β-Sheet Side Chain Polymers Synthesized by Atom-Transfer Radical Polymerization

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Silks are a widely studied class of naturally occurring structural proteins. Dragline spider silk, in particular, is considered to be nature's high-performance material due to its remarkable combination of strength and toughness. These mechanical properties stem from the protein secondary structure, a combination of well-defined β-sheets in a less well-defined glycine-rich matrix. The translation of this structure into a synthetic polymer was the aim of this investigation. To achieve this, a peptide-based monomer containing the sequence alanine−glycine−alanine−glycine, a well-known β-sheet-forming sequence found in silk, was synthesized. Using atom-transfer radical polymerization and a bifunctional initiator, a well-defined peptide-based polymer was prepared. This was then used as the macroinitiator for the polymerization of methyl methacrylate. The resulting well-defined triblock copolymer was analyzed using IR spectroscopy, which clearly showed β-sheet secondary structure had been introduced.

Introduction

Silks are an important class of naturally occurring structural proteins.[1,2] There are many different types of silk, with many combinations of mechanical properties.[3] One of the most extensively studied is dragline spider silk,[4] which is considered to be nature's high-performance fiber, because of its remarkable combination of strength and toughness, which is comparable to that of high-tenacity nylon and steel.[5] Silk fibers attain their unique properties via the characteristic folding pattern of the protein.[6−9] This secondary structure consists of two major parts, crystalline β-sheet domains which are interspersed throughout a less well-defined glycine-rich helical part.[10] There are also some indications of the presence of a third weakly orientated domain.[11]

The remarkable properties of this material make it of interest for a wide range of applications from bullet-proof vests to tissue engineering[12,13] and drug delivery.[14] This has inspired many research laboratories to investigate methods either for reproducing the silk protein using protein engineering or to incorporate elements of the silk peptide sequence into synthetic polymers. Using protein engineering, the major parts of silk proteins have been reproduced in a variety of ways, from expression in tobacco plants, potatoes,[15] and mammalian cells.[16] A major difficulty of this method is that to introduce the correct mechanical properties the silk protein has to be spun into a fiber containing the appropriate secondary structure.[17] A recently developed aqueous spinning process has shown this approach to be promising.[18] A second line of research uses protein engineering to produce only part of the silk protein, namely, the β-sheet domains, which are the most well-defined elements in spider and silkworm silk. Peptide-based materials with β-sheet characteristics can be obtained via this method. A similar, totally synthetic procedure has been recently developed by Rathore and Sogah.[19,20] They synthesized block copolymers in which the common β-sheet-forming sequences in silk were coupled to poly(ethylene glycol) to give multiblock copolymers consisting of a β-sheet area and a random amorphous matrix.

We have developed an alternative, versatile method for the construction of well-defined polymer architectures that mimic the secondary β-sheet structure found in silk. For this purpose we have prepared ABA-type triblock copolymers, in which the B block consists of β-sheet-forming monomeric units with alanylglycine oligopeptides in the side chain, and the A blocks represent the less ordered part of the silklike structure. This is an approach different from that used by Sogah and Rathore in which the β-sheet was included in the main chain. This allows the inclusion of larger β-sheet domains without having to construct longer peptide sequences.

To synthesize these structures, we chose to use atom-transfer radical polymerization (ATRP). ATRP is a robust and versatile technique[21,22] which has been shown to be able to polymerize a wide range of bioinspired monomers, from nucleobases[23] to oligosaccharides.[24] We have also recently shown that ATRP can be used to polymerize peptide-based monomers.[25] The use of ATRP in the construction of these triblock copolymers allows considerable freedom with respect to the length and composition of both A and B blocks. For these reasons ATRP was chosen as the polymerization technique.

Experimental Section

General Procedures. ^{1}H and ^{13}C NMR spectra were measured on a 400 MHz Bruker Inova400 machine with a Varian probe.

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IR spectra were measured on an ATI Mattson Genesis Series FTIR.

MALDI-TOF-MS spectra were measured on a Bruker Biflex III machine, with dihydroxybenzoic acid (DHB) as the matrix. The samples were prepared by dissolving 2 mg of analyte in 1 mL of THF, after which this solution was mixed in a 1:1 ratio with a solution of 10 mg of DHB in 1 mL of H_2O, containing 0.1% trifluoroacetic acid. This was then placed on a MALDI plate.

GPC measurements were performed using a Shimadzu GPC instrument with Shimadzu RI and UV/vis detection, fitted with a Polymer Laboratories Plgel 5 μm mixed-D column and a PL 5 μm Guard column (separation range from 500 to 300000 molecular weight) using THF as the mobile phase at 35 °C. Polymer Laboratories polystyrene calibration kits were used.

Reagents. CuCl (Aldrich, 97%) was purified by washing with glacial acetic acid three times and once with diethyl ether.[26] Boc-alanine-OH (Fluka, 99%), HCl·NH_2-glycine-OEt (Fluka, 99%), methyl methacrylate (MMA; Aldrich, 99%), hydroxyethyl methacrylate (HEMA; Aldrich, 97%), 2-bromoisobutyric acid, (Aldrich, 98%), 2,2′-bipyridyl (bipy; Aldrich, 99%), *N*,*N*-dicyclohexylcarbodiimide (DCC; Fluka, 99%), 4-(dimethylamino)pyridine (DMAP; Across, 99%), DMSO-d_6 (Aldrich, 99.9%), *N*,*N*′-diisopropylethylamine (DIPEA; Fluka, 99%), 1-hydroxybenzotriazole hydrate (HOBt; Fluka, ≥98%), trifluoroacetic acid (TFA; Aldrich, 98%), potassium hydrogen sulfate ($KHSO_4$; Riedel-de haën, 99%), sodium hydrogen carbonate ($NaHCO_3$; Merck, 99.5%), sodium sulfate anhydrous (Fluka, 99%), and ethylenediaminetetraacetic acid tetrasodium salt hydrate (EDTA; Aldrich, 98%) were all used as received.

