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Ultrasensitive Microarray Detection of Short RNA Sequences with Enzymatically Modified Nanoparticles and SPR Imaging Measurements

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Abstract

A novel multiplexed method for short RNA detection that employs an enzymatic capture reaction onto DNA-modified silica nanoparticles (SiNPs) followed by nanoparticle-enhanced surface plasmon resonance imaging (SPRI) is demonstrated. SiNPs functionalized with 5'-phosphorylated single stranded DNA (ssDNA) are used with T4 RNA ligase to capture various short 20–24 base single-stranded RNA (ssRNA) oligonucleotides from a target solution. The ssRNA-modified SiNPs are collected from the target solution, specifically adsorbed onto a complementary DNA microarray and then detected with SPRI. The use of DNA-modified SiNPs to capture ssRNA for profiling has several advantages as compared to a planar SPRI surface bioaffinity adsorption format: (i) the target solution is exposed to a larger total surface area for the RNA ligation reaction, (ii) the SiNPs enhance the diffusion rate of the ssRNA to the surface, (iii) the SiNPs can be collected, washed and pre-concentrated prior to detection, and (iv) the ssRNA-modified SiNPs give an enhanced SPRI signal upon hybridization adsorption to the microarray. Our initial measurements demonstrate that this detection method can be used to detect multiple ssRNA sequences at concentrations as low as 100 fM in 500 μ L.

Keywords

Silica nanoparticles; SPR imaging; DNA detection; microRNA detection; T4 RNA ligase; multiplex detection

I. Introduction

MicroRNAs (miRNAs) are small RNA molecules (19 to 23 base oligonucleotides) that can regulate gene expression in plants and animals either by catalyzing the cleavage of messenger RNA (mRNA) or through direct binding to the 3'-untranslated region of mRNA to block translation.¹ The miRNA registry currently lists over 15,000 miRNA sequences,² and profiling the expression levels of the various miRNA in a biological system has become an important tool for molecular biology.^{3–5} Additionally, a number of researchers have recently identified specific miRNA sequences as cancer biomarkers in tissue samples,^{6, 7} and have detected tumor-related miRNA in serum.⁸ Thus, for both the elucidation of gene expression patterns in biological systems and the early identification of cancer and other diseases, new methods for the rapid simultaneous detection of multiple miRNA at extremely low levels (e.g., femtomolar concentrations) are being actively pursued.

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Microarray-based techniques are particularly attractive for miRNA profiling as they are capable of screening large numbers of miRNAs simultaneously.^{9, 10} RT-PCR methods that employ special stem-loop primers are sensitive enough to be used at femtomolar concentrations,¹¹ but are not easily multiplexed. Direct hybridization to complementary DNA or locked nucleic acid (LNA) microarrays can not be used at femtomolar concentrations;¹⁰ typically, some type of chemical or enzymatic modification of the target miRNA (e.g., ligation, polymerase extension, biotinylation) must be additionally implemented for increased sensitivity.^{10, 12–14} For example, in our own previous work we have employed nanoparticle-enhanced surface plasmon resonance imaging (SPRI) combined with a surface RNA poly(A) polymerase reaction to detect miRNA hybridization onto an LNA microarray at femtomolar concentrations.¹² We have summarized many of the various existing microarray methods for the ultrasensitive detection of miRNA in a recent review.¹⁵

In this paper, we demonstrate a new nanoparticle-enhanced SPRI microarray detection methodology that enables the capture and detection of multiple short ssRNA at sub-picomolar concentrations through the use of enzymatic silica nanoparticles (SiNPs). Using a set of three short (20–24 base) single stranded RNA (ssRNA) oligonucleotides as miRNA mimics, we demonstrate in this paper that this detection methodology can be used to detect multiple ssRNA sequences at concentrations down to 100 fM in a 500 μ L sample. The overall scheme for this detection methodology is shown in Figure 1. A unique feature of this methodology is an initial capture step of target ssRNA by a surface enzymatic ligation reaction with SiNPs that have been functionalized with 5'-phosphorylated single-stranded DNA (ssDNA). The different ssRNA-modified SiNPs are concentrated by centrifugation, separated, washed and reconstituted in a smaller microliter volume format, and then detected by nanoparticle-enhanced SPRI measurements of the specific hybridization adsorption of the SiNPs onto a DNA microarray. The use of enzymatically active SiNPs in this multiplexed ssRNA detection method has three significant advantages over ssRNA hybridization adsorption onto DNA or LNA microarrays. First, the SiNPs present a large total surface area relative to a planar microarray surface, which speeds up the attachment chemistry kinetics. Second, the radial diffusion of ssRNA to the SiNPs shortens the time required to capture the ssRNA as compared to planar diffusion to a microarray surface. And third, as mentioned above, the ssRNA ligated to the SiNPs can be separated, washed and preconcentrated prior to SPRI detection.

