Amphoteric Linear Poly(amido-amine)s as Endosomolytic Polymers: Correlation between Physicochemical and Biological Properties

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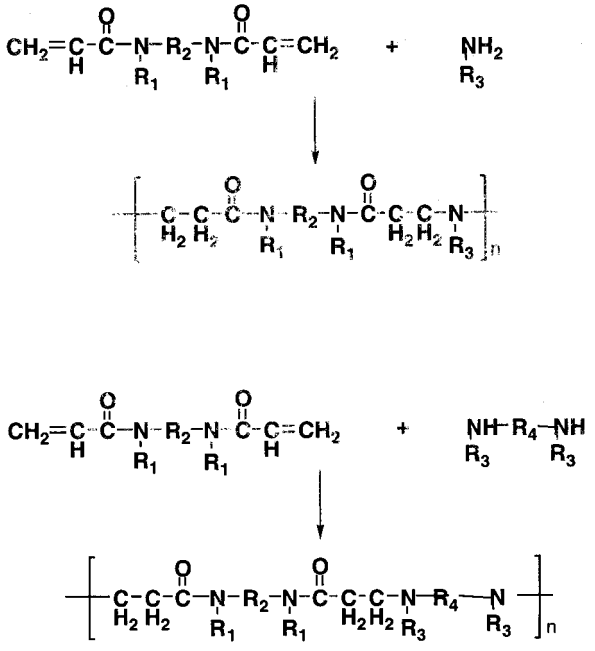
ABSTRACT: Five poly(amido-amine)s (PAAs) carrying two ter-amino groups and one carboxyl group per repeating unit were prepared by hydrogen-transfer polyaddition of 2-methylpiperazine (ISA 23), 1,2-bis(*N*-methylamino)ethane (DMEDA-BAC), 1,2-bis(*N*-ethylamino)ethane (DEEDA-BAC, 1,3-bis(*N*-methylamino)propane (DMEPDA-BAC), or 1,6-bis(*N*-methylamino)hexane (DMEXA-BAC), in each case to 2,2-bis(acrylamido)acetic acid (BAC). The resultant PAAs had an M_n in the range 7 985−24 980 g/mole and an M_w in the range 11 420−42 710 g/mole. Considerable differences were observed in the basicity of the amino groups present (log $K°_1$ = 7.5−9.5; log $K°_2$ = 3.2−8.4), whereas the log $K°_3$ value (2−3) of the carboxyl groups was consistent with that of a fairly strong acid. DEEDA-BAC, DMEDA-BAC, and ISA 23 were nontoxic (IC_{50} > 5 mg/mL). Those PAAs with the highest log $K°_2$ values were more cytotoxic (IC_{50} = 3.55 mg/mL for DMEPDA-BAC, and IC_{50} = 0.23 m/mL for DMEXA-BAC). All the PAAs displayed pH-dependent haemolysis (most lytic at pH 5.5), consistent with their proposed use as endosomolytic polymers.

1. Introduction

To successfully deliver novel therapeutics such as macromolecular drugs (e.g., antisense oligonucleotides, ribozymes, or gene therapeutics), the delivery system must modulate the pharmacokinetics of the therapeutic at both the whole-body and the subcellular level.[1] Because cytosolic access has been shown to be rate-limiting during the polymer-mediated delivery of transgenes,[2] it is important to develop synthetic alternatives to the fusogenic peptides used by viruses and toxins to mediate endosomal escape. Poly(amido-amine)s (PAAs) have already shown potential as endosomolytic polymers for the delivery of genes and toxins.[3,4] PAAs are synthesized by hydrogen-transfer polyaddition of aliphatic amines to bisacrylamides[5−7] according to Scheme 1. The resultant polymers have amido- and tertiary amino groups along the backbone arranged in regular sequences, and diamines with a wide range of basicity can be used as monomers. In addition, other functions can be easily introduced as side substituents. In aqueous media, many PAAs show conspicuous conformational changes upon protonation,[8] and it was this property that prompted us to consider this family of polymers attractive for development as endosomolytic systems.[9]

Here, five PAAs were prepared by hydrogen-transfer polyaddition to carry two ter-amino groups and one carboxyl group per repeating unit. Their structures are shown in Figure 1. As the purpose of this study was to correlate PAA acid−base properties with toxicity and membrane activity—features important for their use as endosomolytic polymers—first the molecular weight, solution behavior, and ionization profile of each PAA were measured. To measure general cytotoxicity, B16F10 mouse melanoma cells were incubated with each PAA, and cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) test.[10] An in vitro haemolysis assay shown previously to correlate well with endosomal/lysosomal rupture in vivo[4] was used to measure pH-dependent PAA−membrane interaction over time. The pH values chosen for these studies were representative of the systemic circulation (pH 7.4), the endosome (pH 6.5), and the

Scheme 1. General Synthetic Process to PAAs



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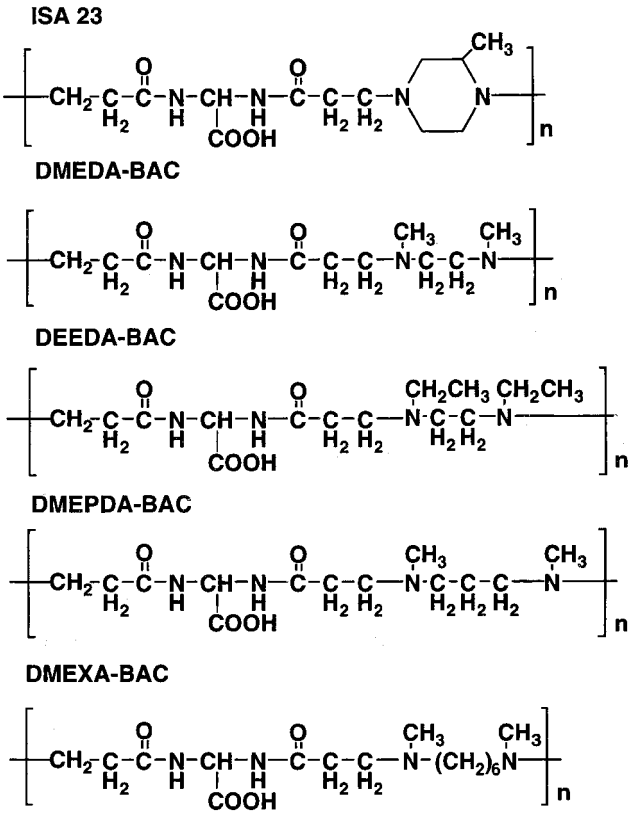


Figure 1. Chemical structure of amphoteric PAAs. In each case, polymers were prepared from 2,2-bis(acrylamido)acetic acid (BAC) by polyaddition reaction with 2-methylpiperazine (ISA 23), 1,2-bis(*N*-methylamino)ethane (DMEDA-BAC), 1,2-bis(*N*-ethylamino)ethane (DEEDA-BAC), 1,3-bis(*N*-methylamino)propane (DMEPDA-BAC), or 1,6-bis(*N*-methylamino)-hexane (DMEXA-BAC).

