Cu/TiO_2-SiO_2 Nanostructured Materials for Brain Cancer Treatment

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ABSTRACT

In the present work, we report the toxic effect exerted on glioblastoma cells of rat (C6 line) by copper complexes released from a binary oxide (TiO_2-SiO_2=TISI). Individually, copper acetylacetonate and ammonium tetrachlorocuprate were added to the oxide during its synthesis procedure using the sol-gel methodology. Several physicochemical techniques were used to characterize the resulting materials. The cell viability was determined by the colorimetric MTT assay. The results of IR and UV-VIS spectroscopies indicate that copper complexes maintained their original structure after being loaded in the oxide. Cell viability results showed high cytotoxic effect of the copper complexes as well as those released from TISI. Pure TISI did not exert cytotoxicity to the C6 cells, indicating its high biocompatibility. Although, COS-7 cells are not cancer cells from the rat brain, these served to determine that a mitochondrial fragmentation (apoptosis) occurred when these cells were treated with $Cu(acac)_2$-TISI during 12 h.

Keywords: copper complexes, binary oxide, brain cancer, nanostructured materials

1 INTRODUCTION

Tumors from the glial are the most frequent primary tumors that develop in the brain. Glioblastoma multiforme (GBM) is the most malignant form of glioma and it is one of the most aggressive incurable human cancers with an average survival of about 12-15 months [1, 2]. At present, the treatment mainly consists of surgery and radiotherapy, however, the local infiltration of high-grade glioblastomas prevents the complete resection of all malignant cells. In addition, it is difficult to remove all the cancerous tissue without severely damaging the brain and healthy brain tissue is less tolerant to conventional radiotherapy than tumor tissue. The administration of chemotherapeutic drugs intravenously has limited use because of their adverse systemic effects and poor blood–brain barrier penetration. Different strategies have been proposed for the brain cancer treatment, such as local drug delivery, which is a promising strategy for achieving localized drug delivery, even in deep-seated brain tumors [3, 4]. We have reported the toxic effect of copper complexes exerted on brain cancer cells, when these were released from a biocompatible nanostructured titania [5, 6]. Therefore, we propose the implantation of a drug eluting material into the tumor brain tissue for direct delivery of the chemotherapy. Titania and silica in their different forms have demonstrated to be appropriate vehicles to many drugs due to their biocompatible properties [6-9]. In the present work we used a binary oxide (TiO_2-SiO_2) as a vehicle for copper complexes to be used as drug delivery systems to the brain cancer treatment.

2 EXPERIMENTAL

The TiO_2-SiO_2 binary oxide was prepared using the sol-gel method and the copper complexes [$Cu(acac)_2$ and $Cu(NH_4)_2Cl_4$] were loaded during its preparation. The molar ratios used were: water/alkoxide 16/1 and ethanol/alkoxide 8/1. The used amount of each copper compound was 10%.

Abbreviations: TEOS= Tetraethyl orthosilicate, GABA= γ-aminobutyric acid, $Cu(acac)_2$= Copper(II) acetylacetonate, $Cu(NH_4)_2Cl_4$= Ammonium tetrachlorocuprate (II) dihydrate.

2.1 Sample preparation

TiO_2-SiO_2 (TISI). One gram of GABA was dissolved in a mixture of water and ethanol under stirring. After, 90 µL of sulfuric acid and phosphoric acid were added respectively. Next, the TEOS amount was dripped to the mixture during two hours. Then, the mixture was cooled at 4 °C and a solution of titanium n-butoxide in absolute

ethanol was added. The mixture was maintained stirring until gel formation. Finally, water and ethanol were removed and the xerogel was dried at 70 °C.

Coppper Complex/TiO_2-SiO_2 (XCC/TISI), X=$Cu(acac)_2$ or $Cu(NH_4)_2Cl_4$. The respective copper complex was dissolved in 50 % water and one gram of GABA was dissolved in the another 50 % of water. After, both solutions were added to the ethanol, which was maintained stirring. Later, 90 µL of sulfuric acid and phosphoric acid were respectively added. Then, the TEOS was added in a lapse of two hours. Immediatly, the temperature was decreased at 4 °C. Afterward, the titanium n-butoxide dissolved in absolute ethanol was added. Later, the mixture was maintained with stirring until the gel formation. Finally, the water and ethanol were removed and the xerogel was dried at 70 °C.

2.2 Sample characterization

The infrared spectra were acquired in an Afffinity-1 FTIR spectrophotometer, using the KBr method. Nitrogen adsorption–desorption isotherms were obtained using a Micromeritics Belsorp IIBell Japan INC apparatus. From the isotherms were calculated the specific surface areas, pore volumes and pore size distributions.

