The AGMA1 polyamidoamine mediates the efficient delivery of siRNA

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AGMA1, a guanidine-bearing linear amphoteric, but prevailingly cationic polyamidoamine is an effective siRNA complexing agent. Here two AGMA1 samples of different molecular weight, i.e. AGMA1-5 and AGMA1-10 were investigated evaluate as siRNA complexing agents and transfection promoters. AGMA1-10 formed stable polyplexes with size lower than 50 nm and positive zeta potential. AGMA1-5 polyplexes were larger, about 100 nm in size. AGMA1-10 polyplexes, but not AGMA1-5 ones, proved effective intracellular siRNA carriers, able to trigger gene silencing in Hela and PC3 cell lines without eliciting cytotoxic effects. In particular, AGMA1-10 knocked down the AKT-1 expression upon transfection with a AKT-1 specific siRNA. The polyplex entry mechanism was investigated and found mediated by macropinocytosis. In conclusion, AGMA1 warrants potential as an efficient, non-toxic tool for the intracellular delivery of siRNA through a peculiar internalization pathway.

Keywords: polyamidoamine, AGMA1; siRNA; AGMA1/siRNA polyplexes; intracellular siRNA delivery; gene silencing

Introduction

Gene therapy, consisting in the transfer of genetic material into specific cells of patients, has been extensively investigated by the scientific community [1,2,3]. This therapeutic approach faces many limitations in the development of a clinical approved protocol. Common drawbacks are lack of safeness and poor delivery efficiency [4]. Recombinant viruses have been extensively employed due to their natural propensity to efficiently deliver nucleic acid inside cells [5,6] . As regards non-viral gene delivery, several progresses have been achieved in the formulation of cationic polymers able to condens negatively charged nucleic acids by electrostatic interaction [7]. Since several physiological and cellular barriers counteract the achievement of an efficient and specific transfection, the design of synthetic cationic polymer able to trigger effective gene delivery was often inspired by the molecular features of viral infection [8], considering that viral vectors are able to elude the mechanisms by which cells protect their genome and the gene expression from external interferences [9]. Nucleic acid

delivery systems based on cationic polymers present several advantages over the viral ones. In particular, they are less immunogenic [10], present fewer risks of erroneous introduction in the genome causing aberrant gene expression and completely avoid the risk of accidentally delivering viral particles capable of triggering active infection [11]. However, cationic polymers are still associated with some shortcomings including low transfection efficiency and poor transgene expression. Major requirements that a transfection agent should meet for being used as a carrier in gene transfection are efficient cell uptake, escape from the endocytic vesicles allowing diffusion of the nucleic acid in the cytosol and, finally, driving the nucleic acid inside the nucleus [12].

The most used non-viral agents employed to trigger transfection can be divided in two main categories, cationic lipids [13] and polymers, both of them presenting enough positive charges to induce complex formation with negatively charged nucleic acids [14].[16] For instance, a typical polymeric transfection promoter of natural origin is the cationic arginine-rich peptide derived from the human immunodeficiency virus type 1 Tat protein [15][25] and, among synthetic polymers, polyethylenimine (PEI) derivatives bearing *prim*- and *sec*-amine groups partially protonated at physiological pH [16] are currently employed for the same purpose [17,18].

Short interfering RNA (siRNA) turned out to be a powerful tool in mediating efficiently gene silencing [19] and hold a great promise in the therapeutic treatment of a variety of human disease. The first step for establishing clinical therapeutics based on siRNA interference is to provide an effective siRNA delivery system [20].

Polyamidoamines (PAAs) are synthetic biodegradable polymers that can be designed to be highly biocompatible. They are obtained by stepwise Michael-type polyaddition of primary or secondary amines to bisacrylamides and contain tert-amine and amide groups regularly arranged along the polymer chain. Many functionalized

amines and bisacrylamides can be used as monomers resulting in an almost endless variety of polymer structures. PAAs were first described in 1970 [21] . Subsequently, their physico-chemical and biological properties were reviewed at intervals [22-24]. As a rule, PAAs are water-soluble and all of them show different charge distribution profiles as a function of pH [25-27]. As regards biocompatibility, most PAAs exhibit LD_{50} values in vitro higher by two orders of magnitude or more than PLL, PEI or PAMAM dendrimers [24]. Amphoteric PAAs, carrying carboxyl groups as side substituents, can be as biocompatible as dextran [26].

The ability of linear PAAs to promote the in vitro transfection of HEPG2 cells by pSV-β galactosidase was first demonstrated for some PAAs [25]. Subsequently, other PAAs were considered as transfection promoters [28-32]. More recently, PAAs carrying primary amine groups as side substituents [15], and bioreducible PAAs containing disulfide linkages in the polymer chain were synthesized and evaluated as non-viral vectors [33]. In a previous paper, we reported on the outstanding ability of a linear amphoteric but prevailing cationic PAA nicknamed AGMA1 [34,25], based on 4-aminobutylguanidine (agmatine), to act as DNA transfection promoter. AGMA1 proved [35] a good gene delivery system for plasmidic DNA (pDNA) leading to efficient expression of pEGFP in HeLa cells and in the liver of tail intravenous injected mouse. The structure of its repeating unit is shown in Figure 1.

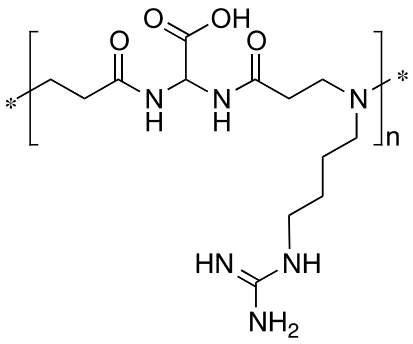

Figure 1

AGMA1 carries guanidine pendants that are completely protonated at physiological pH, that is, present as guanidium ions. Together with the partially ionized *tert*-amine groups typical of all PAAs, the guanidine pendants endow AGMA1 with the cationic density necessary to interact with the negatively charged nucleic acid phosphodiester backbone [27,36].