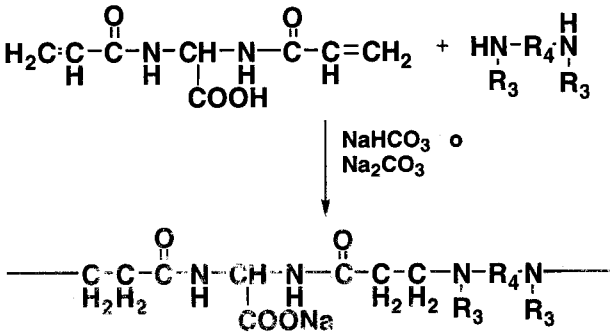
secondary lysosome (pH 5.5), as reviewed by Mellman.[11] For these biological studies, the polycations poly (L-lysine) and poly(ethylenimine), and also the PAA ISA 23[3,10] were used as reference polymers.

2. Experimental Section

2.1. Instruments. Ultrafiltration was performed in aqueous solution acidified to pH 3 with hydrochloric acid, using an Amicon ultrafiltering apparatus (Danvers, MA). NMR spectra were recorded using a Gemini Varian 200 in deuterium oxide, and IR spectra were recorded using a Perkin-Elmer FT-IR 1725 X, as Nujol dispersions.

An original chromatographic multidetector SEC system (obtained by assembling a pulse-free pump and three online detectors) was used for molecular characterization of the PAAs. The analytical system consisted of an Alliance 2690 separation module; a single-capillary viscometer (SCV); a differential refractometer (DRI) from Waters (Milford, MA); and an additional multi-angle laser light scattering (MALS) Dawn DSP-F photometer from Wyatt (Santa Barbara, CA). The multidetector SEC system has been described in detail elsewhere[12−14] and the description will be not reported herein. The wavelength of the MALS detector laser was 632.8 nm. The light-scattering signal was detected simultaneously at 15 scattering angles ranging in the mobile phase from 14.5° to 151.3°. The MALS detector measures the molar mass of the polymer (M_i) and, when the angular dependence is experimentally measurable, also the molecular dimensions: the root-mean-square radius ($<s^2>^{1/2}$) (denoted in the following as gyration radius, *Rg*). The SCV detector capillary had an internal diameter of 0.014 in. and a length of 6 in., and the detector measured the intrinsic viscosity ($[\eta]_i$) at each elution volume. After SEC fractionation, the SEC-MALS-SCV system provided M_i, Rg_i, and ($[\eta]_i$) at each elution volume.

Scheme 2. Synthesis of the Five Amphoteric PAAs Studied Here



The differential refractive index increment, d*n*/d*c*, of the PAAs with respect to the mobile phase at 25 °C was measured by the use of a KMX-16 differential refractometer from LDC Milton Roy (Riviera Beach, FL).

2.2. Materials. 1,2-Bis(methylamino)ethane, 1,2-bis(ethylamino)ethane, 1,3-bis(methylamino)propane, and 1,6-bis(methylamino)hexane were purchased from Fluka and used without further purification. 2-Methylpiperazine, also purchased from Fluka, was recrystallized from *n*-heptane. The purity of all diamines was determined titrimetrically just before use. All solvents were purchased from Aldrich UK or Fluka and were either distilled or stored over molecular sieves prior to use. 2,2-Bis(acrylamido)acetic acid (BAC) was prepared and purified as previously described.[15]

Murine melanoma B16 F10 cells were a kind gift from Prof. I. Hart (St. Thomas Hospital, London, U.K.). RPMI 1640 and fetal bovine serum (FBS) were supplied by Gibco BRL (Paisley, U.K). The MTT, optical-grade dimethylsulfoxide (DMSO), dextran (M_w 72 000 mol/g), poly(L-lysine) (M_w 56 500 mol/g), and poly(ethylenimine) (M_w 70 000 mol/g) were obtained from Sigma Chemicals, (Dorset, U.K.). Male Wistar rats were supplied by Banton and Kingman (Hull, U.K.), and all general reagents were from BDH (Ontario, Canada) or Sigma (Dorset, U.K.) and were of analytical grade.

2.3. Synthesis. In brief, the synthesis of the amphoteric PAAs was performed by hydrogen-transfer polyaddition of the selected bis-secondary amines to BAC according to Scheme 2. The reaction was carried out in water and at room temperature for several days, according to the general method of preparation for this family of polymers.[5−7]

Synthesis of ISA 23 was described previously.[3,15] However, here an improved method was used: powdered BAC (19.82 g, 0.1 mol) was added to a mixture of sodium hydrogen carbonate (8.401 g, 0.1 mol) and water (50 mL). The mixture was stirred until a perfectly clear solution was obtained, cooled to 5 °C, and flushed with nitrogen for 20 min to eliminate carbon dioxide. 2-Methylpiperazine (10.02 g, 0.1 mol) was then added. The resulting mixture was briefly flushed once more with nitrogen, stirred until perfectly homogeneous (while rising to the room temperature), and then maintained at approximately 20 °C, with occasional stirring, for 5 days. After this time, the very viscous reaction mixture was diluted with water to about 500 mL, acidified with hydrochloric acid to pH 3, and ultrafiltered through a membrane of nominal M_w cutoff 10 000 g/mole. The fraction retained was freeze-dried to yield approximately 23 g of polymer.

