FLEth RNA Intercalating Probe Is a Convenient Reporter for Small Interfering RNAs

Ingrid M. van der Wiel,[†] Jenny Cheng,[†] Roger Koukiekolo,[†] Rodney K. Lyn,[‡,†] Nathan Stevens,[§]
Naphtali O'Connor,[§] Nicholas J. Turro,[§] and John Paul Pezacki*,[‡,†]

*Steacie Institute for Molecular Sciences, National Research Council, 100 Sussex Drive, Ottawa, ON,
Canada K1A 0R6, Department of Chemistry, University of Ottawa, 10 Marie Curie, Ottawa, ON,
Canada K1N 6N5, and Department of Chemistry, Columbia University, New York, New York 10027*

Received April 2, 2009; E-mail: John.Pezacki@nrc-cnrc.gc.ca

Small noncoding RNAs play important roles within the eukaryotic cell including templating RNA degradation, inhibition of mRNA translation, and accelerating deadenylation of transcripts.[1] Small noncoding RNAs are usually exogenously introduced, derived from the RNA silencing pathway from pathogen[2−5] or endogenously expressed noncoding genes that give rise to double stranded (ds) RNA intermediates.[6] The latter are converted to small RNAs by the RNase III enzyme Dicer.[1,5,7−10] These small RNAs are generally characterized by their short length (21−25 nt), 2 nt, 3′ overhanging ends and 5′ phosphate groups. The RNA-induced silencing complex or RISC then binds to and unwinds the duplex small RNA and uses one strand to target complementary RNAs.[1,5,7−10] Small interfering RNAs (siRNAs) give rise to the degradation of the complementary strands of RNA and represent important genomic tools for understanding gene function by silencing genes of interest. They also represent novel therapeutics in that they can silence genes associated with human diseases. Herein, we have examined the ability of the small molecule probe FLEth,[11] a small molecule that intercalates with dsRNA and undergoes internal fluorescence resonance energy transfer (FRET), to bind to and report on duplex siRNA (Scheme 1). We have established novel methods based on FLEth for the study of protein−siRNA interactions and the tracking of siRNA entry into mammalian cells. The FLEth probe contains a fluorescein moiety coupled to an ethidium bromide-like intercalator.[11] It is a novel reporter of dsRNA because it can be used to track the FLEth molecule by monitoring fluorescein emission at 520 nm, but the emission from the intercalator at 600 nm is only observed when FLEth is complexed with dsRNA (Scheme 1). Because it retains the fluorescent properties of the fluorescein donor, it can simultaneously be used as both a whole cell marker and a probe for duplex RNA in cellular imaging. To test whether FLEth could serve as a probe for siRNA, we first sought to determine whether FLEth binds to 21 nt duplex siRNAs.

Scheme 1. Interaction of FLEth and siRNA and the FRET That Only Occurs When FLEth Is Bound and Intercalating into the siRNA Duplex
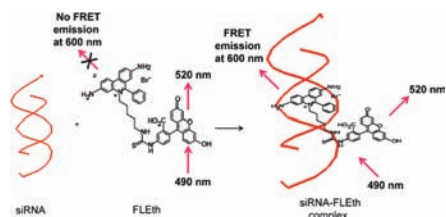
We conducted binding experiments between FLEth and duplex siRNA using a fixed concentration of 1 μM 21 nt duplex siRNA

† Steacie Institute for Molecular Sciences, NRC.
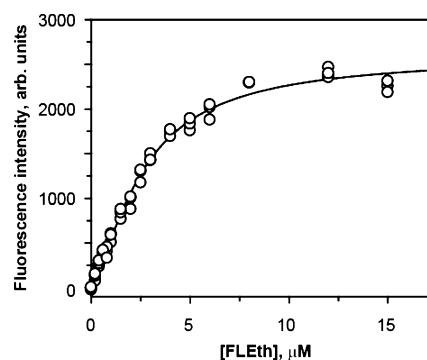‡ University of Ottawa.
§ Columbia University.

Figure 1. Plot of fluorescence intensity arising from FRET (F_{600}) versus FLEth concentration conducted at rt in phosphate buffer, pH 7.4, $I = 0.1$ M. The data were fit to an Adair cooperative binding equation (2 sites) indicating that more than one FLEth molecule binds to each siRNA.

targeting the human CSK gene[12,13] in the presence of various concentrations of FLEth in phosphate buffer (Figure 1). Triplicate experiments were conducted in an opaque 96-well plate with excitation and emission wavelengths of 490 and 600 nm, respectively. FRET fluorescence at 600 nm was detected immediately upon addition of FLEth to CSK duplex siRNA (Figure 1). We observed that the interaction of FLEth with CSK siRNA did not follow a simple one site binding model. Instead the reaction stoichiometry and data analyses indicated that at least two molecules of FLEth bind to each molecule of 21 nt siRNA. Moreover, we observe that the binding of the first FLEth molecule influences the binding of the second, probably by altering the conformation of the siRNA duplex, so that they do not have equivalent binding constants. The data fit best to a two site binding model with two binding constants as shown in eq 1, where F_{600} is the fluorescence signal at 600 nm, $C_{binding}$ is the binding capacity of the siRNA, [FLEth] is the FLEth concentration, and K_d^1 and K_d^2 are the dissociation constants for the two binding sites for FLEth in duplex CSK siRNA.

$$F_{600} = C_{binding} \frac{\left(1 + \frac{2 \times [FLEth]}{K_d^2}\right) \times \left(\frac{[FLEth]}{K_d^1}\right)}{2 \times \left(1 + \left(1 + \frac{[FLEth]}{K_d^2}\right) \times \left(\frac{[FLEth]}{K_d^1}\right)\right)} \quad (1)$$

We determined K_d^1 and K_d^2 to be 1.2 and 4.0×10^{-6} M and $C_{binding}$ to be 2635 arbitrary units of fluorescence. Similar results were obtained for another siRNA targeting the firefly luciferase gene.

