

Trinity University

## Digital Commons @ Trinity

---

Chemistry Faculty Research

Chemistry Department

---

4-2018

### Factors Affecting the Association of Single- and Double-Stranded RNAs with Montmorillonite Nanoclays

A. Gujjari

B. V. Rodriguez

J. Pescador

Corina Maeder

Trinity University, cmaeder@trinity.edu

G. W. Beall

*See next page for additional authors*

Follow this and additional works at: [https://digitalcommons.trinity.edu/chem\\_faculty](https://digitalcommons.trinity.edu/chem_faculty)

 Part of the [Chemistry Commons](#)

---

#### Repository Citation

Gujjari, A., Rodriguez, B. V., Pescador, J., Maeder, C., Beall, G. W., & Lewis, L. K. (2018). Factors affecting the association of single- and double-stranded RNAs with montmorillonite nanoclays. *International Journal of Biological Macromolecules*, 109, 551-559. <https://doi.org/10.1016/j.ijbiomac.2017.12.124>

This Post-Print is brought to you for free and open access by the Chemistry Department at Digital Commons @ Trinity. It has been accepted for inclusion in Chemistry Faculty Research by an authorized administrator of Digital Commons @ Trinity. For more information, please contact [jcostanz@trinity.edu](mailto:jcostanz@trinity.edu).

---

## Authors

A. Gujjari, B. V. Rodriguez, J. Pescador, Corina Maeder, G. W. Beall, and L. K. Lewis



Published in final edited form as:

*Int J Biol Macromol.* 2018 April 01; 109: 551–559. doi:10.1016/j.ijbiomac.2017.12.124.

## Factors affecting the association of single- and double-stranded RNAs with montmorillonite nanoclays

Archana Gujjari<sup>a</sup>, Blanca V. Rodriguez<sup>a</sup>, Jorge Pescador<sup>a</sup>, Corina Maeder<sup>b</sup>, Gary W. Beall<sup>a</sup>, and L. Kevin Lewis<sup>a,\*</sup>

<sup>a</sup>Chemistry and Biochemistry, Texas State University, 601 University Drive, San Marcos, TX, 78666, United States

<sup>b</sup>Department of Chemistry, Trinity University, One Trinity Place, San Antonio, TX, 78212, United States

### Abstract

Montmorillonite (MMT) nanoclays exist as single and stacked sheet-like structures with large surface areas that can form stable associations with many naturally occurring biomolecules, including nucleic acids. They have been utilized successfully as vehicles for delivery of both drugs and genes into cells. Most previous studies have focused on interactions of MMT with DNA. In the current study, we have investigated the binding of small RNAs similar to those used for RNA interference (RNAi) therapy to two major forms of the clay, Na-MMT and Ca-MMT. Association of both forms of MMT with several double-stranded RNAs (dsRNAs), including 25mers, 54mers and cloverleaf-shaped transfer RNAs, was weak and increased only slightly after addition of Mg<sup>2+</sup> ions to the binding reactions. By contrast, ssRNA 25mers and 54mers bound poorly to Na-MMT but interacted strongly with Ca-MMT. The weak binding of ssRNAs to Na-MMT could be strongly enhanced by addition of Mg<sup>2+</sup> ions. The strength of MMT-ssRNA interactions was also examined using inorganic anion competition and displacement assays, as well as electrophoretic mobility shift assays (EMSAs). The aggregate results point to a cation-bridging mechanism for binding of ssRNAs, but not dsRNAs, in the presence of divalent metal cations.

### Keywords

siRNA; Gene delivery; Gene therapy; RNA interference; Nonviral vectors

## 1. Introduction

Nanomaterials are defined as molecules that are 100 nm in at least one dimension [1–4]. These molecules typically have a large surface-area-to-mass ratio, readily bind to many drugs and biomolecules, and can deliver many small molecules into mammalian cells. Nanoclays are typically considered to be layered silicates, usually aluminosilicates. Many are naturally occurring while others are produced synthetically. Studies on these types of nanoclays have been extensively carried out for biomedical purposes [5–9]. Interest in

\*Corresponding author. LL18@txstate.edu (L.K. Lewis).

nanoclays for medicinal purposes arises from their exceptional properties such as low toxicity, high ability to adsorb biomolecules, large surface area, and high ion exchange capacity. This strong ion exchange capacity allows them to be easily modified, thereby altering their surface properties for distinct uses. Small ionic molecules can be exchanged easily on both the surface and the interlayer space of the clay sheets.

Montmorillonite (MMT) is a 2:1 layered aluminosilicate nanoclay consisting of the general formula  $M_y(Al_{2-y}Mg_y)Si_4O_{10}(OH)_2 \cdot nH_2O$  [10]. It exists as a sheet-like structure and is approximately 1 nm thick and can be up to 1000 nm in the other dimensions, with a typical surface area of approximately 700 m<sup>2</sup>/g [11,12]. MMT is classified as a 2:1 layered mineral based upon the number of tetrahedral and octahedral sheets it contains, as well as their structural arrangement. It has two outer silica tetrahedral sheets layered around a central alumina octahedral sheet that form a sandwich-like structure. The octahedral layer contains aluminum and magnesium atoms, which coordinate to oxygen atoms and a small number of hydroxyl groups. The silica atoms coordinate to oxygen atoms. Isomorphous substitutions (replacement of one structural cation for another of comparable size) occur in the octahedral layer by the substitution of the trivalent aluminum ions with divalent cations such as  $Mg^{2+}$ . The charge imbalance arising from this isomorphous substitution is compensated for by exchangeable cations that are displayed as M in the formula. If the exchangeable ion is monovalent then it is  $M_y$  in the chemical formula, but if it is divalent it is  $M_{y/2}$ . In montmorillonite, a typical value for Y is ~0.33.

MMT is a smectite clay, meaning “smeared” in the original Greek, that arises due to the tendency of the clay to form plastic and lubricious masses. The individual plates of MMT are only 1 nm thick but are approximately 150–200 nm in the other two dimensions. These high aspect ratio sheets have a tendency to stack face-to-face, forming tactoids. The individual plates in the tactoids are turbostratic, having no crystallographic relationship between the plates. MMT tactoids are not uniform in size, shape, or charge. The characteristics of the tactoids vary based on the type of cation exchanged and the pH of the environment [10]. The interlayer space within MMT tactoids, also known as the gallery, ranges in size based on the level of hydration. For example, water saturation causes the gallery to expand up to 2 nm, while an anhydrous environment will reduce the gallery to less than 1 nm [13]. The interlayer space of MMT is important, as cations that adsorb can interact with negatively charged particles, such as drugs or biomolecules. The intercalation of biomolecules into nanoclays is an important feature as this strategy can help to protect the drug/biomolecule from chemical and enzymatic degradation.

MMT particles have a high cation (80–150 mEq/100 g) exchange capacity [7–9,14], which allows them to adsorb large numbers of biomolecules. MMT is classified as a cationic nanoclay due to this high exchange capacity of cations. Modified MMT nanocomposites have been used for a variety of applications, including dermatology and cosmetic products, as well as anti-inflammatory agents. They have also been used for food packaging materials and as excipients for pharmaceutical agents. Recently, MMT has shown great promise for the delivery of biomolecules and drugs into mammalian cells, and nanoclays such as MMT could prove to be better delivery vehicles than viral vectors [4–9].

Many studies have been performed to evaluate the ability of anionic biomolecules such as DNA to adsorb to homoionic montmorillonite. Evidence has been presented that DNA adsorption onto MMT occurs through electrostatic interactions, ligand exchange (wherein the hydroxyl groups of the ribose and the phosphate groups at the end of a DNA molecule adsorb to the clay), and cation bridging of the DNA phosphate backbone and exchangeable cations [15,16]. Work in this laboratory found that homoionic montmorillonite can intercalate single-stranded DNA between its sheets, likely through cation bridging [7]. Furthermore, it demonstrated that adding divalent cations such as magnesium to binding reactions can enhance the adsorption of DNA to MMT. Modulating the kind of exchangeable cations in MMT has proven to have significant effects on its ability to bind to biomolecules.

Several studies have assessed montmorillonite for its potential as a drug/biomolecule delivery vehicle. Lin et al. demonstrated the ability of modified montmorillonite to protect DNA from nuclease degradation and deliver it into cells by monitoring the expression of enhanced green fluorescent protein in human dermal fibroblast cells [17]. Kawase et al. performed a series of experiments to evaluate the effectiveness of Na-MMT as a gene delivery system for plasmid DNA encoding EGFP (Enhanced Green Fluorescent Protein) [18]. Their initial *in vitro* studies using intestinal epithelial cells (IEC-6) resulted in expression of EGFP on cells transfected by the Na-MMT/DNA preparations. They also prepared clay/plasmid DNA complexes and administered them orally to mice. EGFP production was detected in the mice that received the MMT/DNA preparations only, no EGFP was detected in mice that received a naked plasmid preparation. This supports the observation that montmorillonite is able to protect DNA from nucleases and from pH changes in the intestine [18]. Kevadiya et al. compared the controlled release of vitamin B1 and vitamin B6 in *in vitro* assays after being intercalated into MMT [19]. Lin et al. reported successful intercalation of a chemotherapy drug, 5-fluorouracil (5-FU), into MMT layers by optimizing time, temperature, pH, and concentration [17]. Many other examples of successful intercalation of drugs such as ibuprofen, promethazine chloride, timolol maleate and paclitaxel and their controlled delivery by montmorillonite can be found in the literature [20–23].

