Halloysite Nanotube-Based Drug Delivery System for Treating Osteosarcoma

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Abstract— **Our previous studies have shown that halloysite nanotubes (HNTs) can be doped with a variety of bioactive agents (antibiotics, growth factors, chemotherapeutics) and the agents can be released in a sustained manner. In this study, anti-tumor drugs were loaded into HNTs and tested for their ability to inhibit/kill osteosarcoma cells. Results demonstrate that drugs released from the HNTs can inhibit proliferation or induce apoptosis in the osteosarcoma cells showing the potential application of doped HNTs as an anti-cancer drug delivery system.**

I. Introduction

Osteosarcoma is the most common malignancy of bone in children [1], and there are about 900 new cases reported in the US every year [2]. Neither surgical abscission nor chemotherapeutics alone is a viable therapy, and a combination of both is required for successful treatment. However, postoperative recurrence rate is very high, and metastasis may lead to an early death. Therefore, new chemotherapeutic agents, strategies and technologies are urgently needed to improve treatment.

Halloysite is a naturally occurring aluminosilicate clay (Fig 1, a and b), and can be mined from natural deposits in many countries such as China, Japan, Brazil, South Korea and America. [3]. It has the chemical formula of $Al_2Si_2O_5(OH)_4 \cdot nH_2O$, which is chemically similar to kaolin, but differs in having a hollow tubular structure (Fig 1c and d) [3, 4]. HNTs typically have a luminal diameter of 10 to 15 nm with an outside diameter ranging from 50 to 70 nm and a length between 500 and 2000 nm [5,6]. The halloysite wall is composed of 10-15 bilayers of aluminum and silicon oxide (Fig 2a). [3] It is different in chemical composition at both the inner and outer surface: the alumina layer is at the inner surface, while the silica layer is confined to the outer surface of the tube [3]. Because the silicon and aluminum oxides have different dielectric and ionization properties, outer and inner surfaces of the tube are oppositely charged in water at pH range from 3 to 8 [7]. The electrical zeta-potential of the silica and alumina colloids reflects this difference. As a whole, the surface charge was predominantly negative at the pH range from 2 to 10 (Fig 2b [3]). The surface area of HNTs is very large (around 57 m^2/g), which indicates HNTs may have a significant potential for binding of biologically functional molecules (drugs, proteins, DNA, growth factors). The luminal porosity is estimated to be about 0.25 ml/g by a mercury intrusion technique.

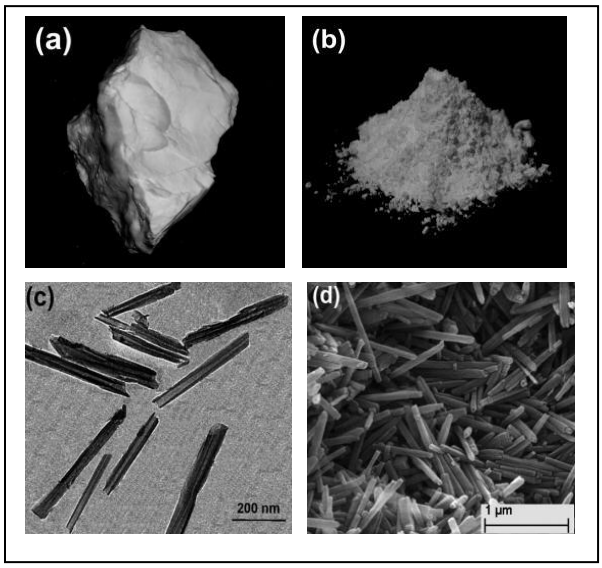

Figure 1. Images of HNTs from Dragon mine, Applied Mineral Inc: (a) raw sample with over 95% pure nanotubes, (b) processed HNTs, (c) TEM and (d) SEM images of HNTs. [3].

Halloysite has high mechanical strength and modulus, together with its low price, making it an ideal material for preparing different polymer based composites. HNTs are being used for many biological and non-biological application: (1) remove environmental contaminants, (2) deliver drugs and various macro molecules, (3) being applied in the fabrication of high quality ceramic white-ware, nanotemplates and nano scale reaction vessels, (4) improve mechanical properties of polymers. With above structural characteristics and properties, HNTs are considered as an ideal nanocarrier for drug delivery.