To determine whether FLEth can report on siRNA that is bound to endogenous proteins in living systems, we conducted competition studies with a viral suppressor of RNA silencing,[14] specifically the p19 protein of the Carnation Italian ringspot virus (CIRV).[12,15−18] Binding studies involving varying concentrations of duplex CSK

siRNA that was preincubated with FLEth (1:4 ratio of siRNA:FLEth) with CIRV p19 were performed as previously described.[12,15−18] No binding of the preassociated FLEth·siRNA complex with p19 was observed. Likewise, binding experiments performed by varying concentrations of the p19 protein preincubated with siRNA (1 h, varying ratio of p19:siRNA) with constant excess concentrations of FLEth yielded little or no binding of FLEth to the p19:siRNA complex. This suggests that the ternary complex involving p19·FLEth·siRNA is high in energy and does not form under the experimental conditions tested (Figure 2). However, FLEth was a good reporter of unbound siRNA and thus could be used to measure p19−siRNA interactions by following the consumption of duplex siRNA. Experiments performed by incubating various concentrations of CIRV p19 with the CSK siRNA·FLEth complex for 2 h at room temperature yielded concentration-dependent changes in FLEth FRET emission. A dissociation constant for the p19−siRNA interaction of $K_d' = 200$ nM (Figure 2) was derived from these data, which is in accordance with values previously determined.[12,15,17] This establishes FLEth as a convenient probe for siRNA−protein interactions.
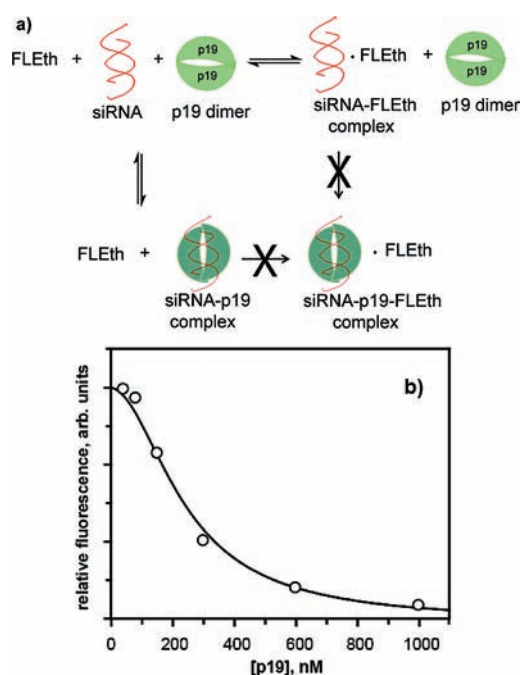

Figure 2. (a) Equilibrium (thermodynamic) cycle for the binding interactions of siRNA, FLEth, and the viral suppressor of RNA silencing protein p19 (dimeric). Neither FLEth can bind to the siRNA−p19 complex nor can p19 bind to the siRNA−FLEth complex so that the trimeric complex involving siRNA, p19, and FLEth does not form to any appreciable extent. (b) Plot of the relative fluorescence intensity (measured at F_{600}, corrected for background) versus p19 concentration with the data fit to a competitive binding model.

To determine whether FLEth can bind siRNAs in living cells we prepared 2:1 complexes of FLEth (900 nM) and CSK duplex siRNA (450 nM). These complexes were then encapsulated in liposomes. The liposomes displayed high levels of fluorescence at 600 nm indicating that the siRNA−FLEth complexes were stable within the liposomes (details provided in the Supporting Information). This suggests that FLEth may be compatible with other modern and elegant approaches for siRNA delivery.[19−21] Huh 7.5 human hepatoma cells were treated with siRNA−FLEth complexes and imaged. Cellular imaging showed that the FRET signal from FLEth is retained in the FLEth−siRNA liposomes, but as the siRNA was delivered to the cells the FLEth was found in the nucleoli (Figure 3) of the treated cells which is where FLEth stains these cells in control experiments with no siRNA present as seen in previous studies.[11] The delivery of siRNA was confirmed

using confocal microscopy with fluorescently tagged CSK siRNA. FLEth likely rapidly dissociates from siRNA once in the cell. This is consistent with its relatively low binding affinity. FLEth FRET signals were not observed in distinct regions in the cytoplasm where components of the RNA silencing machinery are known to be present (Figure 3b).[4,6] One possible explanation is that FLEth strongly prefers to bind to uncomplexed RNA over those directly associated with other biomolecules, as seen with our studies with p19. In conclusion, we demonstrate here that FLEth is a convenient tool for studying siRNA and establish a number of novel methods for which FLEth can be generally applied.

Figure 3. (a) Huh7.5 cells transfected with 300 nM (4 μg) CSK siRNA, 600 nM FLEth, 8 μL Lipofectamine, Red: RNA-bound FLEth, Green: total FLEth. (b) Huh7.5 cells transfected with 300 nM Dy547-labeled CSK siRNA, 8 μL of Lipofectamine, pink: Dy547 with the cell and nuclear boundaries traced in white. (c) Huh7.5 mock transfected cells, 8 μL of Lipofectamine (overlay of normalized images from the 3 channels). (d) Huh7.5 cells stained with FLEth (10 μM FLEth, 24 h incubation), Red: RNA-bound FLEth, Green: total FLEth. Scale bar represents 10 μm.

Supporting Information Available: Additional binding data are available as are complete experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA902636M