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Enhancement of siRNA lipid-based vector stability and siRNA integrity in human serum with addition of anionic polymer adjuvant

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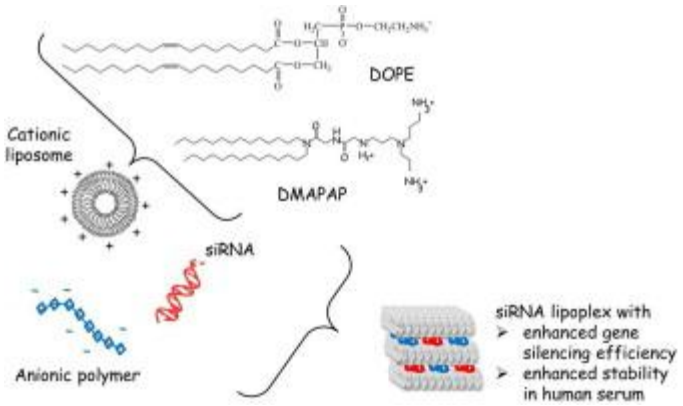
Abstract :

Even though RNA interference is a highly specific technique to selectively silence the expression of any gene, the delivery of RNAi molecules remains a challenge. Recently, we developed a clinically acceptable efficient formulation of siRNA. This delivery system consisted of the cationic lipid 2-{3-[Bis-(3-amino-propyl)-amino]-propylamino}-N-ditetradecyl carbamoyl methyl-acetamide, or DMAPAP, (ii) the neutral lipid 1,2-dioleoyl-sn-glycero-3-phospho-ethanolamine or DOPE and (iii) an anionic polymer that enhances lipoplexes efficiency. We show here that this enhancement of efficiency is due to higher stability of polymer containing-siRNA lipoplexes, leading to longer retention of siRNA integrity. We assayed siRNA integrity upon incubation of siRNA lipoplexes in various biological media using electrophoresis and Fluorescence Correlation Spectroscopy. We show that the addition of anionic polymer provided enhanced stability of incorporated siRNA mainly in saline buffer at room temperature and in human serum at 37 °C and did not interfere with the release of siRNA from lipoplexes in modeled cellular media.

Graphical abstract :



Keywords : siRNA ; Cationic lipid ; Vector ; Integrity ; Fluorescence correlation spectroscopy ; Electrophoresis

1. Introduction

Since its discovery in mammals [1], RNA interference (RNAi) has emerged as one of the promising platforms for therapeutic product development. The advancement of more than 25 therapeutic siRNAs into the clinic illustrates that RNAi-based medicine holds a pivotal place in the future treatment of human diseases [2]. RNAi stakeholders, siRNA, are double stranded RNA oligonucleotides able to induce the silencing of a target gene via RNA interference. The specificity is given by the siRNA sequence. However, there remain several obstacles in their clinical development. One of the greatest challenges in RNAi therapy continues to be the delivery method of the therapeutic siRNA to the target cells [3]. As the site of action of siRNA is cytoplasmic, where the RNAi machinery is housed, there is no requirement to enter nucleus for controlling the expression of target genes. However, naked siRNA molecules have unfavorable physicochemical properties: negative charge that hampers their interaction with the negatively charged cell membrane and instability in biological milieus containing nucleases [4]. Therefore delivery systems are required to protect siRNA from nucleases and to facilitate its access to intracellular sites of action [5]. To date, a large variety of siRNA delivery vectors has been developed, including biodegradable nanoparticles, lipids, bacteria, and attenuated viruses [6]. Major prerequisites for the *in vivo* applicability of siRNAs include favorable blood half-life, adequate tissue bioavailability and efficient transfer across the cell membrane, as well as lack of toxicity. The ideal siRNA vector is able to keep siRNA sheltered from nucleases the entire time the vector is still in the bloodstream or in the extracellular matrix and to release it once it has reached its action site, i.e. the cytoplasm of target cells.

Previously, we developed original siRNA vectors that provide the preservation of siRNA integrity and exhibit high gene silencing efficiency in cultured cells and upon administration to mice, either systematically or locally. In particular, these vectors proved to be efficient in mouse disease models, namely collagen induced arthritis [7-9] and osteosarcoma models [10]. These cationic lipid-based vectors contain, in addition to siRNA, anionic polymers that turned out to enhance the gene silencing efficiency of the vector [11-12], *in vitro* or *in vivo* as well [7]. Interestingly, the addition of anionic polymers to the siRNA vector decreased its cellular toxicity at high siRNA doses or the ability of lipid-based siRNA vectors to induce cytokines expression upon i.v. injection to mouse [13].

So far, the mechanism whereby the addition of anionic polymer into siRNA vector enhances its efficiency is still unknown. Several hypotheses to explain this mechanism might be considered, (i) these vectors could lead to enhanced uptake of siRNA by target cells, or (ii) the particle could be more stable, providing siRNA with a better protection from degradation by enzymes present in biological media, or (iii) the particle could allow an easier release of siRNA in the cytosol of targeted cells.

Here, our objective was finding out whether the addition of anionic polymer to siRNA vector has an impact either on cellular siRNA uptake, or on siRNA integrity when siRNA-containing particles are subjected to incubation in various biological media, including human serum or cell extract. We used electrophoresis and FCS to carry out a quantitative and comparative measurement of the disassembly of siRNA-containing lipoplexes, prepared with or without anionic polymers. Both methods allow quantification of intact siRNA in complex biological media. Maintaining siRNA in intact form, i.e. as a double stranded oligonucleotide, is indeed an absolute prerequisite to keep the molecule active.

2. Materials and methods

2.1. Materials

siRNA (unmodified) specific to luciferase (5' CUU ACG CUG AGU ACU UCG AdTdT 3'), or non-silencing siRNA used as negative control (5' UUC UCC GAA CGU GUC ACG UdTdT 3') were obtained from Eurogentec. TAMRA-labeled double stranded siRNA and TAMRA-labeled single stranded RNA oligonucleotide were obtained from QIAGEN. Anionic polymers sodium salts were obtained from Sigma-Aldrich: sodium poly-L-glutamate (PG; reference P1818), sodium alginate (AA; reference 180947). Triton X100® was provided from Fluka® (Buchs, Suisse).

2.2. Preparation of cationic liposomes and siRNA lipoplexes

Cationic liposomes formed with DMAPAP cationic lipid 2-{3-[Bis-(3-amino-propyl)-amino]-propylamino}-N-ditetradecylcarbamoyl methyl-acetamide (synthesized as in [14], and DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, Avanti Polar Lipids) were prepared as described [11]. siRNA-cationic liposome complexes or lipoplexes were prepared by mixing

an equal volume of the siRNA solution diluted in 150mM NaCl to the cationic liposome suspension prepared also in 150mM NaCl and rapidly mixed by vortexing. When anionic polymer was added, siRNA and polymer solutions were mixed in 150mM NaCl (ratio 1/1 w/w) before adding to cationic liposomes. Lipoplexes were allowed to form for 30min at room temperature before use. The charge ratio was calculated as the molar ratio of positive charges (3 positive charges per molecule of DMAPAP) to the molar ratio of negative charges (from siRNA and anionic polymer molecules, respectively siRNA 3.03, PG 6.58 and AA 5.05 nmoles of negative charges/µg).

In each experiment, the siRNA amount was the same regardless of the assayed formulations, i.e. with or without polymer. The charge ratio of the lipoplexes was also kept constant between formulations since the charge ratio is the key determinant of a lipoplex, especially as regards its efficiency (see Figure 2). Since the added anionic polymers bring anionic charges, lipoplexes prepared with these polymers contained necessarily a higher amount of lipid.