IR (cm^{-1}): 3300 (ν O−H); 2970, 2860 (ν C−H); 1670 (ν C=O); 1520 (ν CO−N−H); 1400 (δ C−H); 1150 (ν C−O).

^{1}H NMR (d, ppm): 1.43 (3H, d, CH_3−C); 2.80 (4H, m, CH_2−CO); 3.38 (6H, m, CH_2−N); 3.80 (5H, m, CH_3−CH−N + CO−C−CH_2−N); 4.71 (2H, broad s, N−H); 8.62 (1H, d N−CH−N).

DMEDA-BAC, DEEDA-BAC, DMEPDA-BAC, and DMEXA-BAC were prepared following exactly the same procedure used to synthesize ISA 23, by substituting 1,2-bis(methylamino)-ethane, 1,2-bis(ethylamino)ethane, 1,3-bis(methylamino)propane, and 1,6-bis(methylamino)hexane, respectively, for the same amount (on a molar basis) of 2-methylpiperazine. The yields were similar. The IR spectra of these PAAs were very

similar to that of ISA 23. Their NMR spectra were also similar, apart for some obvious differences in the multiplets between 3 and 4 ppm (methylenes of the aminic moieties).

2.4. Acid−Base Properties. The acid−base behavior of the PAAs was studied by potentiometric methods at 25 °C in aqueous media with a constant ionic strength (0.1 M NaCl) as described previously,[21] using a digital PHM-84 Radiometer potentiometer, a high-pH glass (pHG 211 Radiometer), a reference electrode (ref 201 Radiometer), and a Metrohm Multidosimat piston buret, all connected to an Olivetti M20 computer that automatically controlled the titrations and stored the potentiometric data.

The glass cell was filled with 100 mL of 0.1 M NaCl containing a weighed quantity of polymer (0.001−0.002 mol/L based on the repeating units) dissolved with magnetic stirring under a presaturated nitrogen stream. Potentiometric data were obtained in the forward and backward titrations with standardized NaOH and HCl solutions (0.1 M), respectively. At least three replicates were obtained for each polymer. The basicity constants were estimated by the APPARK and the SUPERQUAD programs,[16,17] running on an Olivetti M20 and on a Macintosh LC II computer, respectively. The purity of the polymer, as regards the amount of hydrochloride form present and the extent of hydration, was revealed by end-points analysis and by elaboration of the titration curves.

2.5. Solution Properties. A precolumn and three Ultrahydrogel SEC columns of pore sizes 1000, 500, and 120 Å (Waters) were used. The running SEC conditions were as follows: 0.2 M NaCl + 0.1 M Tris buffer pH 8 as mobile phase, temperature 35 °C, flow rate 0.8 mL/min, injection volume 300 μL. All the PAA samples were exhaustively dialyzed against the mobile phase before the dn/dc and SEC measurements. The calibration constant of the light-scattering detector was calculated using toluene as a standard and assuming a Rayleigh factor of 1.406×10^{-5} cm^{-1}. The photodiode's angular normalization was performed by measuring the scattering intensity of a bovine serum albumin globular protein in the mobile phase assumed to act as an isotropic scatterer. Because the signal of an online viscometer detector depends on the intrinsic viscosity and concentration of the solution, to obtain constant signal-to-noise ratio the concentration of the PAA samples was adjusted so that $[\eta]c = 0.1$. The estimation of the molecular properties, M_i, Rg_i, and $[\eta]_i$, requires monodisperse fractions and extrapolation to infinite dilution. When the MALS and SCV detectors are used online with an SEC system, every slice of the chromatogram could be considered homogeneous in molar mass, supposing ideal SEC fractionation, and the concentration could be extremely diluted. Therefore, in an online experiment, contrary to an offline experiment, the extrapolation to infinite dilution is not necessary. Besides, in a multidetector SEC system, accurate value of the interdetectors' delay volume between the absolute detectors, MALS and SCV, and the concentration detector, DRI, must be accounted for. Local properties M_i, Rg_i, and $[\eta]_i$ at each elution volume are very sensitive to an incorrect superimposition of the detector signals. The procedure to determine the interdetectors' delay volume has been described previously.[18]

2.6. Evaluation of Cytotoxicity. B16 F10 cells were grown in RPMI 1640 with 5 mM L-glutamine and 10% (v/v) FBS. Cells were seeded into 96-well microtiter plates at a density of 1×10^4 cells/well, and 24 h later the PAAs were added to give a final concentration range of 0−5 mg/mL. Poly(L-lysine) and dextran and were used as positive and negative reference controls, respectively. The cells were then incubated for 72 h before the assessment of their viability using the MTT assay.[10] Results were expressed as viability (%) relative to a control containing no polymer. The means (±SD) of three experiments, each containing six replicates, are shown in each instance. IC_{50} values were calculated using regression analysis for each of three separate experiments, and the mean (±SD) values are shown.

2.7. Haemolysis Assay. Male Wistar rats were killed and blood was obtained by cardiac puncture. A 2% w/v suspension of red blood cells (RBCs) was made as follows. First, the blood was centrifuged at $1000 \times g$ at 4 °C for 15 min, and the plasma and the top 2−3 mm of the pellet was then removed and discarded. The pellet was resuspended in PBS pH 7.4 that had been prechilled to 4 °C. The suspension was then subject to further centrifugation as before. The wash process was repeated one more time, and the pellet was resuspended in a volume appropriate to a 2% (w/v) suspension using either PBS pH 7.4, PBS pH 6.5, or PBS pH 5.5 (pH attained by addition of HCl). PAAs were dissolved at a concentration of 2 mg/mL in PBS of the appropriate pH and added to an equal volume of 2% (w/v) RBC suspension of similar pH. This solution was then left at 37 °C for 1 or 5 h. Dextran, poly(ethylenimine), and poly(L-lysine), respectively, were used as reference controls and Triton-x100 (1% v/v) was used to give a 100% haemolysis value. Haemoglobin release was determined spectrophotometrically (550 nm) and expressed as a percentage of haemoglobin release relative to the Triton-x100 control.[3,10]

