Supplementary Material (ESI) for Chemical Communications

Preparation of Biohybrid Amphiphiles *via* the Copper Catalysed Huisgen [3 + 2] Dipolar Cycloaddition Reaction

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Supplementary information

1. Methods & Materials

General

Unless otherwise stated, all chemicals were obtained from commercial sources and used without further purification. Analytical thin layer chromatography (TLC) was performed on *Merck* precoated silica gel 60 F-254 plates (layer thickness 0.25 nm) with visualization by ultraviolet (UV) irradiation at λ = 254 nm and/or λ = 366 nm. Purifications by silica gel chromatography were performed using *Acros* (0.035 – 0.070 mm, pore diameter ca. 6 nm) silica gel. Excluding the biological procedures involving BSA all experiments were carried out under an argon or nitrogen atmosphere. Water used in the biological procedures was deionised using a *Labconco Water Pro PS* purification system. THF was distilled under nitrogen from sodium/benzophenone. BSA was obtained from *Sigma*.

Infrared red spectroscopy (IR spectrometry)

IR spectra were recorded on a *ATI Matson Genesis Series FTIR* spectrometer with a fitted ATR cell. The vibrations (ν) are given in cm^{-1}.

UV/vis spectroscopy

UV/vis spectra were measured on a *Varian Cary 50* spectrophotometer using a quartz cuvet.

Nuclear magnetic resonance (NMR)

NMR spectra were recorded on *Bruker DPX300* and *Varian inova 400* spectrometers. ^{1}H NMR chemical shifts (δ) are reported in parts per million (ppm) relative to a residual proton peak of the solvent, δ = 3.31 for CD_3OD, δ = 7.26 for $CDCl_3$ and δ = 2.74 for dimethylformamide-d_7. Multiplicities are reported as: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), dt (doublet of triplets), or m (multiplet). Broad peaks are indicated by the addition of br. Coupling constants are reported as a *J* value in Hertz (Hz). The number of

protons (n) for a given resonance is indicated as nH, and is based on spectral integration values. ^{13}C NMR chemical shifts (δ) are reported in ppm relative to CD_3OD (δ = 49.0).

Transmission electron microscopy (TEM)

TEM images were obtained using a *JEOL JEM 1010* microscope (60 kV) equipped with a CCD camera. Samples were prepared by placing a carbon coated copper grid on top of a droplet of aggregate solution. After 1 minute the excess of water was removed using a filter-paper. Subsequently, the grids were dried in high vacuum for 1 h. The structures were visualised using platinum shadowing at an angle of 45º (for PS-GlyGlyArg-AMC) or without further treatment (for PS-BSA). In case of PS-GlyGlyArg-AMC, the aggregate solution was prepared by injecting 0.2 ml of an amphiphile solution in THF (1 mg/ml concentration) in 1 ml of millipure water. For PS-BSA samples were prepared directly from the purified reaction mixture.

Scanning electron microscopy (SEM)

SEM was performed on a *JEOL JSM-6330F* instrument using the same samples as prepared for TEM. Before measurement a 1.5 nm layer of Pd/Au was sputtered on the grids by using a *Cressington 208 HR* sputter coater fitted with a *Cressington* layer thickness controller.

Size exclusion chromatography (SEC)

Molecular weight distributions were measured with a *Shimadzu* SEC, equipped with a guard column and a PL gel 5 μm mixed D column (*Polymer Laboratories)* with differential refractive index and UV (λ = 254 nm and λ = 330 nm) detection using either THF or $CHCl_3$ as an eluent (1 ml/min at 35°C). In both cases PS standards were used for calibration.

Fast performance liquid chromatography (FPLC)

Protein mixtures were analysed with a *Pharmacia SMART* high performance liquid chromatography (HPLC) system, equipped with a Superdex 75 PC 3.2/30 column (optimal separation 3 – 70 kDa) (*Pharmacia*) or Superose 12 PC 3.2/30 column (optimal separation 1 – 300 kDa) (*Pharmacia*) using phosphate buffer (20 mM, pH 7.2) as an eluent (50 μl/min at room temperature) in combination with UV detection (λ = 254 nm and λ = 280 nm).

