Hetero-Difunctional Dimers as Building Blocks for the Synthesis of Poly(amidoamine)s With Hetero-Difunctional Chain Terminals and Their Derivatives

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ABSTRACT: This article reports on a simple and straightforward preparation method of poly(amidoamine)s (PAAs) with hetero-difunctional chain ends as well as of several up to now hardly obtainable PAA derivatives of biotechnological interest, such as for instance PAAs of controlled molecular weight and narrow polydispersity mono-functionalized at one end with an acrylamide group, PAAs with star-like molecular architecture, graft-PAA-protein conjugates, "tadpole-like" PAA conjugates with hydrophobic moieties able to self assemble into nanoparticles in aqueous media. The key step was to design suitable building blocks consisting of hetero-difunctional dimers (HDDs). In particular, the HDDs considered were the mono-addition products of bis-*sec*-amines and bisacrylamides expected to give PAAs of proven biomedical potential and were obtained as hydrochlorides or trifluoroacetates. In this form, they could be indefinitely kept dormant at 0–5 °C in the dry state, whereas at room temperature and in aqueous media, they polymerized at pH > 7.5. The preparation of the above-cited PAA derivatives did not necessarily involve the preliminary synthesis of hetero-difunctional PAAs but was directly achieved by one-pot polymerization of HDDs in the presence of the substrates of interest. © 2012 Wiley Periodicals, Inc. J Polym Sci Part A: Polym Chem 000: 000–000, 2012

KEYWORDS: amphiphiles; nanoparticles; polyamines; poly(amidoamine)s; protein grafting; star polymers

INTRODUCTION Poly(amidoamine)s (PAAs) are synthetic polymers obtained in linear form by Michael-type polyaddition of *prim*-mono-amines or bis-*sec*-amines to bisacrylamides (Scheme 1).[1]

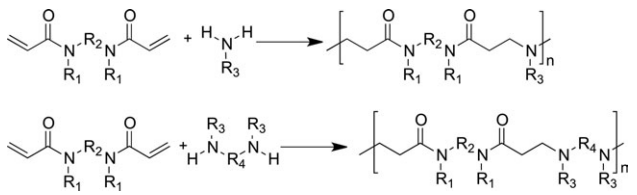
PAAs are characterized by the presence of amide (a) and *tert*-amine groups (b) regularly arranged along the polymer chain according to sequences

a —a —b
or
a —a —b —b

The polymerization reaction takes place in aqueous or alcoholic solutions, at room temperature and without added catalysts. Since under these conditions many functional groups, if present, do not interfere in the Michael reaction, the structure and the physico-chemical properties of PAAs, including acid-base properties, can be tuned within ample limits.[2] Most PAAs are degradable in aqueous media at pH > 7 and biocompatible even as polycations.[3–6] Some purposely planned PAAs are amenable to intracellular localization and act as endosomolytic polymers with potential for the intracellular delivery of genes and toxins.[4,7] Amphoteric PAAs carrying carboxyl groups as side substituents may be nearly as biocompatible as dextran, and one of them, named ISA23, when injected in animals exhibited "stealth-like" properties and passively concentrated in solid tumors by the so-called enhanced permeation and retention effect.[5] Recently, PAAs containing disulphide bonds in the main chain have been prepared from *N,N′*-bis acryloylcystamine and various amines and found to be amenable of bio-reductive degradation, hence useful both in linear and crosslinked form as carriers for selectively delivering bioactive substances in particular body districts.[8–12]

The traditional synthesis of PAAs has a serious limitation. It is apparent from Scheme 1 that to obtain high-molecular weight products the functions involved, that is, activated double bonds and amine hydrogens, must be stoichiometrically balanced. A perfectly balanced reacting mixture will contain three types of macromolecules, a — ····· — a, b — ····· — b, and a — ····· — b in 1:1:2 ratio. Unbalanced mixtures will contain the same molecular species, albeit in different ratios, until the minority function is completely consumed. Only at this point, the product will be entirely

Additional Supporting Information may be found in the online version of this article.



SCHEME 1 Traditional synthesis of linear PAAs by Michael-type polyaddition.

constituted of molecules doubly terminated by the excess function. There is no way, by the traditional method, to straightforwardly obtain PAAs with controlled hetero-difunctional chain terminals, that is, PAAs solely containing molecules of "a — ····· — b" type. This precluded to PAAs the access to the remarkable number of biotechnological applications, for instance liposome preparation, drug conjugation, and protein modification, which have been so far nearly uniquely mastered by hetero-difunctional PEGs that, however, are far to be endowed with the functional versatility of PAAs. In the past, a PAA-albumin conjugate was prepared by employing a large excess of doubly vinyl-terminated, low-molecular weight PAA apt to react with the exposed protein thiol and amine groups. However, despite tedious purification procedures, the presence of "bridged" protein molecules could not be excluded.[13]

Two strategies exist, in principle, for unequivocally preparing hetero-diterminated PAAs, either the ring-opening polymerization of cyclic precursors or the self-polyaddition of hetero-difunctional ("a —*a*—*b*—b" or "a —*a*—b") dimers (HDDs). The first strategy was ruled out because it was thought difficult to design a cyclic PAA precursor amenable to thermal or catalytic ring-opening polymerization, especially in the case of purpose-tailored PAAs carrying reactive side-substituents, as for instance carboxyl groups, liable to interfere in these processes. Therefore, the preparation of HDDs, in particular those of the "a —*a*—*b*—b" type, expected to polymerize according to Scheme 2, was preferred. We limited our investigation to "a —*a*—*b*—b" type HDDs because the procedure adopted in this investigation was not easily applicable to the preparation of the "a —*a*—b" ones. A different approach would be actually needed, hopefully to be reported in a future article.

The synthetic scope of HDDs is manifold. They can be polymerized to high-molecular weight PAAs without bothering with stoichiometric balance. PAAs of controlled average molecular weight and mono-functionalized with an acrylamide- or a *sec*-amine group at one end can be prepared by adding, respectively, a controlled amount of mono-functional acrylamides or *sec*-amines, whereas the addition of multifunctional acrylamides or amines will lead to star-like PAAs. Block PAA-PAA or PAA-PEG copolymers with controlled structure will be easily obtained. "Velvety-like" grafting of PAA chains to properly functionalized surfaces can be achieved. PAA chains of controlled average length can be grafted to proteins with no risk of undesirable side reactions such as protein–protein coupling or crosslinking. "Tadpole-like" PAA conjugates with hydrophobic moieties forming in aqueous media liposomes or nanoparticles can be prepared as functional drug carriers. Based on this premise, the aim of this article is to report on the preparation of some model HDDs corresponding to PAAs extensively studied for biomedical applications and to provide selected factual examples of their synthetic potential.

EXPERIMENTAL

Materials

2,2-Bis(acrylamido)acetic acid (BAC) and 1,4-bis(acryloyl)piperazine (BP) were synthesized as previously described,[14,15] lithium hydroxide monohydrate (98%), piperazine hexahydrate (P) (99%), 2-(R, S)-methylpiperazine (MP) (95%), 1,4,8,11-tetrazacyclotetradecane (Cyclam) (98%), di-(*tert*-butyl)dicarbonate (97%), thiocholesterol (TC), bovine serum albumin (BSA) (96%), 4-acryloylmorpholine (97%), morpholine (99.5%), guanidine hydrochloride (98%), glacial acetic acid, hydrochloric acid (37%), sodium hydroxide (98.5%), sodium chloride (99%), dichloromethane (reagent grade), trifluoroacetic acid, DOWEX® MAC-3 ion exchange resin were purchased from Sigma-Aldrich and used as received.

