Prevailingly Cationic Agmatine-Based Amphoteric Polyamidoamine as a Nontoxic, Nonhemolytic, and "Stealthlike" DNA Complexing Agent and Transfection Promoter

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AGMA1, a prevailingly cationic amphoteric polyamidoamine obtained by polyaddition of (4-aminobutyl)guanidine (agmatine) to 2,2-bis(acrylamido)acetic acid, was studied as a potential DNA carrier and transfection promoter. Fluorescein-labeled AGMA1 was prepared by conjugation with fluorescein isothiocyanate and its cell uptake, blood permanence, and body distribution studied. In spite of its cationic character, AGMA1 is neither toxic nor hemolytic in the pH range 4.0−7.4, circulates for a long time in the blood without preferentially localizing in the liver, easily enters HT-29 cells, gives stable complexes with DNA, and is endowed with good transfection efficiency, suggesting the ability to transport in the cytoplasm a DNA payload without any measurable membranolytic activity. If compared with other transfection promoters, including polyamidoamines of different structures, AGMA1 is apparently endowed with a unique combination of desirable requirements for a nonviral DNA polymer carrier and warrants potential as a transfection agent in vivo.

Introduction

Polyamidoamines (PAAs) are degradable polymers obtained by Michael-type polyaddition of primary or bis secondary amines to bisacrylamides. The first studies on PAA synthesis were published around 1970,[1] and afterward, their chemical properties and biomedical applications were reviewed in several instances.[2−4] All PAAs contain amide and tertiary amine groups. The toxicity of PAAs at pH 7.4 is a function of their average positive charge at the same pH, that is, of their basicity. Highly basic PAAs, having a strong cationic character at physiological pH, are the most toxic.[4] The hemolytic activity of PAAs follows the same trend.

Amphoteric PAAs carrying side carboxyl groups switch from a prevailingly anionic to a prevailingly cationic state in a relatively small pH interval.[4,5] A typical amphoteric PAA named ISA23, obtained by polyaddition of 2-methylpiperazine to 2,2-bis(acrylamido)acetic acid (BAC), contains per repeating unit a single strongly acidic carboxyl group ($pK_a = 2.3$) and two weakly basic tertiary amine groups ($pK_a = 7.48$ and 3.24, respectively). At pH 7.4, the amine groups are poorly protonated, but the carboxyl group is completely present as an anion, and consequently, ISA23 carries excess negative charges. Under these conditions, ISA23 is nontoxic and nonhemolytic and when injected exhibits "stealthlike" properties and circulates for a long time in the blood stream. In tumor-bearing animals ISA23 concentrates in tumor tissues[6] by the EPR (enhanced permeation and retention) effect.[7] ISA23 can be internalized in cells by pinocytosis and localizes in lysosomes, where the pH is about 5.5. Here it strongly increases its polycationic character, becomes membrane-active, and promotes the intracellular trafficking of DNA and proteins.[8−12] Amphoteric PAAs other than ISA23, but belonging to a homologous series, have also been studied. Those that at pH 7.4 are prevailingly anionic proved nontoxic and nonhemolytic. By contrast, those that at the same pH are prevailingly cationic showed significant toxicity and hemolytic activity.[5] Therefore, it seemed possible to predict the biological behavior of amphoteric PAAs by determining their ionization constants and calculating their net average charge as a function of pH. However, recent results obtained with a PAA named AGMA1, prepared by polyaddition of monoprotonated (4-aminobutyl)guanidine (agmatine) to BAC (Scheme 1), disproved this assumption.[13−14]

The repeating unit of AGMA1 contains three ionizable groups, a strong acid ($pK_a = 2.3$), a medium-strength base ($pK_a = 7.4$), and a strong base ($pK_a \geq 12.1$). AGMA1 is prevailingly

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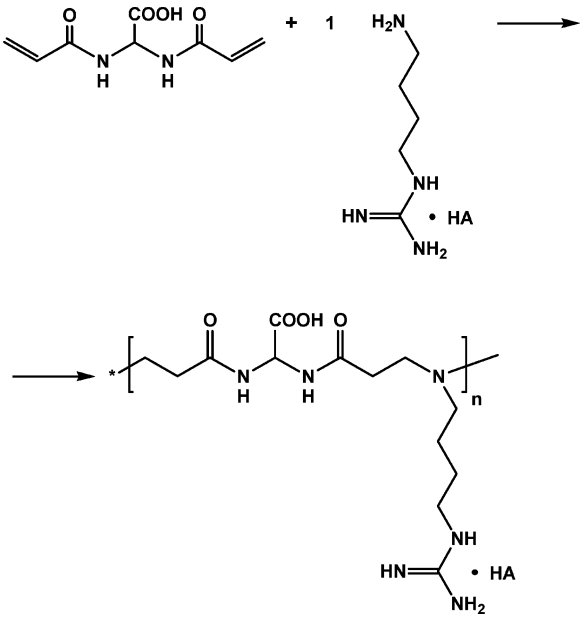
Scheme 1. Synthesis of AGMA1



cationic up to pH 10.5. At pH 7.4 the average excess positive charge is 0.55 per unit.[14] In spite of that, AGMA1 proved nontoxic and nonhemolytic in vitro within the entire pH range tested (4.0−7.4).[14]

In this paper, we prepared AGMA1 labeled with fluorescein isothiocyanate (FITC−AGMA1) and studied its biodistribution in rats and intracellular uptake. Furthermore, we determined the DNA complexing ability and transfection efficiency of AGMA1 in view of its use as a DNA carrier in vivo.