2.3. Gene silencing and lipoplexes uptake in cultured cells

Mouse melanoma (B16-F0, ATCC CRL-6322) cells from LGC Promochem were grown in DMEM with Glutamax (Gibco), 10% fetal calf serum, streptomycin (100µg/ml) and penicillin (100U/ml). B16-Luc cells were obtained and grown as described [11]. Cultures were maintained at 37°C in a 5% CO_2/air incubator.

Gene silencing. B16-Luc cells were seeded onto 24-well plates at 40,000 cells per well the day before transfection. Transfection was performed as described [12].

Uptake. B16 cells were seeded on 96-well culture plates at 10,000 cells/well. Lipoplexes were prepared with TAMRA-labeled siRNA and applied to the cells for 24h at 37°C /5% CO_2. For microscopic analysis, the cells were washed with PBS, and examination on living cells was performed with a Zeiss Axiovert fluorescence microscope (excitation, 546nm; emission, 590nm). For fluorescence measurement, the transfected cells were washed with PBS. Cells were then lysed with Triton X-100 1% in PBS and fluorescence was measured in black 96-microwell plates using a Wallac Victor2 microplate reader (excitation, 546nm; emission, 590nm).

2.4. Preparation of biological media

Human serum. Human serum was purchased from PAA (ref C11-020).

Preparation of cell extract. B16 cells were detached, washed in PBS and pelleted. A volume of pellet (VP) of B16 cells was suspended in 5 VP of lysis buffer (10mM HEPES KOH pH

7.9, 10mM KCl, 1.5 mM $MgCl_2$, 0.5mM PMSF, 1mM DTT) at 4°C. The cells were lysed by sonication and lysates were clarified by centrifugation at 40,000 g at 4°C for 30 min. The volume of the supernatant was measured and mixed with 0.11 of its volume with homogenization buffer (300mM HEPES KOH pH 7.9, 1.4 M KCl, 30 mM $MgCl_2$), the mixture was then centrifuged anew at 100,000 g at 4°C for 1h.

Preparation of microsome suspension. Mouse liver microsome suspension preparation was adapted from [15]. After euthanasia, liver was rapidly removed, placed in ice-cold homogenizing buffer (0.1 M Tris HCl, pH 7.4, 10 mM EDTA, 2mM KCl) and perfused with the same buffer until the effluent was clear. Excess moisture was removed by blotting on paper towels and liver was weighed. Three volumes of ice-cold homogenizing buffer were added and the liver was minced into small pieces with surgical scissors and homogenized with a motor-driven Teflon pestle (Potter type). After centrifugation of the homogenate (12,500 g at 4°C for 15 min), supernatant was ultracentrifuged (105,000 g at 4°C for 70 min). Pellet was suspended in ice-cold pyrophosphate buffer (0.1 M Na pyrophosphate pH 7.4, 10 mM EDTA), homogenized using a hand-held blender and ultracentrifuged (105,000 g at 4°C for 45 min). Finally, supernatant was decanted and pellet was suspended in ice-cold microsome buffer (0.05 M Tris HCl pH 7.5, 10 mM EDTA, 20% glycerol) using a hand-held blender. Total protein concentration of human serum, cell extract and microsome suspension was determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific). The desired protein concentration (1mg/ml) was adjusted with corresponding buffer.

2.5. siRNA integrity assay - Electrophoresis

Lipoplexes were prepared as described above (0.3µg siRNA/10µl), diluted in NaCl 150mM pH7 or pH5 or half diluted in human serum, cell extract or microsome suspension (final siRNA concentration 2µM) and incubated at room temperature (buffer) or 37°C (biological media). At days 0, 3, 7, 10 after incubation two 10µl samples were taken, one of them was treated with 1µl of Triton X100® detergent solution (10%). Both samples (with or without Triton X100) were then mixed with 10µl of buffer (Novex® TBE-Urea sample buffer (2X)) and immediately frozen at -20°C until electrophoresed. Intact siRNA was assessed by gel electrophoresis on 6% acrylamide gels (TBE-Urea, Invitrogen) in TBE buffer at 180 V for 30 min. Gels were incubated in a SYBR® Green II bath (1/10,000) for 15 min and the stained siRNA bands were visualized on an UV trans illuminator and digitalized. Relative band intensities (I) were derived by using Image J to calculate integrated optical density for each band. Results are expressed relative to naked siRNA (designed thereafter as “input”). The

amount of intact released siRNA is given by (1', Figure 1), the amount of intact siRNA remained in particle is given by (2) – (1'). The % of intact siRNA in particles is then given by: 100 x [I(band 2) – I(band 1')] / I(input) while the % of released intact siRNA at any time is given by: 100 x I(band 1') / I(input).

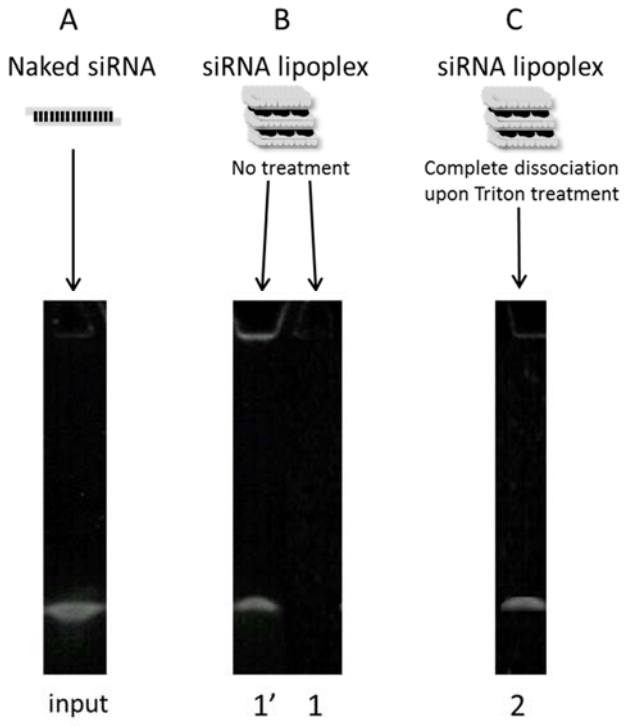


Figure 1*: Diagram of siRNA integrity analysis on acrylamide gel. (A) When analysed by electrophoresis on acrylamide gels, intact naked double stranded siRNA appears as a band. (B) siRNA lipoplexes are loaded on acrylamide gels, (1) if all the siRNAs are complexed in the assayed lipoplexes, no band is detected, (1') whereas a band is detected if some of the siRNA payload leaks out. (C) Treatment of siRNA lipoplexes with Triton X-100 completely dissociates particles, releasing their siRNA payload (2). Band intensity (from the intact band) is then quantified with ImageJ. Results are expressed relative to naked siRNA.*