Montmorillonite has also garnered positive attention as a potential gene carrier system due to its low toxicity. Several previous *in vivo* and *in vitro* studies have demonstrated no significant toxicity of homoionic montmorillonite on animal and human subjects [24–27]. Accordingly, the Federal Drug Administration has classified MMT clay (bentonite containing montmorillonite) as “generally recognized as safe” (GRAS).

While the adsorption capability and binding mode of DNA molecules to MMT have been widely investigated using adsorption assays and structural techniques such as X-ray diffraction and SEM, study of the binding of MMT to RNA has been limited. RNA differs from DNA in several ways, including substitution of uracil for thymine, the presence of an OH group attached to the 2' carbon in the ribose ring, and a natural sequence-dependent propensity to fold onto itself to form complex secondary structures containing duplex stem regions and single-stranded loop regions. Binding to nanomaterial surfaces involves multiple noncovalent interactions with the base, sugar and phosphate moieties of nucleic acids. Some

of these interactions are likely to be different for DNA versus RNA because of these chemical and structural differences. The primary aim of this project was to quantitatively assess the associations of RNA molecules with montmorillonite. Different types of RNA molecules with varying sizes and secondary and tertiary structures were evaluated for their ability to adsorb to MMT.

## 2. Materials and methods

### 2.1. Materials

Powdered sodium montmorillonite (Na-MMT) was obtained from Southern Clay Products, Inc. This form of MMT is Na<sup>+</sup> Cloisite, which is a water-washed homoionic sodium form of the clay having a cation exchange capacity of 95 meq/100 g. Purified *E. coli* transfer RNA(tRNA), tricine, triethanolamine, magnesium chloride, sodium chloride, sodium carbonate, and sodium sulfate were purchased from Sigma-Aldrich. Agarose was obtained from Gold Biotechnology. Boric acid and Tris base were from JT Baker. SYBR Gold was manufactured by Invitrogen Life Sciences. Ethidium bromide was from IBI Scientific. Eppendorf Flex-Tubes (1.5 mL) were purchased from VWR. Electrophoresis was performed using 11 cm × 14 cm Horizon gel rigs (Labrepco) and an EPS 601 power supply (GE Healthcare).

Oligonucleotides were purchased from Integrated DNA Technologies or from Invitrogen/ThermoFisher Scientific. Sequences of the oligomers were as follows:

PvuRNA (AAAUGAGUCACCCAGAUCUAAAUA), cPvuRNA (UUAUUUAGAUCUGGGUGACUCAUUU), Pvu4a (AAATGAGTCACCCAGATCTAAATA), cPvu4a (TTATTTAGATCTGGGTGACTCATTT), dsRNA 54mer RNALoop (AAAUGAGUCACCCAGAUCUAAAUAAGUAAUUAUUUAGAUCUGGGUGACUCAUU), and ssRNA 54mer RNAStr8 (AAAUGAGUCACCCAGAUCUAAAUAAGUAAAAAUGAGUCACCCAGAUCUAAAUA).

### 2.2. Preparation of dsDNA, dsRNA and homoionic Ca-MMT clays

To make double-stranded DNA(dsDNA) 25mers for MMT assays, 1050 ng Pvu4a was mixed with 1050 ng cPvu4a, double-deionized water (ddH<sub>2</sub>O) and 5 mM Tris (pH 7.4) in a total volume of 1 mL. For double-stranded RNA (dsRNA), 600 ng PvuRNA, 600 ng cPvuRNA, ddH<sub>2</sub>O and 5mM Tris (pH 7.4) were mixed in a volume of 1 mL. Each solution was placed into a heating block for 5 min at 100 °C and then allowed to slow-cool and anneal at RT for 30 min. RNALoop was prepared by diluting RNALoop into 5 mM Tris (pH 7.4) in a volume of 500 µL heating for 4 min at 90 °C, and slow-cooling to room temperature for 30 min. Successful dsRNA, dsDNA, and RNALoop formation was confirmed using gel electrophoresis with 3.5% agarose and 0.5× TB (45 mM Tris/45 mM boric acid) as running buffer. Gels were run for 10–15 min at 350 V as described [28] and stained using ethidium bromide or SYBR Gold.

Calcium montmorillonite (Ca-MMT) was prepared by mixing equal volumes of 1 M  $\text{CaCl}_2$  with 6 mg/mL Na-MMT and centrifuging at  $32,000 \times g$  for 30 min at  $4^\circ\text{C}$ . The resulting pellet was subsequently washed with cold water three times and was resuspended in water to match the initial volume. Confirmation of successful recovery was performed by measuring the  $A_{230}$  of the MMT before and after treatment as described [7]. Na-MMT and Ca-MMT suspensions were sonicated using a Sonics & Materials, Inc. Vibracell VC130 Sonicator. The clay was sonicated with the instrument amplitude set at 40 for 4 min.

### 2.3. Centrifugation binding assays

As we have described previously, in order to obtain accurate 260 nm absorbances after centrifugation of MMT solutions, it is necessary to account for the  $A_{260}$  contribution of the small amount of residual MMT in the supernatant [7]. The centrifugation assays therefore included a set of tubes containing MMT and water only, without RNA or DNA. After centrifugation, the  $A_{260}$  of the supernatant of each tube containing MMT only was measured using a spectrophotometer and was subtracted from the  $A_{260}$  measured from the supernatant of each tube containing the same concentration of MMT plus RNA to obtain the absorbance of free nucleic acids. The control tubes contained 75  $\mu\text{L}$  Na-MMT or Ca-MMT (producing final concentrations of 0.2, 0.5, 1.25, or 3.125 mg/mL) + 75  $\mu\text{L}$  ddH<sub>2</sub>O. MMT + ssDNA binding assay solutions were prepared by mixing 75  $\mu\text{L}$  of Na-MMT or Ca-MMT (0.2, 0.5, 1.25, or 3.125 mg/mL final), 7  $\mu\text{L}$  Pvu4a DNA (final  $A_{260} \sim 1.0$ ) and ddH<sub>2</sub>O in a final volume of 150  $\mu\text{L}$ . For dsDNA binding studies, 52.5  $\mu\text{L}$  dsDNA producing an  $A_{260}$  of 1.0 was included in the MMT solutions. MMT-ssRNA and -dsRNA solutions were prepared similarly, and included a constant amount of RNA that was enough to give an  $A_{260}$  of  $\sim 1.0$ . MMT + 54mer RNA and MMT + tRNA mixtures were prepared similarly. Assay solutions were shaken for 5 min at RT and then centrifuged at  $25,000 \times g$  for 1 h at  $4^\circ\text{C}$  using an Allegra 64R centrifuge. Metal ion effect studies were prepared with the same amount of Na-MMT or Ca-MMT and nucleic acids as above, with the addition of 0.1, 1, or 10 mM NaCl or  $\text{MgCl}_2$ . Immediately after centrifugation, the top 60  $\mu\text{L}$  of the supernatant of each sample was transferred into a new Eppendorf brand (non-leaching) microtube. Each assay was performed using 4–5 replicates and averages and standard deviations were calculated.

### 2.4. Salt competition and displacement assays

The salts that were used for these sets of experiments were sodium carbonate and sodium sulfate. For a typical competition assay, 4  $\mu\text{L}$  RNA was mixed with 50  $\mu\text{L}$  6 mg/mL MMT plus salt to give 0 mM, 1 mM, 10 mM, 100 mM or 500 mM final concentrations of each salt in a total volume of 150  $\mu\text{L}$ . The displacement assays were prepared using the same amounts of ssRNA, ddH<sub>2</sub>O, MMT, and salt concentrations. However, the RNA, water, and MMT were mixed and incubated for 5 min first, before adding the salts, for the displacement assays. The binding reactions were shaken for 5 min and then centrifuged for 30 min at  $21,000 \times g$ . The  $A_{260}$  of each sample's supernatant was measured and the percentage of RNA bound calculated.

### 2.5. Electrophoretic mobility shift assays

The EMSA gels were performed using 3.0–3.5% agarose gels and Horizon 11–14 gel rigs as described [28]. Initial gels were prepared in 45–50 mL 0.5X TB (Tris-borate) buffer. Later,

other electrophoresis buffers were tested, including 1×, 0.5× and 0.25× sodium borate (SB) and tricine/triethanolamine (30 mM/30 mM) as described [29,30]. All samples were run at 300–350 V for 5–10 min. SYBR Gold stock solution (10,000×) was diluted to 4× in 40 mL running buffer and layered onto the top of gels. Staining was performed for 15 min, followed by destaining for 20 min in gel buffer with shaking and photography using a ProteinSimple Red Imaging System. Assays utilized 200 ng PvuRNA or RNAstr8 combined with 0.04, 1, 2.5, and 5 mg/mL Ca-MMT in a volume of 20 µL. After letting the solutions sit for 5 min at RT, 4 µL of 30% glycerol was added, followed by mixing and loading of 18 µL of each sample onto the gel.

### 3. Results

#### 3.1. Interaction of single-stranded and double-stranded RNAs with Na-MMT

The major goal of this study was to investigate factors affecting the affinity of single-stranded and double-stranded RNAs for montmorillonite nanoparticles. We have previously analyzed the binding of DNAs, both oligonucleotides and plasmids, to Na-MMT and Ca-MMT [7] and the current project focusing on RNAs has expanded upon that earlier work. RNAs used for this study are depicted schematically in Fig. 1. These molecules include small ssRNA and dsRNA 25mers as well as larger 54 nt RNAs. One of the 54mers, RNAstr8, cannot form stable secondary structures and remains linear, while the other, called RNALoop, has a large region of self-complementary sequence that causes it to fold back on itself and exist as a double-stranded 25mer containing a 4 nt ssRNA loop (Fig. 1B). The double-stranded form of RNALoop is the predominant form in aqueous solutions [31]. The binding of more complex RNAs with extensive secondary structure, cloverleaf-shaped tRNAs, was also evaluated (Fig. 1C).

