UNDERSTANDING THE CHARACTERISTICS OF L-ASCORBIC ACID-MONTMORILLONITE NANOCOMPOSITE: CHEMICAL STRUCTURE AND BIOTOXICITY

BOR-YANN CHEN¹, YUAN-HAUN LEE², WU-CHING LIN², FENG-HUEI LIN², KING-FU LIN²

¹Department of Chemical and Materials Engineering, National I-Lan University, I-Lan, Taiwan

²Department of Material Science and Engineering, National Taiwan University, Taipei, Taiwan

ABSTRACT

*Following the previous study to intercalate a 5-fluorouracil (5-FU) into the interlayer spacing of montmorillonite (MMT) for colorectal cancer treatment, we selected a safe and non-pathological microorganism, *Saccharomyces cerevisiae*, as indicator microorganism to proceed risk assessment on MMT for feasibility study. Dose-response analysis from the perspective in toxicology was conducted to reveal the toxicity rankings of MMT, L-ascorbic acid (LAA) and montmorillonite-L-ascorbic acid (MMT-LAA) composites. This study also tended to intercalate LAA into MMT, observing chemical-structure stabilities of LAA-MMT composites. According to infrared spectroscopic analysis via FTIR, the lattice-vibration peaks of the distorted tetrahedron of SiO_4 for MMT were ranged in $400\text{--}1200\text{ cm}^{-1}$. X-ray diffraction analysis also addressed the layer spacing changes in the MMT lattice with pH changes. The variation in the [001] d-spacing changed from 12.4 to 19.4 Å with the increased pH, allowing higher intercalation of viable compounds (e.g., LAA) for controlled-release applications. Compared to MMT, LAA, the lowest toxicity (e.g., $\text{EC}_{50}=3.77 \times 10^5\text{ ppm}$) of the hybrid MMT-LAA also strongly suggested that MMT-LAA composite is safe to be used for further applications. In contrast, the lowest EC_0 (0.564 ppm) suggested the most sensitive characteristics for MMT-LAA composite to induce the toxic response from no-effect condition. MMT is thus feasible for intercalation of LAA in controlled release to pH changes; particularly, MMT-LAA is considered non-toxic for further applications.*

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1. INTRODUCTION

The structure of montmorillonite (MMT) is an octahedral laminated sheet, sandwiched between

tetrahedral silicate layers [1]. Due to the excellent cation exchange property, it could form many nanocomposites with different organic compounds within the interlayer spacing of ca. 0.9 to 1.2 nm in MMT [2]. In the previous study [3], 5-fluorouracil (5-FU) was successfully intercalated into the interlayer spacing of MMT for the feasibility in colorectal cancer treatment. Apparently, the composite of 5-FU/montmorillonite achieved a promising goal of

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Correspondence: Yuan-Haun Lee, Professor
Department of Material Science and Engineering,
National Taiwan University, Taipei, Taiwan
E-mail: yhlee@ntu.edu.tw

releasing 5-FU in situ to treat colorectal cancer in clinical application [3-5]. Prior to indications of MMT as a good carrier for drug design, Lee et al. [6] also examined acute effects of MMT using wistar rats as an *in vivo* animal model, clearly revealing the harmless nature of MMT. Evidence in biochemical analysis on mice administered with high doses of MMT also indicated that MMT is considered safe for practical use. To provide a quantitative comparison on toxicity of MMT and other related composites, *Saccharomyces cerevisiae* (or baker's yeast) was also used as an indicator microorganism to confirm that MMT alone was less toxic than others MMT composites via dose-response analysis. Apparently, MMT alone could be considered nontoxic in characteristics to *S. cerevisiae* and Wistar rat for myriads of applications. Several studies [7-8] also have been presented the sorption of cation on the MMT, and many of these were conducted over appropriate pH ranges. Owing to these specific characteristics, MMT could thus be very likely feasible to be a candidate drug carrier in medical uses.