2.6. siRNA integrity assay – Fluorescence Correlation Spectroscopy

FCS measurements were performed under two-photon excitation (840 nm) on a home-built system using a 100-fs pulse, 80-MHz mode-locked Mai Tai Ti: Sapphire tunable laser (pulse 100 fs) (Spectra Physics, Mountain View, CA) and a Nikon TE2000 inverted microscope [16-17]. Before entering through the epifluorescence port of the microscope, the laser beam was expanded with a two-lens afocal system to fill the back aperture of the objective (Nikon, Plan Apo, ×100, numerical aperture 1.4, oil immersion). The setup was optimized to obtain a diffraction-limited focal spot. Measurements were typically carried out in a 50µl drop of free siRNA or lipoplexes at the concentration of 10 nM siRNA, deposited on a coverslip pretreated

with dimethyldichlorosilane (to prevent nonspecific binding of materials onto the glass coverslip). Fluorescence of TAMRA was collected by the same objective, separated from the excitation by a dichroic mirror (Chroma 700DCSPXR) and further filtered by a Chroma E700SP-2p filter and a bandpass filter (Semrock FF01-593/40) before being focused onto an avalanche photodiode (SPCM-AQR-14; PerkinElmer Life Sciences). The detector was connected to a digital correlator (ALV 6000, ALV-GmbH, Langen, Germany) for calculation of the normalized correlation function G(τ). Recording times were typically 1min40s (average of 5 cycles of 20s). The analysis of G(τ) allowed the determination of the mean residence time (τ_d) of the TAMRA-labeled siRNA to check the integrity of the siRNA. When the fluorescence fluctuation profiles contained bright fluorescence spikes (accounting for the presence of lipoplexes containing multiple TAMRA-labeled siRNA), the spikes were first analytically removed allowing the determination of the τ_d value characterizing the free TAMRA-labeled siRNA from the resulting G'(τ) function. The percentage of intact siRNA in particles were obtained by either (i) the analysis of the basal fluorescence level (before spikes removal) or (ii) the G'(0) value extracted from the corrected autocorrelation function (after spikes removal) by comparing samples treated or not by Triton X-100 (see text in the Results section and legend of Figure 5).

2.7. Statistical analysis

All the data were expressed as mean ± standard deviation (SD) and statistical differences were analyzed using the ANOVA and Student's t-tests. The acceptable level of statistical significance was set at a p of less than 0.05.

3. Results

3.1. Effect of addition of anionic polymer adjuvant to siRNA lipoplexes on their gene silencing efficiency and cellular uptake

We previously reported an efficient siRNA delivery system that is cationic lipid-based and contains anionic polymer as transfection efficiency adjuvant [12]. Luciferase-specific siRNA lipoplexes were prepared with or without anionic polymers and used to transfect luciferase-expressing B16 cells. As shown in Figure 2A, the addition of polyglutamate (PG) or alginate (AA) leads to increased gene silencing efficiency of siRNA lipoplexes, compared to lipoplexes without anionic polymer, with optimal responses obtained for charge ratio of 2 or

4. No efficiency was observed with a non-silencing siRNA (data not shown). Note that siRNA lipoplexes prepared without adjuvant at charge ratio 2, 6 and 8 had no gene silencing efficiency. We next examined whether this enhanced efficiency was due to better uptake of siRNA by cells.

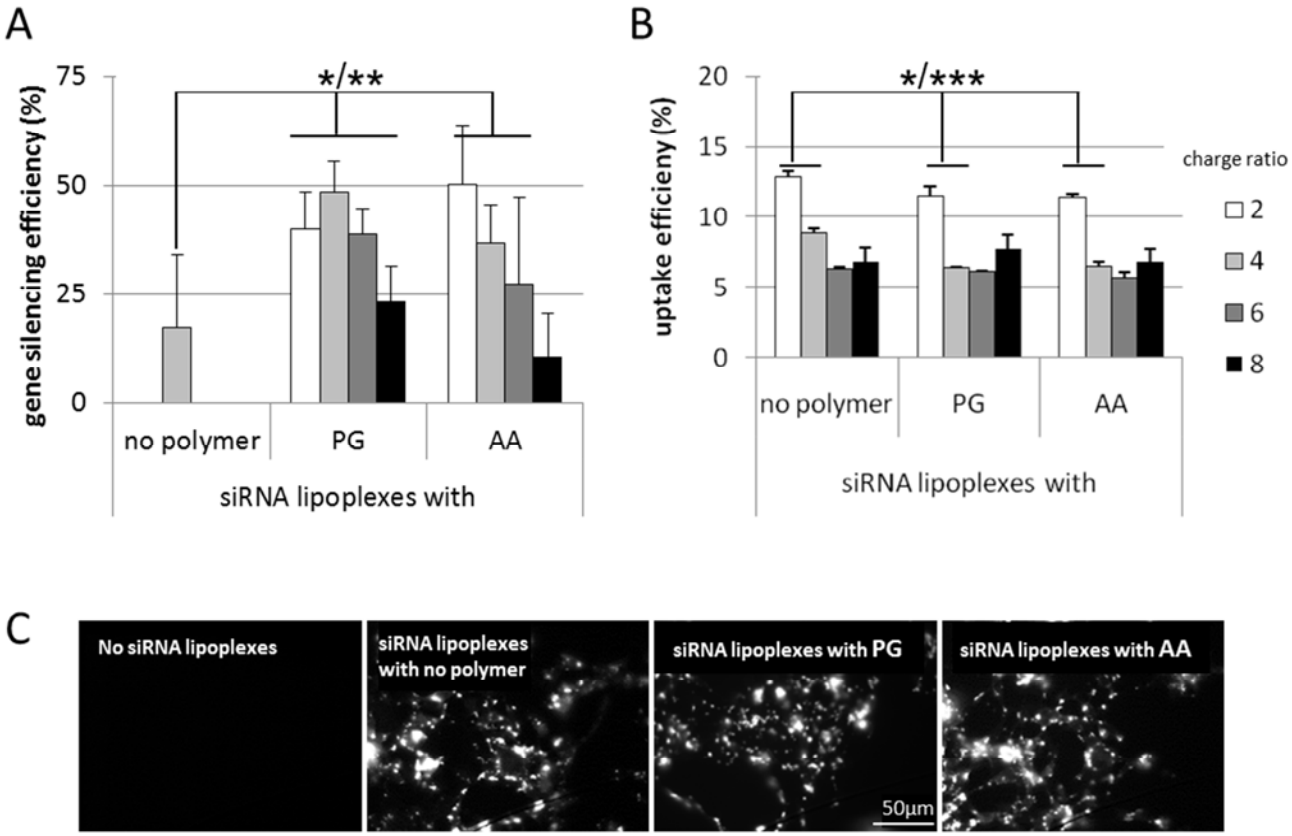


Figure 2*: Comparison of gene silencing and cell uptake efficiencies. siRNA lipoplexes were prepared with siRNA alone (no polymer) or pre associated with polyglutamate (PG) or alginate (AA), and complexed with DMAPAP/DOPE liposome at the indicated charge ratio. (A) Gene silencing efficiencies of siRNA lipoplexes (siRNA specific to luciferase) applied at 20nM of siRNA on B16Luc cells in the presence of fetal calf serum. Results are expressed relative to non-transfected cells. (B) Uptake by B16 cells of fluorescent siRNA lipoplexes, prepared with TAMRA-labeled siRNA and applied to cells at 37°C for 4h. Fluorescence was assayed after extraction with Triton X-100 and expressed as a percentage of added fluorescent lipoplexes. (C) The same fluorescent siRNA lipoplexes (as in B) at charge ratio 4 were applied to cells for 24h at 37°C and after washing with PBS, transfected cells were observed using fluorescence microscopy. Values are the mean ± SEM; n=3;* p<0.05, ** p<0.01, ***p<0.001.*

We chose to use fluorescence to quantify the total amount of siRNA taken up by the transfected cells. However, we know that once complexed in lipoplexes, the intensity of fluorescence emitted by an encapsulated siRNA is rather diminished compared to the same amount of naked siRNA because of the fluorescence quenching observed when fluorophores are highly concentrated (personal data). A way to get rid of fluorescence quenching is to

completely dissociate the lipoplexes using a treatment with Triton X-100 that solubilizes the lipids of the lipoplexes and releases fluorescent siRNA. In this way it is possible to quantify the total amount of fluorescent siRNA taken up by or associated with the cells. We thus transfected cells with TAMRA-labeled siRNA lipoplexes prepared without polymer or with polyglutamate (PG) or alginate (AA), and either quantified fluorescence associated with lysed cells after 4h (Figure 2B) or observed cellular fluorescence pattern of transfected cells with microscopy after 24h (Figure 2C). We found a slightly better uptake for a charge ratio of 2 compared to other charge ratio, regardless of the type of lipoplexes. However no significant difference in fluorescence uptake by cells was observed for the three types of lipoplexes prepared with a charge ratio above 2, neither with the quantitative assay (fluorescence measurement) nor with the qualitative assay (microscopy). Therefore, the addition of anionic polymers in siRNA lipoplexes does not enhance their cellular uptake and the differences in gene silencing efficiency observed – in particular for a charge ratio of 4 – cannot be explained by differences in cellular uptake.