We have previously developed a centrifugation method for analyzing the binding of nucleic acids to montmorillonite in aqueous solutions using small polypropylene microcentrifuge tubes that exhibit minimal leaching of light-absorbing organic molecules upon warming [7,32]. Nucleic acids absorb UV light strongly at 260 nm and reduction of absorbance at this wavelength occurs when nucleic acids bound to clay particles are pelleted by centrifugation. The percentage of free and bound nucleic acid can then be calculated by measuring the  $A_{260}$  of the supernatant, as long as appropriate controls are also performed (see Materials and Methods).

The initial assays for the present study were conducted using different concentrations of the two most widely studied homoionic montmorillonite clays, Na-MMT and Ca-MMT [4–7,9,12,13,17–21,33–35]. Modifying montmorillonite with cations is crucial for binding as the silica surface of MMT is negatively charged and would otherwise encounter electrostatic repulsion with the negatively charged RNA and DNA. Moreover, past reports have shown that binding of nucleic acids to MMT can be impacted by the addition of salts such as NaCl and  $MgCl_2$  [8,15,33]; therefore, assays were performed with and without the addition of metal ions. Both Na-MMT and Ca-MMT were sonicated for 4 min before use to allow the tactoids formed by the clay platelets to disperse. Sonication of MMT is important when binding to biomolecules as it increases the available surface area to which the molecules can



bind and has been proposed as an essential step in preparing MMT that can intercalate DNA [7].

In initial experiments, binding of ssRNA and dsRNA 25mers to different concentrations of Na-MMT was assessed by mixing the nucleic acid and clay in water, shaking for 5 min, centrifuging at 25,000×g, and measuring the  $A_{260}$  of the supernatant. A plot of free, or unbound, RNA versus Na-MMT concentration is shown in Fig. 2A. Binding under these conditions was quite poor, with >80% of the RNA remaining unbound even at the highest concentrations of the clay. In concurrent experiments, binding of ss and ds DNA 25mers with the same sequences as the RNAs (except thymine substituted for uracil) was also tested. Binding of the DNAs was also weak, generating profiles essentially identical to those of the RNAs (Fig. 2A).

### 3.2. Differential effects of $\text{Na}^+$ and $\text{Mg}^{2+}$ ions on binding of small RNAs to Na-MMT

The effect of metal ions on the binding reactions was assessed next. Addition of NaCl up to 10 mM had no effect (Fig. 2B), but inclusion of  $\text{Mg}^{2+}$  ions increased binding (Fig. 2C and D). Strongest enhancement of binding was seen with the single-stranded RNAs and DNAs, which reached 76% and 89.5% bound when 10 mM  $\text{MgCl}_2$  was included in the reaction (Fig. 2D).

### 3.3. Impact of secondary structure on the binding of RNAs to Na-MMT

Binding of longer 54 nt RNAs was assessed using RNAstr8 (single-stranded) and RNAloop, which spontaneously folds into a dsRNA 25mer with a 4 nt loop at one end in aqueous solutions; this structure was previously confirmed by gel electrophoresis analysis [31]. Addition of Na-MMT at concentrations up to 3.1 mg/mL resulted in essentially no binding of RNAstr8 and only modest (~40%) binding of RNAloop (Fig. 3A). As with the 25mers tested previously, addition of NaCl did not increase the amount of bound RNA (Fig. 3B). Interestingly, addition of  $\text{Mg}^{2+}$  ions only modestly affected the affinity of RNAloop dsRNA for Na-MMT but strongly enhanced binding of the long ssRNA RNAstr8. The percentage of bound RNAstr8 reached 87% at 10 mM  $\text{MgCl}_2$ , while only 36% of the ds RNAloop was bound at this concentration (Fig. 3C and D). This finding is in accord with the results in Fig. 2C and D, where ssRNA and ssDNA 25mers bound more strongly than their ds counterparts. These results demonstrate that adsorption depends on the type of cation and also the concentration of the exchangeable cations. Assessment of the binding of larger and more complex cloverleafshaped transfer RNAs revealed that their affinity was weak, both with and without added metal cations (Fig. 4A and B). These ~75–80 nt molecules are largely double-stranded, with multiple duplex stem regions, and therefore the results appear to be consistent with those seen for both of the other primarily double-stranded molecules (the dsRNA 25mer and RNAloop).

### 3.4. Binding of simple and complex RNAs to homoionic Ca-MMT

The next set of experiments employed Ca-MMT as the homoionic clay for the RNA adsorption assays. Due to the previously suggested capability of divalent cations to promote clay:nucleic acid interactions via cation bridging [7], it is possible that bridging could occur even without the addition of  $\text{Mg}^{2+}$  ions, as this clay would already have divalent cations

bound to the outer silica layer. In addition,  $\text{Ca}^{2+}$  is known to form inner-sphere complexes with DNA, unlike  $\text{Na}^+$ , which can only screen the negative charges through cation bridging [36]. This could be important in the formation of strong interactions between the calcium ions and nucleic acids. Fig. 5A shows a plot of bound RNA percentage versus Ca-MMT concentration. Interestingly, the single-stranded RNA 25mers and 54mers showed strong binding to Ca-MMT even without the addition of salts. Notably, these two ssRNAs reached 70–80% binding to Ca-MMT at 3.1 mg/mL clay, but they showed less than 10% binding at this concentration of Na-MMT (Fig. 5A versus Figs 2A and 3A). When increasing amounts of  $\text{MgCl}_2$  were added to reaction mixtures while using a constant concentration of Ca-MMT (0.5 mg/mL), adsorption increased only modestly and only at the highest concentrations of  $\text{Mg}^{2+}$  (10 mM and 100 mM) (Fig. 3B).

The last set of MMT centrifugation assays examined binding between cloverleaf tRNAs and Ca-MMT with or without  $\text{MgCl}_2$ . Addition of increasing amounts of the clay in water revealed only modest binding of the tRNAs (Fig. 5C), similar to the dsRNAs tested previously. At the highest Ca-MMT concentration (3.1 mg/mL) binding only reached 40%. While maintaining a constant 0.5 mg/mL concentration of Ca-MMT, addition of increasing amounts of  $\text{MgCl}_2$  to the binding reactions showed almost no effect on tRNA binding (Fig. 5D). This minimal impact of additional  $\text{Mg}^{2+}$  ions on the mostly double-stranded tRNAs was similar to the small effects seen with the other dsRNAs and Ca-MMT (Fig. 5B).

### 3.5. Assessment of RNA affinity using competition and displacement assays

The possibility that small anions might displace or outcompete the RNAs binding to Ca-MMT inside cells or in other environments led us to conduct a series of centrifugation assays in the presence of two distinct anions, sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) and sodium sulfate ( $\text{Na}_2\text{SO}_4$ ). Competition assays were performed that involved simultaneously mixing 0.5 mg/mL Ca-MMT with ssRNA 25mer PvuRNA ( $A_{260} = 1.0$ ; 3.1  $\mu\text{M}$ ) and increasing concentrations of either sodium carbonate or sodium sulfate (0, 1, 10, or 100 mM). The percentage of RNA able to bind in the presence of each competing anion is plotted in Fig. 6A and B. At the highest concentration of 100 mM, carbonate ions reduced RNA binding from approximately 60% to 10%. Sulfate was less effective, reducing bound RNAs to only 40% at 10 and 100 mM. Displacement studies were performed in a similar manner, except that the anions were added last, after RNA + Ca-MMT complexes had already formed (Fig. 6C and D). These experiments also revealed a larger effect for carbonate ions, which reduced the fraction of bound RNAs from 70% to 30% at 100 mM  $\text{Na}_2\text{CO}_3$  (Fig. 6C). The aggregate data in Fig. 6 indicate that, while anions such as carbonate and sulfate can indeed be exchanged for RNA, it was very inefficient, even at very high concentrations of the anions. This could be explained by the fact that it is difficult for small molecules to displace a large molecule that is attached at multiple sites.