Methods

Nuclear Magnetic Resonance (NMR) Spectra

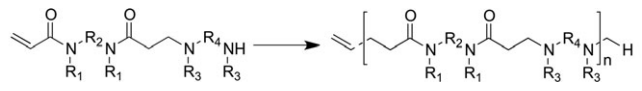
^{1}H NMR, ^{13}C NMR, distortionless enhanced by polarization transfer (DEPT), ^{1}H-^{1}H correlation (COSY), heteronuclear multiple bond correlation (HMBC), and heteronuclear correlation (HETCOR) spectra were run at room temperature on a Brüker Advanced 400 spectrometer operating at 400.132 and 100.623 MHz, respectively.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) traces were obtained using a Knauer Pump 1000 equipped with a Knauer Autosampler 3800, TKSgel G4000 PW, and G3000 PW TosoHaas columns connected in series, light scattering/viscometer Viscotek 270 Dual Detector and a refractive index detector Waters model 2410. The mobile phase was a 0.1 M Tris buffer pH 8.1 ± 0.05 with 0.2 M sodium chloride. The sample concentration was 2% (w/v) and the flow rate 1 mL/min. Triple detector SEC allowed determining, besides the molecular weight, the long chain branching (LCB) frequency of star-like PAAs by means of the dedicated Viskotek software, which compared their Mark–Houwink plots with those of samples of their linear counterparts of approximately the same molecular weight and used common Zimm–Stockmayer equations and methods.[16]

Dynamic Light Scattering

Dynamic light scattering (DLS) analysis was performed to evaluate the hydrodynamic radius of polymeric micelles using a Malvern NanoZS instrument (Malvern Instruments, Worcestershire, UK) with a laser fitted at 532 nm and fixed



SCHEME 2 Self-polymerization of the "a —*a*—*b*—b" type HDDs.

173° scattering angle. DLS measurements were performed on two sets of experiments. In the first, samples were prepared in phosphate buffur solution (PBS) saline in the concentration range of 0.1–1 mg/mL. In the second one, the sample was prepared in PBS saline with concentration of 1 mg/mL, and the measures were performed varying the temperature: 20, 25, 30, 35, 40, 45, 50, 55, and 60 °C. Before each analysis, the samples were filtered through a syringe filter (5 μm pore size; Watmann, UK) to remove the dust. All data were evaluated as numeral distribution of the hydrodynamic diameter.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) analysis of self-assembled polymeric BAC-MP-TC micelles was captured using the negative staining technique. A solution of BAC-MP-TC (1 mg/mL) in ultrapure water at pH 7.4 was prepared; then a 5 μL aliquot was deposited onto a Formvar-coated Cu grid (400 mesh). After adsorption, the grid was stained with 1% uranyl acetate solution (5 μL), wiped away by means of a paper filter and dried at room temperature. Observation was made by using a LEO 912 AB energy-filtering transmission electron microscopy (EFTEM; Carl Zeiss, Obercochen, Germany) operating at 80 kV. Digital images were collected by Proscan 1K slow-scan charge-coupled device camera (Proscan Scheuring, Germany).

Synthesis of 4-N-Boc-2-(R, S)-methylpiperazine (MP-Boc)

Typically, 2-(R, S)-methylpiperazine (MP) (21.1 g, 0.200 mol) was dissolved in tap water (1.2 L) and glacial acetic acid (12.0 g, 0.197 mol) was added. The mixture was gently stirred for 15 min then cooled to 0 °C. After this time, a solution of di-(*tert*-butyl)dicarbonate (34.9 g, 0.160 mol) in methanol (150 mL) was added dropwise in 30 min to the reactive mixture. During this time, the reaction was vigorously stirred and the temperature gradually warmed up to room temperature. The reaction was maintained under this condition for further 20 min, then the precipitated formed filtered and the pH was adjusted to 5.5 with 1 M HCl. The aqueous phase was extracted with CH_2Cl_2 (2 × 100 mL) to separate the disubstituted derivative then the pH was adjusted to 9.5 with 1 M NaOH. The desired product was recovered by repeated extraction with CH_2Cl_2 (5 × 200 mL), the organic layers collected and dried over anhydrous sodium sulfate. The solvent was evaporated to afford the crude solid. Yield: 90%.

^{1}H NMR ($CDCl_3$, δ, ppm): 0.99 (d, $HNCH_2CHC\mathbf{H_3}$), 1.41 (s, $(C\mathbf{H_3})_3C$), 1.57 (br, $N\mathbf{H}CH_2CH$), 2.34 (br, axial $NHC\mathbf{H}_2CH$), 2.64 (m, $NHCH_2C\mathbf{H}$), 2.72 (m, axial $NHCH_2C\mathbf{H}_2NBoc$), 2.90 (m, equatorial $NHC\mathbf{H}_2CH$), 2.95 (br, equatorial $NHC\mathbf{H_2}C\mathbf{H}_2N$). ^{13}C NMR ($D_2O$, δ, ppm): 19.16 ($\mathbf{C}H_3CH$), 28.15 ($(\mathbf{C}H_3)_3CO$), 45.71 ($HN\mathbf{C}H_2\mathbf{C}H_2N$, $CH_3CH\mathbf{C}H_2N$), 51.65 ($CH_3\mathbf{C}H$), 79.51 ($(CH_3)_3\mathbf{C}O$), 154.70 ($N\mathbf{C}O$). Anal. Calcd for: $C_{10}H_{20}N_2O_2$: C, 59.97; H, 10.07; N, 13.99. Found: C, 59.12; H, 10.13; N, 13.75.

Synthesis of N-Boc-piperazine (P-Boc)

P-Boc was synthesized following the same procedure above described for MP-Boc using piperazine hexahydrate (25.0 g, 0.129 mol), glacial acetic acid (7.6 g, 0.127 mol), di-(*tert*-butyl)dicarbonate (23.2 g, 0.103 mol), and tap water (1.2 L). Yield: 87%.

^{1}H NMR ($CDCl_3$, δ, ppm): 1.47 (s, $(C\mathbf{H_3})_3C$), 1.82 (br, $N\mathbf{H}CH_2CH_2NBoc$), 2.81 (m, $HNC\mathbf{H_2}CH_2NBoc$), 3.40 (m, $NHCH_2C\mathbf{H_2}NBoc$). ^{13}C NMR (D_2O, δ, ppm): 28.97 ($(\mathbf{C}H_3)_3CO$), 45.21 ($HN\mathbf{C}H_2\mathbf{C}H_2N$), 79.52 ($(CH_3)_3\mathbf{C}O$), 154.84 ($N\mathbf{C}O$). Anal. Calcd for: $C_9H_{18}N_2O_2$: C, 58.04; H, 9.74; N, 15.04. Found: C, 56.12; H, 10.78; N, 14.46.

Synthesis of BAC-P

To a vigorously stirred solution of *N*-Boc-piperazine (1.3200 g, 7.042 mmol) in bidistilled water (15 mL) cooled at 0 °C, a solution of 2,2-bis(acrylamido)acetic acid (BAC) (2.8000 g, 14.084 mmol) and $LiOH{\cdot}H_2O$ (0.5963 g, 14.084 mmol) in bidistilled water (30 mL) was added dropwise. The reaction was maintained at 0 °C and left under stirring for 2 days. After this period, the crude reaction mixture was purified by ionic exchange chromatography on Dowex Mac-3 (8 × 60 cm) using bidistilled water (500 mL), and then an acid concentration elution gradient passing from 10^{-4} M to 0.1 M hydrochloric acid (1000 mL total). The product obtained was freeze-dried then dissolved in trifluoroacetic acid to obtain a 1 M solution maintained for 3 h under gentle stirring in nitrogen atmosphere. The product was precipitated by addition of diethyl ether (10 mL), washed with fresh solvent (3 × 10 mL), and dried under vacuum affording a white and deliquescent solid. Yield: 55%.

