# Biotin-functionalized targeted polydiacetylene micelles

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1. General

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich and used without further purification. Tetrahydrofuran (THF) and diethyl ether were distilled from sodium/benzophenone before use. Flash chromatography was carried out on Kieselgel 60 (230–240 mesh, Merck). $^1$H and $^{13}$C NMR spectra were recorded on a Bruker Avance DPX 400 spectrometer at 400 and 100 MHz respectively. Chemical shifts ($\delta$) are given in ppm relative to the NMR solvent residual peak and coupling constants ($J$) in hertz.

2. Chemical synthesis

a. Synthesis of biotin-NHS (2)

Under N$_2$, D-biotin (1, 200 mg, 0.819 mmol, 1 equiv.) and N-hydroxysuccinimide (94 mg, 1 equiv.) were dissolved in dry DMF (6 mL). Moderate heating was needed to induce the solubilizing of biotin. DCC (220 mg, 1.3 equiv.) was then added and the solution was stirred at room temperature for 40 h. The formed dicyclohexylurea was filtered off and the filtrate concentrated. The residue was precipitated into diethyl ether and washed two times with isopropanol. After recrystallization in isopropanol, compound 2 (148 mg, 53% yield) was obtained as a white powder.

$^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$(ppm) 6.42 (s, 1H), 6.36 (s, 1H), 4.25–4.33 (m, 1H), 4.15 (m, $J$ = 4.4 Hz, 1H), 3.10 (m, 1H), 2.76–2.87 (m, 5H), 2.67 (t, $J$ = 7.4 Hz, 2H), 2.55 (m, 1H), 1.57–1.70 (m, 3H), 1.34–1.55 (m, 3H).

b. Synthesis of biotin-N$_3$ (3)

Under N$_2$, 2 (37 mg, 0.108 mmol, 1 equiv.) and 2-azidoethanamine (93 mg, 2 equiv.) were dissolved in DMF (2 mL) before triethylamine (160 µL, 11 equiv.) was added. The solution was stirred at room temperature for 21 h. The reaction mixture was concentrated under vacuum and the residue was purified by column chromatography (silica gel, EtOAc/MeOH 5:1). Compound 3 was obtained as a white powder (28 mg, 84% yield) and its spectral data are in agreement with literature (Eur. J. Org. Chem. 2007, 4711).

$^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$(ppm) 8.04 (t, $J$ = 5.49 Hz, 1H), 6.43 (s, 1H), 6.36 (s, 1H), 4.30 (m, 1H), 4.10–4.15 (m, 1H), 3.22 (m, 2H), 3.06–3.12 (m, 1H), 2.82 (dd, $J$ = 5.12, 12.4 Hz, 1H), 2.53–2.57 (m, 1H), 2.07 (t, $J$ = 7.4 Hz, 2H), 1.20–1.66 (m, 6H). $^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$
(ppm) 172.38, 162.67, 61.01, 59.17, 55.39, 49.98, 38.14, 35.12, 28.19, 18.03, 25.14. MS (ESI+), m/z = 313 [M+H]+.

c. Synthesis of DA-PEG-alkyne (5)

Under N₂, DA-PEG-OH 4 (prepared according to Small 2011, 7, 2786) (300 mg, 0.128 mmol, 1 equiv.) was dissolved in dry THF (5 mL). NaH 60 % in oil (15 mg, 3 equiv.) was slowly added. The mixture was stirred for 30 min at room temperature and slowly turned to orange. Propargyl bromide (100 µL, 7 equiv.) was added slowly and the reaction mixture was stirred at room temperature for 22 h. The solution slowly turned to brownish. Water was then added and THF was evaporated under vacuum. The aqueous phase was extracted four times with CH₂Cl₂. Organic layers were dried over MgSO₄, filtered, and evaporated to dryness. The resulting solid was dissolved with CH₂Cl₂, precipitated into cold Et₂O and triturated in Et₂O. Compound 5 was obtained as a solid (214 mg, 70% yield).

