OPEN ACCESS

1st International Electronic Conference on Materials
26 May - 10 June 2014

http://sciforum.net/conference/ecm-1

Article

Synthesization, characterization, and in vitro evaluation of cytotoxicity of biomaterials based on halloysite nanotubes

Antonio Sánchez-Fernández[1], Laura Peña-Parás[2]*, Rodrigo Cué- Sampedro[1], Román Vidaltamayo[2], Ana Mendoza[2], Paulina Riojas[2]

[1] Tecnológico de Monterrey, Eugenio Garza Sada 2501, Col. Tecnológico, Monterrey, N. L., México; E-Mails: asanfer@itesm.mx (A.S.); rodrigo.cue.sampedro@itesm.mx (R.C)

[2] Universidad de Monterrey, Ave. Morones Prieto 4500 Pte., Col. Jesús M. Garza, San Pedro Garza García, N. L., México; E-Mails: laura.pena@udem.edu (L.P.); roman.vidaltamayo@udem.edu (R.V.); ana.mendoza@udem.edu (A.M.); paulina.riojas@udem.edu (P.R.)

* Author to whom correspondence should be addressed; Laura Peña-Parás; E-Mail: laura.pena@udem.edu (L.P.);
Tel.: +52 8182-151000 ext. 1894; Fax: +52 8182-151000 ext. 1228 *P*

Published: 30 May 2014

Abstract: Halloysite is an aluminosilicate clay that has been widely used for controlled drug delivery, bone implants, and for the capture of flowing tumoral cells. Surface modification of halloysite by organosilanes has been explored to improve their properties. In this study halloysite clay nanotubes (HNTs) were functionalized by two different organosilanes: Trimethoxy(propyl)silane, and Triethoxy(octyl)silane. Untreated and modified samples were characterized by scanning electron microscopy (SEM), thermogravimetrical analysis (TGA), and Fourier transform infrared spectroscopy (FTIR). Results showed a strong interaction of the organosilanes with the chemical groups present in HNTs. Biocompatibility and cytotoxicity of materials was determined using C6 rat glioblastoma cells. Reactions combined with HNTs, chitosan, and silica-chitosan were analyzed. Results indicate that HNTs exhibits a high biocompatibility and low cytotoxicity, as well as the sample with HNTs-chitosan. However HNTs functionalized with TEOS and TMPS showed high cytotoxicity caused by apoptosis. Also those samples with silica-chitosan presented a high level of cytotoxicity. These studies allow the identification of potential applications in biomedical areas of the HNTs.

Keywords: HNTs; organosilanes; characterization; functionalization; cytotoxicity

1. Introduction

Halloysite nanotubes (HNTs) have been recently used due to their unique properties such as their hollow tubular structure, high surface area, surface properties, and high biocompatibility [1, 2]. HNTs consist of a two layered aluminosilicate clay ($Al_2Si_2O_5(OH)_4 \bullet 2H_2O$); the tubes have multilayer walls with positively charged Al-OH functional groups on the inner surface, and with negatively charged Si-OH functional groups on the outer surface. These characteristics make HNTs a great absorbent for both cationic and anionic molecules [1]. Furthermore, their nanosized lumen (30 to 190 nm) enables the entrapping a range of active agents such as macromolecules and proteins [3].

HNTs have been studied for diverse biomedical applications, such as for inexpensive drug encapsulation, demonstrated by Price et al. [4, 5, 6]. HNTs are also capable of entrapping drugs and releasing small pharmaceutical molecules [4], they can be used as a template or nanoreactor for biocatalyst [5], as well as for use in personal care and cosmetics [6]. Zhai et al. [2] demonstrated that HNTs can be used to immobilize enzymes, such as the α-amylase and urease with the objective of extending their catalytic lifetime. In this study it was proved that after 15 days, the immobilized enzymes had more than 90% of activity due to the presence of HNTs. It was demonstrated by Zhang et al. [7] that HNTs have also been used for enhanced capture of flowing cells in blood.