Up to now, however, no PAAs seem to have been reported as siRNA delivery systems. The aim of this paper is to report on the ability of AGMA1 to fill this gap.

Results

AGMA1 samples preparation and characterization

Two samples of linear agmatine-containing PAAs of different molecular weights, AGMA1-5 and AGMA1-10, were synthesized by known methods (see Experimental), and studied as intracellular siRNA transfecting agents. Their molecular weight was tuned by adopting different reaction times and finally ultrafiltering through membranes of different nominal cut-off. In particular, AGMA1-5 had $\overline{Mn}$ 3700, $\overline{Mw}$ 5100, PD (polydispersity index, $\overline{Mw}/\overline{Mn}$) 1.38 and AGMA1-10 $\overline{Mn}$ 7800, $\overline{Mw}$ 10100, PD 1.29. It may be observed that the PD values of both polymers were fairly low, due to the selective membrane ultrafiltration adopted in their preparations as the final purification step.

AGMA1-5 and AGMA1-10 samples containing small percentages (4-5%) of units carrying a primary amine group as side substituent, named AGMA1-5NH_2 and AGMA1-10NH_2, were prepared according to a general method for side-aminated PAAs and labelled with FITC following a standard procedure [27]**.** The conjugation of PAAs

with FITC was confirmed by NMR, and the efficiency of the labelling procedure was determined by measuring the fluorescence intensity at λ_{ex}=480 nm and λ_{em}=520 nm of a solution of FITC-PAA of known concentration versus a standard FITC solution. The viscosity and molecular weight values found for AGMA1-5 and AGMA1-10 conjugated with FITC were very similar to those previously determined for the parent polymers, showing that labelling did not significantly modify their properties. Zeta potential measurements demonstrated that AGMA1-5 and AGMA1-10 were positively charged in aqueous solution at pH 7.4 and their overall positive charge is significantly higher at pH 4.0 (Table 1).

Table 1

Polymer	**Zeta Potential pH 4.0 (mV)**	**Zeta Potential pH 5.0 (mV)**	**Zeta Potential pH 6.0 (mV)**	**Zeta Potential pH 7.4 (mV)**
AGMA1-5	32.68±1.37	31.59±0.43	22.59±0.67	17.09 ±0.60
AGMA1-10	21.00±1.16	15.32±0.74	11.10±0.52	2.10±0.37

Biocompatibility assessment

No significant hemolytic activity was observed for both AGMA1-5 and AGMA1-10 after 90 min incubation in blood at pH 7.4 up to a concentration of 15 mg/mL. Moreover the two polymers showed no cytotoxic effects on Hela cells after 48 h incubation up to a concentration of 7 mg/mL.

Preparation of AGMA1-5 and AGMA1-10/siRNA polyplexes

Both AGMA1-5 and AGMA1-10 proved able to form polyplexes with siRNA, with positive zeta potential for all the polymer/siRNA ratios investigated. The size of siRNA/AGMA1-10 polyplexes, determined by dynamic light scattering, was <50 nm. siRNA/AGMA1-5 polyplexes were larger (~100 nm), suggesting lower complexation effectiveness.

Direct TEM observation of polyplexes shows that they are in the form of discrete spherical nanoparticles. Figure 2 shows AGMA1-10/siRNA polyplex nanoparticles with 20/1 w/w polymer/nucleic acid ratio had diameters ranging from 20 to 50 nm.

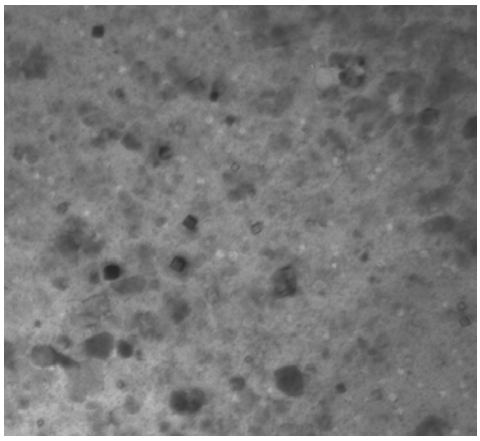

Figure 2

In vitro characterization of AGMA1/siRNA polyplexes

The capacity of AGMA1 to give stable polyplexes with different siRNA oligonucleotides was confirmed by gel retardation assay giving similar results. The preparation of AGMA1 polyplexes was performed at room temperature at pH 5.5 as previously described [35], and then analysed by electrophoresis in agarose gel. Whereas siRNA alone was clearly visible in agarose gel, its polyplex showed reduced ability to migrate dependent on different ratios of AGMA concentration (Fig. 3A). The polyplex non-migratory behaviour was a consequence of the neutralization by AGMA1 of

siRNA's negative charges. In particular, a partial retardation of siRNA was already evident using 1 μg AGMA1. Higher AGMA1 concentrations reduced the amount of free siRNA, reaching maximum efficiency at 6 μg AGMA1 (Fig. 3A).

To evaluate the effect on polyplex stability against competing polyanions, their susceptibility to heparin displacement was assessed. Heparin is one of the negatively charged glycosaminoglycans that are the major components of the extracellular matrix in many tissues and are also found on the cell surface. The displacement of siRNA molecules was evaluated by incubating polyplex samples with increasing amounts of heparin, and the presence of unbound siRNA molecules was monitored running the samples on agarose gel. Unbound siRNA was detected at heparin/siRNA ratio 0.5 (Fig. 3B)

