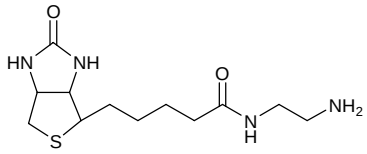
Methyl biotinate (1).



To a solution of biotin (1.50 g, 6.15 mmol) in methanol (150 mL), 1.0 g of Amberlite IR-120 resin was added, and the solution was stirred at ambient temperature for 48 h. After filtration the solvent was removed under reduced pressure to give quantitatively **(1)** as a white solid.

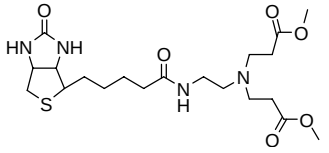
IR (neat): $\tilde{\nu}$ = 3264, 2923, 2850, 1742, 1701, 1463, 1430 cm^{-1}. ^1H NMR (400.03 MHz, CD_3OD, 298 K) δ = 4.51-4.47 (m, 1H, NHC<u>H</u>CH), 4.32-4.29 (m, 1H, NHC<u>H</u>CH_2)), 3.66 (s, 3H, CH_3), 3.23-3.18 (m, 1H, SCH), 2.95-2.69 (dd, 2H, CH_2S, *J* = 12.8, 7.3 *Hz*), 2.35 (t, 2H, CH_2COO, *J* = 7.3 *Hz*), 1.79-1.54 (m, 4H, C<u>H</u>$_2$C<u>H</u>$_2$$CH_2COO$), 1.49-1.43 (m, 2H, SCHC<u>H</u>$_2$) $^{13}C\{^1H\}$NMR (100.59 MHz, CD_3OD, 298 K) δ = 176.78 (COO), 165.83 (NHCONH), 63.38 (NH<u>C</u>HCH), 61.63 (NH<u>C</u>HCH_2), 56.95 (SCH), 52.00 (CH_3), 41.02 (SCH_2), 34.54 (CH_2COO), 29.70 (SCHCH_2<u>C</u>H_2), 29.46 (SCH<u>C</u>H_2), 25.91 (<u>C</u>H_2CH_2COO) Anal. Calcd. for $C_{11}H_{18}N_2O_3S$: C, 51.14; H, 7.02; N, 10.84; S, 12.41. Found C, 51.18; H, 7.02; N, 10.67; S, 11.94. MS: 297 [M+39]

Biotin (2-amino-ethyl)-amide) (2)



To a solution of methyl biotinate (1.50 g, 5.81 mmol) in methanol (50 mL), an excess of ethylene diamine (20 mL) was added. The solution was kept at 60^{o}C for 48h, and the excess ethylene diamine and methanol were removed under reduced pressure. The remaining ethylene diamine was removed by azeotropic distillation using toluene/methanol. The product was obtained quantitatively as light yellow oil and used directly for the next experiment.

Biotin G#0.5

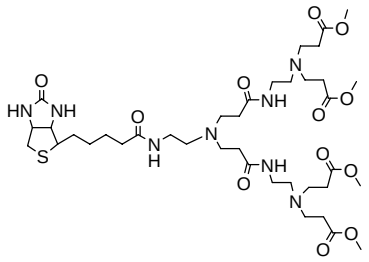


Biotin (2-amino-ethyl)-amide (1.00 g, 3.50 mmol) and an excess of methyl acrylate (20 mL) were dissolved in methanol (50 mL) and the resulting solution was stirred at ambient temperature for 48 h. The volatile small molecules were removed by distillation under reduced pressure. The product was purified by flash chromatography (CC, SiO_2, methanol:ethyl acetate = 1:10) as a pale yellow oil (1.39 g, 87.0%).

IR (neat): $\tilde{\nu}$ = 3315, 2939, 2826, 1732, 1698, 1645, 1359 cm^{-1}. ^{1}H NMR (400.03 MHz, $CDCl_3$, 298 K) δ = 4.50-4.47 (m, 1H), 4.31-4.28 (m, 1H), 3.67 (s, 6H), 3.31-3.27 (m, 2H), 3.17-3.12 (m, 1H), 2.91-2.66 (m, 6H), 2.55 (m, 2H), 2.45-2.42 (m, 4H), 2.22 (t, 2H, *J* = 7.3 *Hz*), 1.79-1.61 (m, 4H), 1.48-1.40 (m, 2H) ^{13}C{^{1}H}NMR (100.59 MHz, $CDCl_3$, 298 K) δ = 173.51, 173.16, 163.77, 61.88, 60.26, 55.57, 53.13, 52.21, 50.81, 40.65, 39.01, 35.91, 32.60, 28.26, 25.64. MS: 459 [M+1]

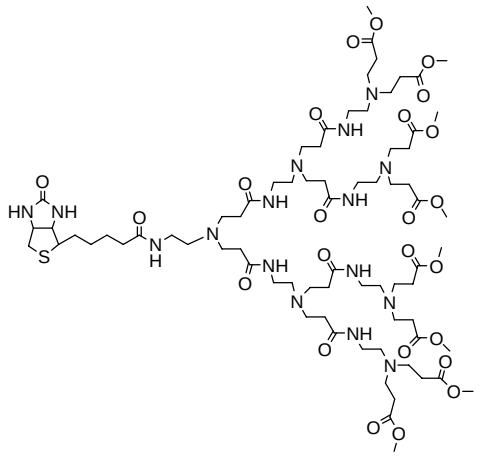
All the following dendrimer generations were prepared by repeated amidation/Michael addition cycles analogous to those employed for the synthesis of **(2)** and **Biotin G#0.5.**