L-ascorbic acid (LAA; i.e., vitamin C, a water-soluble vitamin) contains a variety of biological, pharmaceutical and dermatological functions; for example, it can promote collagen biosynthesis, provide photoprotection, scavenge free radical, cause melanin reduction and enhances the immunity (e.g., anti-viral effect) [9-11]. From the perspective in biochemistry, these functions are closely related to the so-called antioxidant properties of this compound. However, LAA is very unstable to exposures in air, light, moisture, heat, metal ions, oxygen, and base, since it is easily decomposed into biologically inactive compounds such as oxalic acid, L-xylonic acid, L-threonic acid, and L-lyxonic acid [9]. Evidently, applications of vitamin C in various fields (e.g., dermatology and pharmacology) are limited unless these limitations have been overcome. To improve chemical stability of LAA, several extensive attentions have been paid on encapsulation and immobilization of LAA [11, 12-14] using liposome, microemulsions, and liquid crystals.

Herein, we introduced a new method with practical value of intercalating and stabilizing vitamin C within an inorganic layered material MMT. Meanwhile, toxicity assessment also pointed out the margin of safety and toxicity rankings for feasibility of LAA-MMT composites in practical applications.

2. MATERIAL AND METHOD

2.1 Montmorillonite Purification

The crude clay of bentonite in Tai-dong, Taiwan contained several kinds of clay that includes chlorite,

kaolinite, illite, and montmorillonite [15-18]. Montmorillonite is about 30-40% by weight in unearthened bentonite. The purification process is briefly described as follows: One kilogram crude clay was dispersed in 4.0 L of distilled water and then maintained at room temperature for 3 days to be swelled up by all clay minerals. The clay solution was then poured into a filtration tank and gently stirred at a rate of ca. 60 rpm. Particles left on the filtration tank were discarded and all the supernatant was collected. The filtered solution was then centrifuged (700x g) twice. The supernatant was discarded and precipitates were dried by freeze-drying at -40°C for 3 days. The dried powder (size about 2.5 μ m) was classified as pure montmorillonite for later experimental use.

2.2 SEM/EDS Analysis

The surface morphology and element component of montmorillonite was examined by scanning electron microscope (SEM) at 15 kV of accelerating voltage (model number LEO 1530). The samples used for morphology examination were also prepared by compacting the nanocomposite powders into a pellet. The powdered samples placed on a microscope holder were coated with gold in a vacuum (ca. 10^{-4} torr) chamber.

2.3 XRD Analysis

The XRD patterns for the mineralogy of montmorillonite were obtained by a RIGAKU Geigerflex diffractometer equipped with Cu K α X-ray radiation, operating at 40 kV and 30 mA. The diffraction patterns were ranged in between angles (2θ) of 1-10° running at a scanning rate and counting step size of 0.6°/min and 0.01°, respectively.

2.4 Fourier Transform Infrared Spectroscopy

The Fourier transform infrared (FT-IR) spectra using KBr pressed disk technique were conducted by Jasco 410 Fourier transform infrared spectrometer. For each sample, ca. 0.90 mg organo-clay and 63 mg KBr were weighted and then were grounded in an agate mortar for 10 minutes prior to pellet making. The spectra were collected for each measurement over the spectral range of 400-4000 cm^{-1} with a resolution of 4 cm^{-1} .

2.5 Dose-response Analysis

Probit analysis [19] was adopted to reveal dose response curves for toxicity evaluation in response to various montmorillonite solutions. The probit model postulates that the tolerance capacity of individuals with respect to toxic material (e.g., MMT, MMT composites) in a *S. cerevisiae* population is Log-

normal distribution. A semilogarithmic plot of montmorillonite concentration versus the provoked response is thus assumed to reveal a linear relation. The probit model converts sigmoid-shaped dose-response curve into a linear normal equivalent deviation (NED) scale. For example, the 50% and 84.1% response correspond to unit in the model equals NED scale plus 5 (see Table I in [19]). The conversion formulae can be shown as follows:

$$Y = A + B \log Z \quad (1)$$

$$P = \frac{1}{2} \left\{ 1 + \operatorname{erf} \left(\frac{Y - 5}{\sqrt{2}} \right) \right\} \quad (2)$$