We then hypothesized that siRNA lipoplexes prepared with anionic polymer adjuvant protect better their siRNA payload compared to siRNA lipoplexes without adjuvant. It is recognized that once formulated into lipoplexes, siRNA are protected from nucleases [18]. However, upon injection in bloodstream, the particles are subjected to interactions with various components (sera proteins, cells) that may affect the particle structure and release its siRNA payload, giving access to nucleases. We then investigated siRNA integrity when siRNA lipoplexes prepared with or without anionic polymers were subjected to incubation with various biological media. In order to carry out a quantitative measurement of the integrity of siRNA, we performed electrophoresis on acrylamide gels and fluorescence correlation spectroscopy (FCS) experiments.

3.2. Follow-up of siRNA integrity upon incubation of siRNA lipoplexes in various conditions

We first evaluated the minimum detectable amount of siRNA upon migration on TBE-urea acrylamide gels and subsequent staining with SYBR Green II RNA gel stain, a sensitive nucleic acid gel stain. We obtained a minimal detectable amount around 5ng, below the detection limit for ethidium bromide (10ng). Upon digitalization of the gel we also established the conditions for which the staining signal was proportional to the amount of siRNA. The

integrity of the siRNA was provided by its migration distance. Degradation of siRNA can be detected by the presence of a smear, i.e. several shorter bands and/or the decrease of labeling intensity of the band corresponding to intact siRNA. Throughout this work, we never detected a smear, even with naked siRNA incubated in human serum at 37°C. It has been shown by [19], that the electrophoretic degradation profile of siRNA, namely (i) smear, (ii) distinct smaller bands or (iii) complete disappearance, is dependent of siRNA sequence. The siRNA sequences used in this work probably lead to the third type of profile.

We first incubated siRNA lipoplexes, prepared with or without polymer adjuvant, at room temperature in saline buffer pH 7 for several days. Samples were assayed immediately (D0) or after various times of incubation (3, 7, 10 and 14 days). Half of the sample was treated with Triton X100 before loading on gels in order to release complexed siRNA, whereas the other half was loaded untreated, to determine whether some of the siRNA payload has leaked from the particle. The results are expressed in percentage of intact siRNA remaining in the particle or released from the particle, calculated as explained in Materials and Methods section.

When siRNA lipoplexes were incubated for up to 14 days at room temperature in saline buffer, we observed no apparent release of free intact siRNA (without Triton), regardless of the preparation of the particles (Figure 3A). However, we observed a progressive disappearance of intact siRNA remaining in the particles (with Triton), this disappearance being faster for siRNA lipoplexes prepared without polymer adjuvant (Figure 3A). Indeed, siRNA lipoplexes prepared without polymer had a siRNA half-life of 7 days whereas siRNA lipoplexes containing a polymer adjuvant, regardless of the polymer (PG or AA), exhibited a siRNA half-life greater than 14 days. Note that the half-life of naked siRNA in this incubation condition was 5 days. In these experiments, the disappearance of intact siRNA in particles, while no release of these siRNA can be evidenced, could be related to either (i) a binding of released siRNA to tube walls or (ii) a rapid degradation of siRNA upon its release from the particle leading to inhomogeneous degradation in terms of size, each form being under the limit detection or (iii) a combination of these two phenomena (see discussion). Altogether, our results show that the complexation of siRNA with cationic liposome enhances siRNA stability compared to naked siRNA, whereas addition of polymer adjuvant brings an ever better degree of stability.

In this assay, we compared particles bearing the same charge ratio (+/- 4), since as shown in Figure 2A, charge ratio is a decisive efficiency parameter for siRNA lipoplexes. However, these particles contained various amounts of cationic liposome. Indeed polyglutamate and alginate brings 6.67 and 4.77 nmoles of negative charges/µg, respectively; then particles prepared with PG or AA contained 1.6 or 1.34 times more liposome, respectively, than particles with siRNA alone (without polymer). We thus wonder whether the enhanced stability resulted from a larger amount of lipid added in the particle. We prepared siRNA lipoplexes without polymer containing the same amount of cationic liposome as lipoplexes with polymer PG and 3 times more siRNA to compensate for the final amount of lipid and to keep constant the charge ratio (R+/- = 4). When incubated for several days in saline buffer (pH 7) at room temperature, these siRNA lipoplexes exhibited a siRNA half-life similar to the previous one, i.e. 7 days, showing that the amount of lipid did not influence the siRNA half-life. We concluded that the enhanced stability provided by the addition of polymer adjuvant is probably due to stabilization of the particle allowed by the presence of the adjuvant *per se* and not due to the difference in the amount of lipid.

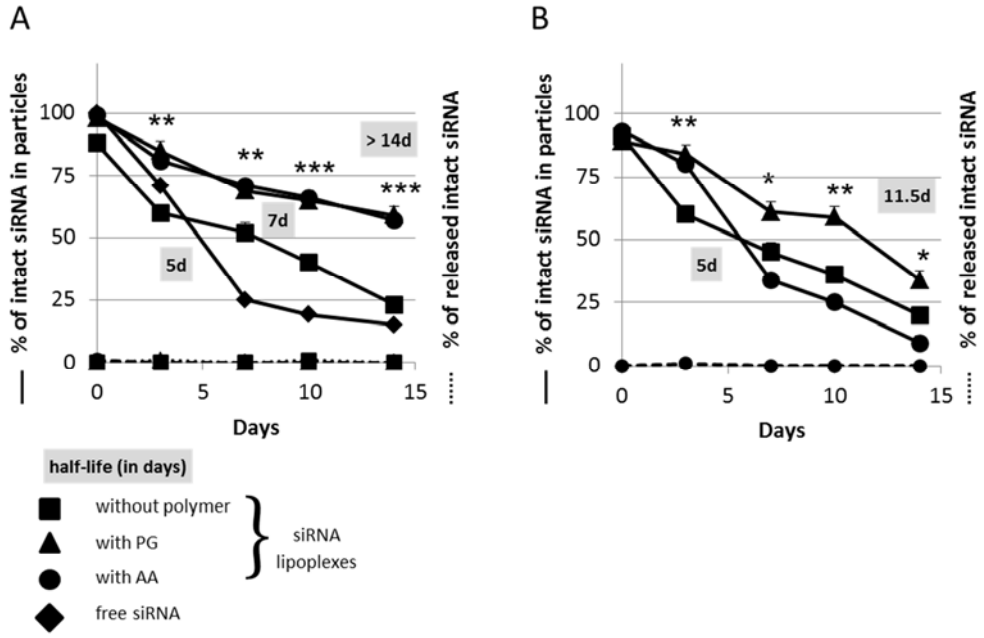


Figure 3*: Follow-up of siRNA integrity when complexed in siRNA lipoplexes in saline buffer using electrophoresis. siRNA lipoplexes prepared without polymer (■), with polyglutamate (PG) (▲), or with alginate (AA) (●) at charge ratio of 4, were incubated for 0, 3, 7, 10 or 14 days at room temperature in buffer pH 7 (A) or pH 5 (B). The percentage of intact siRNA remaining in particles assayed using electrophoresis as described in Figure 1. The percentage of intact siRNA released from particles is indicated by dotted lines. On a grey background, half-life of siRNA in each preparation is indicated. The percentage of intact siRNA incubated as a free molecule (without liposome) was also assayed (♦). Values are the mean ± SEM; n=3; * p<0.05, ** p<0.01, ***p<0.001.*