Mass spectrometry (MS)

Electrospray LC/MS analysis was performed using a *Shimadzu LC/MS 2010A system*. Matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) spectra were measured on a *Bruker Biflex III* spectrometer and samples were prepared from $CHCl_3$ solutions using dithranol (20 mg/ml) as a matrix.

Gas chromatography (GC)

GC analysis was conducted on a *Hewlett/Packard 5890 Series II* gas chromatograph, equipped with capillary columns (HP1, 25 m * 0.32 m * 0.17 μm, HP1707, 25 m * 0.32 m * 0.25 μm), using flame/ionisation detection.

2. Synthesis

ω-Bromo-polystyrene (PS-Br)

Cu(I)Br (72.1 mg, 0.50 mmol) was placed in a Schlenk tube fitted with a stopper, evacuated and back-filled with argon. This procedure was repeated three times. After the evacuating cycles the stopper was replaced by a septum. Anisole (0.6 ml), *N,N,N',N''*-pentamethyl diethylenetriamine (PMDETA, 87.5 mg, 0.50 mmol) and styrene (3.11 g, 29.9 mmol) were added and the reaction mixture was cooled in an ice bath. Subsequently, 1-bromoethylbenzene (92.9 mg, 0.50 mmol) was added and the reaction mixture was purged with argon for five min and placed in a statically controlled oil bath at 90 °C. Samples were taken periodically for conversion analysis by gas chromatography. The polymerisation was stopped after 450 min (64% conversion) by cooling and exposing the catalyst to air. The reaction mixture was diluted with chloroform and washed with an aqueous ethylenediaminetetraacetic acid (EDTA) solution (0.065 M) in order to remove the copper catalyst. The organic layer was dried over anhydrous magnesium sulphate and concentrated under reduced pressure. The polymer was precipitated in methanol, yielding a white solid which was dried under vacuum.

^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 7.36-6.38 (br. m, arom H), 4.48 (br. m, CH_2-C*H*(Ph)-Br), 2.17-1.24 (br. m, backbone C*H*$_2$, C*H*), 1.11-1.02 (br. s, *H*$_3$C-CH(Ph)-CH_2).

FTIR-ATR/cm^{-1} : 3019, 2925, 2837, 2008, 1943, 1865, 1796, 1597, 1493, 1450.

SEC (THF): M_n = 4.15 kDa, PDI = 1.15.

*ω-Azido-polystyrene (PS-N_3, **1**)*

To a solution of PS-Br (1.83 g, 0.44 mmol) in THF (10 ml) were added azidomethylsilane (445.2 mg, 3.86 mmol) and TBAF (4 ml of 1 M solution in THF, 4 mmol). The reaction mixture was stirred overnight at room temperature under a nitrogen atmosphere and the polymer was precipitated in methanol, yielding a white solid which dried under vacuum (yield 1.72 g 94%).

^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 7.36-6.38 (br. m, arom H), 3.91 (br. m, CH_2-C*H*(Ph)-N_3), 2.17-1.24 (br. m, backbone C*H*$_2$, C*H*), 1.11-1.02 (br. s, *H*$_3$C-CH(Ph)-CH_2).

FTIR-ATR/cm^{-1} : 3019, 2925, 2837, 2090 (ν N_3), 2008, 1943, 1865, 1796, 1597, 1493, 1450.

SEC (THF): M_n = 4.15 kDa, PDI = 1.15.

MALDI-TOF MS: see Figure S3A.