Dichloromethane (DCM) and ethyl acetate (EtOAc) were distilled from calcium hydride, and THF was distilled from sodium/benzophenone prior to use. Dimethylformamide (DMF) and isopropyl alcohol were used as received (J.T. Baker).

Monomer Synthesis. (a) Synthesis of Dipeptide Boc-Ala-Gly-OEt. Boc-Ala-OH, 9.47 g (50 mmol), was dissolved in 300 mL of EtOAc in a 500 mL round-bottom flask. To this were added HCl·NH_2-Gly-OEt (13.59 g, 50 mmol), DIPEA (17.4 mL, 100 mmol), HOBt (7.60 g, 50 mmol), and DCC (10.32 g). This mixture was then stirred overnight at room temperature. The precipitated dicyclohexylurea (DCU) was filtered off, and the EtOAc solution was washed, twice with 20 mL of a 1 M solution of $KHSO_4$, twice with 10 mL of distilled water, once with 10 mL of saturated NaCl solution, twice with 20 mL of a 1 M solution of $NaHCO_3$, twice with 10 mL of distilled water, and once with 10 mL of saturated NaCl solution. The EtOAc layer was then dried with Na_2SO_4 and filtered, and after removal of EtOAc pure Boc-Ala-Gly-OEt was obtained in 98% yield. ^{1}H NMR (400 MHz) ($SO(CD_3)_2$): δ 1.1 (NHCH(CH_3)C═O and OCH_2CH_3, 6H, m), 1.4 (OC(O)C(CH_3)$_3$, 9H, s), 3.7 (NHC$H_a$$H_b$C═O, 1H, dd), 3.85 (NHC$H_a$$H_b$C═O, 1H, dd), 3.9−4.1 (NH(CH$_3$)-C*H*C═O and OCH_2CH$_3$, 3H, m), 6.9 (N*H*CH(CH$_3$)C═O, 1H, d), 8.1 (N*H*CH$_2$C═O, 1H, t).

(b) Synthesis of Boc-Ala-Gly-OH. Boc-Ala-Gly-OEt, 6.5 g (23.6 mmol), was placed in 91 mL of a 14:5:1 solution of dioxane/MeOH/NaOH (4 M). This was stirred for 2 h. Next a 1 M $KHSO_4$ solution was added, and then the mixture was concentrated until all MeOH was removed. The product was freeze-dried to yield 5.01 g of Boc-Ala-Gly-OH (86% yield). ^{1}H NMR (400 MHz) ($SO(CD_3)_2$): δ 1.1 (NHCH(CH_3)C═O, 3H, d), 1.4 (OC(O)C(CH_3)$_3$, 9H, s), 3.7 (NHC$H_a$$H_b$C═O, 1H, dd), 3.85 (NHC$H_a$$H_b$C═O, 1H, dd), 3.9−4.1 (NHC*H*(CH$_3$)C═O, 1H, m), 6.9 (N*H*(CH$_3$)CHC═O, 1H, d), 8.1 (N*H*CHC═O, 1H, t).

(c) Synthesis of HCl·NH_2-Ala-Gly-OEt. Boc-Ala-Gly-OEt, 6.5 g (23.6 mmol), was dissolved in 90 mL of 2 M HCl/EtOAc and the resulting solution stirred for 60 min. The EtOAc solution was concentrated to 20 mL, and then 10 mL of *t*BuOH was added. After removal of the solvent the crude product was redissolved in 50 mL of CH_2Cl_2. This solution was extracted twice with 20 mL of water. The water layer was freeze-dried, and 4.96 g of the desired product was obtained quantitatively. ^{1}H NMR (400 MHz) ($SO(CD_3)_2$): δ 1.1 (NHCH(CH_3)C═O and OCH_2CH_3, 6H, m), 3.7 (NHC$H_a$$H_b$C═O, 1H, dd), 3.85 (NHC$H_a$$H_b$C═O, 1H, dd), 3.9−4.1 (NHC*H*(CH$_3$)C═O and OCH_2CH$_3$, 3 H, m), 8.3 (NH_3^+(CH$_3$)CHC═O, 3H, d), 8.9 (N*H*CH$_2$C═O, 1H, t).

(d) Synthesis of Boc-Ala-Gly-Ala-Gly-OEt. Boc-Ala-Gly-OH, 5.01 g (20.3 mmol), was dissolved in 200 mL of EtOAc. To this were added 4.28 g of HCl·H_2N-Ala-Gly-OEt (20.3 mmol), 3.12 g of HOBt (20.3 mmol), 7.06 mL of DIPEA (40.6 mmol), and 4.20 g of DCC (20.3 mmol). The mixture was stirred overnight. The DCU precipitate was filtered off, after which the EtOAc solution was washed, twice with 20 mL of a 1 M solution of $KHSO_4$, twice with distilled water, once with a saturated NaCl solution, twice with 20 mL of a 1 M solution of $NaHCO_3$, twice with distilled water, and once with saturated NaCl solution. The EtOAc layer was then dried with Na_2SO_4 and filtered, followed by removal of the solvent. The crude product Boc-Ala-Gly-Ala-Gly-OEt was purified by column chromatography in 10% MeOH/CH_2Cl_2, resulting in 4.6 g of pure compound, 56% yield. ^{1}H NMR (400 MHz) ($SO(CD_3)_2$): δ 1.1−1.2 (NHCH(CH_3)C═O, 6H, and OCH_2CH_3, 3H, m), 1.4 (OC(O)C(CH_3)$_3$, 9H, s), 3.65 (NHCH_2C═O, 2H, d), 3.75 (NHCH_2C═O, 2H, d), 3.95 (NHC*H*(CH$_3$)C═O, 1H, t), 4.1 (OCH_2CH$_3$, 2H, q), 4.4 (NHC*H*(CH$_3$)C═O, 1H, m), 7.0 (N*H*CH(CH$_3$)C═O, 1H, d), 7.9 (N*H*CH(CH$_3$)C═O, 1H, d), 8.0 (N*H*CH$_2$C═O, 1H, d), 8.3 (N*H*CH$_2$C═O, 1H, s).