We next examined whether siRNA lipoplexes were sensitive to pH variations and incubated them in saline buffer pH5 for several days. As previously observed at pH7, no release of siRNA was observed regardless of the time of incubation or the type of siRNA lipoplexes up to D14 (Figure 3B). Moreover, the progressive disappearance of intact siRNA remaining in the particle, already observed at pH7, was also observed and even more pronounced. The stabilization effect of anionic polymers was still observed for PG, but not for AA. Indeed, the siRNA half-life decreased from 14 to 11.5 or 5 days for siRNA lipoplexes prepared with polyglutamate or alginate, respectively, whereas the siRNA half-life in lipoplexes without anionic polymer decreased from 7 to 5 days. The faster dissociation of particles observed for siRNA lipoplexes prepared with alginate can be explained by the hydrolysis of alginate in acidic environment [20]. Therefore in storage conditions (saline buffer, pH7, room temperature), siRNA lipoplexes prepared with anionic polymer adjuvant preserve siRNA integrity more efficiently than lipoplexes without polymer, probably because the particles formed with anionic polymer are more stable.

To find out whether this enhanced preservation of siRNA integrity is also observed in more complex media, we next incubated siRNA lipoplexes prepared with or without polymer adjuvant in various biological media, namely human serum, cell extract and microsome's suspension. These incubation conditions were not chosen to perfectly model the fate of siRNA upon injection *in vivo*. Our goal was rather to compare the stability of lipoplexes prepared with or without polymer to determine whether the addition of polymer brings an enhancement of stability.

To follow up siRNA integrity, siRNA lipoplexes were prepared as described above except that they were twice concentrated in order to obtain the same final siRNA concentration (0.3µg siRNA/sample in 10 µl) upon a twofold dilution in these media. As shown in Figure 4A, when incubated for several days in human serum at 37°C, siRNA lipoplexes prepared without adjuvant polymer released intact siRNA in the incubation medium from day 7; the siRNA half-life in these particles was 5 days. At day 10 there was no more intact siRNA in the particles. On the contrary, no released siRNA was detected for siRNA lipoplexes prepared with either adjuvant polymer, which exhibit a siRNA half-life of 10-11 days. At D10, about 50-60% of intact siRNA remained in the particles. This assay shows that the addition of polymer adjuvant in siRNA lipoplexes led to enhanced protection against dissociation of the

particles induced by serum proteins and consistently preserved siRNA integrity (compared to lipoplexes without adjuvant polymer) as observed in saline buffer pH7.

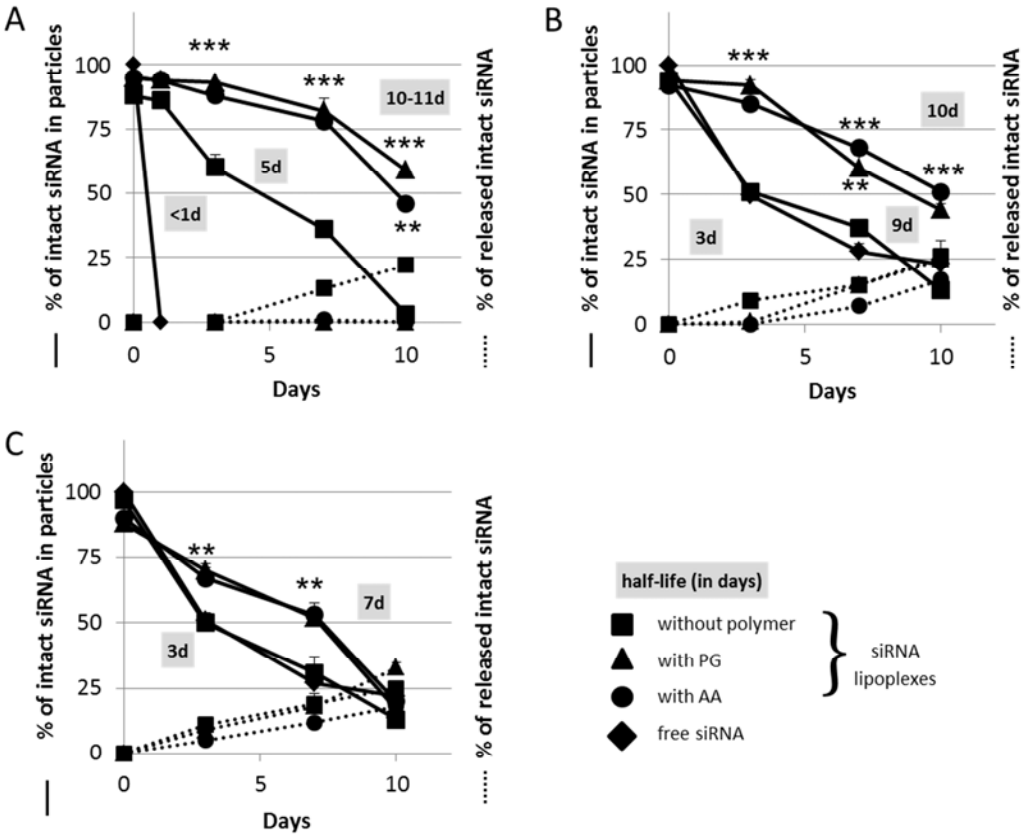


***Figure 4**: Follow-up of siRNA integrity when siRNA lipoplexes were incubated with biological media using electrophoresis. siRNA lipoplexes prepared as in Figure 3 were half-diluted in human serum (A), cell extract (B) or microsome suspension (C) and incubated for 0, 3, 7, or 10 days at 37°C. The percentage of intact siRNA remaining in particles, indicated by solid lines, was assayed using electrophoresis as described in Figure 1. The percentage of intact siRNA released from particles is indicated by dotted lines. The percentage of intact siRNA incubated as a free molecule (without liposome) was also assayed (♦). On a grey background, half-life of siRNA in each preparation is indicated. Note that free siRNA (♦) incubated in human serum is completely degraded in less than 24h. Values are the mean ± SEM, n=3; ** p<0.01, ***p<0.001.*

When incubated in cell extract at 37°C for several days (Figure 4B), siRNA lipoplexes released intact siRNA in the incubation medium regardless of the preparation of the particles, namely with or without adjuvant polymer. The amount of released siRNA is the same with the three tested particles. However, the progressive disappearance of intact siRNA in particles remained slower for siRNA lipoplexes prepared with adjuvant polymers compared with siRNA lipoplexes without polymer, with a siRNA half-life in particles without polymer of 3 days and of 9-10 days in particles with adjuvant polymers. At day 10 about 50% of intact

siRNA remained in the siRNA lipoplexes containing the adjuvant polymers whereas only 25% were detected for particles without adjuvant. Thus, upon interaction with cellular extract the three types of siRNA lipoplexes release equally their siRNA payload, whereas siRNA lipoplexes prepared with anionic polymer adjuvant seems to better protect their siRNA payload than siRNA lipoplexes prepared without polymer.