Experimental Section

Chemistry. *Instruments*. (a) The ^{1}H and ^{13}C spectra were acquired on a Brüker Avance 400 spectrometer, operating at 400.133 MHz (^{1}H) and at 100.623 MHz (^{13}C).

(b) Size exclusion chromatography (SEC) traces were obtained making use of TSK-gel G4000 PW and TSK-gel G3000 PW columns produced by TosoHaas. The two columns were connected in series, and the mobile phase was Tris buffer, pH 8.00, flow rate 1 mL/min (Waters model HPLC pump 515). The UV detector was a Waters model 486, operating at 230 nm. The refractive detector was a Waters model 2410. The samples were prepared in Tris buffer with a 1% concentration in polymer. Molecular weight determinations were based on a calibration curve obtained with pullulan standards. The intrinsic viscosity, $[\eta]$, was obtained in water, at 30 °C, using an Ubbelhode capillary viscometer in dilute solution.

(c) Fluorimetric measurements were performed with an RF 551 Shimadzu fluorimeter.

Materials. FITC and trifluoroacetic acid were purchased from Fluka and used as received. JetPEI was purchased from Polyplus-Transfection (Strasbourg, France). All other reagents (ACS grade) were from Sigma and were used as received. High-performance liquid chromatography (HPLC) solvents were from Carlo Erba (Italy).

(a) AGMA1 was prepared by reacting equimolecular amounts of agmatine and BAC as previously described.[14] In turn, BAC was prepared as previously described.[15]

(b) AGMA1 carrying primary amino groups as side substituents (AGMA1-NH_2) was prepared exactly in the same way by substituting 1 part (7.5% on a molar basis) mono(*tert*-butoxycarbonyl)ethylenediamine for agmatine. The resultant product was deprotected by treatment with 2 M hydrochloric acid in water, ultrafiltered through a membrane with a nominal molecular weight cutoff of 5000, and finally isolated by lyophilization. The complete removal of the protecting group was checked by NMR.

(c) Labeled AGMA1 (FITC−AGMA1) was prepared by treating an AGMA1-NH_2 aqueous solution at pH 7.4 (10 mg/mL) with an FITC solution in methanol (0.2 mg/mL). The resultant mixture was stirred overnight at room temperature and then centrifuged to eliminate insoluble impurities. The resultant clear solution was then dialyzed and the fluorescein-labeled polymer isolated by freeze-drying the retained portion. The recovery was practically quantitative. The product had $[\eta] = 0.17$ dL/g, $\bar{M}_n = 5200$, $\bar{M}_w = 8400$, and PD = 1.62. These values are in line with those previously determined for AGMA1 with the same techniques. The conjugation of AGMA1-NH_2 with FITC was confirmed by NMR, and the efficiency of the labeling procedure was determined by measuring the fluorescence intensity at $\lambda_{ex} = 480$ nm and $\lambda_{em} = 520$ nm of a solution of FITC−AGMA1 of known concentration versus a standard FITC solution.

Biology. *Animal Treatment*. The animals were treated according to the Guiding Principles for the Care and Use of Laboratory Animals, the Recommendation from the Declaration of Helsinki and the European Community, legislation no. 86/605. The protocol was reviewed and approved by the Local Animal Committee.

In Vitro and in Vivo Studies on FITC−AGMA1. (a) Cell cultures and viability: Human colorectal adenocarcinoma cell line, HT-29, was purchased from the American Type Culture Collection (Rockville, MD). HT-29 cells were grown as a monolayer culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 M L-glutamine, and penicillin/streptomycin (100 units/mL), at 37 °C in a 5% CO_2 humidified atmosphere and passaged weekly. At the beginning of the experiments, cells in the exponential growth phase were removed from the flasks with 0.05% trypsin−0.02% EDTA solution. Cells were seeded in 24 wells/plate (25000 cells/well) in RPMI 1640 medium with 10% FCS. The cells were allowed to attach for 72 h, then the seeding medium was removed, and the cells were maintained for 2 days in the experimental medium RPMI 1640 supplemented with increasing concentrations of polymer. The concentrations of AGMA1 varied from 5.8 to 58 μg/mL. All experiments were done three times, each condition being performed in triplicate. Cell viability was assessed by trypan blue exclusion assay. Cytotoxicity was expressed as a percentage of that of the control (cells not exposed to the polymer).

(b) Cell uptake: The cell uptake of FITC−AGMA1 by HT-29, a human colon cancer cell line, was investigated using fluorescence microscopy. A total of 25000 HT-29 cells were seeded in 1 mL of culture medium in 24 wells/plate. HT-29 cells were allowed to attach for 24 h on glass coverslips in the wells. The cells were incubated with three different FITC−AGMA1 concentrations (5.8, 11.6, and 58 μg/mL) for 24 h. After this time they were washed with PBS, and then the coverslips were inverted and mounted on glass slides. The cells were observed and photographed by DIALUX 20 fluorescence miscroscopy (Leitz, Wetzlar, Germany). Three separate experiments were performed in each case, and at least 100 cells were counted in triplicate for each concentration.