*N-butyncarbamate-Gly-Gly-Arg-AMC (**3**)*

To a solution of H-Gly-Gly-Arg-AMC · 2HCl (258.5 mg, 0.5 mmol) in a H_2O/dioxane mixture (5:1, v/v) was added 2M NaOH until the pH reached 9-10. Under a nitrogen atmosphere butyn-chloroformate (62.3 µL 0.55 mmol) was added and the mixture was allowed to stir for 5 hrs at room temperature. Completion of the reaction was monitored by TLC: BuOH/H_2O/AcOH (4:1:1) rf = 0.5. The product was lyophilised and purified by countercurrent chromatography using a BuOH/H_2O system. After purification **3** was obtained as a white solid (yield 185 mg, 63%).

^{1}H NMR: (MeOD, 400 MHz) δ = 7.78 (d, *J* = 2.0 Hz, 1H, arom H AMC) 7.62 (d, *J* = 8.8 Hz 1H, arom H AMC) 7.47 (dd, *J* = 1.9 Hz, 1H, arom H AMC) 6.15 (d, *J* = 1.2 Hz, 1H, (CH_3)C=C*H* AMC) 4.47 (q, *J* = 4.4 Hz, 1H, α-C*H* arginine) 4.03 (dt, 2H CH_2-C*H*$_2$-O-CO) 3.88-3.76 (q, *J* = 12.7 Hz, 2H C*H*$_2$ glycine) 3.71 (s, 2H, C*H*$_2$

glycine) 3.14 (dt, 2H, CH_2-CH_2-C<u>*H*</u>$_2$-NH) 2.41 (dt, 2H, C≡C-C<u>*H*</u>$_2$-CH_2) 2.36 (s, 3H, (C<u>*H*</u>$_3$)C=CH AMC) 2.19 (t, 1H, <u>*H*</u>C≡C-CH_2), 1.93-1.88 (m, 1H, C<u>*H*</u>$_2$-CH_2-CH_2-NH) 1.80-1.71 (m, 1H, C<u>*H*</u>$_2$-CH_2-CH_2-NH) 1.70-1.55 (m, 2H, CH_2-C<u>*H*</u>$_2$-CH_2-NH).

^{13}C NMR (200 MHz, MeOD) δ = 173.50, 172.50, 172.04, 163.19, 159.26, 158.58, 155.23, 143.16, 126.67, 117.30, 113.70, 108.09, 80.98, 71.13, 64.44, 54.98, 45.20, 43.86, 41.97, 29.97, 26.40, 19.89, 18.56.

LC-MS: +99% purity, *m/z* = calc 542.56 (M+H), *m/z* = found 543 (M+H).

*PS-GlyGlyArg-AMC (**4**) via cycloaddition reaction between **1** and **3***

N-butyncarbamate-Gly-Gly-Arg-AMC (6.4 mg, 0.011 mmol) and PS-N_3 (40.2 mg, 0.010 mmol) were placed in a Schlenk vessel. After the vessel was evacuated and back-filled with argon for three times the materials were suspended in THF (0.5 ml). Cu(I)Br (3.2 mg, 0.022 mmol) and PMDETA (5.4 μL, 0.023 mmol) were dissolved in THF (0.5 ml) and argon purging for 5 min prior to addition to the vessel. The mixture was stirred under an argon atmosphere for 18 hrs and from TLC: CH_2Cl_2 (PS rf = 0.95, tripeptide **3** rf = 0.05 and PS-GlyGlyArg-AMC stripes from bottom to front) it was judged that the reaction almost completed. Some Cu(0) was added and the mixture was allowed to stir under an argon atmosphere for another 18 hrs. After dilution with CH_2Cl_2 the mixture was washed twice with an aqueous EDTA solution (0.065 M). The organic phase was concentrated under reduced pressure and filtered over silica gel using CH_2Cl_2 as the eluent (ca. 10 ml). Upon addition of MeOH a milky substance was obtained which was concentrated under reduced pressure yielding an off-white solid (isolated yield 24 mg, 51 %). Note: the moderated yield is due to the difficult isolation of the product.