Last, when incubated in microsome suspension (Figure 4C), 25% of intact siRNA was released in the incubation medium at day 10, depending on the particle. In addition, for the three particles, it remained nearly no intact siRNA in the particles at day 10. Even if the siRNA half-life in siRNA lipoplexes prepared with adjuvant polymers remained higher than the one in particles without adjuvant (7 and 3 days, respectively), siRNA lipoplexes with adjuvant did not bring a better degree of stability to their siRNA payload compared to particles without adjuvant when particles are incubated in microsome suspension.

Taken together, the results obtained with electrophoretic method suggest that the addition of polymer adjuvant to siRNA lipoplexes provided enhanced stability of incorporated siRNA, mainly in saline buffer at room temperature and in human serum at 37°C, and did not interfere with siRNA release in “cell cytoplasm” and “cellular endosome” models. The enhanced stability provided by siRNA lipoplexes with polymer upon incubation in human serum is particularly striking. We then decided to use another technique, also able to evaluate the amount of intact siRNA, in order to confirm this enhanced stability provided by the addition of adjuvant polymer in siRNA lipoplexes, notably upon incubation in human serum.

3.3. Analysis of siRNA integrity in human serum when complexed in siRNA lipoplexes using fluorescence correlation spectroscopy (FCS)

Fluorescence Correlation Spectroscopy measures the translational (Brownian) diffusion of freely diffusing fluorescently labeled molecules, based on the analysis of time-dependent fluorescence intensity fluctuations within a small detection volume (V_d), typically below 1fl. Fluorescence intensity fluctuations are caused by diluted molecules diffusing into and out of the detection volume. It is then possible to extract from the time autocorrelation function, G(t), the average residence time (τ_D), characterizing the average time spent by the labeled molecule in V_d and N, the average number of labeled molecules simultaneously present in V_d, inversely proportional to G(0). However, in the case of TAMRA-labeled siRNA lipoplexes

(charge ratio 4) incubated in human serum, we consistently observed bright fluorescence spikes accounting for the presence of lipoplexes containing multiple siRNA (Figure 5A, trace (2)). These spikes disappeared upon Triton treatment with a concomitant increase in the basal fluorescence intensity (Figure 5A, trace (3)). According to Buyens et al. [21], the concentration of free siRNA can be estimated in a given condition by the direct determination of the basal fluorescence intensity value (basal level of fluorescence counting rate or BCR). Thus the percentage of intact siRNA in lipoplexes (% bound) can be estimated by measuring the BCR values (Figure 5) in absence and presence of Triton and is given by the following formula: % bound = 100X [BCR(trace 3)-BCR(trace 2)]/[BCR(4)-BCR(trace 1)] where BCR(4) is measured using a sample of free naked siRNA (= input) of equivalent concentration as used in experiments with lipoplexes. The percentage of released siRNA from particles is given by: 100X [BCR(trace 2)-BCR(trace 1)] /[BCR(4)-BCR(trace 1)].

Alternatively, by calculating the autocorrelation function G'(t) after removing the spikes from the trace (an example is shown in Figure 5B where the fluorescence fluctuation profile (2') corresponds to the trace (2) after the elimination of spikes), the concentration of free siRNA can also be calculated from G'(0) (G'(0) = 1/N where N is the average number of TAMRA-labeled siRNA in the excitation/detection volume (Vd)). In this way, % bound = 100X [N(trace 3)-N(trace 2')]/[N(4)] where N(4) is measured using a sample of free naked siRNA (= input) of equivalent concentration than used in experiments with lipoplexes and the % of released siRNA from particles is given by: 100X N(trace 2')/N(4).

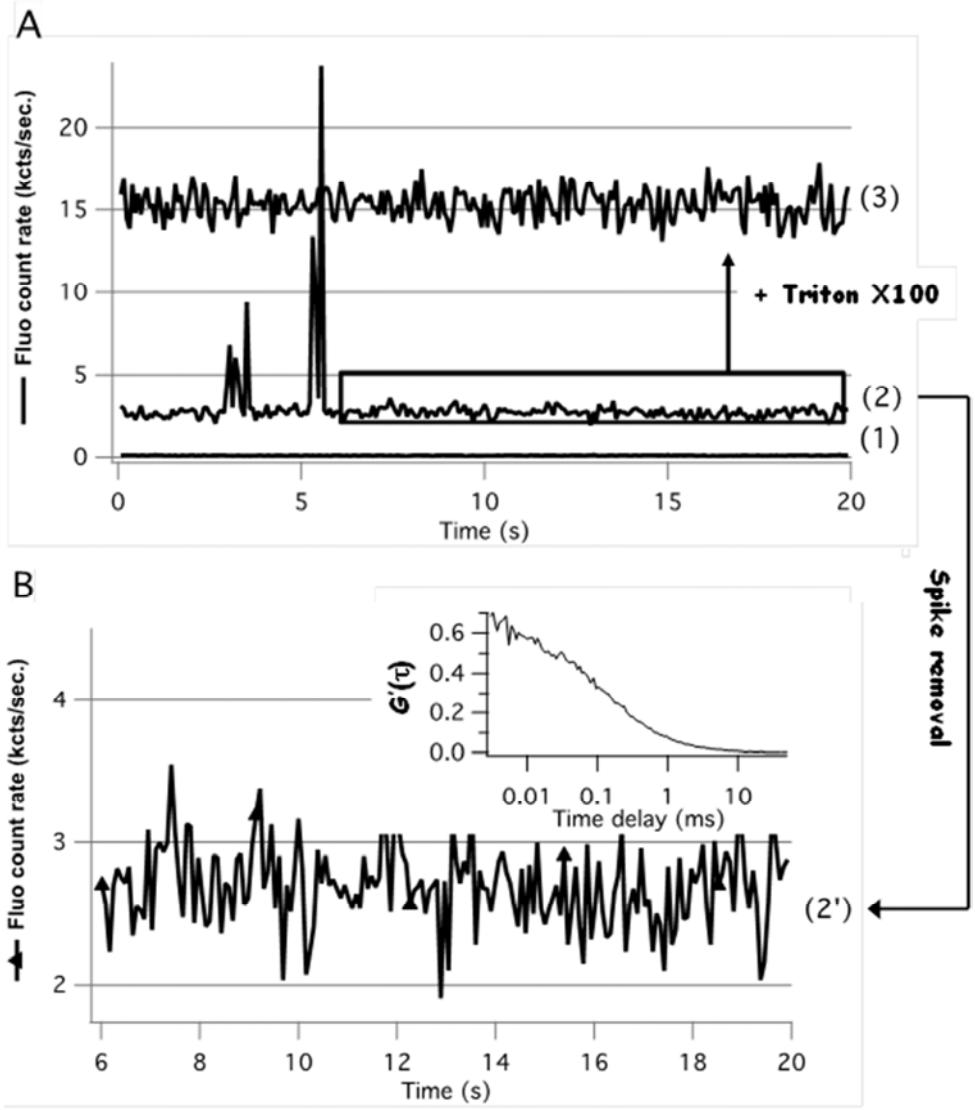


Figure 5*: Principles of the analysis of siRNA integrity when complexed in siRNA lipoplexes using Fluorescence Correlation Spectroscopy (FCS). siRNA lipoplexes were prepared as described in Figure 4, at charge ratio of 4 with TAMRA-labeled siRNA and analyzed by FCS. (A) Examples of fluorescence intensity fluctuation profiles of siRNA lipoplexes before (trace 2) and after (trace 3) Triton treatment (trace 1 corresponds to the "instrumental" baseline). (B) Autocorrelation function analysis. The trace 2' originates from the trace 2 after bright fluorescence spikes removal. The resulting autocorrelation function G'(t) (inset) allows the determination of* τ_D *and G'(0) = 1/N where N is the average number of TAMRA-labeled siRNA in the excitation/detection volume (*V_d*).*

However, before using these two procedures to calculate the percentage of intact siRNA in particles as a function of time, we had to ensure that the detected fluorescent signal was from intact siRNA, especially for samples treated with serum. We first analyzed the τ_D values characterizing the naked TAMRA-labeled siRNA. We were able to distinguish between naked double-stranded siRNA (21nt dsRNA oligonucleotide) and naked single-stranded oligonucleotide (21nt ssRNA oligonucleotide) in saline buffer without cationic liposome based on their respective τ_D values, (0.22 ± 0.01 ms and 0.15 ± 0.01 ms, for siRNA and ssRNA oligonucleotide, respectively). Significant increase of τ_D values was observed in

human serum for viscosity reasons (0.30 ± 0.01 ms and 0.24 ± 0.02 ms for siRNA and ssRNA oligonucleotide, respectively).

