J Membrane Biol (2012) 245:565–571
DOI 10.1007/s00232-012-9468-9

Chemically Modified Oligonucleotide–Increased Stability Negatively Correlates with Its Efficacy Despite Efficient Electrotransfer

Sandrine Pelofy · Justin Teissié · Muriel Golzio · Sophie Chabot

Received: 19 December 2011 / Accepted: 24 June 2012 / Published online: 15 July 2012

Abstract Despite great potential for disease treatment, small interfering RNA (siRNA) development has been hampered due to its poor stability and the lack of efficient delivery method. To overcome the sensitivity, new generations of chemically modified oligonucleotides have been developed such as the locked nucleic acid (LNA). LNA substitution in an siRNA sequence (siLNA) is supposed to increase its stability and its affinity for its complementary sequence. The purpose of this study was to evaluate the potential benefit of an anti-GFP siLNA using the biophysical delivery method electropermeabilization. We used two types of electrical conditions: electrochemotherapy (ECT), a condition for efficient transfer of small molecules in clinics, and electrogenotherapy (EGT), a condition for efficient transfer of macromolecules. We first confirmed that siLNA was indeed more stable in mouse serum than unmodified siRNA. After determining the ECT and EGT optimal electrical parameters for a human colorectal carcinoma cell line (HCT-116) expressing eGFP, we showed that modifications of siRNA do not interfere with electrotransfer efficiency. However, despite its higher stability and its high electrotransfer efficacy, siLNA was less efficient for eGFP silencing compared to the electrotransferred, unmodified siRNA regardless of the electrical conditions used. Our study highlighted the care that is needed when designing chemically modified oligonucleotides.

Keywords Electropermeabilization · Electroporation · Gene silencing · siRNA · siLNA · Locked nucleic acid · Chemically modified oligonucleotide

Introduction

RNA interference (RNAi) is a natural process that allows gene silencing at the transcriptional level (Fire et al. 1998). Short interfering RNAs (siRNAs) are doubled-stranded, noncoding RNAs that, once introduced into cells, are loaded into the cytoplasmic RNA–induced silencing complex (RISC). The complex binds targeted RNA messenger (mRNA), leading to its cleavage (Moazed 2009). Therefore, siRNA offers the possibility to silence the expression of any pathological protein in a specific way. However, its clinical success has been hampered by its poor cellular uptake and stability. To overcome these problems, progress has been made in developing new technologies to optimize the siRNA chemistry, on the one hand, and to achieve its effective delivery, on the other hand (Whitehead et al. 2009).

Electropermeabilization (EP) is a physical delivery method which consists of the application of controlled electric field pulses to induce cell permeabilization (Teissie et al. 2011). Since the first report in 2002 (Hu et al. 2002), numerous publications have demonstrated the potency of this technique for siRNA delivery (Calegari et al. 2002; Pekarik et al. 2003; Golzio et al. 2007; Lewis et al. 2002; Matsuda and Cepko 2004; Paganin-Gioanni et al. 2008). Recently, we demonstrated by time-lapse microscopy that siRNA electrotransfer depends on both the

M. Golzio and S. Chabot contributed equally to this study.

S. Pelofy · J. Teissié · M. Golzio (✉) · S. Chabot
CNRS (Centre National de la Recherche Scientifique),
IPBS (Institut de Pharmacologie et de Biologie Structurale),
BP 64182, 205 route de Narbonne, 31077 Toulouse, France
e-mail: muriel.golzio@ipbs.fr

