Synthesis, Physicochemical Properties, and Preliminary Biological Characterizations of a Novel Amphoteric Agmatine-Based Poly(amidoamine) with RGD-Like Repeating Units

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A linear, amphoteric poly(amidoamine) nicknamed AGMA1, based on 4-aminobutylguanidine, or agmatine, was successfully prepared by Michael-type polyaddition of monoprotonated agmatine and 2,2-bis(acrylamido)acetic acid (BAC). Copolymers between AGMA1 and the biocompatible poly(amidoamine) ISA23 (deriving from the polyaddition of 2-methylpiperazine with BAC) were also prepared. Acid−base titrations gave for AGMA1 three acid dissociation constants, with pK_a values of 2.25, 7.45, and ≥12.1, corresponding to a strong acid, a medium-weak base, and a strong base, respectively. The charge distribution profiles show that this polymer is prevailingly cationic at all physiological pH values, the positive net average charge per unit varying from about 0.5 at pH 7.4 to about 1.0 at pH 5, with an isoelectric point at pH ≈ 10. Zeta-potential measurements confirmed this. Despite that, AGMA1 is nontoxic and nonhemolytic in vitro within all pH ranges tested (4−7.5). This is in contrast with the previously observed behavior of amphoteric PAAs, for instance ISA23, that are weakly hemolytic at pH 7.4 but highly hemolytic at pH 5÷5.5. The lack of hemolytic activity of AGMA1 even at acidic pH values seems typical of the agmatine-BAC sequences and may be ascribed to their RGD-like structure. In fact, AGMA1-ISA23 copolymers behave in a way increasingly similar to that of ISA23; that is, they become hemolytic at low pH values as their ISA23 content increases.

Introduction

4-Aminobutyl guanidine, or agmatine, belongs to the family of biogenic amines involved in many physiological functions. These biomolecules play an important role in cell growth and proliferation, as well as in the synthesis of proteins and nucleic acids.[1] Moreover, agmatine is endowed with a large number of properties, e.g., in the neuronal, vascular, and metabolic fields.[2] For these reasons, agmatine is increasingly being studied as a bioactive molecule but has seldom been considered so far as a comonomer or side-substituent in the synthesis of polyelectrolytes.[3]

Agmatine derives from the arginine decarboxylase (ADC)-mediated decarboxylation of L-arginine, a semi-essential amino acid with interesting properties mostly attributed to its guanidine group. The tripeptide arginylglycylaspartic acid (RGD) is an oligopeptide capable of reproducing the receptorial sites of proteins, such as fibronectin, vitronectin, and others playing a fundamental role in cell adhesion.[4]

Grafted on a material's surface, RGD is capable of promoting a strong cell adhesion even at very low surface densities.[5] The RGD sequence gained much interest in the past decade for its astounding properties. Many examples can be found in the literature.[6]

Based on this premise, we thought it interesting to study the introduction of agmatine-deriving units in poly(amidoamine)s (PAAs).

PAAs are a family of synthetic polymers containing *ter*-amino groups and amido groups regularly arranged along the polymer chain.[7−10] Many PAAs exhibit a combination of properties imparting them a considerable potential in the biomedical field. They are usually degradable in water at a rate depending on their structure. Therefore, if injected, they are bioeliminable.[11] Most PAAs are only moderately toxic despite their polycationic nature. According to a number of tests, the toxicity of most PAAs is significantly lower than that of poly-L-lysine (PLL) or polyethyleneimine (PEI).[12] Moreover, amphoteric PAAs carrying carboxyl groups as side substituents may exhibit an interesting combination of properties.[13] In particular, an amphoteric PAA nicknamed ISA23, deriving from the polyaddition of 2-methylpiperazine to BAC, is approximately as biocompat-

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ible as dextran, and after intravenous injection, it exhibits the so-called "stealth" properties, which means that after intravenous injection it is not captured by the reticulo-endothelial system but has a prolonged permanence in the blood circle and is passively concentrated in tumor tissues, if present, by the EPR (enhanced permeation and retention) effect.[7]

Moreover, ISA23 is poorly ionized as a polycation at physiological pH (~7.4), but following internalization into cells, it localizes in lysosomes, where the pH is 5.5 or whereabouts. Here it strongly increases its polycationic character and becomes membrane-active, hence promoting the intracellular trafficking of DNA and proteins.[14]

In a previous paper, we have reported on cross-linked ISA23-based hydrogels containing some agmatine-deriving units, showing remarkable ability to promote cell adhesion and proliferation.[15] Here we report on the Michael-type polyaddition of agmatine with BAC, leading to a linear amphoteric PAA nicknamed AGMA1. The repeating units of AGMA1 carry guanidine- and carboxyl groups and show a strong structural resemblance to the RGD sequence. In addition, linear AGMA1-ISA23 copolymers were prepared. All of these polymers were studied for some relevant physicochemical properties, such as solubility, hydrolytic stability at physiological pH, and acid−base behavior. Preliminary biological characterizations, namely cell toxicity and hemolytic activity, were also performed.

Experimental Section

Instrumentation and General Methods. ^{1}H NMR and ^{13}C NMR spectra were run in deuterated water on a Bruker 400 MHz instrument operating at 400.133 (^{1}H) and at 100.00 MHz (^{13}C).

Size exclusion chromatography (SEC) traces were obtained using a Waters 515 HPLC Pump instrument, with Toso-Haas 486 columns, using 0.1 M Tris buffer pH 8.00 ± 0.05 as mobile phase. Conditions: sample concentration 10 mg/mL; flow rate 1 mL/min; detector UV Knauer model, wavelength 230 nm; temperature 30 °C, pullulans reference standards.

Intrinsic viscosities were measured by a capillary viscometer at 30°C in 0.1 M Tris buffer pH 8.00 ± 0.05.

