Supporting Information

"Inverse" Synthesis of Polymer Bioconjugates using Soluble Supports.

Anna Meszynska[1], Nezha Badi[1], Hans Börner[2] and Jean-François Lutz[1*]

[1] Precision Macromolecular Chemistry Group, Institut Charles Sadron, UPR-22 CNRS, BP 84047, 23 rue du Loess, 67034 Strasbourg Cedex 2, France

[2] Laboratory of Organic Synthesis of functional Systems, Humboldt-Universität zu Berlin, Brook-Taylor-Str. 2, 12489 Berlin, Germany

*E-Mail: jflutz@unistra.fr

Content:

A. Experimental procedures.

A.1. Chemicals. 5-(Fmoc-amino)-1-pentanol (Aldrich, 98%), triethylamine (Aldrich, 99.5%), 2-bromoisobutyrylbromide (Alfa Aesar, 97%), 3-(Fmoc-amino)-1-propanol (Iris Biotech, 98%), 4-dimethylaminopyridine (DMAP, Acros Organics, 99%), sodium sulphate anhydrous (Na_2SO_4, SDS, 99.6%), 4,4'-di-*n*-nonyl-2,2'-bipyridine (Alfa Aesar, 97%), piperidine (Sigma-Alrdich, 99%), 4-hydroxymethylphenoxyacetic acid (Novabiochem/Merck, 97%), *N*-hydroxy succinimide (Aldrich, 98%), *N*,*N*-dicyclohexylcarbodiimide (DCC, Aldrich, 99%), Fmoc-Gly-OH (Novabiochem/Merck, 98%), Fmoc-L-Lys(Boc)-OH (Polypeptide Lab., 99%), Fmoc-L-Lys(Mtt)-OH (Polypeptide Lab., 98.5%), Fmoc-Tyr(tBu)-OH (Novabiochem/Merck, 100%), Fmoc-Tyr(2-ClTrt)-OH (Novabiochem/Merck, 98%), *N*,*N*-diisopropylethylamine (Sigma-Aldrich, >99%), (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP, Novabiochem/Merck, 99%), trifluoroacetic acid (Sigma-Aldrich, 99%), methanol (Carlo Erba, 99.9%), dichloromethane (DCM, Carlo Erba, 99.9%), dry DCM (Sigma-Aldrich, anhydrous, with amylene), dimethylformamide (DMF, Sigma-Aldrich, >99%), tetrahydrofuran for SEC (THF, Sigma Aldrich, 99.8% HPLC, stabilizer-free) and *N*-Methyl-2-pyrrolidone for SEC (NMP, Sigma Aldrich, CHROMASOLV® Plus ≥99% HPLC), lithium bromide (LiBr, Sigma-Aldrich, ≥99.0%, dried material) were used as received. THF (99%, Aldrich, stabilized with BHT) was dried and distilled over sodium-benzophenone. Copper-(I)-bromide (Sigma-Aldrich, 98%) was washed with glacial acetic acid in order to remove any soluble oxidized species, filtered, washed with ethanol, and dried. Styrene (Sigma-Aldrich, 99%) was distilled over calcium hydride under vacuum and stored under argon at -15 °C before use.

A.2. Synthesis of ATRP initiator 1. 2.5 g of 3-(Fmoc-amino)-1-propanol (8.2 mmol) and 0.2 g of DMAP (1.65 mmol) were dissolved in 100 mL dry THF in a 250 mL-two neck round bottom flask and cooled down to 0 °C. 2.87 mL of triethylamine (20.6 mmol) was then added to the mixture. Subsequently, 2.55 mL of 2-bromoisobutyrylbromide (20.6 mmol) was added dropwise to the solution. The experimental mixture was slowly allowed to warm up to room temperature and stirred for 22 h. After reaction, triethylamine hydrobromide was removed by filtration and the solvent by rotary evaporation at 40 °C. The crude reaction mixture was mixed with water (100 mL, sodium chloride saturated) and extracted with dichloromethane (100 mL and 2 x 50 mL). The organic phases were combined, washed with water (20 mL), dried over Na_2SO_4, and concentrated on rotavapor. The product was purified by column chromatography using *n*-hexane/ethyl acetate (volume ratio 4:1) as eluent. Yield: 84%. ^{1}H NMR (400 MHz, $CDCl_3$): δ(ppm) = 1.26 (t, 2H, NH-CH_2-$\underline{CH_2}$-CH_2-O), 2.05 (s, 6H, CH_3), 3.32 (m, 2H, $\underline{CH_2}$-NH), 4.26 (m, 2H, CH_2-$\underline{CH_2}$-O), 4.41 (d, 1H, $\underline{\text{Fmoc (cyclopentane)}}$-$CH_2$-O), 5.05 (broad s, 2H, CH (cyclopentane)-$\underline{CH_2}$-O), 7.32 (t, 2H, 2H aromatic Fmoc I) 7.40 (t,