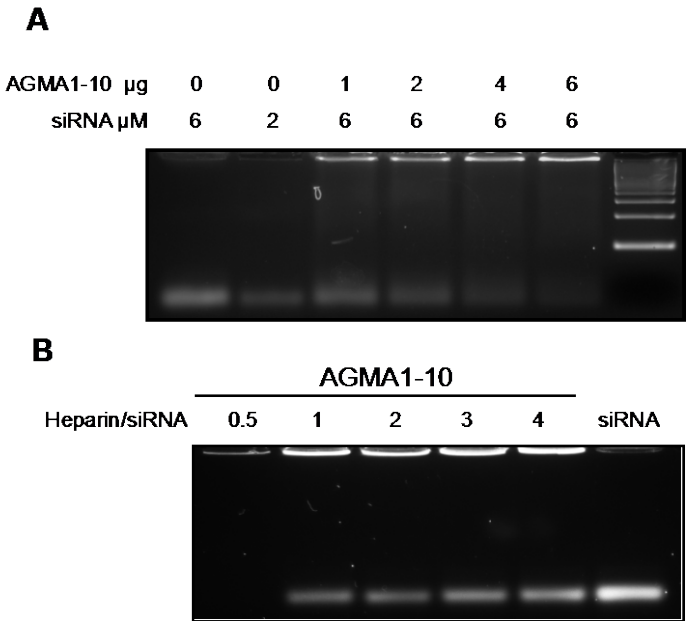

Figure 3

AGMA1-mediated siRNA transfection

It was previously demonstrated that AGMA1/pDNA polyplexes were efficiently delivered into mammalian cells [35]. In this work, AGMA1-10 ability to deliver small nucleic acids, such as siRNA, and to cause gene silencing was investigated. AGMA1-10 siRNA polyplex internalization in Hela cells was assessed by transfecting them with fluorescent siRNA complexed with both AGMA1-10 and commercial transfection agents taken as reference compounds. The number of fluorescent cells transfected with AGMA1-10 was similar to that of cells transfected with PEI reagent (JetPEI®) and larger than that of Oligofectamine® transfected (Fig. 4A). Moreover, AGMA1-10/siRNA polyplex was more efficiently internalized than JetPEI®, as demonstrated by the mean fluorescence values (Fig. 4A).

The gene silencing ability of AGMA1-10/siRNA polyplex was assayed by western blot analysis on HeLa cells transfected with siRNA targeting siAKT1. Two different amounts of AGMA1-10, 5 μg and 50 μg, were tested in the presence of 1 μg siRNA and compared with the silencing efficiency achieved with Oligofectamine®-transfected cells. Cells transfected with siAKT1 complex with 50 μg of AGMA1-10 exhibited reduced levels of Akt1, similar to cells transfected with Oligofectamine®, while complex with 5 μg of AGMA1-10 was unable to silence the expression of Akt1 (Fig. 4B). No significant differences were observed at 24 or 48 h after transfection. By contrast, under the same conditions AGMA1-5 was unable to silence the Akt1 expression.

A major challenge encountered employing a synthetic polymer for in vivo delivering nucleic acid inside cells is that it must be well tolerated by the organism when present in the bloodstream. Accordingly, AGMA1-10 was tested for its biocompatibility for gene delivery by measuring the cell viability with MTT compound.

Cells treated with AGMA1-10 or AGMA10/siRNA polyplex underwent MTT test 72 h post-treatment. AGMA1-10 had no effect on cell viability, whereas AGMA1-10/siRNA polyplex showed only low toxicity levels (Fig. 4C). Interestingly, the viability of cells transfected with 50 µg of AGMA1-10/siRNA polyplex was comparable to that of Oligofectamine® (Fig. 4C) transfected cells.

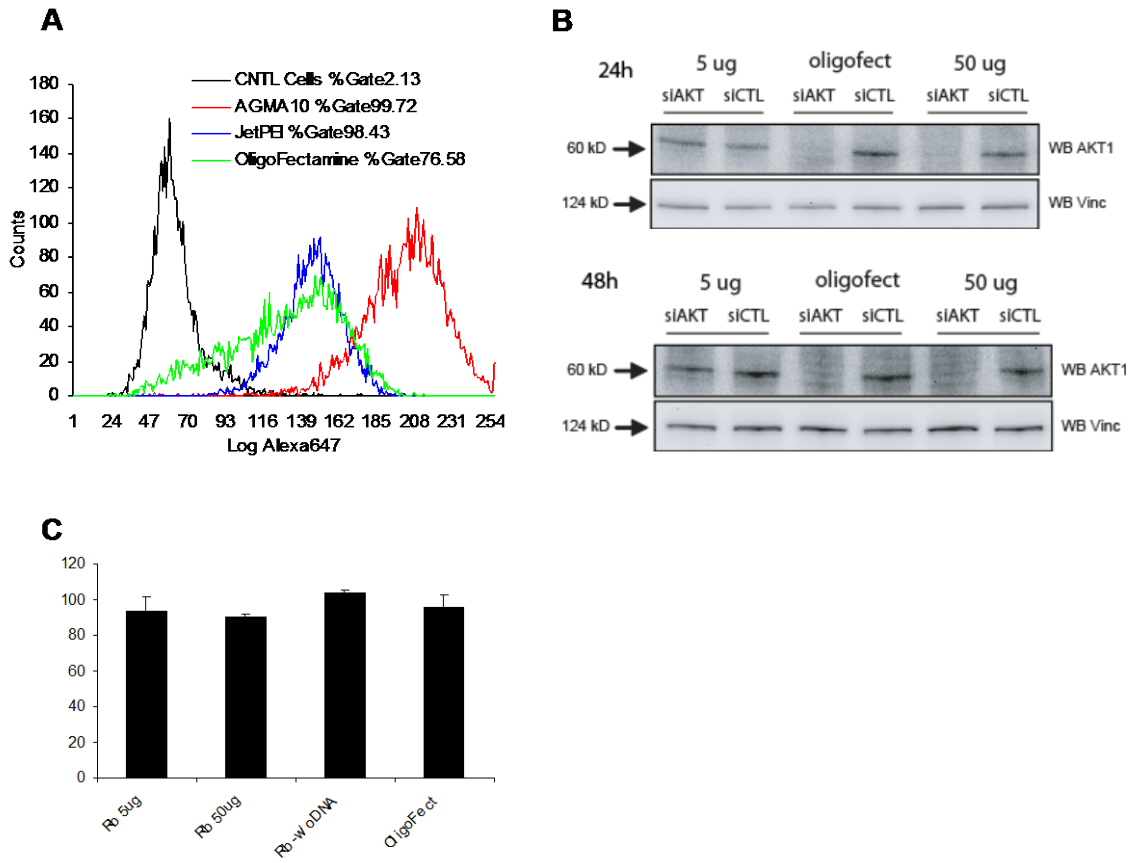

Figure 4

Gene silencing efficiency of AGMA1-10 polyplexes

We further investigated the ability of AGMA1-10 to silence endogenous genes by transfecting cells with low siRNA concentration in a time course experiment. In the same experiment, another gene, PDK1, was targeted with a specific siRNA (siPDK1).