Starting Materials. All solvents were of analytical grade purchased from Aldrich, Fluka, or LAB−SCAN and used as received. Lithium hydroxide monohydrate (99%) and 37% hydrochloric acid were purchased from Fluka and used as received. 4-Aminobutylguanidine sulfate (97%) (agmatine) was purchased from Aldrich. 2,2-Bis-(acrylamido)acetic acid (BAC) was synthesized as previously described and its purity determined titrimetrically.[8] 2-Methylpiperazine was purchased from Fluka and used after re-crystallization from *n*-heptane. The final purity was determined with acidimetric titration.

For potentiometric measurements, potassium nitrate, potassium tetraoxalate, sodium chloride, soium hydrogencarbonate, sodium carbonate, and phosphoric acid were purchased from Fluka and used as received. 0.1 M Sodium hydroxide and 0.1 M hydrochloric acid standard solutions were purchased from MERCK. Certipur Merck buffers at pH = 4, 7, and 9 were adopted.

Synthesis of AGMA1. Agmatine sulfate (2.000 g, 8.5 mmol) and lithium hydroxide monohydrate (0.360, 8.5 mmol) were added to a solution of BAC (1.689 g, 8.5 mmol) and lithium hydroxide monohydrate (0.360 g, 8.5 mmol) in distilled water (2.8 mL). This mixture was maintained under nitrogen atmosphere and occasionally stirred for 72 h. After this time, it was diluted with water (2.8 mL), acidified with hydrochloric acid to pH 4−4.5, and then ultrafiltered through a membrane with nominal cutoff 3000. The fraction retained was freeze-dried and the product obtained as a white powder. Yield: 2.1 g.

^{1}H NMR (D_2O): δ (ppm) = 1.61 (br, $NHCH_2CH_2C\mathbf{H}_2$), 1.76 (br, $NHCH_2C\mathbf{H}_2$), 2.79 (br, $NHCOCH_2C\mathbf{H}_2$), 3.19 (m, $NHC\mathbf{H}_2$, $NC\mathbf{H}_2$), 3.44 (br, $NHCOC\mathbf{H}_2$), 5.55 (s, $COOHC\mathbf{H}$).

Table 1. Comparison of Some Structural Features of AGMA1 Repeating Unit and RGD Sequence

structural feature	AGMA1 unit	RGD sequence
no. of guanidine groups	1	1
no. of carboxyl groups	1	1
no. of amidic groups	2	2
distance between guanidine and carboxyl groups	sequence of 10 atoms including 2 amidic groups	sequence of 11 atoms including 2 amidic groups

Table 2. Intrinsic Viscosities and SEC Apparent Molecular Weights of AGMA1 and AGMA1−ISA23 Copolymers

sample	percent AGMA1 content (on a molar basis)	[η] (dL/g)	$\bar{M}_n$	$\bar{M}_w$	d
AGMA1	100	0.16	4800	7200	1.50
AGMA0.5	50	n.d.[a]	15 200	19 500	1.56
AGMA0.2	20	n.d.[a]	13 800	20 300	1.47

[a] n.d. = not determined.

^{13}C NMR (D_2O): δ (ppm) = 22.3 ($NHCH_2CH_2\mathbf{C}H_2$), 25.0 ($NHCH_2\mathbf{C}H_2$), 28.9 ($NHCOCH_2\mathbf{C}H_2$), 40.4 ($CH_2N\mathbf{C}H_2$), 49.1 ($NHCO\mathbf{C}H_2$), 52.5 ($NH\mathbf{C}H_2$), 56.0 ($COOH\mathbf{C}H$), 155.1 ($NH_2\mathbf{C}NNH$), 171.3 ($NH\mathbf{C}O$), 173.5 ($CH\mathbf{C}OOH$).

Molecular weight values by SEC chromatography are reported in Table 2.

Synthesis AGMA1-ISA23 Copolymers. *(a) AGMA0.5.* BAC (2.640 g, 12.8 mmol) and lithium hydroxide monohydrate (0.542 g, 12.8 mmol) were dissolved in water (4.3 mL) at room temperature, under nitrogen flow and gentle stirring. When the solids were completely dissolved, agmatine sulfate (1.500 g, 6.4 mmol), lithium hydroxide monohydrate (0.271 g, 6.4 mmol), and 2-methylpiperazine (0.667 g, 6.4 mmol) were added, and the mixture left in the dark for 72 h. After this time, the reaction mixture was diluted with water (one volume), acidified with hydrochloric acid to pH 4−4.5, and ultrafiltered through a membrane with nominal cutoff 3000. The fraction retained was freeze-dried and the product obtained as a white powder. Yield: 3.12 g.

^{1}H NMR (D_2O): δ (ppm) = 1.37 (d, $CHC\mathbf{H}_3$), 1.59 (br, $NHCH_2$-$CH_2C\mathbf{H}_2$), 1.75 (br, $NHCH_2C\mathbf{H}_2$), 2.79 (br, $NHCOCH_2C\mathbf{H}_2$), 3.04 (br, $CH_3CHC\mathbf{H}_2$) 3.19 (br, $NHC\mathbf{H}_2$, $NC\mathbf{H}_2$), 3.44 (br, $NHCOC\mathbf{H}_2$), 3.55 (br, CH_3CH), 3.66 (br, $CH_2NC\mathbf{H}_2$), 5.53 (br, $COOHC\mathbf{H}$).

The copolymer composition was determined by calculating the integrals' ratio of the peaks placed at 1.38 (methyl group of 2-methylpiperazine), 1.62 plus 1.76 (two agmatine methylene groups), and 5.54 (BAC hydrogen α to the carboxyl group) ppm, respectively. Theoretical values: 0.5:0.5:1; calculated values: 0.59:0:51:1

^{13}C NMR (D_2O): δ (ppm) = 13.6 ($CH\mathbf{C}H_3$), 22.3 ($NHCH_2CH_2\mathbf{C}H_2$), 25.0 ($NHCH_2\mathbf{C}H_2$), 28.9 ($NHCOCH_2\mathbf{C}H_2$), 29.5 ($CH_3CH\mathbf{C}H_2$), 40.4 ($CH_2N\mathbf{C}H_2$), 48.2 ($CH_3\mathbf{C}H$), 49.1 ($NHCO\mathbf{C}H_2$), 52.5 ($NH\mathbf{C}H_2$), 54.4 ($CH_2N\mathbf{C}H_2$), 56.0 ($COOH\mathbf{C}H$), 155.1 ($NH_2\mathbf{C}NNH$), 171.3 ($NH\mathbf{C}O$), 173.5 ($CH\mathbf{C}OOH$).