2.3 *"In vitro"* release tests

The resulting XCC/TISI powder was compressed into a tablet and emerged in 100 ml of artificial cerebrospinal fluid. At predetemined times an aliquot of 4 ml was removed for its measurament by ultraviolet. After each reading, the aliquot was returned at the release solution. To determine the amount released each copper complex a calibration curve of known concentrations of each copper complex vs absorbance was used.WCu(acac)2-TISI=37.9 mg and $W_{Cu(NH4)2Cl4\text{-}TISI}$=21.0 mg. $\lambda_{Cu(aca)2}$=293 nm and $\lambda_{Cu(NH4)2Cl4}$=205 nm.

2.4 Biological tests

"In Vitro" Cell Viability Test. The C6 rat glioma cell line, was used to determine the toxic properties of TISI, each copper complex and XCC-TISI, taking as referring to Cis-Pt and as reference untreated cells. $1x10^5$ cells were treated with increasing concentrations (15.7 to 1000 µg/mL) of each aforementioned compound for 24 hours at 37º C. The effect of the compounds on the cell survival was determined by the MTT assay (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, which measures mitochondrial activity. The absorbance was measured at 570 nm using a multiwall scanning spectrophotometer reader. Six experiments were made for each concentration of each compound used. Data were obtained as the mean of the total of the determinations (X + SD) and expressed in % of absorbance. The percentage of surviving cells with respect to untreated cells (control) was calculated using the following formula: % viability = [(At/As) × 100] %, where At and As indicate the absorbance of the sample and control, respectively.

Staining of cells and epifluoresce microscopy. The COS-7 fibroblast-like cells (from monkey kidney tissue) were labeled in vivo by including the fluorescent mitochondrial marker MitoTracker Deep Red (Molecular Probes) at a concentration of 1µg/ml for 30 min at 37 °C. The cells were analyzed by epifluorescence through 60x (numerical aperture (NA):1.00 W). The images were obtained and recorded by using a Nikon digital sight-DG-Ri1 camera controlled with the Nikon NIS-Elements AR-3.0- SP7 software included in the system.

3 RESULTS

The spectra of TISI, $Cu(acac)_2$ and $Cu(acac)_2$-TISI are reported in figure 1. From the TISI spectrum a peak is located at 3428 cm^{-1} correspond to the stretching vibrations of O–H bonds, while the peak seen at around 1629 cm^{-1} has been asignated to the bending vibration of the O–H bond, which was ascribed to the adsorbed water molecules. The peaks located at 796 and 1086 cm^{-1} were attributed to the symmetric and asymmetric stretching vibrations of the Si–O–Si bonds, respectively. The peak observed at 944 cm^{-1} corresponds to the Ti–O–Si asymmetric stretching vibration and which confirms the formation of Ti–O–Si linkages. Indicative that some silicon atoms in the silica network were replaced by titanium atoms, producing the TiO_2-SiO_2 binary oxide.

The spectrum corresponding to the $Cu(acac)_2$ complex was recently analyzed and reported [9]. For this work, we are only repeating the most important findings. The small peaks located between 3099 and 2850 cm^{-1} were generated by CH_3 stretching vibrations of the acetylacetonate ligand. The peaks at 1577, 1555 and 1533 cm^{-1} correspond to C=C and C=O stretching vibrations respectively. The peak seen at 684 cm^{-1} is attributed to Cu-O stretching vibration between the copper atom and the oxygen atoms in the acetylacetonate ions. The peak at 610 cm^{-1} was due to ring vibrations and Cu-O stretching vibration modes. Finally, the peaks located at 451 cm and 431 cm^{-1} were due to Cu-O and ring deformation modes respectively.

When the $Cu(acac)_2$-TISI spectrum was analyzed, several similarities with the TISI and $Cu(acac)_2$ spectra were found. The peaks that identify to $Cu(acac)_2$ were seen in the $Cu(acac)_2$-TISI spectrum. Also, the peaks that identify TISI were observed.

In figure 2 we display the spectra of $Cu(NH_4)_2Cl_4$ and $Cu(NH_4)_2Cl_4$-TISI. The $Cu(NH_4)_2Cl_4$ spectrum shows several peaks that are mainly derived from the ammonium ion (3167, 1592, and 1413 cm^{-1}). The first peak is attributed to stretching vibrations of the N-H bond, while the other peaks are related with bending and librational vibrations of the ammonium ion.