^{1}H NMR and ^{13}C-NMR (D_2O) are reported in Supporting Information (Fig. S1, Table S1). Anal. Calcd for: $C_{12}H_{20}N_4O_4$: C, 50.69; H, 7.09; N, 19.71. Found: C, 49.46; H, 7.31; N, 18.83.

Synthesis of BAC-MP

The reaction and purification of BAC-MP were performed as previously described for BAC-P using BAC (2.8000 g, 14.084 mmol), $LiOH{\cdot}H_2O$ (0.5963 g, 14.084 mmol) and 4-*N*-Boc-2-methylpiperazine (1.4100 g, 7.042 mmol) as reagents. Yield: 60%.

^{1}H NMR and ^{13}C-NMR (D_2O) are reported in Supporting Information (Fig. S2, Table S1). Anal. Calcd for: $C_{13}H_{22}N_4O_4$: C, 52.34; H, 7.43; N, 18.78. Found: C, 50.47; H, 7.62; N, 17.89.

Synthesis of MBA-P

To a solution of MBA (4.7438 g, 30.77 mmol) in bidistilled water (220 mL) a solution of *N*-Boc-piperazine (1.9297 g, 10.46 mmol) in bidistilled water (50 mL) was added dropwise under stirring at 0 °C. The reaction was maintained at 0 °C for 48 h and monitored by thin layer chromatography (TLC) using 2-propanol/water/0.15 M acetate buffer pH 5.5 = 2:2:6 (v/v) as eluent. During this time, a precipitate was formed that was filtered off and the solution acidified at pH 3.0 using 1 M hydrochloric acid. Unreacted MBA was successfully eliminated by repeated extraction with diethyl ether (5 × 100 mL) and ethyl acetate (2 × 150 mL). The aqueous solution was treated with 5% hydrochloric acid (4.6 mL) and stirred for 24 h. The mixture was then freeze dried and the product obtained as a white solid. Yield: 70%.

^{1}H NMR and ^{13}C-NMR (D_2O) are reported in Supporting Information (Fig. S3, Table S1). Anal. Calcd for: $C_{11}H_{20}N_4O_2$: C, 54.98; H, 8.39; N, 23.32. Found: C, 49.98; H, 8.83; N, 21.14.

Synthesis of MBA-MP, BP-P, and BP-P

The same procedure above described for MBA-P was followed.

MBA-MP. Yield: 65%.

^{1}H NMR and ^{13}C-NMR (D_2O) are reported in Supporting Information (Fig. S4, Table S1). Anal. Calcd for $C_{12}H_{22}N_4O_2$: C, 56.67; H, 8.72; N, 22.03. Found C, 51.92; H, 9.11; N, 19.74.

BP-P. Yield: 81%.

^{1}H NMR and ^{13}C-NMR (D_2O) are reported in Supporting Information (Fig. S5, Table S1). Anal. Calcd for $C_{14}H_{24}N_4O_2$: C, 59.98; H, 8.63; N, 19.98. Found: C, 59.43; H, 8.73; N, 19.59.

BP-MP. Yield: 75%.

^{1}H NMR and ^{13}C-NMR (D_2O) are reported in Supporting Information (Fig. S6, Table S1). Anal. Calcd for $C_{15}H_{26}N_4O_2$: C, 61.20; H, 8.90; N, 19.03. Found: C, 61.25; H, 9.02; N, 18.98.

Synthesis of BAC-MP Homopolymer (ISA23)

To a solution of BAC-MP (0.4 g, 1.3408 mmol) in bidistilled water (450 μL), 1 M NaOH was added dropwise until pH 9, and the reaction kept under stirring at room temperature for 5 days in nitrogen atmosphere. After this period, the mixture was acidified to pH 4.4–5.5 with 37% HCl, filtered through a paper filter then ultrafiltered through a membrane with nominal cut-off of 3000. The product was obtained after freeze-drying as a yellowish solid. Yield: 79%.

M_n = 38,000, M_w = 50,000, M_w/M_n = 1.32, R_h = 7.4 nm, [η] = 0.567 dL/g, a = 0.773, log K = −3.855. ^{1}H NMR (D_2O, δ, ppm): 1.26 (d, C**H$_3$**CH), 2.64 (m, C**H$_2$**CONH), 2.85 (m, C**H**CH$_3$), 3.07 (br, NC**H$_2$** MP ring), 3.28 (br, equatorial NC**H$_2$**CH$_2$ MP ring), 3.28 (br, axial NC**H$_2$**CH$_2$N MP ring, NC**H$_2$**CH$_2$), 5.51, 5.45 (ds, C**H**COOH and C**H**COO$^-$).

Synthesis of BP-P Homopolymer

The reaction was performed as above reported, starting from BP-P (0.7200 g, 5.569 mmol) dissolved in bidistilled water (1.4 mL). After 5 days under gentle stirring, the mixture was acidified with concentrated HCl, filtered through a paper filter then ultrafiltered through a membrane with nominal cut-off of 3000. The pure product was recovered after freeze-drying. Yield: 72.5%.

M_n = 30,900, M_w = 38,000, M_w/M_n = 1.13, R_h = 3.808 nm, [η] = 0.294 dL/g, a = 1.01, log K = −4.703. ^{1}H NMR (D_2O, δ, ppm): 2.58 (br, C**H$_2$**CONH), 3.06 (s, NC**H$_2$** piperazine ring), 3.12 (m, NC**H$_2$**CH$_2$), 3.31 (br, C**H$_2$**NCO piperazine ring), 3.38 (br, C**H$_2$**NCO piperazine ring).

Synthesis of BAC-MP-Acryloylmorpholine Conjugate

Acryloylmorpholine (4.4 mg, 0.035 mmol) was dissolved in 0.1 M sodium hydroxide (150 μL) and BAC-MP (100.0 mg, 0.335 mmol) was added under vigorous stirring until a clear solution was obtained. Then, 1 M NaOH was dropped into the reaction mixture until pH 8.5 was reached, and the reaction was kept 3 days at room temperature under nitrogen atmosphere. After this time, the mixture was acidified at pH 5 by the addition of 1 M HCl. The final product was obtained after freeze drying. Yield: 97%.

M_n = 3200, M_w = 4100, M_w/M_n = 1.28, R_h = 6.8 nm, [η] = 0.234 dL/g, a = 0.781, log K = −3.74. ^{1}H NMR (D_2O) is reported in Supporting Information (Fig. S7).

Synthesis of BAC-P-grafted-BSA

Guanidine hydrochloride (0.5056 g, 5.190 mmol) was dissolved in bidistilled water (5 mL), BSA (1.5065 g, 0.023 mmol) was added, and the mixture was vigorously stirred for 10 min until a clear solution was obtained. BAC-P (0.3190 g, 1.122 mmol) was then added to the mixture, and the pH was adjusted at 8.0 by the addition of solid Na_2CO_3. The reaction was kept under nitrogen atmosphere and gentle stirring for 5 days. The mixture was purified by dialysis against water (membrane cut-off 50,000) and the pure product collected after freeze-drying. Yield: 72%.

Molecular weight and viscometric data are reported in Table 2.

Synthesis of BAC-MP-grafted-BSA

The reaction was performed following the same procedure described for BAC-P-grafted-BSA using guanidine hydrochloride (0.5056 mg, 5.190 mmol), BSA (1.5065 g, 0.023 mmol), BAC-MP (0.3347 g, 1.122 mmol). The pure product was obtained after the usual ultrafiltration procedure and freeze drying. Yield: 84%.

Molecular weight and viscometric data are reported in Table 2.