### 3.6. In contrast to RNAs bound to hydrotalcite clays, RNA-montmorillonite complexes are unstable during gel electrophoresis

In a previous study of the interactions of DNAs and RNAs with the nanoclay hydrotalcite [31], we developed an electrophoretic mobility shift assay (EMSA) system for analysis of binding. In the most common application of this biochemical assay, increasing amounts of a

DNA or RNA binding protein are mixed with a set amount of nucleic acid, followed by an incubation period to allow binding to occur [37,38]. The products of each binding reaction are then run in different lanes of a gel, separating the molecules by size, and the nucleic acid bands are visualized. Unbound nucleic acid migrates quickly and produces a single band low in the gel. As the protein concentration is increased, some of the nucleic acid molecules become bound and a new slower-migrating band usually appears higher up in the gel that represents the protein:nucleic acid complex. Sometimes multiple complexes of different sizes are formed that do not produce a single discrete upper band. The process can be monitored by analyzing the formation of the new band(s) or the progressive loss of the lower, free nucleic acid band. We employed 3.5% agarose gels to analyze complexes formed between Ca-MMT and RNA by following the parameters set forth previously [28,31]. The gels were run at high voltage (250–300 V with 14cm long gels) for 5–10min using 0.5× TB (Tris-borate) buffer and stained using SYBR Gold as described [38]. The SYBRGold stained the RNA but not the clay, allowing visualization of the free RNA band as it migrated down the gel. As an initial control experiment, PvuRNA was mixed with increasing amounts of the nanoclay hydrotalcite (HT), incubated at RT for 10 min, and analyzed using EMSAs. A progressive loss of the free RNA band was observed as clay concentration was increased from 0.04 mg/mL to 0.64 mg/mL (Fig. 7A). When the same experiment was performed using PvuRNA and Ca-MMT at concentrations between 0.4 and 5.0 mg/mL, no progressive reduction of band intensity was seen for the ssRNA (Fig. 7B) suggesting that the MMT-RNA complexes were not stable under these conditions. The 45 mM Tris in the electrophoresis buffer is an amine that can potentially bind strongly to the MMT particles [39,40], so the experiment was repeated using other electrophoresis media in an effort to find one that did not destabilize the complexes. Examples of this approach are shown in Fig. 7C and D, where 1X and 0.25× sodium borate (SB) solutions were used [30]. No stable Ca-MMT:RNA complexes could be detected using this non-Tris buffer. Several other attempts were made to improve the stability of the complexes, including using other weak acid:weak base buffers described by Liu et al. [29], adding divalent metals to the electrophoresis buffer and gel, and running the gels at lower voltages to slow migration (data not shown), but the MMT:RNA complexes remained labile under all conditions tested.

## 4. Discussion

Many past studies have focused on understanding the role of homoionic montmorillonite in the origins of life, with most studies focused on interactions with DNA [33–35]. Additional reports have investigated the use of MMT as gene delivery vehicle for transport of DNA molecules into cells [1,7,15–18]. By contrast, there has been little focus on probing MMT as a carrier for RNA molecules for therapeutic purposes. A large body of work in recent years has shown that small RNAs such as those analysed in the current study can function in RNA interference (RNAi) therapy, which can be employed to inactivate mRNAs and other types of RNA molecules inside cells [42–47]. Such RNAi-based therapies have been used successfully to target mutant genes causing human genetic disorders as well as genes whose protein products are overexpressed inside cells [42–47]. In the current study we quantitatively assessed the binding of single-stranded and double-stranded RNAs to two major forms of MMT in aqueous solutions. The association of RNA 25mers, 54mers and

75–80 nt long tRNAs with homoionic Na-MMT was weak, even at clay concentrations as high as 3 mg/mL. Thus, despite the overall positive charge from the sodium ions on Na-MMT, this monovalent cation was not sufficient to mediate the adsorption between MMT and the negatively charged RNAs. Furthermore, increasing the concentration of  $\text{Na}^+$  ions in the binding reactions did not improve RNA:clay interactions. In contrast to results with the monovalent cations, addition of  $\text{Mg}^{2+}$  modestly increased binding of dsRNAs and strongly promoted the association of all single-stranded RNAs to Na-MMT.

Adsorption of RNAs to Ca-MMT was much stronger than to Na-MMT, without inclusion of additional cations in the binding reactions (Fig. 5). Possible contributing factors to this difference is the smaller ion size and hydration energy of  $\text{Na}^+$  ions when compared to calcium or magnesium and also a reduced ability of  $\text{Na}^+$  to participate in cation bridging.  $\text{Na}^+$  ions are not able to open up the interlayer space of MMT tactoids to the same extent that divalent cations can, thereby retaining fewer  $\text{H}_2\text{O}$  molecules in the interlayer space [41] and this may be a contributing factor as well. It is possible that nucleic acids are limited in accessing the interlayer space when sodium is the exchangeable cation.

The strength of the interaction between ssRNA and Ca-MMT was also assessed using anion competition and displacement experiments. Sulfate ions were largely ineffective in these assays, but carbonate ions showed competitive and displacement activity, albeit only at high concentrations (100 mM) (Fig. 6). These data suggest that binding of RNAs to the clay is not likely to be disrupted significantly by the presence of small anions in complex solutions *in vitro* or *in vivo*. Although interactions between RNAs and the synthetic clay hydrotalcite could readily be monitored using mobility shift assays (Fig. 7A) [31], we were unable to assess RNA:montmorillonite complex formation using this method. Several changes were made to the electrophoresis gels and conductive media, including elimination of buffer components such as Tris known to interact with MMT [39,40] and addition of  $\text{Mg}^{2+}$  ions to the gel and electrophoresis buffer, but no loss of free RNA or gain of shifted higher molecular weight bands could be detected. It is unclear why the MMT complexes were so labile during electrophoresis. One possibility is that the clay and RNA have opposite charges and are drawn in different directions during electrophoresis, disrupting the complexes. However, we have observed that MMT can be loaded onto and separated on agarose gels, where it retains a net negative charge in standard TAE and TBE electrophoresis buffers (pH 7.5–8.0) and migrates toward the positive electrode, similar to RNA and DNA (the clay particles were visualized by staining with methylene blue; LKL and GWB, unpublished).

Previous work established that Na-MMT has high affinity for small ssDNAs in the presence of divalent salts [7,34] and the current study demonstrates that this is also true for small ssRNAs. The precise reason that ssRNAs are able to bind more strongly than dsRNAs is not known and may involve factors such as the nucleic acid's size, net charge, capacity for intercalation into the interlayer spaces between clay platelets, and ability to form bridging associations with  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  ions. The phosphodiester bonds and glycosidic linkages in single-stranded RNAs have more freedom of rotation than those in their double-stranded counterparts. In addition, both the negatively charged phosphates and the nitrogenous bases of nucleic acids have been shown to be capable of associating with montmorillonite [48–50]. It is likely that individual components of ssRNA such as phosphates and bases can be

oriented toward clay surfaces and cations in ways that are not possible for double-stranded RNA and this may contribute to the enhanced affinity. The 54mers used in this study, single-stranded RNAstr8 and double-stranded RNALoop, have the same molecular weight and the same potential net charge of  $-54$ , but the ssRNA form bound much more strongly to MMT than the double-stranded form. Interactions between anionic polymers and clay have widely been speculated to occur via cation bridging [7,33]. These past reports and the current study's data suggest that total charge and size are not major factors and imply that the potential for forming bonds with divalent metal ions, especially via cation bridging, is the most important determinant of binding affinity for small RNAs.

## Acknowledgments

BVR was supported, in part, by NIGMS funds from the South Texas Doctoral Bridge program (R25-GM-102783) and by a fellowship from the Robert A. Welch Foundation. All authors declare no conflict of interest.

## Abbreviations:

<b>Nt</b>	nucleotide
<b>bp</b>	base-pair
<b>Na-MMT</b>	sodium montmorillonit
<b>Ca-MMT</b>	calcium montmorillonite