In these experiments, we utilize DNA-modified SiNPs that have been optimized for the capture and nanoparticle-enhanced SPRI detection of ssRNA. The SiNPs are DNA-modified with a robust aminosilane/polyGlutamic acid/EDC/NHSS coupling chemistry that we have demonstrated previously on planar surfaces¹⁶ and the ssRNA capture uses a T4 RNA ligase surface enzymatic reaction that we have implemented previously in a planar format for array fabrication purposes.¹⁷ Here we use an excess of SiNPs to capture ssRNA in a sample with at most one ssRNA ligated to a given SiNP. The SiNPs had an average 120 ± 5 nm diameter (see Supplementary Information); this size was chosen because: (i) the SiNPs are easily separated from the target solution by centrifugation, and (ii) they provide an enhanced SPRI signal upon adsorption to the DNA microarray that is comparable to that observed in other nanoparticle-enhanced SPRI measurements.¹⁸

II. Experimental Considerations

Materials

11-amino-1-undecanethiol hydrochloride (MUAM; Dojindo), poly-L-glutamic acid (pGlu; MW=2,000–15,000; Sigma), 1-ethyl-3-(3-(dimethylamino) propyl)carbodiimide hydrochloride (EDC; Pierce), N-hydroxysulfosuccinimide (NHSS; Pierce), T4 RNA ligase 1 (20,000 units/mL, New England BioLabs), T4 RNA ligase reaction buffer (50 mM Tris-

HCl, 10 mM MgCl₂, 10 mM Dithiothreitol and 1 mM ATP, pH=7.8, Tris buffer, New England BioLabs), silica nanoparticles (mean sizes 0.15 μ m, Bangs Laboratories, Inc., Fishers, IN), 3-aminopropyltrimethoxysilane (APTMS, 97%, Aldrich) and ethanol (Gold Shield, absolute) were used as received. All water used was Millipore water. DNA oligonucleotides and RNA oligonucleotides were obtained from Integrated DNA Technologies (IDT) and Thermo Scientific respectively, and used as received. A PBS buffer (100 mM Na₂HPO₄, 0.3M NaCl, 5mM MgCl₂, 1mM EDTA, pH=7.4) was used for all DNA array fabrication. A phosphate buffer (0.1 M NaCl, 10 mM sodium phosphate, pH=7.4) was used for DNA/DNA and DNA/RNA hybridization measurements.

DNA and RNA Sequences

The DNA and RNA sequences used are summarized in Table I. All the DNA sequences were checked using mfold RNA/DNA folding calculations and showed no hairpin formation.¹⁹ Three short ssRNA sequences, R₁, R₂ and R₃, with lengths of 24, 24 and 20 bases respectively were used as mimics of miRNA. These sequences were chosen as a reliable non-interacting set of ssRNA; we have used these sequences previously in other SPRI measurements.²⁰

Synthesis of 5'-phosphorylated ssDNA-modified SiNPs

The average size of SiNPs was characterized by electron microscopy (TEM) (see Supporting Information). The concentration of particles was determined to be 220 nM. (i) Amino-functionalization. Amino-functionalized SiNPs were synthesized as follows. A solution of SiNPs (0.05 mL), APTMS (4 μ L) and ethanol (8 mL) was mixed, gently stirred at room temperature for 24 h, and then centrifuged at 5000 rpm for 10 min. The supernatant was removed and the nanoparticles were resuspended in ethanol. This wash cycle was repeated three times, and finally the nanoparticles were redispersed in water (1 mL). (ii) pGlu-modification. pGlu (2.5 mg) was added to the above solution and reacted for 1 day at 37 °C. Then the excess pGlu was removed by discarding the supernatant after centrifugation, and the nanoparticles were redispersed in water (1 mL). (iii) EDC coupling reaction. 5'-phosphorylated and 3'-amino-terminated ssDNA (2 μ L, 1 mM), EDC (3 mg) and NHSS (0.9 mg) were added to water (30 μ L) and then combined with above solution of pGlu-modified SiNPs. The mixture was held at room temperature for 24 h. Unreacted DNA was removed by centrifuging the nanoparticle solution, removing the supernatant, and re-suspending nanoparticles in water. This process was repeated three times before a re-dispersion in phosphate buffer. The final concentration of particles was estimated to be 10 nM by UV-visible absorption (Details are available in the Supporting Information).