¹H-NMR (400 MHz, CDCl₃) δ (ppm) 4.18–4.22 (d, J = 2.5 Hz, 2H), 3.42–3.81 (m, 180H), 3.41–3.49 (m, 2H), 2.42–2.45 (t, J = 2.3 Hz, 1H), 2.24 (t, J = 6.9 Hz, 4H), 1.45–1.61 (m, 6H), 1.22–1.36 (m, 28H), 0.88 (t, J = 6.6 Hz, 3H). MS (ESI+), m/z = 2403 [M+Na]+.

d. Synthesis of DA-PEG-biotin (6)

Compound 5 (50 mg, 0.021 mmol, 1 equiv.), biotin-N₃ (7.9 mg, 1.2 equiv.), ligand L (prepared according to J. Am. Chem. Soc. 2010, 132, 16893) (1 mg, 0.1 equiv.) and sodium ascorbate (4.2 mg, 1 equiv.) were introduced into a flask. Degassed water (65 µL) and degassed tBuOH (275 µL) were added, the mixture was sonicated in a bath. Triethanolamine in degassed water (60 µL of a 50 mg mL⁻¹ soln, 1 equiv.) and pentahydrated copper sulfate in degassed water (100 µL of a 5 mg mL⁻¹ soln, 0.1 equiv) were added. The reaction mixture was stirred under N₂ for 16 h at room temperature. The mixture was then washed with saturated NH₄Cl and extracted with CH₂Cl₂. The organic layer was washed with water and brine, dried over MgSO₄, filtered and evaporated to dryness. The crude was purified by column chromatography (pure CH₂Cl₂ to CH₂Cl₂/MeOH 9:1) to afford a solid corresponding to compound 6 (14 mg, 25% yield).

¹H-NMR (400 MHz, DMSO-d₆) δ (ppm) 8.04 (s, 1H), 7.96 (t, J = 5.6 Hz, 1H), 6.41 (s, 1H), 6.36 (s, 1H), 4.51 (s, 2H), 4.39 (t, J = 6.2 Hz, 2H), 4.27–4.34 (m, 1H), 4.11 (m, 1H), 3.41–3.72 (m, 182H), 3.08 (m, 1H), 2.82 (dd, J = 5.1, J = 12.2 Hz, 1H), 2.57–2.63 (m, 1H), 2.27 (t, J = 6.9 Hz, 4H), 2.02 (t, J = 7.5 Hz, 2H), 1.13–1.51 (m, 40H), 0.80–0.88 (m, 3H). MS (ESI+) m/z = 1347 [M+2H]⁺.
3. Micelle preparation and characterization

a. Preparation of micelles PDA-PEG-alkyne/PDA-PEG-OMe in variable ratio (10:90, 25:75, 50:50 and 0:100)

A mixture of DA-PEG-alkyne and DA-PEG-OMe amphiphiles in the desired molar ratio was solubilized into deionized water at a final 10 mg mL\(^{-1}\) concentration. The solution was sonicated with an ultrasonic probe (300 ms pulses per second, 25 W output power) for 30 min. The solutions were then subjected to UV irradiation (254 nm – low pressure mercury UV lamp – Heraeus) for 6 h. The volume of the solution was adjusted to the initial volume by adding deionized water. The solution was then filtered over a 0.2 µm nylon membrane to yield the PDA-micelles.

![DLS profile of pDA-PEG-OMe/alkyne (75:25) micelles.](image)

b. Post-functionalization of the polymerized micelles with biotin

The functionalization of micelles with 50% surface biotin is given as a representative example. Biotin-azide 3 (1.2 mg, 0.004 mmol, 1.3 equiv.), ligand (BimC4A)\(_3\) (0.3 mg, 0.0006 mmol, 0.2 equiv.), and Na-ascorbate (1.8 mg, 0.009 mmol, 3 equiv.) were introduced in a round-bottom flask. Micelles composed of DA-PEG-OMe/DA-PEG-alkyne 50:50 (1.5 mL of a 10 mg mL\(^{-1}\) colloid corresponding to 0.003 mmol of alkyne groups, 1 equiv.) were then added. The mixture was bath-sonicated to solubilize biotin-azide, and the solution was degassed. Triethanolamine (90 µL of a 5 mg mL\(^{-1}\) solution in degassed water, 0.45 mg, 0.003 mmol, 1 equiv.) and CuSO\(_4\) pentahydrate (0.08 mL of a 1 mg mL\(^{-1}\) solution in degassed water, 0.08 mg, 0.0003 mmol, 0.1 equiv.) were added to the solution. The mixture was stirred at room temperature under N\(_2\) for 16 h before 10 equiv. of HEDTA (0.6 mL of a 0.05 M solution, 8.35
mg, 0.03 mmol, 10 equiv.) was added. The reaction mixture was further stirred at room temperature for 24 h before micelles were purified by size exclusion chromatography over a NAP-25 column from GE-Healthcare. The micelles concentration was calculated according to the diluting factor after the purification. Absence of free biotin was confirmed by LCMS and full functionalization of the free alkynes was observed by $^1$H-NMR of the micelles in DMF-d$_7$.