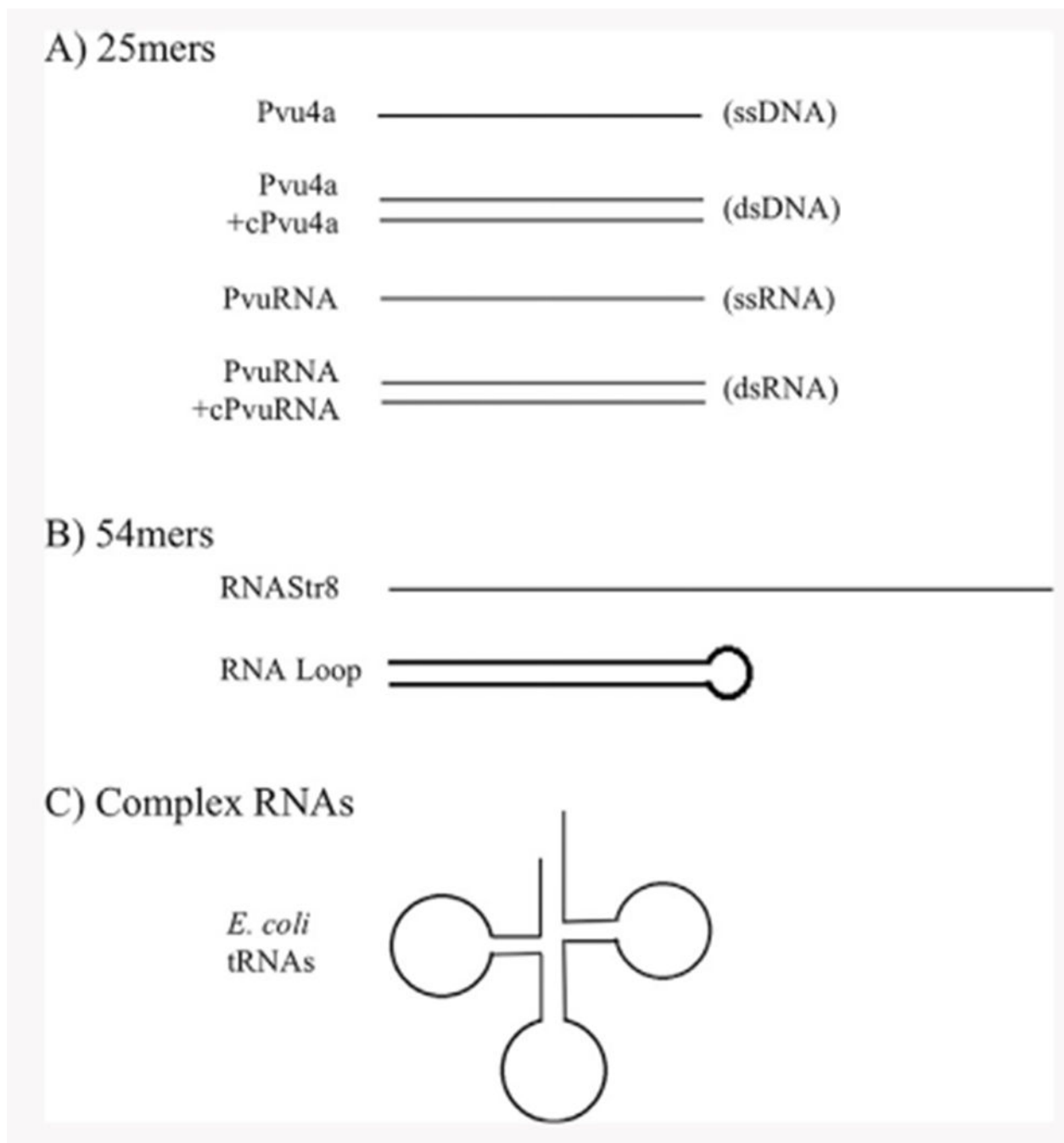
## References

- [1]. Sokolova V, Epple M, Inorganic nanoparticles as carriers of nucleic acids into cells, *Angew. Chem* 47 (2008) 1382–1395. [PubMed: 18098258]
- [2]. Wilczewska AZ, Niemirowicz K, Markiewicz KH, Car H, Nanoparticles as drug delivery systems, *Pharm. Rep* 64 (2012) 1020–1037.
- [3]. De Jong WH, Borm PJA, Drug delivery and nanoparticles: applications and hazards, *Int. J. Nanomed* 3 (2008) 133–149.
- [4]. Suresh R, Borkar SN, Sawant VA, Shende VS, Dimble SK, Nanoclay drug delivery system, *Int. J. Pharm. Sci. Nanotechnol* 3 (2010) 901–905.
- [5]. Choy Jin-Ho, Choi Soo-Jin, Oh Jae-Min, Park Taeun, Clay minerals and layered double hydroxides for novel biological applications, *Appl. Clay Sci* 36 (2007) 122–132.
- [6]. Aguzzi C, Cerezo P, Viseras C, Caramella C, Use of clays as drug delivery systems: possibilities and limitations, *Appl. Clay Sci* 36 (2007) 22–36.
- [7]. Beall GW, Sowersby DS, Roberts RD, Robson MH, Lewis LK, Analysis of oligonucleotide DNA binding and sedimentation properties of montmorillonite clay using ultraviolet light spectroscopy, *Biomacromolecules* 10 (2008) 105–112.
- [8]. Sanderson BA, Sowersby DS, Crosby S, Goss M, Lewis LK, Beall GW, Charge density and particle size effects on oligonucleotide and plasmid DNA binding to nanosized hydrotalcite, *Biointerphases* 8 (2013) 8. [PubMed: 24706120]
- [9]. Lin FH, Chen CH, Cheng WT, Kuo TF, Modified montmorillonite as vector for gene delivery, *Biomaterials* 27 (2006) 3333–3338. [PubMed: 16488006]
- [10]. Cadene A, Durand-Vidal S, Turq P, Brendle J, Study of individual Na-montmorillonite particles size morphology, and apparent charge, *J. Colloid Interface Sci* 285 (2005) 719–730. [PubMed: 15837491]
- [11]. Helmy AK, Ferreira EA, De Bussetti SG, Surface area evaluation of montmorillonite, *J. Colloid Interface Sci* 210 (1999) 167–171. [PubMed: 9924120]

- [12]. Winter D, Zubay G, Binding of adenine and adenine-related compounds to the clay montmorillonite and the mineral hydroxylapatite, *Origins Life Evol. Biosphere* 25 (1995) 61–81.
- [13]. Lal R, *Encyclopedia of Soil Science*, Taylor & Francis, New York, 2006.
- [14]. Pusch R, Yong RN, *Microstructure of Smectite Clays and Engineering Performance*, CRC Press, 2006.
- [15]. Cai P, Huang Q, Zhang X, Microcalorimetric studies of the effects of MgCl<sub>2</sub> concentrations and pH on the adsorption of DNA on montmorillonite, kaolinite and goethite, *Appl. Clay Sci* 32 (2006) 147–152.
- [16]. Hou Y, Wu P, Zhu N, The protective effect of clay minerals against damage to adsorbed DNA induced by cadmium and mercury, *Chemosphere* 95 (2013) 206–212. [PubMed: 24047649]
- [17]. Lin FH, Lee YH, Jian CH, Wong JM, Shieh MJ, Wang CY, A study of purified montmorillonite intercalated with 5-fluorouracil as drug carrier, *Biomaterials* 23 (2002) 1981–1987. [PubMed: 11996039]
- [18]. Kawase M, Hayashi Y, Kinoshita F, Yamato E, Miyazaki JI, Yamakawa J, Ishida T, Tamura M, Yagi K, Protective effect of montmorillonite on plasmid DNA in oral gene delivery into small intestine, *Biol. Pharm. Bull* 27 (2004) 2049–2051. [PubMed: 15577232]
- [19]. Kevadiya BD, Joshi GV, Patel HA, Ingole PG, Mody HM, Bajaj HC, Montmorillonite-alginate nanocomposites as a drug delivery system: intercalation and in vitro release of vitamin B1 and vitamin B6, *J. Biomat. Appl* 25 (2010) 161–177.
- [20]. Zheng JP, Luan L, Wang HY, Xi LF, Yao KD, Study on ibuprofen/montmorillonite intercalation composites as drug release system, *Appl. Clay Sci* 36 (2007) 297–301.
- [21]. Fejer I, Kata M, Eros I, Berkesi O, Dekany I, Release of cationic drugs from loaded clay minerals, *Colloid Polym. Sci* 279 (2001) 1177–1182.
- [22]. Joshi GV, Kevadiya BD, Patel HA, Bajaj HC, Jasra RV, Montmorillonite as a drug delivery system: intercalation and in vitro release of timolol maleate, *Int. J. Pharm* 374 (2009) 53–57. [PubMed: 19446759]
- [23]. Dong Y, Feng SS, Poly (d,l-lactide-co-glycolide)/montmorillonite nanoparticles for oral delivery of anticancer drugs, *Biomaterials* 26 (2005) 6068–6076. [PubMed: 15894372]
- [24]. Maisanaba S, Gutiérrez-Praena D, Pichardo S, Moreno FJ, Jorda M, Camean AM, Aucejo S, Jos A, Toxic effects of a modified montmorillonite clay on the human intestinal cell line Caco-2, *J. Appl. Toxicol* 34 (2014) 714–725. [PubMed: 24122917]
- [25]. Phillips TD, Afriyie-Gyawu E, Williams J, Huebner H, Ankrah NA, Ofori-Adjei D, Jolly P, Johnson N, Taylor J, Marroquin-Cardona A, Xu L, Reducing human exposure to aflatoxin through the use of clay: a review, *Food Addit. Contam* 25 (2008) 134–145.
- [26]. Robinson A, Johnson NM, Strey A, Taylor JF, Marroquin-Cardona A, Mitchell NJ, Afriyie-Gyawu E, Ankrah NA, Williams JH, Wang JS, Jolly PE, Calcium montmorillonite clay reduces urinary biomarkers of fumonisin B1 exposure in rats and humans, *Food Addit. Contam.: Part A* 29 (2012) 809–818.
- [27]. Wiles MC, Huebner HJ, Afriyie-Gyawu E, Taylor RJ, Toxicological evaluation and metal bioavailability in pregnant rats following exposure to clay minerals in the diet. Nanoclay drug delivery system, *J. Toxicol. Environ. Health, Part A* 67 (2004) 863–874. [PubMed: 15205041]
- [28]. Sanderson BA, Araki N, Lilley JL, Guerrero G, Lewis LK, Modification of gel architecture and TBE/TAE buffer composition to minimize heating during agarose gel electrophoresis, *Anal. Biochem* 454 (2014) 44–52. [PubMed: 24637158]
- [29]. Liu Q, Li X, Sommer SS, pK-matched running buffers for gel electrophoresis, *Anal. Biochem* 270 (1999) 112–122. [PubMed: 10328772]
- [30]. Brody JR, Kern SE, Sodium boric acid: a Tris-free cooler conductive medium for DNA electrophoresis, *Biotechniques* 36 (2004) 214–217. [PubMed: 14989083]
- [31]. Rodriguez BV, Pescador J, Pollok N, Beall GW, Maeder C, Lewis LK, Impact of size, secondary structure, and counterions on the binding of small ribonucleic acids to layered double hydroxide nanoparticles, *Biointerphases* 10 (2015) 041007. [PubMed: 26620852]
- [32]. Lewis LK, Robson M, Vecherkina Y, Ji C, Beall G, Interference with spectrophotometric analysis of nucleic acids and proteins by leaching of chemicals from plastic tubes, *Biotechniques* 48 (2010) 297–302. [PubMed: 20569206]

