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MicroRNA Detection by DNA-Mediated Liposome Fusion

Coline Jumeaux, Olov Wahlsten, Stephan Block, Eunjung Kim, Rona Chandrawati, Philip D. Howes, Fredrik Höök,* and Molly M. Stevens*

Abstract: Membrane fusion is a process of fundamental importance in biological systems, involving highly selective recognition mechanisms for trafficking of molecular and ionic cargos. Mimicking natural membrane fusion mechanisms for the purpose of biosensor development holds great potential for amplified detection, since relatively few highly discriminating targets lead to fusion and an accompanied engagement of a large payload of signal-generating molecules. In this work, we use sequence specific DNA-mediated liposome fusion for highly selective detection of microRNA. We demonstrate detection of miR-29a, a known flu biomarker, down to 18 nM within 30 minutes with high specificity using a standard laboratory microplate reader. Further, we demonstrate one order of magnitude improvement in the limit of detection by using a novel imaging technique combined with an intensity fluctuation analysis, coined 2-color fluorescence correlation microscopy.

Detection of target molecules in a specific and sensitive manner is of critical importance for the development of efficient disease diagnostic devices. Nature is a great source of inspiration for the design of such platforms, as over the course of evolution highly sensitive and specific sensing or signaling processes have emerged, using refined components made of only a few molecular building blocks.¹ One of these mechanisms is the fusion of lipid bilayers, an essential process allowing the transfer of chemicals through an otherwise impervious barrier,^{2,3} facilitating inter- and intracellular communication.⁴ In the case of neuronal fusion, where Ca²⁺-triggered neurotransmitter release into the synaptic cleft from a vesicle occurs at sub-millisecond timescale,⁵ complex molecular machinery facilitates this highly regulated process, and proteins belonging to the SNARE family (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) have emerged as the key components facilitating the molecular recognition and fusion of bilayers.^{6–8}

The increased understanding of biological processes and of the design principles underlying the recognition enables the progression of the field of biomimetic materials design, which harnesses the power and efficiency of natural processes,

combined with biological building blocks (for example DNA, RNA, peptides, proteins, lipids) to create hierarchically organized materials, and the development of a new generation of sensors.¹

Membrane fusion has been extensively studied using model systems such as proteoliposomes, in which native fusogenic proteins are exposed on the surface of artificial lipid vesicles (liposomes).^{9–14} Another approach involves the design of biologically-inspired constructs to trigger liposome docking and membrane fusion. Simplified synthetic analogues are an excellent tool to increase the understanding of the fusion process at the atomistic level, and each segment of the synthetic construct can be varied to study its role in the occurrence of fusion.² Synthetic analogues enabling specific molecular recognition used to trigger membrane fusion include coiled-coil forming peptides,^{3,15} and DNA.^{16–19} Cholesterol-terminated double-stranded DNA (dsDNA) has been shown to facilitate efficient self-insertion into liposomes,²⁰ and also that their hybridization, if designed to occur in a zipper-like fashion, can induce liposome-liposome fusion (Figure S1).^{18,19} DNA, thanks to its high selectivity in sequence-guided self-assembly, has great potential for biosensing applications. Attractive properties of liposomes, such as ease of functionalization and biocompatibility, endow them with promising applications in biomedical and biotechnology fields.^{21–23} Further, with relatively few targets leading to fusion and engagement of a large payload of signaling molecules (for example FRET-active dyes), it is reasonable to assume that target-mediated liposome fusion could lead to highly amplified detection. Inspired by this reasoning, we have engineered a sensing platform for detecting a clinically relevant biomarker for influenza virus infection,²⁴ microRNA-29a (miR-29a), in a highly specific and sensitive manner by DNA-mediated liposome membrane fusion. This work, conjointly with a recent report demonstrating the possibility to trigger peptide nucleic acid-mediated liposome fusion with oligonucleotides,²⁵ constitute the first examples of biomarker-triggered liposome fusion.