(e) Synthesis of Boc-Ala-Gly-Ala-Gly-OH. Boc-Ala-Gly-Ala-Gly-OEt, 4.6 g (11.4 mmol), was placed in 62.7 mL of a 14:5:1 solution of dioxane/MeOH/(4 M) NaOH. The reaction mixture was stirred for 40 min and then quenched with 1 M $KHSO_4$. The solution was concentrated until all MeOH was removed. The pure product was obtained by freeze-drying, in quantitative yield (4.2 g). ^{1}H NMR (400 MHz) ($SO(CD_3)_2$): δ 1.1−1.2 (NHCH(CH_3)C═O, 6H), 1.4 (OC(O)C(CH_3)$_3$, 9H, s), 3.65 (NHCH_2C═O, 2H, d), 3.75 (NHCH_2C═O, 2H, d), 3.95 (NHC*H*(CH$_3$)C═O, 1H, t), 4.4 (NHC*H*(CH$_3$)C═O, 1H, m), 7.0 (N*H*CH(CH$_3$)C═O, 1H, d), 7.9 (N*H*CH(CH$_3$)C═O, 1H, d), 8.0 (N*H*CH$_2$C═O, 1H, d), 8.3 (N*H*CH$_2$C═O, 1H, s).

(f) Synthesis of Boc-Ala-Gly-Ala-Gly-Ethyl Methacrylate (1). Boc-Ala-Gly-Ala-Gly-OH (11.4 mmol) was placed

in 120 mL of DMF. To this were added 1.43 mL (11.4 mmol) of HEMA, 139 mg (1.14 mmol) of DMAP, 1.754 g (11.4 mmol) of HOBt, and 2.37 g (11.4 mmol) of DCC. The reaction mixture was stirred overnight, after which the DCU precipitate was filtered off, and DMF was removed under reduced pressure. The product was then dissolved in EtOAc and washed twice with 20 mL of a 1 M $KHSO_4$ solution, twice with 10 mL of distilled water, once with 10 mL of a saturated NaCl solution, twice with 20 mL of a 1 M $NaHCO_3$ solution, twice with 10 mL of distilled water, and once with 10 mL of saturated NaCl solution. The EtOAc layer was then dried with Na_2SO_4 and filtered, followed by evaporation of EtOAc. The crude product was purified by column chromatography in 10% MeOH/CH_2Cl_2, resulting in 2.22 g of pure **1**, in 40% yield. ^{1}H NMR (400 MHz) ($SO(CD_3)_2$): δ 1.1 (NHCH(CH_3)C═O, 6H,), 1.4 (OC(O)C(CH_3)$_3$, 9H, s), 1.85 (C(O)C(CH_3)═CH_2, 3H, s), 3.65 (NHCH_2C═O, 2H, d), 3.75 (NHCH_2C═O, 2H, d), 3.95 (NHCH(CH$_3$)C═O, 1H, t), 4.15−4.3 (NHCH(CH$_3$)C═O and OCH_2CH_2O, 5H, m), 5.6 and 6.05 (C(O)C(CH$_3$)═CH_aH$_b$, 1H, s, and 1H, s), 7.0 (NHCHC═O, 1H, d), 7.9 (NHCHC═O, 1H, d), 8.0 (NHCH$_2$C═O, 1H, d), 8.3 (NHCH$_2$C═O, 1H, s). ^{13}C NMR (300 MHz) ($SO(CD_3)_2$): δ 172.5, 172.1, 171.8, 169.1, 167.8, 165.9, 135.1 125.8, 77.9, 62.3, 62.2, 49.7, 47.8, 41.9, 28.2, 18.2, 18.08, 18.01. IR: ν 3313 (N−H str); 2979 (C−H str); 1752 (C═O str, ester) 1658 (C═O str, amide I); 1529 (N−H vib, amide II); 1452 (C−H vib) cm^{-1}. MALDI-TOF-MS: *m*/*e* 429 (M^+ − ^{t}Bu + Na); 476 (M^+ − ^{t}Bu + 2Na); 508 (M^+ + 2Na).

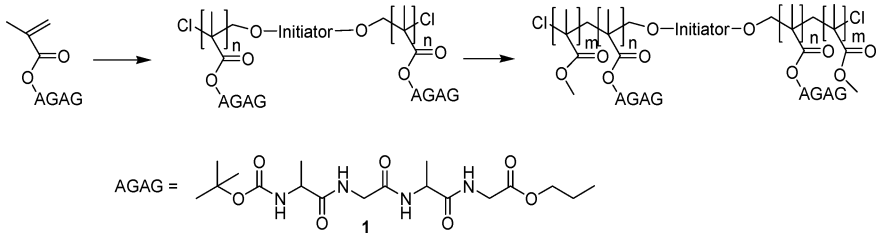
Synthesis of 1,4-(2′-Bromo-2′-methylpropionato)benzene (2). An excess of 2-bromoisobutyryl bromide (10.129 g, 44.06 mmol) was added dropwise to a round-bottomed flask containing a solution of hydroquinone (2.174 g, 19.75 mmol) and Et_3N (4.390 g, 43.38 mmol) in THF (80 mL), which was purged with N_2, and cooled in an ice bath. After complete addition of the acid bromide the reaction mixture was stirred for 4 h at room temperature. With TLC (heptane/EtOAc, 5:1) completion of the reaction was determined. The excess of acid bromide was quenched with MeOH. Triethylammonium bromide was removed by filtration over Hiflo and the solvent removed in vacuo. The product was recovered as a yellow oil that was recrystallized three times from MeOH, to give 5.36 g of a white crystalline product which was dried under vacuum, 67% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.18 (arom *H*, s, 4H), 2.07 ($O_2CC(CH_3)_2Br$, s, 12H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.08 (O*C*(═O)C(CH$_3$)$_2$Br), 148.39 (arom *C*−O), 122.06 (arom *C*), 55.19 ($O_2CC(CH_3)_2Br$), 30.60 ($O_2CC(CH_3)_2Br$). IR: 1747 (C═O, ester) cm^{-1}.