S. Pelofy · J. Teissié · M. Golzio · S. Chabot
IPBS, Université Paul Sabatier de Toulouse, 31077 Toulouse, France

permeabilization of the cell membrane and the electrophoretic drag of the negatively charged oligonucleotide (Paganin-Gioanni et al. 2011). In addition, we showed that, contrary to most of the carrier systems (Decuzzi and Ferrari 2008), EP allows siRNA to enter directly into the cell, avoiding endolysosomal compartmentalization and keeping it localized in the cytoplasm, where its enzymatic machinery and mRNA target are located (Paganin-Gioanni et al. 2011). Therefore, EP is particularly well adapted for siRNA use. Several electric settings have been described in the literature as efficiently electrotransferring siRNA. Basically, two major electric conditions exist for EP supporting oligonucleotide delivery. The first one, electrochemotherapy (ECT), is commonly used in clinics and mainly allows transfer of small hydrophilic molecules (Mir et al. 1997). It corresponds to pulse duration in the microsecond range. The second electric condition is electrogenotherapy (EGT) and usually leads to a more efficient plasmid DNA electrotransfer. It is based on the application of pulses with duration in the millisecond range (Aihara and Miyazaki 1998; Rols and Teissie 1998).

Despite efficient siRNA electrodelivery, its silencing effect is transient and short (25 days for electrotransferred muscle and 2–4 days for in vitro and in vivo electrotransferred tumor cells) (Golzio et al. 2005, 2007; Paganin-Gioanni et al. 2008). The transient effect of siRNA could be mainly explained by its low biostability as it is quickly degraded by extra- and intracellular ribonucleases (Layzer et al. 2004; Raemdonck et al. 2006).

To address this problem and improve siRNA potency and efficacy, approaches based on the introduction of chemical modifications in its sequence have been developed. Locked nucleic acid (LNA) containing a methylene bridge that connects the 2′-carbon of the ribose with the 4′-carbon (Kumar et al. 1998) is the third generation of such nucleotide analogues. Due to its methylene bridge, the sugar moiety is conformationally locked in an RNA/mimicking C3′endo/N-type conformation that preorganizes the base for hybridization (Petersen et al. 2000). Consequently, oligonucleotides containing LNA nucleotides exhibit very high thermal stability when hybridized with its RNA target molecule (Braasch and Corey 2001) and improved mismatch discrimination compared to unmodified oligonucleotides (Kaur et al. 2007). In addition, oligonucleotides containing LNA nucleotides are highly resistant to nuclease degradation (Crinelli et al. 2002) and display low toxicity in biological systems (Wahlestedt et al. 2000). Therefore, LNA-modified siRNA (siLNA) appears to be a promising tool for siRNA therapeutic application.

The goal of the present study was to evaluate the potential benefit of siLNA compared to unmodified siRNA using the EP technique. Electrotransfer efficiency was quantified using cyanine 5 (Cy5)–labeled oligonucleotides. To analyze electrotransferred oligonucleotide silencing potency, we targeted enhanced green fluorescent protein (eGFP) because it allows a simple quantitative readout for gene-specific knockdown over time. We first confirmed that LNA substitutions in the oligonucleotide sequence increased half-life in serum. After determining the optimal electrical parameters, we showed that siLNA and siRNA were equivalently electrotransferred using Cy5-labeled oligonucleotides. However, we observed that electrotransferred siLNA was less efficient for eGFP silencing than electrotransferred siRNA in vitro. Our study highlighted the care that is needed when designing chemically modified oligonucleotides.

Materials and Methods

Cell Culture

HCT-GFP cells are HCT-116 cells (human colorectal carcinoma) expressing eGFP that have been transferred by retroviral transduction. HCT-GFP cells were maintained in DMEM, 4.5 g/l of D-glucose, L-glutamine, 110 mg/l of sodium pyruvate (GIBCO-Invitrogen, Grand Island, NY), containing 1 % antibiotic penicillin/streptavidin (GIBCO-Invitrogen) supplemented with 10 % inactivated fetal calf serum (Boehringer Ingelheim, Paris, France) in a 5 % CO_2 humidified incubator at 37 °C.