The silencing effect was already evident 24 h after transfection and remained constant up to 72 h (Fig 5A). Reduced concentration of siAkt1 (50 nM) gave similar results than higher concentration of the same siRNA (100 nM), suggesting that the polyplex was efficiently delivered to silencing machinery. Moreover, PDK1 expression level was strongly reduced in cells transfected with 100 nM solution of siPDK1 but not with siAkt1.

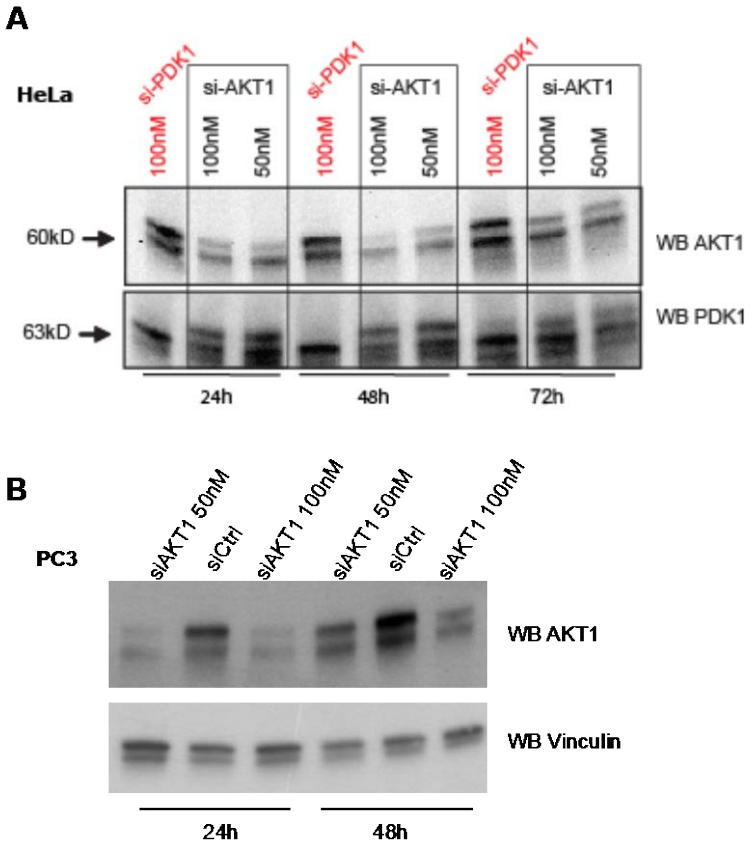

Figure 5

In addition, we evaluated the ability of AGMA1 to transfect and silence gene in a different tumor cell line. Prostate cancer cells, PC3, were transfected with AGMA1-10/siRNA polyplex showing reduced levels of AKT1 when transfected with specific siRNA but not with scambled siRNA used as control. However, the silencing efficiency already decreased after 48 h post-transfection.

Intracellular localization of AGMA1- 10/siRNA polyplexes

To better characterize the transfection process of AGMA1-10/siRNA polyplexes their intracellular localization was investigated. Fluorescent siRNA became observable inside cells 1 h after transfection, and assumed a specific localization in vesicular structures after 3 h, mainly in the peri-nuclear region (Fig. 6A). Unexpectedly, the intracellular localization pattern of the Oligofectamine®/siRNA polyplex was completely different, large structures appearing as vesicular aggregates (Fig. 6B). These different intracellular localizations suggested that AGMA1-10 polyplex was internalized through a different way. To exclude that it passed through the plasmamembrane by passive diffusion, a transfection experiment maintaining cells for the first hour of incubation at 4 °C was performed. No fluorescent siRNA was observed inside cells under this condition, showing that the polyplex was cell internalized by endocytosis, which is known to be temperature dependent. Similar results were obtained with Oligofectamine®, even if at 4 °C a certain amount of siRNA was anyway internalized.