3. Results and Discussion

PAAs are a family of polymers with interesting properties that can be exploited in biomedical and other applications (reviewed in refs 5−7). They are usually water-soluble and contain hydrolyzable bonds in their main backbone that allow degradation in aqueous media.[19] This makes them particularly attractive for use as drug carriers in that they should not accumulate in the body after repeated administration.[20] PAAs have already been used to deliver anticancer platinates[15] and complex polyanions such as heparin,[21] and their ability to promote pH-dependent membrane lysis[9] has been used to enhance intracellular delivery of DNA and toxins.[4,22]

The need to better understand PAA features that facilitate pH-dependent membrane interaction led to the synthesis and characterization of the PAA structures described here (Figure 1). Typically, the repeating unit of PAA polymers comprises a combination of an amidic moiety and an aminic moiety. In this study, the amidic comonomer BAC (containing a single carboxyl group) was conserved in each polymer synthesized: ISA 23, DEEDA-BAC, DMEDA-BAC, DMEPDA-BAC, and DMEXA-BAC. In contrast, the aminic comonomers used were differentially substituted to prepare polymers that would be expected to display similar acid−base properties as regards the carboxyl group, but that would vary in terms of the acid−base properties of the amino groups. Although DMEDA-BAC and DEEDA-BAC have similar acid−base properties, overall they display a different hydrophobic−hydrophilic balance.

3.1. Synthesis. Optimization of the synthetic procedure was necessary. The BAC comonomer is a fairly strong acid and forms salts with amines (including the aminic monomers). Protonated amines do not react with bisacrylamides under the usual conditions used for PAA synthesis.[23] In addition, partially protonated polyamines react with acrylamides only to the extent of the unprotonated amino groups present, after which the reaction becomes extremely sluggish.[24] Therefore, BAC must be used as a salt. First reactions used BAC as a triethylamine salt. Although the results were fair in the case of ISA 23, because of the 2-methylpiperazine comonomer, which has the weakest basicity of all the aminic monomers used, the results were poor for all the other polymers. This possibly was due to the fact that the basic strength of the bis-secondary amines used as monomers was not far from that of triethylamine. A water-soluble BAC calcium salt was also explored. However, the calcium salts of all PAA polymers were water-insoluble, and they precipitated long before a conveniently high molecular weight was attained. A

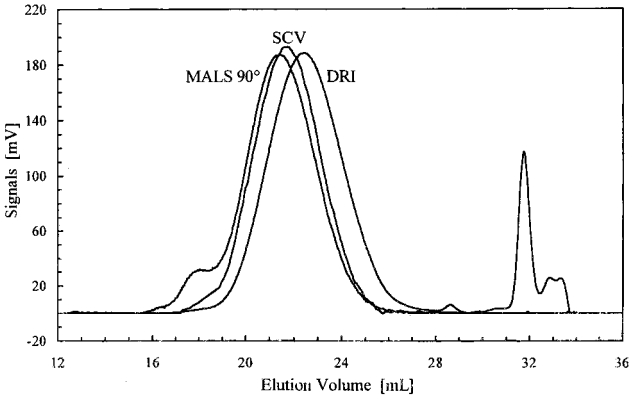


Figure 2. SEC of ISA 23. The signals arising from the MALS° 90, SCV, and DRI are shown.

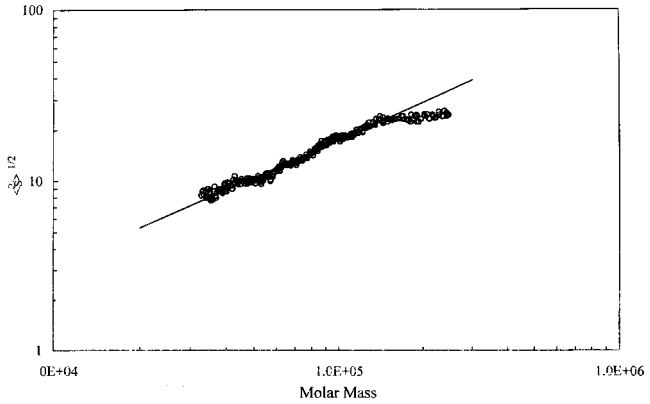


Figure 3. $Rg = f(M)$ power law for ISA 23.

Table 1. Characterizations of the PAAs

PAA code	dn/dc (mL/g)	$M_n \cdot 10^{-3}$ (g/mol)	$M_w \cdot 10^{-3}$ (g/mol)	D	$[\eta]$ (dL/g)
ISA 23	0.193	25.0	42.7	1.7	0.69[4]
DMEDA-BAC	0.195	16.1	29.0	1.8	0.33[5]
DEEDA-BAC	0.191	16.4	30.3	1.9	0.22[3]
DMEPDA-BAC	0.193	8.0	11.4	1.4	0.22[6]
DMEXA-BAC	0.196	19.4	30.9	1.6	0.51[5]

BAC sodium salt gave the best results. This salt could be prepared by several methods. The first is BAC neutralization in water using aqueous sodium hydroxide (the salt being recovered by lyophilisation). A second potential method is BAC neutralization in methanol with methanolic sodium hydroxide. In this case, the salt was recovered by precipitation with a mixture of acetone and ether. Polymerization occurred using the BAC sodium salt prepared by either of the above methods, provided that the salt content was determined by NMR or elemental analysis to preserve the stoichiometric balance of the comonomers.

Best results were obtained by the use of a third method, in which the BAC sodium salt was prepared by adding solid sodium hydrogen carbonate to a stoichiometric amount of solid BAC, and then adding just enough water to obtain, after stirring for a few minutes, a homogeneous solution which was flushed with nitrogen to eliminate the carbon dioxide produced. The BAC sodium salt was not isolated before adding the aminic monomers. Highly concentrated reaction mixtures prepared as described above rapidly became very viscous; the mixtures had to be diluted at least 1:10 before processing. However, they were preferred, because more diluted reaction mixtures invariably lead to lower-molecular-weight products even if the reaction time is prolonged.

