Polyamidoamines as Drug Carriers: Synthesis of Polymers Featuring Extrachain-type Primary Amino Groups as Drug-anchoring Sites

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ABSTRACT

The versatile polymerization of bisacrylamides with mono- and difunctional amines, first investigated and greatly expanded in Ferruti’s laboratory,1–3 is utilized in the present project for the synthesis of macromolecular drug carriers. Specifically, we report on the preparation of linear polyamidoamines possessing primary amino groups as terminals of short side chains, designed to function as drug attachment sites. In the first reaction step, performed in aqueous medium, methylenebisacrylamide (MBA) is copolymerized with two types of comonomer: (1) primary amines bearing solubilizing functionality, such as tert-amine or hydroxyl groups, and (2) a variety of mono-N-Boc-protected primary diamines (Boc = tert-butoxycarbonyl). In other reactions, MBA is allowed to react with a mono-N-protected diamine to give a macromonomer, which is polymerized with an oligo- or poly(ethylene oxide) terminated at both ends by a primary amino group. The intermediary polymers so obtained, as yet featuring N-protected amino side groups, are treated with trifluoroacetic acid for deprotection. Further work-up by aqueous dialysis (25 000 mwco tubing) and freeze-drying affords the target polymers as water- and methanol-soluble solids in ultimate yields of 10–25%. 1H NMR spectroscopy serves to confirm the structural assignments1–12. In order to demonstrate the drug-carrying potential of these polymers, an exemplifying polyamidoamine (11) is allowed to react with an active ester of 4-ferrocenylbutanoic acid.

KEYWORDS

Polyamidoamines., methylenebisacrylamide, macromolecular drug carriers, primary amine side functionality, 4-ferrocenylbutanoic acid.

1. Introduction

For a forthcoming polymer-drug conjugation project we were in need of carrier polymers that would provide primary amino groups as side-chain terminals for drug attachment, while possessing complete solubility not only in aqueous media but also, quite critically for certain drug coupling reactions, in methanol. The synthetic versatility inherent in polyamidoamines of the structural type pioneered by Ferruti1–3 suggested that polymer type to serve our purpose. This prompted a study aiming at the synthesis of bisacrylamide-derived polymers containing various solubilizing groups as side-chain or main-chain components in addition to short side chains terminated with a primary amine functionality as the drug conjugation site. These synthetic efforts are reported in the present communication. While this work was in progress, a recent paper from Ferruti’s laboratory describing similar synthetic approaches came to our attention1

2. Experimental

2.1. General Procedures

Aqueous polymer product solutions were dialysed in Spectra/Por 4 cellulose membrane tubing (12 000–14 000 molecular mass cut-off) and Spectra/Por 6 wet tubing (25 000 molecular mass cut-off) against stirred and repeatedly exchanged batches of H2O. Solutions were freeze-dried in a Virtis Bench Top 3 freeze-drier operating at ~30°C, 10–15 Pa. Freeze-dried material was routinely post-dried in a Sartorius Thermo Control Infrared Drying System programmed for 2 × 2 min at 65°C. Analytical samples were additionally dried in an Abderhalden tube at 65°C under reduced pressure. 1H NMR spectroscopy was performed on D2O solutions adjusted to pH 10 (NaOH) in order to eliminate protonation effects; they were referenced against sodium 3-(trimethylsilyl)-2,2,3,3-d4-propionate (integration error limits ±12%). In the spectra of polymeric compounds, the brackets contain the expected proton counts. Viscometric measurements were conducted in Cannon-Fenske viscometers at 30.0 ± 0.1°C (c = 0.2 g/100 mL); inherent viscosities, ηinh, are given in mL/g.

2.2. Solvents, Reagents and Reactants

Deionized H2O was used for all preparative and work-up operations. N,N-Dimethylformamide (DMF) was distilled under reduced pressure in a faint stream of N2; a forerun (5%) was discarded. Methylenebisacrylamide (MBA), puriss., was used as received, and so were di-tert-butyl dicarbonate and the amine monomers, ethylenediamine, 1,3-diaminopropane, diethylenetriamine, 1,2-bis(2-aminoethoxy)ethane, 4,7,10-trioxo-1,13-tridecanediamine, and O,O’-bis(3-aminopropyl) poly(ethylene glycol) 1500 (Fluka Chemie AG, Aldrich Chemie GmbH).