ATRP of 1 Using Bifunctional Initiator 2. The ATRP of monomer **1** was carried out using bifunctional initiator **2** in a solution of DMSO-d_6. **1** (486 mg, 1 mmol) was placed in a Schlenck vessel along with 19.8 mg of **2** (0.05 mmol), 20 mg of CuCl (0.2 mmol), and 63.3 mg of bipy (0.4 mmol). The vessel was evacuated and filled with N_2. This procedure was repeated three times. Then 2 mL of DMSO-d_6 was added, and the solution was purged with N_2. The reaction mixture was placed into an oil bath preheated to 40 °C. The polymerization was followed by ^{1}H NMR spectroscopy with samples taken every 30 min. Conversion was measured by comparing the NMR resonances of the amide signal at δ 8.3 ppm with the methacrylate vinylic proton signal at δ 5.6 ppm. After 75 min the polymerization had reached 81% conversion. After polymerization the polymer was precipitated in an aqueous solution of EDTA (25 g/L). The solid was washed twice with 10 mL of CH_2Cl_2, dried, and redissolved in THF. The polymer was reprecipitated from THF in aqueous EDTA solution, filtered, and taken up in THF, which was subsequently removed under reduced pressure. Yields could not be determined due to the fact that samples were removed during polymerization; however, 260 mg of polymer **3** was obtained. GPC: M_n = 6.5 kg/mol, PDI = 1.12. ^{1}H NMR (400 MHz) ($SO(CD_3)_2$): δ 0.5−1 (−CH_2C(R)(CH$_3$)−, m), 1.1−1.3 (NHCH(CH_3)C═O, m), 1.4 (OC(O)C(CH_3)$_3$, s), 1.75 (−CH$_2$(C(R)(CH_3)−, m), 3.55−4.35 (NHCH_2C═O, NHCH(CH$_3$)C═O, and OCH_2CH_2O, m), 7.0 (NHCHC═O, s), 7.9 (NHCHC═O, s), 8.0 (NHCH$_2$C═O, s), 8.3 (NHCH$_2$C═O, s).

Typical Macroinitiation of MMA from Poly(Boc-Ala-Gly-Ala-Gly-ethyl methacrylate). Poly(Boc-Ala-Gly-Ala-Gly-ethyl methacrylate) (**3**) (35 mg, 5 μmol) was placed in a Schlenck vessel. CuCl (3.96 mg, 0.040 mmol) and bipy (13 mg, 0.08 mmol) were added, and the vessel was evacuated and filled with N_2. This procedure was repeated three times. Then MMA (0.47 mL 0.5 mmol) and 1 mL of DMSO-d_6 were added. This mixture was purged with N_2 and then placed in an oil bath which had been preheated to 70 °C. The polymerization was followed using ^{1}H NMR spectroscopy, by comparing the signals of the amide protons from the macro initiator at δ 8.3 ppm with the vinylic protons of the methacrylate at δ 5.6 ppm. After 2 h a conversion of 64% was reached. The polymer was worked up by precipitation in an aqueous EDTA solution (25 g/L), followed by filtration and extraction of the desired product with CH_2Cl_2. The solvent was then removed under reduced pressure. No yield could be determined, as samples were removed during the polymerization; however, 46 mg of polymer was obtained. GPC: M_n = 11.5 kg/mol, PDI = 1.29. ^{1}H NMR (400 MHz) ($SO(CD_3)_2$): δ 0.5−1 (−CH_2C(R)-(CH$_3$)−, m), 1.1−2.0 (NHCH(CH_3)C═O, OC(O)C(CH_3)$_3$, and −CH$_2$(C(R)(CH_3)−, m), 3.55−4.35 (NHCH_2C═O, (NHCH(CH$_3$)C═O, OCH_3, and OCH_2CH_2O, m), 7.0 (NHCH(CH$_3$)C═O, s), 7.9 (NHCH(CH$_3$)C═O, s), 8.0 (NHCHC═O, s), 8.3 (NHCH$_2$C═O, s).

In Situ Formation of pMMA−p(Boc-Ala-Gly-Ala-Gly-EMA)−pMMA (4). 1 (247 mg, 0.5 mmol) was placed in a Schlenck vessel. To this were added **2** (10.8 mg, 0.025 mmol), CuCl (9.9 mg, 0.1 mmol), and bipy (31.2 mg, 0.2 mmol). The vessel was evacuated and filled with argon. This procedure was repeated three times. To this was added 1 mL of DMSO-d_6, followed by purging with argon. The reaction mixture was then placed in a preheated oil bath at 40 °C and polymerized. Samples were taken to follow the conversion by ^{1}H NMR spectroscopy; conversion was determined by comparing the signals of the amide at δ 8.3 ppm with the vinylic protons of the methacrylate at δ 5.6 ppm. In a separate Schlenck vessel, 220 μL of MMA (0.2 mmol) was placed in 1 mL of DMSO-d_6, and this solution

Scheme 1. Schematic Representation of the Synthesis of an AGAG-Based Triblock Copolymer Using a Bifunctional Initiator