3.2. Molecular Characterization and Solution Behavior. The molar mass averages, M_n and M_w; the dispersity index, D; and the intrinsic viscosity, $[\eta]$, of the PAAs are summarized in Table 1. In each case the dn/dc values were high and the differences among the five PAA polymers were very low, within experimental error. A typical SEC-MALS-SCV profile (MALS 90°, SCV, and RI detectors) is shown in Figure 2 (for ISA 23). The initial shoulder seen using the light-scattering detector indicates the presence of aggregates, but these are less visible with the SCV detector and are not visible using the DRI detector. Under the running conditions (pH 8.0), some of the PAAs did show a small amount of aggregation. The online MALS detector is very sensitive to the presence of aggregates. The presence of a little shoulder in the chromatogram demonstrates that in the mobile phase there was only a small amount of aggregates.

Although all the PAAs were prepared using the same conditions their molecular weights were very different (M_w in the range between 11 and 42K g/mol; Table 1). ISA 23 had the highest molecular weight, followed DMEDA-BAC, DMEPDA-BAC, and DMEXA-BAC, the latter three all deriving from *N*-methyl-substituted diamines. DEEDA-BAC, deriving from an *N*-ethyl-substituted diamine, had by far the lowest molecular weight. PAA molecular weight did not correlate with the basic strength of the aminic monomers, but it was related to the inverse of the steric hindrance on the nitrogen atoms. Even taking into account the fractionating effect of ultrafiltration, these data reasonably reflect the difference in reactivity of the diamines used, and steric hindrance is apparently the dominant factor. The effect of nucleophilicity of the nitrogen atoms, if any, is clearly overshadowed. These observations are in agreement with early data derived from the polyaddition of different diamines to bisacrylamides other than BAC.[23]

The molar mass distribution of the four PAA samples was quite narrow. Excluding the DMEPDA-BAC sample D, they ranged between 1.6 and 1.9. $[\eta]$ values were relatively high considering the molar mass range; these values ranged between 0.22 and 0.7 dL/g. The relationship between $[\eta]$ and the molar mass of the PAA samples shows important differences in the stiffness of the polymer chain. Thus ISA 23, which had a higher molar mass than the other PAA samples, was more carefully characterized in terms of its conformational properties. The experimental $Rg = f(M)$ power law for ISA 23 is shown in Figure 3. The plot shows two different behavior patterns over the molar mass range. At a molar mass lower than 15 kg/mol, nonaggregated ISA 23 macromolecules can be seen, whereas at higher molar mass a small fraction of ISA 23 aggregates are seen. Nonaggregated PAA fractions, that is, discarding the initial shoulder, have a slope of the $Rg = f(M)$ power law of approximately 0.74. This slope value is unusually high, confirming the high stiffness of the ISA 23 polymer chain.

The experimental $[\eta] = f(M)$ power law, Mark–Houwink–Sakurada (MHS) plot, for the ISA 23 is shown in Figure 4. Estimating the slope of the MHS plot is not simple, as it shows a significant downward curvature. This curvature is not unusual for stiff chains with the molar mass ranging from low to medium values. The initial slope α of the MHS plot is very high:

Table 2. Protonation Constants of Amphoteric PAAs[a]

	$\log K°_1$ $L^- + L^+ = LH^\pm$	n_1	$\log K°_2$ $LH^\pm + H^+ = LH_2^+$	n_2	$\log K°_3$ $LH_2{+}{+} + H^+ = LH_3{+}2$
ISA 23	7, 48(5)	1, 19(3)	3, 24(4)	1, 03(5)	2, 3(2)
DMEDA-BAC	8, 25(5)	1, 10(7)	4, 85(10)	1, 20(4)	2, 0(3)
DEEDA-BAC	8, 65(16)	1, 15(4)	4, 62(15)	1, 34(8)	2, 6(2)
DMEPDA-BAC	8, 997(4)		6, 849(6)		1, 91(3)
DMEXA-BAC	9, 527(4)		8, 367(5)		2, 17(1)

[a] Figures in parentheses are the standard deviations relating to the last significant figure.

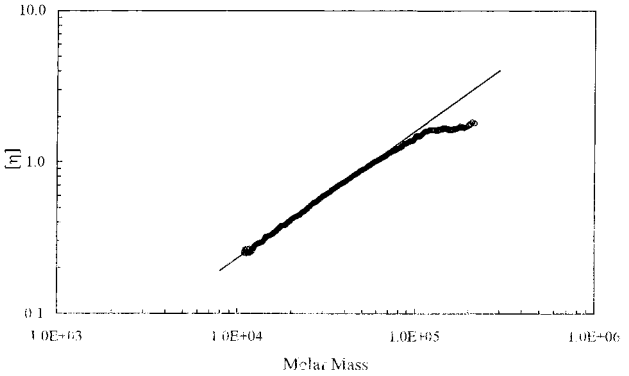


Figure 4. $[\eta] = f(M)$ power law, MHS plot, for ISA 23.

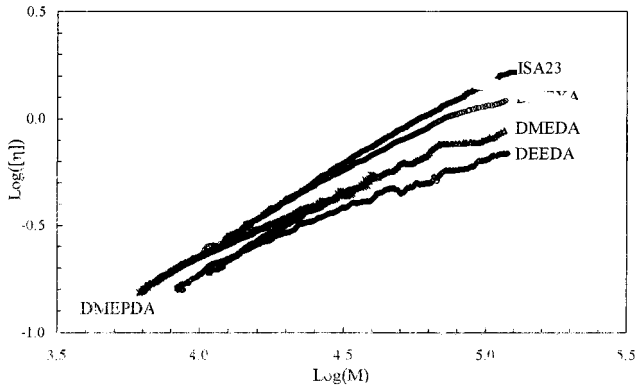


Figure 5. Comparison of the $[\eta] = f(M)$ power law for all the PAA polymers

$\alpha > 1$ with M lower than 20K g/mol. When the molar mass of the ISA 23 sample increases, the slope of the MHS plot slowly decreases, with an asymptotic value close to 0.85. Using a molar mass range that is fairly linear, 20K g/mol $< M <$ 60K g/mol, the following values of the MHS constants can be obtained for ISA 23: $k = 9.53 \times 10^{-5}$ dL/g, $\alpha = 0.85$. This result, the high slope of the $Rg = f(M)$ power law, and the high $[\eta]$ with regard to the molar mass confirm the stiffness of the ISA 23 polymer chain.