2H, 2H aromatic Fmoc II) 7.59 (d, 2H, aromatic Fmoc III), 7.76 (d, 2H, aromatic Fmoc IV). ^{13}C NMR (400 MHz, $CDCl_3$): δ(ppm) = 30.7 (1C, NH-CH_2-$\underline{CH_2}$-CH_2-O), 32.5 (2C, CH_3), 38.0 (1C, $\underline{CH_2}$-NH), 47.3 (1C, Fmoc-CH_2-O), 55.8 (1C, $\underline{C}$-(CH_3)Br), 63.7 (1C, CH_2-$\underline{CH_2}$-O), 66.6 (1C, Fmoc-$\underline{CH_2}$-O), 120.0 (2C, aromatic Fmoc I), 124.8, 127.0, 127.7 (6C, aromatic Fmoc II-IV), 141.3, 144.0 (4C, Fmoc cyclopentane), 156.4 (1C, CO-NH), 171.7 (1C, CO-C-(CH_3)Br).

A.3. Synthesis of ATRP initiator 2. 2.5 g of 5-(Fmoc-amino)-1-pentanol (7.7 mmol) was dissolved in 100 mL of dry THF in a 250 mL-two neck round bottom flask and cooled down to 0 °C. 3.21 mL of triethylamine (23 mmol) was then added to the mixture. Subsequently, 2.85 mL of 2-bromoisobutyrylbromide (23 mmol) was added dropwise to the solution. The experimental mixture was slowly allowed to warm up to room temperature and stirred for 20 h. After reaction, the solution was filtered to remove the triethylamine hydrobromide and concentrated by rotary evaporation at 40 °C. The crude reaction mixture was mixed with water (100 mL, sodium chloride saturated) and extracted with dichloromethane (1 x 100 mL and 2 x 50 mL). The organic phases were combined, washed with water (20 mL), dried over Na_2SO_4, and concentrated on rotavapor. The product was purified by column chromatography using *n*-hexane/ethyl acetate (volume ratio 4:1) as eluent. Yield: 44%. ^{1}H NMR (400 MHz, $CDCl_3$): δ(ppm) = 1.38 (m, 2H, NH-CH_2-CH_2-$\underline{CH_2}$-CH_2-CH_2-O), 1.51 (m, 2H, NH-CH_2-$\underline{CH_2}$-CH_2-CH_2-CH_2-O), 1.66 (m, 2H, NH-CH_2-CH_2-CH_2-$\underline{CH_2}$-CH_2-O), 1.90 (s, 6H, CH_3), 3.15 (m, 2H, $\underline{CH_2}$-NH), 4.13 (t, 2H, CH_2-$\underline{CH_2}$-O), 4.34 (m, 1H, Fmoc (cyclopentane)-CH_2-O), 4.75 (broad s, 2H, CH (cyclopentane)-$\underline{CH_2}$-O), 7.28 (t, 2H, 2H aromatic Fmoc I) 7.33 (t, 2H, 2H aromatic Fmoc II) 7.53 (d, 2H, aromatic Fmoc III), 7.70 (d, 2H, aromatic Fmoc IV). ^{13}C NMR (400 MHz, $CDCl_3$): δ(ppm) = 28.0 (1C, NH-CH_2-CH_2-$\underline{CH_2}$-CH_2-CH_2-O), 29.5 (1C, NH-CH_2-$\underline{CH_2}$-CH_2-CH_2-CH_2-O), 30.7 (1C, NH-CH_2-CH_2-CH_2-$\underline{CH_2}$-CH_2-O). 32.3 (2C, CH_3), 40.2 (1C, $\underline{CH_2}$-NH), 47.3 (1C, Fmoc-CH_2-O), 56.0 (1C, $\underline{C}$-(CH_3)Br), 65.7 (1C, CH_2-$\underline{CH_2}$-O), 66.5 (1C, Fmoc-$\underline{CH_2}$-O), 120.0 (2C, aromatic Fmoc I), 125.0, 127.0, 127.7 (6C, aromatic Fmoc II-IV), 141.3, 144.0 (4C, Fmoc cyclopentane), 156.4 (1C, CO-NH), 171.7 (1C, CO-C-(CH_3)Br).

A.4. Synthesis of cleavable support P4.

A.4.1. ATRP (P1). Mixture of **1** (1 g, 1 Eq.), copper (I) bromide (0.34 g, 1 Eq.) and 4,4'-Di-n-nonyl-2,2'-bipyridine (1.95 g, 2 Eq.) were added into a 25 mL round bottom flask equipped with a magnetic stirrier and sealed with a rubber septum. The mixture was degassed and purged with dry argon for a few minutes. Then, degassed styrene (10.9 mL, 40 Eq.) was added using a degassed syringe and the flask was introduced into an oil bath thermostated at 110 °C. After 5 h and 15 min of reaction, the polymer was precipitated in methanol. The precipitate was collected by filtration, washed with methanol and dried in a vacuum oven at room

temperature overnight. The purified polymer **P1** was characterized by ^{1}H NMR in $CDCl_3$ and SEC in THF (M_n = 4095 g·mol^{-1}, M_w/M_n = 1.16).