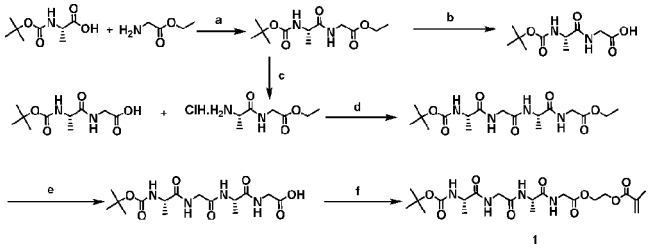
was purged with argon. Two hours after the polymerization of **1** was initiated, and a 71% conversion was reached, the MMA solution was added to the Schlenck vessel, using a siphon. The polymerization was then continued for 3 h until an MMA conversion of 56% had been achieved. The polymerization was then stopped, and the polymer was worked up by precipitation in 50 mL of a solution of EDTA in water (25 g/L), followed by filtration and redissolution of the polymer in CH_2Cl_2. The yield could not be determined due to the removal of samples during the polymerization, but 284 mg of polymer was obtained. GPC: $M_{n,macroinitiator} =$ 4.6 kg/mol, PDI = 1.17; $M_{n,ABA\ block\ copolymer} =$ 6.1 kg/mol, PDI = 1.19. ^{1}H NMR (400 MHz) ($SO(CD_3)_2$): δ 0.5−1 ($-CH_2C(R)(CH_3)-$, m), 1.1−2.0 ($NHCH(CH_3)C{=}O$, $OC(O)C(CH_3)_3$, and $-CH_2(C(R)(CH_3)-$, m), 3.55−4.35 ($NHCH_2C{=}O$, $NHCH(CH_3)C{=}O$, OCH_3, and OCH_2CH_2O, m), 7.0 (NHCHC=O, s), 7.9 (NHCHC=O, s), 8.0 (NHCHC=O, s), 8.3 ($NHCH_2C{=}O$, s). IR: ν 3284 (N−H str); 2933 (C−H str); 1725 (C=O str, ester); 1669 (C=O str, amide I); 1524 (N−H vib, amide II); 1451 (C−H vib) cm^{-1}.

Synthesis of pMMA−p(NH$_2$-Ala-Gly-Ala-Gly-EMA)−pMMA (5). The removal of the Boc group from pMMA−p(Boc-Ala-Gly-Ala-Gly-EMA)−pMMA was performed by stirring 200 mg of the polymer in 10 mL of a 50:50 mixture of TFA and CH_2Cl_2 for 1 h. The solvent was removed under reduced pressure. The yield was quantitative. To remove residual trifluoro acetic acid salts the polymer was dissolved in butanol and washed twice with a 1M $NaHCO_3$ solution. The solvent was removed under reduced pressure. ^{1}H NMR (400 MHz) ($SO(CD_3)_2$): δ 0.5−1 ($-CH_2C(R)(CH_3)-$, m), 1.1−2.0 ($NHCH(CH_3)C{=}O$ and $-CH_2(C(R)(CH_3)$, m), 3.45−4.35 ($NHCH_2C{=}O$, $NHCH(CH_3)C{=}O$, OCH_2CH_2O, and OCH_3, m), 7.0 (NHCHC=O, s), 7.9 (NHCHC=O, s), 8.0 (NHCHC=O, s), 8.3 ($NHCH_2C{=}O$, s). IR (before TFA removal): 3284 (N−H str); 2953 (C−H str); 1724 (C=O str, ester); 1669 (C=O str, amide I); 1542 (N−H vib, amide II); 1436 (C−H vib) cm^{-1}. IR (after TFA removal): 3321 (N−H str); 2917 (C−H str); 1683 w and 1625 s (C=O str, amide I); 1537 (N−H vib, amide II); 1468 (C−H vib) cm^{-1}.

Results and Discussion

The approach that was used for the construction of β-sheet-containing ABA triblock copolymers using ATRP can be divided into three stages. First, a monomer containing a β-sheet-forming peptide sequence in the side chain had to be synthesized. This was followed by polymerization of this peptide-based monomer into a bifunctional macroinitiator, and finally the polymerization of MMA from the ends of this peptide-based macroinitiator was performed to give the desired ABA-type block copolymer (see Scheme 1). On the basis of earlier experiments in our laboratories, for the polymerization of peptide-based monomers methacrylate handles are the most appropriate, as they can be polymerized under dilute conditions. To keep the backbone of the block copolymer consistent and to facilitate macroinitiation, we chose to use pMMA as the end block of our polymer. Although pMMA has a higher T_g than can be expected from the silk matrix material, it was still preferred over other polymers with lower T_g values, such as poly(methyl acrylate) (pMA), or polymers with a higher degree of biocompatibility for this reason. Via this approach we want to demonstrate the possibility of introducing β-sheet functionality into a block copolymer by incorporation of functional peptide sequences via a versatile and controlled polymerization approach.

Scheme 2. Synthesis of Boc-Ala-Gly-Ala-Gly-Ethyl Methacrylate[a]

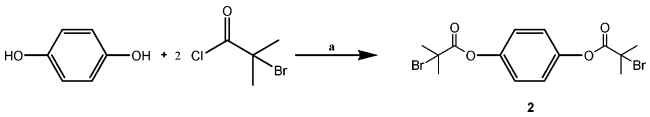


[a] Reagents and conditions: (a) DCC, HOBt, DCM, 98%; (b) 4 M NaOH, MeOH, dioxane, 86%; (c) EtOAc/2 M HCl, quantitative; (d) DCC, HOBt, DCM, 56%; (e) 4 M NaOH, MeOH, dioxane, quantitative; (f) DCC, DMAP, HOBt, DMF, HEMA, 40%.

Synthesis of a β-Sheet-Based Monomer. To introduce β-sheet functionality into a synthetic polymer, it is necessary to synthesize a monomer based on known β-sheet-forming sequences, such as polyalanine repeats, as found in dragline spider silk, and alanylglycine[27] repeats, more commonly observed in silkworm silk. It was known from previous work carried out in our laboratory that polyalanine repeats are difficult to synthesize due to insolubility at higher molecular weights; therefore, it was decided to prepare monomers based on alanine and glycine repeats. As a first model compound the Ala-Gly-Ala-Gly tetrapeptide was chosen, which was easily synthesized via solution-phase peptide chemistry as depicted in Scheme 2. After the peptide synthesis the free carboxylic acid end group was functionalized with HEMA to give monomer **1**. HEMA was chosen as a functional handle as the addition of the ethyl spacer allows the peptide more flexibility in the side chain.