Oligonucleotides

Cy5-labeled and unlabeled siRNA and siLNA directed against eGFP mRNA were purchased from Qiagen Xeragon (Germantown, MD) and Ribotask (Odense, Denmark), respectively. The siLNA sequences were, sense, 5′-GcaagcugacccugaaguucaTT-3′ and, antisense, 5′-gaacuucagggucagcuugcCG-3′ (LNA nucleotides are depicted in uppercase letters). The position of LNA was chosen with respect to previous results (Mook et al. 2007). The same exact sequences were used for siRNA as previously described (Caplen et al. 2001). To determine oligonucleotide uptake, Cy5-labeled siRNA (Cy5-siRNA) and Cy5-siLNA were used. Labeling of siRNA does not modify its global negative charge.

Stability Test

We incubated siRNA or siLNA (10 µM) at 37 °C in 30 µl of PBS or in 30 µl of fresh mouse serum. Aliquots of 4 µl were withdrawn after 1, 2, 3, 4 and 7 days of incubation and stored at −20 °C until used. Samples were analyzed on 2 % agarose gel. Oligonucleotides were visualized with ethidium bromide (Sigma-Aldrich, St. Louis, MO) staining.

Electropermeabilization

Cell suspension (5×10^5) in 100 µl of pulsing buffer (10 mM phosphate, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4) containing 100 µM of propidium iodide (Sigma-Aldrich) was placed in an electropulsation chamber, which was designed using stainless-steel parallel plate electrodes (10 mm length, 0.5 mm thickness and 4 mm interelectrode distance; IGEA, Carpi, Italy) brought into contact with the bottom of a 35-mm Petri dish (Nunc, Roskilde, Denmark). Electropulsation was operated using the S20u electropulsator (βtech, l'Union, France), which delivered square-wave electric pulses. The βtech monitored pulse shape online on its integrated display. A uniform electric field was generated when the voltage pulse was delivered (Gehl et al. 1999). Pulses with controlled duration and frequency of 1 Hz were applied at preset electric field intensities at room temperature (25 °C). Cells were analyzed by flow cytometry using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ) to determine the percentage of permeablized cells (i.e., propidium iodide–positive cells). A minimum of 10^4 events were acquired on the FL-2 channel and analyzed with Cellquest software (Becton Dickinson).

Cell Viability Analysis

Cell viability was determined by the ability of cells to grow and divide over 24 h (Gabriel and Teissie 1995). Viability was measured by counting cells by crystal violet staining (Merck, Darmstadt, Germany). Briefly, cells were stained with 1 ml crystal violet (0.1 % in pulsing buffer) for 20 min, rinsed with PBS and then lysed with 500 µl acetic acid (10 %) for 5 min. Cell density was evaluated by 595 nm OD measurement. Nonpulsed cells were referred to as 100 % viable cells.

Confocal Fluorescence Microscopy

For fluorescence microscopy, cells (8×10^4) were seeded on a glass coverslip chamber (Nalge Nunc, Illkirch, France) overnight at 37 °C in a humidified atmosphere with 5 % CO_2. The electropulsation chamber was designed using two stainless-steel parallel rods (1 mm diameter, 10 mm length, 5 mm interelectrode distance) that were connected to the electropulsator. The chamber was set on the stage of an inverted confocal microscope (Zeiss LSM510; Carl Zeiss MicroImaging, Göttingen, Germany) equipped with a 40 × Zeiss objective (1.3 numerical aperture, oil immersion). Adherent cells were then electrotransfected in 500 µl of pulsing buffer in the presence of 250 nM (final) Cy5-labeled siLNA using the following electrical parameters: 300 V/cm, 10 pulses of 5 ms, 1 Hz. Cy5 was visualized using a 633-nm laser (emission filter 640–710 nm). Eight-bit images were recorded with Zeiss LSM510 software (EMBL, Heidelberg, Germany). LSM images were processed with ImageJ software (NIH, Bethesda, MD) as described in Paganin-Gioanni et al. (2011).