For the preparation of N-(tert-butoxycarbonyl)-1,2-diaminoethane, 13 (Scheme 1), the solution of di-tert-butyl dicarbonate,
1.6 m, 2H (CH2C
1.4 s, 9H (CH3).

and the filtrate was extracted with several 40-mL portions of ethane as the most hydrophilic constituent in the aqueous phase. From the combined extracts, dried over anhydrous MgSO4, crude mono-Boc-protected amine was removed by filtration, and the filtrate was extracted with several 40-mL portions of methylene chloride, leaving any residual unreacted diamino-derivative as an oily liquid in a yield of 6.72 g (84%).

Table 1: Molar feed ratios and compositions of polyamidoamines 1–12.

<table>
<thead>
<tr>
<th>Reactants (equivalents)</th>
<th>Polymers</th>
</tr>
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<tbody>
<tr>
<td>MBA</td>
<td>R1</td>
</tr>
<tr>
<td>(2) –(CH3)2NMe2</td>
<td>(1)</td>
</tr>
<tr>
<td>(2) –(CH3)2NMe2</td>
<td>(1)</td>
</tr>
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<tr>
<td>(5) –(CH3)2NMe2</td>
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<td>(2) –(CH3)2NH(CH2)2</td>
<td>(1)</td>
</tr>
<tr>
<td>(2) –(CH3)2NH(CH2)2</td>
<td>(1)</td>
</tr>
</tbody>
</table>

* R1 in Scheme 2 for 1 to 10; in Scheme 3 for 11 and 12.
* R2 in Scheme 2 for 1 to 10.

Base molecular mass.

10.9 g (50 mmol) in 70 mL of dioxan, was added dropwise to 1,2-diaminoethane, 21 g (350 mmol), predissolved in 60 mL of the same solvent. The mixture was stirred for 1 d at ambient temperature, and the solvent, together with excess 1,2-diaminoethane, was distilled off by rotary evaporation at 50°C bath temperature. Upon the addition of 50 mL of H2O, a small portion of insoluble N,N'-bisprotected amine was removed by filtration, and the filtrate was extracted with several 40-mL portions of methylene chloride, leaving any residual unreacted diamino-derivative as an oily liquid in a yield of 7.55 g (95%).

1H NMR, δ/ppm: 3.1 t, 2H (CONHC
2); 2.65 t, 2H (C
2NH2); 1.45 s, 9H (CH3).

In an analogous fashion, N-(tert-butoxycarbonyl)-1,3-diaminopropane, 14 (Scheme 1), was prepared from di-tert-butyldicarbonate, 10 g (45.8 mmol), and 1,3-diaminopropane, 30.4 g (410 mmol), in a total of 130 mL of dioxan. Work-up as in the preceding experiment afforded crude mono-Boc-protected amine 13 was obtained after solvent removal as a yellow, oily liquid in a yield of 6.72 g (84%). The compound, just like the subsequently described Boc derivatives 14–16, gave a very clean 1H NMR spectrum and was used without further purification.

1H NMR, δ/ppm: 3.7 s, 4H (O-CH2CH2O); 3.6 m, 4H (N-CH2CH2O); 3.2 t, 2H (CONCH2CH2); 2.75 t, 2H (CH2NH); 1.45 s 9H (CH3).

Di-tert-butyl dicarbonate, 10 g (45.8 mmol), and diethylene-triamine, 32.0 g (310 mmol), in 130 mL of dioxan, treated as before, gave 8.8 g (94.5%) of crude N-(tert-butoxycarbonyl)-1,4,7-triaza-heptane, 16 (Scheme 4), as an oily liquid.

1H NMR, δ/ppm: 3.2 t, 2H (CONCH2CH2); 2.6 m, 6H (remaining CH2); 1.45 s, 9H (CH3).

The ferrocenylation agent, 4-ferrocenylbutanoic acid N-succinimidylester, was synthesized as previously described12–14.

2.3. Polyamidoamines 1–10

Amounts of polymeric educts and products are given as base moles and thus refer to the simplest recurring units, defined by structures 1–10 normalized to y = 1.

Polymer 1. The procedure given in the following for the synthesis of 1 is representative of the experiments providing the first ten target polymers of this study.