(c) FITC−AGMA1 intravenous administration to rats: The in vivo experiments were performed on albino male rats (Charles River, Italy) weighing 300−350 g. Under general anesthesia, a catheter was introduced into the rat's jugular vein, positioned subcutaneously with the tip in the interscapular region. The rat was allowed to recover for 24 h and the catheter flushed with 0.9% NaCl solution and heparin to avoid blood clot obstruction. The day of the experiment, 1 mL of FITC−AGMA solution at pH 7.4 (0.6 or 1 mg/mL) was injected into the catheter of four rats. Blood samples were collected 1, 15, 30, 45, and 60 min and 2, 3, 6, 12, and 24 h after the intravenous injection. All plasma samples were analyzed by the reversed-phase HPLC method described below, using a 5 μM Lichrosorb RP-18 column (250 × 4.6 mM).

Noncompartmental analysis (NCA) was used to compute derived pharmacokinetic parameters from raw individual FITC−polymer time−concentration data sets, employing the commercially available software

WinNonLin 4.0 Pro (WNL, Pharsight Inc.). All calculations were done following standard rules. The first-order rate constant associated with the terminal (log-linear) portion of the curve (λ_z) was calculated via linear regression of time vs log concentration and the terminal half-life ($t_{1/2\beta}$) by $[\ln(2)]\lambda_z^{-1}$. The $t_{1/2\beta}$ value was calculated with the noncompartmental analysis and was obtained by ln(2) on the terminal log-linear portion of the curve.

(d) Tissue distribution: To study the tissue distribution, eight rats received an injection in the tail vein of a 1 mg/mL FITC−AGMA1 aqueous solution at pH 7.4. Four rats were killed at 3 h and four rats at 24 h after the intravenous administration to collect samples of liver, spleen, heart, kidney, and brain. All tissues were analyzed by the HPLC method described below.

(e) Quantitative determination of FITC−AGMA1: FITC−AGMA1 was determined by a reversed-phase HPLC method either in vitro or in biological samples. The mobile phase was an 88:12 (v/v) phosphate buffer/acetonitrile mixture adjusted at pH 7.25, with a 1.1 mL/min flow rate, and using a fluorescence detector (RF-551, Shimadzu) set at λ_{ex} = 480 nm and λ_{em}= 520 nm. A linear correlation was obtained between the detector response and the concentration of FITC−AGMA1 with r^2 values in the range of 20 ng/mL to 5 μg/mL, with $r^2 = 0.995$.

(f) Determination of FITC−AGMA1 plasma concentration: A 0.2 mL volume of plasma was added to 0.8 mL of water and 0.5 mL of methanol and centrifuged and the surnatant analyzed by HPLC.

(g) Determination of FITC−AGMA1 tissue distribution: The tissues (0.5 g) were first homogenized in filtered water (3 mL) for 3 min using a Polytron homogenizer. Tissue extracts were then prepared by adding 25 μL of trifuoroacetic acid to 0.250 mL of homogenized tissue. After vortexing, three extractions were performed with 0.5 mL of diethyl ether followed by evaporation of the combined organic phases. The residues were reconstituted with 0.5 mL of eluent and analyzed by HPLC. For tissue analysis the eluent was corrected to an 88:12 (v/v) phosphate buffer/acetonitrile mixture adjusted at pH 7.25.

DNA Complex Formation and Characterization. (a) Complex preparation: The DNA−AGMA1 complexes were prepared at different pH values (4.0, 6.0, and 7.4) to evaluate the AGMA1 capacity to interact with DNA. A DNA solution (1 μg/mL) in 10 mM HEPES buffer at different pH values was added to AGMA1 aqueous solutions at different pH values in the required amounts to obtain the following ratios: 1:1, 1:2.5, 1:5, 1:10, 1:15, and 1:20 (w/w). After mixing, the complexes were incubated for 30 min at room temperature before analysis.

(b) Complex size analysis: The average diameters and polydispersity indices of DNA−AGMA1 complexes at different DNA:AGMA1 ratios were determined by photocorrelation spectroscopy (PCS) using a 90 Plus instrument (Brookhaven, New York) at a fixed angle of 90° and at 25 °C. Each reported value is the average of 10 measurements. The polydispersity index reflects the distribution size of the nanoparticle population.

(c) Complex surface charge determination: The electrophoretic mobility and ζ potential were measured using a 90 PLUS instrument (Brookhaven Instrument Corp., New York). To determine the ζ potential, the DNA complexes were diluted with 0.1 mM KCl and placed in the electrophoretic cell, where an electric field of about 15 V/cm was applied. Each sample was analyzed at least in triplicate. The measured electrophoretic mobility was converted into the ζ potential by using the Smoluchowsky equation.

(d) Agarose gel electrophoresis: A series of DNA−AGMA1 complexes were prepared by mixing at different ratios (1:2, 1:5, 1:10, 1:15 (w/w)) HEPES buffer (pH 7.4, 20 mM) solutions of plasmid DNA (20 μg/mL) and AGMA1 (330 μg/mL). Thirty minutes after preparation, the complexes were subjected to electrophoresis on an agarose gel (0.7%, w/v) with ethidium bromide (0.25 μg/mL) for 1 h at 70 V. The banding pattern was obtained using a UV transilluminator and photographed with a Polaroid camera.

(e) TEM analysis: The morphology of DNA complexes was determined by transmission electron microscopy (TEM) analysis using a Philips CM10 (Eindhoven, The Netherlands) instrument. For this purpose the DNA complexes in aqueous solution were sprayed on a copper grid before analysis.