Electrotransfer of Oligonucleotides

Cells were pulsed or not in the presence of oligonucleotides (2 µg), as described above, using optimal electric parameters as determined by cell permeabilization and viability analysis. After 5-min incubation at room temperature, cells were analyzed by flow cytometry or recultivated in 35-mm Petri dishes, supplemented with 2 ml of DMEM and then incubated for 24 h to 7 days at 37 °C under 5 % CO_2. To determine oligonucleotide uptake, the percentage of $Cy5^+$ cells was quantified (FL-4 channel). To determine the efficiency of electrotransferred oligonucleotides, the percentage of cells expressing eGFP was analyzed (FL-1 channel).

Statistical Analyses

Quantitative data (presented as means ± SD) were analyzed with GraphPad (San Diego, CA) Prism 4 software. Before carrying out statistical tests, we determined whether the data were normally distributed and evaluated their variance. We then carried out appropriate tests as indicated. We report the actual P value for each test. For in vitro time course experiments, we used two-way ANOVA with the Bonferroni post hoc test. $P < 0.05$ was considered statistically significant.

Results

Oligonucleotide Stability

We first compared siRNA and siLNA stability. For that purpose, oligonucleotides were incubated at 37 °C in saline buffer or in fresh mouse serum. In PBS, both oligonucleotides were detectable for at least 7 days (Fig. 1a). However, in serum, siRNA was undetectable within 2 days of incubation, while siLNA was detectable for 3 days (Fig. 1b). Our result confirmed that siLNA was more stable in mouse serum than the unmodified siRNA.

Optimal Electric Parameter Determination

EP is modulated by the electric parameters used and may vary as a function of cell type. Therefore, we determined the optimal electric parameters (i.e., high transfection level in accordance with good cell viability) for HCT-GFP cells

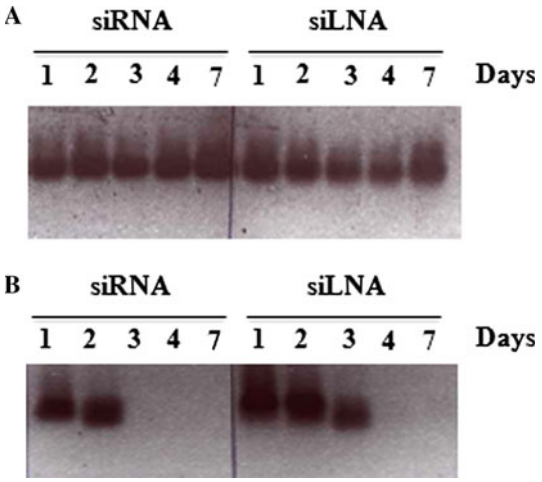

Fig. 1 Oligonucleotide stability. SiRNA and siLNA were incubated at 37 °C in PBS (**a**) or in fresh mouse serum (**b**) for the indicated times. Oligonucleotide electrophoresis was run on an agarose gel and visualized with ethidium bromide staining. This gel is representative of three independent experiments

for the two main EP conditions. Permeabilization was analyzed by entry of propidium iodide. Cell viability was analyzed 24 h later by crystal violet staining.

In both conditions, an increase of EP was induced by an increase of the field intensity. On the contrary, cell viability decreased as the field intensity increased. In ECT condition, we obtained 81 ± 5 % of viable cells for 84 ± 1 % of permeabilized cells at 1,500 V/cm. Thus, the optimal electric parameter (eight pulses of 100 µs duration with a field intensity of 1,500 V/cm at 1 Hz frequency) led to 65 ± 7 % of viable and permeabilized HCT-GFP cells (Fig. 2a). Under the EGT condition, we obtained 71 ± 7 % of viable cells for 71 ± 6 % of permeabilized cells at 600 V/cm. Optimal electric parameters (10 pulses of 5 ms duration at 1 Hz frequency and a field intensity of 600 V/cm) led to 42 ± 10 % of viable and permeabilized HCT-GFP cells (Fig. 2b). The percentage of viable permeabilized cells was determined under the assumption that dead cells must have been permeabilized: [permeabilization (%) + viability (%)] − 100 (Gabriel and Teissie 1997).