RNA ligation reaction on DNA-functionalized SiNPs

Different concentrations of ssRNA were added into the tris buffer solution containing 5'-phosphorylated ssDNA-modified SiNPs (0.5 mL, 1 nM of SiNPs) along with T4 RNA ligase (5 μ L). The mixture was gently shaken for several seconds and kept at 37 °C for two hours. The enzyme and unreacted ssRNA were removed by centrifuging, discarding the supernatant, and resuspending the nanoparticles in the phosphate buffer. This wash process was repeated three times to yield a final particle concentration of 1 nM.

DNA Microarray Fabrication

DNA microarrays were fabricated using a three-step process as described previously.¹⁶ Briefly, gold thin film spots (1.0 mm diameter, 45 nm thickness with a 1-nm underlayer of chromium) were deposited onto SF-10 glass (Schott Glass, 18 $\times$ 18 mm²) using a Denton DV-502A metal evaporator. The slides were immersed in a 1mM ethanolic MUAM solution overnight. pGlu (2 mg/mL) in PBS buffer was electrostatically absorbed onto the amino-

terminated MUAM to form a pGlu monolayer. Finally, each gold spot was exposed to a 250 μ M amino-modified ssDNA solution in a PBS buffer that contained 75 mM EDC and 15 mM NHSS for 4 h.

SPRI apparatus

The SPRI measurements were taken with an SPRImager from GWC Technologies (Madison, WI) as described previously.²¹

III. Results and Discussion

In this multiplexed ssRNA detection methodology, ssDNA-modified silica nanoparticles (SiNPs) are used to capture ssRNA sequences from a target solution using a surface enzymatic ligation reaction. The ssRNA are then detected with nanoparticle-enhanced SPRI measurements that measure the sequence specific SiNP hybridization adsorption onto a DNA microarray.

A. Fabrication and Characterization of DNA-Functionalized SiNPs

DNA attachment chemistry on the SiNPs—The SiNPs used in these measurements had an average diameter of 120 ± 5 nm as measured by TEM measurements (see the Supporting Information for more details). The SiNPs were biofunctionalized with amino-modified ssDNA molecules through an attachment chemistry that we have used previously to functionalize planar gold¹⁶ and silica-modified gold surfaces.²² Briefly, the multi-step attachment chemistry is shown in Figure 2a: (i) amino groups are first attached to the SiNP surface by reaction of aminopropylsilane (APTMS) with surface silanol groups; (ii) a monolayer of the polyanion poly-L-glutamic acid (pGlu) is then electrostatically adsorbed onto the amino-modified SiNP surface; (iii) in a single reaction step, the carboxylate groups of the pGlu are covalently linked with an amide-forming reaction using EDC and NHSS to both the amino moieties on the particle surface and 5'-phosphorylated, 3'-amino-modified 24-base DNA oligonucleotides (molecule D0 in Table I). The ssDNA-modified SiNPs created by this attachment chemistry are stable in the presence of DTT (a stabilizing component of the enzyme buffer solutions), are stable at temperatures up to 80°C, and can be repeatedly hybridized and denatured. A more detailed description of the reaction conditions for these coupling chemistries is given in the Experimental Section.

Hybridization and Ligation Characterization—Two different experiments were performed in order to characterize the hybridization activity and ligation efficiency of the DNA-modified SiNPs. The average number of hybridization-active ssDNA molecules attached to the SiNPs was first estimated by fluorescence wash-off measurements in a procedure described in detail in the Supporting Information. Briefly, fluorescently labeled ssDNA complementary to the DNA-modified SiNPs were captured, separated, denatured, collected and detected. From these measurements, an average of 150 ± 16 ssDNA molecules per SiNP were estimated due to base-pairing interactions; this corresponds to an active surface coverage of 3.3×10^{11} ssDNA molecules cm^{-2} . A series of hybridization adsorption SPRI experiments were also performed on these ssDNA-modified SiNPs. This surface coverage was chosen as optimal in terms of hybridization adsorption kinetics and surface hybridization efficiency (please see the Supporting Information for more details).