^{1}H-NMR (DMF-d_7, 400 MHz): δ = 7.40-6.20 (br. m, arom H), 6.10 (br. s, (CH_3)C=C<u>*H*</u> AMC), 5.18 (br. m, triazole-C<u>*H*</u>(Ph)-CH_2), 4.09 (br. m, CH_2-C<u>*H*</u>$_2$-O-CO), 2.27 (br. s, (C<u>*H*</u>$_3$)C=CH AMC) 2.20-1.20 (br. m, backbone C<u>*H*</u>$_2$, C<u>*H*</u>), 1.00-0.90 (br. s, <u>*H*</u>$_3$C-CH(Ph)-CH_2).

SEC ($CHCl_3$, UV detection λ = 330 nm): M_p = 3.9 kDA, PDI > 1.45. (Figure S2).

MALDI-TOF MS: see Figure S3B.

*Alkyne functionalised bovin serum albumin (BSA) **6***

To a 10 ml plastic culture tube was added 1 ml of BSA solution (in 20 mM phosphate buffer (PB) pH 7.2, 1 μmol) and 0.6 ml of *N* alkyne functionalised maleimide solution (in 20 mM PB pH 7.2, 63 μmol). The reaction mixture was shaken for 18 hrs with exclusion of light. Subsequently, the mixture was dialysed against 20 mM PB pH 7.2 (200 ml) for 12 hrs (again with exclusion of light) using a molecular weight cut-off (MWCO) of 12 – 14 kDa. This procedure was repeated twice and the mixture was stored at 4 °C.

*Cycloaddition reaction between **6** and coumarin dye **7** affording **8***

To a 10 ml plastic culture tube was added 2 ml of **6** solution (in 20 mM PB pH 7.2, 0.63 μmol), 400 μl of **1** solution (in THF, 29.8 μmol), 1 ml of catalyst solution (in 20 mM PB pH 7.2, 4.0 μmol $CuSO_4 \cdot 5H_2O$, 20 μmol ascorbic acid) and 100 μl of 20 mM PB pH 7.2 affording a final volume of 3.5 ml (11 %) THF/PB. The reaction mixture was shaken for 18 hrs and then dialysed against 20 mM PB pH 7.2 (200 ml) for 12 hrs using a MWCO of 1000 Da. Subsequently, the mixture was passed over a sephadex G75 column for further purification.

*Cycloaddition reaction between **6** and PS-N_3 (**1**) affording **9***

To a 10 ml plastic culture tube was added 1 ml of **6** solution (in 20 mM PB pH 7.2, 0.63 μmol), 400 μl of **1** solution (in THF, 31.5 μmol), 1 ml of catalyst solution (in 20 mM PB pH 7.2, 4.0 μmol $CuSO_4 \cdot 5H_2O$, 20 μmol ascorbic acid) and 100 μl of 20 mM PB pH 7.2 affording a final volume of 2.5 ml (16 %) THF/PB. The reaction mixture was shaken for 24 hrs. Subsequently, the mixture was diluted with THF (1 ml) and dialysed against 20 mM PB pH 7.2 (200 ml) for 24 hrs using a MWCO of 66 kDa. Finally, the mixture was dialysed against 20 mM PB pH 7.2 (200 ml) for 1 week at 4 °C (again using a MWCO of 66 kDa), yielding a white precipitate and a slightly turbid phase. SEC analysis confirmed that the precipitate was formed by the excess of PS. The slightly turbid phase was used for further characterization (FPLC and TEM).

3. Characterization

*FPLC analysis of BSA-PS (**9**)*

The crude reaction mixture of the click reaction between PS-N_3 (**1**) and alkyne functionalised BSA (**6**) was analysed by FPLC using a Superdex 75 column (Figure 2, article). Further characterisation of the purified product (**9**) was carried out using a Superose 12 column which has a broader range of separation (Figure S1).