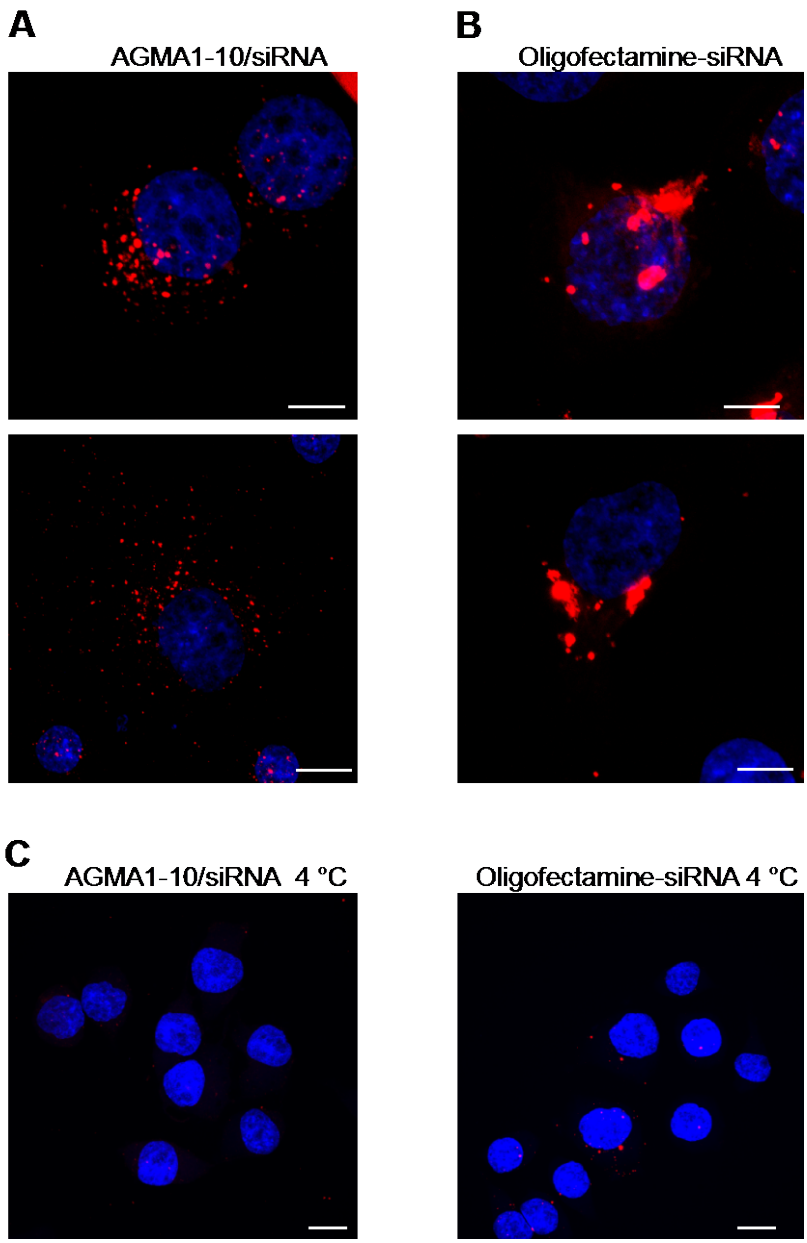

Figure 6

AGMA10/siRNA polyplex micropinocytic internalization

To elucidate the endocytic pathway involved in the uptake of AGMA1-10/siRNA polyplexes we employed a number of inhibitors specific for different endocytic pathways. The internalization of AGMA1-10 was affected by treatment with the micropinocytosis inhibitor amiloride that blocked the intracellular delivery of

siRNA (Fig. 7). Cell treatment with methyl-β-cyclodextrin, a lipid-mediated endocytosis inhibitor, such as caveolae, reduced but not completely eliminated siRNA cell entry (Fig. 7). By contrast, the inhibition of dynamine-dependent internalization processes, including clathrin and caveolae endocytosis, did not substantially modify the intracellular delivery of fluorescent siRNA (Fig. 7).

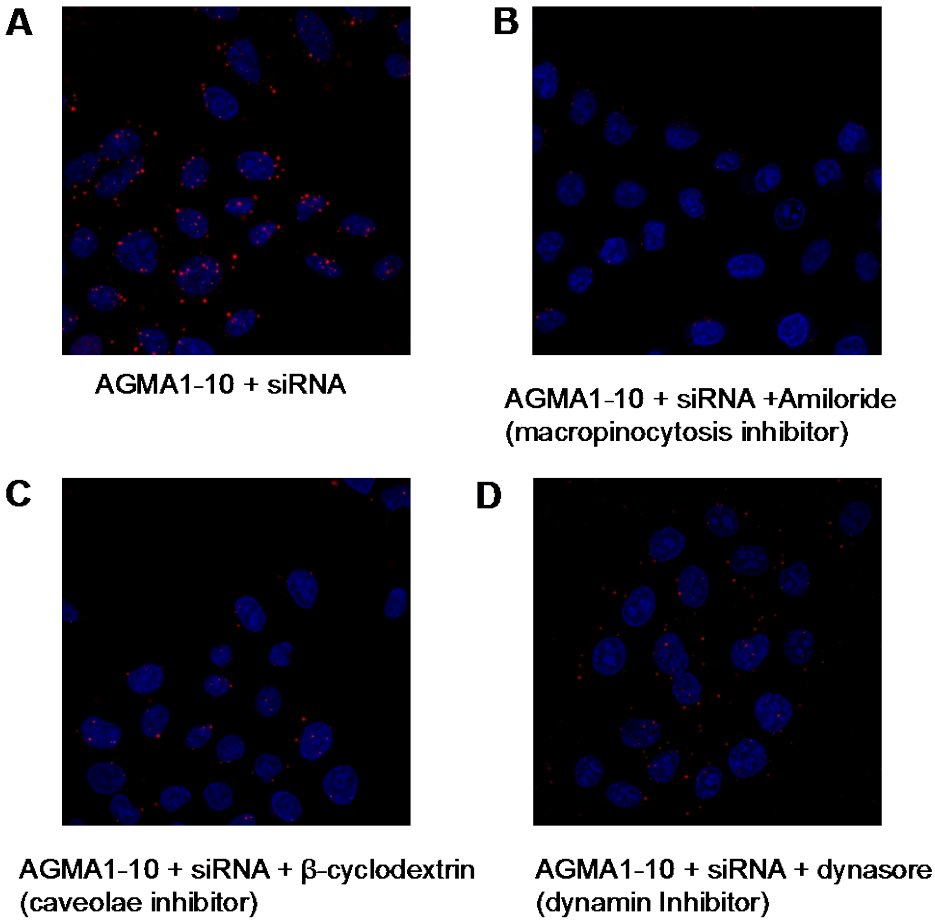

Figure 7

Taken together these results suggested that micropinocytosis, blocked by amiloride and partially sensitive to methyl-β-cyclodextrin, was the main endocytic internalization pathway of AGMA1-10/siRNA polyplexes. To substantiate this hypothesis, internalization experiments in the presence of micropinocytically

internalized fluorescent dextran were performed. AGMA1-10/siRNA polyplexes entirely localized in dextran-positive vesicles (Fig. 8A). Moreover, AGMA1-10/siRNA polyplex transfection in cells treated with amiloride did not silence the target gene, indicating that both delivery and gene silencing were dependent on micropinocytosis (Fig. 8B).