Molecular weight values by SEC chromatography are reported in Table 2.

(b) AGMA0.2. BAC (2.640 g, 12.8 mmol) and hydroxide monohydrate (0.542 g, 12.8 mmol) were dissolved in water (4.3 mL) at room temperature, under nitrogen flow and gentle stirring. When the solids were completely dissolved, agmatine sulfate (0.6 g, 2.56 mmol), and hydroxide monohydrate (0.108 g, 2.56 mmol) were added and the mixture left in the dark for 6 h. After this time, 2-methylpiperazine (1.067 g, 10.24 mmol) and hydroxide monohydrate (0.432 g, 10.24 mmol) were added and the mixture left in the dark for 72 h. After this time, the reaction mixture was diluted with water (4.3 mL), acidified with hydrochloric acid to pH 4−4.5, and ultrafiltered through a membrane with nominal cutoff 3000. The fraction retained was freeze-dried and the product obtained as a white powder. Yield: 3.05 g.

Table 3. Acid Dissociation Constants of AGMA1

protonation step	assignments	direct titration	back-titration
pK_{a1}	carboxyl group		2.25
pK_{a2}	*ter*-amino group	7.30	7.45
pK_{a3}	guanidine group	11.6	≥12.1

Table 4. Zeta Potential (ZP) of Polymers Synthesized in This Study

polymer	ZP (mV) pH = 4.0	ZP (mV) pH = 7.4
AGMA1	+21.00	+ 17.30
AGMA0.5	+18.42	+ 8.13
AGMA0.2	+15.70	+ 5.03

Molecular weight values by SEC chromatography are reported in Table 2.

The ^{1}H NMR and ^{13}C NMR spectra in D_2O of AGMA0.2 were qualitatively equal to those of AGMA0.5. The copolymer composition was determined as for AGMA0.5. Theoretical values: 0.2:0.8:1; calculated values: 0.24:0.87:1

Potentiometric Determination of the Acid Dissociation Constants. AGMA1 samples (about 0.0013 M in highly deionized water (Millipore Milli-Q system), with the addition of 0.1 M sodium chloride as a ionic strength stabilizer), disaerated by continuous ultrapure nitrogen bubbling and thermostated at 298 °K, have been pH-metrically titrated forward (with 0.1 M sodium hydroxide) and backward (with 0.1 M hydrochloric acid). The operating cell (including a 211/S6G/12 AMEL glass electrode and an AMEL 3842 saturated calomel electrode with a double bridge filled with 0.1 M potassium nitrate, both connected to an AMEL 631 differential electrometer) was calibrated against a multiple pH standard buffer set, (including 0.05 *m* potassium tetraoxalate [pH=1.68]; three buffers at pH = 4, 7, and 9; and 0.025 *m* sodium hydrogencarbonate + 0.025 *m* sodium carbonate [pH = 10.012]) in order to cover a wide pH range. Moreover, the calibration was refined (and the whole apparatus checked) by closely fitting experimental forward- and back-titration curves of 0.175 *m* phosphoric acid, adopted as a reference on account of its having three dissociation constants being very similar to the target compound ones, with the corresponding to theoretical ones, calculated by our nonapproximated treatment of multiple acid/base equilibria following the De Levie approach.[16] The same modeling was afterward applied to the simulation of the experimental AGMA1 titration curves, with concomitant optimization of the sought pK_a values. A preliminary analysis of the flat portion of the titration curves corresponding to the buffer region was also performed according to the classic, approximated treatment of Katchalsky and Spitnik,[17] in order to evaluate the β parameter accounting for possible nonequivalence of a given functional group located on adjacent monomeric units.

Determination of the Zeta Potential. Electrophoretic mobility and zeta potential were measured using a 90 PLUS instrument (Brookhaven Instrument Corporation, NY). To determine the zeta potential, polymer aqueous solutions at pH 7.4 were diluted with a solution of KCl 0.1 mM and placed in the electrophoretic cell, where an electric field of 15.2 V/cm was applied. Each sample was analyzed at least in triplicate. The measured electrophoretic mobility was converted into zeta potential by using the Helmholtz-Smoluchowsky equation.[18]

Cell Lines and Culture Conditions. HT-29 cell lines were obtained from the Interlab Cell Line collection (ICLC, Genova, Italy). Cells were grown in monolayer culture in RPMI 1640 supplemented with 2mM L-glutamine, penicillin/streptomycin (100 units/ml) and 10% heat-inactivated fetal calf serum (FCS). All cell lines were cultured at 37° C in a humidified atmosphere of 5% CO_2-air.

Cytotoxicity Assay. Appropriate dilution was made in order to obtain a concentration of 200 kcell/mL in culture medium. In each well containing 1 mL of this suspension, varying amounts of polymers (see Table 5) were added in solution and a row was left untreated as the control. The plates were incubated at 37 °C for 24, 48, and 72 hours, respectively. Cell viability was assessed by trypan blue exclusion assay. Cell survival was expressed as the percentage of control cells. The 50% inhibition concentration (IC_{50}) was interpolated from the growth curves thus obtained. For all assays, the experiments were performed in triplicate.

Determination of Hemolytic Activity. A total of 250 μL of human blood is added to different amounts of polymers (see Table 6 and Table 7), and the volume is adjusted to 1 mL with sterile PBS 10 mM at requested pH. In each experiment, a vial containing 250 μL of human blood and 750 μL of the same PBS and a vial containing 250 μL of human blood and 750 μL of the same PBS containing an excess of ammonium chloride to obtain complete hemolysis are added as controls. Vials are incubated at 37 °C for 90 min and then centrifuged at 2000 rpm for 10 min. A total of 250 μL of the supernatant is removed and placed in quartz cells containing 2.5 mL of the same PBS used before.