(f) Protection from enzymatic degradation: DNA (10 μg/mL) and DNA−AGMA1 complexes were incubated at 37 °C with DNasi I (1000 units/mL) in phosphate buffer, pH 7.4. At specific intervals (up to 1 h) samples were withdrawn and centrifuged and the supernatants analyzed at 260 nm using a Perkin-Elmer Lambda II UV spectrophotometer. Results were expressed as a percentage of the control degradation (naked DNA).

(g) In vitro transfection experiments: Transfection experiments were performed on Hela cell lines. Complexes of plasmidic DNA and AGMA1 were prepared at three different ratios (1:5, 1:15, and 1:30 (w/w)). Briefly, 2 μg of plasmidic DNA of pEGFP was incubated with three different amounts of AGMA1 for 30 min, and the mixture was added to the cells in a six-well tissue culture. The JetPEI, a linear polyethylenimine, was used as a positive control, according to the manufacturing instructions, with a N/P ratio of 5.

The expression of GFP was observed after 24 h by fluorescence microscopy, and the quantitative determination of the transfection efficiency was obtained by flow cytometric analysis with a FACScan (Beckton-Dickinson).

(h) Statistical analysis: Experimental data have been represented as the mean and standard deviation of different independent determinations. The significance of the plasma concentrations was calculated using the Student's *t* test, and the difference was considered statistically significant at $P < 0.01$.

Results

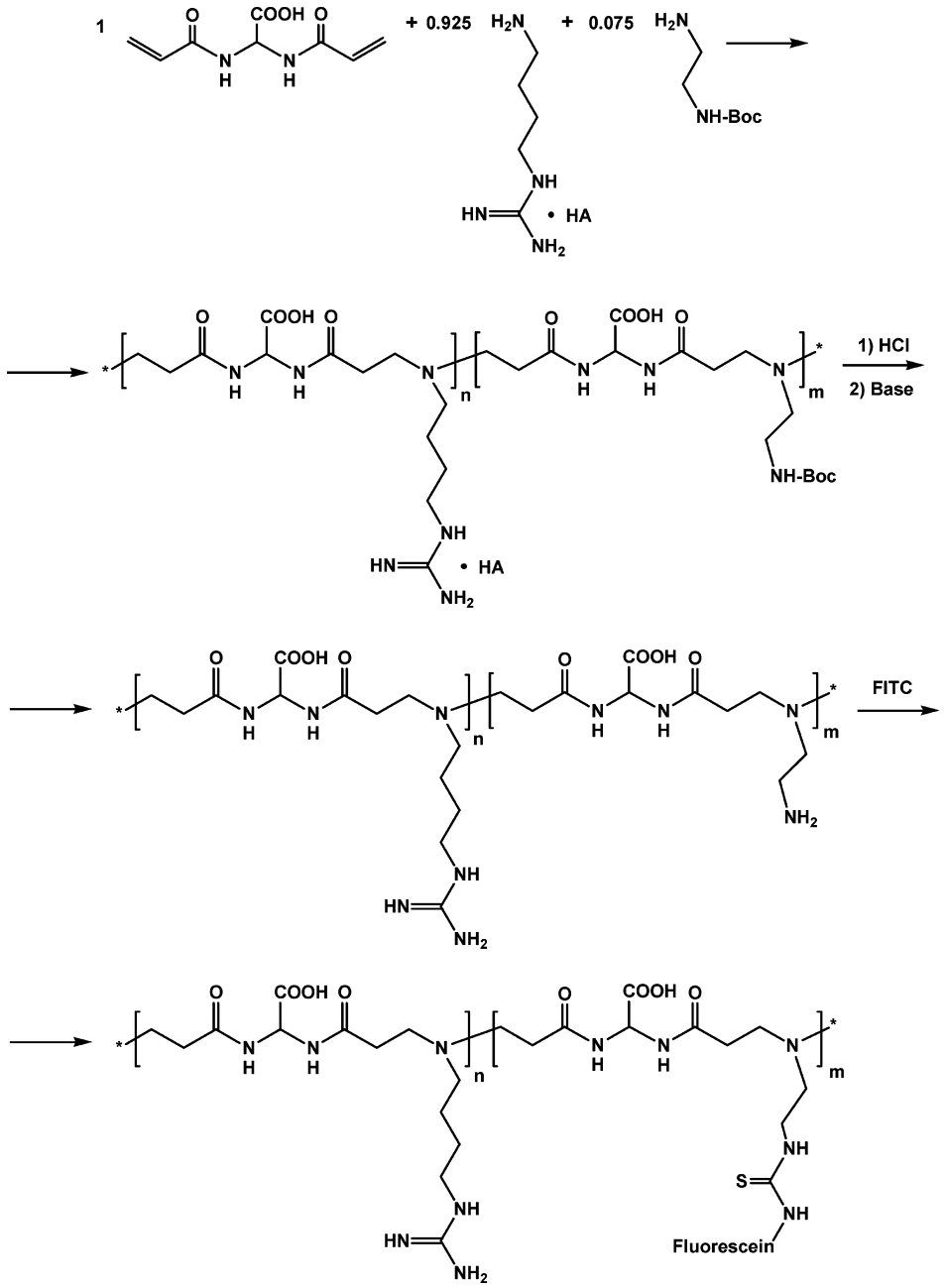
Preparation and Properties of Fluorescein-Labeled AGMA1. The preparation of FITC−AGMA1 involved first the introduction in AGMA1 of a small amount of side primary amine groups. This was achieved by introducing a small amount of mono(*tert*-butoxycarbonyl)ethylenediamine as a comonomer in the preparation recipe of AGMA1 and then removing the protecting group with hydrochloric acid (Scheme 2). Labeling was then performed by reacting the side primary amino groups so introduced with FITC according to a procedure usually employed for labeling proteins. The [η] and molecular weight values found for FITC−AGMA1 were very similar to those previously determined for AGMA1 (0.17 dL/g, $\bar{M}_n = 5200$, $\bar{M}_w = 8400$, and PD = 1.62 versus 0.16 dL/g, $\bar{M}_n = 4800$, $\bar{M}_w = 7200$, and PD = 1.50).