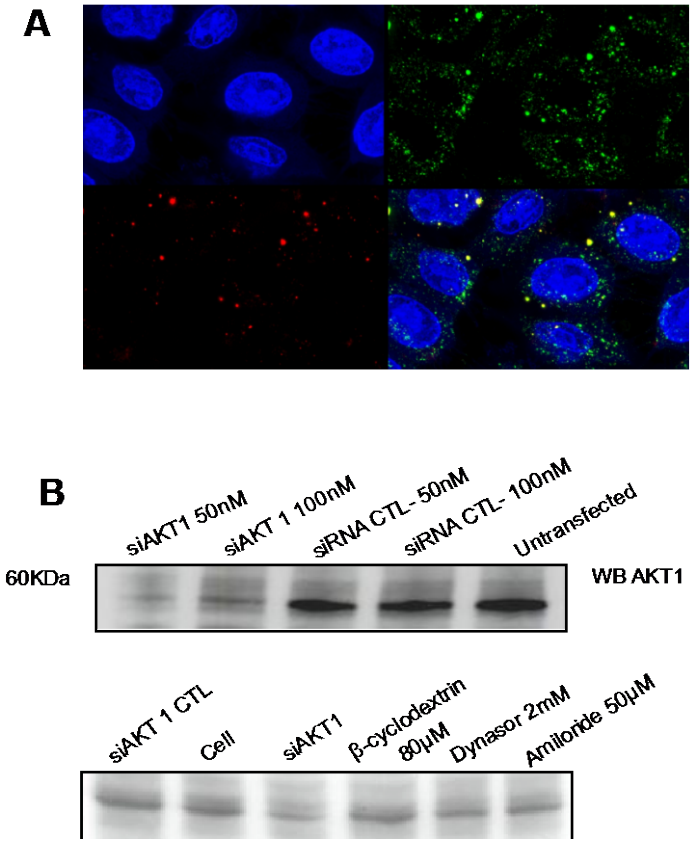

Figure 8

Effective siRNA delivery systems are needed to overcome the severe limitations of free siRNA, such as rapid degradation, fast renal clearance and poor cellular uptake. In the last years, many siRNA delivery strategies based on cationic polymers have been proposed. Good results were obtained with PEI, PLL and PAMAM [37-42]. In some cases, lipophilic moieties were introduced to increase efficiency by facilitating crossing of the cell membranes. AGMA1 represents an alternative nucleic acid polymer carrier.

It is a linear, amphoteric, but prevailingly cationic polymer soluble in water and biodegradable. It is only moderately basic and carries guanidine pendants attached to the polymer chain by 1,4-butylene groups. Its overall structure is reminiscent of the arg-gly-asp (RGD) motif, a common element in cellular recognition [43].

This polymer was successfully studied as DNA delivery system [27]. In this work AGMA1 of molecular weight 10100 (AGMA1-10) proved to interact with siRNA, forming polyplexes less than 50 nm in diameter. The release ability of siRNA from its AGMA1-10 polyplexes was assessed with heparin competition test. Polyplexes effectively promoted siRNA cytoplasmatic delivery and achieved a remarkable degree of knockdown effectiveness in HeLa and PC3 cell lines without eliciting significant toxicity.

It was demonstrated that siRNA/AGMA1-10 polyplexes were internalized in cells by macropinocytosis, a pathway known to be predominant in tumour cells. This finding let to envisage that after administration in tumour-bearing animals might mainly target to tumour the cells where the macropinocytosis is magnified and, therefore, exploited for selective siRNA delivering.

Conclusions

AGMA1 is a prevailingly cationic polyamidoamine based on 4-aminobutylguanidine and bearing guanidine groups as side-substituents. In this paper, it was demonstrated that AGMA1-10, an AGMA1 sample of $\overline{M}_w$ 10100, was an effective siRNA complexing agent and transfection promoter, whereas AGMA1-5, an AGMA1 sample of $\overline{M}_w$ 5100 was ineffective. In particular, siRNA/AGMA1-10 polyplexes proved able to induce Akt1 gene silencing in HeLa and PC3 cells, with transfection efficiency comparable with that of other widely used promoters, such as JetPEI® and

Oligofectamine®. In particular, they effectively knocked down the expression of Akt1 upon transfection with a siRNA specific for Akt1 transcript without eliciting toxicity. In addition, it was demonstrated that the the polyplex entry inside cells was mediated by macropinocytosis.

Recently it has been demonstrated that cancer cells seem to possess increased macropinocytic activity to fulfill diverse functions that include metastasis, metabolism, and signal transduction. For this reason, there has been an increased interest to investigate whether and how macropinocytosis could be exploited as cancer therapeutics strategy. In this contex, AGMA-mediated siRNA delivery could be developed as novel therapeutics against cancers [44,45].

Materials and methods

Instruments

^{1}H and ^{13}C NMR spectra were run on a Bruker Advance 400 spectrometer operating at 400.132 (^{1}H) and 100.623 (^{13}C) MHz. Size exclusion chromatography (SEC) traces were obtained with a Knauer Pump 1000 equipped with a Knauer Autosampler 3800, TSKgel G4000 PW and G3000 PW TosoHaas columns connected in series, Light Scattering (LS) Viscotek 270 Dual Detector, UV detector Waters model 486, operating at 230 nm and a refractive index detector Waters model 2410. The mobile phase was a 0.1 Tris buffer pH 8.00 ± 0.05 with 0.2 M sodium chloride. The flow rate was 1 mL min $^{-1}$ and sample concentration 1% solution. Fluorimetric measurements were performed with an RF 551 Shimadzu fluorimeter.

Materials

Ultra-pure water was obtained using a system 1-800 Milli-Q (Millipore, F). Fluorescein 5- isothiocyanate (FITC), ethidium bromide and Etoposide were purchased from Fluka (CH). DNase I was supplied by Sigma (UK). Plasmidic DNA was purified with Qiagen Midiprep Kit. siRNA duplex was purchased by Ambion Applied Biosystem (ID #s695).

Agarose was purchased from BIO-RAD Laboratories S.r.l (Milan) and JetPEI® from Polyplus-Transfection (Strasbourg, France). Oligofectamine® was purchased from Invitrogen. All other reagents (ACS grade) were from Sigma and were used as received. High-performance liquid chromatography (HPLC) solvents were from Carlo Erba (Italy).