A solution was prepared from MBA, 2.47 g (16 mmol), in 15 mL of hot H2O. Upon the addition of the mono-Boc derivative 13, 1.28 g (8 mmol), the N2-saturated solution was stirred for 1 d at room temperature and for another 1 d at 60°C. 3-(Dimethylamino)propylamine, 817 mg (8 mmol), was added. Stirring of the solution, resaturated with N2, was continued for 3 d at 60°C and, upon the addition of ethanolamine, 49 mg (0.8 mmol), for another 2 h at that temperature. The last step served to eliminate terminal vinyl groups as potential causes of delayed cross-linking. The volatiles were now removed by rotating evaporation (60°C bath temperature), and the residual intermediary polymer was treated with 5 mL of trifluoroacetic acid (1 h, room temperature). Removal of the acid under reduced pressure at 60°C bath temperature was followed by precipitation of the product polymer with Et2O-EtOH-hexane (2:1:2), thorough washing with hot toluene, and redissolution in 20 mL of H2O. The pH was adjusted to 7, and the solution was dialyzed for 2 d in Spectra/Por 4 tubing and for another 2 d in Spectra/Por 6.
For the last 4 h of this operation, the pH of the tube contents was raised to 8.5–9 (NH₄OH) to eliminate protonation effects. The retentate was freeze-dried and post-dried, to give 0.68 g (18.1%) of solid, water- and methanol-soluble 1; \( \eta_{inh} \), 16.0 mL g⁻¹.

1H NMR, δ/ppm: 4.55, 10H (10H; NHCH₂NH₂); 2.8, 21H (20H; NHCOCH₂); 2.75–2.25, 49H (44H; CH₂N(CH₂)(CH₃), CH₂NH₂C(NH₂)CH₂N(CH₂)(CH₃), CH₂NH₂C(NH₂)CH₂N(CH₂)(CH₃), 2.2, 21.5H (24H; CH₂); 1.6, 7.7H (8H; CH₂CH₂CH₂H).

Polymers. In the two polyamidoamines 7 and 8, 2-(dimethylamino)ethylamine was employed in lieu of the propylamine derivative serving as the solubilizing factor in 1–6. Polymer 7 was thus prepared as described for 3, except that 3-(dimethylamino)propylamine was replaced by 2-(dimethylamino)ethylamine, 1.13 g (12.8 mmol). Conventional work-up gave 850 mg (22.2%) of water- and methanol-soluble, solid 7; \( \eta_{inh} \), 24.3 mL g⁻¹.

1H NMR, δ/ppm: 4.55, 10H (10H; NHCH₂NH₂); 2.8, 21H (20H; NHCOCH₂); 2.7–2.3, 41.7H 40H; CH₂N(CH₂)(CH₃), CH₂NH₂C(NH₂)CH₂N(CH₂)(CH₃), 2.2, 23H (24H; CH₂); 1.6, 2.3H (2H; CH₂CH₂CH₂H).

Polymers. This polymer was synthesized as described for 4, except that 3-(dimethylamino)-propylamine was replaced by 2-(dimethylamino)ethylamine, 1.13 g (12.8 mmol). The target polymer was isolated as a water- and methanol-soluble solid in a yield of 636 mg (15.6%); \( \eta_{inh} \), 21.0 mL g⁻¹.

1H NMR, δ/ppm: 4.55, 10H (10H; NHCH₂NH₂); 3.7–3.5, 8.9H (8H, CH₂OCH₂); 2.8, 23H (20H; NHCOCH₂); 2.7–2.3, 39.7H (40H; CH₂N(CH₂)(CH₃), CH₂NH₂C(NH₂)CH₂N(CH₂)(CH₃)); 2.2, 24.4H (24H; CH₂).

Polymers 9. Polyamidoamines 9 and 10 are counterparts to 2 and 4, with the 3-(dimethylamino)-propylamine solubilizing group replaced by 2-(2-hydroxyethoxy)ethylamine. Thus, 9 was prepared from MBA, 2.47 g (16 mmol), Boc-derivative 14, 1.39 g (8 mmol), and the hydroxyethoxylamine, 841 mg (8 mmol). The water- and methanol-soluble solid 9 was obtained in a yield of 430 mg (11.0%); \( \eta_{inh} \), 16.3 mL g⁻¹.

1H NMR, δ/ppm: 4.5, 4H (4H; NHCH₂NH₂); 3.7–3.5, 6.3H (6H; CH₂OCH₂CH₂OH); 2.8–2.2, 23H (22H; NHCOCH₂); 1.6, 1.55H (2H; CH₂CH₂CH₂H).

Polymers 10. The reaction of MBA, 2.47 g (16 mmol), Boc-derivative 15, 1.99 g (8 mmol), and 2-(2-hydroxyethoxy)ethylamine, 841 mg (8 mmol), gave 10 as a water- and methanol-soluble solid in a yield of 475 mg (10.8%).

