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

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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^{2+} 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^{2+} 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.

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 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 $M_y(Al_{2-y}Mg_y)Si_4O_{10}(OH)_2{*}nH_2O$ [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^2/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.

Abbreviations: nt, nucleotide; bp, base-pair; Na-MMT, sodium montmorillonite; Ca-MMT, calcium montmorillonite.

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MMT is a smectite clay, meaning "smeared" in the original Greek, that arises due to the tendancy of the clay to form plastic and lubitious 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 moeities 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^+ 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 (AAAUGAGUCACCCAGAUCUAAAUAA), cPvuRNA (UUAUUUAGAUCUGGGUGACUCAUUU), Pvu4a (AAATGAGTCACCCAGATCTAAATAA), cPvu4a (TTATTTAGATCTGGGTGACTCATTT), dsRNA 54mer RNALoop (AAAUGAGUCACCCAGAUCUAAAUAAGUAAUUAUUUUAGAUCUGGGUGACUCAUU), and ssRNA 54mer RNAStr8 (AAAUGAGUCACCCAGAUCUAAAUAAGUAAAAAUGAGUCACCCAGAUCUAAAUAA).

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_2O) 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_2O and 5 mM 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