In order to improve the interaction with polymer matrices, HNTs may be modified either with salts or organosilanes [8]. It has been proved that coating HNTs with organic molecules on its surface may reduce agglomeration due to its interaction with organic media. According to Girones et al. [9], organosilanes are widely used because of their low cost and availability. Functionalized HNTs with silanes can be incorporated to different polymer matrices, such as polypropylene [10], epoxy resin [11], and natural rubber [12]. Yuan et al. [3] modified HNTs with γ-Aminopropyltriethoxysilane (APTES) by direct grafting of the organosilane onto the surface hydroxyl groups. Similarly, Shi et al. [13] functionalized HNTs with APTEs to use them as carriers of therapeutic gene ASODNs (antisense oligodeoxynucleotides). Due to the large aspect ratio, good biocompatibility, and high mechanical strength of the modified HNTs, it was demonstrated that these nanotubes are promising vector for gene therapy applications.

In this study HNTs were functionalized with two different organosilanes and characterized by TGA and FTIR. The biocompatibility and cytotoxicity of these materials was determined with C6 rat glioblastoma cells.

2. Methods

2.1. Materials

HNTs with dimensions of 30-70 nm × 1-3 μm, Trimethoxy(propyl)silane (TMPS), and Triethoxy(octyl)silane (TEOS) were purchased from Sigma-Aldrich (St. Louis, MO). Acetone analytical grade from CTR was also used for sample preparation. Hybrid polymers of silica-chitosan were developed by Sánchez-Fernández and Peña-Parás in "Green chemistry synthesis of chitosan-silica hybrid materials for biomedical applications" (article in preparation). Low molecular weight Chitosan, Collagen from calf skin, acetic acid, and Phosphate buffered saline (PBS) were obtained from Sigma-Aldrich (St. Louis, MO). DMEM medium, fetal bovine serum (FBS), L-glutamine, trypsin, penicillin-streptomycin from Life Technologies (NY).

2.1. Functionalization of HNTs

HNTs were functionalized with 2 different organosilanes, namely Trimethoxy(propyl)silane and Triethoxy(octyl)silane. Functionalization was carried out by mixing in a flask ball 10g of HNTs, 50 ml of acetone and 2 ml of organosilane material. The samples were heated at 50°C for 48 h. in order to eliminate unreacted materials. Finally, the suspensions were vacuum filtered in order to obtain a solid phase.

2.2. Characterization methods

FTIR-ATR spectra were obtained with a Perkin Elmer SPECTRUM 400 spectrometer using ZnSe trapezoidal shaped ATR elements. Sample spectrum and background were acquired with the coated ATR element and the clean ATR element, respectively. The spectra were acquired with a resolution of $4cm^{-1}$ and 16 scans. A High Vacuum Tescan Vega3 SEM with a 30kV was used to characterize the morphology of the untreated and functionalized HNTs. Decomposition temperatures of samples were determined using thermogravimetric analysis (TGA) TA Instruments SDT Q600. All samples were heated al $10^{o}C$/min from room temperature to $850^{o}C$ under 100 mL/min nitrogen purge.

2.3. Cell culture

C6 Rat glioblastoma cells were obtained from American Type Culture Collection. The cell line was cultured under control conditions in cell culture Petri dishes (Corning) with DMEM medium supplemented with FBS (10%), L-glutamine (5%), and Pep Strep (5%). Cells were cultured for 11 days.

2.4. Cytotoxicity analysis

Materials were autoclaved for 15 minutes at 100°C, followed by a 30 minute drying cycle. Then, they were left to cool and stored at room tempereature (25°C).
Cell-cultures treated 96-well fluorescence microplates (Corning, NY, cat. no 3916) were covered with a 0.05 mg/ml solution of collagen in 0.1 M acetic acid overnight. After this incubation step, the wells were washed 3-times with phosphate buffered saline solution (PBS, 0.1 M phosphate buffer, 0.9 % w/v sodium chloride, pH 7.2). After washing, 20,000 C6 cells were plated per well and incubated overnight to reach 60% confluence. At this moment, the different treatments were administered and cells were incubated overnight. Control experiments have demostrated that after these incubations cells achieve

80% confluence, and at this stage citotoxicity was determined using an ApoTox-Glo Triplex assay (Promega, WI, cat. no. G6320) following the manufacuter's reccomendations.
Assays were read in a GloMax automated plate fluorometer at 402 nm / 505 nm (excitation/emission) for cell viability, and at 485 nm/ 520 nm for citotoxicity assessments.