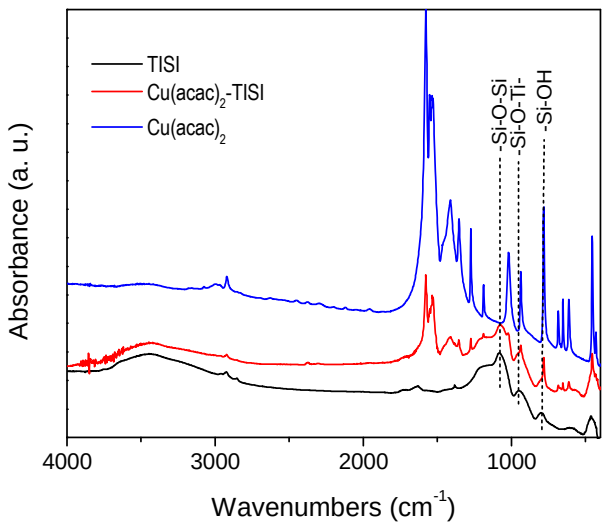

Figure 1: FT-IR spectra of $Cu(acac)_2$, $Cu(acac)_2$-TISI and TISI.

These signals, which were marked with a green dotted line, are seen in the $Cu(NH_4)_2Cl_4$-TISI spectrum. It was also posible to observe the peaks corresponding to TISI as is denoted in figure 2.

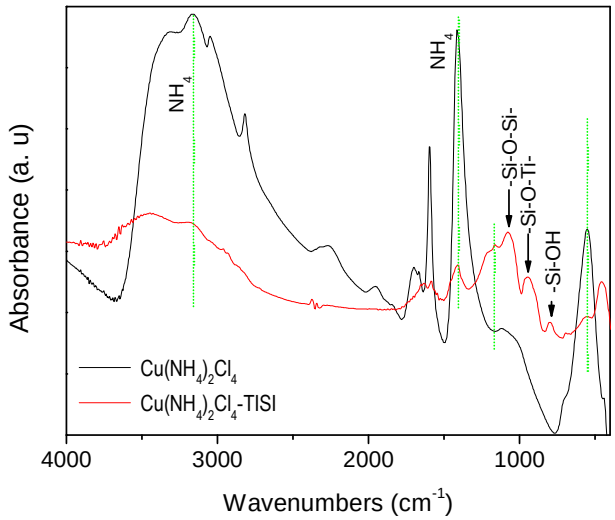

Figure 2: FT-IR spectra of $Cu(NH_4)_2Cl_4$ and $Cu(NH_4)_2Cl_4$-TISI.

The surface area, pore volume and pore diameter values were determinated from the nitrogen adsorption-desorption isotherms and these values are reported in table 1. The TISI sample has a surface area of 320 m^2/g, a pore diameter of 5.28 nm, and a pore volume of 0.423 g/cc respectively. However, these values were drastically reduced in samples containing any copper complex (see table 1). This phenomenon is due to copper complexes are occuping those spaces.

Sample	S_{BET} (m^2/g)	D_p (nm)	V_p (g/cc)
TISI	320	5.28	0.423
$Cu(acac)_2$-TISI	61	3.28	0.200
$Cu(NH_4)_2Cl_4$-TISI	206	2.22	0.106

Table 1: Textural properties of TISI and it loaded with any copper complex. Surface area (S_{BET}), Pore diameter (D_p) and Pore volume (V_p).

The *in vitro* release of $Cu(acac)_2$ and $Cu(NH_4)_2Cl_4$ from the binary oxide (TISI) was assessed in artificial cerebrospinal fluid. As shown in Figure 3, $Cu(NH)_4Cl_2$ is released faster than $Cu(acac)_2$, due to it is higer solubility soluble. At the end of the experiment about 2% of $Cu(acac)_2$ was released, while a 5 percent of $Cu(NH_4)_2Cl_4$ was released. From the textural properties (table 2), it is posible to infer that $Cu(acac)_2$ occupied the surface more efectively than pores. In the other hand, Ammonium tetrachlorocuprate was occluded into the pores.

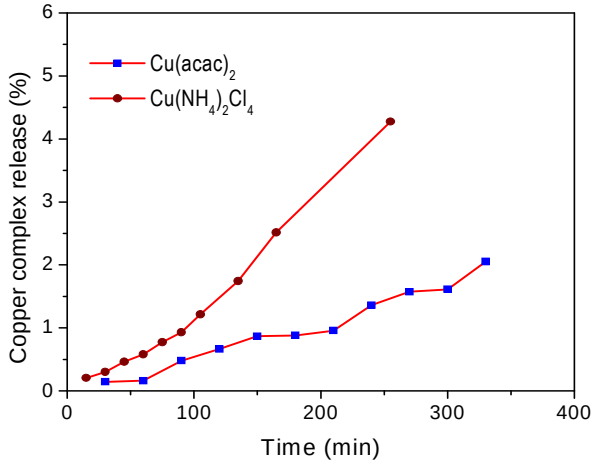

Figure 3: Copper complex release profiles.