The experimental MHS plot allows comparison of the conformational properties and the chain stiffness for all five PAAs (Figure 5). There was a significant difference in the stiffness of the chain of the PAA samples studied. At constant molar mass, the $[\eta]$ values were very different. ISA 23 showed systematically higher $[\eta]$ values at each molar mass, and DMEXA-BAC behavior was essentially similar, although a little lower. DEEDA-BAC had consistently lower $[\eta]$ values. The behavior of DMEDA-BAC and DMEPDA-BAC samples was intermediate between the ISA 23 and DEEDA-BAC, but they were similar despite their different molar mass. In addition, the slope of the experimental MHS plot for the different PAAs was quite different. Figure 5 shows that the influence of the differences in the repeating units of the PAAs (Figure 1) on the stiffness of the polymer chains is meaningful. It should be noted that the above data were obtained for all at pH 8, which is not very far from the PAA isoelectric points, with the exception of ISA 23. The last point, together with the presence of cyclic structures in its polymer chain, might explain why ISA 23 shows consistently higher $[\eta]$ values at each molar mass than any other amphoteric PAA studied here.

3.3. Acid−Base Properties. The protonation constants relative to the protonation of the basic groups of the amphoteric PAAs considered are summarized in Table 2. Three constants are reported for each polymer, relating to the protonation of the two aminic groups and of the carboxylate anion of the repeating units. The third constant relates to the protonation of the carboxylate anion, and this is in all cases very low, with little variation among the different polymers. This indicates that at all physiological pH's (ranging from 7.4 in extracellular fluids to 5 in some intracellular compartments), the PAA carboxylate anion does not protonate and is always present as an anion.

The second constant relates to the second amino group, and this shows considerable variation, from 3.24 for ISA 23 to 8.37 for DMEXA-BAC. This indicates that at pH 7.4 this group is highly ionized in the case of DMEXA-BAC, and almost fully un-ionized in ISA 23. At pH 5, even the second amino group of ISA 23 undergoes protonation to some extent. Values for the other PAAs lie at intermediate positions. The first constant relates to the first amino group. Variation in this value was seen from sample to sample, but to a lesser degree than in the second constant. All the PAAs studied would protonate of this group at pH 7.4, albeit not completely for the less basic members, and it would be fully ionized at pH 5.

As regards "polyelectrolyte" behavior, the log K values of the two basic nitrogens of ISA 23, DMEDA-BAC, and DEEDA-BAC can be considered as borderline between "real" and "apparent". They follow the modified Henderson−Hasselbalch equation with n values slightly higher than 1.

$\log K = \log K° + (n-1) \log[(1 - \alpha)/\alpha]$ (where α is the protonation degree)

In contrast, DMEPDA-BAC and DMEXA-BAC show a "real" behavior; thus it is possible to evaluate log K values as for nonmacromolecular compounds.

Each repeating unit of the amphoteric PAAs can exist in four states of ionization: purely anionic (L^-), neutral zwitterionic ($L^\pm$), positively charged zwitterionic ($L^{\pm+}$), and doubly positively charged (L^{2+}), which is equal to unprotonated (L^-), monoprotonated (LH), diprotonated (LH_2), and triprotonated (LH_3) conditions. To allow correlation between the PAA structure and biological behavior, it is interesting to express the relative distribution of the different ionic species for each polymer as a function of pH (Figure 6). The different PAAs exhibited widely different distribution patterns as regards the