Table 5. Cytotoxicity and Net Average Charge of AGMA1 and Selected Amphoteric PAAs at pH 7.4[a]

polymer	pK_a	isoelectric point	net average charge per unit (- or +) at pH 7.4	cytotoxicity IC_{50} (mg/mL)	ref
ISA23	7.48 3.24 2.00	~ 5.4	~ 0.40 (−)	> 5 B16F10, Cos-1, HT-29 and ML-1 cells	13 this paper
DMEDA-BAC	8.25 4.85 2.0	~ 6.8	~ 0.08 (−)	> 5 B16F10 cells	13
DMEPDA-BAC	8.99 6.85 1.91	~ 7.9	~ 0.20 (+)	3.55 ± 0.31 B16F10 cells	13
DMEXA-BAC	9.53 8.37 2.17	~ 8.9	~ 0.75 (+)	0.23 ± 0.06 B16F10 cells	13
AGMA1	12.50 7.30 1.80	≥ 10	~ 0.50 (+)	> 5 Cos-1, HT-29 and ML-1 cells	This paper
dextran				> 5 B16F10 cells	13
PEI				0.01 ± 0.01 B16F10 cells	13

[a] Legend: Amphoteric PAAs other than ISA 23 derived from the polyaddition to 2,2-bis(acrylamidoacetic) acid (BAC) of 1,2-bis (*N*-methylamino ethane) (DMEDA), 1,3-bis(*N*-methylamino propane) (DMEPDA), and 1,6-bis(*N*-methylamino)hexane (DMEXA). Dextran and poly(ethyleneimine) (PEI) were reported for comparison purposes.

Table 6. Hemolytic Activity of AGMA1 and AGMA1-ISA23 Copolymers in Phosphate Buffer at pH 7.4

conc. (mg/mL)	AGMA1 (% lysis)	AGMA0.5 (% lysis)	AGMA0.2 (% lysis)	ISA 23 (% lysis)[a]	dextran (% lysis)[a]	PEI (% lysis)[a]
1.0	0	0	0	0	0	40
1.5	0	0	0	n.d.[b]	0	n.d.[b]
5	0	0	2	20	0	5[c]
7	0	0	5	n.d.[b]	n.d.[b]	n.d.[b]
10	0	1.5	5	n.d.[b]	n.d.[b]	n.d.[b]
15	1	1.5	8	n.d.[b]	n.d.[b]	n.d.[b]
20	2	2	9	n.d.[b]	n.d.[b]	n.d.[b]

[a] Reference 25. [b] n.d. = not determined. [c] The apparent fall in Hb release was probably due to Hb precipitation after extensive hemolysis.[11]

Table 7. Hemolytic Activity of AGMA1 and AGMA1-ISA23 Copolymers in Phosphate Buffer at pH 6.5 and 5.5

pH	AGMA1	AGMA0.5	AGMA0.2	ISA 23[a]	dextran[b]	PEI[b]
6.5	0% at 7.5 mg/mL	20% at 7.5 mg/mL	>30% at 7.5 mg/mL	n.d.[c]	0% at 1.0 mg/mL	30% at 1.0 mg/mL
5.5	0% at 5 mg/mL	12% at 5 mg/mL	19% at 5 mg/mL	30% at 1 mg/mL	0% at 1.0 mg/mL	10% at 1.0 mg/mL

[a] Reference 13. [b] Reference 25. [c] n.d. = not determined.

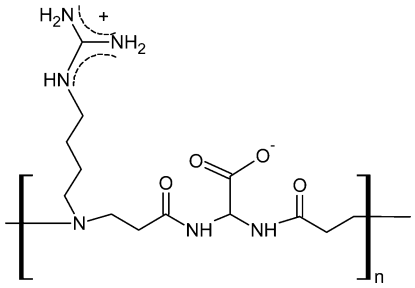
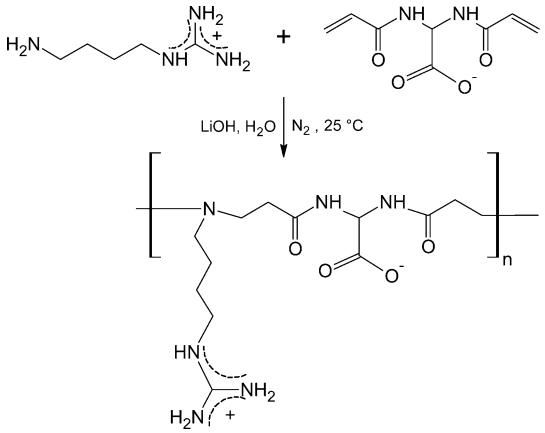


Figure 1. Structure of AGMA1.

All samples are analyzed using a Lamba2 Perkin-Elmer spectrophotometer at a wavelength of 543 nm. The percentage of hemolysis is calculated using the control with 100% hemolysis.

Scheme 1. Synthesis of AGMA1 by Polyaddition of Agmatine to BAC



Results

Structural Features. The repeating unit of AGMA1 derives from the polyaddition of agmatine to BAC (Figure 1). It is immediately apparent from Figure 1 that the repeating unit of AGMA1 bears a strong resemblance to the RGD sequence. In particular, the main features of both structures are reported in Table 1.

The repeating units of the AGMA1-ISA23 copolymers are reported in Figure 2. Two copolymers, AGMA0.5 and AGMA0.2, with $x:y = 1$ and 0.25, respectively, were prepared.