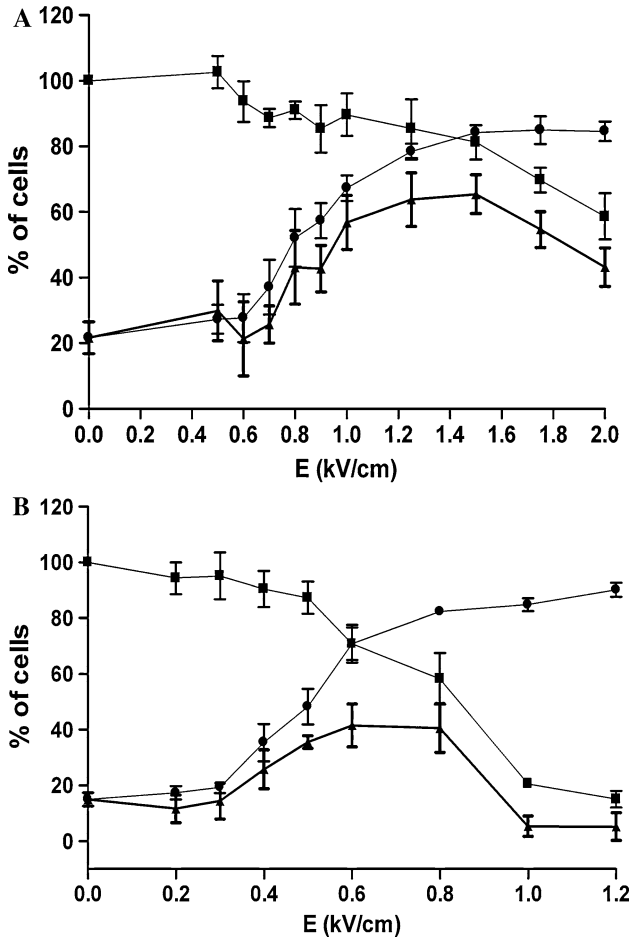

Fig. 2 Optimal electric parameter determination. Percentages of viable cells and permeabilized cells were plotted as a function of the electric field intensity according to (**a**) ECT condition (eight pulses of 100 µs duration at 1 Hz) and (**b**) EGT condition (10 pulses of 5 ms duration at 1 Hz). Permeabilization was assayed by propidium iodide entry into cells and analyzed by flow cytometry (*filled circle*). Cell viability was determined 24 h after EP by crystal violet staining (*filled square*). Values are means ± SD of three experiments

Oligonucleotide Electrotransfer Efficiency

We first visualized the electrotransferred siLNA at the single-cell level, as we previously did for siRNA (Paganin-Gioanni et al. 2011). We observed that, just after pulse application, siLNA is dispersed in the cytoplasm of the electrotransfected cells (Fig. 3a) in the same way as siRNA (Paganin-Gioanni et al. 2011). We then compared siRNA and siLNA electrotransfer efficiency in ECT (1,500 V/cm, 8 × 100 µs, 1 Hz) and EGT (600 V/cm, 10 × 5 ms, 1 Hz) conditions, using Cy5-labeled oligonucleotides. A nonsignificant 10 % augmentation of $Cy5^+$ cells was observed for EGT condition (Fig. 3b), and the cell-associated Cy5 fluorescence intensity was doubled for this electrical condition (Fig. 3c). In ECT condition, 67 ± 7 and 73 ± 6 % of cells were $Cy5^+$ when siRNA or siLNA was present in the pulsing buffer, respectively (Fig. 3b). In EGT conditions, we observed that 79 ± 5 and 83 ± 4 % of cells were $Cy5^+$ when pulsed in the presence of siRNA or siLNA, respectively (Fig. 3b). No significant difference was observed between the two oligonucleotides (Fig. 3b, c). Both electrical conditions led to efficient oligonucleotide electrotransfer, with similar efficiencies for both constructs.