In this work, we encapsulated Förster Resonance Energy Transfer (FRET) donor-acceptor pairs, Dil and DiD (Figure S2), in the membrane of DNA-functionalized liposomes. In this assay, liposomes containing either Dil or DiD FRET pairs in the membrane are functionalized with self-inserting, cholesterol-terminated dsDNA (Figure 1). A hairpin DNA (H, green strand in Figure 1) is designed to block the sticky end of dsDNA A/B (ds-A/B), preventing their hybridization with dsDNA C/D (ds-C/D) and inhibiting liposome docking and fusion. The hairpin DNA is also specially designed to include a region containing a complementary sequence with miR-29a. Hence, in the presence of target miRNA, hybridization with hairpin DNA reveals the sticky end of ds-A/B, which can then hybridize with ds-C/D, initializing liposome docking and membrane fusion. This is then detected by an increase in FRET signal. The reported liposome fusion mechanism for oligonucleotides detection presents several key advantages in its experimental design. It is a homogeneous assay, operated at room temperature, and involving relatively few experimental steps. Notably, other liposome-based assays for oligonucleotide detection require

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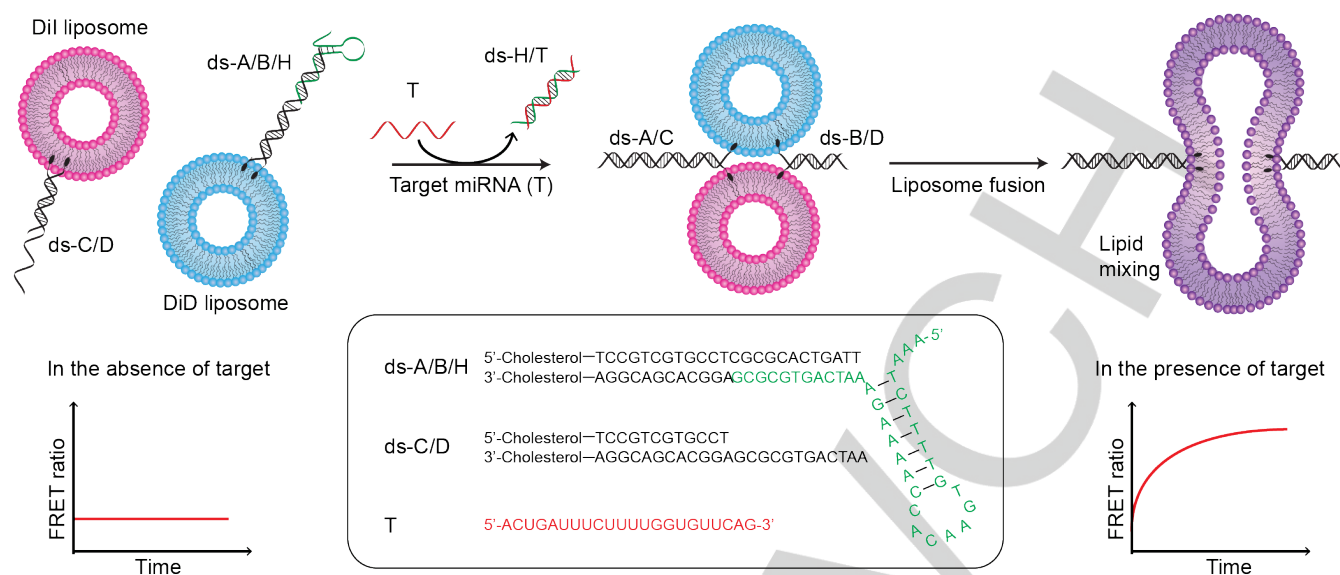


Figure 1. Schematic for miRNA detection based on promotion of DNA-mediated liposome fusion. Target miRNA (red strand) is complementary to the hairpin (green strand) and hybridizes to it; this displacement reveals the sticky end of ds-A/B. In the presence of target miRNA, liposome fusion is promoted by ds-A/B and ds-C/D hybridizing in a zipper-like manner, resulting in an increase in FRET signal. In the absence of target miRNA, liposome fusion is inhibited, as hairpin DNA stays hybridized on ds-A/B, and no FRET increase is observed.

the destruction of liposomes by the addition of membrane-disrupting agents.^{26,27} Here, similarly to assays developed by Jakobsen *et al*, the readout is obtained without the need for additional separation, amplification, and washing steps, which has great potential for further developing this assay towards point-of-care applications.^{28,29}