$$\operatorname{erf}(x) = \frac{2}{\sqrt{\pi}} \int_0^x e^{-\xi^2} d\xi \quad (3)$$

where A and B denote the intercept and slope of dose-response relation, Z and Y are montmorillonite concentration (ppm) and probit unit, respectively; P is the response (%) corresponding to montmorillonite concentration, erf(x) is an error function. Note that the response variable is normalized to be located between 0 and 1. The conversion relation between the probit unit and provoked response is listed elsewhere (Table I in [19])

Table I . The component of montmorillonite
(Two major metal element of montmorillonite are Si(34.42%) and Al(22.64%))

Element	wt%	Element	wt%
O	25.26%	Si	34.42%
Na	1.44%	Ca	7.52%
Mg	1.67%	K	4.13%
Al	22.64%	Fe	2.93%

3. RESULT AND DISCUSSION

3.1 XRD Analysis

As shown in Fig. 1(a), montmorillonite is one kind of bioinert clay minerals with fine grain and large inter-planar spacing, in particular located in the (001) plane, indicating superior capability to intercalate large molecules into the interlayer space at the (001) plane. It was shown that the [001] diffraction peak of the montmorillonite appears at $2\theta = 7.2^\circ$. The characteristic basal spacing d is 12.4 Å as calculated from Bragg's Law (Eq. (4)) as follows:

$$n\lambda = 2d \sin \theta, \quad (4)$$

where n , λ , θ , and d are an integer, the wavelength, the glancing angle of incidence and the interplanar spacing of the clay layers, respectively. For example, plugging $n = 1$, $\lambda = 1.54$ nm, $\theta = 7.2^\circ$ into eq. (4), we might obtain $d = 12.4$ Å. This value is consistent with the reported d_{001} which characterized a single layer of hydrates-12.5 Å in natural Na-montmorillonite [20]. The d -spacing difference is as calculated from Eq. (5) as follows:

$$d_{001}(\text{at pH } 3, 7, 10) - d_{001} = \Delta d \quad (5)$$

For example, plugging d_{001} (at pH 3) = 15.4 Å, $d_{001} = 12.4$ Å into eq. (5), we might obtain $\Delta d = 3.0$ Å. As shown in Fig. 1(b), the XRD pattern of MMT-LAA composite at pH 3 revealed that the diffraction peak was shifted from $2\theta = 7.2^\circ$ (i.e., MMT alone) to 5.8° and the d -spacing increased from 12.4 Å to 15.4 Å, since L-ascorbic acid particles were intercalated within montmorillonite layers. Fig. 1(c), (d) also showed a layer with the basal spacing of 15.5 Å ($2\theta = 5.7^\circ$) and 19.3 Å ($2\theta = 4.7^\circ$) at pH 7 and 10, respectively. These results suggested that L-ascorbate anions might be absorbed or intercalated within the inorganic layers, particularly in wider basal d -spacing at higher pHs (e.g., from pH 7 to 10). Fig. 2 clearly showed that the Δd of the MMT-LAA expanded with the increasing pH at 3, 7, 10. The d -spacing increased slightly at pH increased from 3 to 7, but significantly increased from 7 to 10. Due to such a specific characteristics, it is appropriate to be selected as the drug carrier (refer to [3] for an example) at pH alterations.

Fig 1(a). X-ray diffraction pattern of montmorillonite (MMT) alone at pH 7 : (b) MMT-LAA at pH 3; (c) MMT-LAA at pH 7; (d) MMT-LAA at pH 10.

Fig 2. The relationship for Δd of MMT versus pH.