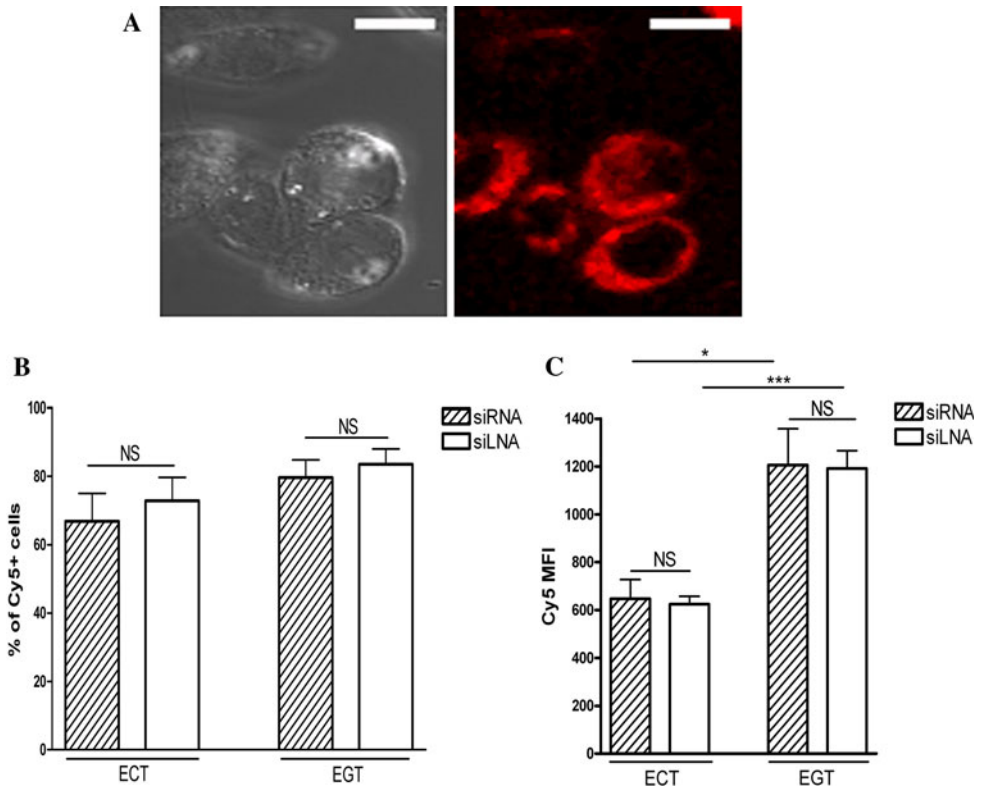

Fig. 3 Oligonucleotide electrotransfer efficiency. **a** Visualization of Cy5-siLNA electrotransfer by confocal fluorescence microscopy. Plated cells were pulsed on a coverslip under a confocal microscope in EGT (300 V/cm, 10 pulses of 5 ms, 1 Hz) condition in the presence of Cy5-siLNA (250 nM). Acquisition was performed after pulse application. *Left* Light transmission acquisition. *Right* Cy5 fluorescence acquisition. Scale bar = 10 µm. **b**, **c** HCT-GFP cells were pulsed in the presence of Cy5-siRNA or Cy5-siLNA using ECT condition (eight pulses of 100 µs duration, 1,500 V/cm at 1 Hz) and EGT condition (10 pulses of 5 ms duration, 600 V/cm at 1 Hz). The percentage of Cy5-positive cells was determined by flow cytometry a few minutes after the electrotransfer (**b**), and the cell-associated Cy5 mean fluorescence intensity was determined (**c**). Values are means ± SD of three experiments. *NS* nonsignificant; $*P = 0.0175$, $***P = 0.0004$ (Student's *t*-test)