The ζ potential of FITC−AGMA1 at pH 7.4 was +7.8 mV, confirming its overall positive charge. The hemolytic activity and cell toxicity of FITC−AGMA1 were determined in triplicate following the same procedure previously described for AGMA1.[14] No hemolytic activity was observed up to a polymer concentration of 15 mg/mL (for AGMA1, 0% lysis at 10 mg/mL and 1% at 15 mg/mL). No significant toxic effect was observed on HT-29 cells up to 58 μg/mL after 24−48 h of exposure (for AGMA1, $IC_{50} > 5$ mg/mL). No toxic side effects were observed in animal experiments. For this purpose, we may observe that amphoteric PAAs usually have a very low toxicity[4] and agmatine has no toxic effect in normal animals in doses up to 100 mg/kg.[16]

These values were consistent with those previously found for AGMA1, showing that labeling did not induce any significant alteration of properties.

Intracellular Uptake and Biodistribution in Rats of FITC−AGMA1. In vitro experiments on HT29 cells showed that FITC−AGMA1 was internalized by HT-29 cells. After 24 h of incubation, in preliminary qualitative results, the polymer uptake was approximately 80% of the administered amount

Scheme 2: Synthesis of FITC−AGMA1



(Figure 1). Experiments are in progress to quantify the internalized amount of FITC−AGMA1.

The plasma concentration versus time of FITC−AGMA1 in rats is reported in Figure 2. The peak plasma concentrations were about 8.5 ± 1.0 and 16.0 ± 2.0 μg/mL after the iv administration of 0.6 and 1.0 mg/mL, respectively, and the polymer was still circulating in the plasma after 24 h. The mean residence time (MRT) and the $t_{1/2\beta}$ were 4.35 ± 1 and 4.89 ± 0.7 h, respectively. The initial loss was mainly due to kidney excretion of low molecular weight fractions, as the polymer sample used was not fractionated and had a rather broad molecular weight distribution.

The tissue distribution of FITC−AGMA1 was also determined and is reported in Figure 3. In liver and spleen the polymer concentration was low, reaching 1.27 and 0.33 μg/g, respectively, after 3 h, while after 24 h a very low FITC−AGMA1 concentration (0.12 μg/g) was detected only in the liver. No blood−brain barrier crossing was observed, as expected for a highly charged hydrophilic polymer. On the basis of plasma and tissue concentrations, we can speculate that the clearance by the reticuloendothelial system (RES) is negligible; that is, AGMA1 is endowed with the so-called stealthlike properties. Remember, however, that AGMA1 is ultimately bioeliminable because it degrades in aqueous media to nontoxic products.[13,14]

DNA Complexing Ability of AGMA1 and Transfection Experiments. Not surprisingly, owing to its polycationic character, AGMA1 forms stable complexes with DNA in the entire pH range considered (4.0−7.4). The ζ potentials of DNA−AGMA1 mixtures at different weight ratios are reported in Figure 4. A moderate excess of AGMA1 (5:1) shifts the negative DNA charge to positive ζ potential values already at pH 7.4. At acidic pH, a slightly positive charge is observed even at a 2:1 ratio (data not shown). These data are confirmed by the strong electrophoretic retardation, as shown in Figure 5, where it can be observed that increasing the DNA−AGMA1 ratio leads to a progressive fading of the DNA band. This band is not detected at a 15:1 DNA:AGMA1 ratio, indicating that

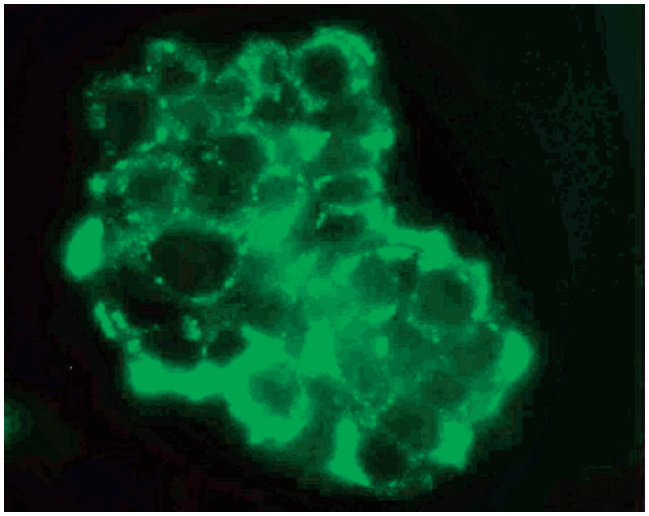


Figure 1. Uptake of FITC−AGMA1 by HT-29 cells after 24 of incubation detected by fluorescence microscopy (magnification 60×).