A.4.2. Deprotection of Fmoc group (P2). Polymer **P1** (8.8 g) was dissolved in a mixture of piperidine/DCM (1/1, 20 mL) and the solution was stirred for 1.5 h at RT. After the reaction, the polymer was precipitated in methanol. The precipitate was collected by filtration, washed with methanol and dried in a vacuum oven at room temperature overnight. The deprotected polymer **P2** was characterized by ^{1}H NMR in $CDCl_3$.

A.4.3. Attachment of Wang linker (P3). Polymer **P2** (7.5 g, 1 Eq), 4-hydroxymethylphenoxyacetic acid (0.5 g, 1.5 Eq.), *N*-hydroxy succinimide (0.25 g, 1.2 Eq.), dicyclohexylcarbodiimide (0.42 g, 1.1 Eq), and dry THF (100 mL) were added into a 250 mL round bottom flask. The mixture was stirred for 21 h at RT. After reaction, the mixture was filtrated and the polymer was precipitated in methanol. The precipitate was collected by filtration, washed with methanol and dried in vacuum oven at room temperature overnight. The purified polymer **P3** was characterized by ^{1}H NMR in $CDCl_3$ and SEC in THF (M_n = 4127 g·mol^{-1}, M_w/M_n = 1.13).

A.4.4. Loading of glycine (P4). Fmoc-Glycine (1 g, 10 Eq.) was dissolved in 10 mL dry DCM (several drops of DMF were added in order to complete dissolution). DCC (0.34 g, 5 Eq.) was then added to the solution and the mixture was stirred for 15 min at 0 °C. Next, P3 (1.36 g, 1 Eq.) and DMAP (0.004 g, 0.1 Eq.) were added and the reaction mixture was stirred for 1 h at RT. After reaction, dicyclohexylurea was removed by filtration and the polymer was precipitated in methanol. The precipitate was collected by filtration, washed with methanol and dried in a vacuum oven at room temperature overnight. The polymer support loaded with glycine was characterized by ^{1}H NMR in $CDCl_3$ and SEC in THF (M_n = 4471 g·mol^{-1}, M_w/M_n = 1.10).

A.5. Synthesis of permanent support P5. Mixture of **2** (0.2 g, 1 Eq.), copper (I) bromide (0.063 g, 1 Eq.) and 4,4'-di-*n*-nonyl-2,2'-bipyridine (0.36 g, 2 Eq.) were added into a 25 mL round bottom flask equipped with a magnetic stirrer and sealed with a rubber septum. The mixture was degassed and purged with dry argon for few minutes. Then, degassed styrene (2 mL, 40 Eq.) was added using a degassed syringe and the flask was introduced into an oil bath thermostated at 110 °C. After 6 hours of reaction, the polymer was precipitated in methanol The precipitate was collected by filtration, washed with methanol and dried in a vacuum oven at room temperature overnight. The purified polymer was characterized by ^{1}H NMR in $CDCl_3$ and SEC in THF (M_n = 4390 g·mol^{-1}, M_w/M_n = 1.18).

A.6. General procedure for peptide coupling. The present example corresponds to coupling of Fmoc-Lys(Mtt)-OH to the polymer support loaded with glycine (P4) and can be understood

as a general procedure for peptide coupling. The Fmoc-protected PS-*b*-Gly polymer (0.975 g, 1 Eq) was dissolved in 4 mL of piperidine/DCM (1/1) and the mixture was stirred for 15 min at RT. The deprotected polymer was then precipitated in methanol. The precipitate was collected by filtration, washed with methanol and dried in vacuum oven at room temperature for 2 h. Next, the deprotected polymer, Fmoc-Lys(Mtt)-OH (0.681 g, 5 Eq.), PyBOP (0.556 g, 4.9 Eq.) and DIPEA (0.38 mL, 10 Eq.) were dissolved in DCM (several drops of DMF were needed in order to complete dissolution) and the mixture was stirred for 2 h at room temperature. The polymer was then obtained following the same procedure as after Fmoc deprotection. The polymer was characterized by ^{1}H NMR in $CDCl_3$ and SEC in THF after each step of new amino acid coupling.