1H NMR, δ/ppm: 4.5, 4H (4H; NHCH₂NH₂); 3.7–3.5, 13.3H (14H; CH₂OCH₂CH₂OH); 2.6–2.2, 22.4H (22H; NHCOCH₂); 1.6, 1.55H (2H; CH₂CH₂CH₂H).

Polymers 11. MBA, 2.47 g (16 mmol), was dissolved in 25 mL of hot isopropanol-H₂O (4:1). The mono-N-Boc derivative 14, 1.39 g (8 mmol), dissolved in 5 mL of isopropanol, was added. Upon saturation with N₂, the resulting solution was stirred for 3 d at ambient temperature. Following solvent removal by rotatory evaporation, the residual macromonomer was redissolved in 80 mL of the same solvent blend, thus providing the high dilution ([MBA] = 0.2 M) required for the second reaction step. After cooling in an ice bath, 4,7,10-trioxaoctadecanediimi, 1.76 g (8 mmol), was added, followed by NEt₃, 810 mg (8 mmol), and 3-(dimethylamino)propylamine, 817 mg (8 mmol). The target macromonomer was isolated as a water- and methanol-soluble solid in a yield of 841 mg (8 mmol), gave 10 as a water- and methanol-soluble solid in a yield of 841 mg (8 mmol), gave 10 as a water- and methanol-soluble sol in a yield of 475 mg (10.8%).

1H NMR, δ/ppm: 4.55, 10H (10H; NHCH₂NH₂); 2.8, 21H (20H; NHCOCH₂); 2.7–2.25, 49H (44H; CH₂N(CH₂)(CH₃), CH₂NH₂C(NH₂)CH₂N(CH₂)(CH₃), 2.2, 21.5H (24H; CH₂); 1.6, 7.7H (8H; CH₂CH₂CH₂H).

Polymers 11. MBA, 2.47 g (16 mmol), was dissolved in 25 mL of hot isopropanol-H₂O (4:1). The mono-N-Boc derivative 14, 1.39 g (8 mmol), dissolved in 5 mL of isopropanol, was added. Upon saturation with N₂, the resulting solution was stirred for 3 d at ambient temperature. Following solvent removal by rotatory evaporation, the residual macromonomer was redissolved in 80 mL of the same solvent blend, thus providing the high dilution ([MBA] = 0.2 M) required for the second reaction step. After cooling in an ice bath, 4,7,10-trioxaoctadecanediimi, 1.76 g (8 mmol), was added, followed by NEt₃, 810 mg (8 mmol), and stirring of the solution, resaturated with N₂, was continued for 1 d at room temperature and another 2 d at 60°C. The solvent was removed again under reduced pressure, 10 mL of trifluoroacetic acid was added, and stirring was continued for 1 h at ambient temperature. The acid was removed under reduced pressure, and the residual material was washed with hot toluene to remove traces of unreacted oligo(ethylene oxide). Polymer precipitation and further work-up was as described for polymer 1. There was obtained 516 mg (10.7%) of water- and methanol-soluble solid 11; \( \eta_{inh} \), 19.8 mL g⁻¹.

1H NMR, δ/ppm: 4.55, 4H (4H; NHCH₂NH₂); 3.7–3.5, 12H (12H; CH₂OCH₂); 3.1, 7.5H (8H; NHCOCH₂); 2.7–2.4, 14H (14H; CH₂OCH₂); 1.6, 8.1H (8H; CH₂CH₂CH₂H).

1H NMR, δ/ppm: 4.55, 4H (4H; NHCH₂NH₂); 3.6–3.5, 8.8H (8H; CH₂OCH₂); 2.8–2.25, 62H (60H; NHCOCH₂); 2.15, 6.5H (6H; CH₂); 1.55, 2H (2H; CH₂CH₂CH₂H).

The standard synthesis method was used for the preparation of 6, except that isopropanol-water (4:1), 25 mL, was employed as the solvent, and the heating period of the second step was reduced to 36 h. The following amounts of reactants were used: MBA, 2.47 g (16 mmol), N-Boc derivative 16, 651 mg (3.2 mmol), and 3-(dimethylamino)propylamine, 1.31 g (12.8 mmol). The water- and methanol-soluble, solid 6 was collected in a yield of 0.72 g (17.5%); \( \eta_{inh} \), 16.8 mL g⁻¹.
Polymer 12. For the preparation of 12 the same procedure was used as in the preceding experiment, except that the trioxatridecanediamine was replaced by O,O’-bis(3-amino-propyl)poly(ethylene glycol) 1500, 12 g (8 mmol), and the solvent volume in the second reaction step was increased to 100 mL. The solid, water- and methanol-soluble 12 was collected in a yield of 3.79 g (25.2%).