Polymerization of Boc-Ala-Gly-Ala-Gly-ethyl methacrylate. The polymerization of monomer **1** was carried out

Scheme 3. Synthesis of Bifunctional Initiator 1,4-(2′-Bromo-2′-methylpropionato)benzene[a]



[a] Reagents and conditions: (a) THF, Et_3N, 0 °C.

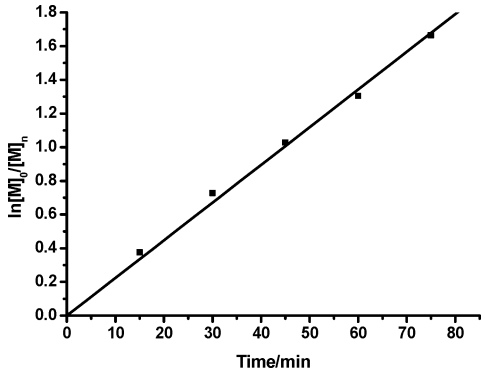


Figure 1. Polymerization of Boc-Ala-Gly-Ala-Gly-ethyl methacrylate in DMSO-d_6 at 40 °C for 75 min. An 81% conversion was observed.

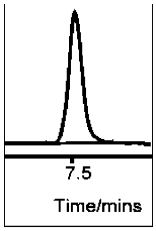


Figure 2. GPC trace for the polymerization of monomer **1**. M_w was 6.5 kg/mol, and the polydispersity was 1.2.

in DMSO-d_6 at 40 °C for 75 min, after which a conversion of 81% was reached. As bifunctional initiator the commonly used **2** was used (see Scheme 3). DMSO is not a common solvent for ATRP;[28] however, we have recently shown that it is suitable for the polymerization of peptide-based monomers, and it allows monitoring of the kinetics of the polymerization via ^{1}H NMR spectroscopy.[25] The plot of the natural logarithm of conversion vs time (see Figure 1) showed that the kinetics of polymerization are clearly first order, indicating a living polymerization. This was further confirmed by the GPC data (see Figure 2), which showed a PDI of 1.12. Therefore, it can be concluded that ATRP of monomer **1** proceeded without much difficulty.

Macroinitiation of MMA from Poly(Boc-Ala-Gly-Ala-Gly-ethyl methacrylate). To synthesize the desired ABA block copolymer architecture, it was necessary to reinitiate the polymerization of the second monomer, MMA, from the halogen moieties that should be present at both chain ends of the synthesized polymer poly(Boc-Ala-Gly-Ala-Gly-ethyl methacrylate). First, the peptide-based polymer was worked up after polymerization by precipitating the polymer in a solution of EDTA in water, followed by washing with dichloromethane. The purified polymer was then used as the macroinitiator for ATRP of MMA. This process was again carried out in DMSO-d_6 to ensure solubility of the macroinitiator and the resulting block copolymer. To obtain the optimal conditions for macroinitiation, several experiments were performed, in which the ligand, catalyst:initiator ratio, and temperature were varied (see Table 1).

Although in some cases livingness was observed, on the basis of the kinetics of polymerization and GPC results, it is clear that the reproducibility of the reinitiating experiments was rather poor. The increase in polydispersity observed in many experiments, and in one case even the occurrence of a bimodal distribution, indicates that the polymerization does not initiate properly, probably due to the loss of functional halide end groups. As the ATRP of the peptide-based block was performed in a well-controlled manner, it was thought that the loss of control during the second polymerization was due to partial removal of the halide end groups during the aqueous workup procedure. To test this hypothesis, the macroinitiation was therefore performed in situ.

In Situ Macroinitiation of MMA from Boc-Ala-Gly-Ala-Gly-Ethyl Methacrylate. Boc-Ala-Gly-Ala-Gly-ethyl methacrylate was polymerized as before at 40 °C, using initiator **2** in DMSO-d_6 with CuCl/bipy as a catalyst. This time after 2 h, when the polymerization had reached 71% conversion, an MMA solution in DMSO-d_6 was added. This second polymerization reached 56% conversion after 3 h. From the plots of the natural logarithm of conversion vs time (Figures 3 and 4) it was observed that both polymerizations were living. The GPC data (Table 2 and Figure 5) showed that the PDI was low and a monomodal distribution was obtained for the triblock copolymer, indicating that both parts of the polymerization were controlled and that the in situ macroinitiation was successful. This confirms the idea that the loss of control during the first macroinitiation procedure is a result of the workup procedure.

Using an in situ macroinitiation procedure to build up our block copolymer has advantages and disadvantages. The main disadvantage is that it is not possible to reach 100% conversion while maintaining a living ATRP polymerization, meaning that there will be some monomer **1** left after the addition of the second monomer MMA. This will result in some incorporation of monomer **1** into the A block of our desired block copolymer. This however was expected not to have a pronounced influence on any β-sheet structure present in the final polymer. The main advantage is that there is no workup procedure, and therefore, the loss of end groups and formation of dead chains can be minimized.

IR Characterization of pMMA-*b*-p(Boc-Ala-Gly-Ala-Gly-EMA)-*b*-pMMA. To investigate whether the introduction of AGAG peptides leads to the formation of β-sheet elements in the triblock copolymer, secondary structure analysis had to be performed. One technique which is simple, reliable, and not influenced by the presence of the polymer backbone is IR spectroscopy, which was therefore used for the analysis of the prepared triblock copolymers. Other techniques such as CD spectroscopy, electron microscopy, and AFM were also applied. Unfortunately, CD spectroscopy was hampered due to scattering of the polymer films, and with electron microscopy no specific structures could be detected. Preliminary AFM results showed some structural formation which has to be further investigated in more detail to be conclusive.