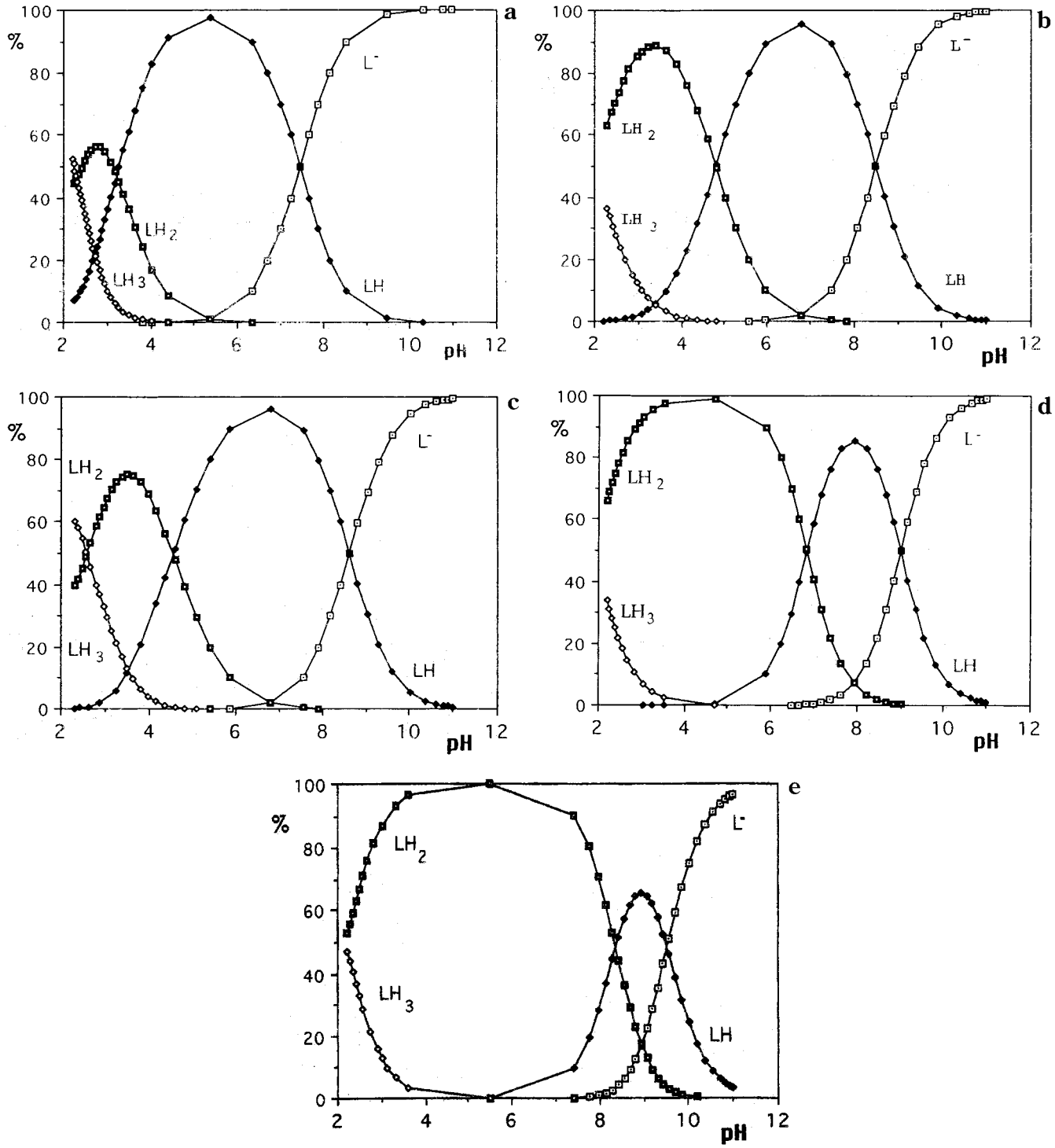


Figure 6. Species distribution curves (percentage) in relation to pH as calculated by the basicity constants obtained in aqueous 0.1 M NaCl at 25 °C (polymer concentration 0.004 mol/L based on the repeating unit). Panel (a), ISA 23; panel (b), DMEDA-BAC; panel (c), DEEDA-BAC; panel (d), DMEPDA-BAC; panel (e), DMEXA-BAC.

species L^-, $L^{\pm}$, and $L^{\pm+}$, with the obvious exceptions of DMEDA-BAC and DEEDA-BAC. The data in Figure 6 allow estimation of the pH at which the polymer is electrically neutral, that is, its isoelectric point. This corresponds to the intersection of the curves relative to LH_2 and L^-. The isoelectric points for ISA 23, DMEDA-BAC, DEEDA-BAC, DMEPDA-BAC, and DMEXA-BAC were ~ 5.4, 6.8, 6.8, 7.9, and 8.9, respectively. However, it is noteworthy that at the isoelectric point for each PAA, the relative proportion of L^-, $L^{\pm}$, and $L^{\pm+}$ species was not identical. Specifically, the percentage of electrically neutral units (i.e., LH units) was ~ 99%, 96%, 96%, 86%, and 67% for the above-mentioned PAAs, respectively. The remainder in all cases were equally divided between positively (LH_2) and negatively (L^-) charged units, consistent with their truly amphoteric nature. Thus, at pH intervals close to their isoelectric point, all the PAAs studied would exhibit both positively and negatively charged units localized in different positions along the polymer chain, which might facilitate electrostatic interactions. Moreover, viscometric studies showed that, not unexpectedly, the PAAs studied exhibited a minimum hydrodynamic volume at their isoelectric point. The curves of η_{spec}/C as a function of

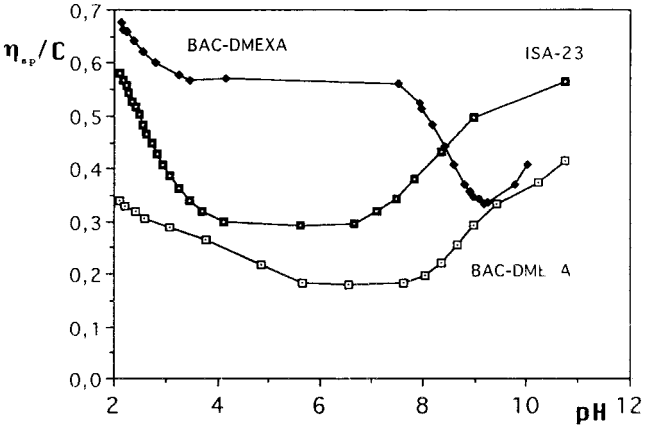


Figure 7. Reduced viscosity of amphoteric PAAs as a function of pH.

Table 3. Cytotoxicity of PAAs against B16F10 Melanoma Cells

polymer	$M_w \cdot 10^{-3}$ (g/mol)	IC_{50} (mg/mL ±SD)
Dextran	72.0	>5
ISA 23	42.7	>5
DMEDA-BAC	29.0	>5
DEEDA-BAC	30.3	>5
DMEPDA-BAC	11.4	3.55 ± 0.31
DMEXA-BAC	30.9	0.23 ± 0.06
Poly(L-lysine)	56.5	0.05 ± 0.01
Poly(ethylenimine)	70.0	0.01 ± 0.01

pH for ISA 23, DMEDA-BAC, and DMEXA-BAC are shown in Figure 7.

3.4. Biological Properties. Previous studies have shown that PAAs generally display low cytotoxicity against many different cell lines.[3,19] The novel PAAs described here were all less toxic than poly(L-lysine) and poly(ethylenimine) (Table 3). In the case of the high-molecular-weight ISA 23 (compared to the ISA 23 previously reported[3]), DMEDA-BAC, and DEEDA-BAC, the PAAs were more than 100 times less cytotoxic than the nonamphoteric aminic polymers used as a reference. At pH 7.4, ISA 23 would have ~50% of its units in L^- and ~50% in $L^\pm$ form, so the polymer would have a relatively large negative average charge. Similarly, at pH 7.4, DMEDA-BAC and DEEDA-BAC would be prevailingly negatively charged, with more than 90% of units in the $L^\pm$ form. This may explain their lack of toxicity. This conclusion is supported by the observation that DMEPDA-BAC and DMEXA-BAC, which are positively charged at pH 7.4, are more cytotoxic than the other PAAs but less toxic than the purely cationic poly(L-lysine) and poly(ethylenimine). Comparing the IC_{50} values of DMEDA-BAC and DEEDA-BAC, it would appear that a modest increase in hydrophobicity does not significantly induce toxicity. In addition, the pattern of cytotoxicity observed for the PAAs suggests that as the log $K°_2$ relating to the protonation of the second amino group approaches the physiological pH (Table 2), so does polymer-mediated toxicity.