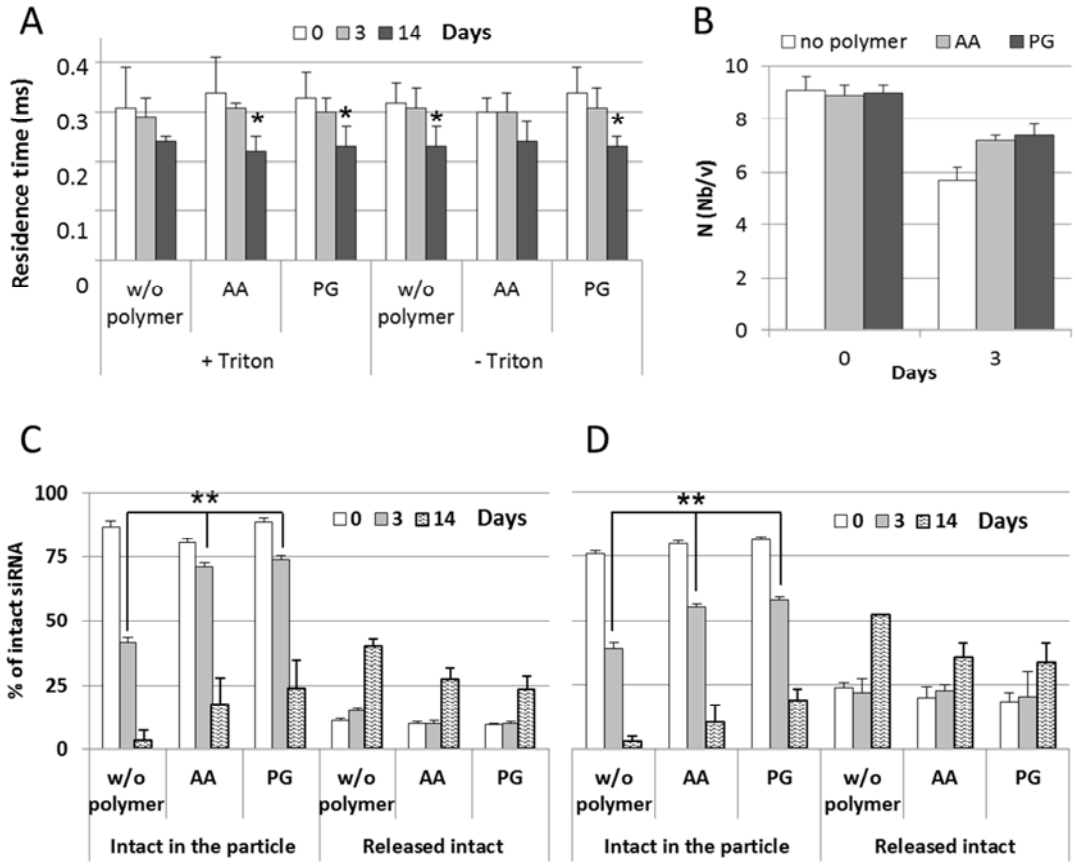


Figure 6*: Follow-up of siRNA integrity when complexed in siRNA lipoplexes in human serum using Fluorescence Correlation Spectroscopy (FCS). siRNA lipoplexes were prepared as described in Figure 4, at charge ratio of 4 with TAMRA-labeled siRNA, incubated in human serum at 37°C for 3 and 14 days and analyzed by FCS. (A) Residence times (τ_D) characterizing free TAMRA-labeled siRNA deduced from autocorrelation functions: G(t) and G'(t) in the presence or absence of Triton, respectively. (B) N, the average number of labeled molecules simultaneously present in the detection volume from autocorrelation functions in the presence of Triton. (C-D) Percentage of intact siRNA remaining in lipoplexes or released as a function of time (0, 3 and 14 days) incubation at 37°C in human serum. The percentage of intact siRNA remaining in particles or released from particles was calculated (C) using BCR or (D) using N (as described in text). The values calculated at D14 are given for information (see explanations in text). Values are mean ± SEM, n=3; **p<0.005, *p<0.05. All the values (BCR, N, τ_D) are reported in Table S1.*

In experiments with TAMRA-labeled siRNA lipoplexes in human serum, the integrity of the free siRNA was derived from the τ_D value calculated in each condition (directly from G(t) or G'(t) in the presence or absence of Triton, respectively). The τ_D values were in accordance with intact siRNA up to day 3 (0.29-0.34ms), regardless of the nature of the lipoplex or the

presence or absence of Triton, whereas the τ_D values obtained at day 14 were significantly lower (0.22-0.24ms) (Figure 6A; see also Table S1). The lower τ_D value at D14 may be interpreted as a mixture of degraded molecules; alternatively, we cannot exclude a de-hybridization (double strand to single strand conversion) concomitant to the dissociation of the siRNA from the particle as this value was similar to the one characterizing ssRNA oligonucleotide. From these results, only values (BCR or N) obtained at shorter times, i.e. at day 0 and 3, can be used to calculate the percentage of intact siRNA remaining in the particle or released siRNA.

For this calculation, we used the two independent procedures described above (Figure 6C-D, respectively). We first observed that serum treatment induced the release of around 10-20% of siRNA even for siRNA lipoplexes prepared with anionic polymer adjuvant, by contrast to what we observed using electrophoretic method. Nevertheless, we still observed a better protection provided by siRNA lipoplexes prepared with polymer to their siRNA payload compared with lipoplexes without polymer. At D3, 60-70% of siRNA was still intact in lipoplexes with polymer whereas were only 40% remained intact in lipoplexes without polymer (Figure 6C-D).

These results confirm the above-mentioned results based on gel-electrophoresis analysis. To note, the N value (corresponding to the average number of TAMRA-labeled siRNA simultaneously present in the detection volume, deduced from the autocorrelation function) of samples treated with Triton significantly decreased from D0 to D3 (Figure 6B), indicating a disappearance of TAMRA-labeled molecule as already observed in gel-electrophoresis experiments. This point will be further discussed in the next section.

4. Discussion

siRNA vector stability can be investigated by incubating it in various biological preparations, mimicking the interactions of the vector with various biological components, and by tracking the integrity of siRNA throughout incubation. Several approaches are available to detect siRNA but only a few allow the detection of intact siRNA and its quantification. Most approaches use electrophoretic methods in agarose gels [22-24] followed or not by Northern

Blot [25-26]. Other methods use fluorescently labeled siRNA like FRET [27-29], FCS [30] or qPCR-based techniques [31].

