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ULTRAFAST DETERMINATION OF THE STABILITY OF siRNA COMPLEXES IN SERUM AT THE NANOMOLAR LEVEL

Kevin Buyens, Stefaan C. De Smedt, Joseph Demeester and Niek N. Sanders

Laboratory of General Biochemistry and Physical Pharmacy, Department of Pharmaceutics, Ghent University
Harelbekestraat 72, 9000 Ghent, Belgium

Introduction

In literature, gel electrophoresis is considered the golden standard for determining the stability of naked siRNA and siRNA-carrier complexes. However, this technique is hampered by several disadvantages that delay the development of therapeutic siRNA-carrier complexes. First, it is a time-consuming technique. Second, its applicability in complex media like full serum is not straightforward. Third, it is difficult to determine the amount of siRNA in the bands on the gel. Fourth, siRNA that becomes completely degraded after dissociation will not be detected. Moreover, this technique is not suitable to determine the physicochemical properties of siRNA-carriers at low concentrations that occur *in vivo* after e.g. intravenous application. To overcome the above-mentioned shortcomings, we recently developed a fast analytical tool that allows us to characterize siRNA-carrier complexes at nanomolar concentrations in complex media like serum.

Materials and methods

siRNA-carrier complexes

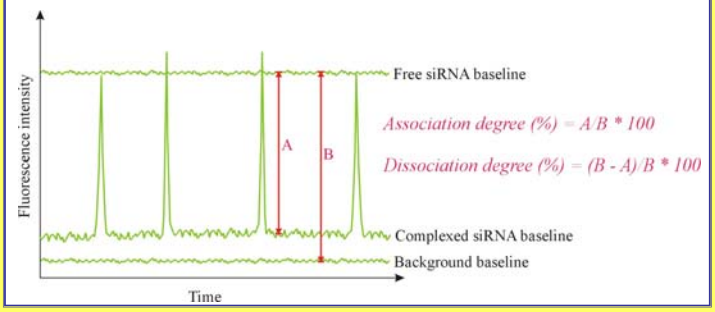
siRNA-liposome complexes were prepared in Hepes buffer (20mM, pH 7.4) by adding Alexa-488 labelled siRNA to extruded cationic DOTAP/DOPE (D/D) (1/1 mol ratio) liposomes or to D/D liposomes that contained 5 mol% DSPE-PEG2000. The siRNA and cationic liposomes were mixed at a +/- ratio (ratio of the total number of positive charges over the total number of negative charges) of 1 and 10.

Fluorescence Fluctuation Spectroscopy (FFS)

FFS monitors fluorescence intensity fluctuations in the small excitation volume of a confocal microscope (Fig 1). These fluorescence fluctuations are due to the diffusion of fluorescent molecules in and out of the excitation volume. Free fluorescent siRNAs give rise to small fluorescence fluctuations and a baseline intensity that is proportional to the siRNA concentration. In contrast, the baseline of siRNA-liposome complexes is much lower. Indeed, most of the siRNAs are bound in the complexes. This generates high fluorescent peaks that originate from the movement of siRNA-liposome complexes bearing many fluorescent siRNAs, through the confocal volume. By using the fluorescence intensity of the baselines one can quantify the amount of dissociated siRNA (Fig 1).

The dissociation of the siRNA-carrier complexes was determined in Hepes buffer and 90% Fetal Bovine Serum (FBS) at a final siRNA concentration of 20nM by recording the fluorescence fluctuation profile for 30s.

Figure 1. Schematic outline of the calculation of the association and degradation degree of siRNA-complexes based on an FFS profile



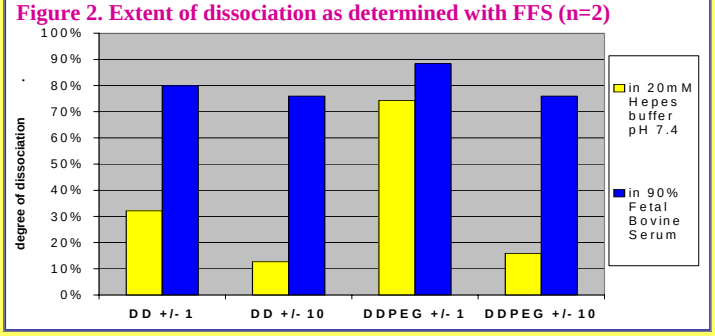
Gel electrophoresis

Non-denaturing Polyacrylamide Gel Electrophoresis (PAGE) was performed in 20% gels loaded with 0.2 µg of free or complexed siRNA per lane. siRNA was visualized with SYBR Green after 2hr of electrophoresis at 100V.

Results and discussion

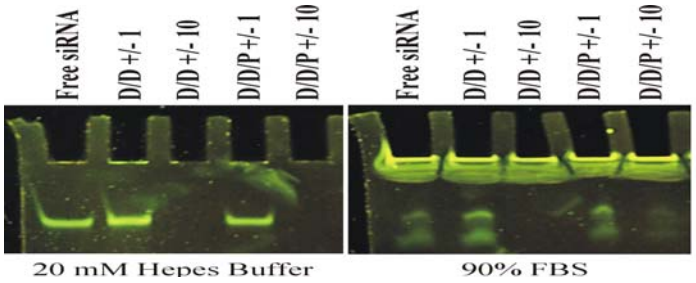
The dissociation of the siRNA-carrier complexes in buffer and 90% FBS as determined with FFS is shown in Fig 2. In buffer, about 90% of the siRNA is bound to the liposomes when a +/- ratio of 10 was used. At a +/- ratio of 1, 70% of the siRNA was complexed to the D/D liposomes, while the pegylated liposomes (D/D/P) could only bind 30% of the siRNA. The presence of polyethylene glycol (PEG) seems to hinder the binding of the siRNAs.

Figure 2. Extent of dissociation as determined with FFS (n=2)



Subsequently, the dissociation of the siRNA-complexes was studied via non-denaturing PAGE. To obtain an acceptable amount of staining a minimum concentration of 600 nM siRNA, i.e. 30-fold higher than the concentration expected in *in vivo* experiments, is required. Fig 3 shows, as in Fig 2, that a much better complexation of siRNA at the highest +/- ratios. But a quantitative analysis is hard to achieve. In contrast to the FFS method, no free siRNA was detected in siRNA-liposome complexes in HB made at a +/- of 10 (Fig 3, left). Additionally, no or small amounts of free siRNA were observed when respectively non-pegylated and pegylated siRNA-liposomes (+/- 10), were incubated with 90% FBS (Fig 3, right). The different dissociation behaviour observed via FFS and non-denaturing PAGE is most likely due to the higher concentrations of siRNA-complexes and the less sensitive detection of free siRNA in the non-denaturing PAGE experiments.

Figure 3. Complexation experiment with non-denaturing PAGE



Conclusions

As summarized in Table 1 we can conclude FFS is a faster, cheaper and less labour-intensive method than PAGE for determining the stability of siRNA-carrier complexes. Moreover, it is able to generate a sensitive quantification of stability at realistic *in vivo* concentrations, even in complex media like serum.

Table 1. Comparison of PAGE and FFS for determining the stability of siRNA-complexes

	PAGE	FFS
siRNA concentration	600 nM	20 nM or lower
Measurement time	> 2 hours	30 seconds/sample
Quantification	Difficult	Easy
Sensitivity	Moderate	High
Serum compatibility	Not straightforward	Good