A.7. Cleavage of the peptide from the cleavable support. 0.1 g of PS-*b*-Gly-Lys(Mtt)-Tyr(2-ClTrt)-Gly-Lys(Mtt)-Tyr(2-ClTrt)-OH was treated with 1 mL of TFA/CH_2Cl_2 mixture (1/1) for 4 h at RT. After reaction, the polymer support was isolated by selective precipitation in methanol. The precipitate was collected by filtration and the remained solution was then removed by rotavapor, giving the peptide as a result. The polymer support was characterized by ^{1}H NMR in $CDCl_3$ and SEC in THF (M_n = 4785 g·mol^{-1}, M_w/M_n = 1.28), and the formation of the desired peptide sequence was determined by ESI-MS (exact mass of the peptide: 714.3701 g/mol; m/z found: $[M-H]^-$ = 713.383 g/mol).

A.8. Selective deprotection of the side groups. 0.1 g of PS-*b*-peptide containing a cleavable linker was treated with 1 mL TFA/CH_2Cl_2 mixture (3/97) for 0.5 h at RT. In parallel, 0.1 g PS-*b*-peptide containing a non-cleavable linker was treated with 1 mL TFA/CH_2Cl_2 mixture (1/1) for 4 h at RT. After the reactions, the deprotected PS-*b*-peptide copolymers were isolated by selective precipitation in methanol. The precipitates were collected by filtration, washed with methanol and dried in a vacuum oven at room temperature overnight. The deprotected copolymers were characterized by ^{1}H NMR in $CDCl_3$ and SEC in NMP (PS-*b*-peptide containing a cleavable linker: M_n = 5043 g·mol^{-1}, M_w/M_n = 1.16) and in THF (PS-*b*-peptide containing a non-cleavable linker: M_n = 5443 g·mol^{-1}, M_w/M_n = 1.22).

A.9. Solid-phase automated synthesis of the control peptide GKYGKY. The peptide synthesis was performed on an Applied Biosystems ABI 433a peptide synthesizer in a 0.1 mmol scale, using a Gly preloaded Wang-PS resin as solid support. Fmoc-amino acid derivatives were coupled following standard ABI-Fastmoc protocols (single coupling, no capping) in NMP facilitated by HBTU/DIPEA. After final Fmoc removal the resin was washed with NMP and DCM and dried overnight under vacuum at 25 °C. Cleavage of the control peptide from the resin was performed with TFA/water/TES 90:9:1 v/v for 2 h, to obtain the fully deprotected peptide. The peptide was isolated by diethylether precipitation,

centrifugation, and lyophilization from water. Raw product: 153 mg; yield = 85%. Molecular characterization was performed by MALDI-TOF-MS. m/z 715.350 $[M+H]^+$ (100%); 737.338 $[M+Na]^+$ (2%); and 772.382 (4%) $[M+tBu+H]^+$.

B. Measurements and analysis.

B.1. Size exclusion chromatography (SEC).

B.1.1. SEC in THF. Molecular weights and molecular weight distributions were determined using a SEC system equipped with a Shimadzu RiD_10A refractive index detector and five PLgel 10µ Mixed-B columns. The mobile phase was THF with a flow rate of 1 $mL \cdot min^{-1}$ using a Shimadzu LC20AD pump. Toluene was used as internal reference. The molecular weight calibration was based on sixteen narrow molecular weight linear polystyrene standards from Polymer Laboratories.

B.1.1. SEC in NMP. Molecular weights and molecular weight distributions of deprotected PS-*b*-oligopeptide molecules were determined using a SEC system equipped with viscometer, RD and RALS (90 °) detector (TDA Viscotek) and three PLgel 10µ Mixed-B columns. The mobile phase was NMP with 0.1M LiBr with a flow rate of 0.5 $mL \cdot min^{-1}$ at 60 °C using a Shimadzu LC20AD pump. Toluene was used as internal reference. The molecular weight calibration was based on fourteen narrow molecular weight linear polystyrene standards from Polymer Laboratories.

B.2. Nuclear Magnetic Resonance (NMR). ^1H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ or in CD_3OD on either a Bruker Avance 400 MHz or on a Bruker Avance 600 MHz spectrometers equipped with Ultrashield magnets.

B.3. Electrospray Liquid Chromatography Mass Spectrometry (ESI-LC-MS). ESI-LC-MS was carried out on a Waters Acquity UPLC-SQD apparatus equipped with a PDA detector (190-500 nm, 80Hz), using a reverse phase column (Waters, BEH C18 1.7 mm, 2.1mm x 50 mm) and the MassLynx 4.1 – XP software. The mobile phase was methanol/water with 0.005% ammonia, and a solution of the peptide in methanol was prepared for the analysis.