Synthesis of BP-P-grafted BSA

The same procedure as in the previous case was followed using guanidine hydrochloride (0.5056 mg, 5.190 mmol), BSA (1.5065 g, 0.023 mmol), and BP-P (0.3146 mg, 1.122 mmol). The usual work-up afforded the pure product. Yield: 79%.

Molecular weight and viscometric data are reported in Table 2.

Synthesis of Star-like BAC-MP

To a solution of cyclam (2.0 mg, 0.001 mmol) in 0.5 M NaOH (150 μL), BAC-MP (100.0 mg, 0.335 mmol) was added, and the reaction left 5 days at pH 8 under gentle stirring. After this time, the mixture was freeze-dried and the dry product recovered. Yield: 98%.

M_n = 10,800, M_w = 11,660, M_w/M_n = 1.08, [η] = 0.125 dL/g, a = 0.607, log K = −3.706, branching frequency = 2.17. ^{1}H NMR (D_2O) is reported in Supporting Information (Fig. S8).

Synthesis of Star-like BP-MP

The same procedure above described was followed using cyclam (20.4 mg, 0.102 mmol), 0.5 M sodium hydroxide (550 μL), and BP-MP (300.0 mg, 1.020 mmol). The reaction was filtered through a paper filter and the crude product freeze-dried. Yield: 97%.

M_n = 3300, M_w = 3900, M_w/M_n = 1.21, [η] = 0.128 dL/g, a = 0.521, log K = −3.823, branching frequency = 2.08. ^{1}H NMR (D_2O) is reported in Supporting Information (Fig. S9).

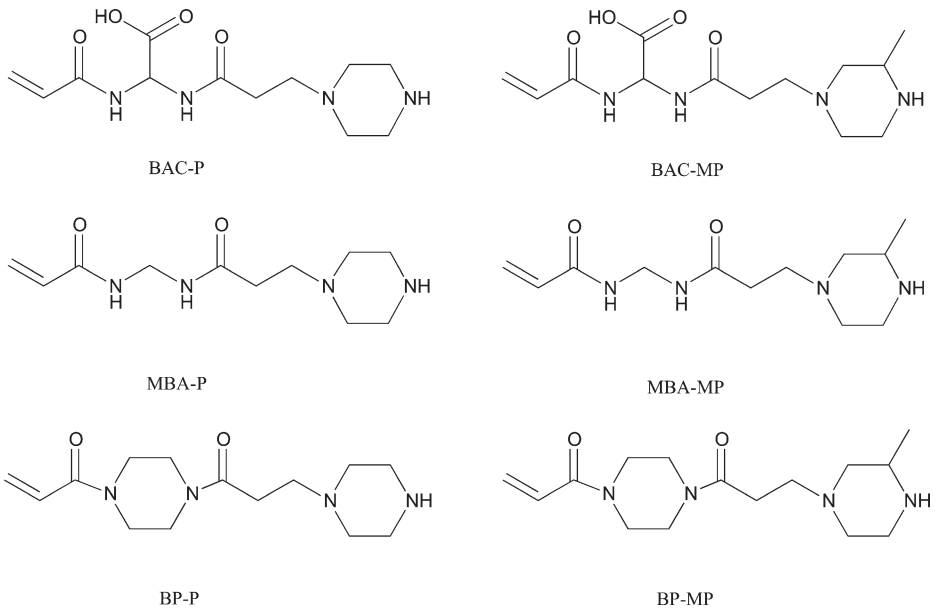


FIGURE 1 Structures of the HDDs synthesized.

Synthesis of Hetero-difunctional BAC-MP-TC

To a solution of BAC-MP (103.3 mg, 0.251 mmol) in bidistilled water (1 mL), TC (10.1 mg, 0.025 mmol) dissolved in diethyl ether (0.1 mL) was added, and the formation of a macroemulsion was observed. The pH was adjusted to 7.0 by the addition of 3 M NaOH, and the mixture vigorously stirred for 2 days. Then, 1 M NaOH was added until pH 9 and the reaction maintained in this condition under stirring for further 5 days. After this time, the mixture was acidified to pH 5 by the addition of 1 M HCl and purified by dialysis against water (membrane cut-off 3500) for 2 h. The crude product collected after freeze drying was suspended in diethyl ether (2 mL) to remove unreacted TC, and dried in vacuum. Yield: 38%.

Anal. Calcd for $(C_{13}H_{22}N_4O_4)_{10}$-$(C_{27}H_{46}S)$: C, 55.69; H, 7.92, N, 16.55. Found: C, 51.98; H, 8.46, N, 18.15.

Synthesis of Hetero-difunctional MBA-P-TC

The reaction was performed following the same procedure as in the previous case using MBA-P (60.3 mg, 0.251 mmol) and TC (10.1 mg, 0.025 mmol). Yield: 29%.

Anal. Calcd for $(C_{11}H_{20}N_4O_2)_{10}$-$(C_{27}H_{46}S)$: C, 58.64; H, 8.84; N, 19.97. Found: C, 58.00; H, 8.76; N, 20.56.

Synthesis of Hetero-difunctional BP-P-TC

The reaction was performed following the same procedure as in the case of ISA23-TC using: BP-P (70.4 mg, 0.251 mmol) and TC (10.1 mg, 0.025 mmol). Yield: 44%.

Anal. Calcd for $(C_{14}H_{24}N_4O_2)_{10}$-$(C_{27}H_{46}S)$: C, 62.56; H, 8.99; N, 17.48. Found: C, 61.42; H, 8.83; N, 18.60.

Synthesis of Hetero-difunctional BPMP-TC

The reaction was performed following the same procedure as in the case of ISA23-TC using: BP-MP (73.9 mg, 0.251 mmol) and TC (10.1 mg, 0.025 mmol). Yield: 36%.

Anal. Calcd for $(C_{15}H_{26}N_4O_2)_{10}$-$(C_{27}H_{46}S)$: C, 63.53; H, 9.21; N, 16.74. Found: C, 62.75; H, 9.11; N, 17.50.

RESULTS AND DISCUSSION

The aim of this work was to prepare hetero-diterminated PAAs as well as some novel PAA derivatives not previously obtained by simple methods. HDDs were the simplest building blocks capable of exactly reproducing the same sequence of amide-and *tert*-amine groups along the polymer chain of PAAs, while unequivocally ensuring hetero-difunctional chain terminals. As mentioned in Introduction, linear PAAs can be prepared by the stepwise polyaddition of bisacrylamides and either *prim*-monoamines or *sec*-bisamines, which yields PAAs with different chain structures. The investigation reported in this paper concerned only HDDs leading to PAAs with "a —*a* —*b* —b" sequence of amide- and amine groups along the polymer chain, that is, HDDs obtained by the mono-addition of bis-*sec*-amines to bisacrylamides (Scheme 2). Six HDDs were prepared starting from 2,2-bisacrylamidoacetic acid (BAC), 1,4-bisacryloylpiperazine (BP), and *N,N′*-methylenebisacrylamide (MBA) as bisacrylamides and piperazine (P) and 2-methylpiperazine (MP) as amines. BAC was selected because, in combination with MP, it gives ISA23, the highly biocompatible "stealth-like" amphoteric PAA mentioned in Introduction. BP and MBA were chosen because with P and MP they give PAAs extensively studied as components of bioactive hydrogels (data not shown). Moreover, P and MP are endowed with high reactivity toward Michael polyaddition, coupled with limited basicity leading, as a rule, to good biocompatibility of the resultant PAAs.[1,5,6,7,14] The six HDDs obtained by combining the above building blocks were named BAC-P, BAC-MP, BP-P, BP-MP, MBA-P, and MBA-MP after their parent compounds. Their structures, together with some relevant characterizations, are reported in Figure 1 and Table 1.