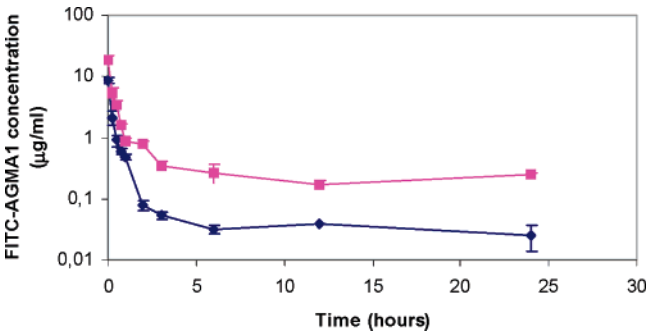


Figure 2. Plasma concentrations of FITC−AGMA1 versus time after 1 mL of iv administration at two different concentrations: ◆, 0.6 mg/mL; ■, 1 mg/mL. Data are given as the mean ± SD, $P < 0.01$.

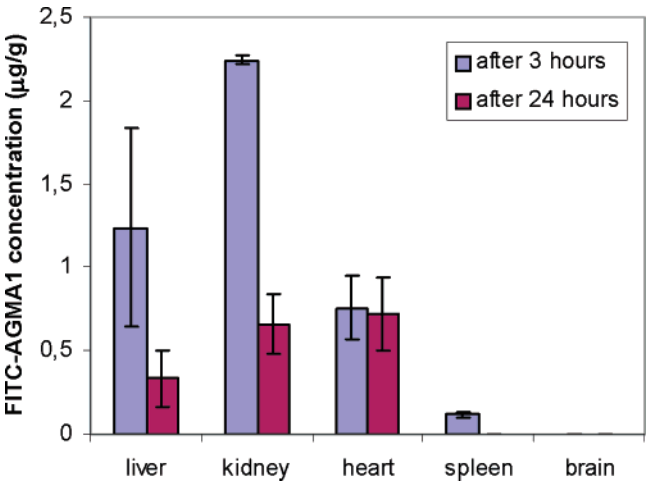


Figure 3. FITC−AGMA1 concentration in tissues after iv administration of 1 mL of 1 mg/mL FITC−AGMA1 solution. Data are given as the mean ± SD.

no detectable DNA is present; that is, DNA is entirely shielded by AGMA1.

AGMA1 significantly protects DNA from degradation by DNase I. After 1 h, less than 40% of the DNA in the 1:10 DNA−AGMA1 complex is degraded, compared with 100% degradation of free DNA (Figure 6). Direct observation by TEM reveals that the DNA−AGMA1 complexes are obtained in the form of discrete nanospheres (Figure 7), whose average size and polydispersity depend on the DNA:AGMA1 ratio (Table 1). The complex sizes have been confirmed by PCS. The

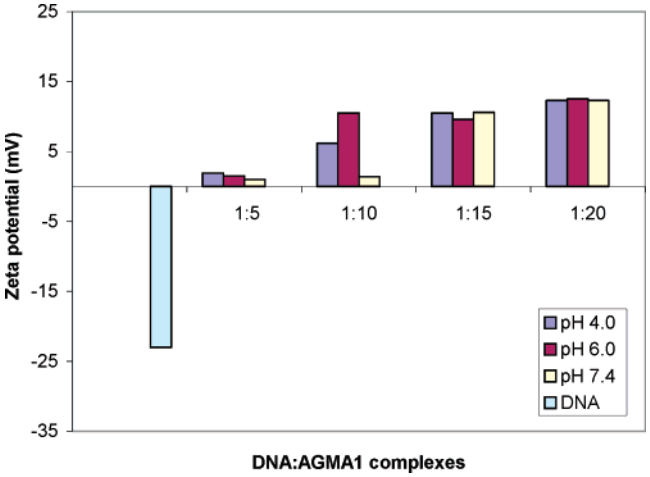


Figure 4. ζ potential values of DNA−AGMA1 complexes.

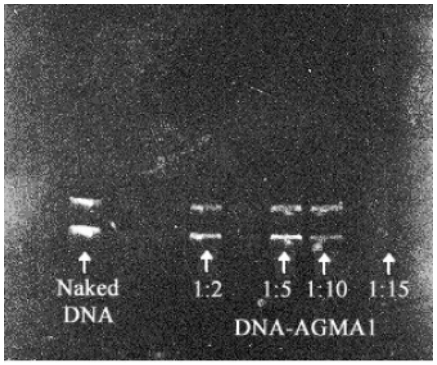


Figure 5. Electrophoretic mobility analysis of DNA−AGMA1 complexes: from the left naked DNA and 1:2, 1:5, 1:10, and 1:15 (w/w) DNA−AGMA1 complexes. The band of the 1:15 (w/w) complex is not visible because the DNA complex is completely shielded by the polymer.