Preparation of AGMA1-5 and AGMA1-10 samples

The preparation of AGMA1-5 had been previously reported [27]. Briefly, agmatine sulfate (2.000 g, 8.5 mmol) and lithium hydroxide monohydrate (0.360 g, 8.5 mmol) were added to a solution of 2,2-bisacrylamidoacetic acid (BAC) (1.689 g, 8.5 mmol) and lithium hydroxide monohydrate (0.360 g, 8.5 mmol) in distilled water (2.8 mL). The reaction mixture was maintained for 72 h at 20 °C under nitrogen atmosphere with occasional stirring. After this time, it was diluted with water (2.8 mL), acidified with hydrochloric acid to pH 4-4.5, then ultrafiltered through a membrane with nominal cut-off 3000. The fraction retained was freeze-dried and the product obtained as a white powder with $\overline{M}_n$ 3700, $\overline{M}_w$ 5100 and *PD* 1.38. Yield: 2.1 g.

AGMA1-10 had the same structure as AGMA1-5, but higher molecular weight and narrower distribution. It was obtained by adopting the same recipe, but the reaction

time was prolonged to 168 h, the resultant mixture ultrafiltered through a membrane of nominal cut-off 5000, and the product retrieved by freeze-drying the retained fraction. $\overline{M}_n$ 7800, $\overline{M}_w$ 10100 and *PD* 1.29. Yield 1.98 g.

The structure of both AGMA1-5 and AGMA1-10 was confirmed by NMR spectra in comparison with those reported in literature [27].

Preparation of FITC-AGMA1-10 and FITC-AGMA1-5

Labelled FITC-AGMA1-10 was prepared by treating with a FITC solution in methanol (0.2 mg/mL) a 10 mg/mL solution in buffer pH 7.4 of an AGMA1-10-NH_2 carrying amine groups as side substituents. In turn, AGMA1-10-NH_2 was obtained by a general procedure established for preparing aminated PAAs, as previously described [27]. The resultant mixture was stirred overnight at room temperature and then centrifuged to eliminate insoluble impurities. The resultant clear solution was then dialyzed through a membrane with nominal cut-off 3000, and the fluorescein-labelled polymer isolated by freeze-drying the retained portion. The recovery was practically quantitative. The conjugation of AGMA1-10-NH_2 with FITC was confirmed by NMR spectroscopy and fluorescence microscopy. A similar procedure was followed for preparing FITC-labeled AGMA1-5.

Characterization of AGMA1-5 and AGMA1-10 samples

Zeta potential determination

The zeta potential values (ZP) of AGMA1-5 and AGMA1-10 were determined in aqueous solutions at increasing pH values, ranging from 4.0 to 7.4, to verify the

polymer charge distribution as function of the pH. A 90 Plus instrument (Brookhaven, NY, USA) was used to determine the electrophoretic mobility and the zeta potential of the two polymers. For the determinations, the aqueous solutions of AGMA1-5 and AGMA1-10 were placed in the electrophoretic cell, where an electric field of about 15 V/cm was applied. Each value reported is the average of ten measurements. The electrophoretic mobility measured was converted into zeta potential using the Smoluchowsky equation [46].

Determination of AGMA1 polyplex size

The average diameter and polydispersity index of siRNA polyplexes were determined by photocorrelation spectroscopy (PCS) using a 90 Plus instrument (Brookhaven, NY, USA) at a scattering angle of 90° and a temperature of 25 °C. The morphology of formulations was evaluated by Transmission Electron Microscopy, using a Philips CM10 (Eindhoven, NL) instrument. The siRNA polyplexes aqueous nanosuspensions were sprayed on Formwar-coated copper grid and air dried before observation

Biocompatibility assessment

Hemolytic activity of AGMA1-5 and AGMA1-10 was studied on human blood. Increasing amounts of the two polymers up to 15 mg/mL were added to a suspension of erythrocytes (30 % v/v) in phosphate buffer pH 7.4 and then incubated for 90 min at 37 °C. A suspension containing only a 30 % v/v of erythrocytes in phosphate buffer pH 7.4 was used as blank. A similar suspension added with excess ammonium chloride gave the complete hemolysis as 100% hemolytic control. After centrifugation at 2000 rpm for 5 min the supernatants were analyzed using a Lambda 2 Perkin-Elmer spectrophotometer at a wavelength of 543 nm. The percentage of hemolysis was calculated versus the 100% hemolysis control.

Preparation of the siRNA polyplexes and in vitro transfection studies

Commercial oligonucleotides not-targeting siRNA, anti-AKT1, anti-PDK1 were purchased by AMBION-Thermofisher.

Two siRNA polyplexes were prepared using AGMA1-5 or AGMA1-10 at two different ratio siRNA:polymer, 1:5 and 1:50 by weight. To obtain the siRNA/AGMA polyplexes different amount of AGMA1-5 or AGMA1-10 solution (1 mg/mL)in NaCl 150 mM at pH of 5.5 were added to siRNA solution in NaCl 150 mM (50 µM). After mixing, siRNA complexes were incubated under mild stirring for 30 min at room temperature before adding to the cell cultured. Before transfection, the cell medium was replaced by serum-free medium, the siRNA/AGMA1 added and after 3 h the western blot and viability assays were performed. Transfection experiments were then performed in HeLa and PC3 cells lines grown in DMEM (Cambrex) supplemented with 10% FCS, 2 mM L-glutamine (Cambrex) and antibiotics.

Cell cytotoxicity

The cytotoxicity of AGMA1-5 and AGMA1-10 was assessed on Hela cells seeded in 24 wells plate. The toxicity of the polymer was evaluated by MTT assay [MTT=(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide)]. The measurement was performed at 24 and 48 h post-incubation. The viability of the treated cells was compared to untreated cells and to cells treated with Etoposide (0.1 □g/□L) a toxic compound. All experiments were performed in triplicate.