In our study, drug loaded HNTs were used to inhibit cell proliferation and to induce apoptosis, thus showing its potential as a tool to delivery chemotherapeutic drug loads to tumor sites. In this way, a focal and sustained drug release can be delivered to the targeted tumor sites and therefore, prevent

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potential postoperative surgical tumor recurrence and metastasis.

II. METHOD

A. Preparation of HNTs/drug composites

HNTs were immersed individually in solutions containing either methotrexate, artermisinin, quercetin and taurolidine, and solutions were stirred overnight. Solutions were then centrifuged and the supernatant was removed. The sediment was dried and crushed into a fine power. Pristine HNTs were used as a control and the drug-loaded HNTs were characterized via SEM to show presence of the loaded drugs.

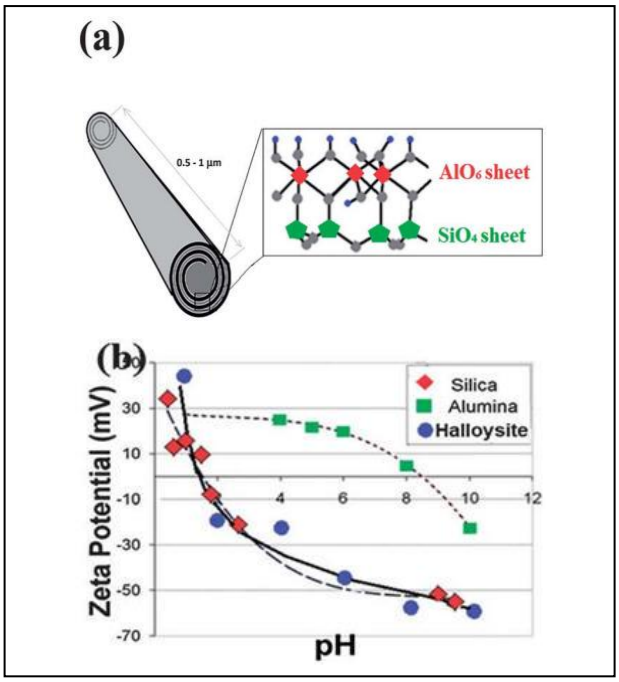


Figure 2 (a) Representation of the halloysite tubular structure and wall chemistry. (b) Variation of the silica and alumina surface potentials by pH of the solution. [3].

B. Release test of HNTs/methotrexate composite

Among the four tested drugs, only methotrexate is a drug used widely in clinical practice, and it has much better solubility in water than the others. Because of convenience and lab equipment limit, only the HNTs/methotrexate composite was tested to show sustained release. 10 mg powders of HNTs/methotrexate composite were immersed within 1ml DI water. The turbid liquid was stirred immediately and then kept being shaken until the collection of sample. At specific time points (5m, 10m, 15m, 30m, 1h, 5h, 12h and 22h), the turbid liquid was centrifuged, the supernate was collected, and fresh DI water was added. Sample was measured by Nanodrop 2000 at the wavelenth of 370nm. Release of methotrexate from HNTs into water can be calculated.

C. Osteosarcoma cell culture and cytotoxicity tests

Osteosarcoma cells (OC, UMR-106, ATCC) were cultured and incubated in 96-well plates and maintained in Dulbecco's Modified Eagle's Medium (D-MEM, Quality Bio. INC.) at 95% air and 5% CO_2. After 24 hours, the premade HNTs/drug composites were added to osteosarcoma cell cultures. After 48 hours, the Live/Dead Cytotoxicity kit (Invitrogen) was used to test for cellular viability and proliferation.

III. RESULT

A. Drug loaded HNTs

All of the tested model drugs were easily doped into HNTs (Fig. 3). Compared to the pristine HNTs, all the HNTs/drug composites have a bright layer of drug on the outside surface of HNTs. Although it is obvious that a layer of drug could be doped on the HNTs, there are still many nanotubes which were not coated. HNTs clumping due to the existence of drug on the outside surface was also observed.