Synthesis. In principle, the Michael-type polyaddition of agmatine to BAC is not straightforward. Agmatine contains several mobile hydrogens belonging to the amino- and guanidine groups and, therefore, is a potential cross-linking agent in hydrogen-transfer polyadditions to bisacrylamides. It is common knowledge that a large difference in basicity exists between amines and guanidines. Therefore, we thought it was possible to employ partial protonation as a tool for protecting the guanidine group of agmatine in the preparation of linear AGMA1 and agmatine-containing PAA copolymers. Agmatine is usually sold as sulfate. By adding a single mol of a strong base per mol agmatine sulfate only the amino group lost its proton, and monoprotonated agmatine, in which the proton was localized on the guanidine group, was obtained. Lithium hydroxide was found particularly convenient as added base for the ease in dosage and of elimination from the end product. Under these conditions, the guanidine group did not participate in the polyaddition reaction and linear AGMA1 was obtained (Scheme 1).

AGMA1 structure was confirmed by ^{1}H and ^{13}C NMR analysis (see the Experimental Section). The ^{1}H spectrum is reported in Figure 3 together with assignments.

It may be mentioned that a linear PAA with pendant primary amino groups had been previously prepared from monoprotonated 1,2-diaminoethane and BAC.[19] The difference existing

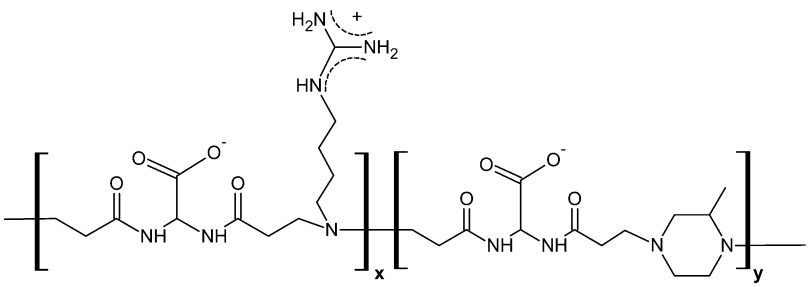


Figure 2. Structure of AGMA1−ISA23 copolymers.

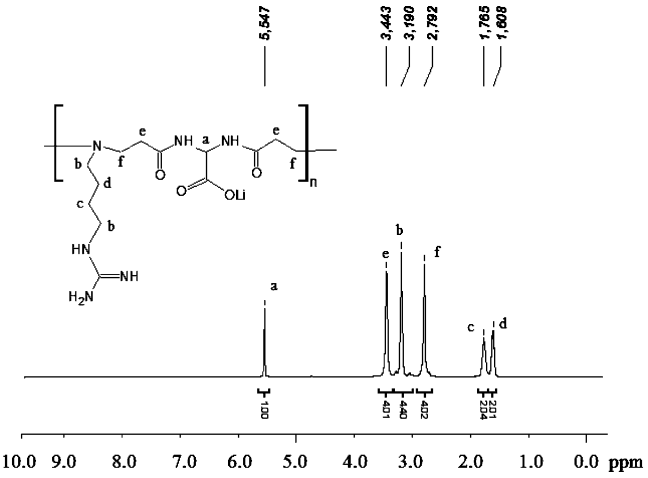


Figure 3. ^{1}H NMR spectrum of AGMA1 in D_2O.

between the first and the second protonation constants of EDA is sufficiently high to allow this.

This technique is hardly suitable for preparing linear copolymers of 1,2-diaminoethane with other amines, since the addition of a second aliphatic amine in the monomer mixture leads to partial deprotonation of monoprotonated 1,2-diaminoethane. On the contrary, linear AGMA1-ISA23 copolymers as well as AGMA1 copolymers with other PAAs can be easily obtained from mixtures of monoprotonated agmatine and 2-methylpiperazine or other primary or secondary amines, since no amine is capable of appreciably deprotonating a protonated guanidine group.

We prepared two AGMA1-ISA23 copolymers nicknamed AGMA0.5 and AGMA0.2 with agmatine:2-methylpiperazine ratios of 1:1 and 1:4 (on a molar basis), respectively. Their composition was checked by NMR and corresponded to the composition of the preparation recipe. The ^{1}H spectra of AGMA0.5 and AGMA0.2 are reported in Figures 4 and 5, respectively.

The aim was to compare the effect on biological properties of varying amounts of ISA23 units inserted in the structure of AGMA1.

The primary amino group of agmatine, at least in its second step, is probably less reactive than the amino groups of 2-methylpiperazine, as it is usually the case with primary amines.[7] Therefore, if agmatine and 2-methylpiperazine were added together in the reactant mixture, the latter would react first, yielding copolymers with an uneven distribution of the two units along the polymer chain. To avoid this, we first treated agmatine with excess BAC and then, after several hours, added the required amount of 2-methylpiperazine to saturate the residual double bonds (Scheme 2).

Physicochemical Properties. *Solubility.* The solubility of AGMA1 was tested in the most common solvents. AGMA1 is soluble in water within the range of pH values tested (2−12) and in the presence of most common acids and bases. As hydrochloride salt, it is also partially soluble in ethylene glycol and formamide but insoluble in methanol, ethanol, acetone, ethyl acetate, dimethylformamide, dimethyl sulfoxide, and chlorinated solvents. The solution properties of AGMA1 are similar to those of ISA23. Not unexpectedly, also the solution properties of AGMA1-ISA 23 copolymers are similar.

Molecular Weight Determination. The intrinsic viscosity, [η], and the apparent molecular weight of AGMA1 are reported in Table 2. Both values are not very high, if compared with those attainable for most PAAs.[7]

It should be observed, however, that the SEC standards adopted (see the Experimental Section) might be inadequate for AGMA1. For instance, intramolecular associations due to the presence of positive and negative charges in relatively distant positions may reduce the hydrodynamic volume of AGMA1. Moreover, the SEC mobile phase adopted as well as the solvent for [η] determination was a saline solution buffered at pH 8, that is, not very far from the isoelectric point of AGMA1, where its hydrodynamic volume is expected to reach the minimum, as observed for other amphoteric PAAs.[13] Therefore, the real