B.4. MALDI-TOF Mass Spectrometry. Mass measurements were carried out on an Ultraflex™ MALDI-TOF/TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). This instrument was used at a maximum accelerating potential of 25 kV in positive mode and was operated in mode reflector at 26 kV. The delay extraction was fixed at 110 ns and the frequency of the laser (nitrogen 337 nm) was set at 20 Hz. The acquisition mass range was set to 400-800 m/z with a matrix suppression deflection (cut off) set to 400 m/z. The

equipment was first externally calibrated with a standard peptide calibration mixture that contained 7 peptides (Bruker Peptide Calibration Standard #206196, Bruker Daltonics GmbH, Bremen, Germany). Sample preparation was performed with the dried droplet method using a mixture of 0.5 µl of sample with 0.5 µl of matrix solution dry at room temperature. The first matrix solution was prepared from a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) in water/acetonitrile 50/50 diluted three times in water/acetonitrile/trifluoroacetic acid 50/49.9/0.1.

C. Additional data and Figures.

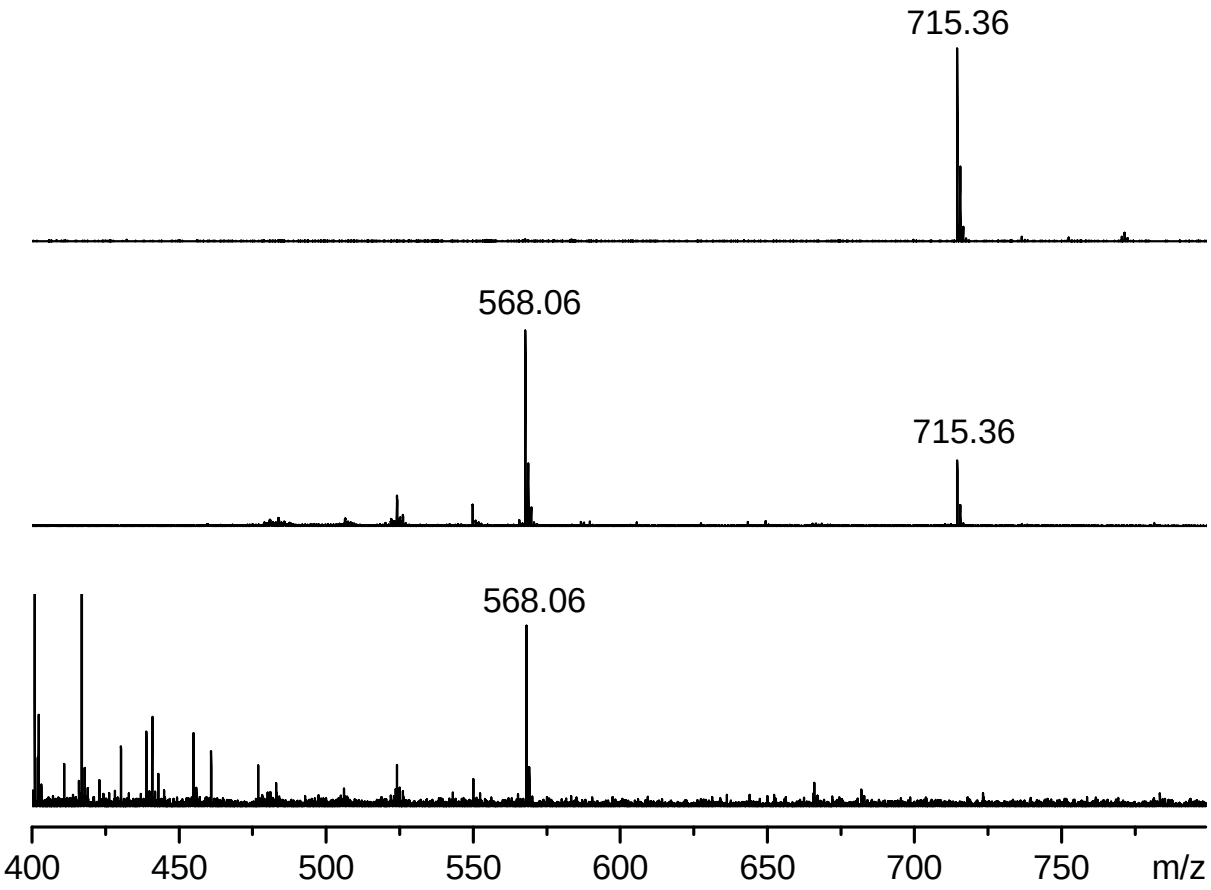

Figure S1. MALDI-TOF spectra recorded for the hexapeptide GKYGKY ($[M+H]^+$, m/z = 715.36) prepared on a solid support (top) or on the soluble support **P4** (middle). The bottom spectrum was recorded for the bare CHCA matrix. The peak at m/z = 568.06 is due to the matrix and is a consequence of a low concentration of the analyzed sample.