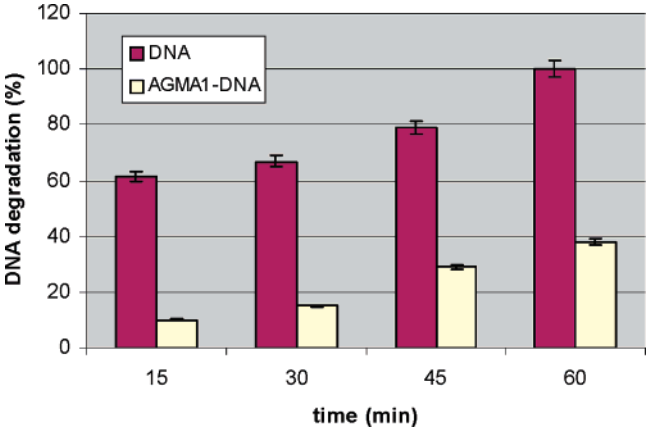


Figure 6. Enzymatic degradation of DNA and DNA−AGMA1 complexes with time. Data are given as the mean ± SD.

complex size decreases with increasing AGMA1 concentration, thus showing evidence of DNA compactation.

The ability of AGMA1 to transduce DNA into cells was evaluated with the expression of the green fluorescent protein (GFP) gene in Hela cells. The high number of fluorescent cells, observed by fluorescence microscopy (Figure 8a) and flow cytometric analysis, indicates a good in vitro transfection efficiency for AGMA1. The best results were obtained with a DNA:polymer ratio of 1:15 (w/w). In this experimental condition we observed by cytofluorimetric analysis 60% GFP expressing cells. The transfection ability of AGMA1 is comparable to that

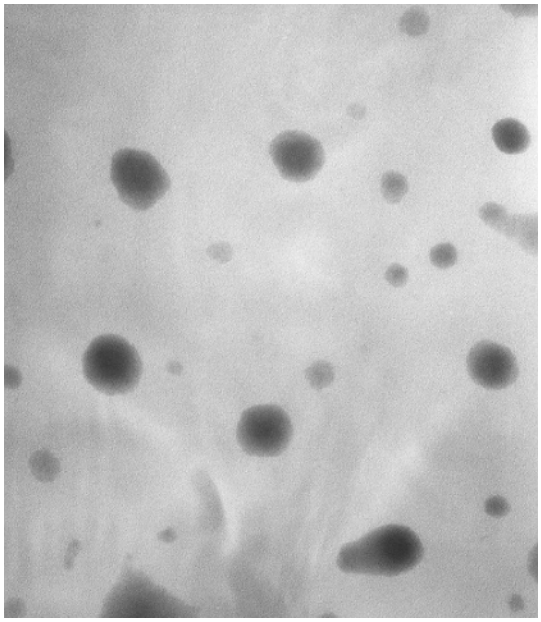


Figure 7. TEM photomicrograph of the 1:10 (w/w) DNA−AGMA1 complex (magnification 36000×).

Table 1. Average Diameter and Polydispersity Index of DNA−AGMA1 Nanoparticles at pH 7.4

DNA:AGMA1 ratio in the complex	av diam (nm)	polydispersity index
1:15	310 ± 5	0.22 ± 0.01
1:10	340 ± 10	0.25 ± 0.02
1:5	450 ± 10	0.23 ± 0.02

of other commercial polymeric transfection agents such as the JetPEI (63% GFP expressing cells) (Figure 8b), and the transfection capacity was maintained in the FCS even if to a lower extent (about 40% GFP expressing cells). No cytotoxicity was observed in all experiments.

Discussion

As pointed out in the Introduction, it is a generally confirmed rule that the toxicity of PAAs is related to their polycationic character. Most purely cationic PAAs are only moderately basic and are incompletely protonated at physiological pH; hence, they are less cytotoxic against many different cell lines than other popular polycationic polymers such as polyethylenimine (PEI) and poly-L-lysine (PLL).[4,17] The same rule applies also to amphoteric PAAs. Amphoteric PAAs that at pH 7.4 are prevailingly negatively charged were found to be nontoxic, whereas structurally related amphoteric PAAs positively charged at the same pH displayed appreciable cytotoxicity.[5] However, AGMA1 behaves in differently from the other amphoteric PAAs previously studied, since it is not appreciably toxic in vitro in spite of having at pH 7.4 an excess average positive charge of 0.55 per unit.[14]

Moreover, in contrast to PLL and PEI, amphoteric PAAs showed pH-dependent hemolysis. In particular, they caused more hemolysis at pH 5.5 (the lysosomal pH) than at pH 7.4. The given explanation, strongly supported by the literature describing polycation-mediated membrane damage,[18−21] was that lowering the pH led to protonation of the polymer backbone, increasing its capacity to interact with the anionic red blood cell membrane and, consequently, to cause concentration-dependent membrane perturbation. For this reason, it was