In this assay, schematically illustrated in Figure 1, the hairpin DNA strand (H) is designed to hybridize on the sticky end of a duplex and to be displaced in the presence of target miRNA. This novel mechanism of a dual-function hairpin DNA is an advantageous strategy as it offers a unique site for target hybridization, which is independent from the zippering regions; therefore its design is universal and can be tailored to adapt to a broad spectrum of target biomarkers. It is crucial to optimize the design of H, to meet the following three requirements: (i) the unbound H must maintain its structural stem-loop feature (not a random coil structure), and have at least one arm of the stem that is part of the recognition sequences, (ii) H must be strongly hybridized to ds-A/B in absence of target, preventing the hybridization of A with C and further unzipping of the dsDNAs, and (iii) H must be displaced only by the specific target miRNA (T), revealing the sticky end of ds-A/B, which will initiate liposome docking by hybridizing with ds-C/D.

We designed three hairpin structures, H₆, H₇ and H₁₃ (sequences in Table S1). The optimization of the hairpin design is summarized in Section S5 (SI). Briefly, first we visualized the predicted secondary structures formed by the hybridization of H, A, and B (Figure S3) using algorithms provided in the NUPACK software,³⁰ which also showed that out of the three predicted constructs, ds-A/B/H₇ had the highest probability of formation (Table S2). Furthermore, the hybridization and displacement of H in solution were characterized using native PAGE analysis (Figure S4). We demonstrated that only H₇ showed both efficient

hybridization with ds-A/B and displacement by T, therefore H₇ was chosen as the hairpin structure for the liposome fusion assay.

Following selection of H₇, we translated the hairpin strand displacement mechanism in the presence of target miR-29a into a liposome fusion assay. A majority of the methods for detecting membrane fusion are based on fluorescence generation or FRET, and careful choice of reporter assay allows selective discrimination between lipid mixing (inner or outer lipid leaflet) and content mixing.³¹ To maximize signal generation during the assay, we selected a lipid-mixing assay to report fusion events, as studies have shown that a high level of lipid mixing can occur with a limited degree of content mixing.¹⁷ Dil and DiD FRET pairs are based respectively on Cy3 and Cy5 cyanine dyes (Figure S2), and contain long alkyl chains rendering them highly lipophilic; therefore they will be included in the membrane of liposomes. Two populations of liposomes containing either Dil or DiD were mixed; upon membrane fusion, these fluorescent dyes mixed, resulting in FRET signal generation.

DiD and Dil liposomes were functionalized with ds-A/B/H₇ and ds-C/D respectively, and we studied the ability of miR-29a to displace H₇ and trigger liposome fusion (Figure 2). DiD liposomes were incubated with various quantities of miR-29a. Then, equal volumes of Dil and DiD liposomes were mixed and the evolution of the FRET ratio was measured over time (Figure 2a, Figure S5). In the absence of miR-29a, the FRET ratio slightly increased during the first 20 minutes of measurement then plateaued, showing that H₇ was successfully hybridized on ds-A/B and was able to prevent liposome fusion. As the quantity of miR-29a increased, the FRET ratio and its gradient in the first 20 minutes increased, demonstrating that miR-29a was able to displace H₇ and trigger liposome fusion. In order to study the sensitivity of the assay, we plotted the dose-response curve