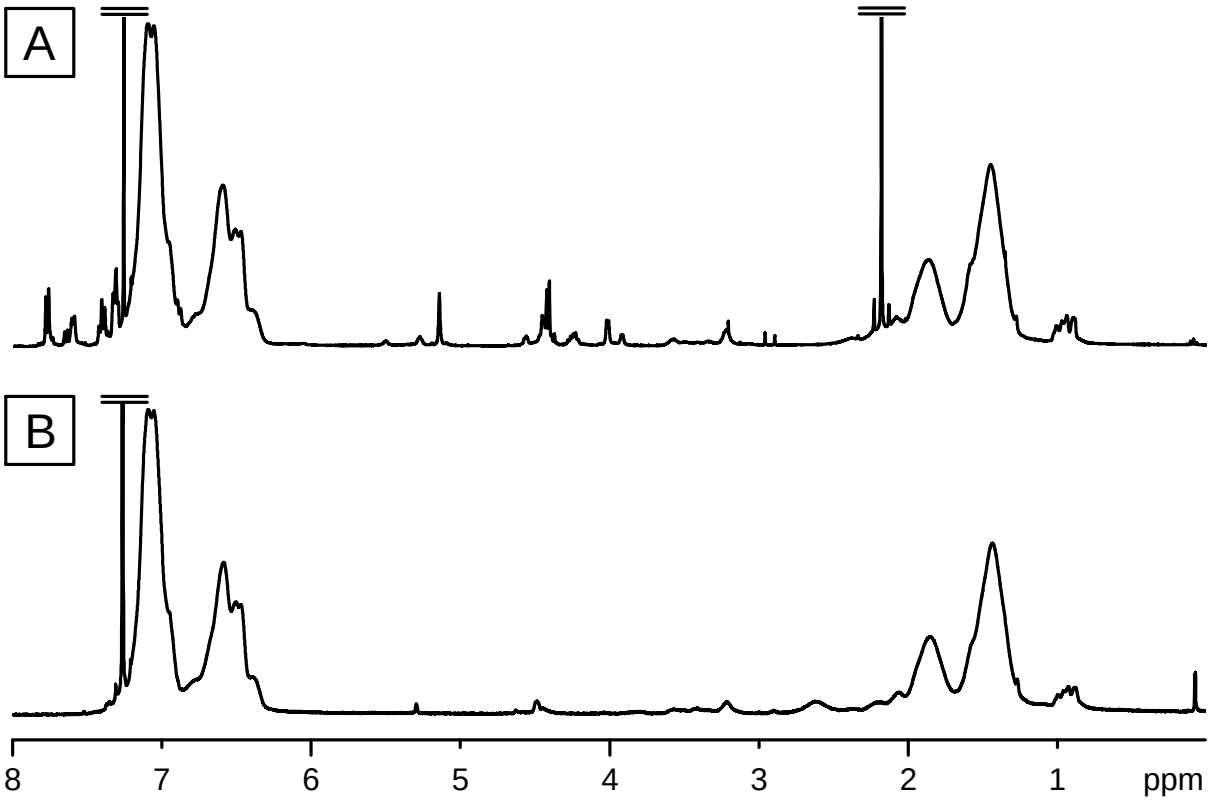

Figure S2. ^{1}H NMR spectra recorded in $CDCl_3$ for: (A) the fully protected peptide-polymer bioconjugate prepared using approach **a**, (B) the final peptide-polymer bioconjugate PS-*b*-GKYGKY after complete deprotection.