Biotin G#1.5



IR (neat): $\tilde{\nu}$ = 3280, 2948, 2826, 1730, 1698, 1643, 1541, 1436, 1198 cm^{-1}. ^{1}H NMR (400.03 MHz, $CDCl_3$, 298 K, selected peaks) δ = 4.50-4.47 (m, 1H), 4.35-4.32 (m, 1H), 3.67 (s, 12H), 3.36-3.27 (m, 6H), 3.19-3.14 (m, 1H), 2.94-2.74 (m, 14H), 2.62-2.53 (m, 6H), 2.45-2.42 (m, 12H), 2.27-2.24 (m, 2H), 1.72-1.68(m, 4H), 1.49-1.46 (m, 2H) $^{13}C\{^{1}H\}$NMR (100.59 MHz, $CDCl_3$, 298 K, selected peaks) δ = 173.53, 173.09, 163.33, 61.86, 60.18, 53.07, 51.86, 49.91, 49.39, 37.66, 37.36, 32.84, 27.31 MS: 898 [M+39]

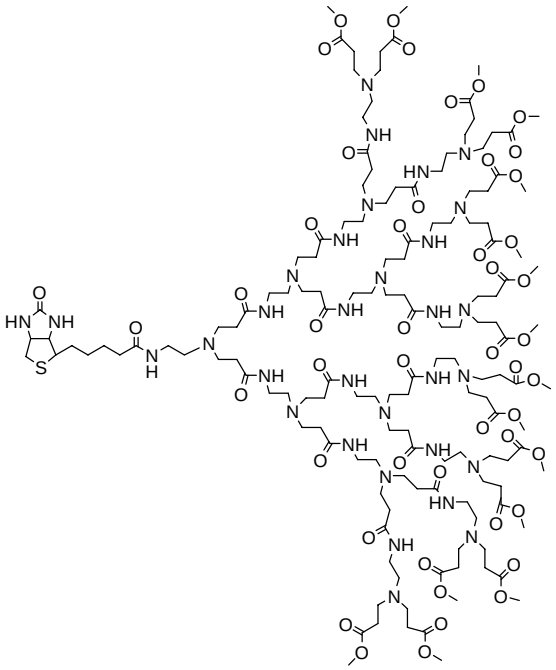
Biotin G#2.5



IR (neat): $\tilde{\nu}$ = 3278, 2849, 2845, 1730, 1641, 1541, 1437, 1359, 1198, 1045 cm^{-1}.
^{1}H NMR (400.03 MHz, $CDCl_3$, 298 K) δ = 4.50-4.47 (m, 1H), 4.34-4.31 (m, 1H), 3.66 (s, 24H), 3.27 (m, 14H), 3.17-3.14 (m, 1H), 2.94-2.74 (m, 30H), 2.57-2.52 (m,

14H), 2.40-2.23 (m, 28H), 2.23 (m, 2H), 1.72-1.66(m, 4H), 1.47-1.45 (m, 2H) $^{13}C\{^{1}H\}$NMR (100.59 MHz, $CDCl_3$, 298 K, selected peaks) δ = 174.34, 173.57, 173.26, 172.89, 172.68, 163.56, 61.88, 60.20, 55.65, 53.02, 51.81, 50.87, 49.94, 49.38, 40.68, 37.57, 37.36, 34.16, 33.97, 32.79, 27.95 MS: 1662 [M+2]

Biotin G#3.5



IR (neat): $\tilde{\nu}$ = 3289, 2950, 2825, 1733, 1644, 1543, 1437, 1359, 1199, 1053 cm^{-1}.
^{1}H NMR (400.03 MHz, $CDCl_3$, 298 K) δ = 4.48-4.47 (m, 1H), 4.32-4.30 (m, 1H), 3.65 (s, 48H), 3.27-3.25 (m, 30H), 3.18-3.14 (m, 1H), 2.91-2.72 (m, 62H), 2.56-2.53 (m, 32H), 2.51-2.22 (m, 60H), 2.23-2.20 (m, 2H), 1.70-1.66(m, 4H), 1.46-1.44 (m, 2H). $^{13}C\{^{1}H\}$NMR (100.59 MHz, $CDCl_3$, 298 K, selected peaks) δ = 173.22, 172.79, 172.63, 163.72, 61.86, 55.76, 53.01, 52.60, 51.78, 50.81, 50.06, 49.36, 37.63, 37.35, 33.93, 32.79, 32.42, 27.98 MALDI-TOF: 3283 [M+23].

2.0 Preparation of the Avidin-biotinated PAMAM dendrimer bioconjugate.

1.0 mL of an aqueous solution of **Biotin G#3.5** dendron (3.0 mg, 9.2×10^{-4} mmol in 2.0 mL of water) was added to an aqueous solution of avidin (3.0 mg, 4.4×10^{-5} mmol

in 3.0 mL of water). The solution was kept at ambient temperature overnight, then the excess of dendron was removed by spin dialysis (VIVASPIN, 0.5 mL, Concentrator, membrane: MWCO 30,000). The resulting solution was freeze-dried to give the desired avidin- **Biotin G#3.5** conjugate as white powder.

3.0 HABA test on the Avidin- Biotin G#3.5 dendron bioconjugate.

0.1 mL of saturated HABA aqueous solution was added into an avidin aqueous solution (1 mg mL^{-1}): the colourless avidin solution became pale red immediately, and the UV-Vis spectrum was recorded. Subsequently, 0.1 mL of **Biotin G#3.5** dendron aqueous solution (1 mg mL^{-1}) was added into the pale red solution of the avidin-HABA complex. The colour of the solution disappeared instantaneously, and an UV-Vis spectrum was recorded again.