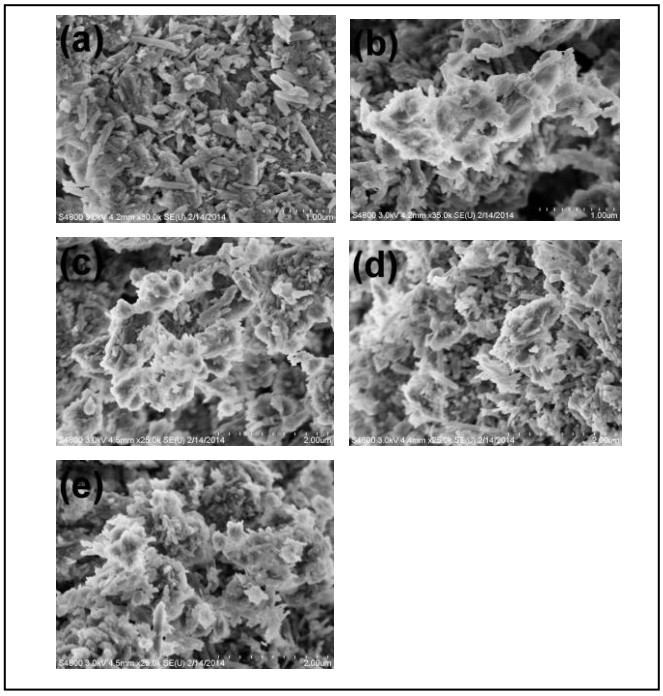


Figure 3. Images of HNTs and HNTs/drug composites: (a) pristine HNTs, (b)HNTs/quercetin, (c)HNTs/taurolidine, (d)HNTs/methotrexate, (e)HNTs/artermisinin