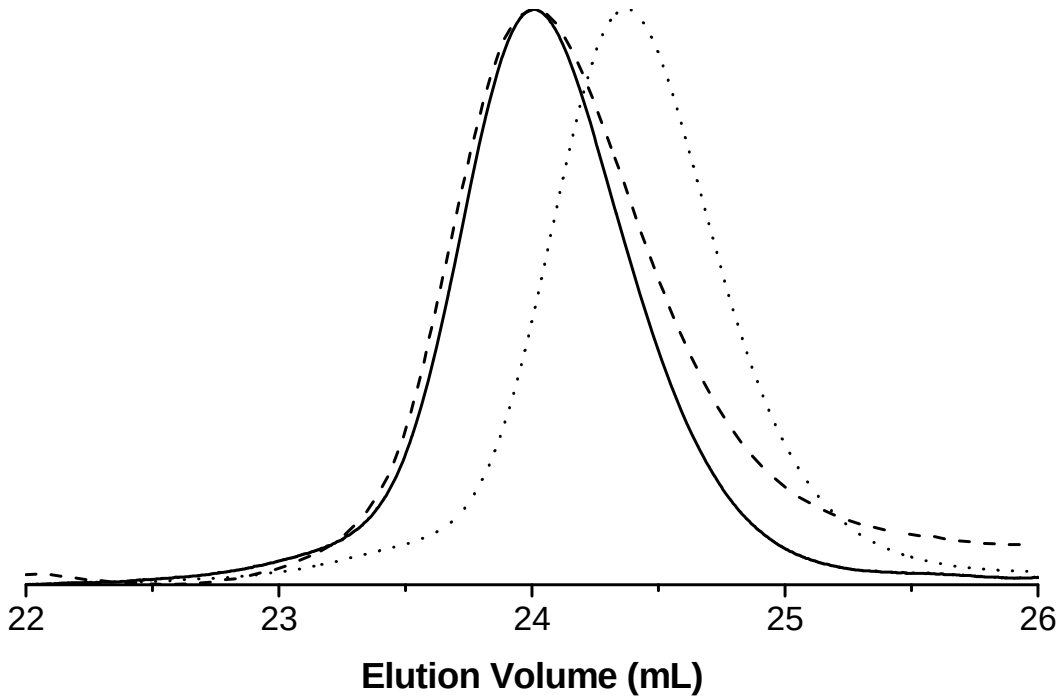

Figure S3. SEC chromatograms recorded in NMP for: the cleavable soluble support **P3** (dotted line), the corresponding peptide-polymer bioconjugate after removal of the Fmoc protecting group (solid line), and the final peptide-polymer bioconjugate PS-*b*-GKYGKY after complete deprotection (dashed line).

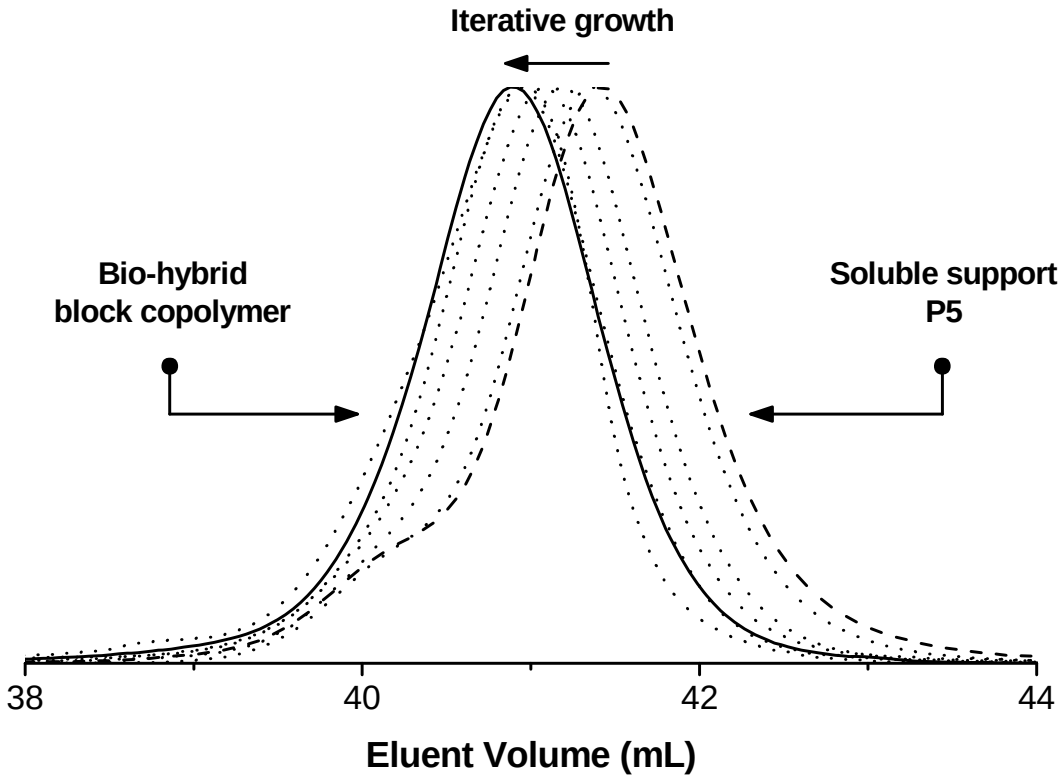

Figure S4. SEC chromatograms recorded in THF for the polystyrene support **P5** (dashed line) and the resulting peptide-polymer conjugate (solid line). The latter still possess side-chain protecting groups and a Fmoc terminal moiety. The dotted traces show intermediate situations recorded after each amino acid coupling.