The primary reason for creating stable, homogeneous ssDNA monolayers on the SiNPs was to maximize the ligation efficiency of the RNA onto 5'-phosphorylated ssDNA-modified SiNPs. The ssRNA 24 mer R₁ was attached to the SiNPs by a T4 RNA ligation reaction as described in the Experimental section; no template oligonucleotide was required for this RNA-DNA ligation reaction. The efficiency of this ligation reaction was then optimized and

measured with fluorescence wash-off measurements; sequence R₁ was attached to $80 \pm 15\%$ of the 5'-phosphorylated ssDNA on the SiNPs by reaction with T4 ligase at 37 °C for two hours (details of the reaction conditions and fluorescence data are given in the Supporting Information). This ligation efficiency is comparable to that observed previously on planar surfaces ($85 \pm 10\%$ after 4 hours at 25°C).¹⁷

SPRI Enhancement Characterization—In addition to the enzymatic capture of target ssRNA molecules, the SiNPs were also used to enhance the SPRI measurements of the sequence-specific hybridization and adsorption of ssRNA (this process is termed “hybridization adsorption”) onto the complementary ssDNA microarrays. Although gold nanoparticles (AuNPs) or nanorods are typically used in nanoparticle-enhanced SPRI, larger non-metallic nanoparticles can produce a comparable SPRI response that is independent of the distance from the gold thin film surface.^{23–25} To quantitatively measure the SiNP enhancement, a series of SPRI experiments were performed to examine the adsorption of ssDNA-modified SiNPs onto partially complementary mixed monolayer DNA microarrays. These two component SPRI measurements are in a similar form as studied previously.¹⁶ Briefly, DNA microarrays were constructed in which each of the array elements contained a two component mixed DNA monolayer (sequence A and B in Table I). The percentage of sequence A in the monolayer in an element was varied from 0 % to 60 %. Upon exposure of the DNA microarray to SiNPs functionalized with ssDNA complementary to A (sequence Ac in Table I), different amounts of SPRI signal were observed from the various microarray elements. Figure 2c summarizes the results and plots the amount of differential reflectivity ($\Delta\%R$) as a function of the percentage of sequence A (%A). At %A value of 18% or higher, a maximum $\Delta\%R$ of 30% was observed. This maximum reflectivity is attributed to the formation of a full SiNP monolayer. The inset of Figure 2c shows the linear dependence of $\Delta\%R$ values for very low values of %A (less than 1%). These measurements show that the SPRI can be reliably used to detect the presence of DNA-modified SiNPs at surface coverages from 1–2% down to as low a value as 0.01%. This minimum detectable surface coverage is comparable to that observed previously with nanoparticle-enhanced SPRI utilizing 13 nm AuNPs,¹⁸ and suggests that we should be able to detect ssDNA-modified SiNPs down to a concentration of 500 fM to 1 pM.²⁶

B. SPRI Measurements of ssRNA Detection Sensitivity

Having thoroughly characterized both (i) the ability of the DNA-modified SiNPs to capture ssRNA, and (ii) the amount of nanoparticle enhancement that the SiNPs can provide for the SPRI measurements, a series of experiments were performed to determine the sensitivity of this method for detecting ssRNA. Multiple 500 μ L samples were prepared, each containing 5'-phosphorylated ssDNA SiNPs (1 nM), T4 RNA Ligase (0.38 μ M) and ssRNA (sequence R₁ in concentrations varying from 100 fM to 10 pM) in a tris buffer solution (see the Experimental Section for details). After the surface ligation reaction, the ssRNA-labeled SiNPs were centrifuged and washed several times to remove any enzyme and other proteins/impurities, and then re-suspended in 500 μ L of phosphate buffer for the hybridization adsorption measurements.