TABLE 1 *C*/*N* Ratio of HDDs

	C/*N* Ratio	
HDD	Calculated	Found
BAC-P	2.57	2.63
BAC-MP	2.79	2.82
MBA-P	2.35	2.36
MBA-MP	2.57	2.63
BP-P	3.00	3.04
BP-MP	3.22	3.23

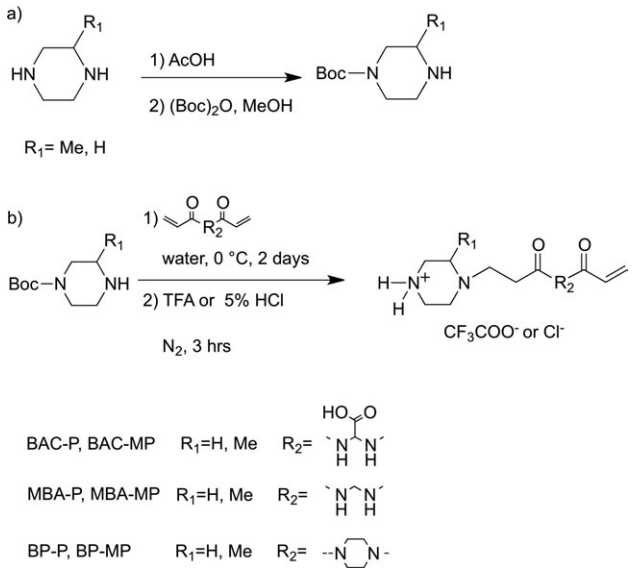
The general synthetic procedure adopted for preparing HDDs is depicted in Scheme 3. It involved the synthesis of mono-Boc-protected P and MP [Step (a)] followed by reaction with moderate excess bisacrylamide, isolation and purification of the mono-addition product, and cleavage of the protecting group [Step (b)].

Both steps deserve comments. In Step (a), Boc-P and Boc-MP were prepared from quasi-stoichiometric amounts of di-(*tert*-butyl)dicarbonate and mono-protonated P and MP, in turn obtained by *in situ* adding acetic acid to the amine in 1:1 molar ratio. Only a slight excess of amine mono-acetate was required to maximize the yield of the mono-substituted products, since the two pK_a values of both amines differ by $\sim$ four orders of magnitude,[17] and under the conditions adopted the amount of doubly protonated molecules and, correspondingly, of uncharged molecules amenable to react twice was minimal. In Step (b), the addition reactions of Boc-P and Boc-MP to BAC, BP, and MBA were performed for 2 days in water at 0 °C, using two- to threefold excess bisacrylamide. Under these conditions, the double addition to the same bisacrylamide molecule was minimized. It may be noticed that the excess bisacrylamide required to achieve this result was significantly lower than expected from purely statistical calculations. It would appear that at 0 °C the amine addition to one of the two double bonds biased the reactivity of the other notwithstanding the fairly long distance between them. However, at room temperature and with the same stoichiometric ratios, significant amounts of the double-addition products were formed. The product work-up depended on the bisacrylamide used. In the case of BAC, the reaction mixtures contained excess BAC, protected BAC-P (or BAC-MP) and small amounts of di-addition products, that is, di-protected trimers of the "b —*b* —*a* —*a* —*b* —b" type. All these products were highly polar and hardly extractable from water with organic solvents. Therefore, they were isolated by ion-exchange chromatography using a weakly acid (carboxylated) resin and following a two-step elution protocol. The first step was performed with bidistilled water as eluent, the second step with an acid concentration elution gradient passing from 10^{-4} M to 0.1 M hydrochloric acid (see Fig. 2). Unreacted BAC was eluted in the first step. In the second step, the mono-addition products were eluted first, followed by the di-substituted products, which were discarded. The combined fractions containing the mono-addition products were then lyophilized and pure BAC-P and BAC-MP finally obtained by deprotection with trifluoroacetic acid.

The addition reactions of MBA or BP with Boc-P or Boc-MP gave protected MBA-P, MBA-MP, BP-P, and BP-MP, respectively. The reaction mixtures, containing dimers and residual bisacrylamide, were acidified to pH 3 and the bisacrylamides fractionally extracted with organic solvents. Under these conditions, the protected dimers, being ionized, remained in the aqueous phase and were retrieved by freeze-drying. As the products were insoluble in TFA, the deprotection step was performed with 5% hydrochloric acid.

All protected HDDs were characterized by ^{1}H NMR. All deprotected HDDs were fully characterized by elemental analysis, ^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY, HMBC, and HETCOR (see Experimental and Supporting Information, Table S1). The results of these characterizations were in agreement with the proposed structures. *C*/*N* ratios by elemental analysis are reported in Table 1. As all HDDs were isolated as trifluoroacetates or hydrochlorides and vacuum dried or lyophilized, some loss of acid was inevitable and, therefore, the absolute values of elemental analyses were not fully reliable. However, the *C*/*N* ratios were not liable to be significantly affected by the isolation procedures and provided a reliable confirmation that the product structures were as expected.

All HDDs, as either hydrochlorides or trifluoroacetates, could be stored for a long time at 0–5 °C, if protected from moisture and did not polymerize at room temperature. In aqueous solution, polymerization was triggered by rising pH above 7.5 with bases inert toward Michael addition, such as inorganic hydroxides or tertiary amines. The molecular weights of the resultant polymers were usually high and



SCHEME 3 General synthetic pathway for the preparation of HDDs. Step (a): synthesis of mono-Boc-protected P and MP. Step (b): Michael-type reaction with bisacrylamide, and cleavage of the protecting group.

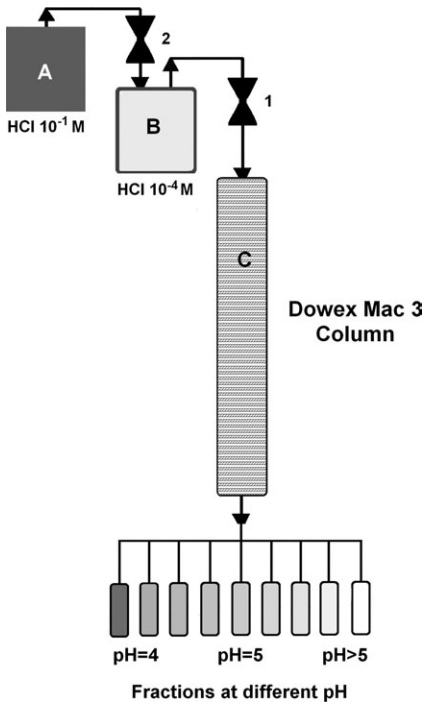


FIGURE 2 Equipment adopted to obtain the hydrochloric acid gradient used in the second elution step of BAC-P and BAC-MP isolation. Column C was connected through Valve 1 to Flask B containing 10^{-4} M hydrochloric acid, in turn connected through Valve 2 to Flask A containing 10^{-1} M hydrochloric acid. Each drop flowing out the column drew a drop from flask B, immediately replaced by a drop from flask A, thus obtaining the desired acid concentration gradient.

obviously related to the reaction time, whereas all practical difficulties to achieve a precise balance among the reactant functions were got round.

The synthetic potential of HDDs was assessed by performing model reactions, namely self-polyaddition, preparation of monofunctional PAA oligomers with controlled molecular weight, of star-like PAA architectures, of protein-PAA conjugates and of tadpole-like PAA-cholesterol amphiphiles. The aim was to test the general aptitude of HDDs to be used for these reactions and was limited to a few examples involving only some of the prepared dimers at a time. It may be noticed that most of the reaction products were hardly obtainable by the traditional PAA preparation method.