In contrast to poly(L-lysine) and poly(ethylenimine), all the PAAs studied showed pH-dependent haemolysis (Figure 8). All of the PAAs cause more haemolysis at pH 5.5 than at pH 7.4, and protonation of the polymer backbone at lower pH would be expected to increase its capacity to interact with the anionic RBC membrane. This in turn would cause concentration-dependent membrane perturbation. This observation is strongly

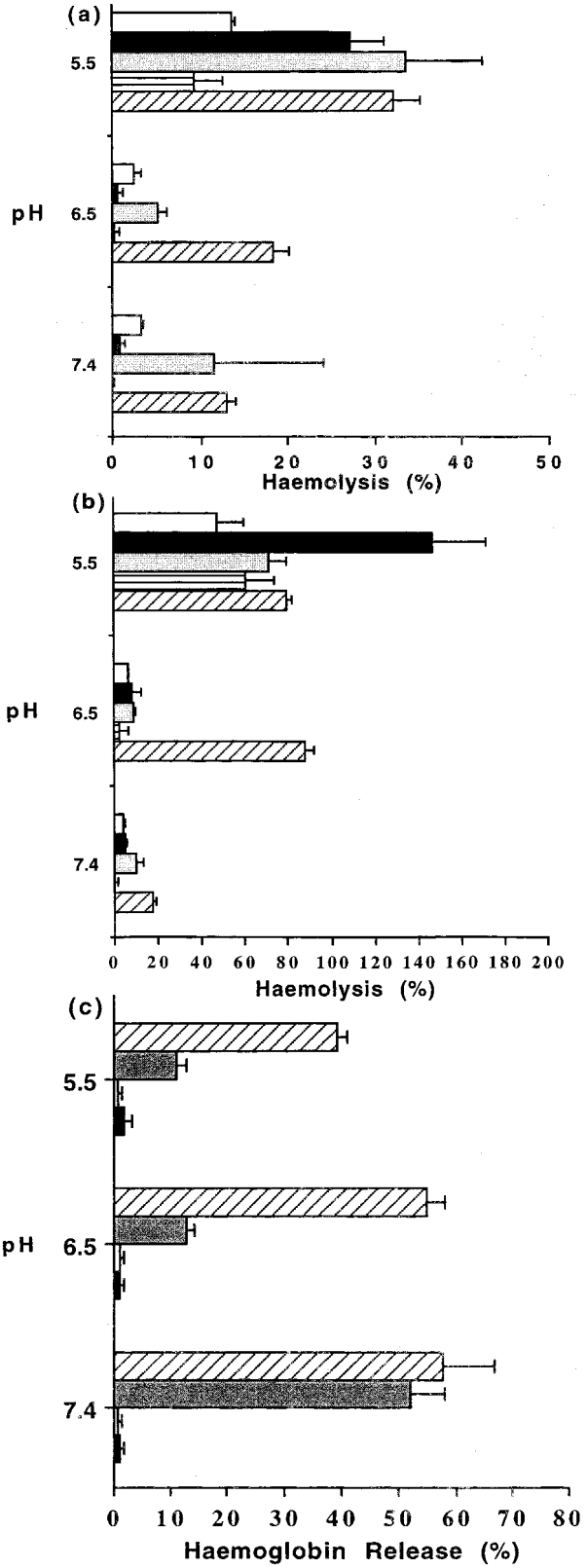


Figure 8. Haemoglobin release mediated by PAAs and reference control polymers. Panel (a), haemolysis caused by the PAAs at 1 h; ISA 23 (bars with slanted lines); DMEPDA-BAC (open bar); DMEXA-BAC (solid bar); DEEDA-BAC (gray bar); and DMEDA-BAC (bar with vertical lines) after 1 h. Panel (b), haemolysis caused by PAAs at 5 h (the key is the same). Panel (c) shows the haemolysis caused by poly(L-lysine) (gray bar); poly(ethyleneimine) (bar with slanted lines); dextran (open bar); and phosphate buffered saline PBS (solid bar), all at 1 h. In each case, the polymer concentration used was 1 mg/mL (n = 12), and the results show mean (±SD) PBS.

supported by the literature describing polycation-mediated membrane damage.[25−28]

Comparing the haemolytic profiles of DMEDA-BAC and DEEDA-BAC at 1 h (Figure 8a), it is obvious that the increased hydrophobicity of DEEDA-BAC leads to increased haemolysis. Interestingly, ISA 23 shows the greatest haemolysis at pH 6.5, which becomes even more obvious as the length of incubation increases from 1 to 5 h. This may be related to the high rigidity of the ISA 23 polymer chain (Figures 3 and 4), and it is interesting to note that ISA 23 is not recognized by the liver when administered intravenously. The so-called "stealth" properties of ISA 23 have been reported previously.[3] Although they are often used as transfection agents, neither poly(L-lysine) nor poly(ethylenimine) showed haemolytic activity that was dependent on pH (Figure 8c), and this observation might give further credence to the proton sponge hypothesis,[29] which suggests that poly(ethylenimine) swelling causes endosomolytic properties of poly(ethylenimine).

4. Conclusions

(1) The synthesis reported is a general method that uses BAC as an amidic comonomer and provides procedures for a new family of amphoteric PAAs.

(2) These amphoteric PAAs were designed to contain repeating units with different basicity constants of the ter-amino groups. It was possible to determine, for each PAA at any given pH, the relative percentages of units in different protonation conditions, i.e., the distribution of the different ionic species, as well as the isoelectric point. All the amphoteric PAAs studied here have considerable chain stiffness in aqueous media, and consequently exhibit hydrodynamic volumes that are particularly high in relation to their relatively modest average molecular weights.

(3) The amphoteric PAAs were throughout less cytotoxic than poly(L-lysine) and poly(ethylenimine). Their toxicity can be correlated with their log $°K_2$ (relating to the second amino group present in the repeating unit).

(4) Amphoteric PAAs display unique pH-dependent membrane activity evidenced by their haemolytic activity at different physiological pHs. This opens interesting perspectives as regards their potential as totally synthetic fusogenic agents.

(5) Those properties exhibited by amphoteric PAAs described here, together with their known susceptibility to degradation[19] and "stealth" properties in vivo,[3] would suggest that PAAs are particularly promising both as polymeric drug-carriers and as endosomolytic polymers that can facilitate cytoplasmic access. Their ability to deliver genes[22] and toxins[30] will be descibed elsewhere.

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