3. Results and Discussion

3.1. Characterization results

Figure 1(a) shows that HNTs have diameters ranging from 130-180 nm. However, functionalized nanotubes showed an increase in diameter; Figure 1(b) shows that the diameters of HNTs-TMPS ranged from 150-230 nm. Similarly HNTs-TEOS had diameters of 140-230 nm (Figure 1(c)). This increase is attributed to the presence of organosilanes on the outer wall.

Figure 1. (a) HNTs**, (b)** HNTs-TMPS, **(c)** HNTs-TEOS.

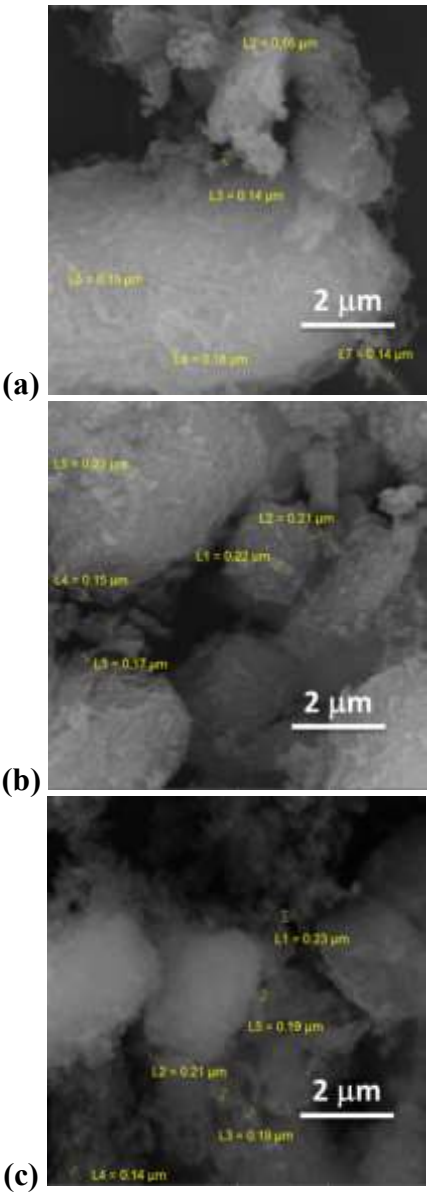

Figure 2 depicts the FTIR spectrums of HNTs, HNTs-TMPS, HNTs-TEOS. The vibrational mode at 2924 cm^{-1} on Figure 2(b) and Figure 2(c) corresponds to the methyl and methylene groups, confirming the chemical interaction with Al-O or Si-O functional groups of HNTs.

Figure 2. (a) HNTs, **(b)** HNTs-TMPS**, (c)** HNTs-TEOS.