The self-polyadditions of BAC-MP and BP-P were performed as model syntheses of PAAs via the corresponding HDDs. The reaction progress was monitored by ^{1}H NMR until signals at 5.78, 6.13, and 6.67 ppm, relative to the acrylic hydrogens, fell below the detection threshold of the instrument. The products had, respectively, M_n 38,000 and 30,900, with M_w/M_n 1.32 and 1.13. These molecular weight values were significantly higher than those of the corresponding PAAs obtained by the traditional methods after the same reaction times, with lower polydispersities.

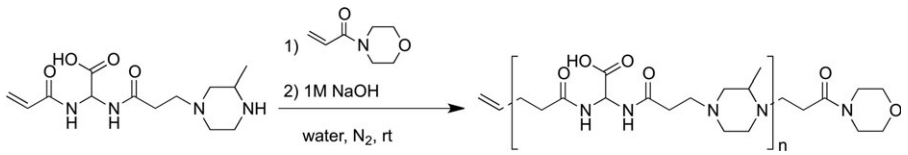
The polymerization of HDD mixtures with either monofunctional acrylamides or amines with approximately the same reactivity as the corresponding function present in HDDs, leads at 100% conversion to an average polymerization degree equal to:

$$\overline{X}_n = \frac{N_{\mathrm{HDD},0}}{N_{\mathrm{monofunctional},0}} \quad (1)$$

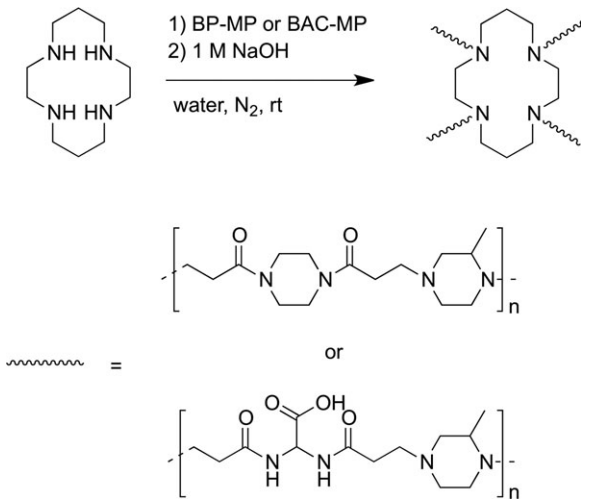
where $N_{\mathrm{HDD},0}$ is the initial number of HDD molecules and $N_{\mathrm{monofunctional},0}$ is the initial number of mono-functional compound. Obviously, M_n is given by the product of $\overline{X}_n$ times the molecular weight of the HDD used, added by the molecular weight of the mono-functional compound. The resultant polymer will be terminated at one terminus with the unreactive group and at the opposite terminus with the excess reactive function. The preparation of an ISA23 oligomer monofunctionalized at one terminus with an activated double bond by polymerization of BAC-MP in the presence of 4-acryloylmorpholine is reported in Scheme 4.

The reaction was performed adopting a 10/1 BAC-MP/4-acryloylmorpholine molar ratio. The reaction progress was monitored by ^{1}H NMR by comparing the signals at 6.22 and 5.62 ppm, relative to the end-chain acrylic group and the CH-COO$^-$ hydrogen of BAC, respectively (see Supporting Information Fig. S7). The reaction was stopped when in the NMR spectrum, the ratio of the reference peaks' integrals remained constant with time and was consistent with the presence of a double bond every 10 BAC-MP units, that is, the reaction yield had approached 100%. The M_n of the product was 3200, with M_w/M_n 1.28. This molecular weight value was very close to the expected value of 3150, as calculated from eq 1.

The same derivative, as well as the cholesteryl-terminated PAA (see below) could be also prepared by reacting 4-acryloylmorpholine and TC with presynthesized HDDS polymers. However, the direct polymerization of HDDs in presence of the same monofunctional compounds presented two distinct advantages: it could be performed in one-pot, one step reaction; moreover, both 4-acryloylmorpholine and TC acted in the mean time as chain regulators.



SCHEME 4 Synthesis of the mono-functionalized ISA23 oligomer by polymerization of BAC-MP in the presence of 4-acryloylmorpholine.



SCHEME 5 Synthesis of four-arm star-like PAAs from BP-MP and BAC-MP and cyclam.

Four-arm star-like PAAs (BP-MP-C and BAC-MP-C) were synthesized from BP-MP and BAC-MP and 1,4,8,11-tetraazacyclotetradecane (cyclam; Scheme 5). The polymerization reactions were performed, as usual, in aqueous solution at pH 9 and monitored by ^{1}H NMR. The products were isolated when the signals attributed to the double bond hydrogens fell below the instrument's detection threshold.

In the case of BP-MP-C, the adopted HDD/cyclam molar ratio was 10. The M_n of the product was 3300, with M_w/M_n 1.21. These values were in close agreement with the values of 3400 and 1.29 calculated from the classic literature's formulas[18,19] extrapolated for $p = 1$ (eqs 2 and 3):

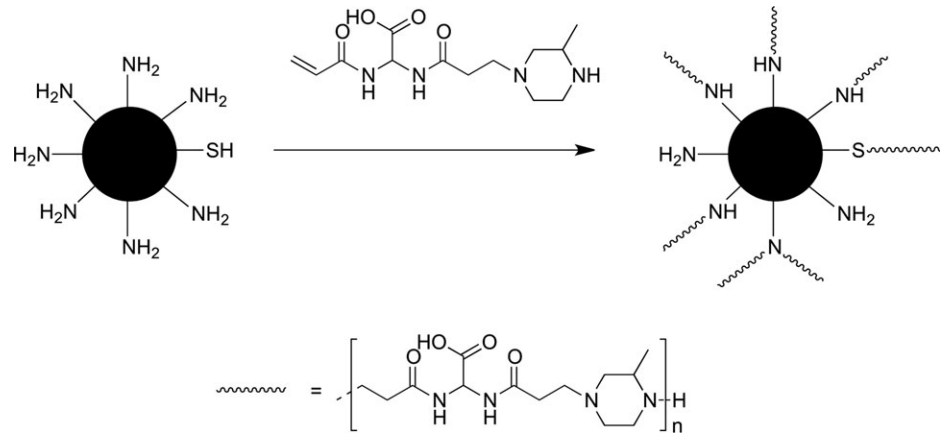
$$\bar{X}_n = \frac{(frp + 1 - rp)}{(1 - rp)} \quad (2)$$

$$\frac{\bar{X}_w}{\bar{X}_n} = 1 + \frac{frp}{(frp + 1 - rp)^2} \quad (3)$$

where f is the number of reactive functions of the multifunctional monomer (four for cyclam), $r = \frac{N_a}{N_b + fN_{b'}}$, $N_a = N_b$ is the molar amount of HDD and $N_{b'}$ is the molar amount of the multifunctional monomer. In the case of BAC-MP-C, the adopted HDD/cyclam molar ratio was 34.2. The product had M_n 10,800 with M_w/M_n 1.08, to be compared with the calculated values of 10,700 and 1.26. It may be observed that M_n was in excellent agreement with the expected value, whereas the M_w/M_n ratio was remarkably lower. In fact, the product obtained was quasi-monodisperse. Interestingly, this effect was not observed with BP-MP, suggesting that the zwitterionic nature of BAC-MP was largely responsible for it. Possibly, BAC-MP reversibly interacted with cyclam, resulting in a local catalytic activity of the latter favoring the growth of the chains attached to it over that of "free" molecular species. Thus, the behavior of the BAC-MP/cyclam system as regards the molecular weight distribution of the resultant polymer approached that of a cyclic monomer growing on a multifunctional center. The LCB frequency of BP-MP-C and BAC-MP-C was determined by SEC connected in series with refractive index (RI), light scattering, and viscometric detectors (see Experimental) and found in both cases of $\sim$ two side chains per macromolecule. This LCB frequency value was consistent with the expected four-arm architecture originating from a cyclam core.