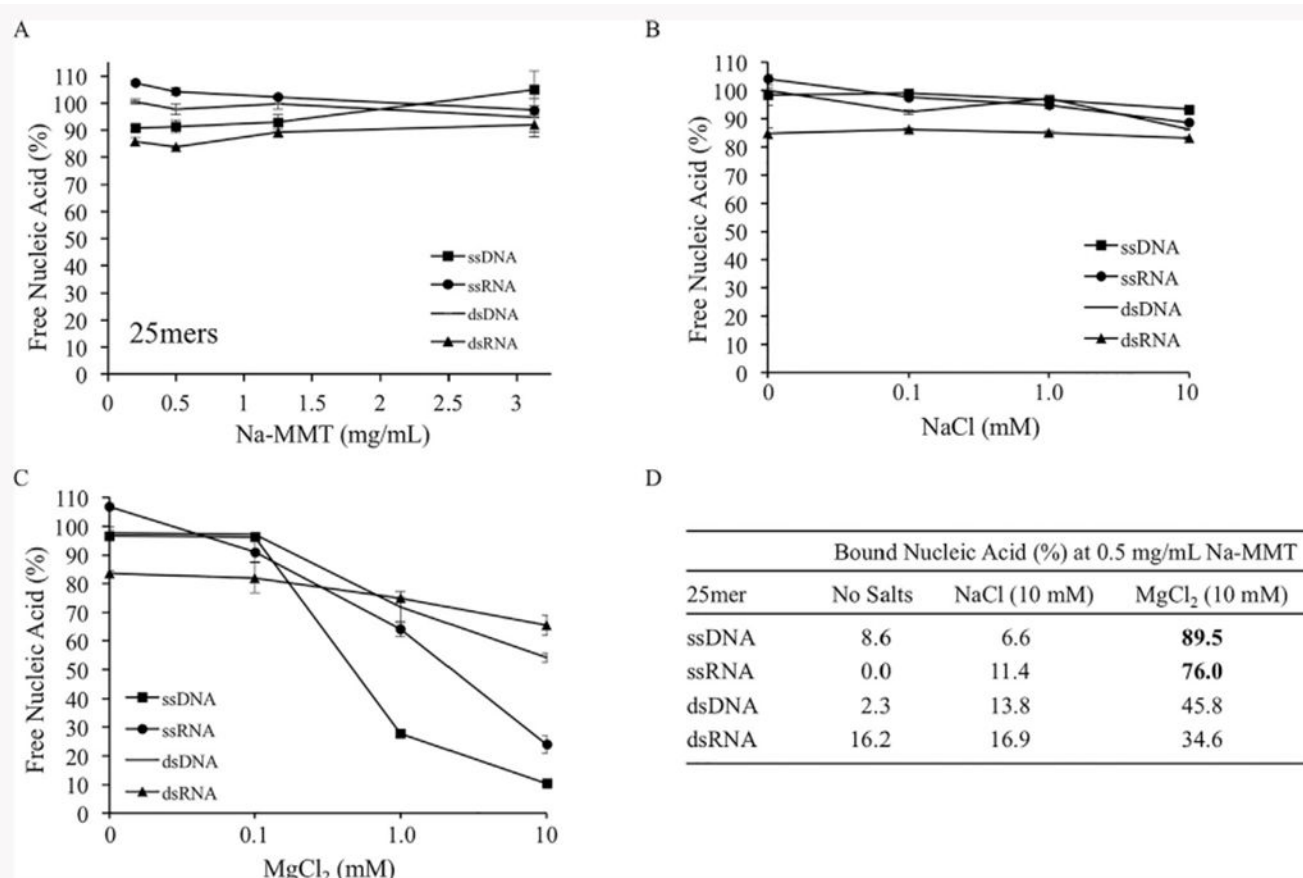


- [33]. Franchi M, Ferris JP, Gallori E, Cations as mediators of the adsorption of nucleic acids on clay surfaces in prebiotic environments, *Origins Life Evol. Biosphere* 33 (2003) 1–16.
- [34]. Hashizume H, Van der Gaast S, Theng BK, Interactions of clay minerals with RNA components, in: *Evolutionary Biology: Exobiology and Evolutionary Mechanisms*, 2013, pp. 61–79.
- [35]. Ferris JP, Mineral catalysis and prebiotic synthesis: montmorillonite-catalyzed formation of RNA, *Elements* 1 (2005) 145–149.
- [36]. Nguyen TH, Chen KL, Role of divalent cations in plasmid DNA adsorption to natural organic matter-coated silica surface, *Environ. Sci. Technol* 41 (2007) 5370–5375. [PubMed: 17822104]
- [37]. Altschuler SE, Lewis KA, Wuttke DS, Practical strategies for the evaluation of high-affinity protein/nucleic acid interactions, *J. Nucleic Acids Invest* 4 (2013) 19–28.
- [38]. Ream JA, Lewis LK, Lewis KA, Rapid agarose gel electrophoretic mobility shift assay for quantitating protein: RNA interactions, *Anal. Biochem* 511 (2016) 36–41. [PubMed: 27495142]
- [39]. Saeki K, Kunito T, Sakai M, Effect of Tris-HCl buffer on DNA adsorption by a variety of soil constituents, *Microbes Environ* 26 (2011) 88–91. [PubMed: 21487209]
- [40]. Keren R, O’connor GA, Effect of exchangeable ions and ionic strength on boron adsorption by montmorillonite and illite, *Clays Clay Miner* 30 (1982) 341–346.
- [41]. Xu W, Johnston CT, Parker P, Agnew SF, Infrared study of water sorption on Na-, Li-, Ca-, and Mg-exchanged (SWy-1 and SAz-1) montmorillonite, *Clays Clay Miner* 48 (2000) 120–131.
- [42]. Mollaie HR, Monavari SHR, Arabzadeh SAM, Shamsi-Shahrabadi M, Fazlalipour M, Afshar RM, RNAi and miRNA in viral infections and cancers, *Asian Pacif. J. Cancer Prev* 14 (2013) 7045–7056.
- [43]. Uchino K, Ochiya T, Takeshita F, RNAi therapeutics and applications of microRNAs in cancer treatment, *Jap. J. Clin. Oncol* 43 (2013) 596–607. [PubMed: 23592885]
- [44]. Lee JM, Yoon TJ, Cho YS, Recent developments in nanoparticle-based siRNA delivery for cancer therapy, *Bio Med Res. Intl* (2013) 782041.
- [45]. Mansoori B, Shotorbani SS, Baradaran B, RNA interference and its role in cancer therapy, *Adv. Pharm. Bull* 4 (2014) 313. [PubMed: 25436185]
- [46]. Ho W, Zhang XQ, Xu X, Biomaterials in siRNA delivery: a comprehensive review, *Adv. Healthcare Mat* 5 (2016) 2715–2731.
- [47]. Dowdy SF, Overcoming cellular barriers for RNA therapeutics, *Nat. Biotechnol* 35 (2017) 222–229. [PubMed: 28244992]
- [48]. Mignon P, Ugliengo P, Sodupe M, Theoretical study of the adsorption of RNA/DNA bases on the external surfaces of Na<sup>+</sup>-montmorillonite, *J. Phys. Chem. C* 113 (2009) 13741–13749.
- [49]. Sciascia L, Liveri MLT, Merli M, Kinetic and equilibrium studies for the adsorption of acid nucleic bases onto K10 montmorillonite, *Appl. Clay Sci* 53 (2011) 657–668.
- [50]. Perezgasga L, Serrato-Diaz A, Negron-Mendoza A, De Pablo Galan L, Mosqueira FG, Sites of adsorption of adenine uracil, and their corresponding derivatives on sodium montmorillonite, *Orig. Life Evol. Biosph* 35 (2005) 91–110 [PubMed: 16010992]