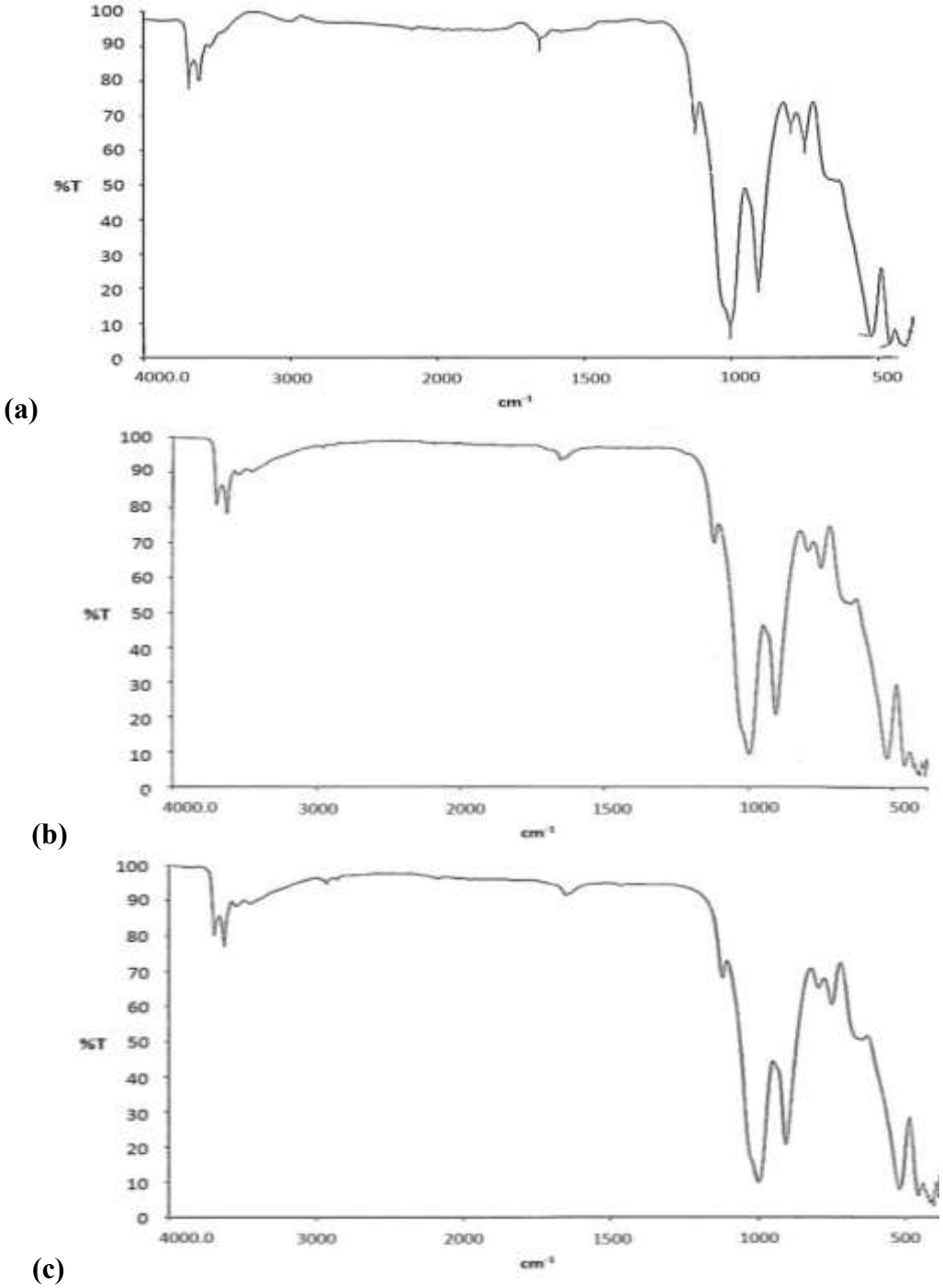

Table 1 shows the weight loss % at 200-320°C for functionalized and untreated HNTs. The higher weight loss shown from HNTs-TEOS compared to HNTs-TMPS is consistent with the higher hydrocarbon chain of the octyl group from Triethoxy(octyl)silane. Also shows the strong chemical interaction of the organosilanes with the Si-O and Al-O groups of HNTs.

Table 1. Weight loss by TGA of HNT and functionalized HNTs

Sample	Weight loss in TGA, 200–320 °C (%)	Difference relative to HNTs (%)
HNTs	1.86	-
HNTs-TMPS	1.94	0.08
HNTs-TEOS	2.04	0.18

3.1. Cytotoxicity results

The results obtained from the cytotoxicity tests were analyzed using the Sigma Stat 3.5 program, which gave statistical analysis with the mean, standard deviation and error of each test. These results are shown in the Table 2 where the three cytotoxicity assay results are compared.

Table 2. Comparison of the three cytotoxicity assay results.

Sample	Cytotoxicity	Apoptosis	Viability
HNTs	-	-	-
HNTs-TEOS	x	x	↑
HNTs-TMPS	x	x	-
Silica-chitosan	-	-	↓
Chitosan	-	-	↑
HNTs/Silica-chitosan	-	-	↓
HNTs-TEOS/Silica-chitosan	-	-	↓
HNTs-TMPS/Silica-chitosan	-	-	↓
HNTs/ Chitosan	-	x	-
HNTs-TEOS/ Chitosan	-	x	↑
HNTs-TMPS/ Chitosan	-	-	↑

Comparing the results of the three cytotoxicity assays is possible to conclude that HNTs are a highly biocompatible material. A similar reaction showed HNTs with chitosan prevented cell proliferation by increasing apoptosis. On the other hand it was determined that both TEOS and TMPS functionalization exhibited high cytotoxicity, killing cells by apoptosis. The results of the samples based on silica chitosan showed a decrease in viability but no significant difference in the cytotoxicity assays. Thus it is concluded that silica-chitosan is a highly cytotoxic compound that causes the cell death at a high speed.