The re-suspended solutions of ssRNA-labeled SiNPs were then analyzed by SPRI measurements of hybridization adsorption onto a 16 element DNA microarray. For these single ssRNA sequence detection measurements, a two-component ssDNA microarray was fabricated that consisted of eight complementary D1 elements and 8 non-complementary D4 microarray elements as a negative control (see Table I for the sequences D1 and D4). The hybridization adsorption kinetics was followed in real time with SPRI measurements. A small amount of nonspecific adsorption was detected onto the non-complementary control sequences (D4) corresponding to a $\Delta\%R$ of 0.2% after 1000 s (see Supporting Information).

This non-specific adsorption is probably caused by a small amount of residue enzyme on the particle.²² The SPRI kinetic curves for the other microarray elements were corrected for this nonspecific adsorption.

The corrected SPRI kinetic curves for the adsorption of the ssRNA-modified SiNPs are shown in Figure 3 for various target R_1 concentration from 0–10 pM. These curves were analyzed using Langmuir adsorption/desorption kinetics and thermodynamics as describe elsewhere;^{26, 27} an easy method for comparing the relative adsorption strength is the value of $\Delta\%R$ after a longer time (e.g., 1800 seconds or 30 min). The inset of the Figure plots the $\Delta\%R$ observed in the SPRI after 1800 s versus ssRNA concentration; a linear slope for $\Delta\%R$ is observed over the entire concentration range. The lowest concentration detected in these experiments was 500 fM.

Also shown in Figure 3 is a red curve labeled 1 pM*. This data was obtained from a 100 fM sample that was resuspended in only 50 μ L of phosphate buffer instead of 500 μ L. This ten-fold post-ligation concentration resulted in a 1 pM solution that yielded an SPRI kinetic curve identical to that obtained from the 500 μ L sample with the same concentration. This additional measurement demonstrates that we can concentrate the separated ssRNA-modified SiNPs into smaller volumes and achieve even lower detection limits. The ten-fold post-ligation concentration allowed us to detect 100 fM ssRNA in a 500 μ L sample volume. Using microfluidic channels, we should be able to reduce the detection volume down to 1 μ L,²⁸ corresponding to an ultimate limit of detection of approximately 5 fM.

C. Multiplexed ssRNA Capture and SPRI Detection Measurements

In a final experiment, we demonstrated that this ssRNA detection methodology can be used to simultaneously detect multiple ssRNA sequences from a single target solution. For these experiments, between a 100-fold to 2000-fold excess of SiNPs as compared to target ssRNA is used; this excess was necessary to insure that there was typically just a single ssRNA attached to any given SiNP. To verify that we can detect multiple ssRNA, the DNA-functionalized SiNPs were used to simultaneously capture three different ssRNA sequences from a single sample, each at different concentrations. The three ssRNA sequences (R_1 , R_2 and R_3) used in these experiments are listed in Table I. We captured these three ssRNA from a single target solution solution at three different picomolar concentrations ($R_1 = 10$ pM, $R_2 = 5$ pM and $R_3 = 1$ pM) using 5'-phosphorylated ssDNA modified-SiNPs (1 nM) and the enzyme T4 RNA ligase (Figure 1 and Figure 4a). Since the particle concentration is in excess compared to RNA concentration, three types of RNA-labeled SiNPs are obtained from the ligation reaction between single ssDNA modified-SiNPs and the RNA in one solution (Figure 4a). As before, the RNA-modified SiNPs were then collected, washed and resuspended into 500 μ L of a phosphate buffer solution. SPRI measurements were then performed to detect the hybridization adsorption of ssRNA-modified SiNPs onto a four-component DNA microarray containing the three complementary ssDNA sequences D1, D2 and D3 as well as one non-complementary D4 control sequence. The 16 element pattern of this four element microarray is shown in Figure 4b. Both SPRI images and SPRI multiplexed kinetic curves were obtained in these hybridization adsorption measurements. A representative SPRI difference reflectivity image is shown in Figure 4c inset, and matches the 16 element pattern presented in Figure 4b. The normalized real-time kinetic data for each three complementary ssDNA elements are shown in Figure 4c; the adsorption of R_1 , R_2 and R_3 labeled-SiNPs yielded SPR reflectivity increases proportional to the different ssRNA concentrations.