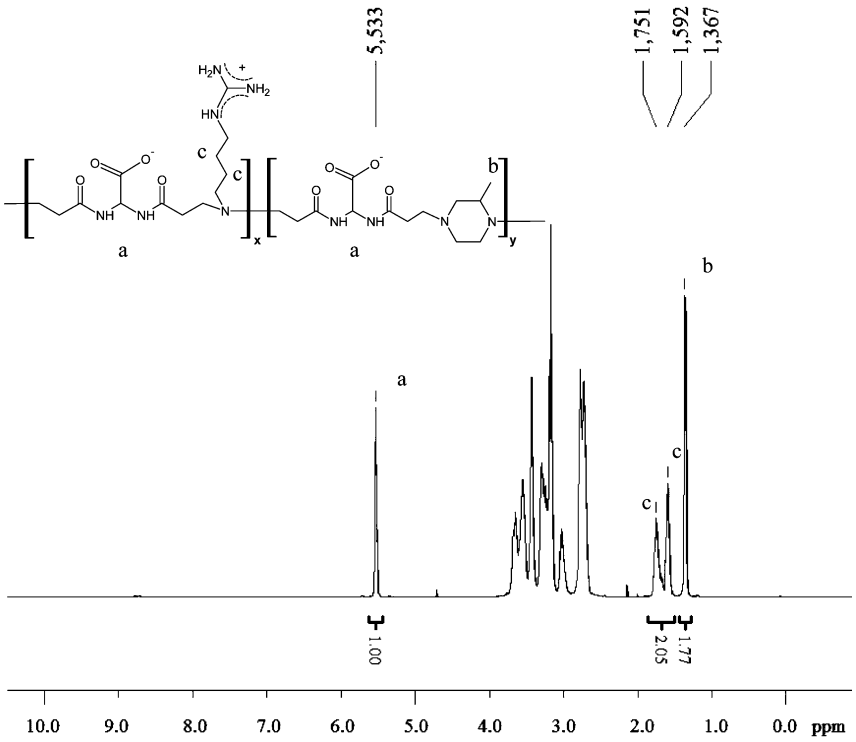


Figure 4. ^{1}H NMR spectrum of AGMA0.5 in D_2O. The marked peaks were used in the determination of copolymer composition.

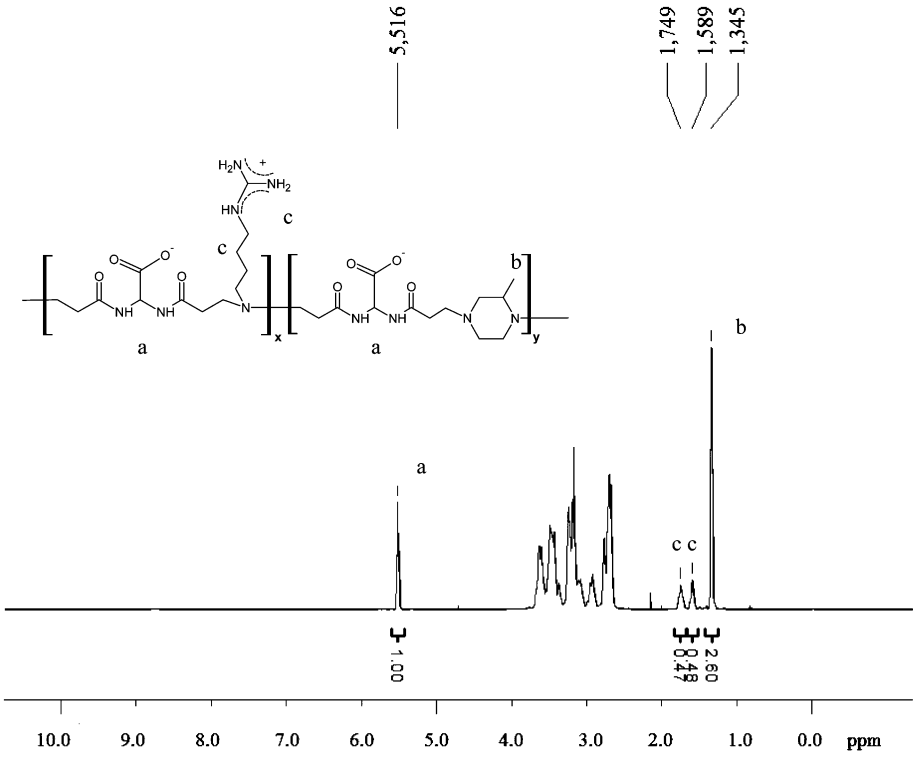
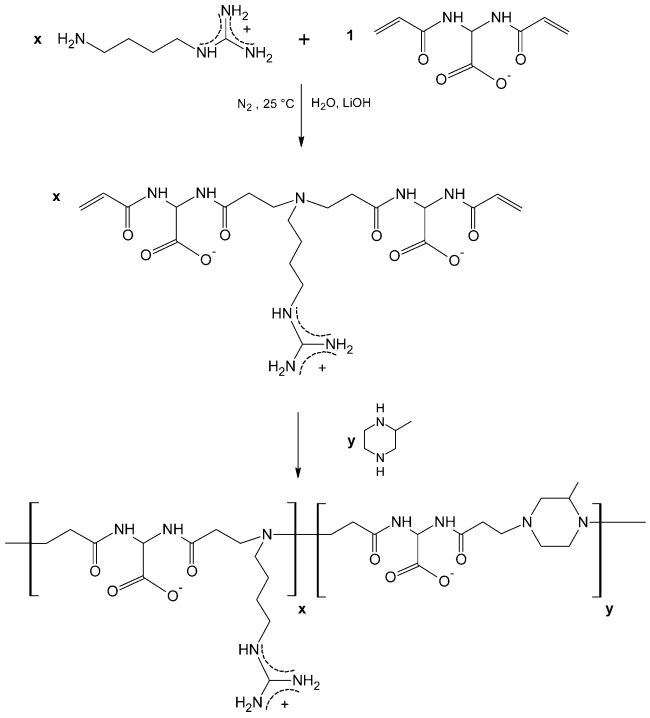


Figure 5. ^{1}H NMR spectrum of AGMA0.2 in D_2O. The marked peaks were used in the determination of copolymer composition.