4. Conclusions

Functionalization of HNTs was achieved by modification with TMPS and TEOS organosilanes. Characterization by FTIR and TGA showed the strong interaction of organosilanes with the chemical groups present in HNTs. This functionalization may be useful to improve the properties of HNTs for several applications, including drug encapsulation and delivery, biocatalysis, and for nanocomposites with enhanced mechanical properties. Citotoxicity of untreated and functionalized HNTs was characterized. Results showed that at the selected concentrations HNTs showed to be a highly biocompatible material, however, functionalization by selected organosilanes exhibited high cytotoxicity, showing cell death by apoptosis.

Acknowledgments

The authors acknowledge the support from UDEM VIAC grant UIN14019.

Conflicts of Interest

The authors declare no conflict of interest.

References

[1] Zhao, Y.; Abdullayev, E.; Vasiliev, A.; Lvov, Y. Halloysite nanotubule clay for efficient water purification. *Journal of Colloid and Interface Science* **2013**, 406, 121-129.

[2] Vergaro, V.; Abdullayvev, E.; Lvov, Y.M.; Zeitoun, A. Cytocompatibility and Uptake of Halloysite Clay Nanotubes. *Biomacromolecules* **2010**, 11, 3, 820-826.

[3] Yuan, P.; Southon, P.D.; Liu, Z.; Green, M.E.R.; Hook, J.M.; Antil, S.J.; Kepert, C.J. Functionalization of Halloysite Clay Nanotubes by Grafting with γ-Aminopropyltriethoxysilane. *Journal of Physical Chemistry C* **2008**, 112, 40, 15742-15751.

[4] Price, R.; Gaber, B.; Lvov, Y. Release Characteristics of Tetracycline Khellin and NAD from Halloysite: A Cylindrical Mineral for Delivery of Biologically Active Agents. *J. Microencapsulation* **2001**, 18, 713-723.

[5] Shchukin, D.; Price, R.; Sukhorukov, G.; Lvov, Y. Biomimetic Synthesis of Vaterite in the Interior of Clay Nanotubules. *Small* **2005**, 1, 510-513.

[6] Kelly, H.; Deasy, P.; Ziaka, E.; Claffey, N. Formulation and Preliminary in Vivo Dog Studies of a Novel Drug Delivery System for the Treatment of Periodontitis. *Int J. Pharm* **2004**, 274, 167-183.

[7] Levis, S.; Deasy, P. Characterisation of halloysite for use as a microtubular drug delivery system. *Int J Pharm.* **2002**., 243, 1, 125-134.

[8] Shchukin, D.; Sukhorukov, G.; Price, R.; Lvov, Y. Halloysite nanotubes as biomimetic nanoreactors. *Small* **2005**, 1, 5, 510-513.

[9] Kamble, R.; Ghag, M.; Gaikawad, S.; Panda, B.K. Halloysite Nanotubes and Applications: A Review. *Journal of Advanced Scientific Research* **2012***,* 3, 2, 25-29.

[10] Zhai, R.; Zhang, B.; Liu, L.; Xie, Y.; Zhang, H.; Liu, J. Immobilization of enzyme biocatalyst on natural halloysite nanotubes. *Catalysis Communications* **2010***,* 12, 4, 259-263.

[11] Carli, L.N.; Daitx, T.S.; Soares, G.V.; Crespo, J.S.; Mauler, R.S. The effects of silane coupling agents on the properties of PHBV/halloysite nanocomposites. *Applied Clay Science* **2014***,* 87, 311-319.

[12] Gironès, J.; Méndez, J.A.; Boufi, S.;, Vilaseca, F.; Mutjé. P. Effect of silane coupling agents on the properties of pine fibers/polypropylene composites. *Journal of Applied Polymer Science* **2007***,* 103, 6, 3706-3717.

[13] Du, M.; Guo, B.; Jia, D. Thermal stability and flame retardant effects of halloysite nanotubes on poly(propylene). *European Polymer Journal* **2006***,* 42, 6, 1362-1369.