The *in vitro* cytotoxicity assay was performed on C6 cells via MTT assay by incubating the different concetrations of TISI, XCC-TISI, Cis-Pt and untreated cells taken as reference for 24 h. TISI sample did not show any significant cytotoxic effect after 24 h incubation even at higher concentration (1000 µg/mL). $Cu(acac)_2$ exerted very potent cytotoxic effect at the low concentrations used. The cell death was nearly 100 % when 62.5 µg/mL of $Cu(acac)_2$ was used. After this concentration the cell viability was the same (figure 4). Also, $Cu(NH_4)_2Cl_4$ showed significant toxic effect. However, it was necessary to use 125 µg/mL to have the same effect. When the cells were treated with the materials containning the copper complex a sustained cell death was observed. It has been attribuited to low concentrations of the copper complex is released from the TISI material to excert its toxic effect. Individual copper complexes as well as those loaded on titania had higher toxic effect than Cis-Pt.

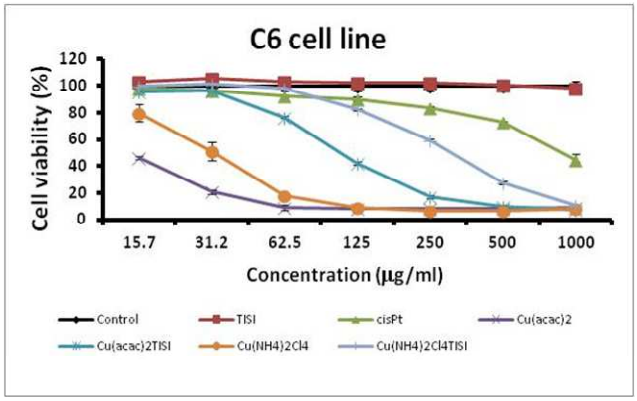

Figure 4: % Cell viability as a fuction of the concentratration of each used compound.

Figure 5 shows staining with the MitoTracker DeepRed dye which stains metabolically active mitochondria. It can be observed in the controls that the normal shape of the mitochondria is maintained (see asterisks), whereas in cells that were incubated with $Cu(acac)_2$-TISI the mitochondrial network is lost, concentrating on the area surrounding the cell nucleus also present a more elongated morphology almost as fibers (see arrow).

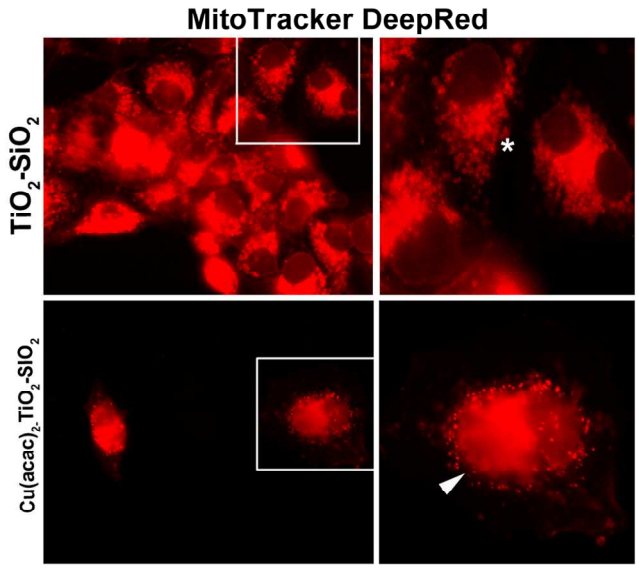

Figure 5: Mitochondrial alterations induced by $Cu(acac)_2$-TiO_2-SiO_2 (arrow), while TiO_2-SiO_2 does not induce damage (asterisks).

4 CONCLUSIONS

The binary oxide TiO_2-SiO_2 was successfully obtained and it served as a carrier for copper complexes. The copper complexes released from the binary oxide remained structurally unchanged and they exerted their toxic effect to the C6 cells. Mitochondrial alterations iduced by $Cu(acac)_2$-TiO_2-SiO_2 were observed in COS-7 cells, while TiO_2-SiO_2 did not induce damage in COS-7 cells. Therefore, the probable death mechanism is by apoptosis via mitoclondrial.

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