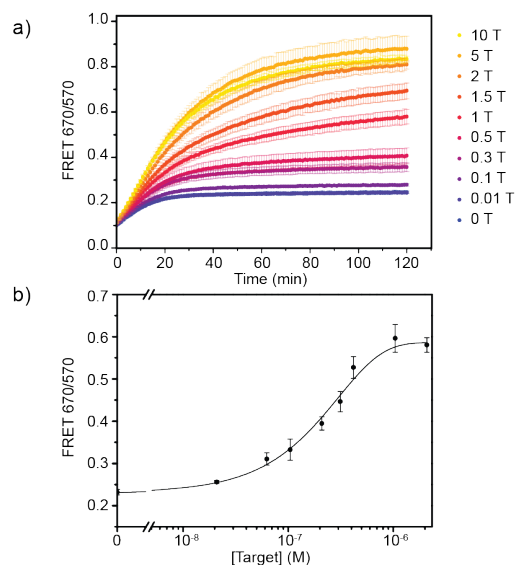


Figure 2. Evaluation of the sensitivity of the DNA-mediated liposome fusion assay for miR-29a detection: a) evolution of the FRET signals over time showing the kinetics of DNA-mediated liposome fusion in the presence of different concentrations of target miR-29a (where 1 T corresponds to 2.1×10^{-7} M) and b) corresponding dose-response curve obtained after 30 minutes of mixing DiI- and DiD-labeled DNA-functionalized liposomes ($n=3$). Error bars represent standard deviation.

obtained after 30 minutes of incubating DiI and DiD liposomes (Figure 2b). The limit of detection (LOD) was calculated by first determining the z-value ($z = \text{blank} + 3\sigma$), where σ is the standard deviation of the blank. The LOD was determined to be 18 nM by reporting the z-value in the equation of the curve fitted to the dose-response measurements.

Next, the specificity of the assay was studied (Figure 3). The miR-29 family members are important regulators of human diseases,³² and include miR-29a, miR-29b-1, miR-29b-2, and miR-29c. In a microarray assay, the expression levels of both miR-29a and miR-29b were significantly different between critically ill patients with H1N1 infection and healthy controls.²⁴ We tested the ability of the assay to discriminate between the fully complementary target, miR-29a, and its miR-29 family counterparts, miR-29b and miR-29c. The sequences of miR-29b and miR-29c differ from miR-29a by several mismatching and additional nucleotides (Table S1). Therefore, to study the specificity of our assay, we designed artificial sequences, differing from miR-29a by 1, 2 or 3 nucleotides: miR-29a-1MM, miR-29a-2MM and miR-29a-3MM (Table S1). DiD liposomes functionalized with ds-A/B/H₇ were incubated with 1 molar equivalent of different miRNAs. Subsequently, equal volumes of DiI and DiD liposomes were mixed, and the evolution of the FRET signal was measured over time (Figure 3a, Figure S6). Only the fully complementary target, miR-29a, was able to trigger liposome fusion, while all the other sequences did not show a significant increase in the FRET ratio compared to the signal measured in absence of target. Figure 3b summarizes the relative values of the FRET ratios at 30 minutes of incubation obtained with each miRNA compared to the control, and showed

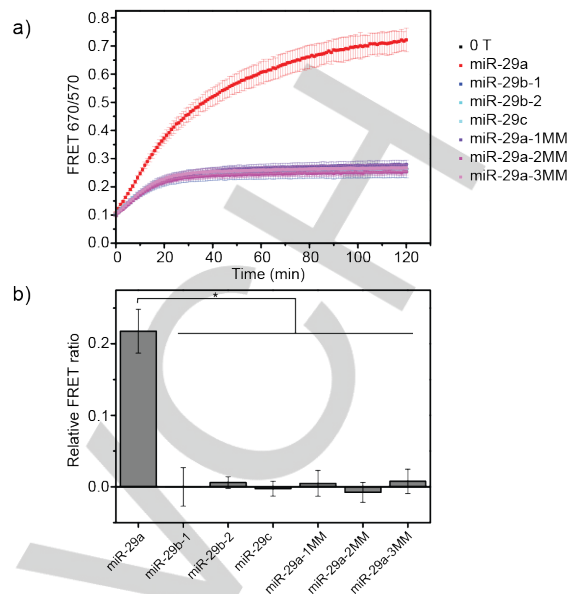


Figure 3. Evaluation of the specificity of the DNA-mediated liposome fusion assay for miR-29a detection: a) evolution of FRET signals over time showing the kinetics of DNA-mediated liposome fusion in the presence of various miRNA sequences at a concentration of 2.1×10^{-7} M. b) Corresponding bar chart representing the FRET ratio values relative to the control for each miRNA sequence 30 minutes after mixing DiI- and DiD-labeled DNA-functionalized liposomes. $n=3$, * $p < 0.05$ (ANOVA followed by Tukey *post-hoc* test). Error bars represent standard deviation.

that our assay has a high specificity, as we are able to discriminate between sequences with one nucleotide mismatch.