(c. Encapsulation of DiO)

DiO (100 µg) in EtOH (20 µL) was added to the as prepared micelle colloid (1 mL, 10 mg mL$^{-1}$) and the mixture was sonicated (ultrasonic probe, 300 ms pulses per second, 25 W output power) for 3 × 10 min. The clear orange colloid was filtered through a 0.22 µm membrane.

d. Absorption/fluorescence profile of the micelles

![Figure S2](image_url)  
**Figure S2.** Absorption (blue curve) and emission (red curve, exc. 490 nm) of 1wt% DiO-loaded micelles (incorporating 25% biotine).
**4. Cell proliferation/survival assay**

MCF-7 (human breast adenocarcinoma cell line) cells were routinely maintained in RPMI (Sigma) supplemented with 10% (v/v) Fetal Bovine Serum (PAA), Penicillin (100 U/mL, Sigma) and Streptomycin (100µg/mL, Sigma). MCF-7 cells suspended in culture medium were seeded at a final concentration of 8000 cells/well into the wells of an optical 96-well culture plate (Costar Ref: 3904) pre-coated with rat tail collagen (Sigma). Plates were incubated for 4 h at 37 °C, 5% CO₂ to allow cell adhesion. Micelles (or PBS as vehicle) were diluted in OptiMEM (Invitrogen) to achieve the indicated final concentrations and layered on top of the culture wells. Plates were incubated for 72 h at 37 °C, 5% CO₂. Cells were then fixed by addition of Paraformaldehyde (4% [w/v] final, Sigma) and nuclear DNA was fluorescently labeled by Hoechst 33342 (1 µg mL⁻¹ final, Sigma). After an overnight incubation at 4 °C, supernatants were removed by aspiration, replaced by 100 µL PBS, and plates were imaged on a high-content imaging device (Operetta, Perkin Elmer) in the blue channel (Ex: 360–400 nm, Em: 410–480 nm, 10 x magnification, 9 fields acquired/well). Using a high-content imaging analysis software (Harmony 3.0, Perkin-Elmer), DNA-labeled nuclei were segmented, and the absolute amount of nuclei per condition was quantitated. Results are expressed as the relative amount of cells in micelle-treated wells relative to their vehicle treated controls.

![Graph](image)

**Figure S3.** Cell proliferation/survival assay of MCF-7 cells treated with pDA-PEG micelles incorporating 25% of biotin. For each experimental condition, results are expressed as the averaged amount of cells relative to its respective vehicle controls (3 replicates per condition). Student’s t-test: ** for p < 0.01 ; *** for p < 0.001.
5. **Cellular internalization assays**

MCF-7 cells derived from human breast adenocarcinoma were purchased from ATCC (Manassas, VA). They were grown at 37 °C with 5% CO₂ until approximately 80% of confluence in 12-well plates in RPMI 1640 GlutaMAX medium (ref 61870-010, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal calf serum (Thermo Fisher Scientific) and 1% antibiotic/antifungal solution (ref A5955, Sigma-Aldrich). After aspirating the culture media, adherent cells were washed with 1 mL of DPBS containing magnesium and calcium (ref 14040117, Thermo Fisher Scientific) before being incubated with 500 µL of the same DPBS supplemented with DiO-labeled micelles (100 µg mL⁻¹). The incubation was performed at 37 °C with 5% CO₂ during different times (30, 60, 90, 120, 180, 240 min). Then, the medium was removed and cells were washed two times with 1 mL of DPBS without magnesium and calcium (ref 14190136, Thermo Fisher Scientific). Finally, cells were detached from the plate by an incubation of 3 min at 37 °C with 100 µL of trypsin-EDTA solution (ref 25300054, Thermo Fisher Scientific) before being analyzed by flow cytometry on a BD Accuri C6 instrument (BD Biosciences). The analysis was performed by analyzing the fluorescence of living cells that was recovered with a 533/30 filter after excitation with a 488 nm laser.

For competition experiments in the presence of biotin, a similar protocol was used but cells were first pre-incubated for one hour with 500 µL of DPBS supplemented with free biotin (2 mM). The medium was aspirated and cells were incubated for 90 min in DPBS supplemented with both biotin (2 mM) and fluorescently labeled (DiO) micelles (100 µg mL⁻¹). All experiments were performed in triplicate.