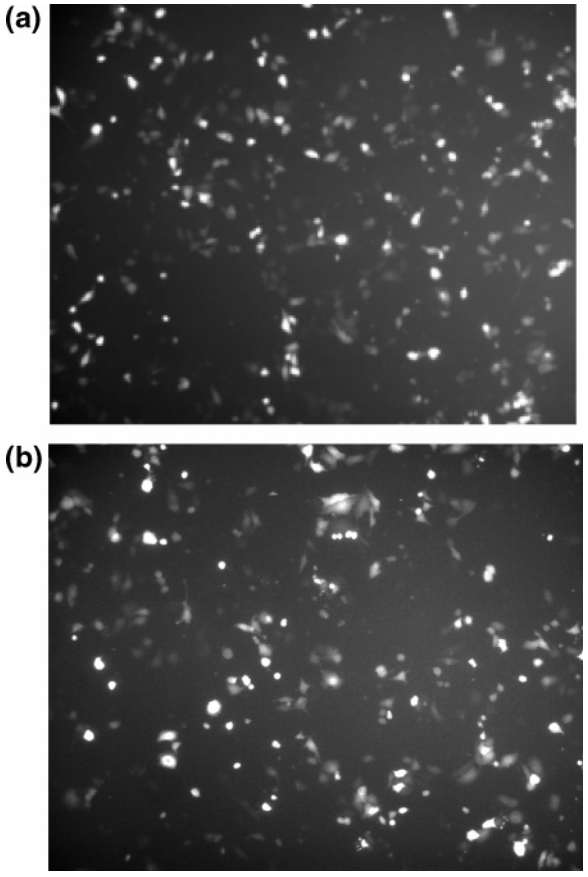


Figure 8. (a) Hela cells transfected with AGMA1 and a plasmid carrying the fuorescent protein GFP. (b) Hela cells transfected with JetPEI and the same plasmid.

supposed that the same PAAs could make easier the intracellular trafficking of DNA and proteins by promoting their escape from the lysosomes into the cytosol, and in fact, this was experimentally confirmed in several instances.[8−12] AGMA1 behaves unusually in regard to hemolysis, since it is not appreciably hemolytic both at pH 7.4 and at pH 4.0, where the excess average positive charges are, respectively, 0.55 and 1.1 per unit.[14] It may be argued that the lack of hemolytic properties does not necessarily imply the absence of membrane interactions, but only the lack of capacity of inducing membrane damage with or without interactions. Reconsidering the previous data on linear AGMA1,[14] we think that for this polymer membrane interaction does not equal membrane destabilization. This is in agreement with the high capacity of AGMA1-based cross-linked hydrogels of inducing cell adhesion and proliferation, but not cell damage.[13] The same effect is exerted also by linear AGMA1 solutions when added to dishes for culturing chromaffin cells (data not shown): patch-clamp recordings were performed to test cell adhesion and functionality. When the chromaffin cells plated on dishes pretreated with AGMA1 solution were compared with those plated on laminin- and poliornithine-treated dishes, the same properties of calcium channel gating, modulation, and calcium-evoked secretion were found. These data suggest that, besides being nontoxic, the AGMA1 solution could be used as a powerful tool for facilitating neuronal cell adhesion.

In the present study, the body distribution experiments carried out in vivo with fluorescent-labeled AGMA1 confirmed its exceptional properties. As a rule, cationic PAAs when injected are rapidly cleared out of the blood stream and preferentially localize in the liver, as expected for positively charged

polymers.[6] On the contrary, the previously studied amphoteric PAAs that are prevailingly anionic at pH 7.4 may exhibit stealthlike properties, circulate for a long time in the blood stream, and do not preferentially localize in the liver.[6] This may be ascribed to the absence of membrane interaction, as indirectly confirmed by the fact that ISA23 hydrogels are biocompatible, but exhibit antiadhesive properties toward the same cells that eagerly adhere and proliferate on AGMA1 hydrogels.[22] At pH 7.4, AGMA1 is definitely cationic, yet when injected it apparently behaves like the prevailingly anionic PAAs. We hypothesize that AGMA1 is able to localize on the blood cell membranes without destabilizing them, thus escaping clearance. However, further experiments are needed to confirm this.

AGMA1 gives stable complexes with DNA, as expected for a positively charged polymer, and acts as a transfection promoter with a transfection efficiency, in the system adopted, comparable with that of JetPEI, a popular and highly active PEI-based transfection agent. By comparing AGMA1 with other PAAs studied as transfection agents, we observe that to be membrane-active or to become so as a consequence of decreasing pH inside lysosomes (as, for instance, ISA23) has been considered a requirement for acting as an intracellular carrier of DNA and proteins.[8−12] Rather puzzlingly, AGMA1 acts as an effective transfection promoter; that is, it is apparently capable of transporting in the cytosol a DNA payload, without exerting any measurable membranolytic activity in a pH interval including the pH values of both extracellular and intracellular liquids.

Conclusions

AGMA1, an agmatine-based PAA water-soluble at all pH values, in spite of being fairly strongly cationic at physiological pH, is neither toxic nor hemolytic and when injected in rats circulates for a long time in the blood stream with minimal preferential localization in the liver.

Nonamphoteric PAAs studied as transfection agents [9−12] are significantly toxic, albeit less than other cationic polymers such as PEI or PLL, and when injected in animals are rapidly cleared out of the blood stream and localize up to about 90% in the liver. Also amphoteric PAAs such as ISA23 that are prevailingly anionic at pH 7.4, but not at pH 5, can act as transfection promoters.[8] AGMA1 gives stable complexes with DNA at pH 7.4 or lower and exhibits a good transfection efficiency, suggesting a significant ability of transporting a DNA payload in the cytoplasm of HT-29 cells not related to any measurable membranolytic activity. This surprising finding is against the common, well-founded belief that to be membranolytic or (as ISA23 and other amphoteric PAAs) to become so at the lysosomal pH as a consequence of protonation is a condition, besides being capable of complexing DNA, for acting as effective transfection agents. We do not have, at present, a theory explaining this.

As the final conclusion, AGMA1 is endowed with a unique combination of desirable requirements for a nonviral DNA polymer carrier and warrants attention as a transfection agent in vivo. For this purpose, animal studies are presently being undertaken and will be reported in a forthcoming paper.

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