BAC-P, BAC-MP, BP-P, and BSA were chosen as model HDDs and protein for the preparation of PAA-protein graft copolymers. This was simply achieved by triggering the HDD polymerization in aqueous medium in the presence of BSA that participated in the polymerization through its exposed NH_2 and SH groups. To give an example, the synthesis of BAC-MP-*g*-BSA is reported in Scheme 6.

The occurrence of PAA grafting onto BSA was evaluated by SEC connected in series with RI, light scattering, and viscometric detectors. The data, reported in Table 2, provided clear evidence of the increasing of the molecular weight of copolymers with respect to native BSA, always accompanied by low polydispersities and higher values of the Mark–Houwink constants, hence higher hydrodynamic radii. A further



SCHEME 6 Synthesis of BAC-MP-*g*-BSA by polymerization of BAC-MP in the presence of BSA.

TABLE 2 Molecular Weight and Viscometric Data of PAA-Grafted BSA Samples Compared with BSA

Sample	M_n[a]	M_w/M_n[b]	$[\eta]$[c] (dL/g)	a[d]	log K[e]	R_h[f] (nm)
BSA	67,000	1.04	0.054	0.034	−1.45	3.92
BAC-MP-*g*-BSA	1,01,000	1.07	0.067	0.203	−2.74	6.1
BAC-P-*g*-BSA	84,900	1.17	0.053	0.265	−2.58	4.11
BP-P-*g*-BSA	88,100	1.14	0.083	0.163	−2.04	5.90

[a] Number average molecular weight.
[b] Polydispersity index.
[c] Intrinsic viscosity.
[d,e] Mark–Houwink constants.
[f] Hydrodynamic radius.

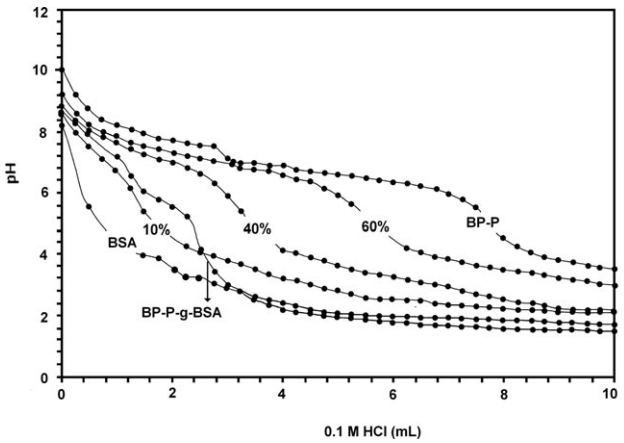


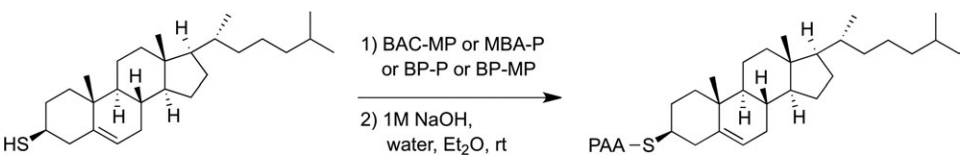
FIGURE 3 Potentiometric titration curves of BP-P-*g*-BSA copolymer and of BSA/linear BP-P mixtures of known composition.

confirmation was provided by the far higher solubility in aqueous media of the copolymers compared with virgin BSA.

The BP-P-*g*-BSA copolymer was further characterized by comparing the tracing of its potentiometric titrations with those of virgin BSA/linear BP-P mixtures of known composition (Fig. 3), as previously reported.[13] The titration curves of the copolymer and of the BSA/BP-P mixtures exhibited two evident inflections at pH values close to the inflections of the BP-P titration curve, whereas the BSA curve was characterized by a semicontinuous decrease of pH on acid addition without clearly detectable inflections. Considering that the amine character of the albumin —NH_2 groups involved in the grafting reaction is preserved, we may reasonably suppose that PAA/BSA mixtures are good models for PAA-grafted albumin, as far as titration is concerned. Therefore, from the family of titration curves reported in Figure 3, the PAA content in the grafted product can be determined as $\sim$ 20% by weight. This value was consistent with the molecular weight difference of the BP-P-*g*-BSA copolymer with respect that of BSA (calculated 18,000, experimentally found 21,100). Assuming that no BP-P was present, as the copolymer had been extensively ultrafiltered through a membrane with molecular weight cut-off 50,000, this correspondence confirmed that grafting reaction occurred as expected. No titration experiments were performed with BAC-MP-*g*-BSA and BAC-P-*g*-BSA as the acid–base properties of BAC-MP and BAC-P are close to those of BSA.

"Tadpole-like" PAA-cholesterol amphiphiles were obtained by performing the polymerization of HDDs in the presence of TC, which participates in the Michael polyaddition through its SH group (Scheme 7).

These reaction systems closely resembled the preparation of mono-functional PAAs by copolymerization of HDDs with monofunctional amines or acrylamides, as the SH group reacts with activated double bonds in the same way as the amine groups.[20,21] Being TC insoluble in water, the preparations of HDD-TC conjugates were performed in a diethylether/water slurry. TC resided in the former solvent and the HDDs in the latter. The two solutions were vigorously stirred under nitrogen at room temperature in a closed vessel until the signals due to the acrylic hydrogens of bisacrylamides were no longer detectable in the ^{1}H NMR spectra of the aqueous phases. The feasibility of this process was ascertained with BAC-MP, MBA-P, BP-P, and BP-MP. A 10:1 HDD/TC molar ratio was used for obtaining polymers with 10 repeating units of HDD per TC molecule. This value was chosen because literature data[22,23] suggested it as leading to a suitable hydrophilic/lipophilic balance to form and hydrophobically stabilize polyelectrolyte emulsions. In the case of BAC-MP-TC and MBA-P-TC, their amphiphilic nature led to phase segregation both in aqueous media and organic solvents. Consequently, their ^{1}H NMR spectra showed neither traces of the PAA backbone in deuterated water, nor of the cholesterol moiety in deuterated chloroform, as expected. BP-P turned to be hardly soluble in both solvents, probably due to the tendency of the BP-P chain to crystallize (data not shown). Only in the case of BP-MP-TC both components were soluble in deuterated chloroform. This allowed obtaining clear homogeneous solutions and running ^{1}H NMR spectra in this solvent. These spectra did not significantly differ from those of a previously synthesized BP-MP PAA carrying pendant TC moieties[24] and showed the presence of the diagnostic peaks of both components. The calculated and experimentally



SCHEME 7 Synthesis of "tadpole-like" PAA-cholesterol amphiphiles by polymerization of HDDs in the presence of TC.

TABLE 3 C/N Ratio of "Tadpole-like" PAA-Cholesterol Amphiphiles

Sample	*C/N*[a]	*C/N*[b]	HDD/TC[c]
BAC-MP-TC	3.21	2.98	4.52
MBA-P-TC	2.94	2.82	7.97
BP-P-TC	3.58	3.30	5.17
BP-MP-TC	3.80	3.59	6.61

[a] Calculated.
[b] Found.
[c] Molar ratio between the HDD repeat unit and the TC moiety as calculated from C/N found values, to be compared with a 10:1 HDD/TC molar ratio in the reaction feeding.