Scheme 2. Synthesis of AGMA0.5 and AGMA0.2 Copolymers



molecular weight of AGMA1 might be higher than the figures reported in Table 2. The absence of significant NMR peaks attributed to residual double bonds seems to confirm this. It is not easy to perform absolute molecular weight determinations of AGMA1 for the possibility, along with intramolecular, of intermolecular associations in solution, as already observed in the case of ISA23 and related polymers.[20]

The apparent molecular weight of AGMA1−ISA23 copolymers is higher than that of AGMA1. The polydispersity is in all cases narrower than expected for products of a stepwise polyaddition. This is most probably due to the purification procedure adopted, involving an ultrafiltration step through a membrane.

Degradation. The degradation of AGMA1 was studied in 0.1 M phosphate buffer pH 7.4 at 37 °C, that is, in aqueous solutions mimicking biological conditions. Degradation data were obtained by monitoring the molecular weight decrease on time with SEC chromatography. On the whole, the degradation pattern of AGMA1 was similar to that of most PAAs, that is, it degraded to small molecules within few weeks.[7] Under the same conditions, the degradation pattern of AGMA0.5 and AGMA0.2 was similar to that of AGMA1.

Acid−Base Behavior. Under physiological conditions, AGMA1 has three ionizable functions per repeating unit: a carboxyl group, a tertiary amino group, and a guanidine group. Their acid dissociation constants have been determined by the pH-metric titration procedure described in the Experimental Section. Preliminary estimation of the β parameter from the analysis of the buffer region yielded 1.01 for the forward titration and 0.99 for the backward titration (which is quite reasonable considering the distance between same functional groups on adjacent monomer units in the polymer chain); therefore, the subsequent nonapproximated modeling of the titration curves was performed assuming $\beta = 1$. The values obtained for the three pK_as are summarized in Table 3.

Each repeating unit of AGMA1 can exist in four ionization states: purely anionic L(−), neutral zwitterionic L(±), positively charged zwitterionic L(+), and doubly positively charged L(2+), which is equal to unprotonated L, monoprotonated (LH), diprotonated (LH_2), and triprotonated (LH_3) conditions. Owing to the correlation normally existing between protonation and biological behavior, as previously found for instance in the case of amphoteric PAAs, it is interesting to express the relative distribution of the different ionic species for each polymer as a function of pH (Figure 6). It may be reminded that, although the pH of blood is 7.4, the pH inside lysosomes, that is, where

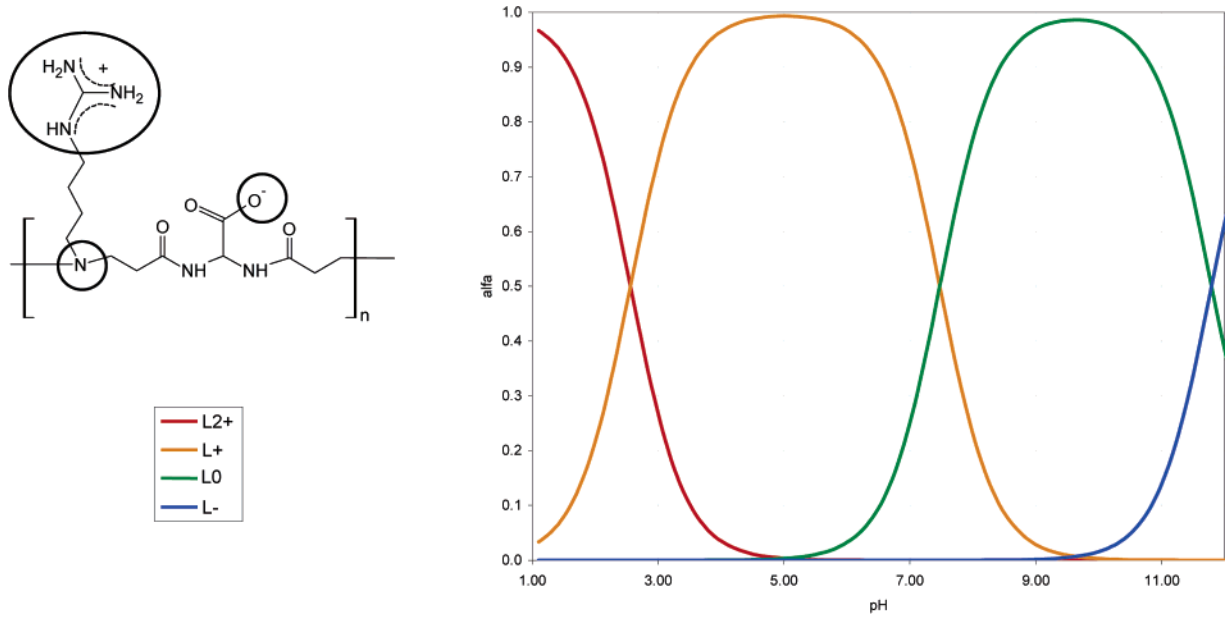


Figure 6. Relative distribution of different ionic species in AGMA1 as a function of pH.

normally high polymers locate after internalization in cells is 5.5 or whereabouts.

Three constants are reported (Table 3), relating to the acid dissociation of the carboxylic group and of the two protonated amino groups of the repeating unit. The first pK_a is very low, which indicates that at all physiological pHs (ranging from 7.4 in extracellular fluids to 5 in some intracellular compartments) the AGMA1 carboxylate anion does not protonate and is always present as an anion. The second constant relates to the *ter*-amino group and corresponds to a medium-weak base, incompletely ionized at pH 7.4, but fully ionized at pH 5.5. The third constant relates to the guanidine group, which is fully protonated at both pH 7.4 and 5. Charge distribution profiles show that this polymer is prevailingly cationic at all physiological pH values, the net average charge per unit varying from about 0.5 at pH 7.4, to about 1.0 at pH 5. 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^+ and L^- and is located close to pH 10. It is apparent that AGMA1 carries a remarkable excess of positive charges at pH 7.4 and even more so at pH 5.5.