Table 1. Different Polymerization Conditions Used for the Macroinitiation of MMA from Poly(Boc-Ala-Gly-Ala-Gly-ethyl methacrylate)[a]

ligand	conv/%	time/h	temp/°C	concn/M	I:Cu	M_n/kg/mol	$M_{n,th}$/kg/mol	MWD	first order
bipy	64	2	70	0.5	1:8	11.5	13	1.26	no
bipy	57	4	60	0.5	1:8	11.1	13	1.29	no
bipy	56	4	50	0.5	1:8	11.9	13	1.29	no
bipy	3	4	60	0.25	1:8	NA	10	NA	no
bipy	64	6	60	0.5	1:4	13.0	13	1.07	no
bipy	16	24	60	0.5	1:8	NA	10	NA	no
PMDETA	73	15	80	0.5	1:8	bimodal	13	bimodal	yes
PMDETA	51	2.5	70	0.5	1:4	N/A	13	1.3	no
PMDETA	77	23	60	0.5	1:4	7.0	13	1.3	no
PMDETA	50	4	50	0.5	1:8	N/A	11	N/A	no

[a] Bipy (bipyridine)and PMDETA (pentamethyldiethyltriamine) were both used as ligands (in 2:1 and 1:1 ratios with copper chloride, respectively) along with different initiator:catalyst ratios and different temperatures.

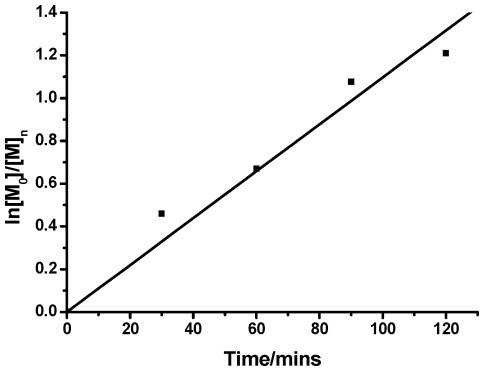


Figure 3. Natural logarithm of conversion vs time for the polymerization of Boc-Ala-Gly-Ala-Gly-ethyl methacrylate. This is the first part of the in situ macroinitiation of MMA from poly(Boc-Ala-Gly-Ala-Gly-ethyl methacrylate).

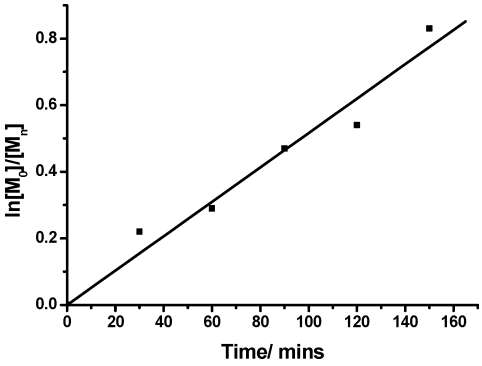


Figure 4. Natural logarithm of conversion vs time for the polymerization of MMA. This is the second part of the in situ macroinitiation of MMA from poly(Boc-Ala-Gly-Ala-Gly-ethyl methacrylate).

Table 2. Experimental Data for the Polymerization of **1** and the in Situ Macroinitiation of MMA from Poly(Boc-Ala-Gly-Ala-Gly-ethyl methacrylate)[a]

monomer	time/h	conv/%	$M_{n,th}$/kg/mol	M_n/kg/mol	PDI
1	2	71	7.3	4.6	1.17
MMA	3	56	12.9	6.1	1.19

[a] Polymerization was performed at 40 °C.

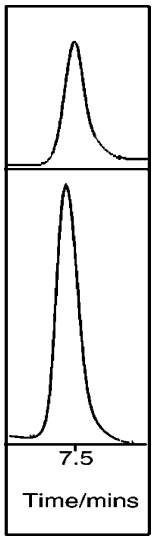


Figure 5. GPC trace for the in situ macroinitiation of MMA from poly(Boc-Ala-Gly-Ala-Gly-ethyl methacrylate): (a, top) part 1, polymerization of poly(Boc-Ala-Gly-Ala-Gly-ethyl methacrylate), (b, bottom) part 2, polymerization of MMA.

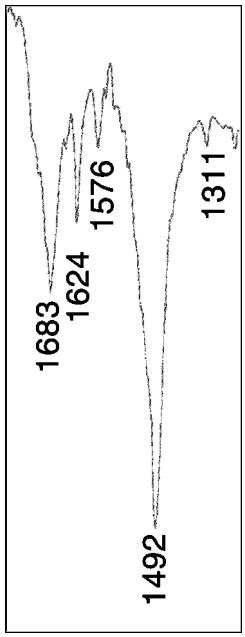


Figure 6. IR spectra of the amide I and amide II regions for pMMA-*b*-p(Ala-Gly-Ala-Gly-EMA-*b*-pMMA.

The different Ala-Gly-Ala-Gly-containing structures were characterized using FT-IR (ATR) (see Figure 6), and the amide I and amide II values were compared to values reported in the literature for β-sheet structures.[29,30] First, both the monomer and Boc-protected polymer were analyzed. Both of these showed no indication of β-sheet formation; instead, the amide I and amide II values were indicative of a random coil structure (see Table 3). A possible explanation for the absence of β-sheet formation within the polymer could

Table 3. IR Values (cm^{-1}) Obtained for Monomer **1** and the Respective Block Copolymers before and after Removal of the Boc Protecting Group and TFA Salts

compound	amide I	amide II
random coil[a]	1656	1535
antiparallel β-sheet[a]	1632 s/ 1685 w	1530
Boc-AGAG-EMA (monomer)	1655	1526
pMMA-*b*-p(Boc-AGAG-EMA)-*b*-pMMA	1662	1534
pMMA-*b*-p(AGAG-MA-EMA·TFA)-*b*-pMMA	1670	1545
pMMA-*b*-p(AGAG-MA-EMA)-*b*-pMMA	1624 s/ 1683	1537

[a] Standard IR values for the amide I and II bands.[29,30]

be the presence of the bulky Boc protective group at the N terminus of the tetrapeptide moieties. This was therefore investigated by cleavage of this protective moiety. After removal of the Boc group by treatment with trifluoroacetic acid (TFA) a sharp shift in the amide I and amide II signals was observed, which were now indicative of an antiparallel β-sheet conformation. The suggestion that β-sheet formation was sterically hindered in the case of the protected triblock copolymer was furthermore substantiated by the observation that when TFA was still present in the block copolymer as a counterion for the free amine groups β-sheet structures were also disrupted. Only after a thorough washing procedure was β-sheet folding observed.