IV. Conclusions

The experiments in this paper conclusively demonstrate that sub-picomolar concentrations of multiple short ssRNAs can be detected using enzymatic silica nanoparticles combined with SPRI measurements of DNA microarrays. Short ssRNA target molecules were captured onto SiNPs with an efficient enzymatic ligation reaction that utilized T4 RNA ligase and 5'-phosphorylated ssDNA attached to the SiNPs. The ssRNA-modified SiNPs were then separated, washed and preconcentrated prior to nanoparticle-enhanced SPRI detection of hybridization adsorption onto a DNA microarray. These results should be immediately applicable to the multiplexed capture and detection of miRNA; we have used similar DNA microarrays previously to detect multiple miRNA sequences from biological samples.¹² Using enzymatic reactions on nanoparticles to capture miRNA has several distinct advantages as compared to capturing miRNA on a microarray surface: enhanced diffusion to the SiNP surface, an increased surface area for capture that leads to an increase in surface ligation kinetics, and the ability to wash and pre-concentrate the miRNA prior to SPRI detection. In addition to SiNPs, silica-coated gold nanoparticles or nanorods that are impervious to degradation by DTT or heat can also be used in these experiments.²⁹ The experiments shown in this paper demonstrate the detection of ssRNA in solutions as dilute as 100 fM at 500 μ L; by using small volume microfluidic detection chambers,²⁸ we should be able to reduce the limit of detection into the single femtomolar range. Additionally, other more sensitive methods for detecting the sequence specific hybridization adsorption of SiNPs such as SPR phase imaging³⁰ or fluorescence imaging with dye-labeled SiNPs should also lower the detection limits of this enzymatic ssRNA capture method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic of enzymatic SiNPs using T4 ligation reaction for ssRNA detection with nanoparticle-enhanced SPRI measurements.

Figure 2.

(a) Schematic of pGlu attachment chemistry for the creation of ssDNA-modified SiNPs. (b) SPRI measurements of SiNP adsorption onto mixed ssDNA monolayers. The A_C-modified SiNPs hybridization adsorption onto a complementary ssDNA (A) and a non-complementary ssDNA (B) microarray. (c) Quantitative plot of SPRI reflectivity change (Δ%R) versus %A for the hybridization adsorption of A_C.

Figure 3.

Normalized real-time SPRI kinetic curves for the detection of ultra-low concentration of ssRNA (R_1 of 0 pM, 0.5 pM, 1 pM and 10 pM, 1 pM* concentrated from R_1 of 100 fM) collected by 5'-phosphorylated ssDNA-SiNPs through T4 ligase reaction. A plot of the SiNPs-enhanced SPR response versus different ssRNA concentrations is shown in the inset with corresponding linear fit. A schematic representation of the R_1 -modified SiNPs hybridization adsorption onto a complementary ssDNA (D1) microarray is shown at the bottom right.

Figure 4.

(a) Schematic representation of a multiple ssRNAs detection methodology using enzymatic nanoparticles. (b) The arrangement of a four-component DNA microarray consisting of D1-3 and D4 (as control). (c) Normalized real-time SPRI kinetic curves for the detection of R_1 of 10 pM, R_2 of 5 pM and R_3 of 1 pM. SPR difference image of detection a SiNPs (1nM) solution containing R_1 , R_2 and R_3 (inset).

Table I

Summary of all DNA and RNA used for the DNA/DNA and DNA/RNA hybridization SPRI measurements

Amino-modified ssDNA used with planar surfaces:
A: 5'-NH ₂ -(CH ₂) ₁₂ -(T) ₃₀ -3'
B: 5'-NH ₂ -(CH ₂) ₁₂ -TTC GGT TCG TGC TTA TGT GTC TGG ATT TCG-3'
D1: 5'-TTC CCC GTC GTT AGT GTG AG-NH ₂ -3'
D2: 5'-CAA GAG GCG AAG CTATTG AG-NH ₂ -3'
D3: 5'-TAA TGT GTG TGT GTG TGT AG-NH ₂ -3'
D4: 5'-CTG GGG AAA AGG GTG GCA CG-NH ₂ -3'
Amino-modified ssDNA used with nanoparticles:
D0: 5'-PO ₄ -(T) ₂₄ -NH ₂ -3'
A _C : 5'-NH ₂ -(CH ₂) ₁₂ -(A) ₃₀ -3'
RNA:
R ₁ : 5'-CUC ACA CUA ACG ACG GGG AAU UUU-3'
R ₂ : 5'-CUC AAU AGC UUC GCC UCU UGU UUU-3'
R ₃ : 5'-CUA CAC ACA CAC ACA CAU UA -3'