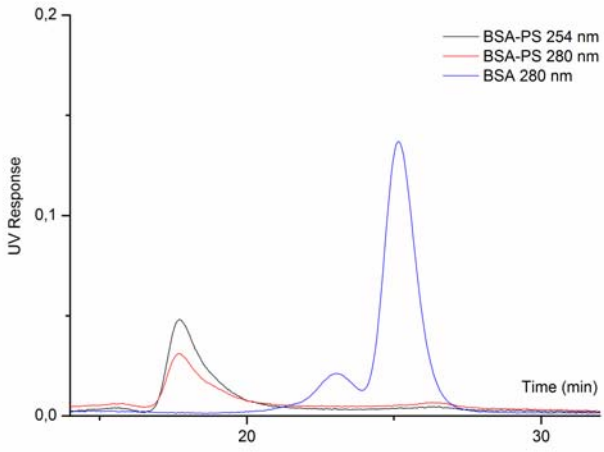

Figure S1 FPLC analysis of BSA-PS after purification using PB 20 mM pH 7.2 as an eluent.

*SEC analysis of PS-GlyGlyArg-AMC (**4**)*

In Figure S2 SEC traces of PS-N_3 and PS-GlyGlyArg-AMC at λ = 254 nm and λ = 330 nm are depicted. PS-N_3 clearly shows no absorption at λ = 330 nm while for PS-GlyGlyArg-AMC the opposite is true. For the latter, however, the molecular weight distribution was broadened and slightly shifted to lower molecular weight. This change might be attributed to the amphiphilic character of the product causing a different hydrodynamic volume with respect to the apolar PS-N_3.

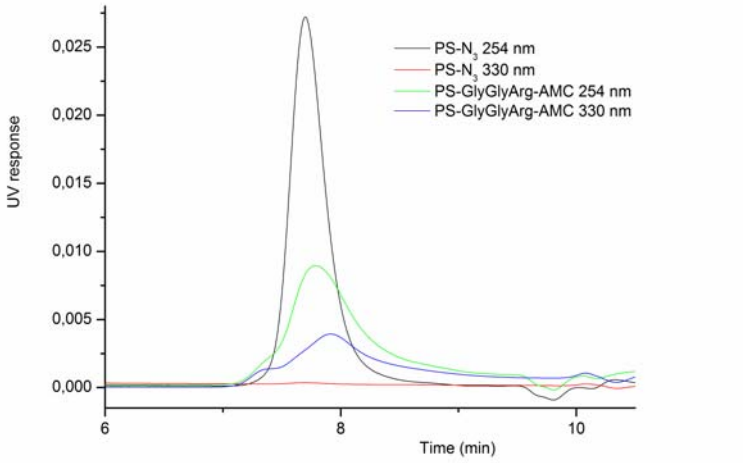

Figure S2 *SEC ($CHCl_3$) traces of PS-N_3 and PS-GlyGlyArg-AMC at λ = 254 nm and λ = 330 nm.*

*MALDI-TOF analysis of PS-N_3 (**1**) and PS-GlyGlyArg-AMC (**4**)*

MALDI-TOF spectra of both PS-N_3 and PS-GlyGlyArg-AMC are presented in Figure S3. Although PS-GlyGlyARg-AMC was much easier characterised than PS-N_3, it can clearly be observed that the molecular weight distribution of the biohybrid (Figure S3B) is shifted upward with respect to starting material (Figure S3A).