This high specificity is attributed to the fact that displacing the hairpin requires high stringency.³³ Although the LOD value reported here is much higher than that obtained with other miRNA detection methods based on enzymatic target amplification,^{34,35} this assay remarkably distinguished strong sequence homologies, which is typically difficult to achieve for homogeneous assays that do not incorporate washing, amplification or separation steps.³⁶ Additionally, the DNA-mediated liposome fusion assay reduces the complexity and the risk of errors that would otherwise be associated with enzymatic target amplification.³⁷

To further the understanding of the assay for miRNA detection and potentially gain in detection limit and/or minimize material consumption, a suspension-based 2-color fluorescence correlation microscopy method was developed, allowing extraction of FRET signals from imaging low numbers of liposomes (< 50).³⁸ The experimental setup is presented in Section S7 (SI). In brief, it comprises a regular fluorescence microscope equipped with a beam splitter (Figure S7a), which enables the simultaneous separation of the emitted light originating from DiI liposomes (centered around 570 nm; denoted as "green channel" in the following) and fused DiI and DiD liposome complexes (centered around 670 nm; "red channel") upon direct excitation of DiI liposomes by a laser source (488 nm).

We exploited the increased sensitivity in terms of liposome concentration of the 2-color fluorescence microscopy setup and

mixed liposomes solutions at 10 pM concentration for incubating with the target, and then diluted the solutions to 1 pM before performing the measurements (compared to 1.7 nM liposomes concentration used for the microplate reader setup).

Due to its large conceptual similarity to fluorescence correlation spectroscopy (FCS),^{39–41} we tried to assess FRET characteristics from the intensity fluctuations of $I_g(t)$ and $I_r(t)$ (intensity traces integrated over the entire the green and red channel, respectively). In FCS, the average number N of fluorescent particles within the readout volume (usually a diffraction-limited spot due to usage of a confocal excitation scheme) can be obtained by calculating the autocorrelation function g of the recorded fluorescence intensity trace $I(t)$,

$$g(\tau) = \frac{\langle I(t) \cdot I(t + \tau) \rangle}{\langle I(t) \rangle^2} - 1,$$

and employing $N=1/g(0)$.⁴² As shown in Section S7 (SI), the same methodology can be applied to the intensity traces obtained from our microscope setup, if the autocorrelation function of the intensity ratio $I_r(t)/I_g(t)$ is calculated. This normalization was necessary since fluctuations in $I_r(t)$ are caused by two processes, fused FRETing liposomes and bleed-through of Dil liposomes, making $g(0)$ more sensitive to the number of Dil liposomes than fused FRETing DiD liposomes (Section S7). Since Dil liposomes cause correlated fluctuations in $I_g(t)$ and $I_r(t)$, using $I_r(t)/I_g(t)$ eliminates the sensitivity of Dil liposomes but boosts FRET-based fluctuations (Figure 4a), making $g(0)$ mainly sensitive to the number of fused FRETing DiD liposomes (Section S7, Figure S8). Representative examples for the autocorrelation function of $I_r(t)/I_g(t)$ are shown in Figure 4b, clearly indicating a decrease in $g(0)$ with increasing miR-29a concentration, which is, due to $N \sim 1/g(0)$, indicative for an increase in the number of fused FRETing DiD liposomes N_{FRET} in the field of view. Calibration of this approach using solutions containing only FRETing vesicles of known bulk concentration C_{FRET} (Figure S9) allowed us to extract a dose-response curve (Figure 4c) similar to the microplate reader assay. The LOD obtained with the 2-color fluorescence correlation microscopy setup was determined to be 1.2 nM, one order of magnitude improvement in sensitivity compared with the ensemble measurements using the microplate reader setup. Hence, 2-color fluorescence correlation microscopy has in its current implementation an improved performance as the microplate reader assay (in terms of LOD), and consumes three orders of magnitude less material thanks to its ability to operate at liposome concentrations as low as 1 pM (in contrast to the 1.7 nM used in the microplate reader assay).