Silencing Efficacy of Electrotransferred Oligonucleotides

Finally, we compared the silencing efficacy of electrotransferred siRNA and siLNA under ECT and EGT conditions. For both conditions, maximal eGFP silencing was observed at day 3 for the electrotransferred siRNA and at day 2 for the electrotransferred siLNA (Fig. 4). Using the ECT condition, we obtained 46 ± 4 and 66 ± 3 % of GFP^+ cells with electrotransferred siRNA and siLNA, respectively (Fig. 4a). In the EGT condition, we obtained 38 ± 6 and 63 ± 4 % of GFP^+ cells with electrotransferred siRNA and siLNA, respectively (Fig. 4b). One week after oligonucleotide electrotransfer, the percentage of eGFP-expressing cells returned to its control value. In summary, we observed a transient decreased eGFP expression with both electrotransferred anti-eGFP oligonucleotides, this effect being larger under the EGT conditions. However, in both electric conditions, electrotransferred siLNA led to a significantly weaker time effect of eGFP silencing compared to the unmodified, electrotransferred siRNA ($P < 0.001$, two-way ANOVA). In conclusion, electrotransferred siLNA was around 20 % less effective and its maximal silencing effect was shorter (2 vs. 3 days) compared to unmodified, electrotransferred siRNA.

Discussion

LNA nucleotides are proposed to offer a high resistance to nucleases. We indeed observed that siLNA was more stable in mouse serum than unmodified siRNA (Fig. 1). We then determined the optimal electric parameters (i.e., high transfection level in accordance with good cell viability) for HCT-GFP electrotransfer as follows: eight pulses of 100 µs duration at 1,500 V/cm for ECT condition and 10 pulses of 5 ms duration at 600 V/cm for EGT condition (Fig. 2). We observed for both oligonucleotides a significant increase in the cell-associated Cy5 fluorescence intensity with EGT parameters compared to ECT (Fig. 3c). This increase could be explained by the electric drift,