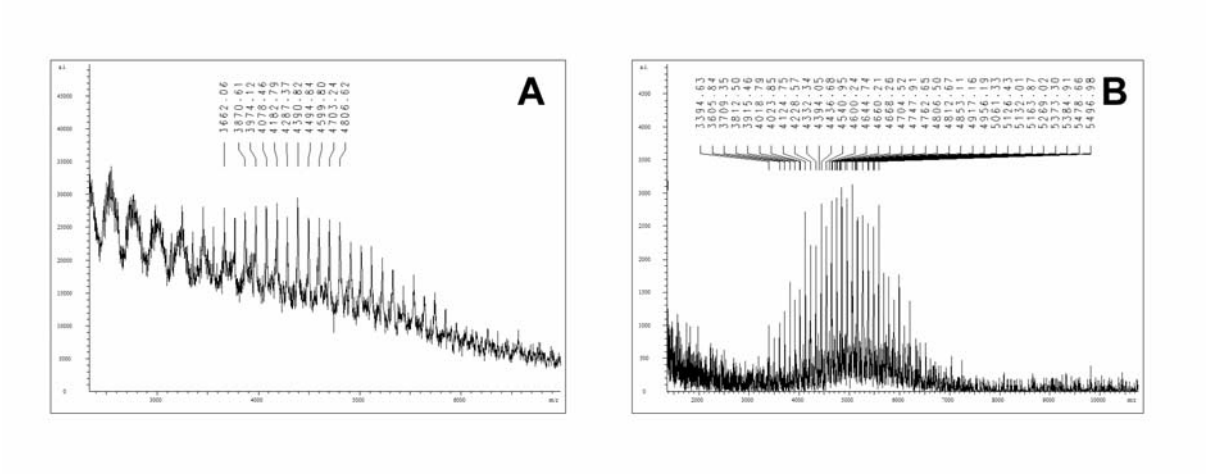

Figure S3 *MALDI-TOF spectra of PS-N_3 (A) and PS-GlyGlyArg-AMC (B).*

In principle the mass of a single polymer chain can be defined as the sum of masses of the end groups and the number (N) of repeating units. Therefore, the mass of the end groups can be derived from a molecular weight distribution by plotting the molecular mass against N. Consequently, the intercept (N = 0) affords the mass of the end groups. Prior knowledge to the likely nature of these end groups gives an appropriate choice of N, which in case of the obtained PS-GlyGlyArg-AMC was defined as 26 for the lowest peak assigned. Using this peak as a

reference also N for the other peaks was determined and a plot of mass versus N was constructed. Linear regression (using *Origin 6.1* software) of this dataset resulted in the following equation:

*Mass = 104.07 * N + 689.98*

Herein 104.07 nicely corresponds to the mass of one repeating unit of polystyrene (calc 104.06) and the intercept of 689.98 is in line with the initiator (calc 105.07) plus the GlyGlyArg-AMC end group (calc 583.24) and an additional proton.

*UV spectroscopy of BSA-coumarin (**8**)*

The UV spectra of purified BSA-coumarin (**8**) and alkyne functionalised BSA (**6**) are depicted in Figure S4. Furthermore, UV spectra of purified mixtures of blank experiments are included. These blank experiments were carried out under similar conditions as the previously described cycloaddition between BSA and coumarin dye but leaving out either the catalyst or dye. For click product **8** the absorbance at λ = 330 nm (maximum absorption of the coumarin dye) is significantly higher than for the starting material (**6**) and blank experiments.

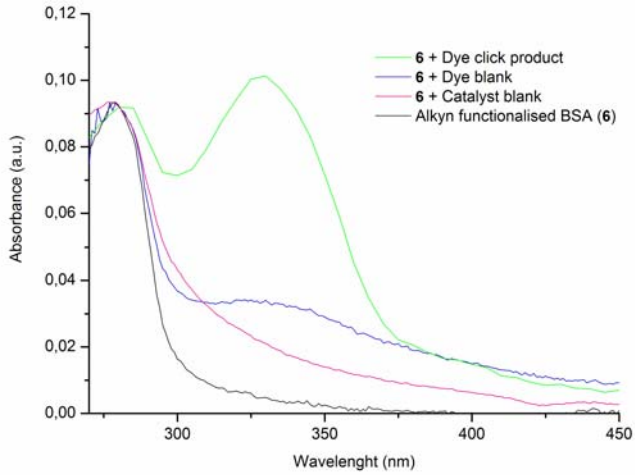

Figure S4 *UV/vis spectra of alkyne functionalised BSA (**6**), **6** + coumarin dye click product, **6** + Dye blank and **6** + catalyst blank experiments.*