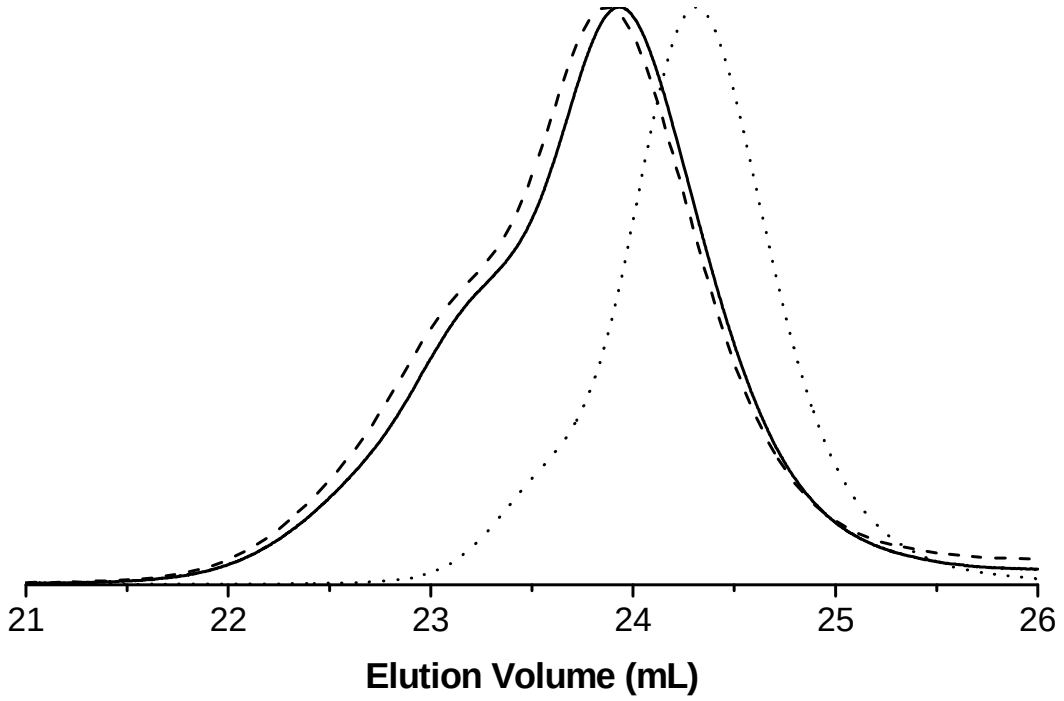

Figure S5. SEC chromatograms recorded in NMP for: the non-cleavable soluble support **P5** (dotted line), the corresponding peptide-polymer bioconjugate after removal of the Fmoc protecting group (solid line), and the final peptide-polymer bioconjugate PS-*b*-GKYGKY after complete deprotection (dashed line).

Table S1. Yields obtained after each step of the synthesis of a polymer bioconjugate on a cleavable Wang soluble support (Approach **a** in Scheme 1). In each case, these yields were calculated after reaction and precipitation of the conjugate in methanol.

	Isolated polymer	**Yield [%]**
1	PS-x-Gly-Fmoc (**P4**)	78
2	PS-x-Gly-NH_2	90
3	PS-x-Gly-Lys(Mtt)-Fmoc	99
4	PS-x-Gly-Lys(Mtt)-NH_2	72
5	PS-x-Gly-Lys(Mtt)-Tyr(2ClTrt)-Fmoc	94
6	PS-x-Gly-Lys(Mtt)-Tyr(2ClTrt)-NH_2	91
7	PS-x-Gly-Lys(Mtt)-Tyr(2ClTrt)-Gly-Fmoc	95
8	PS-x-Gly-Lys(Mtt)-Tyr(2ClTrt)-Gly-NH_2	90
9	PS-x-Gly-Lys(Mtt)-Tyr(2ClTrt)-Gly-Lys(Mtt)-Fmoc	99
10	PS-x-Gly-Lys(Mtt)-Tyr(2ClTrt)-Gly-Lys(Mtt)-NH_2	76
11	PS-x-Gly-Lys(Mtt)-Tyr(2ClTrt)-Gly-Lys(Mtt)-Tyr(2ClTrt)-Fmoc	92

Table S2. Yields obtained after each step of the synthesis of a polymer bioconjugate on a non-cleavable soluble support (Approach **b** in Scheme 1). In each case, these yields were calculated after reaction and precipitation of the conjugate in methanol

	Isolated polymer	**Yield [%]**
1	PS-Gly-Fmoc	98
2	PS-Gly-NH_2	96
3	PS-Gly-Lys(*t*Boc)-Fmoc	78
4	PS-Gly-Lys(*t*Boc)-NH_2	94
5	PS-Gly-Lys(*t*Boc)-Tyr(*t*Bu)-Fmoc	97
6	PS-Gly-Lys(*t*Boc)-Tyr(*t*Bu)-NH_2	72
7	PS-Gly-Lys(*t*Boc)-Tyr(*t*Bu)-Gly-Fmoc	99
8	PS-Gly-Lys(*t*Boc)-Tyr(*t*Bu)-Gly-NH_2	97
9	PS-Gly-Lys(*t*Boc)-Tyr(*t*Bu)-Gly-Lys(*t*Boc)-Fmoc	79
10	PS-Gly-Lys(*t*Boc)-Tyr(*t*Bu)-Gly-Lys(*t*Boc)-NH_2	87
11	PS-Gly-Lys(*t*Boc)-Tyr(*t*Bu)-Gly-Lys(*t*Boc)-Tyr(*t*Bu)-Fmoc	89