Here, we first used electrophoretic method to follow up the integrity of siRNA once complexed in lipoplexes treated with various conditions. We first identify the more sensitive stain and verified that the quantification process was accurate. We were able to show that the addition of anionic polymer in siRNA lipoplexes gave more stable particles when incubated in neutral buffer at room temperature or in human serum at 37°C. We also used FCS. In contrast to electrophoresis, FCS does not require any physical separation step and then is able to estimate in a simple way the amount of free/bound RNA species as well as their molecular size; however, unlike electrophoresis FCS requires fluorescent-labeled molecules.

We have shown in this study that the addition of anionic polymer to siRNA lipoplexes enhanced their gene silencing efficiency without modifying their cellular uptake. We then addressed the integrity of siRNA over time upon various treatments of the particles which contained siRNA. Four conditions were chosen for modeling (i) vector storage condition (neutral buffer at room temperature), (ii) injection in blood (human serum), (iii) interaction with cytosol (cellular extract) and (iv) interaction with endosomal membrane (microsome suspension), considered as relevant for characterizing the particles in terms of interactions with different components of biological media.

Using electrophoresis, we were unable to recover 100% of the amount of siRNA used to prepare the siRNA lipoplexes upon treatment with Triton, even when lipoplexes were treated immediately after preparation. Indeed, at Day 0 only 90% of intact siRNA were recovered upon Triton treatment of siRNA lipoplexes without anionic polymer while no siRNA was detectable when untreated lipoplexes were loaded on gel. However, 10% of siRNA corresponds to 30ng, which is far above the minimal detectable amount we determined (5ng). This can be explained either by the inability of Triton treatment to completely dissociate siRNA lipoplexes, or by a decreased staining sensitivity due to the presence of Triton and lipids when the mixture was loaded to gel. On the contrary, using FCS analysis of siRNA lipoplexes without Triton treatment at D0, we observed that 10% of siRNA was not associated to lipoplexes and we recovered 100% of the amount of added siRNA upon Triton treatment. These findings point to (i) enhanced sensitivity of FCS compared to electrophoresis in terms of detection and show that (ii) Triton treatment efficiently dissociates siRNA lipoplexes, but

siRNA is not completely complexed inside the particles. Taking into account that the concentration of siRNA lipoplexes used in FCS is lower than the one used in electrophoresis experiments, we can hypothesize that an equilibrium exists between bound and free siRNA and the low concentrations used in FCS account for an equilibrium displacement toward the free siRNA form.

We performed a follow-up of siRNA integrity once complexed inside the particle in the presence of human serum at 37°C during 14 days. We detected enhanced siRNA integrity in human serum when siRNA lipoplexes were prepared with anionic polymer compared to siRNA lipoplexes without polymer with either gel-electrophoresis or FCS. We also observed with both methods a disappearance of intact siRNA from D0 to D14. However, even if this disappearance could be explained for electrophoresis, since with this method we detect only oligonucleotides above a certain size compatible with RiboGreen binding, such an explanation is not valuable regarding FCS experiments. Indeed, in FCS, only the fluorescent probes (TAMRA), covalently bound at the extremity of one siRNA strand, accounts for the detected signal. Even if siRNA is degraded by nucleases present in human serum, it is unlikely that the emission signal of TAMRA will disappear. Nevertheless, from D0 to D14, we consistently observed a decrease in the basal level of fluorescence (from 17 kHz at D0 to 8 kHz at D14) and, most importantly, in N (from 9 at D0 to 5 at D14) regardless of the type of siRNA lipoplexes. We also verified that the TAMRA fluorescence yield remained the same between single stranded and double stranded RNA TAMRA-labeled oligonucleotides (data not shown). These results suggest that a part of fluorescent siRNA is lost in tubes, by binding of siRNA and/or lipoplexes to tube walls, where the incubation with serum occurred before deposit of the samples onto coverslip. This putative binding to tubes is of concern if long-term storage of siRNA lipoplexes is considered. Lyophilisation rather than storage in suspension would be much better [32].

FCS has been shown by others as a sensitive method for measuring the integrity of siRNA once siRNA lipoplexes were incubated in human serum [21, 30, 33]. In this work, the authors observed a huge dissociation of pegylated siRNA lipoplexes in less than 1h incubation, whereas we still detected around 70% intact siRNA in particles after 3 day-incubation in human serum. The discrepancy between these observations could be explained by the use by Buyens et al [21] of pegylated lipoplexes that did not allow complexation of siRNA inside the complex anymore, but only loose attachment to the surface.

Conclusion

In the present work, our main objective was comparing the siRNA protection brought by siRNA lipoplexes prepared with or without anionic polymer, in order to determine whether their difference in gene silencing efficiency was due to a difference in stability or protection of their siRNA payload. Anionic polymers such as poly-L-glutamate or alginate significantly improved the silencing effect of siRNA. We found that the primary effect of these adjuvant polymers did not concern the uptake step of siRNA into target cells but was rather to ensure better stability of lipoplexes and thus in turn a better protection against degradation of siRNA by nucleases in biological fluids such as human serum. Note that PG appeared to be less sensitive to modulation of physico-chemical environment than AA, in particular to pH variation. Finally, while both polymers improved lipoplexes stability and siRNA integrity in the serum context, they remained compatible with siRNA release in the cytosol as judged by experiments with cell extracts, an interesting profile for further developments and pharmacological applications.

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Table S1: Values of BCR, τ_D and N obtained with FCS analysis.

	siRNA lipoplexes	D0	D3	D14	
No Triton	w/o polymer	2.0 ± 0.1	2.7 ± 0.1	7.2 ± 0.5	BCR (kHz)
		0.32 ± 0.04	0.31 ± 0.04	0.23 ± 0.04	τ_D (ms)
		2.1 ± 0.2	2.0 ± 0.2	4.7 ± 0.3	N (nb/v)
	AA	1.8 ± 0.1	1.8 ± 0.1	4.9 ± 0.8	BCR (kHz)
		0.3 ± 0.03	0.3 ± 0.04	0.24 ± 0.04	τ_D (ms)
		2.0 ± 0.4	2.1 ± 0.1	3.3 ± 0.5	N (nb/v)
	PG	1.7 ± 0.1	1.8 ± 0.2	4.2 ± 0.9	BCR (kHz)
		0.34 ± 0.05	0.31 ± 0.04	0.23 ± 0.02	τ_D (ms)
		1.8 ± 0.1	1.9 ± 0.3	3.1 ± 0.6	N (nb/v)
With Triton	w/o polymer	17.6 ± 0.4	10.1 ± 0.4	7.8 ± 0.7	BCR (kHz)
		0.31 ± 0.08	0.29 ± 0.04	0.24 ± 0.01	τ_D (ms)
		9.1 ± 0.5	5.7 ± 0.5	5.1 ± 0.6	N (nb/v)
	AA	16.2 ± 0.3	14.5 ± 0.3	8.0 ± 1.9	BCR (kHz)
		0.34 ± 0.07	0.31 ± 0.01	0.22 ± 0.03	τ_D (ms)
		8.9 ± 0.4	7.2 ± 0.2	4.4 ± 1.1	N (nb/v)
	PG	17.6 ± 0.3	15.0 ± 0.3	8.5 ± 1.9	BCR (kHz)
		0.33 ± 0.05	0.30 ± 0.03	0.23 ± 0.04	τ_D (ms)
		9.0 ±0.3	7.4 ± 0.4	4.9 ± 1.0	N (nb/v)

Free naked siRNA (input)	17.9 ± 0.43	BCR (kHz)
	0.30 ± 0.01	τ_D (ms)
	9.09 ± 0.1	N (nb/v)