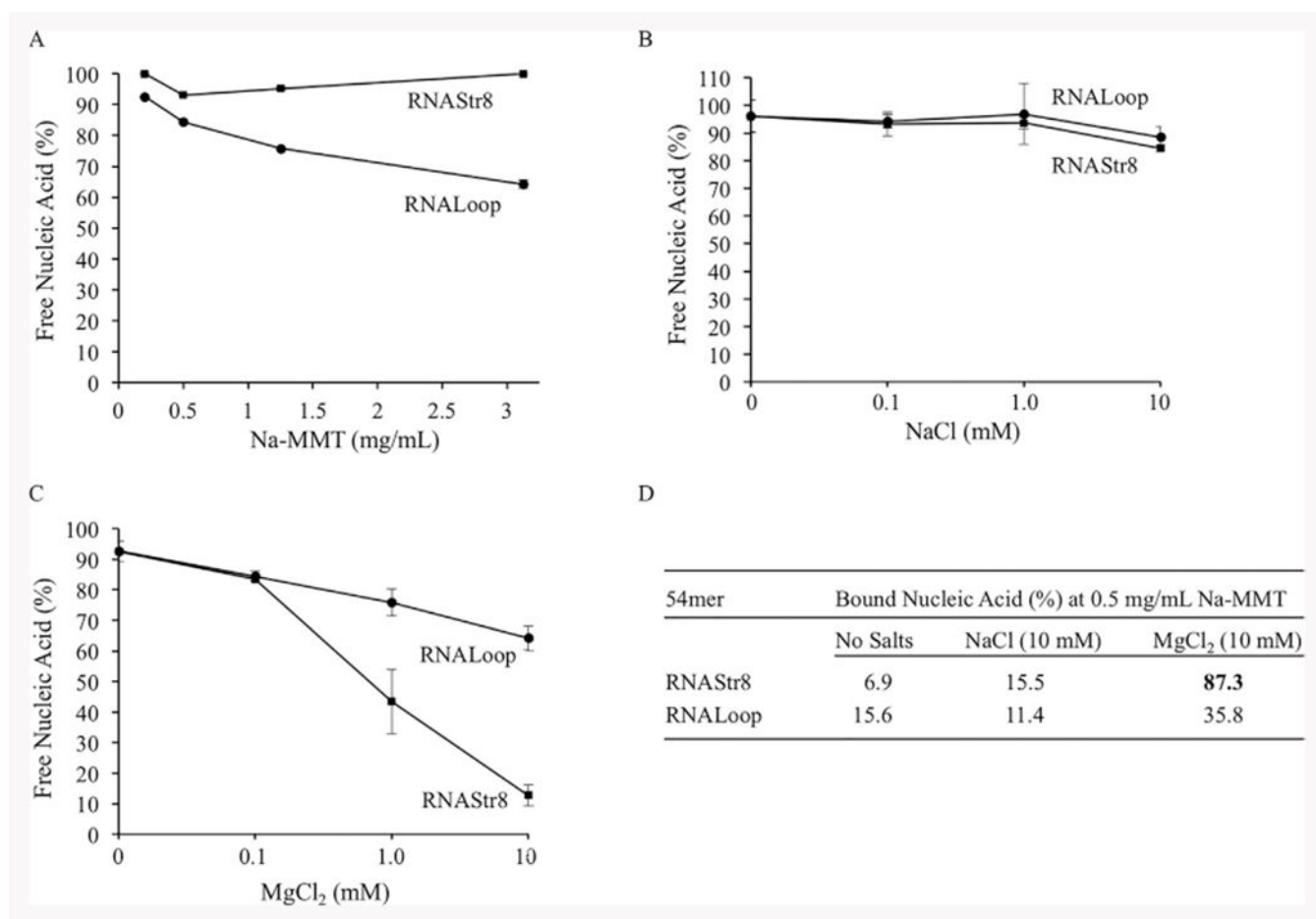
**Fig. 1.**

Schematic representations of the different types of nucleic acids used in the study. (A) Oligonucleotide 25mer names are listed on the left side and single-stranded (ss) or double-stranded (ds) structure is indicated on the right side. Double-stranded 25mers were prepared by annealing complementary single-stranded 25mers and confirming by gel electrophoresis. (B and C) Structural representations of 54mers and 75–80 nt transfer RNAs. The 54mer RNAstr8 cannot form stable secondary structures while the RNALoop 54mer naturally forms a 25 bp double-stranded stem region with a 4 nt ssRNA loop at one end.

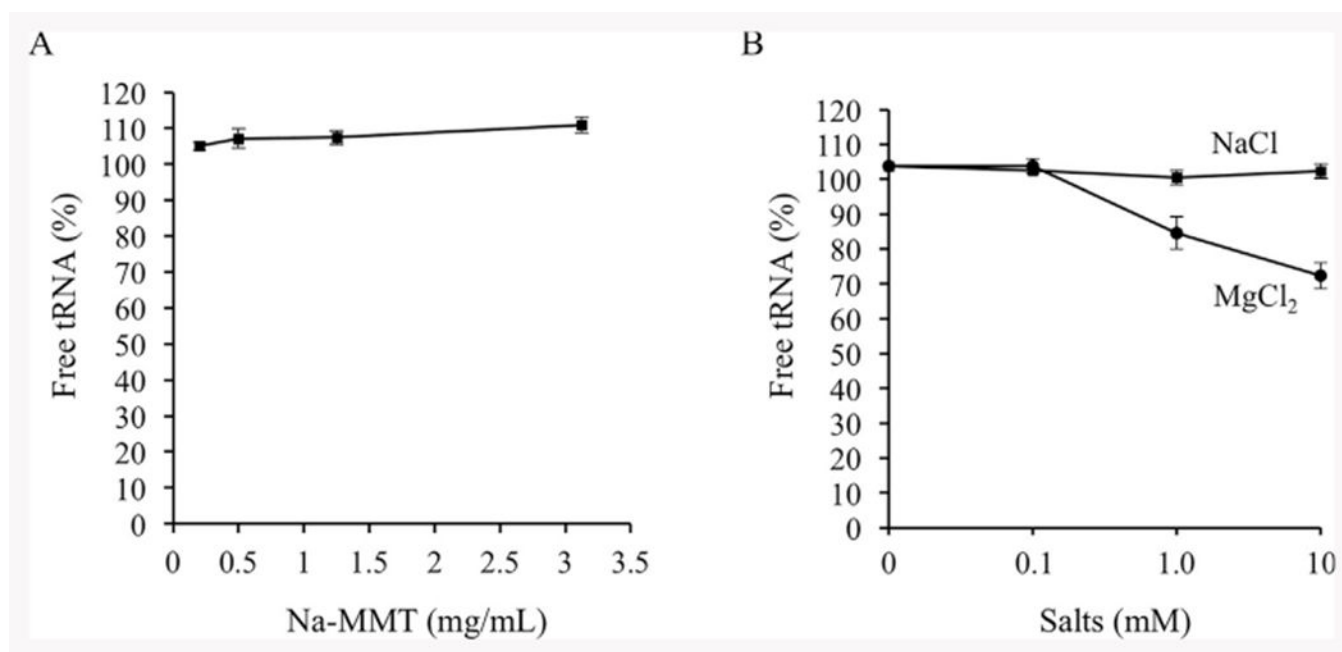




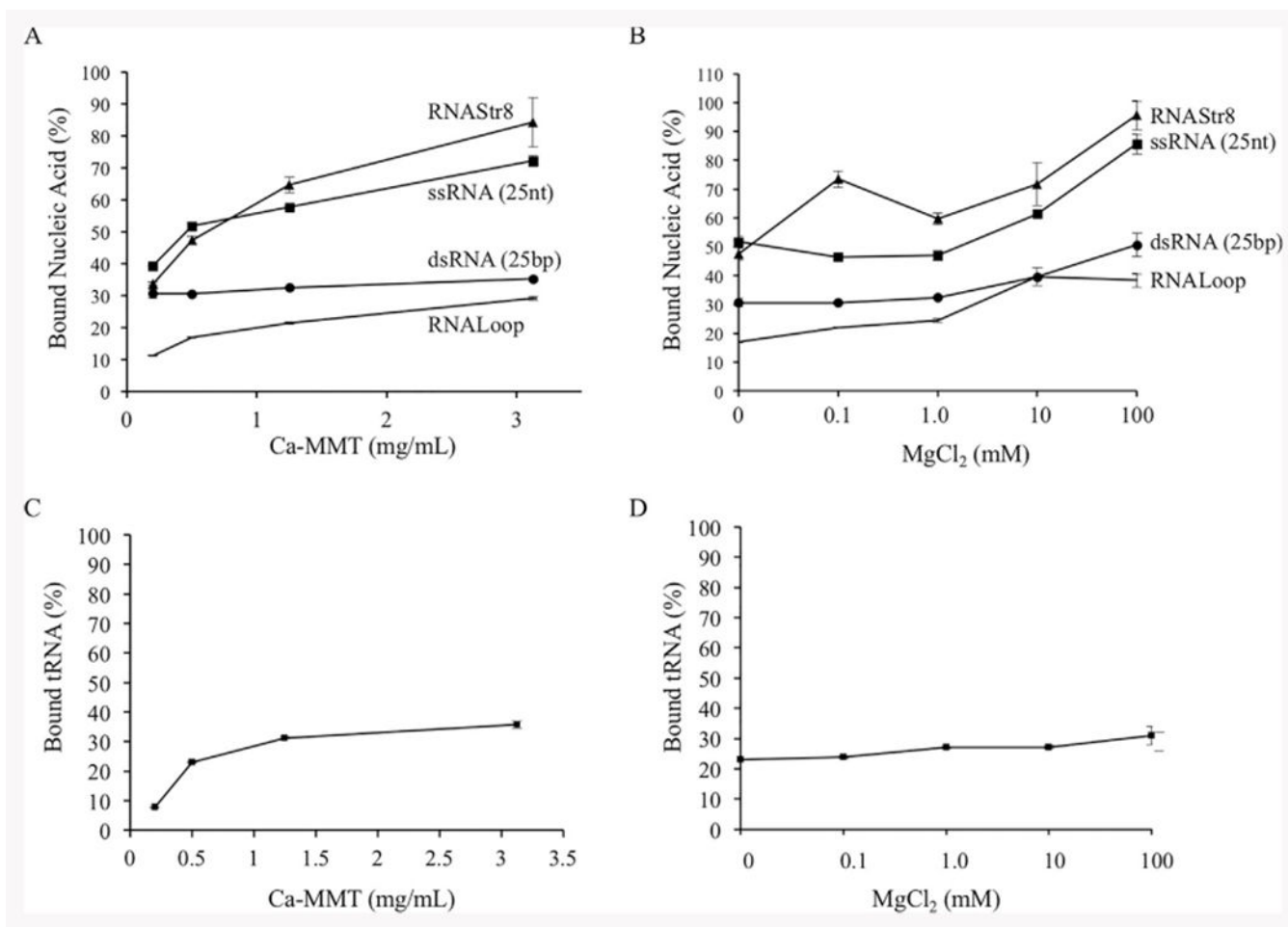
**Fig. 2.** Na-MMT centrifugation assays performed with ssDNA, ssRNA, dsRNA, and dsRNA 25mers. (A) Binding of four nucleic acids to increasing concentrations of Na-MMT. The amount of DNA or RNA remaining free (unprecipitated) is plotted on each y-axis. (B and C) Impact of adding NaCl and MgCl<sub>2</sub> on binding of nucleic acids to 0.5 mg/mL clay. (D) Percentage of free nucleic acids in the supernatant with and without 10 mM NaCl or 10 mM MgCl<sub>2</sub>.