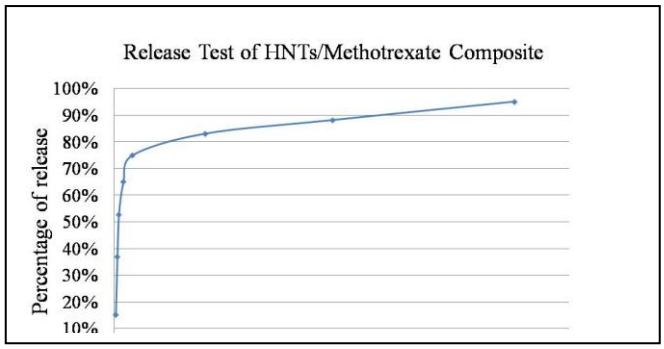


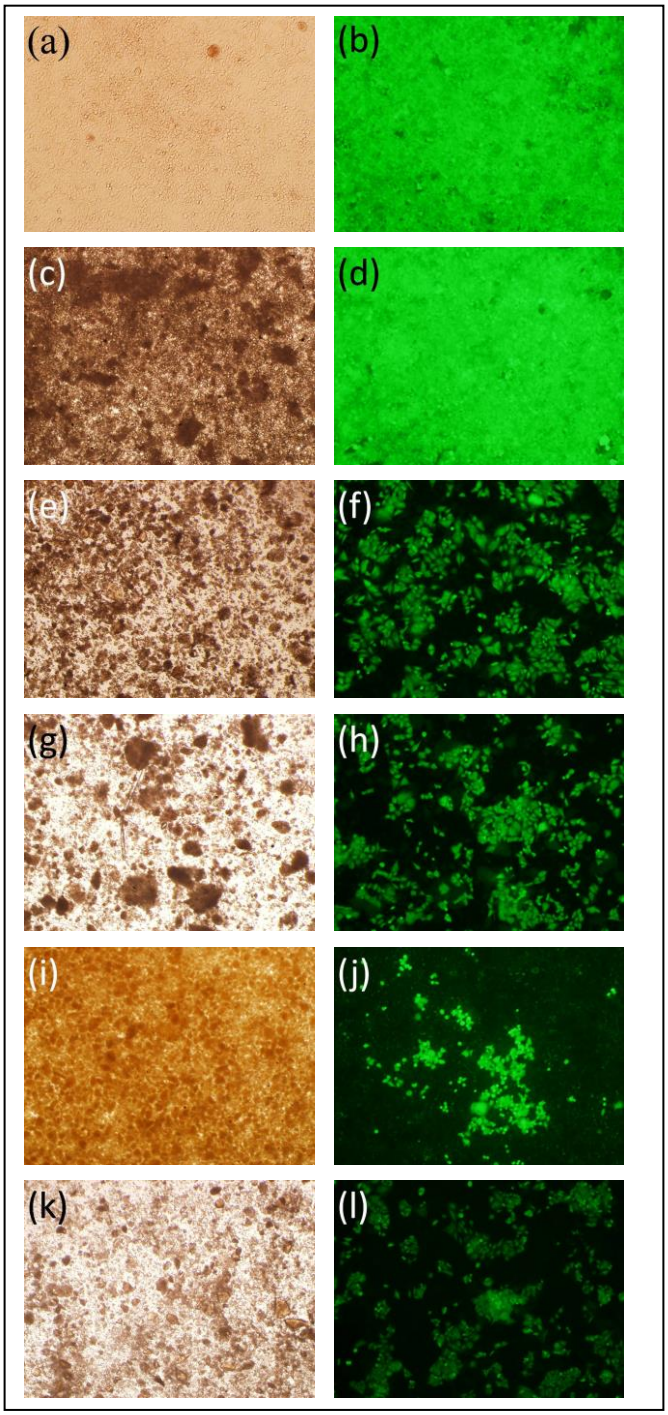
Figure 4. Release test of HNTs/methotrexate composite in DI water.

B. Release test of HNTs/methotrexate composite

As it is shown in Fig 4, there was a burst release at the early stage, and then it entered a relatively mild phase.

During a period of 24h, almost over 95% of the coated methotrexate, was released into the DI water.

Figure 5. Live/dead cytotoxicity test of HNTs/drug composite on osteosarcoma cells: (a) and (b) control, (c) and (d) HNTs, (e) and (f) HNTs/ methotrexate, (g) and (h) HNTs/ artermisinin, (i) and (j) HNTs/ quercetin, (k) and (l) HNTs/ taurolidine. (Images of completely dead cells were not provided, as dead cells which were not attached to the bottom of the well should have been washed away during process.)



C. *In vitro cytotoxicity test*

Similar to the result from the release test, all of the tested model drugs were released into the culture medium over the experimental period, which was demonstrated by the response of osteosarcom cells to the released drug. Addition of undoped HNTs at concentrations up to 8 mg/ml showed no observable cytotoxicity on osteosarcoma cells and did not impair their normal growth. Cellular growth in the HNTs group stayed the same as the control, while the cell densities in all the other groups were decreased significantly (Fig. 5). In the HNTs/methotrexate group, a few dying cells were observed, but proliferation was significantly inhibited. In the HNTs/artermisinin and HNTs/taurolidine groups, apoptosis was observed and proliferation was also arrested when compared to the control as shown after testing with an apoptosis immunohistochemical kit. Apoptosis was also observed in the HNTs/quercetin group as evidenced by the round and bright apoptotic bodies. (data not shown).

According to different test drugs, cells in different groups showed a response corresponding to that suggested by each drug, providing strong evidence that drug loaded HNTs can be used to alter the cellular behavior of osteosarcoma cells.

IV. Discussion

A. *Advantages and novelty*

In the field of nanomedicine, both halloysite nanotubes (HNTs) and carbon nanotubes (CNTs) are proving to be ideal candidates for new drug delivery system. Both nanomaterials possess advantages and disadvantages for drug delivery, and a comparison between them is shown in Table 1. Compare to CNTs, HNTs have a larger lumenal diameter and can be loaded with heavier biomolecules. Besides, HNTs are biocompatible, at lease at low rage, while CNTs have been proved to be poisonous. The major advantage of CNTs over HNTs is the tremendous amount of publications regarding to their properties and potential uses in nanomedicine. However, the cheap price is still able to make HNTs a strong competitor to CNTs.