Zeta Potential Measurements. We then assessed the amount of charge of the polymers in aqueous solution using zeta potential measurements at different pHs (Table 4). The values reported in Table 4 confirm that AGMA1 is positively charged at pH 7.4 and that the positive charge increases lowering the pH to 4, thus confirming the evidence of Figure 6. As expected, the copolymers show lower (but still positive) charge quantities at pH 7.4 as the agmatine content decreases.

Preliminary Biological Evaluations. *Cell Toxicity.* The cytotoxicity of AGMA1 at pH 7.4 was evaluated and compared to that of other amphoteric PAAs previously determined by R. Duncan and her colleagues at the Schools of Pharmacy of London and of Cardiff Universities.[7,13] The results are shown in Table 5. In the same table, the net average charge per unit of the same polymers at pH 7.4 is also reported.

The data of Table 5 refer to different cell lines but show the same tendency for all of the cell lines tested. It can be clearly seen that at pH 7.4 amphoteric PAAs may or may not be toxic depending on their isoelectric point. Only those with an isoelectric point lower than 7.4, being prevailingly anionic at the same pH value, are not cytotoxic.

AGMA1 is approximately as biocompatible as ISA23 and dextran and yet at pH 7.4 is prevailingly cationic, its isoelectric point being about 10.

Hemolytic Activity. Table 6 shows the hemolytic activity of AGMA1 and AGMA1-ISA23 copolymers in phosphate buffer at pH 7.4.

It may be observed that at pH 7.4 AGMA1 and AGMA0.5 are not hemolytic, whereas a modest hemolytic activity is shown by AGMA0.2.

The hemolytic activity of the same polymers was also determined at pH 6.5 and pH 5.5 (Table 7).

A higher positive average charge, as a consequence of lowering pH, did not affect the behavior of AGMA1 but induced significant hemolytic activity in AGMA1-ISA23 copolymers.

Discussion

Synthetic polycations, such as for instance polyethyleneimine (PEI), poly-L-lysine (PLL), and the structurally related to PAAs PAMAM dendrimers, have been developed for several biological applications. Among these, the lysis of cell membranes, the transport of material into cells, and the synthesis as nonviral vectors for gene therapies can be mentioned.[21,22] The high therapeutic potential of polycations for these applications is fully recognized in the scientific community, but very few of them have been approved so far for a clinical use. The main problem is the high toxicity usually associated with the polycationic character. Even in the case of amphoteric PAAs, it has been previously demonstrated that they are toxic, even if less than PLL or PEI, when on the average the charge of their repeating units at pH 7.4 is positive instead of neutral or negative.[13]

This paper reports on a polymer, AGMA1, that between pH 5 and 7.5, carries on the average a significantly high positive charge per unit and is nevertheless devoid of significant toxicity, at least toward the cell lines tested.

Even more striking is the difference in hemolytic activity between AGMA1 and most synthetic polycations, including PAAs. Hemolysis is usually a function of the overall positive charge and is related to cell membrane perturbation. A typical example is provided by ISA23, which is only weakly hemolytic at pH 7.4 but becomes highly hemolytic at pH values 5−5.5.[23]

This suggests that a high degree of hemolytic activity is observed for ISA23 only once the second amino group starts protonating. AGMA1, despite being always positively charged, is not hemolytic in the pH range 5.5−7.4. Interestingly, AGMA1-ISA23 copolymers showed a small, but noticeable, hemolytic activity that increased with decreasing the pH of the medium. Moreover, their hemolytic activity seemed to increase with increasing the ISA23 content. In other words, the behavior in this respect of AGMA1-ISA23 copolymers approached that of ISA23.

In a previous paper, we have shown that hydrogels obtained by cross-linking ISA23 were highly biocompatible, but did not exhibit appreciable cell adhesion.[24] The introduction of agmatine-deriving units in the ISA23 hydrogels did not affect biocompatibility but rendered them capable of strongly promoting cell adhesion and proliferation, like the RGD sequence does when grafted on surfaces.[5] This finding suggests that the repeating unit of AGMA1 resembles the RGD sequence not only in structure but also in biological activity, at least as regards cell membrane interaction. Moreover, this might provide a hint for explaining the lack of hemolytic activity of AGMA1 at all pH values. Further experiments are obviously needed before drawing reliable statements, but we think it not unreasonable to postulate that in AGMA1 the RGD-like repeating units exert a stabilizing action on cell membranes, overshadowing the membranolytic effect of the excess positive charges. In AGMA1-ISA23 copolymers the stabilizing action of the AGMA1 portion is partially superseded by the known hemolytic activity at low pH values of the ISA23 portion.[13]

Conclusions

1. A linear, amphoteric agmatine-containing PAA, nicknamed AGMA1, was successfully prepared starting from monoprotonated agmatine, where the positive charge is confined on the guanidine group that is therefore inert toward polyaddition under the reaction conditions adopted.

2. Copolymers between AGMA1 and ISA23 were easily prepared under the same conditions by substituting in part 2-methylpiperazine for agmatine.

3. Acid−base titrations gave for AGMA1 three acid dissociation constants, with pK_a values 2.25, 7.45, and ≥12.1, corresponding to a strong acid, a medium-weak base, and a strong base, respectively. Charge distribution profiles show that AGMA1's isoelectric point is at pH 10 or whereabouts, and therefore, AGMA1 is prevailingly cationic both inside cells and in extracellular fluids. The excess average positive charge per unit varies from about 0.5 at pH 7.4 to about 1.0 at pH 5. Zeta-potential measurements confirmed this.

4. Despite that, AGMA1 is, in vitro, nontoxic and nonhemolytic within the pH range tested (4−7.5). To our present knowledge, this is a unique feature in the polyelectrolyte domain.

5. The lack of hemolytic activity of AGMA1 even at acidic pH seems typical of the AGMA−BAC units and could be ascribed to their RGD-like structure. Subsequent papers will ascertain to which extent this structural resemblance leads to similar biological performances.

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