3.2 SEM/EDS Analysis

After MMT purified from the crude clay of bentonite, it still kept its layer structure and uniformed fine grains to be observed from scanning electron micrograph (SEM) as shown in Fig.3 (a). This photograph showed crumpled platelets that looked like gypsum flower [21]. In addition, as shown in Fig. 3(b)-(d), SEM images of MMT-LAA powders at different pH value (i.e., 3, 7, 10) indicated spherical aggregates on the surface of MMT. At pH 3, XRD patterns showed that owing to the tightly closed structure in MMT, fewer particles could penetrate into the interlayer. At pH 10, the layers of MMT opened widely and thus much fewer particles covered on the surface of MMT due to significant intercalation within layers. These also pointed out a marked rise in the capacity for intercalation over pH changes. As indicated in Table II, the most abundant chemical composition of purified MMT analyzed by EDS are metallic Si (34.42%) and O (25.26%), suggesting SiO_2 as a major building block in structure. The chemical formula of the purified MMT could be termed $[(\text{Al}_{0.61}\text{Fe}^{\text{III}}_{0.04}\text{Mg}_{0.12})\text{Si}_{0.88}\text{O}_{2.68}] \text{Na}_{0.1}\text{K}_{0.05}$.

3.3 FT-IR Analysis

The Fourier transform infrared spectra of MMT, MMT-LAA were shown in Fig. 4. As shown the Fig. 4(a) in, the typical bands in spectra of MMT usually appeared to be under 1500 cm^{-1} due to the absence of H-bond (ca. $3200\text{--}3600 \text{ cm}^{-1}$) in MMT. Fig. 4(b)-(d) showed the spectra of MMT-LAA composites for various pHs at 3, 7, 10. The bands of 520 and 470 cm^{-1} (arrow marked at ca. 480 cm^{-1}) were assigned to Si-O-Al (octahedral Al) and Si-O-Si bending vibrations, respectively. The OH bending band appears at ca. 915 cm^{-1} (i.e., Al-OH) [22]. Gradual decreases in the

(a)

(b)

(c)

(d)

Fig 3. SEM image of (a) montmorillonite surface (25,000X) at pH 7; (b) MMT-LAA surface (25,000X) at pH 3; (c) MMT-LAA surface (25,000X) at pH 7; (d) MMT-LAA surface (25,000X) at pH 10.

Table II. Critical effective concentrations of different additives to *Saccharomyces cerevisiae* predicted from the probit model.

	EC ₀	EC ₂₀	EC ₅₀	EC ₁₀₀	Y=A+B logZ
Montmorillonite (MMT)	34.1	4.11×10 ³	5.41×10 ⁴	7.36×10 ⁸	Y=1.45+0.751(logZ)
L-Ascorbic acid (LAA)	21.5	3.92×10 ³	6.48×10 ⁴	2.02×10 ⁹	Y=1.68+0.690(logZ)
MMT-LAA	0.546	3.41×10 ³	3.77×10 ⁵	1.32×10 ¹³	Y=2.71+0.411(logZ)

Note: The response variable P is defined as $1 - \mu / \mu_0$ and Z denotes the supplemental concentration (ppm).

intensities of OH bending bands as well as those of the Si-O-Al band with increased pHs reflected marked decreases in octahedral cations due to deprotonation of LAA to L-ascorbate anion. According to Le Châtelier's principle, an increase in pH (i.e., hydroxide ion increased) enforced the proton on LAA to be released for neutralization with OH⁻, causing significantly decrease in intensities of OH bending band. The broad bands near 1010-1100 cm⁻¹ (e.g. 1016 cm⁻¹) were assigned to complex Si-O stretching vibrations in the tetrahedral sheets [23]. The intensity of the Si-O band for amorphous SiO₄ [22] near 1100 cm⁻¹ increased at lower pHs. The peaks at 1340-1530 cm⁻¹ denoted C-H bond appeared and thus caused the adsorbed vibrations. Literature [24-25] indicated that the C-H stretching bands most occurred in the 3020-2800 cm⁻¹ region, thus the increase in the bands (ca. 3053 cm⁻¹) intensity indicated the more protons and ascorbate ions released to interact with C-H bond due to deprotonation of LAA with the increased pH [22]. Thus, the results might suggest that the L-ascorbate molecules were bounded to the cation in tetrahedron or octahedron, and L-ascorbic acid intercalated into the layers. As shown in Fig. 4(e) for the typical L-ascorbic acid spectrum, the spectra of C-H bond stretching vibrations appeared at ca. 2915 cm⁻¹. Peaks at 1275, 1140 cm⁻¹ denoted the fingerprint regions for the C-2-O stretch vibration mode and the C-5-O adsorbed band, respectively [26].