Gel retardation assay

Gel electrophoresis assay was used to evaluate the formation of the siRNA/AGMA1-10 complex. The freshly made polyplex was subjected to electrophoresis on a 4% agarose gel with SYBR safe dye for 1 h at 100 V to confirm the

nucleic acid complexation. The banding pattern was obtained using a UV transilluminator.

Western Blot experiments

Total proteins were extracted in Laemli buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol) and quantified. Equal amounts of each sample were resolved by SDS-PAGE and transfected to PVDF membrane. After blocking with TBS/0.1% Tween20/5% BSA, membranes were incubated with primary antibody overnight at 4 °C. Primary antibodies used are: α-Akt1, α-PDK1 (Cell Signalling), α-Tubulin (Santa Cruz Biotechnology). Immunoreactive proteins were identified with secondary antibody coupled to horsereadish peroxidase (HRP) antibody and visualized by ECL.

Confocal microscopy

Transfected cells, previously plated on glass coverslips, were fixed and as previously described [47]. Analysis was performed using a confocal laser-scanning microscope (TCS SP2 with DM IRE2; Leica) equipped with a 63×/1.40 HCX Plan-Apochromat oil-immersion objective. Confocal images are the maximum projections of a *z*-section of ~1.50 µm. The images were arranged and labelled using Photoshop software (Adobe) and are representative of three independent experiments.

Cytofluorimetric analysis

HeLa cells were transfected as above described, in order to check the siRNA internalization level a Cyanine3 labelled RNA oligonucleotide was employed. After 3 h incubation with the polyplex the cells were detected by trypsinization, and washed twice with phosphate saline buffer then finally incubated with DAPI dye. Unfixed cells were cytofluorimetrically analysed looking at the CY3 signals, and the level of positive cells

was gated based on untransfected cells. The same protocol was applied to cells treated with endocytosis inhibitors.

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Table and Figure captions

Table 1. Zeta potentials with standard deviation at different pH values. The values reported is the mean of ten measurements.

Figure 1. Chemical structure of AGMA1 repeating unit.

Figure 2. TEM microphotograph of the complex siRNA/AGMA1-10 1:20 w/w (Magnification 130000X).

Figure 3. Agarose gel electrophoresis retardation assay of siRNA complexed with AGMA1-10. **A.** Increasing ratio of AGMA1-10/siRNA showed a concomitant reduction of free siRNA visualized as electrophoretic mobility. **B.** Displacement of siRNA from AGMA1-10 by the negative charged heparin occurred for heparin/siRNA molar ration above 1.

Figure 4. Transfection efficiency and cytoxicity. **A.** Comparison of AGMA1-10 transfection efficiency to the commercially available JetPEI and Oligofectamine transfection agents. Transient transfection has been carried out in HeLa cells using Cyanine 3 fluorescence labelled siRNA (100 nM) complexed with 50 μg of AGMA1-10. Percentage of transfected cells has been evaluated with cytofluorimetric analysis, AGMA transfection gave higher percentage of positive cells with more intense signal. **B.** Western blot analysis of protein knockdown in HeLa cells transfected with siRNA against AKT1 100 nM, efficiency of silencing has been observed at 24 (top) and 48 (bottom) h post transfection and 50 μg of AGMA1-10 resulted in a knockdown comparable to Oligofectamine knockdown efficiency. **C.** Cytotoxicity of AGMA1-10 transfection in HeLa cells, no significant different in toxicity has been observed among cells treated with AGMA1-10 with or without RNA and Oligofectamine.

Figure 5. Silencing efficiency on HeLa and PC3 cells. **A.** Western Blot analysis of HeLa cells transiently transfected with siRNAs targeting AKT1 and PDK1 messengers (siAKT1 and siPDK1). Knockdown efficiency has been evaluated at three different time points (24, 48 and 72 h) with two concentration of siRNA: 50 nM and 100 nM in complex with 50 μg of AGMA1-10. **B.** Same transfection condition as for HeLa cells has been tested on PC3 prostate cancer cell line.

Figure 6. Intracellular localization of siRNA/ AGMA1-10 complex. **A.** Representative confocal images of HeLa cells transfected with 50nM of Alexa-647 labelled siRNA (red) in complex with 50 μg AGMA1-10 polymer, cells were prepared for microscopy observation after DAPI nuclear staining (blue). **B.** HeLa cells transfected with 50 nM siRNA Alexa-647 labelled in complex with Oligofectamine reagent. **C.** Temperature dependency of AGMA and Oligofectamine siRNA cell internalization in HeLa cells. Cells have been incubated for 30 min at 4 °C after complexes addition to the transfection medium.

Figure 7. siRNA/AGMA1-10 complex is internalized by macropinocytosis. Fixed-cell confocal microscopy images showing the cellular uptake of Alexa-647 labelled siRNA (red) complexed with AGMA1-10 in the presence of different endocytosis inhibitors. Before siRNA transfection HeLa cells were incubated for 30 min with endocytosis inhibitors. **A.** Untreated cells transfected with siRNA/ AGMA1-10 polyplex. Transfection carried out in the presence of: **B.** Amiloride for macropinocytosis inhibition pathway, **C.** β-cyclodextrin for caveolae dependent endocytosis inhibition, **D.** Dynasore for dynamine dependent endocytosis inhibition. Each image is representative of the transfected cell population. The nucleus is stained with DAPI (blue).

Figure 8. siRNA/AGMA1-10 complex colocalizes with dextran. **A.** Co-localization of Alexa-647 labelled siRNA (red) complexed with AGMA1-10 together with FITC labelled Dextran 10000 kDa (green) as a specific marker for micropinocytosis internalization compartment. HeLa cells have been transfected with 50 nM siRNA

complexed with 50 μg of AGMA1-10 polymer in combination with Dextran in the transfection medium. Bottom right panel represent superimposition of green (Dextran) and red (siRNA) signal, yellow spots indicate endocytic vesicles within the micropinocytosis compartment where the two molecules co-localized. **B.** Western blot analysis of HeLa transiently transfected with 50 nM of siRNA targeting AKT-1 complexed with 50 μg of AGMA1-10 in presence or absence of endocytosis inhibitors.