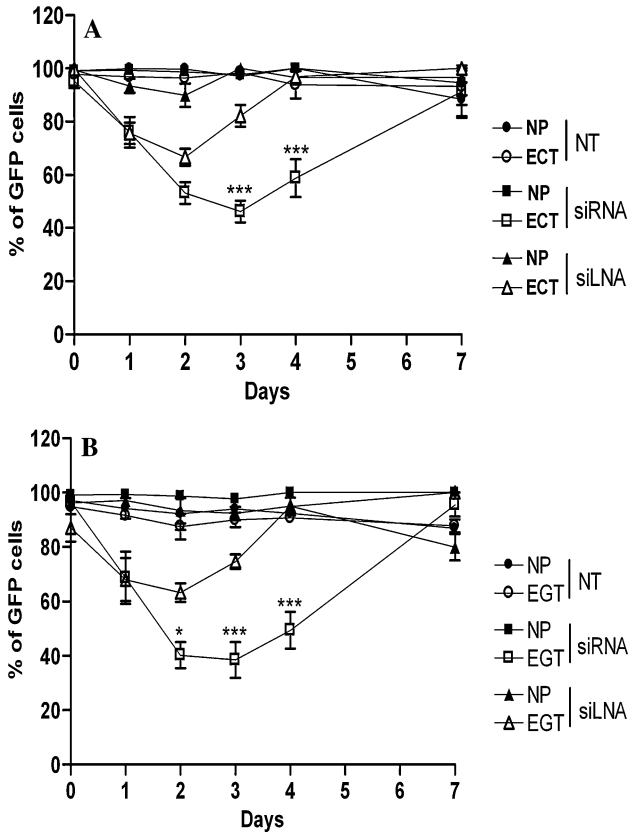

Fig. 4 Silencing efficiency of electrotransferred oligonucleotides. HCT-GFP cells were pulsed or not (*NP*) in the presence of siRNA or siLNA or not untreated (*NT*) using (**a**) the optimal ECT condition (eight pulses of 1.5 kV/cm, 100 µs, 1 Hz) and (**b**) the optimal EGT condition (10 pulses of 0.6 kV/cm, 5 ms, 1 Hz). The percentage of eGFP-positive cells was analyzed over time by flow cytometry. Values are means ± SD of three experiments. $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$ (two-way ANOVA)

which is more important with EGT parameters compared to ECT parameters. In consequence, electrophoretic movement of the electrotransferred, negatively charged oligonucleotide during the pulse is more important and therefore could lead to its more efficient uptake. We further showed that siRNA and siLNA were equivalently electrotransferred, although a slight but nonsignificant increase was observed with siLNA in both ECT and EGT conditions (Fig. 3b). Despite these positive results, electrotransferred siLNA was less efficient for eGFP silencing regardless of the electric conditions that were used (Fig. 4).

The positioning and number of LNA nucleotides in the oligonucleotide sequence are crucial for siLNA activity (Kauppinen et al. 2006). As internal modification in the antisense strand may hamper siLNA activity (Elmen et al. 2005), we designed a gapmers endblock siLNA, meaning that LNA substitutions were located at the extremities of the oligonucleotides. The 3′-end LNA modifications in both the sense and antisense strands have been described as increasing oligonucleotide stability (Elmen et al. 2005), as confirmed by our result (Fig. 1). A limitation in siRNA use is that both strands might be incorporated into the RISC. Loading of the sense strand leads to unwanted off-target effects. LNA substitution could also be useful in this context as LNA modification in the sense strand 5′ end favors loading of the antisense strand. In fact, the thermodynamic stabilities of the 5′ ends of the two siRNA strands in the duplex determine the identity of the guide strand and the passenger strands (Schwarz et al. 2003). The guide strand directs the silencing, whereas the passenger strand is ultimately destroyed. Considering these data, the 5′ end of the antisense strand of our siLNA was devoid of LNA nucleotides, and LNA substitutions were added in the 5′ end of the sense strand. This design was thought to increase the binding energy of the sense 5′ base pair, preventing its loading into the RISC. In addition, the 5′ end LNA in the sense strand impaired nucleotide phosphorylation and, thus, RISC activation (Kaur et al. 2007). Thus, according to published data, our construct should encourage antisense strand loading and increase eGFP silencing. This was not supported by the experiments; we found that electrotransferred siLNA was less efficient for eGFP silencing (Fig. 4). More surprising, despite an enhanced serum half-life, the siLNA effect was less sustained compared to unmodified siRNA. Thus, the lowered siLNA efficacy was not compensated for by its increased stability, contrasting with previous suggestions (Mook et al. 2007). The lowered siLNA efficacy may be due to its less efficient recruitment by the RISC complex and/or its low processing as suggested by Bramsen et al. (2007). An interesting alternative will be the use of small internally segmented interfering RNAs (Bramsen et al. 2007).

It is noteworthy that the EGT condition that led to a slightly better cellular uptake of both oligonucleotides was associated with a better oligonucleotide eGFP silencing efficacy.

To conclude, we demonstrated that increased stability and efficient electrotransfer did not correlate with chemically modified oligonucleotide gene silencing efficacy.

Acknowledgement This work was supported by grants of the Ligue contre le Cancer, CNRS and FP7 Oncomirs (Grant 201102). Flow cytometry was performed at the TRI (funded by the region Midi-Pyrénées, the communauté du Grand Toulouse and the FEDER) platform at the IPBS. We thank Bettina Couderc (Institut National de la Santé et de la Recherche Médicale U563, Toulouse) for providing the HCT-GFP cell lines.

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