3.4 Dose-response Analysis

Comparative differences in toxicity of various MMT composites were determined via dose-response analysis in Fig. 5. According to Ottoboni [26], if effective concentrations EC_x (the effective concentration to provoke x % toxic response; e.g., EC₀, EC₅₀ for $x=0, 50$) for chemical A are much less than those for chemical B, A is inevitably much more toxic than B. That is to say, a dose-response curve located on the left side clearly indicate high toxicity characteristic of the chemical. The value of EC₀ indicated the maximum dose of toxicants showing

Fig 4. (a) FT-IR spectra of Montmorillonite at pH 7; (b) FT-IR spectra of MMT-LAA at pH 3; (c) FT-IR spectra of MMT-LAA at pH 7; (d) FT-IR spectra of MMT-LAA at pH 10; (e) FT-IR spectra of LAA.

insignificant inhibition to microorganisms. This term may be defined as an indicator variable for revealing "margin of safety" [26]. The toxicity ranking in medium effect dose EC₅₀ (in ppm; shown in Table II) : MMT-LAA (EC₅₀=3.77×10⁵) > LAA (EC₅₀=6.48×10⁴) > MMT (EC₅₀=5.41×10⁴) clearly suggested that once LAA was added to MMT, this composite was significantly less toxic than both MMT and LAA alone to *S. cerevisiae*. Meanwhile, the ranking of EC₀ (in ppm): MMT (EC₀=34.1) > LAA (EC₀=21.5) > MMT-LAA (EC₀=0.546) simply suggested that MMT-LAA was more susceptible to induce toxic response from no-effect condition compared to MMT and LAA alone. As shown in the slope factor B of dose-response curve, all curves owned slope factors B less than unity (Table II; Fig. 5; 1.0 > 0.751 (MMT) > 0.690 (LAA) > 0.411 (MMT-LAA)), suggesting sharper response curves in

Fig 5. The dose-response curve for *Saccharomyces cerevisiae* in response to different amended solutions using probit model.

the toxic characteristics for all cases [27-29]. These sharp response curves suggested a broader tolerance range to have toxic response from no-effect concentration (EC_0) to 100% response concentration (EC_{100}), implying wider administered dose ranges for practical applications. In addition, the smallest value (0.411) of slope B for MMT-LAA directly implied the widest range of tolerance and highest adaptation to our model microorganism, suggesting the most beneficial characteristics of this composite due to significant attenuation in combined toxicity.

It was concluded that using montmorillonite as a composite base for practical uses (e.g., LAA-MMT) was inevitably biologically-feasible due to the non-toxic characteristics to *S. cerevisiae*. This result was indeed supported that montmorillonite as the "safe" carrier of anticancer drug, 5-FU in previous study [3] was very likely viable. Therefore, this might reveal the significant capability to develop montmorillonite as a newly natural nano-biomaterial for drug release and control in practice.

4. CONCLUSION

SEM and XRD results showed that as the pH increased from 3 to 10, the interlayer of montmorillonite gradually expanded allowing more L-ascorbic acid molecules to penetrate within the interlayer. In addition, results of FT-IR also indicated that a marked rise in ascorbate molecules might be bound to the tetrahedron Si-O bonds, leading to the decreased peaks due to formation of ascorbate anion from LAA molecules at increased pHs (e.g., 3, 7, 10). Dose-response analysis revealed that once MMT was

combined with LAA, the EC_{50} of MMT-LAA was significantly larger than that of MMT and LAA, implying that MMT-LAA was much less toxic than LAA and MMT. In conclusion, MMT might be scientifically feasible to be developed a new form of nanocomposites for further applications in various fields.

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