**Fig. 3.**

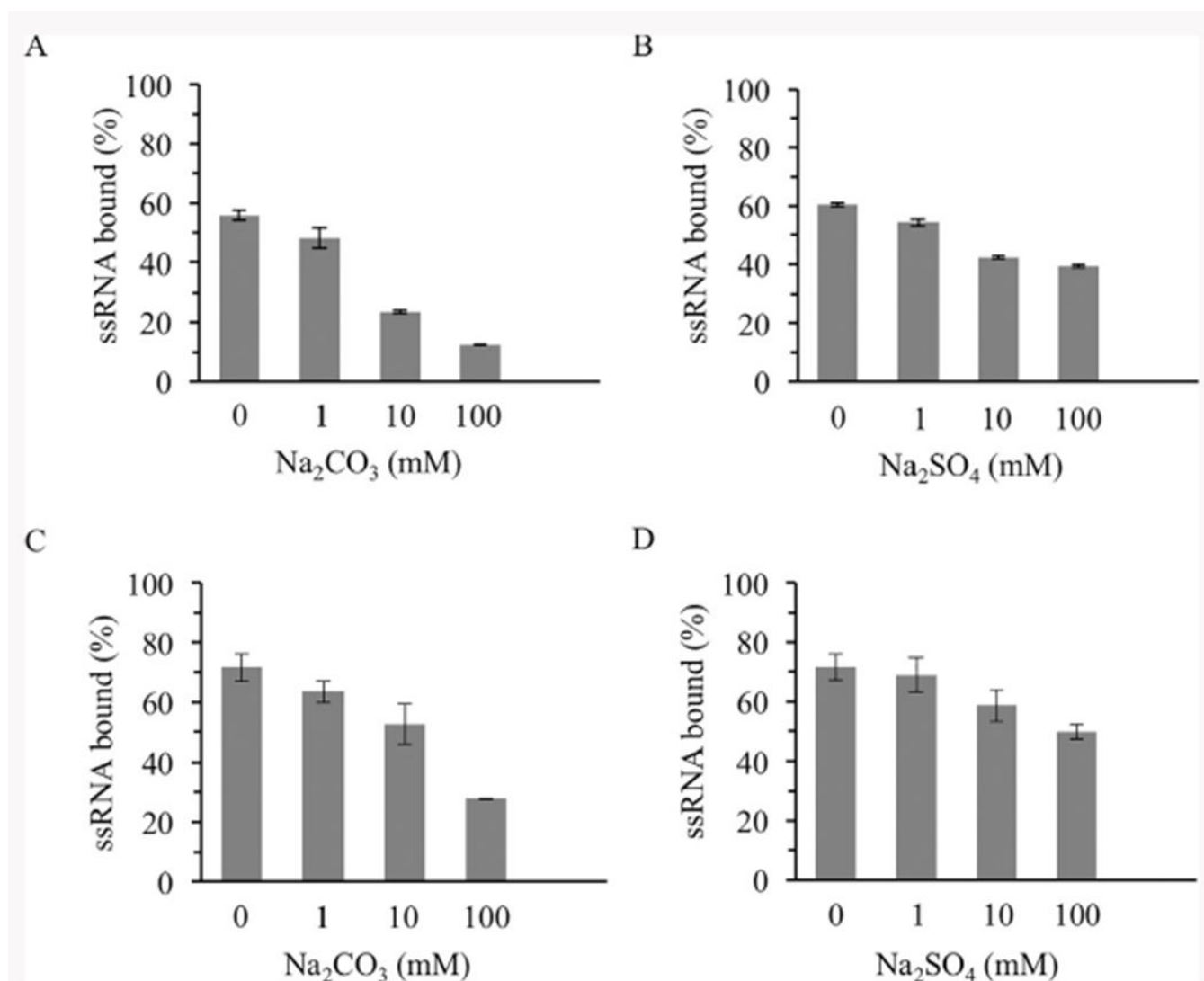
Adsorption assays using RNAstr8 and RNAloop 54mers and Na-MMT. The percentage of free RNA remaining in the supernatant after centrifugation is shown in A-C. (A) The effect of adding increasing amounts of clay on RNA binding. (B and C) Impact of adding either NaCl or MgCl<sub>2</sub> on binding of RNA to 0.5 mg/mL clay. (D) Comparison of the amounts of RNAstr8 and RNAloop that are bound at 0.5 mg/mL Na-MMT with or without NaCl or MgCl<sub>2</sub>.



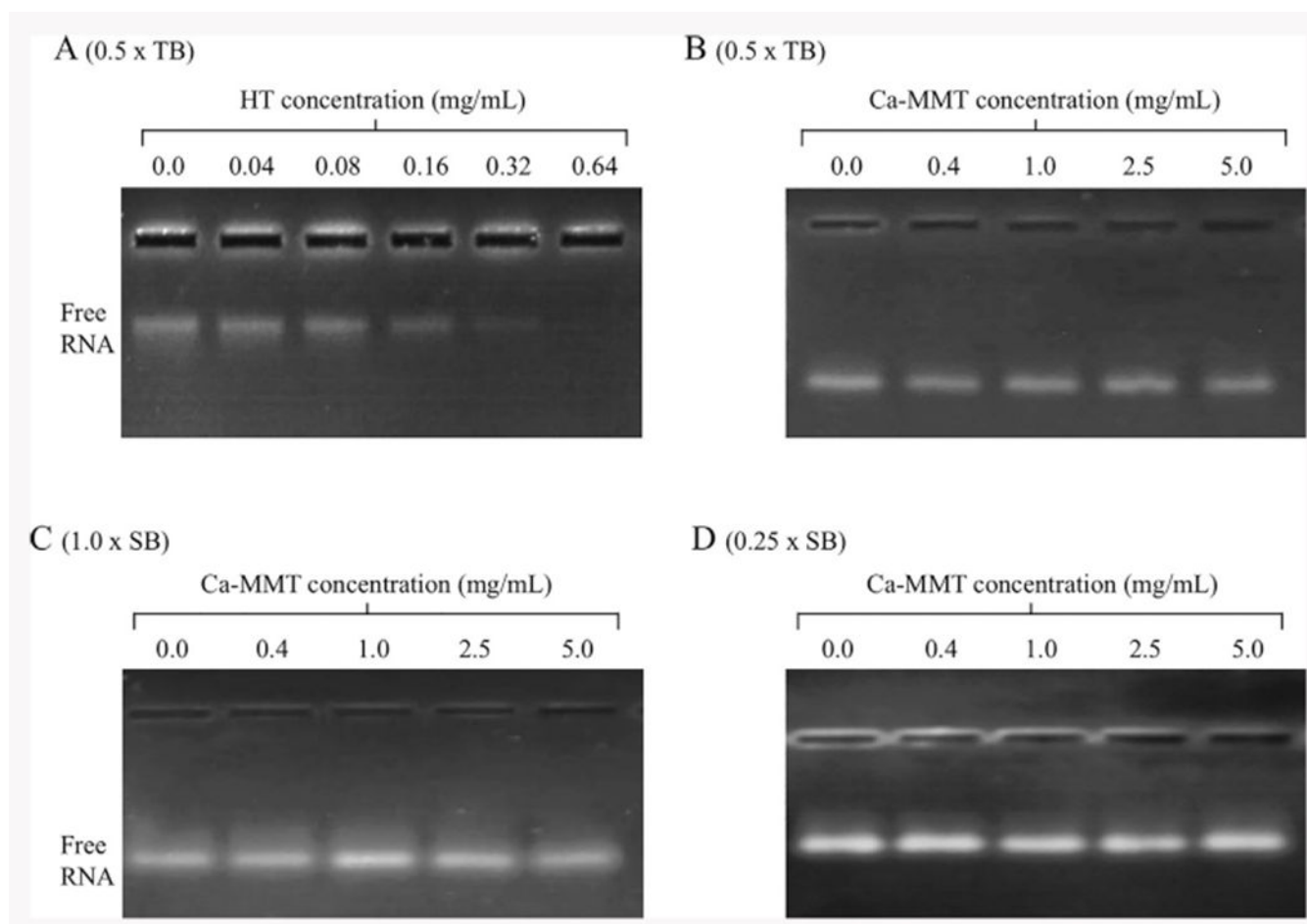
**Fig. 4.** Adsorption of complex tRNA molecules to Na-MMT. (A) Percentage of RNA remaining free when increasing amounts of clay are added to the binding reactions. (B) Assessment of binding to 0.5 mg/mL clay with and without addition of 10 mM NaCl or MgCl<sub>2</sub>.



**Fig. 5.** Binding of small RNAs to homoionic Ca-MMT. (A) Measurement of free RNA remaining after incubation of 25mers and 54mers with Ca-MMT. (B) Effect of adding MgCl<sub>2</sub> to Ca-MMT:RNA binding reactions. (C and D) tRNA adsorption to Ca-MMT without additional metal cations and after addition of MgCl<sub>2</sub>.



**Fig. 6.** Effect of inorganic anions on binding of ssRNA 25mer PvuRNA to Ca-MMT. (A and B) Centrifugation assays were performed to determine the ability of carbonate or sulfate anions to compete with ssRNA for binding to CaMMT. (C and D) Displacement of RNA from RNA:Ca-MMT complexes upon addition of carbonate or sulfate ions.

**Fig. 7.**

Electrophoretic mobility shift assays can readily monitor formation of RNA:HT complexes but not RNA:Ca-MMT complexes. Assays were performed using 3.5% agarose gels and ssRNA 25mer PvuRNA. (A) Addition of increasing amounts of hydrotalcite (HT) to 200 ng ssRNA leads to disappearance of the free RNA band as HT:RNA complexes are formed. (B-D) In contrast to results with HT, MMT:RNA complex formation could not be monitored using conventional Tris-borate (TB) or other electrophoresis buffers such as sodium borate (SB).