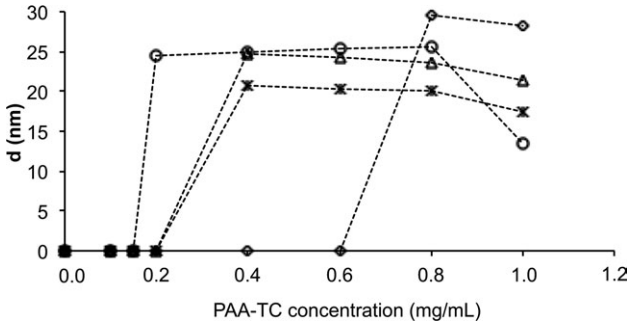


FIGURE 5 PAA-TC nanoparticle diameters in PBS solution pH 7.4 at 25 °C as a function of concentration: BAC-MP (- - -◇- - -); MBA-P (- - -△- - -); BP-P (- - -○- - -); BP-MP (- - -✳- - -).

determined *C/N* ratios for all the PAA-TC polymers synthesized are reported in Table 3. It may be observed that the experimental values were invariably lower than the theoretical ones, pointing to cholesterol content somewhat lower than expected, doubtless due to the heterogeneous reaction conditions adopted. All PAA-TC samples formed nanoparticles in aqueous media, as confirmed by DLS and TEM analyses. As an example, the DLS curves of BAC-MP-TC are reported in Figure 4.

It may be noticed that the correlogram [Fig. 4(a)] is consistent with a neat homogeneous monomodal size distribution. The other PAA-TC samples gave similar results. The average PAA-TC nanoparticle sizes in PBS solution pH 7.4 as a function of concentration and temperature are reported in Figures 5 and 6, respectively.

It may be observed that particle formation at 25 °C in PBS started at concentrations in the range 0.15–0.60 mg/mL, their size increased sharply, reached a maximum at about twice these values, then remained approximately constant and finally decreased for concentrations higher than 1 mg/mL. Consequently, the critical micelle concentration (CMC) deduced from the graphs varied from 0.16 to 0.7 mg/mL, following the trend BP-P < BP-MP ≤ MBA-P << BAC-MP. This trend is consistent with the relative hydrophilicities of the PAA portions. The apparent exception provided by BP-P-TC can be explained by the previously mentioned structure-forming tendency of its PAA portion. Nanoparticle average sizes at 1 mg/mL concentration varied in the range 13–28 nm and, not surprisingly, followed the same trend as the CMC, the most hydrophilic ones giving larger nanoparticles. The temperature dependence of nanoparticle average size in the range 25–60 °C are reported in Figure 6. All curves, leaving apart BAC-MP, show qualitatively the same trend. The average size initially decreased until about 30 °C, remained approximately constant in the range 30–45 °C and then increased until the maximum tested temperature of 60 °C. In the case of BAC-MP, the trend was initially the same and the average size decreased until about 30 °C, but immediately after started to increase until 60 °C. TEM analysis showed a quasispherical shape with no evidence of dual distribution, as for example in the case of BAC-MP reported in Figure 7.

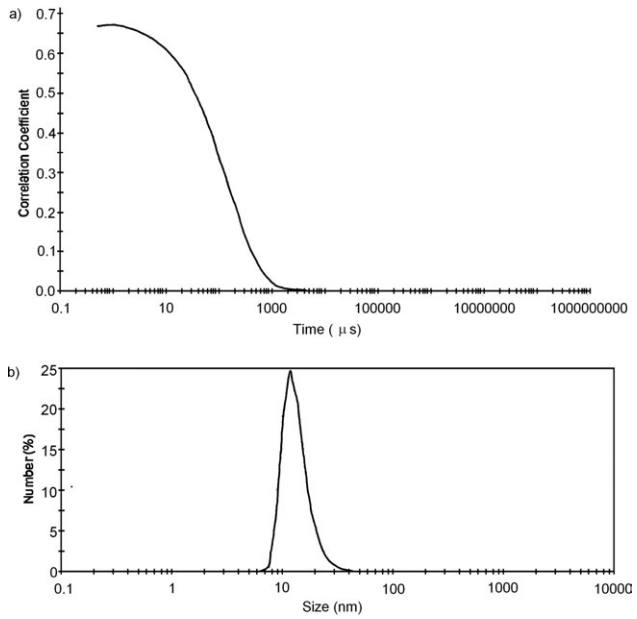


FIGURE 4 DLS analysis of BAC-MP-TC nanoparticles in PBS (1 mg/mL) at 25 °C. Panel (a): correlogram; panel (b): number size-distribution.

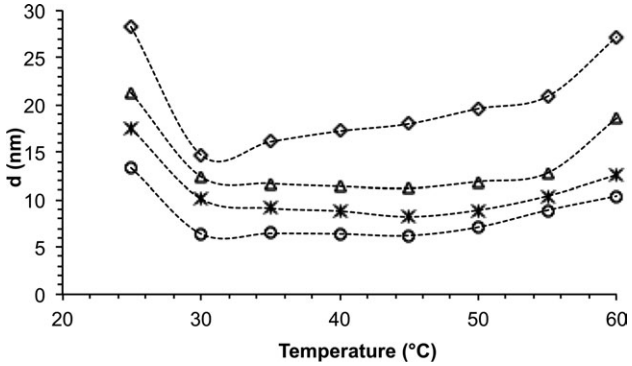


FIGURE 6 PAA-TC nanoparticle diameters in PBS solution pH 7.4 (1 mg/mL concentration) as a function of temperature: BAC-MP (- - -◇- - -); MBA-P (- - -△- - -); BP-P (- - -○- - -); BP-MP (- - -✳- - -).

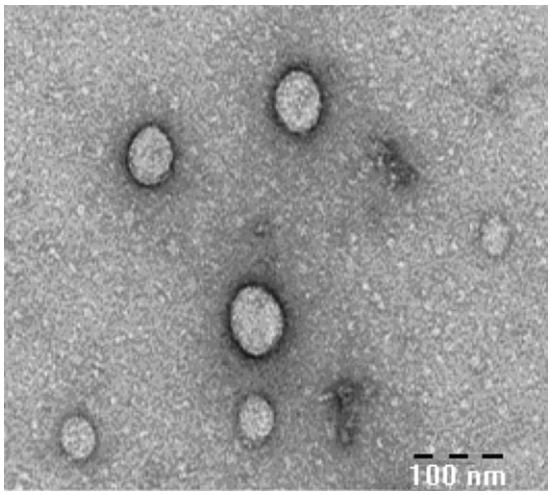


FIGURE 7 TEM micrograph of BAC-MP-TC conjugate obtained by negative staining microscopy using uranyl acetate.

CONCLUSIONS

Up to now, there was no simple method to straightforwardly prepare PAAs with differently functionalized chain termini, that is, PAAs solely containing molecules of a —·····— b type. This precluded to PAAs the access to a number of derivatives, such as for instance PAAs of controlled average molecular weight and mono-functionalized with an acrylamide- or a *sec*-amine group at one end, PAAs with a star-like molecular architecture, graft-PAA-protein conjugates, "tadpole-like" PAA conjugates with hydrophobic moieties able to self-assemble into nanoparticles in aqueous media. The devised strategy to overcome this problem was to prepare HDDs of "a —*a*—*b*—b" type, that is, the mono-addition products of a bis-*sec*-amine to a bisacrylamide, which as strong acid salts could be indefinitely kept dormant in the dry state at 0–5 °C but polymerized in aqueous media at pH $>$ 7.5. In this article, we report on the preparation of a selected number of HDDs and provide evidence of their synthetic potential by performing model reactions following the above cited lines. It should be noticed that the specificity of the Michael addition of amines to bisacrylamides in aqueous media, shared only by thiols under the PAA preparation conditions, allowed preparing most of the above derivatives by simply performing the polymerization of HDDs in the presence of the substrates of interest with no need of presynthesizing PAAs mono-functionalized at one end with an activated double bond. This greatly simplified the synthetic processes negligibly affecting the products' characteristics. On the whole, it can be concluded that the aim of this research has been adequately fulfilled, thus demonstrating the wide synthetic potential of HDDs.

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