## 3. Results and Discussion

Methylenebisacrylamide (MBA), used in some of our earlier\textsuperscript{5,6} and more recent\textsuperscript{7} investigations, was chosen as the bifunctional acrylic acid derivative, to be copolymerized in various feed ratios with functionalized monoamines and diamines mono-N-protected by the tert-butoxycarbonyl substituent. Deprotection of the primary amino groups in the intermediary structures so obtained would then give the target polymers 1–10 (Scheme 2).

For comparison a hydroxyl-terminated side chain incapable of adding to the polymers’ cationic behaviour has been introduced in 9 and 10. Various short-chain aliphatic spacers designed to provide spacing between main chain and drug represent the segment R1 as depicted in Scheme 1.

The polymerizations, conducted in aqueous medium, were performed in two steps. The first step involved reaction of MBA with the protected amine, NH$_2$-R$_2$-NH-BOC, for 2 d at 25–50°C. Upon the addition of the second amine comonomer, NH$_2$-R$_1$, the experiments were then continued for 2–3 d at 50–60°C. A brief treatment with ethanolamine to eliminate any terminal unsaturation was followed by solvent removal, treatment with trifluoroacetic acid for N-deprotection, and acid removal under
reduced pressure. The crude target polymers were worked up by aqueous dialysis in tubing with 12 000–14 000 and 25 000 molecular mass cut-off and were isolated in the solid state upon freeze-drying. The products possessed complete solubility in water and, as demanded, in methanol. They were structurally characterized by comparison of the NH-CH2-NH methylene proton resonance near 4.5 ppm with other prominent bands in the 1H NMR spectra.

Contrasting with polymers 1–10, in which side groups served as the solubilizing entities, polymeric structures comprising the solubilizing units as main chain segments are exemplified by 11 and 12 (Scheme 3). The synthesis again involved a two-step process. In the first step, MBA was treated with 0.5 equivalents of a mono-N-protected diamine. The bis(acrylamido)-terminated macromonomer so generated was allowed in the second step to copolymerize with primary diamines of the type NH2-CH2(CH2CH2O)n(CH2)3NH2, where n = 3 and 32, thus giving polymers 11 and 12, respectively. Polymerization and work-up conditions were similar to those leading to 1–10, and the ultimate polymer products retained water and methanol solubility.

The ultimate yields in these polymerization experiments were quite low, ranging from 10 to 25%. It was emphasized previously1,5–7 that the polymerization process of bisacrylamides with amines by a Michael addition mechanism is inherently inefficient. Aqueous or partly aqueous media are necessary for an efficacious addition step, and in this solvent hydrolytic chain fission involving the rather labile amide links invariably militates against the propagation sequence. As a result the growing chain will become increasingly vulnerable to fragmentation, and the ensuing molecular mass distribution of the polymeric product will be unduly wide. The rigorous fractionation step by dialysis in 25 000 tubing will therefore remove the lower-molecular material as the predominant product portion, leaving a very minor fraction in the retentate. As in previous work, we accept the resultant low yields as a price to be paid for having at hand a collection of polymer samples with molecular masses in the desired range of 20 000–40 000, sufficiently high to retard renal excretion, yet low enough to avoid toxicological problems.

In order to demonstrate the proneness of these NH2-substi-
tuted polyamidoamines to conjugation with bioactive agents in methanolic solution, polymer 11 was chosen to serve as a drug carrier. The compound, dissolved in methanol, was treated (72 h, room temperature) with 1.2 equivalents of N-succinimidyl 4-ferrocenybutanoate\textsuperscript{12–14} (Scheme 4). Ferrocene, di-η\textsuperscript{5}-cyclopentadienyl-iron(II), is an experimental drug, which in polymer-bound form has shown highly promising antiproliferative properties,\textsuperscript{15} and the active ferrocenylbutanoate ester has been the ferrocenylation agent of choice in this laboratory. Following solvent removal, an aqueous work-up similar to that employed for the isolation of 1 to 12 afforded the ferrocene conjugate 11-Fc as a water-soluble polymer in 72\% yield. A ferrocene content corresponding to 93\% acylation of primary amino groups was determined from \textsuperscript{1}H NMR data.

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Supplementary Material
The proton NMR spectra of the different polymers are available as supplementary material.

References