TABLE 1. Comparison between HNTs and CNTs

Parameters	HNTs	CNTs
Diameter/length	50/1000 nm	2/1000nm
Inner lumen diameter	10-15 nm	1 nm
Biocompatibility	Biocompatible	Poisonous
Price	Cheap	Expensive
Patents	15-20	About 600
Publications	About 100	About 40,000
Researchers/companies	Louisiana Tech Univer. And 2 companies in the US	Hundreds of labs and companies

In this study, the research about HNTs/drug composite is the first step towards the final target in the treatment of osteosarcoma. The original idea is to use HNTs/drug containing medical products (like sutures and bone cement) to passively deliver chemotherapeutics to the tumor sites where postoperational recurrence or metastasis would be most likely to happen. In this way, a local and sustained release of antitumor drugs could be applied to kill the potentially residual tumor cells, or at least to decrease the amount of systemic chemotherapeutics required for postoperational treatment and the corresponding harm to healthy tissues. Similarly, biomolecules like growth factors and antibiotics can be delivered in the same way to help rebuild normal tissues and/or prevent infections after operations.

B. *Factors affecting loading and release*

Generally speaking, an idea delivery system should be able to carry enough amounts of the targeted molecules and release them in a controllable manner. Many factors can affect the process of loading and releasing, including molecular size and charge, surface area of the delivery system, specific fabrication method, and physical properties of the targeted molecules. Halloysite is a compatible material and has many properties which make it an ideal candidate for drug delivery. HNTs have a huge surface area (including both lumen and outer surfaces) that can be modified to be combined with biofunctional molecules. At certain rage of pH, the lumen surface and the outer surface may show different charges, and can correspondingly be doped with oppositely charged particles. The tubular structure makes it a natural nanocontainer for small molecules.

When it comes to the release profile, the situation becomes more complicated, as there are more factors, besides the HNTs, that can affect the combined result, like the solubility of the delivered molecule to the test solvent, and the frequency of sample collection and replacement of fresh solvent. In this study, the major factor that drives the release of methotrexate is the concentration gradient between the HNTs/methotrexate composite and the DI water. Although it showed a burst release (more than 70%) during the first hour, the result was significantly affected by the high frequency of sample collection and replacement of fresh DI water. Once the HNTs/methotrexate composite was immersed into water, the high concentration gradient would immediately drive the drug start to be released into water until a balance was reached. Then, the concentration difference disappeared and no more drug was released. After the supernate was collected and fresh water was replaced, new concentration gradient was rebuilt and additional release of the loaded drug was started again until another balance was reached. So, the release of methotrexate was more determined by how often the fresh water was replaced. To be more specific, it was determined by how fast the released drug was consumed.

Fortunately, additional fabrication method can be applied to gain more control over the release. One common method is to use the layer-by-layer technique to hold the drug layer between scheduled layers of polymers. Then, the release of targeted molecules would be more controlled by the degradation of the biocompatible polymers. Another method which is related to the original idea in this study is to mix the HNTs/drug composite into other materials to affect the release profile. A potential application may be the sutures and the bone cement.

C. *Future study*

Although results from this study were promising, this is only the first step, and more research need to be done to enhance the potential of using HNTs as a delivery tool in the future medical practice. New fabrication methods need to be developed to increase the dose of loaded drug, and to extend the period of release. New polymers are in great need to modify the surface of HNTs for targeting delivery, especially in the treatment of cancers. Also, more effort is required for loading and releasing multiple drugs. Besides, new materials and fabrication method should be developed to use HNTs based drug delivery system for potential clinical practice.

V. CONCLUSION

This study showed a potential for using HNTs to deliver a variety of chemotherapeutics to targeted tumors, and to provide sustained drug release. HNTs can be added to bone cement, polymer scaffolds, sutures, and hydrogels and used as a nanocomposite drug delivery system.

ACKNOWLEDGMENT

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