Conclusions

We have successfully demonstrated the preparation of ABA triblock copolymers containing β-sheet-forming peptide sequences. For this purpose, the synthesis of an alanine−glycine tetrapeptide-based monomer was performed and an in situ macroinitiation technique was developed to construct the desired architecture. We have established a strategy with which we can take simple peptide sequences which have been isolated from structural proteins and place them into a synthetic polymer. By doing this, we have shown that it is possible to reproduce the secondary structure associated with these structural proteins in the polymer. Because of the versatility and robustness of ATRP, this method can be extended to different peptide sequences in combination with a wide range of monomers.

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References and Notes

(1) Atkins, E. *Nature* **2003**, *424*, 1010.
(2) Purves, W. K. *Sci. Am.* **2002**, *287*, 107.
(3) McGrath, K., Kaplan, D., Eds. *Protein-based Materials*; Birkhauser: Boston, 1997.
(4) Warner, S. B.; Polk, M.; Jacob, K. *J. Macromol. Sci., Rev. Macromol. Chem. Phys.* **1999**, *C39*, 643−653.
(5) Kaplan, D.; Wade Adams, W.; Farmer, B.; Viney, C. *Silk polymers: Materials Science and Biotechnology*; American Chemical Society: Washington, DC, 1994; Vol. 544.
(6) Perez Rigueiro, J.; Elices, M.; Llorca, J.; Viney, C. *J. Appl. Polym. Sci.* **2001**, *82*, 53−62.
(7) Perez Riguero, J.; Elices, M.; Llorca, J.; Viney, C. *J. Appl. Polym. Sci.* **2001**, *82*, 2245−2251.
(8) Perez Rigueiro, J.; Elices, M.; Llorca, J.; Viney, C. *J. Appl. Polym. Sci.* **2001**, *82*, 1928−1935.
(9) Lewis, R. V. *Biopolymers* **2003**, *8*, 1−24.
(10) van Beek, J. D.; Hess, S.; Vollrath, F.; Meier, B. H. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 10266−10271.
(11) Simmons, A. H.; Michal, C. A.; Jelinski, L. W. *Science* **1996**, *271*, 84−87.
(12) Altman, G. H.; Diaz, F.; Jakuba, C.; Calabro, T.; Horan, R. L.; Chen, J. S.; Lu, H.; Richmond, J.; Kaplan, D. L. *Biomaterials* **2003**, *24*, 401−416.
(13) Altman, G. H.; Horan, R. L.; Lu, H. H.; Moreau, J.; Martin, I.; Richmond, J. C.; Kaplan, D. L. *Biomaterials* **2002**, *23*, 4131−4141.
(14) Megeed, Z.; Cappello, J.; Ghandehari, H. *Pharm. Res.* **2002**, *19*, 954−959.
(15) Scheller, J.; Guhrs, K. H.; Grosse, F.; Conrad, U. *Nat. Biotechnol.* **2001**, *19*, 573−577.
(16) Lazaris, A.; Arcidiacono, S.; Huang, Y.; Zhou, J.; Duguay, F.; Chretien, N.; Welsh, E. A.; Soares, J. W.; Karatzas, C. N. *Science* **2002**, 472−477.
(17) Vollrath, F.; Knight, D. *Biopolymers* **2003**, *8*, 25−46.
(18) Vollrath, F.; Knight, D. P. *Nature* **2001**, *410*, 541−548.
(19) Rathore, O.; Sogah, D. Y. *Macromolecules* **2001**, *34*, 1477−1486.
(20) Rathore, O.; Sogah, D. Y. *J. Am. Chem. Soc.* **2001**, *123*, 5231−5239.
(21) Matyjaszewski, K., Ed. *Controlled radical polymerization*; American Chemical Society: Washington, DC, 1998; Vol. 685.
(22) Matyjaszewski, K. *Curr. Org. Chem.* **2002**, *6*, 67−82.
(23) Marsh, A.; Khan, A.; Garcia, M.; Haddleton, D. M. *Chem. Commun.* **2000**, 2083−2084.
(24) Haddleton, D. M.; Ohno, K. *Biomacromolecules* **2000**, *1*, 152−156.
(25) Ayres, L.; Vos, M. R. J.; Adams, P.; Shklyarevskiy, I. O.; van Hest, J. C. M. *Macromolecules* **2003**, *36*, 5967−5973.
(26) Keller, R. N.; Wycoff, H. D. *Inorg. Synth.* **1946**, *2*, 1−4.
(27) Kishi, S.; Santos, A.; Ishii, O.; Ishikawa, K.; Kunieda, S.; Kimura, H.; Shoji, A. *J. Mol. Struct.* **2003**, *649*, 155−167.
(28) Godwin, A.; Hartenstein, M.; Muller, A. H. E.; Brochini, S. *Angew. Chem., Int. Ed.* **2001**, *40*, 594−597.
(29) Krimm, S.; Bandekar, J. In *Advances in Protein Chemistry*; Academic Press: New York, 1986; Vol. 38, pp 282−361.
(30) Miyazawa, T.; Blout, E. R. *J. Am. Chem. Soc.* **1961**, *83*, 712−719.

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