In summary, in this work we set out to explore whether DNA-mediated liposome fusion could be controlled by interaction with specific nucleic acid targets, and whether this mechanism could form the basis of a detection assay. We successfully demonstrated sensitive and specific detection of miR-29a at nanomolar concentrations using a FRET-based fluorescence output, and explored two methods of analyzing the fluorescence signal. Using a standard laboratory microplate reader, a limit of detection of 18 nM was obtained. Using 2-color fluorescence correlation microscopy combined with an intensity fluctuation analysis, we observe a limit of detection improved by one order of magnitude, while employing liposome and analyte

concentrations that are 3 orders of magnitude lower than those used in the microplate reader assay, and demonstrate the utility of such a setup in both the study of liposome fusion and application in a biosensing system. Further, our system has potential in the development of new diagnostic platforms targeting miRNA and other nucleic acids. By optimizing the highly promising concept of relatively few targets leading to fusion and activation of a large payload of signal-generating molecules, the LOD could be much improved in subsequent iterations. Factors such as assay temperature, dsDNA coverage, and choice of reporter signal are expected to improve the sensitivity in our assay, and will be the subject of further research. Also, by tuning the design of the DNA hairpin, detection of diverse molecular targets with high specificity, combined with a simple experimental workflow, is possible.

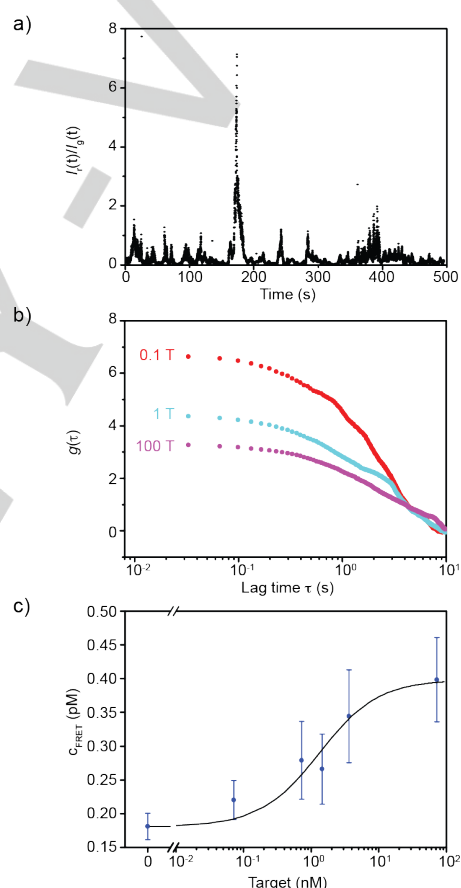


Figure 4. a) Ratio $I_r(t)/I_g(t)$ of the total intensity of the red channel over the green channel, b) representative autocorrelation functions of $I_r(t)/I_g(t) - \gamma$ (with $\gamma = 0.065$ the average bleed-through factor) (see Section S7 (SI)), for miR-29a concentrations as indicated (where 1 T corresponds to 7.2×10^{-10} M), and c) dose-response curve obtained using the 2-color fluorescence correlation microscopy setup (averaged over 3 independent sample sets covering at least 5 measurements each). Error bars represent SEM. (See Section S7 (SI) for details on the entire process.

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Conflict of interest

The authors declare no conflict of interest

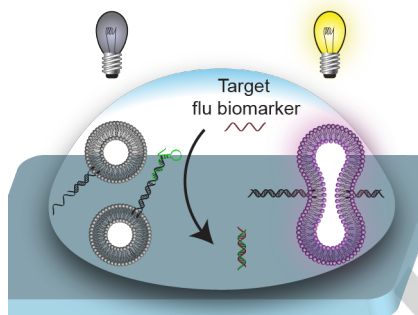
Keywords: biosensors • fluorescence • liposomes • membrane fusion • nucleic acids

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Entry for the Table of Contents

COMMUNICATION

A new biomimetic sensor for microRNA is developed based on a liposome fusion mechanism. The presence of miR-29a, a flu biomarker, triggers fusion between DNA-functionalized liposomes, which can be monitored by an increase in FRET signal. The liposome fusion events offer high selectivity and can detect nanomolar concentrations of miR-29a.



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Page No. – Page No.

Title